FUNCTIONAL CLASSES OF NATURALLY OCCURRING HUMAN ANTIBODIES DEVELOPED AGAINST GRAM-NEGATIVE BACTERIAL PATHOGENS

By

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DEDICATION

To my parents and grandparents, who always fostered my curiosity, even when it made a mess.

To my wife, who supports me when life is great and when it is a struggle.

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CHAPTER I

I. Antibacterial antibodies have diverse targets and functions.

Antibodies arise after bacterial exposures and infections and have diverse functions at the host-pathogen interface. In my dissertation, I describe the ways with which human monoclonal antibodies (mAbs) interact with different Gram-negative pathogens or their secreted effectors. In this first chapter, I review the possible targets of mAbs that bind to bacteria. In the second chapter, I report the isolation and functional characteristics of a panel of antibodies to *Escherichia coli* outer membrane protein A. In chapter three, I assess the capsular specificities of antibodies to *Klebsiella pneumoniae* and discuss how these antibodies may function to alter *K. pneumoniae* infection. Finally, in the fourth chapter I report on the development of a novel assay to assess antibodies to pertussis toxin produced by *Bordetella pertussis* and describe use of the assay to probe a large pre-existing panel of anti-toxin antibodies. I conclude my dissertation by summarizing these works and examining future directions.

Overview

The mammalian immune system is responsible for protecting the host organism from myriad pathogenic or opportunistic microbes. The immune system is classically divided into innate and adaptive components, and adaptive immunity is further subdivided into cellular (or T-cell) and humoral (or B-cell) immunity. Humoral immunity technically encompasses all the secreted molecules found in the “body humors”, or fluids, including complement, antimicrobial

peptides, and secreted antibodies, as well as the B cells necessary for antibody production (1). Antibodies are generally regarded as the most significant component of humoral immunity, to such an extent that humoral immunity is interchangeably called antibody-mediated immunity.

An antibody is the product of complex selection and maturation processes that act upon and occur within B cells to generate a cell that produces a single antibody (2, 3). These cells circulate in their host and may encounter antigens to which their antibody binds, for example, during an infection or following vaccination. After recognizing its cognate antigen, a parental B cell proliferates, and the descendent B cells undergo affinity maturation by somatic hypermutation. This process, which introduces random changes to the DNA encoding the antibody of that cell, ultimately generates higher affinity antibodies that have critical roles in responding to disease and preventing subsequent recurrences of disease (4). Monoclonal antibodies can be generated from these B cells using hybridoma technology (5, 6). Because the antibody maturation process is dynamic and continually ongoing, it is optimal to study fully human antibodies that arose because of exposure, infection, or vaccination. These naturally occurring human antibodies more accurately model how the human immune system responds to antigenic stimuli and the antibodies provide critical insights into disease processes. Furthermore, by studying the human antibody response, we can develop insight into how therapeutic and preventative strategies, like vaccination, can be used to minimize and control human illnesses.

Classes of antibodies specific to bacteria

The critical interaction that enables all antibody-mediated functions is the binding of a specific antibody to a specific antigen. The interactions of antibodies with viral proteins have

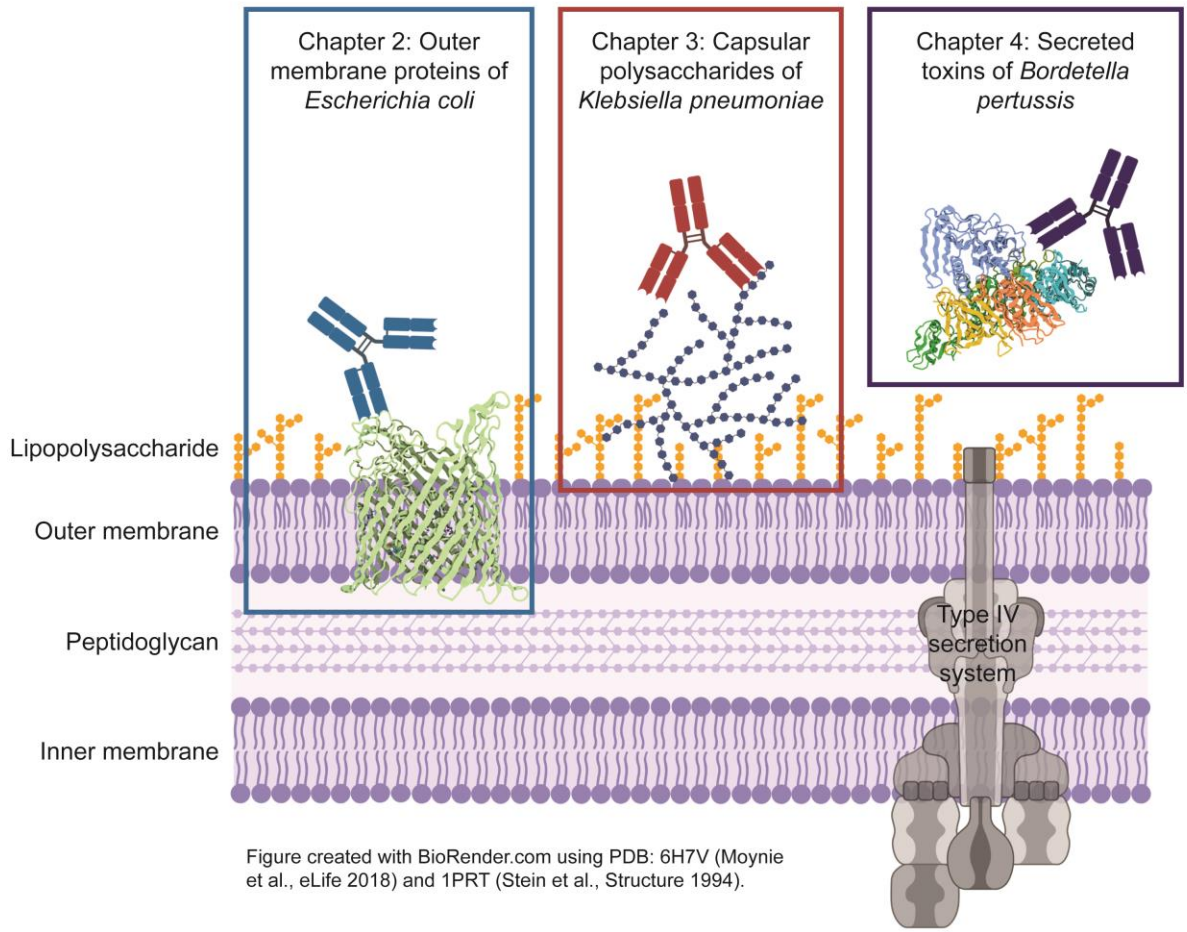

Figure 1: Graphical overview of dissertation. Gram-negative bacteria are defined by the multilayer cell envelope, consisting of an inner membrane, peptidoglycan within the periplasmic space, and an asymmetric glycolipid bilayer outer membrane decorated with lipopolysaccharide and other antigens. In my dissertation, I discuss antibodies to three antigens produced by Gram-negative bacteria. Chapter 2 details antibodies to outer membrane protein A of *Escherichia coli*. Chapter 3 describes the identification of antibodies to *Klebsiella pneumoniae* that target diverse antigens, including capsular polysaccharide. Chapter 4 presents the development of a novel assay to assess antibodies that neutralize pertussis toxin secreted using a Type IV secretion system from *Bordetella pertussis*.

been well-characterized for many viruses over the past decades, although much remains to be learned (7, 8). Bacteria are orders of magnitude more complex than viruses, and as a result, the manners with which antibodies can interact with bacteria are also complicated (9). In parallel to viruses, bacteria have outer membrane proteins exposed to the extracellular environment that an antibody may bind to. Many bacteria produce capsular polysaccharides to create an extracellular matrix. This capsule can prevent antibodies from binding to surface proteins while at the same time serving as a unique class of antigens for antibody targeting (10, 11). Many successful

bacterial vaccinations target capsular polysaccharides. Bacteria also frequently produce secreted toxins that diffuse throughout a host organism and cause harmful effects. Many of these toxins can be neutralized by the binding of an antibody (12). Bacterial toxins have also successfully been used as vaccine immunogens and as conjugates for other vaccines (13, 14). Anti-bacterial antibodies can act through a variety of non-exclusive functions. The most common correlate of protection for antibacterial Abs is opsonophagocytic killing (OPK), also called antibody-dependent cellular phagocytosis (ADCP) (15). Complement-dependent antibody-mediated bactericidal activity is another mechanistic correlate of protection, most described for serogroup A, C, W, & Y *Neisseria meningitidis* (16, 17). Antibodies may also inhibit bacterial disease or pathogenesis by altering specific bacterial protein functions through binding interactions.

Outer membrane proteins

The Gram-negative outer membrane is vitally important for the persistence and pathogenesis of Gram-negative organisms. It is a highly discriminatory interface that protects the organism from external influences, including detergents and antibiotics. The membrane is an asymmetric bilayer, composed of a phospholipid inner leaflet and a glycolipid outer leaflet (18). These glycolipids are lipopolysaccharide (LPS) or lipooligosaccharide (LOS), depending upon the synthetic machinery of the species, and the saccharide backbones of these molecules interact with divalent cations (Mg^{++} and Ca^{++}) to form a densely packed largely impermeable barrier (19). To survive, Gram-negative bacteria require mechanisms to import and export compounds and with which to interact with their extracellular environment. These roles are predominantly filled by β-barrel outer membrane proteins (OMPs), which act as passive porins or as specific

effectors of various functions (18, 20). Importantly for this dissertation, OMPs have been found to be potent immunogens that elicit antibody responses in murine models and in humans after infection.

Early studies of the human immune response to *Escherichia coli* identified several distinct but unidentified OMPs as targeted by antibodies induced after infection of humans and inoculation of mice (21, 22). Interestingly, some of these antigenic *E. coli* OMPs were found to be regulated by intracellular iron levels (23). Much of the knowledge of OMPs as antibody targets are derived from studies of immunization trials in mice, done with the goal of identifying promising vaccine candidates. The immune response to *Acinetobacter baumannii*, an important opportunistic pathogen of humans, has been primarily characterized through the study of immunization trials. Many unique *A. baumannii* OMPs have been used as immunogens in mice, and several have shown the importance of passive serum transfer for protection from disease, indicative of antibody-mediated protective mechanisms. Oligoantigenic immunization with whole bacteria, extracted outer membranes, isolated outer membrane vesicles, or generated outer membrane complexes generated antibody responses in mice (24-27). Outer membrane vesicles and complexes were shown to protect naïve animals by passive transfer from immunized animals (25, 27). The most commonly identified antigenic components of these oligoantigenic immunizations were OMPs, best evidenced by the elimination of antigenicity by proteinase K treatment of extracted outer membranes (28). Some individual antigenic proteins have been identified through these diverse immunizations. Outer membrane protein 22 (Omp22) from *A. baumannii* was found to be a dominant antigen, and most intriguingly, iron-regulated proteins of *A. baumannii* were also identified as important antigens to which immune responses were

elicited (29, 30). Since iron is an essential nutrient for all forms of life, altering metal metabolism with antibodies has been further studied and was an initial focus of this thesis work.

In *A. baumannii*, iron is acquired through three siderophore-dependent iron acquisition systems. These systems utilize three groups of secreted siderophores, high-affinity iron-binding molecules, called acinetobactin, baumannoferrins, and fimsbactins (31-33). Siderophores are secreted and bind iron in the extracellular environment. Each system is dependent upon a single OMP that binds siderophores and traffics iron-bound siderophore into the periplasmic space. FbsN binds the fimsbactins, BfnH binds the baumannoferrins, and BauA binds pre-acinetobactin and is also the best-studied as an antibody-eliciting antigen (34-36). The acinetobactin system, including BauA, is critically important for *A. baumannii* pathogenesis and virulence in *in vivo* models of pneumonia and sepsis (35). Several studies have used peptides from or full-length BauA as an immunogen, although so far, no immunization trials have demonstrated *in vitro* verification of protein conformational integrity. Multiple similar studies have immunized mice and rabbits with rBauA and demonstrated protection from *A. baumannii* challenge by active immunization and passive transfer of serum to naïve animals (37-41). No studies have further evaluated any potential protective mechanisms due to antibodies specific to BauA. Production of recombinant OMPs is very challenging, has significantly limited the ability to study these proteins further, and was a major obstacle to this dissertation work. To address those difficulties, there has been some interesting work engineering loops from OMPs, including BauA, onto a "loopless C-lobe" derived from *Neisseria meningitidis* transferrin binding protein B, TbpB. These chimeric proteins are not trafficked to the outer membrane and remain soluble in the cytoplasm, greatly simplifying recombinant purification (40, 42). However, these chimeras are not as immunogenic as the full-length protein, suggesting they fail to recapitulate the full

spectrum of antigenicity of OMPs. Despite being far from comprehensive, these studies demonstrate that antibodies to iron-regulated OMPs protect mice from bacterial challenges, yet it is not known whether anti-iron receptor antibodies are elicited in humans. Determining this will require the recombinant production and purification of conformationally intact OMPs and the identification and recruitment of human donors who have had prior infection with, or significant exposure to, the organisms of interest.

Study of other OMPs has provided insight into the possible functions that anti-OMP antibodies may perform. One of the most important proteins in the outer membrane is BamA, which is responsible for folding and inserting all other OMPs into the outer membrane (43). Antibodies to *E. coli* or *A. baumannii* BamA are induced after immunization and are highly specific and some antibodies cross-react across species as *bamA* is conserved across many Gram-negative organisms (44-46). Because BamA is essential for basic bacterial functions, antibodies are in rare instances able to inhibit growth of *E. coli* by binding to BamA (44). One murine antibody, MAB1, binds to the extracellular loops of BamA and inhibits the capacity of BamA to mediate protein insertion into the outer membrane (47). Subsequent work found that MAB1 also increases outer membrane fluidity, altering membrane dynamics and activating bacterial stress responses (48). Critically however, MAB1 only bound and functioned against *E. coli ΔwaaD*, a strain that produces and displays a truncated LPS upon its surface. BamA is one of the most important OMPs, but it is not the most abundant protein on the Gram-negative outer membrane. Both OmpA and OmpC are found at high levels on the bacterial surface and serve a variety of different porin functions (49-51). Both OmpA and OmpC have been found to be immunogenic and induce antibody formation in mice. Murine antibodies to OmpC have recently been described that exhibit a variety of functions when expressed as secreted IgA. These mAbs coated

the *E. coli* surface and induced aggregation and addition of OmpC mAbs to an *in vivo* intestinal microbiome culture model altered *E. coli* gene expression and metabolism (52). Other murine mAbs to OmpC and OmpA have been discovered, but very little is known about what functions they might have (53-55). Polyclonal antibodies most likely to OmpA have been identified in humans who recovered from *E. coli* infection (22, 56, 57). Yet no mAbs from humans have been described and the field currently does not understand the significance of human OmpA humoral immunity. In summary, OMPs are important for bacterial pathogenesis and can serve as potent antigens through immunization, infection, and possibly commensal exposure. The functions of antibodies to OMPs are myriad and encompass antigen-specific functions such as inhibition of the insertion functions of BamA as well as antigen-agnostic functions such as antibody-dependent opsonophagocytosis or complement-mediated killing.

Capsular polysaccharides

One mechanism used by bacteria to protect themselves from extracellular insults is the production of mucoid, sugar-based encapsulation. Bacterial capsules, also known as the glycocalyx, are fragile structures covalently linked to the outer membrane or peptidoglycan and are composed of polysaccharide chains that link together forming a protective matrix (58). These capsular polysaccharides (CPS) are one of the main interfaces between a pathogen and its host's immune system. The interactions between CPS and antibodies are a balance. On one hand, some of the most successful vaccines use CPS as the primary immunogen, such as the vaccinations for *N. meningitidis*, *Haemophilus influenzae*, or *Streptococcus pneumoniae* (15). On the other hand, capsules can undergo phase variation, or variable induction, by some bacteria; and when induced

or constitutively present, CPS can obscure antibody epitopes upon OMPs (59-61). Furthermore, CPS is involved in biofilm formation and enhances bacterial clumping, which may physically exclude antibodies from access to antigens within a biofilm or bacterial cluster (62). Overall, antibodies against bacterial CPS will not behave uniformly against all organisms, and it is necessary to study the nuances of CPS-antibody interactions for each specific organism to understand the roles antibodies have in pathogenesis.

For some pathogens, anti-capsular antibody responses are important determinants of protection and prevention and many have been described (63). The first vaccine against a Gram-negative bacterium used *H. influenzae* CPS, but CPS vaccination did not become successful until the polysaccharides were conjugated to protein carriers. These protein carriers greatly enhance the ability of small CPS molecules to elicit protective antibody responses (14, 64, 65). Although protein carriers greatly enhance anti-capsular immunity, there is evidence that natural polysaccharides or purified polysaccharides can induce antibody-mediated immunity, possibly through natural killer T cell assistance or through naturally occurring conjugation of capsular polysaccharides to bacterial proteins (66). Several human or murine monoclonal antibodies to *H. influenzae* Type B (*Hi*B) CPS have been discovered and studied. Two human mAbs, one unnamed and the other 5M1H9, were separately shown to increase complement-mediated bacterial killing and to increase neutrophil-mediated killing (67, 68). Murine mAbs B10 and 12E7 also reacted to *Hi*B CPS and enhanced *in vitro* bacterial killing (69, 70). *N. meningitidis* is also highly susceptible to antibody-mediated complement fixation and killing, also called serum bactericidal activity. Antibodies to CPS are protective for *N. meningitidis* of groups A, C, W, and Y, though most monoclonal antibodies generated have primarily been tested for ability to diagnostically discriminate between capsular groups (71). Two human monoclonal antibodies,

8C7Br1 and 9B10, both IgM isotype, induce bactericidal activity and prophylactically protect mice from *N. meningitidis* disease (72, 73). CPS binding murine mAbs have also been identified and studied against *Klebsiella pneumoniae, A. baumannii,* and *Salmonella enterica*, but the roles antibodies play in naturally occurring infections with these organisms is understudied (10, 74-76). Likewise, many additional bacterial diseases may have significant protective contributions from CPS but have not been evaluated thoroughly.

The most successful vaccinations against bacterial diseases depend heavily upon antibody responses to capsular polysaccharides. The understanding of the contributions of anti-CPS antibodies to the pathogenesis and prevention of other bacterial diseases has greatly expanded in the past two to three decades. However, for some pathogens, data have emerged that suggest CPS may be a significant obstacle to antibacterial antibody functions and prophylactic or therapeutic efficacy. As discussed previously, antibodies to CPS can induce complement mediated killing and antibody dependent cellular phagocytosis by macrophages or neutrophils. The capsules of *K. pneumoniae or N. meningitidis* have been implicated in resistance to complement fixation, reducing the effects of anti-CPS antibodies (77, 78). Additionally, *K. pneumoniae* sometimes sloughs its capsule, leaving cell-free capsule. These sloughed globules of CPS serve as antibody sinks, since antibody binds to the cell-free CPS but there are no cells to fix complement to and kill (77). A similar phenomenon is used by *Vibrio cholerae*, an epidemic organism responsible for significant disease burdens in under-resourced areas. *V. cholerae* responds to antibody-mediated aggregation by increasing production of its CPS, resulting in significant levels of extracellular matrix formation. This polysaccharide matrix effectively shields the organism from further binding by antibodies, limiting the ability of antibody to bind and induce any form of bacterial killing (79). Within a single species of bacteria, there can be a

vast variety of CPS structures and thicknesses. Antibodies to bacterial proteins can be identified and are functional against some strains, but when tested against strains that produce different forms of CPS or produce greater amounts of CPS, the antibodies may be unable to bind their antigens. This CPS inhibition of binding is the case for *A. baumannii*, where antibodies that exhibit strong binding to the strain 19606 are unable to bind to an encapsulated strain AB307. It was then shown that an isogenic capsule negative mutant, AB307.30, was bound by mAb and killed by ADCP (11). Furthermore, in *A. baumannii*, it has been reported that a mAb to K2 CPS, 8E3, may enhance disease, increasing mortality, bacterial dissemination, and organ bacterial burdens in a murine pneumonia model (80). The authors speculate that this is due to the formation of immune complexes that inhibit bacterial clearance, but do not show data to support this claim. There data do suggest antibody enhances invasion of host epithelial and phagocytes, which may better explain the enhancement they observed. Antibody-dependent enhancement is an extremely concerning observation, but it is important to note that these findings were observed using 200 mg/kg of mAb, an extremely high dose. For comparison, the COVID-19 mAb cocktail of tixagevimab-cilgavimab (Evusheld or AZD7442) is dosed at 300 mg per individual, or approximately 4 mg/kg for the average adult human (81). Many of these possible complications have only so far been reported for specific organisms or under specific conditions. Significant work remains to be done to understand the roles that anti-capsular mAbs have in human disease.

Toxins

Toxins are molecules produced and often secreted by living organisms that are harmful to other organisms. By definition, toxins are antigenic and can generate protective antibody responses, a phenomenon that has been known, by phenotype, not mechanism, for over a century. The first targeted antimicrobials were antisera from animals, initially from guinea pigs but later typically from horses (82). Antisera offered some protection against diseases including tetanus, diphtheria, *S. pneumoniae* pneumonia, *N. meningitidis* meningitis, as well as exposure to some venoms (83, 84). Since that era, significant research has been done to understand the mechanism of toxin antisera, and the critical roles of antibodies quickly became apparent.

Numerous monoclonal antibodies to toxins have been produced and studied, yet most clinical uses of antitoxins still use polyclonal sera (12, 85). Dozens of mAbs to *E. coli* and *Shigella* sp. Shiga toxin have been described and several have been clinically evaluated with moderate success (86). Shiga toxin is produced by enterohemorrhagic *E. coli* and is responsible for causing severe gastrointestinal disease, sometimes leading to hemolytic uremic syndrome resulting in permanent kidney damage (87). An AB toxin, Shiga toxin is a complex of an enzymatically active A subunit and a B subunit responsible for entry and trafficking into targeted cells (88). Many described mAbs neutralize Shiga toxin killing of cells *in vitro* and some protect mice from lethal Shiga toxin exposure (89, 90). It is thought that most Shiga toxin mAbs neutralize toxin by inhibiting the ability of the toxin to interact with its effectors and by altering the intracellular distribution of toxin within host cells (91, 92). Two nanobodies, Nb113 and NbStx2e1, have been structurally characterized and found to bind to the B subunit of Shiga toxin. These B subunit epitopes block the receptor binding site for glycolipids responsible for

triggering endocytosis to bring the toxin into the eukaryotic cell (93, 94). Similar interactions have been found to be protective against *V. cholerae* cholera toxin, another AB toxin. MAbs to cholera toxin such as CTB IgA-1, -2, or -3, are predominantly functional when expressed as IgA, which is consistent with the gut specificity of *V. cholerae*. These mAbs were able to inhibit chloride secretion from *in vitro* cell monolayers, indicating that they may have activity against the mechanism responsible for the hallmark watery, chloride-rich diarrhea of cholera disease (95). Toxins secreted by Gram-negative bacteria that serve significant roles in bacterial diseases are important targets of antibodies. Shiga toxin antibodies reinforce the importance of understanding the structure to function correlations to elucidate antibody-dependent protective mechanisms. Research in these areas can shed light upon the often overlooked field of anti-toxin antibodies and provide new fodder for next-generation vaccines and targeted monoclonal therapies and preventatives.

Summary

Antibodies are just one component of the immune response to a pathogen; but they are a powerful multifunctional and multitargeted class of molecules. As conventional antimicrobial therapeutics lose efficacy in the face of pandemic antimicrobial resistance, antibodies can serve many roles. MAbs act as tools to enhance mechanistic understanding of bacterial pathogenesis, as guides to inform upon naturally immunogenic bacterial elements to advise vaccine design, and as intrinsically prophylactic and therapeutic molecules to supplement small molecule antibiotics. The targets of antibacterial antibodies are as diverse as the pathogens they bind. Outer membrane proteins are perhaps the most conventional of the antibody targets, as protein-antibody

interactions have been extensively assessed in the context of viral disease and cancer. Antibodies to capsular polysaccharides are a heralded success of vaccine science, vastly decreasing the burden of childhood illnesses in the past 75 years. As molecular biology techniques have advanced, so too has our ability to understand the mechanisms of established therapies, such as toxin antisera. Our ability to rationally interrogate bacterial toxins and secreted effectors to discover, understand, and take advantage of inhibitory or modulatory antibodies will continue to greatly enhance the field of antitoxin antibodies in the near future. The myriad antibodies discussed so far act through many different antibacterial mechanisms. Some, for example the anti-Shiga toxin mAbs and anti-BamA mAbs, possess intrinsic functions that inhibit the basic functions or pathogenesis of an organism. MAbs to *N. meningitis* capsular polysaccharides act by facilitating the fixation of complement, activating a cascade resulting in the formation of membrane attack complexes that lyse the attacking organism. Antibodies also can enhance the interactions of host phagocytic macrophages and neutrophils with bacterial pathogens through opsonophagocytosis, as shown to be important for the antibacterial functions of mAbs to *K. pneumoniae* CPS and *A. baumannii* CPS or outer membrane proteins. These unique targets and individual functions of human antibodies are interrogated in the remaining chapters of this dissertation for three representative organisms, *E. coli*, *K. pneumoniae*, and *B. pertussis*.

CHAPTER II

II. Human monoclonal antibodies to *Escherichia coli* outer membrane protein A porin domain cause aggregation but do not alter *in vivo* bacterial burdens in a murine sepsis model.[1]

Introduction

Escherichia coli is a versatile Gram-negative bacterial organism that can be a human enteric commensal, a widely used laboratory reagent, and in some settings a dangerous human pathogen. The latter of these is especially concerning because pathogenic *E. coli* frequently exhibit antimicrobial resistance (AMR), even to the most advanced antibiotics in clinical use. These carbapenem-resistant or extended spectrum beta lactamase (ESBL)-producing *E. coli* cause over 200,000 infections and more than 10,000 deaths annually in the United States (96). These infections lead to extensive morbidity, mortality, and more than 1 billion USD of excess healthcare costs annually (97). Treating *E. coli* infections and stopping the spread of AMR bacteria requires a multi-pronged approach involving public health containment, clinical care improvement, and novel therapeutic strategies.

The utility of monoclonal antibodies (mAbs) as an antibacterial strategy has only recently begun to be systematically evaluated, yet numerous groups have espoused the promise of this class of therapeutics (98-102). Although interest in antibacterial mAbs has recently increased,

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[1] Adapted from Fowler BD, Kose N, Reidy JX, Handal LS, Skaar EP, Crowe JE Jr. Human monoclonal antibodies to *Escherichia coli* outer membrane orotein A porin domain cause aggregation but do not alter *in vivo* bacterial burdens in a murine sepsis model. *Infect Immun.* 2022 May 18:e0017622. doi: 10.1128/iai.00176-22. Epub ahead of print. PMID: 35583347. It is republished here with permission from the publisher and my co-authors.

several mAbs have been tested in clinical trials with varying degrees of success, including MEDI4983 (103), 514G3 (104), and AR301 (105) against *Staphylococcus aureus* and MEDI3902 (106, 107) against *Pseudomonas aeruginosa*. MAbs to *E. coli* have not been extensively evaluated, although previous work identified an outer membrane porin, likely outer membrane protein A (OmpA), as a dominant antibody target following *E. coli* infection (21, 22, 56, 57, 108). More recently, a murine mAb, 49.4-15, was identified that is specific for *E. coli* OmpA (55). Multiple studies of OmpA protein variants as vaccine candidates have also demonstrated the development of OmpA-specific humoral immunity in immunized animals, although the usefulness of OmpA as a vaccine may be limited by the high level of conservation of *ompA* across commensal bacteria (109-112). It is evident that antibodies to *E. coli* are induced in humans after infection, and the murine antibody studies suggest these human antibodies may impact the virulence or pathogenesis of *E. coli* infection by targeting OmpA.

In this study, I identified and evaluated naturally occurring human mAbs that specifically bind to OmpA. I investigated the binding of these antibodies to protein and intact bacteria under various growth conditions and determined functional properties of mAb-OmpA binding upon *E. coli* pathogenesis. I assessed the characteristics of this mAb against K12 MG1655 and the well-characterized urinary tract isolate UTI89. Urinary tract infections represent a large burden of disease caused by *E. coli*, and OmpA has previously been reported as important for *E. coli* uropathogenesis (113, 114). This work provides a foundation for experiments to obtain a better understanding of the interaction of *E. coli* outer membrane proteins and the human immune system.

Methods

Human subjects. Peripheral venous blood was collected at Vanderbilt University Medical Center (VUMC) with informed written consent from healthy individuals with exposure to Gram-negative pathogens through laboratory work, but no known history of infection with *E. coli*. Heparinized peripheral blood was processed to isolate peripheral blood mononuclear cells (PMBCs) using SepMate-50 tubes, and PBMCs were cryopreserved and stored in the vapor phase of liquid nitrogen until used. The studies were approved by the Vanderbilt University Medical Center (VUMC) Institutional Review Board.

Generation of human mAbs. Human PBMCs were thawed and transformed with Epstein-Barr virus and plated in 384-well plates in the presence of CpG DNA, Chk2 inhibitor, and cyclosporine as previously described (115), expanded into 96-well plates containing a feeder layer of irradiated human PBMCs from an unrelated donor, then supernatants were screened by ELISA for binding to *E. coli* outer membrane proteins. Cells from reactive wells were electrofused with HMMA2.5 myeloma cells to generate oligoclonal hybridoma cell lines. These cell lines were cultured, stained with propidium iodide and single-cell sorted using fluorescence-activated cell sorting with an SH800S cytometer. Monoclonal hybridomas were cultured and mAb IgG protein purified from cell supernatants with MabSelect or Protein G affinity columns using fast protein liquid chromatography (FPLC) on an ÄKTA pure chromatography system. RNA was extracted from monoclonal hybridoma cells, and the antibody transcript sequenced using 5′ rapid amplification of cDNA ends and cloned into a monocistronic expression vector by Twist Biosciences. Antibodies were expressed by transfection of these constructs into ExpiCHO

cells and purified from supernatants using affinity chromatography as above for hybridoma mAbs. All data presented were generated using recombinant mAb proteins.

Bacterial strains. *E. coli* strains BL21(DE3), BL21(DE3) *ΔA*, and BL21(DE3) *ΔABCF* were gifts from Dr. Jack Leo (Addgene # 102256 and 102270) (116). Strain UTI89 was a gift from Dr. Maria Hadjifrangiskou, MicroVU, and the VUMC Center for Personalized Microbiology. All wild-type bacterial strains were grown for 14 to 16 hours on Miller LB agar (LBA) plates or in Miller LB Broth at 37°C. Mutant bacterial strains were cultured for 24 hours at 30°C in the same medium. Bacterial lysates were prepared using bacterial protein extraction reagent (BPER) with DNase and lysozyme.

***E. coli* outer membrane protein isolation.** Outer membranes from *E. coli* were purified using previously described techniques (36, 117). Briefly, 1 L cell pellets were lysed in buffer containing lysozyme and EDTA followed by buffer with magnesium, RNase/DNase, and EDTA-free protease inhibitor and homogenized using a Microfluidics LM20. Unlysed cells were removed by centrifugation followed by ultracentrifugation at 100,000 x *g* to pellet inner and outer membranes. Inner membranes were solubilized with 1% N-lauroylsarcosine and 10% glycerol, and afterwards outer membranes were pelleted at 100,000 x *g* before being solubilized in β-octyl glucoside or Fos-choline-12. Membrane separation was verified with SDS-PAGE.

Enzyme linked immunosorbent assay (ELISA). ELISA plates were coated with 1 to 10 µg/mL *E. coli* outer membrane proteins or crude lysate in carbonate buffer, then blocked with non-fat milk. Antigens were probed with cell supernatants or purified mAb followed by HRP-conjugated

secondary antibodies and developed with 3,3′,5,5′-tetramethylbenzidine and read at 450 nm absorbance on a Biotek Synergy H1 or Powerwave HT microplate reader.

Multiple sequence alignment. OmpA amino acid sequences were extracted from or translated from deposited genomes (BL21: ACT29660.1; K12: NP_415477.1; UTI89: ABE06507.1). Sequences were aligned using Clustal Omega (118). Multiple sequence alignment graphics were developed using ESPript 3.0 (119) and PDB 1BXW for the N-terminal porin domain (120) and PDB 2MQE for the C-terminal periplasmic domain (121).

Immunofluorescence. Bacterial cultures were grown overnight or sub-cultured and then rinsed with PBS and normalized to an $OD_{600\ nm}$ of 1.0. Cells were stained with Hoechst 33342 at 5 µL/mL for 2 minutes and excess was removed with a PBS wash. Bacteria then were incubated with mAb for 30 minutes, rinsed, then incubated with mouse anti-human IgG Alexa Fluor 647 for 30 minutes. Stained bacteria were dried overnight on a #1.5 cover glass and then fixed to glass slides with Prolong Gold Antifade and imaged on a Nikon Structured Illumination microscope with an Andor iXon Ultra DU-897 EMCCD monochrome camera and reconstructed in NIS-Elements.

Reverse transcriptase quantitative PCR. Bacterial RNA was extracted in triplicate from exponential or stationary phase *E. coli* cultured in LB using lysing matrix B tubes and RNeasy kits . Carryover DNA was removed by DNase digestion, and 1 µg of RNA was used to synthesize cDNA . Synthesized cDNA was diluted in nuclease-free water, and qPCR was performed on a BioRad CFX96 instrument using SYBR green chemistry and primers as shown

in **Table 1**. Target gene expression was normalized to *16S* housekeeping gene expression by ΔΔCt.

Table 1: RT qPCR primers.

Primer Target	Primer Sequence	Source
ompA	CTGGGTGGTATGGTATG	This publication
ompA	TAGCGATTTCAGGAGTG	This publication
16s	TGATCATGGCTCAGATT	This publication
16s	CAGTTTCCCAGACATTAC	This publication

Western blot. Overnight or sub-cultured bacteria were rinsed with PBS and pellets were lysed with BPER + DNase + lysozyme. Protein content was measured using the bicinchoninic acid assay and 2 µg of lysate were prepared with NuPAGE Sample Buffer and reducing agent then heated at 70°C for 10 minutes before loading on 4-12% bis-tris protein gels. SDS-PAGE run in MOPS buffer for 60 minutes at constant 200 V. Gels were transferred to polyvinylidene fluoride (PVDF) then stained for total protein with Revert 700 stain and imaged with a LI-COR Odyssey CLx. Membranes were then blocked with Intercept blocker, probed with rECOL-4 IgG1 followed by IRDye800CW goat anti-human IgG, and then membranes imaged again.

Capsule staining. A 10 to 20 µL volume of bacterial culture was added to 20 µL of 1% Congo red aqueous solution on a glass slide and allowed to air-dry. The slide was then flooded with Maneval solution (acid fuchsin, ferric chloride, acetic acid, and phenol) for 5 minutes and then

gently rinsed with distilled water. Slides were air-dried and then imaged with an Olympus brightfield microscope and camera. Four randomly selected regions of each slide were photographed and the capsule thicknesses of eight individual bacteria were measured for each replicate and condition.

Aggregation assays. Overnight or sub-cultured bacteria were rinsed with and resuspended in PBS to a $OD_{600\ nm}$ of 1.0, and then 50 µL was transferred to U-bottom 96-well plates. MAb or Fab was added to each well and gently mixed before the plates were allowed to sit at room temperature for 16 to 24 hours. Plates were then imaged on an ImmunoSpot plate reader. Alternatively, 15 µL of rinsed bacteria were applied to glass slides, treated with mAb for 15 to 30 minutes, protected with cover glass, and imaged with an Olympus brightfield microscope and camera. Eight randomly selected regions of each slide were photographed, and aggregated clumps of bacteria were counted.

Adhesion assays. RAW 264.7 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% heat inactivated fetal bovine serum and 10 mM sodium pyruvate at low passages (<15 passages). Cells were cultured in 24-well plates until confluent. Overnight or sub-cultured bacteria were rinsed with PBS and resuspended to $OD_{600\ nm}$ of ~0.150 (~1.0×10^8 CFU/mL) and opsonized with mAb for 60 minutes. Cell culture medium was refreshed with medium as above except with ultra-low IgG heat inactivated fetal bovine serum and 50 µL of opsonized bacteria were added and incubated for 15 min at either 37°C to measure adhesion and phagocytosis or 4°C to measure adhesion but prevent phagocytosis. Cell layers were rinsed four times with PBS to remove non-adhered bacteria and then lysed with 0.25% sodium deoxycholate

in PBS supplemented with Benzonase nuclease. Cell lysates were serially diluted and plated on LB agar to enable counting of colony forming units.

Mouse experiments. All animal studies were approved by and performed according to the guidelines of the VUMC Institutional Animal Care and Use Committee. A murine model of septic *E. coli* disease was developed. After a 16- to 18-hour overnight culture, *E. coli* UTI89 was sub-cultured 1:100 and grown to mid-exponential phase for 3 hours at 37°C, 225 RPM before being pelleted and rinsed with PBS. Bacteria were normalized in PBS using $OD_{600\ nm}$ to approximately 5.0 x 10^7 colony forming units (CFU)/mL and stored on ice until inoculation. Female, 6- to 8-week-old, BALB/cJ mice were weighed and filter sterilized mAb in PBS was administered intraperitoneally 90 minutes prior to inoculation at 10 mg/kg or 25 mg/kg. After treatment, mice were anesthetized by intraperitoneal injection of avertin (~400 mg/kg) and then inoculated retro-orbitally with ~5.0 x 10^6 CFU of *E. coli* UTI89. Mice were monitored for 24 hours then euthanized by CO_2 inhalation. Blood was collected by cardiac puncture and organs were collected by dissection in a sterile environment. Organs were homogenized in sterile PBS using a bead beater then homogenates were serially diluted, and spot plated in technical duplicate on LBA plates for enumeration of CFUs.

Results

Isolation of human mAbs that bind to OmpA.

Peripheral blood samples were collected after written informed consent from healthy laboratory workers working in microbiology research laboratories. Peripheral blood mononuclear cells

(PBMCs) were isolated by gradient fractionation and used to make human B cell hybridoma cell lines secreting naturally occurring mAbs that bound to *E. coli* outer membrane fractions by ELISA. Most mAbs were found to be of the IgG1 isotype, and the antibody variable gene sequences were obtained and used to produce recombinant IgG1 proteins. If the native isotype was not IgG1, we recombinantly produced and tested both the native isotype IgG as secreted by the hybridoma cell line and the IgG that was isotype-switched to IgG1. We also produced recombinant LALA-PG Fc region variant IgG proteins (which do not bind to mouse nor human FcγR (122)), Fab variants (which only contain a single binding site instead of the paired binding arms of IgG mAbs), and rSTAU-228 IgG1 (a negative control antibody specific for *Staphylococcus aureus* IsdA (123)). To determine the antigen specificity of these mAbs, they were assessed for binding to bacterial lysates of several strains of *E. coli*, including strains in

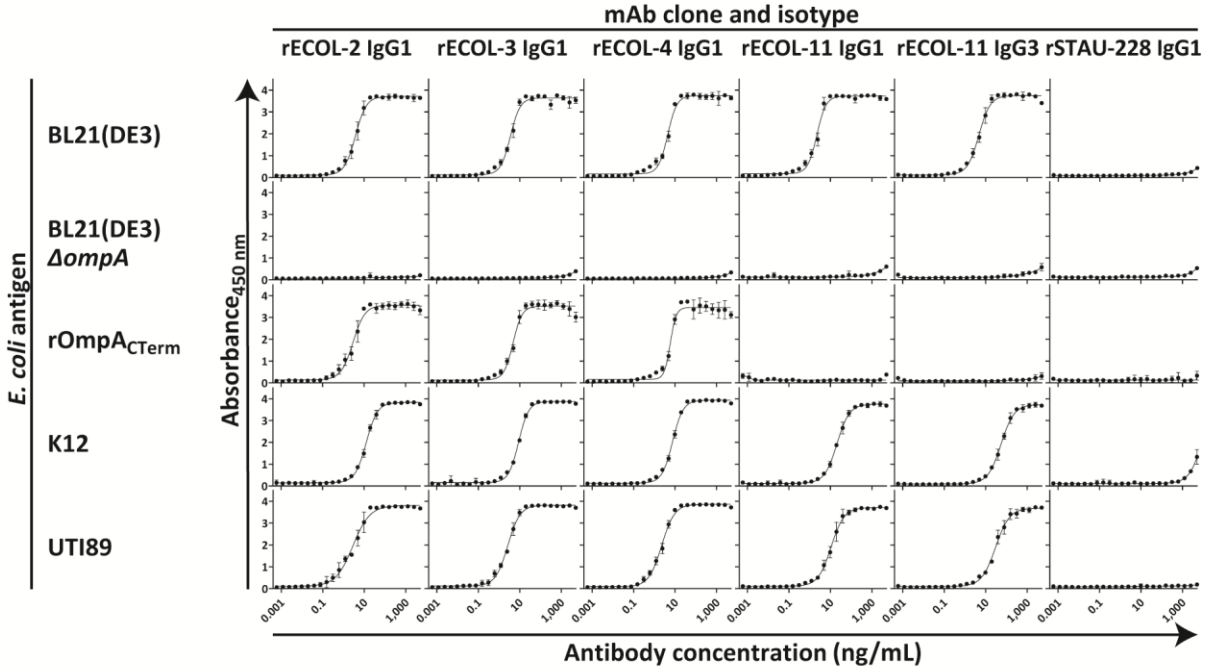

Figure 2: rECOL mAbs bind to the conserved outer membrane protein A (OmpA). Enzyme-linked immunosorbent assays (ELISA) of recombinant monoclonal antibody binding to *E. coli* lysate (BL21(DE3), K12, & UTI89) or recombinant protein (rOmpA$_{Cterm}$). Points represent error bars represent standard deviation, curves indicate four-parameter variable slope logistic analysis, data are representative of three independent replicates performed in quadruplicate.

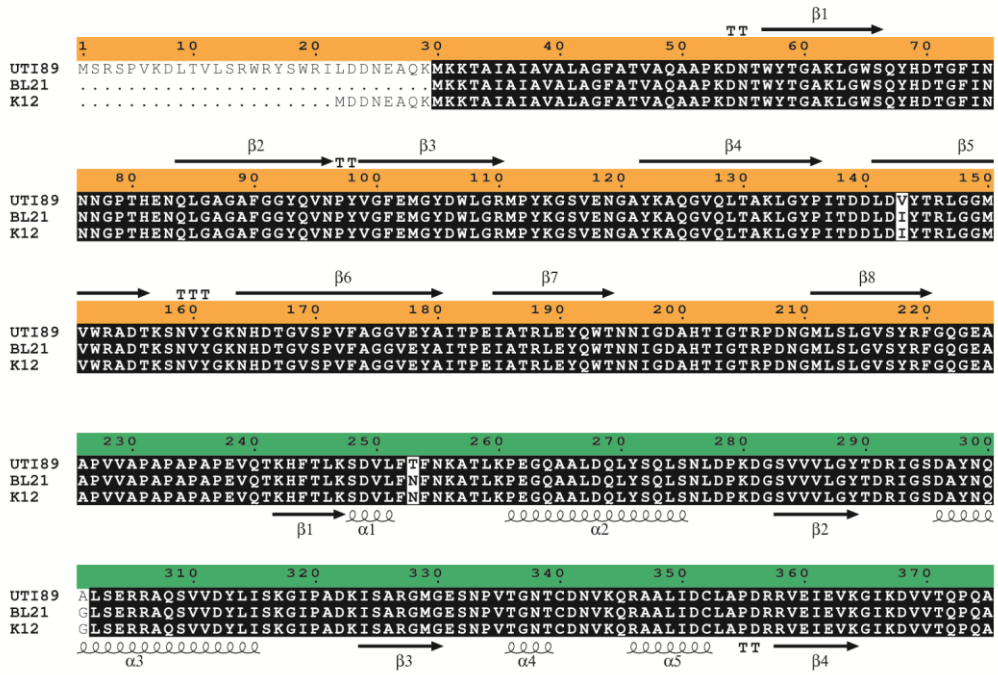

Figure 3: OmpA is highly conserved across *E. coli* isolates. Clustal Omega alignment of OmpA from the strains tested by ELISA, an engineered *E. coli* (BL21), a laboratory or commensal strain (K12 MG1655), and a clinical uropathogenic isolate (UTI89), shows 99% identity (black = conserved, white = variable). Orange indicates the N-terminal porin domain with secondary structure above mapped from PDB 1BXW using ESPript 3; green indicates the C-terminal periplasmic domain with secondary structure below mapped from PDB 2MQE.

which the genes encoding the most common outer membrane proteins (*ompA*, *ompC*, *ompF*, and *lamB*) were inactivated (116). Four mAbs bound to BL21(DE3) WT but not to BL21(DE3) *ΔompA*, indicating specificity for the gene product OmpA (**Figure 2**). Furthermore, when I tested binding to a recombinant partial OmpA protein that includes only the C-terminal periplasmic domain, three mAbs (rECOL-2, rECOL-3, and rECOL-4) bound, but one (rECOL-11) did not. This result suggests that three of four mAbs are specific for the C-terminal periplasmic domain of OmpA ($OmpA_{CTerm}$) and that one of four mAbs is specific for the N-terminal porin domain ($OmpA_{NTerm}$). All mAbs retain binding to lysates from both the laboratory strain *E. coli* K12 and the clinical isolate *E. coli* UTI89 (**Figure 2**). This degree of conserved binding is not surprising, as OmpA is generally highly conserved in sequence. Most *E. coli* strains exhibit >90% OmpA identity, and the three strains I tested only differ at three amino acid positions (**Figure 3**). Using

a small panel of *E. coli* clinical isolates we identified specific *ompA* genes that produced protein to which rECOL-11 did not bind. I cloned the wild-type UTI89 *ompA* into pET15b and then used site-directed mutagenesis to introduce the N-terminal domain single amino acid changes observed in clinical isolates. When N46P, V131F, or Y132D are introduced into UTI89 *ompA* and the resulting OmpA expressed in BL21(DE3) *ΔompA*, rECOL-4 binds identically, indicating the protein is produced and folded (**Figure 4**). However, rECOL-11 significantly loses binding when those variants are introduced, confirming rECOL-11 is specific for the N-terminal domain of OmpA.

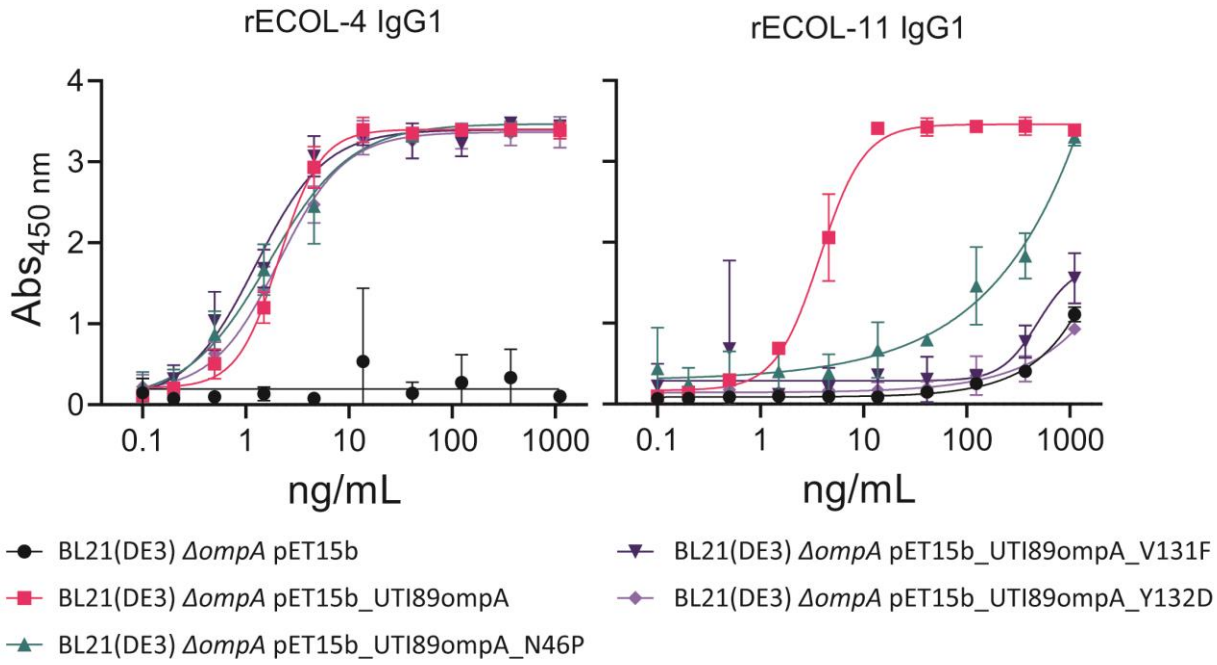

Figure 4: Clinical $OmpA_{Nterm}$ variants eliminate ECOL-11 binding. ELISA to *E. coli* expressing OmpA with specific variations from known clinical isolates. Wild-type UTI89 *ompA* was cloned into pET15b and then individual variations were introduced. All proteins were expressed in BL21(DE3) *ΔompA* and binding assessed by ELISA to bacterial lysates normalized to protein concentration.

$OmpA_{NTerm}$ mAb ECOL-11 binds whole *E. coli.*

Having demonstrated binding of four mAbs to OmpA in bacterial lysate, I next sought to examine the capacity of these mAbs to bind intact whole *E. coli*. Using bacteria cultured to mid-exponential phase or late stationary phase, I performed immunofluorescence imaging using human mAbs. $OmpA_{CTerm}$ mAbs such as rECOL-4 did not stain *E. coli* of any growth phase. However, the $OmpA_{NTerm}$ mAb rECOL-11 stained exponential phase K12 *E. coli* (**Figure 5A**) and strongly stained stationary phase K12 *E. coli* (**Figure 5B**). Binding to a uropathogenic isolate UTI89 was also evident, but the level of binding was inconsistent (**Figure 5C**). To evaluate potential causes of differential binding, I assessed expression of OmpA across multiple time points. Messenger RNA transcripts for OmpA were assessed by RT qPCR and found to be more abundant in exponential phase *E. coli* than in stationary phase *E. coli*, consistent with previously reported differential *ompA* mRNA stability during growth (**Figure 6**) (124). OmpA protein levels were assessed at multiple time points during growth by immunoblot. While statistically significant differences were noted between stationary phase *E. coli* and exponential phase *E. coli*, these differences were small and OmpA levels in both K12 (**Figure 7A)** and UTI89 (**Figure 7B**) *E. coli* were relatively consistent across all time points. I observed two bands stained by rECOL-4 IgG1, based upon relative molecular weight and previously reported studies of OmpA, I predict the 37 kDa band is consistent with full-length OmpA and the 25 kDa band is consistent with a partially denatured protein or isolated C-terminal domain. Neither *ompA*

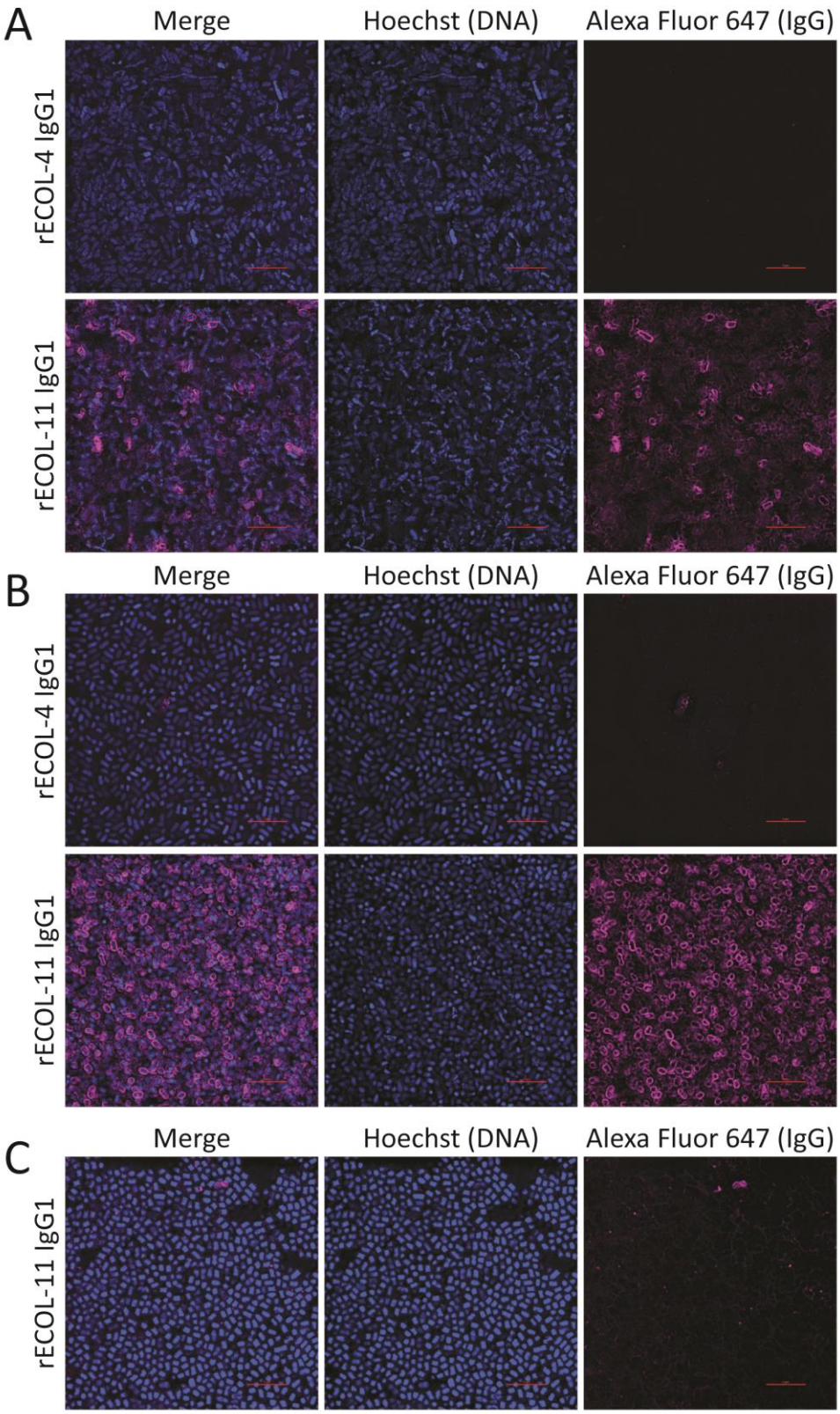

Figure 5: Immunofluorescence staining of K12 or UTI89 *E. coli*. Staining of (A) exponential or (B) stationary phase K12 E. coli or (C) stationary phase UTI89 E. coli with Hoechst (blue), recombinant mAb and mouse anti-human IgG Alexa Fluor 647 (magenta). Image locations were randomly selected from slides, gathered with identical image and laser settings using a 100× oil objective, and processed with identical look-up tables. Red scale bars are 5 µm.

mRNA nor OmpA protein levels explain the minimal binding of ECOL-11 to intact UTI89 by immunofluorescence. Previous literature has reported that polysaccharide capsules may impair the binding of antibodies to bacteria such as *A. baumannii* (11), and the K1 capsule of UTI89 has been found to be an important virulence factor (125, 126). I performed Maneval's capsule staining of K12 and UTI89 *E. coli* to determine whether greater capsule production might explain the decreased binding of ECOL-11 to UTI89. Our data show that UTI89 exhibits a thicker capsular polysaccharide layer than K12 *E. coli* (**Figure 8**), and I hypothesize that this finding explains the differential ECOL-11 binding observed by immunofluorescence.

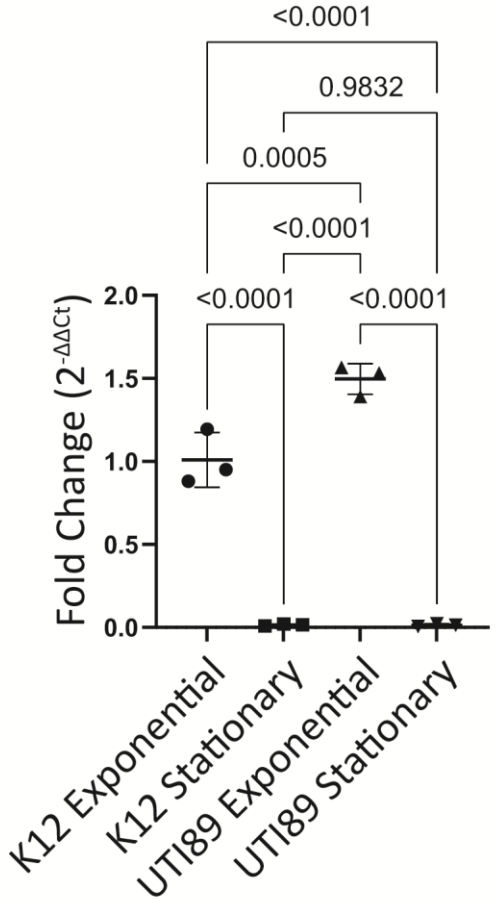

Figure 6: OmpA transcript levels. Transcript level expression of *ompA* by RT qPCR normalized to *16s* housekeeping gene expression relative to exponential phase K12 *E. coli*. RT qPCR was analyzed by one-way ANOVA. Error bars show standard deviation.

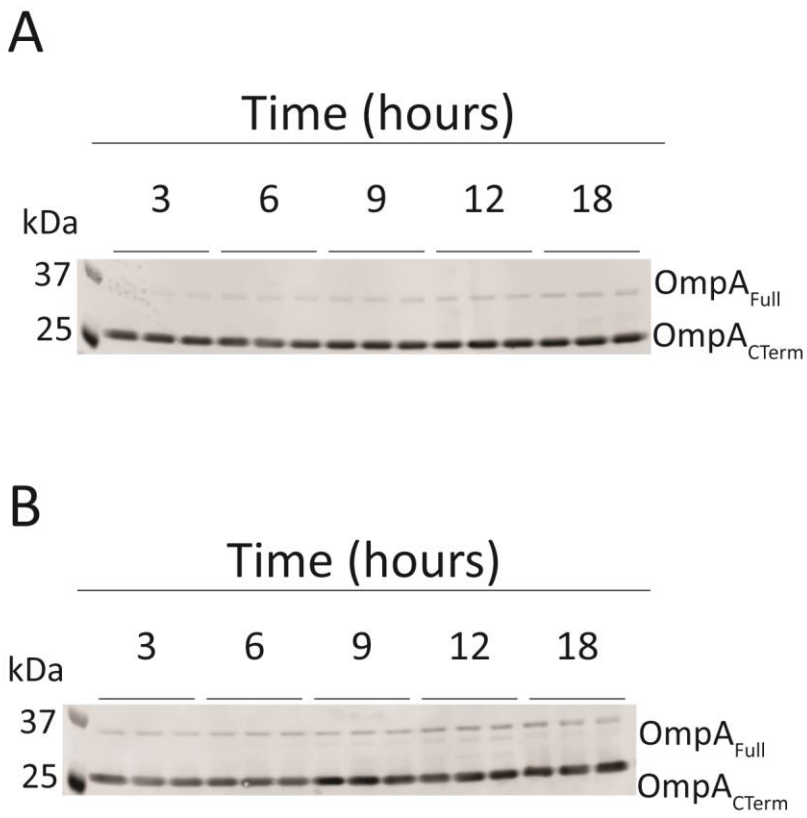

Figure 7: Western blot of OmpA. Protein level expression of OmpA by immunoblot to (**A**) K12 or (**B**) UTI89 *E. coli*. Two bands are observed; based upon molecular weight they are predicted to be two forms of OmpA. Data are representative of at least three independent experiments.

Bivalent IgG aggregates stationary phase *E. coli.*

Murine mAbs to *E. coli* have been demonstrated previously to induce aggregation or clumping of *E. coli* (52). I incubated IgG1 or fragment antigen binding (Fab) forms of mAbs with exponential or stationary phase K12 or UTI89 *E. coli* and imaged bacterial pellet morphology after allowing the bacteria to settle. Aggregation, or crosslinking, of bacteria prevents the cells from settling. The images shown in **Figure 9** reveal that rECOL-11 IgG1 causes stationary phase K12 *E. coli* to aggregate, but the rECOL-11 Fab does not, indicating that bivalency is essential for aggregation to occur. rECOL-11 IgG1 does not cause aggregation of UTI89 *E. coli*, likely due to

decreased mAb binding to this strain as described in **Figure 5**. Furthermore, when bacteria are imaged immediately after addition of antibody, rECOL-11 IgG1 causes the formation of visible clumps that are absent in bacteria treated with rECOL-4 IgG1 or other $OmpA_{CTerm}$-specific mAbs (**Figure 10**). This observation shows the activity depends upon accessibility of the antigen on the bacterial surface and could be used as an assay to quickly assess antibodies for binding to clinical isolates in an antigen-independent screen.

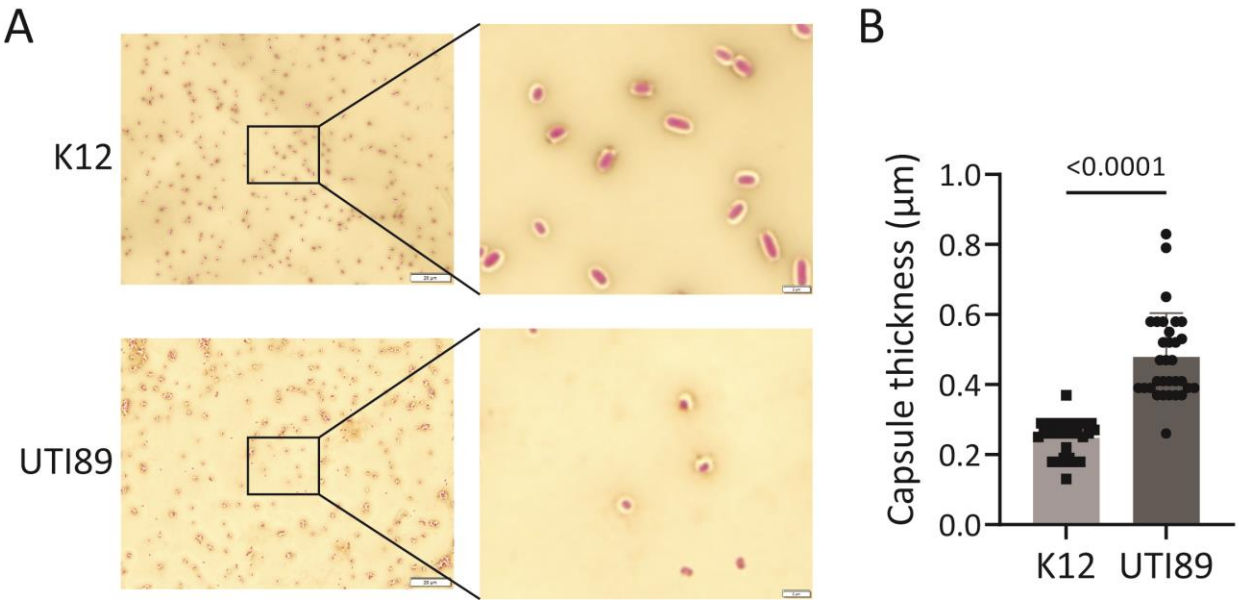

Figure 8: UTI89 produces more capsule than K12 *E. coli*. (A) Maneval's capsule stain of K12 or UTI89 *E. coli*. Background is counter stained with Congo red and cells are stained with Maneval's solution (acid fuchsin). Images taken with 100× objective, primary image scale bar is 20 µm, inset scale bar is 2 µm. (**B**) The zone of stain exclusion by capsule was measured from eight cells in three independent replicates. Data were pooled, each data point represents once cell, data were analyzed by Mann-Whitney U test, and error bars show standard deviation.

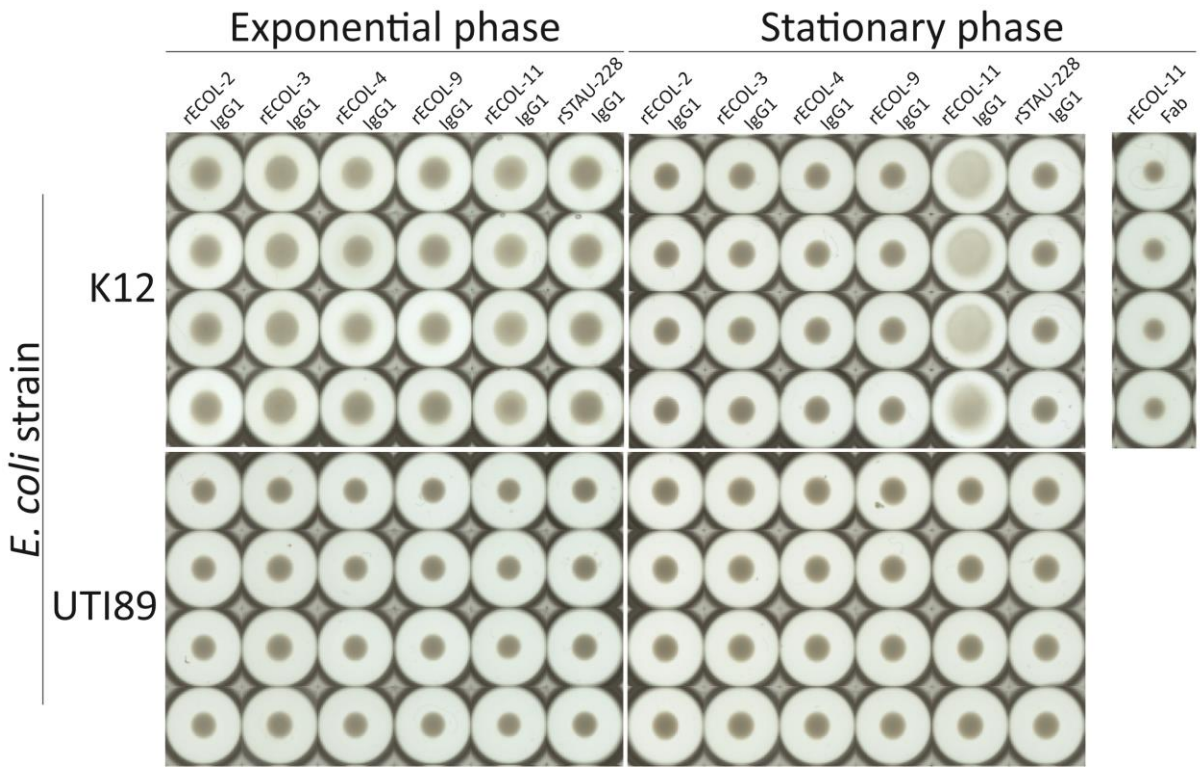

Figure 9: Aggregation of *E. coli* by mAb treatment. Exponential or stationary phase *E. coli* were mixed with mAb and allowed to aggregate in 96-well plates. Aggregation was imaged on a CTL Immunospot device to visualize bacterial pellets. Aggregation of bacteria prevents formation of a denser pellet by crosslinking between organisms. Images were performed in quadruplicate (shown) and are representative of two independent experiments.

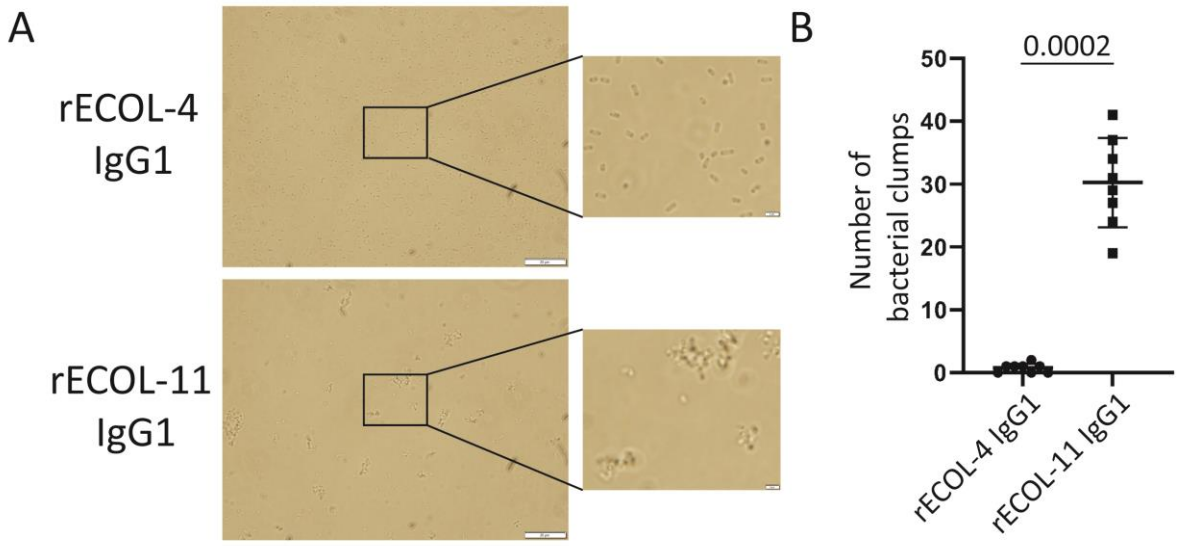

Figure 10: Clumping of *E. coli* by mAb treatment. (**A**) Aggregation of stationary phase K12 *E. coli* was imaged by brightfield microscopy and clumps are visible when treated with rECOL-11 IgG1. Images were taken with 100× objective, primary image scale bar is 20 µm, inset scale bar is 2 µm. (**B**) Number of clumps of five or more bacteria in direct contact per 100× objective field were analyzed by Mann-Whitney U test (n=8 random images per condition) corrected for multiple comparisons. Data are representative of two independent experiments and error bars show standard deviation.

MAb treatment of *E. coli* alters macrophage phagocytosis *in vitro.*

Aggregation has been associated with increased opsonophagocytosis (127). I used a temperature-dependent model with RAW264.7 macrophage-like cells to test opsonophagocytosis *in vitro*. Opsonized K12 *E. coli* were incubated with cells at 4°C to allow adhesion but prevent phagocytosis or at 37°C to allow adhesion and phagocytosis. Cells were then rinsed, lysed, and plated to count adhered or phagocytosed bacteria. Opsonization of *E. coli* with rECOL-11 expressed in the format of an IgG1, IgG3, IgG1-LALA-PG Fc variant, or Fab resulted in decreased adhesion to and phagocytosis by RAW 264.7 cells (**Figure 11**). Interestingly, this decrease persists despite eliminating Fcγ receptor (FcγR) interactions with a LALA-PG variant IgG and persists despite eliminating aggregatory activity with a Fab. To explain this phenotype,

I propose that under the tested conditions, OmpA is an important adhesion factor and rECOL-11 binding inhibits this adhesion. This finding is consistent with prior work indicating that OmpA is important for adhesion or entry to eukaryotic cells (55, 113, 128). This interaction complicates *in vitro* assessment of opsonophagocytosis.

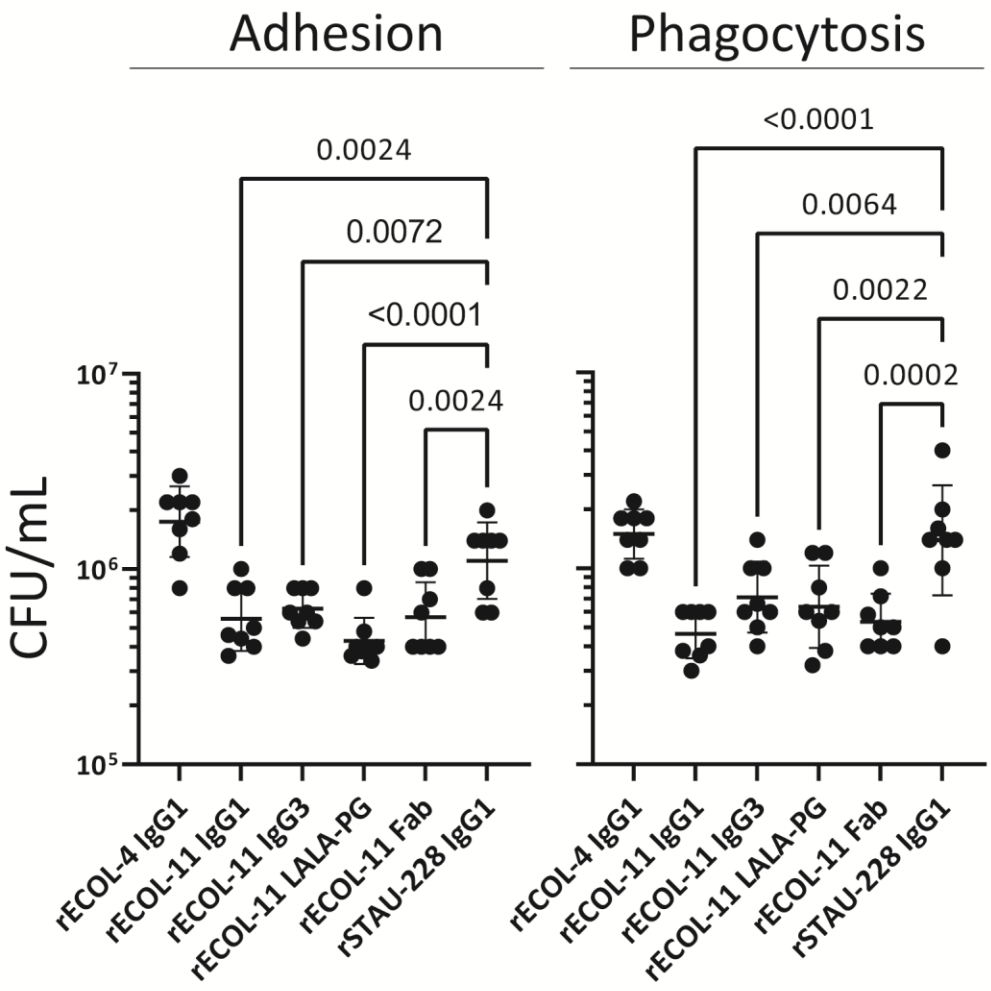

Figure 11: rECOL-11 alters adhesion and phagocytosis. rECOL-11 opsonization alters K12 *E. coli* adhesion to and phagocytosis by RAW 264.7 macrophage-like cells. Adhesion is the bacterial count after co-incubating opsonized bacteria and RAW 264.7 cells at 4°C and phagocytosis is the bacterial count after co-incubating at 37°C. Data are representative of three independent experiments and were analyzed in comparison to rSTAU-228 IgG by one-way ANOVA and comparisons where $p<0.01$ are shown. Error bars show standard deviation.

MAb treatment does not reduce bacterial burdens *in vivo.*

To better understand the interactions of OmpA mAbs, *E. coli*, and the mammalian immune system, I adapted a systemic infection model of *E. coli* in mice. Mice were pre-treated with mAb, inoculated with UTI89 *E. coli*, and then organ bacterial burdens were enumerated at 24 hours post-inoculation. If disease is allowed to progress, mice reach terminal endpoints due to septic shock at around 36 hours post-inoculation. When pre-treated with 10 mg/kg of mAb, organ bacterial burdens at 24 hours post-inoculation did not differ significantly between treatments using any of the OmpA-specific mAbs compared to a control antibody (the similarly prepared human IgG rSTAU-228, directed to a *Staphylococcus aureus* protein (123)) (**Figure 12A**). Increasing the dose of mAb to 25 mg/kg also did not have an impact upon bacterial burdens in this stringent model of *E. coli* disease (**Figure 12B**). Thus, we conclude that despite altering phagocytosis, mAb treatment is not sufficient to alter acutely lethal systemic *E. coli* disease in mice.

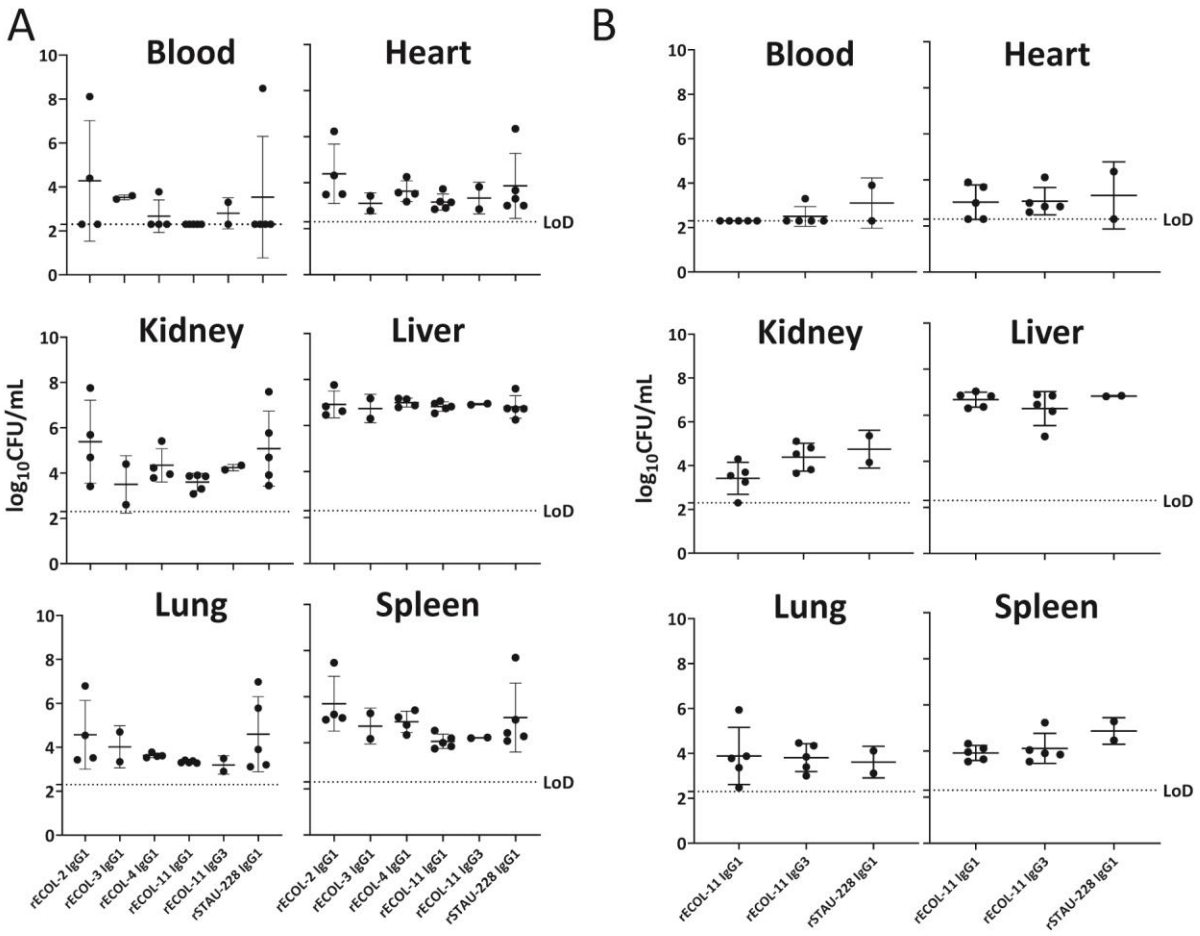

Figure 12: *In vivo* assessment of mAb prophylaxis. Six- to 8-week-old female BALB/cJ mice were pre-treated with (**A**) 10 mg/kg or (**B**) 25 mg/kg of mAb then inoculate with ~6.5 x 10^6 CFU of UTI89 *E. coli* and monitored for 24 hours before euthanasia and enumeration of organ bacterial burdens. Limit of detection (LoD) was 200 CFU/mL. All groups began with 5 mice, mice that did not recover from anesthesia were excluded and account for any groups with <5 data points. Data were compared to control mAb (rSTAU-228 IgG1) by Kruskal-Wallis testing with Dunn's correction for multiple comparisons. No conditions exhibited statistically significant differences. Error bars show standard deviation.

Discussion

E. coli, particularly MDR strains, are frequent human pathogens that can be challenging to prevent or treat. While a variety of factors contribute to these difficulties, one reason is the lack of development of new antibacterial strategies, and another reason is the minimal progress towards identifying promising vaccine candidates. One possible antigen that might serve as a

target of protective immunity is OmpA, a multifunctional virulence factor found almost universally in pathogenic *E. coli* strains and exhibiting remarkable conservation across widely divergent strains. OmpA is composed of an N-terminal porin domain that resides within the bacterial outer membrane joined by a trypsin-digestible region to a C-terminal domain that is found in the periplasmic space (120, 121, 129). Although OmpA has previously been studied as a vaccine candidate and found to elicit some degree of immunity, little has been done to study the functions and mechanisms of OmpA-specific immunity in humans. In this study, we sought to isolate and study naturally occurring antibodies obtained from healthy human donors and identified four OmpA-targeting mAbs. Most interestingly, one of these mAbs, ECOL-11, was specific for the N-terminal porin domain of OmpA, a region of the protein that is essential for a variety of OmpA-dependent effector functions (128, 130, 131). Manipulating the interactions of OmpA with host immune effectors provides insight into host-pathogen relationships and may prove to be a potent strategy to combat *E. coli* pathogenesis.

The utility of this strategy has already been demonstrated by using cyclic peptides to inhibit OmpA (132). We took a similar approach to assess the ability of naturally occurring mAbs binding to OmpA to alter *E. coli* biology. It is important to note that the mAbs we evaluated came from healthy laboratory workers with no specific history of *E. coli* infection, and yet we were still able to isolate OmpA-specific, somatically-mutated, class-switched antibodies. This finding may be due to the ubiquity of *E. coli* as a commensal organism in the intestine or could be a result of prior infection with *E. coli* that was not reported by the donors upon sample collection. We assessed the impact of these mAbs upon a subset of antigen-agnostic and OmpA-specific possible effector functions. Aggregation, which we observed with rECOL-11 treatment of *E. coli*, is an antigen-agnostic mAb function that has sporadically been reported after exposing

bacteria to antibody (52). Aggregation has varying effects upon interactions between bacteria and host phagocytes. It may impede phagocytosis due to large aggregate size or irregular shape, or it may enhance phagocytosis due to increased complement fixation (133, 134). Coupling this complex effect with the known role of OmpA as an adhesin and the interactions of mAb with Fc receptors suggests that assessing phagocytosis *in vitro* is challenging. We observed decreased adhesion to and decreased phagocytosis of rECOL-11-treated *E. coli* by RAW 264.7 macrophage-like cells. This phenotype persists with a LALA-PG Fc variant IgG, suggesting it is independent of FcγR interactions, and with Fab, indicating it is not a result of aggregation as the Fab does not induce aggregate formation. Although we have not directly shown that ECOL-11 blocks the adhesin activity of OmpA, by eliminating other possible causes, our data support this model as the best explanation of this observed phenotype.

Regardless of underlying cause, when tested for effects upon bacterial burdens in a lethal model of *E. coli* septic shock, these mAbs did not alter organ bacterial counts. Our study design euthanized mice well before they begin to exhibit significant signs of disease and was not designed to assess survival. The statistical power of our experiments was limited by expected anesthesia-related mortality that was unexpectedly concentrated in specific groups and reduced the sample size of some groups; however, it is unlikely we missed a significant phenotype in this severe infection model. The systemic model of *E. coli* septic shock that we used is highly stringent and may only detect extreme phenotypes. Other research has demonstrated functional utility of mAbs to *E. coli* in highly nuanced *in vivo* models of microbiome interactions (52). Overall, our *in vivo* data do not show reduced bacterial burdens in mAb-prophylaxed septic mice. However, further evaluation of these mAbs using additional *E. coli* strains, greater numbers of replicates, milder disease models, or microbiome interaction models such as competitive

intestinal infections are likely necessary to fully understand the interactions of antibodies with *E. coli* within a mammalian host.

In addition to the antibody-mediated effector functions we analyzed, OmpA has been implicated in many different aspects of *E. coli* biology and pathogenesis, including cell adhesion, immunomodulation, membrane stability, bacteriophage binding, and biofilm formation (128, 135-140). The extracellular loops of OmpA have been directly implicated in meningeal invasion of *E. coli* by association with GlcNAcβ1-4GlcNAc on endothelial cells (128, 136). OmpA also triggers dendritic cell activation in intestinal cell co-culture and is responsible for binding to polarized intestinal epithelial cells (138). Taken together, these studies support the further exploration of OmpA as a potent immunogen and potential therapeutic target. Importantly, although *E. coli* is frequently a pathogen, it is even more commonly a commensal (141-143). In this work, we assessed some of the roles mAb may have during pathogenesis, but the roles that human mAbs exhibit in *E. coli* commensalism remain largely unknown. The data presented here indicate that antibodies naturally arise in humans against OmpA, and different clones bind to multiple antigenic sites on the surface of OmpA. Furthermore, mAbs that bind extracellular portions of outer membrane proteins may have effects that impact host-pathogen interactions, such as aggregation or altering phagocytosis, that are worth studying as tools to understand membrane protein biology and may yet be found to have impacts upon *E. coli* pathogenesis or commensalism.

Our work has also recapitulated some of the challenges of understanding membrane protein biology and antibody interactions with bacteria. We noted growth phase-dependent and strain-dependent differences in antibody binding to *E. coli*. We found minor differences in OmpA expression between growth phases that could explain variable binding during growth.

Strain-dependent binding differences are critically important to understand in the context of vaccination and therapeutic strategy development. Our antibodies bound identically to protein isolated from disparate *E. coli* strains, such as UTI89 and K12, but exhibited significantly less binding to UTI89 *E. coli* by immunofluorescence. OmpA was expressed in UTI89 to similar levels as K12, so we conclude that additional factors must be contributing to this strain dependent difference. We observed increased capsule thickness of UTI89, which could explain this differential binding between clinical and laboratory isolates, or it may be due to different lipopolysaccharide structures, as has previously been reported for an antibody targeting *E. coli* BamA (44, 47). Elucidating the biological processes that explain sequence-independent strain-to-strain mAb binding variability will be valuable follow-up studies. rECOL-11 may provide a critical tool to enable increased understanding of the mechanisms for strain dependent binding of antibacterial mAbs that will guide future vaccine and therapeutic development.

CHAPTER III

III. Antibodies to *Klebsiella pneumoniae* bind capsular polysaccharides but are specific to individual capsule subtypes.[2]

Introduction

Klebsiella pneumoniae is a Gram-negative, opportunistic bacterial pathogen historically linked with healthcare associated infections but more recently found to be increasingly community acquired. The hallmark of *K. pneumoniae* virulence is the extensive production of capsular polysaccharide, leading to the organism's distinctive hyper-mucoid phenotype when cultured on solid media. Infections with *K. pneumoniae* are often divided into two categories, classical and hypervirulent. The first hypervirulent *K. pneumoniae* were described in 1986 as disease with pyogenic liver abscesses in the presence of severe disseminated endophthalmitis (144). Hypervirulent *K. pneumoniae* have continued spreading in the ensuing decades and have acquired extensive antimicrobial resistance as they encounter new sources of extrinsic resistance factors in the environment (145-147). The carbapenem-resistant *K. pneumoniae* are the most concerning, as patients must be placed on antimicrobials of last resort, often colistin, which have significant side effects and only moderate efficacy. Case-control studies suggest carbapenem-resistant *K. pneumoniae* infections have mortality rates between 33 and 48% (148, 149). Novel

[2] Adapted from work performed in collaboration with Kasturi Banerjee, Ph.D., Michael Motley Ph.D., and Bettina Fries, M.D., Ph.D..

therapeutic and prophylactic strategies to combat antimicrobial resistant hypervirulent *K. pneumoniae* are urgently required.

Capsular polysaccharide (CPS) production is strongly associated with *K. pneumoniae* hypervirulence, although CPS is neither required for, nor exclusive to, hypervirulence (150). The tissue tropisms of *K. pneumoniae* are broad and heavily dependent upon the individual patients. Liver abscesses and biliary infections are particularly pathognomonic (151), but *K. pneumoniae* may present as pneumonia (152), meningitis (153), bacteremia (154), osteomyelitis (155), soft tissue infection (156), or septic arthritis (157), and any of these occurrences may metastasize to involve additional organs. To deconvolute the broad spectrum of *K. pneumoniae* isolates, multi-locus sequence typing has been used to generate sequence type (ST) classifications that represent genetically related isolates. *K. pneumoniae* isolates also produce a variety of CPS subtypes which classically were defined by K numeric serotyping, but increasingly are determined by similarities in *wzi* CPS biosynthetic genes. At least 130 K loci have been described in *K. pneumoniae*.

The ST258 lineage is the predominant carbapenem-resistant hypervirulent lineage in the United States and this lineage produces only a few K loci in two clades (1 and 2) (158, 159). Three genes, *wzi29, wzi50*, and *wzi154* are responsible for CPS anchoring in the large majority of ST258 isolates, although they may anchor different polysaccharide chains (160, 161). While these CPS provide ST258 isolates with intrinsic resistance to human neutrophils, strains are susceptible to complement-mediated killing (162, 163). When rabbits were immunized with ST258 CPS, the generated antisera bound to *K. pneumoniae* isolates by flow cytometry. These isolates also were more effectively killed by complement and by neutrophils when opsonized with anti-CPS polyclonal antisera (164). Monoclonal murine mAbs to *K. pneumoniae* CPS have

been found to have a similar effect. Two mAbs, 17H12 and 8F12, agglutinate clade 2 ST258 strains, they promote neutrophil and macrophage opsonophagocytic killing, and reduce bacterial dissemination in a murine pneumonia model of *K. pneumoniae* disease (75). Human serum from patients with positive carbapenem-resistant *K. pneumoniae* also exhibit potent anti-CPS IgG titers and these polyclonal IgG pools increase phagocytosis of multiple clinical isolates of various serotypes (165). Importantly, early human monoclonal IgA to *K. pneumoniae* CPS were found to cross-react between multiple serotypes of bacteria (74). IgA cross-reactivity supports the hypothesis that human IgG mAbs can be identified from human donors and that these mAbs will broadly bind to unique clinical isolates to increase antibacterial effector immune functions by opsonization of *K. pneumoniae*.

In this chapter, I describe my work isolating and testing human antibodies generated against *K. pneumoniae*. We used a target agnostic approach to generate four *K. pneumoniae* (KPNA) mAbs, which we produced recombinantly and tested further. Two of these mAbs appear to be specific for CPS or a related antigen, while the antigens of the remaining two mAbs are unknown. Our collaborators have begun to functionally characterize these antibodies using *in vitro* opsonophagocytosis and complement-mediated killing assays and *in vivo* models of *K. pneumoniae* in mice.

Methods

Human hybridoma generation. Peripheral venous blood was collected from laboratory workers with confirmed sero-reactivity to *K. pneumoniae* isolates and anticoagulated with lithium heparin until processing. Peripheral blood mononuclear cells (PBMCs) were isolated from anticoagulated

blood using SepMate-50 tubes and the PBMCs were cryopreserved with DMSO in liquid nitrogen until use. These studies were approved by the Vanderbilt University Medical Center Institutional Review Board. Thawed PBMCs were transformed with Epstein-Barr virus and plated with CpG DNA, Chk2 inhibitor, and cyclosporine before being expanded upon irradiated feeder PBMCs using previously published techniques to generate lymphoblastoid cell lines (LCLs) (115). Supernatants were screened by ELISA to whole-cell methanol fixed *K. pneumoniae*. The fusion partner HMMA2.5 myeloma cells were prepared and LCLs from ELISA-reactive wells were fused to create oligoclonal hybridoma pools. After culturing to allow expansion, hybridomas were screened again by ELISA and then reactive hybridomas were stained with propidium iodide and single cell sorted using an SH800S sorting cytometer. Clonal hybridoma cell lines were screened again by ELISA to verify reactivity and then expanded for mAb isolation.

Monoclonal antibody purification. Hybridomas were cultured in G-Rex bioreactors and supernatants harvested for IgG purification. Human IgG were purified using MabSelect or Protein G affinity chromatography on fast protein liquid chromatography systems. Hybridoma antibody transcript sequences were also obtained by RNA extraction, cDNA generation, and 5' rapid amplification of cDNA ends and cloned into proprietary monocistronic protein expression vectors by Twist Biosciences. If an antibody native sequence was not IgG1, that sequence was expressed both as the native isotype and as a converted IgG1. Antibodies were also converted to Fab and FcγR binding deficient LALA-PG versions. MAbs were expressed recombinantly by transfection of these constructs in ExpiCHO cells and IgG was purified from supernatants as above using FPLC. Recombinant mAb binding was compared with hybridoma mAb binding to

verify the recovery of the correct sequence and maintenance of activity when expressed in CHO cell lines. Unless specifically noted, all data presented were generated using recombinant mAb.

Bacterial strains. *K. pneumoniae* strains 33576, 33576*Δ*, MMC34, MMC36, MMC38, and MMC39 were provided by Dr. Bettina Fries at Stony Brook Medicine. Bacteria were cultured on Miller LB agar plates for 14-16 hours at 37°C then overnight cultures were prepared and cultured at 37°C 225 RPM for 14-16 hours to reach stationary phase.

***K. pneumoniae* whole-cell methanol ELISA.** Overnight bacterial cultures were pelleted at 4000 x g for 10 minutes. The pellets were gently rinsed twice with PBS and then resuspended in distilled water equal to the culture volume and the normalized to 8.0 x 10^8 CFU/mL by $OD_{600\ nm}$. ELISA plates were coated with 100 µL of normalized bacteria for 96-well plates or 25 µL for 384-well plates and the solution allowed to evaporate for 2-3 days at room temperature. After all water had evaporated, wells were fixed with 100 µL or 25 µL of methanol which was allowed to evaporate for 24 hours at room temperature. Plates were then blocked with 3% blotting grade blocker for 1 hour at room temperature. ELISA plates were washed between steps using a Biotek 406EL washer. ELISAs were probed with either hybridoma supernatant or purified IgG and developed with mouse anti-human IgG HRP secondaries then Ultra-TMB substrate followed by a hydrochloric acid quench before being read at 450 nm absorbance on a Biotek Synergy H1.

Results

Murine mAbs have limited binding to diverse *K. pneumoniae* isolates.

Two murine mAbs, 17H12 mIgG3 and 24D11 mIgG3, were developed by collaborators (75). Binding of these mAbs and a control antibody, rSTAU-228 IgG1, to multiple *K. pneumoniae* clinical isolates was assessed by methanol fixation ELISA. Methanol fixation largely preserves intact bacteria, but also likely forms pores that allow for detection of binding to intracellular proteins as well (166, 167). *K. pneumoniae* produce diverse CPS, which may be defined by *wzi* sequence typing; the strains used in this chapter are defined in **Table 2**.

Table 2: *K. pneumoniae* strains. Strains used in this study express unique CPS as defined by *wzi* sequence typing.

Strain	*wzi* gene
33576	154
MMC34	154
MMC36	29
MMC38	50
MMC39	154

17H12 binds to 33576 but not the isogenic capsule mutant 33576*Δ* (a CPS biosynthesis deficient strain as a result of deleting *wzy*). Another *wzi154* strain, MMC34, is also bound by 17H12 while MMC38 (*wzi50*) is not bound (**Figure 13**). Neither 24D11 nor rSTAU-228 bind to any *K. pneumoniae* strains by this ELISA. 17H12 mAb binds effectively to strains of identical CPS,

however it does not cross-react between CPS isolates to any significant degree. Cross-reactive mAbs are desirable as therapeutic agents, and mAbs to *wzi29* nor *wzi50* have not been described and characterized. Furthermore, understanding whether anti-CPS mAbs are elicited in humans is an important consideration when assessing the utility of CPS-based vaccinations for *K. pneumoniae*.

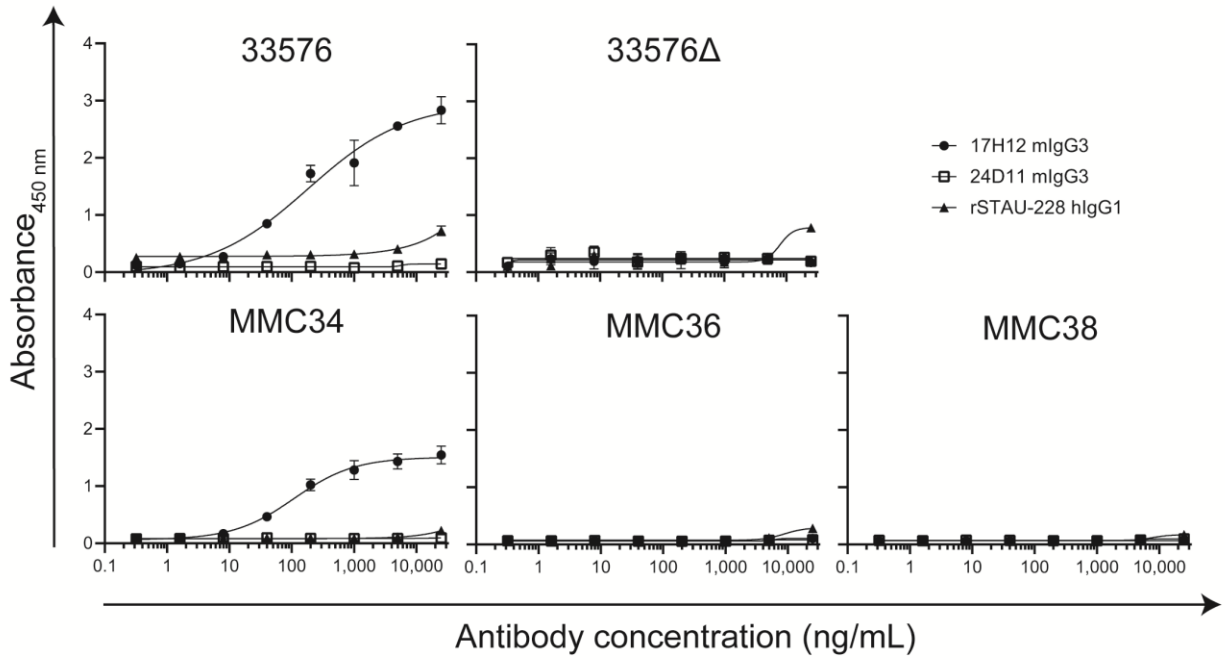

Figure 13: Murine mAbs to *K. pneumoniae*. Two murine IgG3 and one human IgG1 were assessed for binding to *K. pneumoniae* isolates by ELISA. Data were collected in quadruplicate and are representative of two independent experiments. Points represent arithmetic mean, error bars show standard deviation, curves show four-parameter logistic regressions of logarithmically transformed data.

Human donor serum reacts to *K. pneumoniae*.

To understand the serological landscape of humoral immunity to *K. pneumoniae*, human serum from a PBMC donor and a commercially available pooled human serum were tested for reactivity to *K. pneumoniae* by methanol fixation ELISA. The human PBMC donor had been reported by collaborators to have sero-reactivity to numerous *K. pneumoniae* CPSs. The ELISA

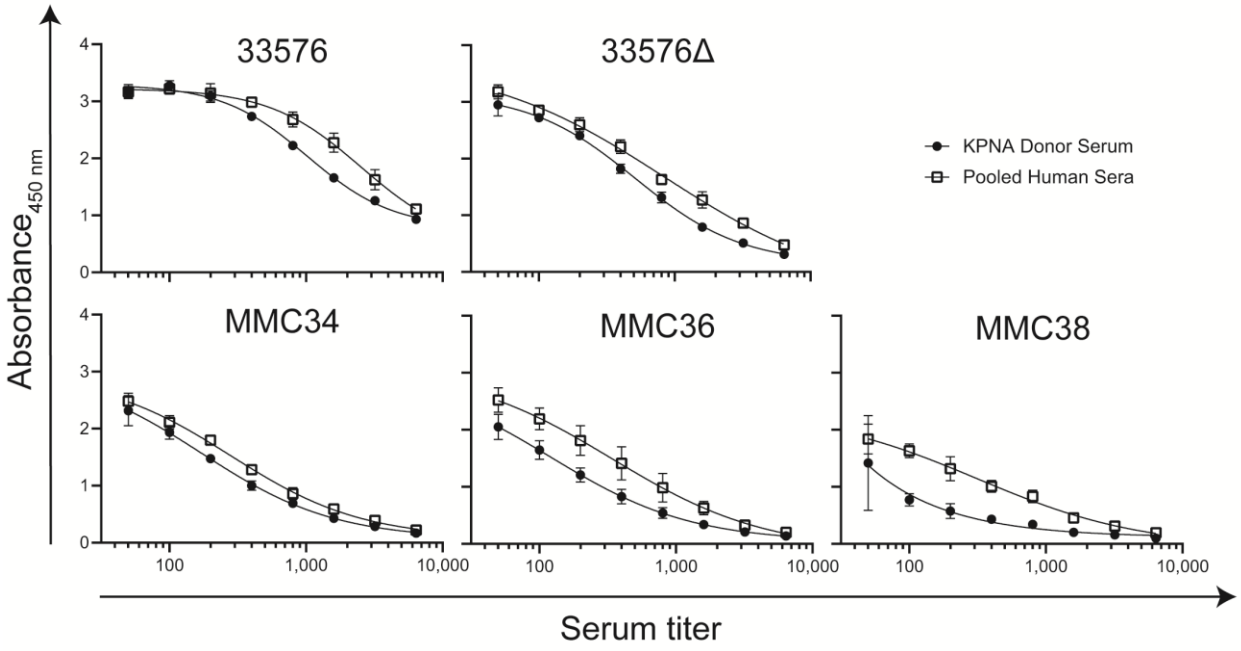

Figure 14: Serum reactivity to *K. pneumoniae*. All tested clinical isolates were bound by both a human PBMC donor serum and by pooled human sera. Data were collected in quadruplicate and are representative of two independent experiments. Points represent arithmetic mean, error bars show standard deviation, curves show four-parameter logistic regressions of logarithmically transformed data.

data confirm that the PBMC donor serum reacts to all tested CPS variants; interestingly, the ELISA also demonstrated repeatably that pooled human sera also possessed significant reactivity to *K. pneumoniae* (**Figure 14**). This result is not entirely unexpected and may be explained several ways. First, this assay is a methanol-fixation ELISA, which exposes intracellular antigens for binding. Some housekeeping proteins like polymerases and ribosomes exhibit significant conservation across numerous species that may be targeted by naïve antibodies or antibodies generated against other organisms. Second, bacteria are often thought to exhibit a high degree of "stickiness", which may increase the degree of non-specific or minimally specific binding of antibodies found in serum. Third, *K. pneumoniae* is a commensal organism in a small but still frequent portion of the population (168, 169). Regular exposure to *K. pneumoniae* through intestinal colonization may trigger some degree of B-cell activation that results in the generation of somatically-mutated IgG specific for *K. pneumoniae* even in the absence of disease.

Generation of human mAbs to *K. pneumoniae*.

An estimated 14,000 EBV-transformed human memory B cells were screened for antibodies that bound *K. pneumoniae* by ELISA. Oligoclonal cells from 19 pools were fused with HMMA2.5 cells and further screened. Four mAbs with unique sequences were obtained, KPNA-5, KPNA-13, KPNA-14, and KPNA-16. Three mAbs were natively IgG2, while KPNA-13 was IgG1; all mAbs used κ light chain genes. The specific genes as well as the complementarity determining region 3 (CDR3) lengths are in **Table 3**.

Table 3: *K. pneumoniae* human mAb descriptors.

mAb	Heavy chain isotype	Heavy chain gene usage	CDR3 amino acids
KPNA-5	IgG2	V: *3-23*01* D: *5-18*01* J: *3*02*	14
KPNA-13	IgG1	V: *4-61*01* D: *1-1*01* J: *3*02*	16
KPNA-14	IgG2	V: *3-30-3*01* D: *1-7*01* J: *3*02*	16
KPNA-16	IgG2	V: *4-59*01* D: *2-2*01* J: *6*03*	19

All mAb clone transcripts were sequenced and recombinantly produced as both the native IgG isotype and converted to an IgG1 backbone. Fab and IgG LALA-PG FcγR binding deficient mutants were also produced and purified. All recombinant mAbs were verified to bind similarly

to hybridoma produced mAb and then recombinant mAb was used for all studies unless otherwise noted.

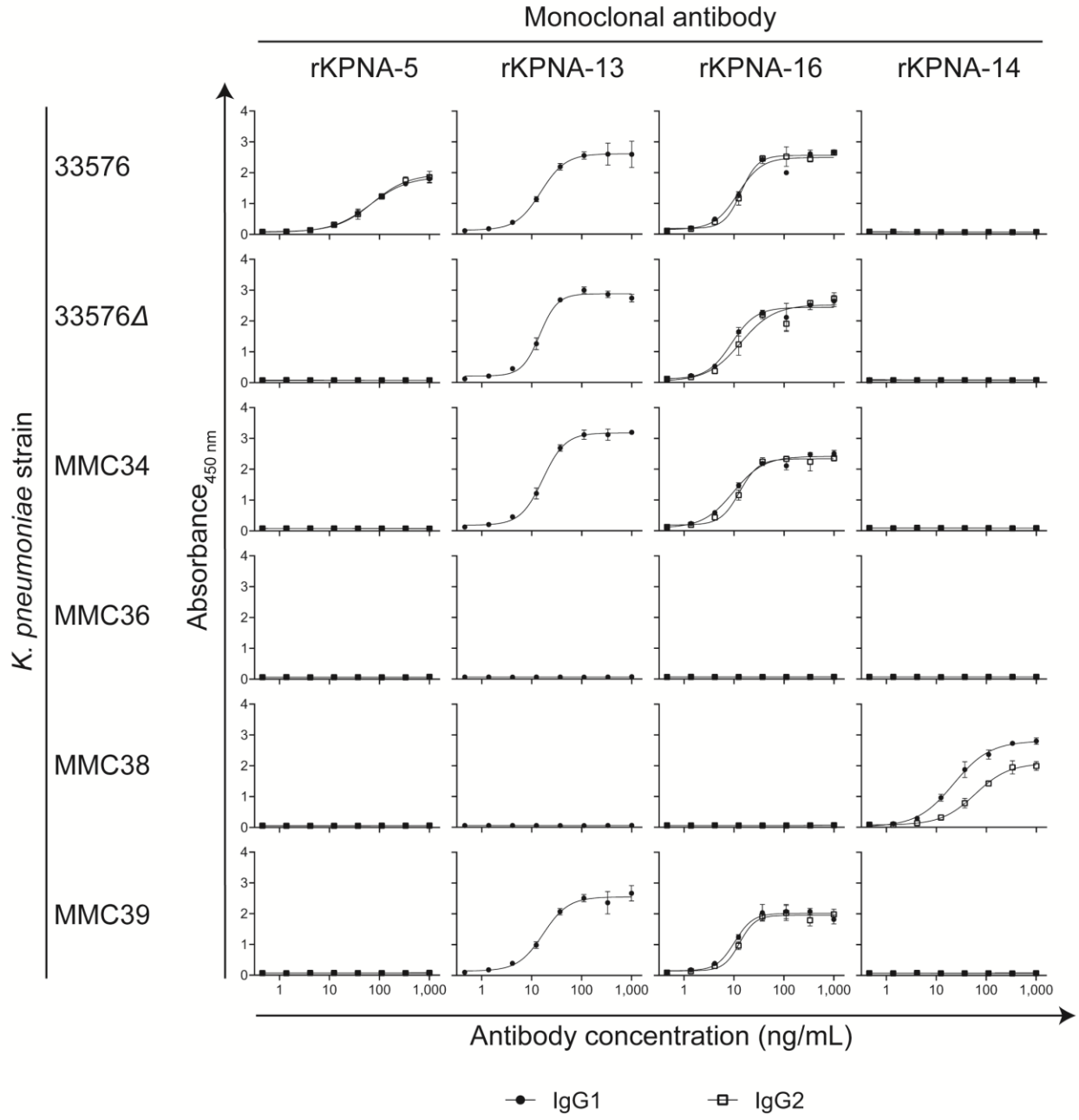

Figure 15: ELISA of human *K. pneumoniae* mAbs. Recombinant antibodies were tested as native (IgG1 or IgG2) and as converted IgG1 for binding to whole *K. pneumoniae* by ELISA. Points show the arithmetic mean of four replicates. Lines show the four-parameter logistic regression curve. Error bars represent standard deviation. Data shown are representative from two independent experiments.

Human *K. pneumoniae* mAbs bind diversely.

All recombinantly produced mAbs were assessed for binding to whole *K. pneumoniae* by methanol-fixation ELISA. Three unique binding patterns are observed (**Figure 15**). KPNA-5 binds to wild-type 33576 (*wzi154*), but not to the isogenic *Δwzy* strain that lacks a component of CPS biosynthetic machinery (164). However, KPNA-5 does not bind to MMC34 nor MMC39, which also use *wzi154* synthetic machinery and thus are of the same predicted CPS type. KPNA-5 not binding to MMC34 and 39 could mean that KPNA-5 is specific for *wzy* since the mAb does not bind to 33576*Δ*. *wzy* is also involved in O-antigen linkage though, so KPNA-5 could alternatively bind to *K. pneumoniae* LPS and we cannot eliminate this possibility using these mutants. A second binding pattern is exhibited by KPNA-13 and KPNA-16, which bind broadly to 33576, 33576*Δ*, MMC34, and MMC39. All these strains are of the same *wzi* type (*wzi154*), however since KPNA-13 and KPNA-16 bind to 33576*Δ*, it is unlikely that either antibody is binding to CPS. Instead, the antigens may be proteins that are conserved across these strains and not the more divergent MMC36 and MMC38 isolates. Finally, KPNA-14 binds only to MMC38, which produces capsule with *wzi50* linkage. Although not definitive, the lack of binding to any other strain that produce similar proteins but have unique CPS suggests that KPNA-14 may be specific to *wzi50* CPS. Data from our collaborators supports the conclusion that KPNA-14 is specific for CPS. All mAbs bound when expressed as their native isotype (IgG1 or IgG2) and all IgG2 mAbs retained binding when converted to and expressed as IgG1.

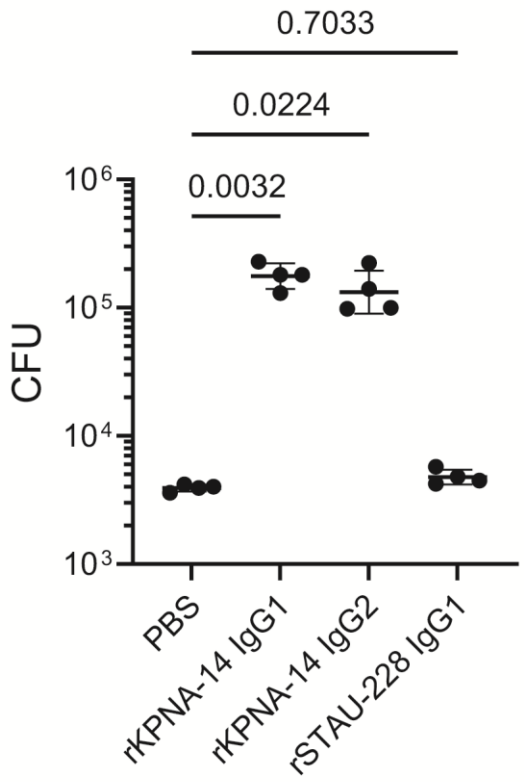

Figure 16: Opsonophagocytosis of *K. pneumoniae*. Macrophage-like J774A.1 cells were incubated with *K. pneumoniae* strain MMC38 that was pre-opsonized with mAb and normal human serum. Phagocytosed bacteria were then plate for enumeration of CFUs. Data were collected by Kasturi Banerjee, Stony Brook University. Bacterial counts were log transformed and analyzed by one-way ANOVA. Error bars show standard deviation.

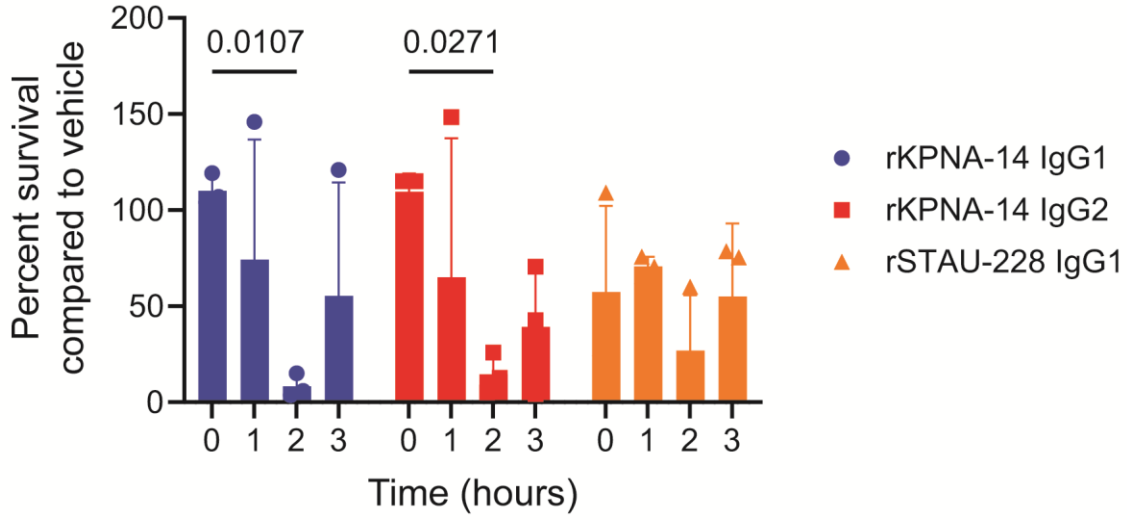

Figure 17: Complement killing by KPNA mAbs. *K. pneumoniae* strain MMC38 was incubated with mAb and normal human serum. Replicates were taken hourly for three hours and plated for enumeration of colony forming units. Data were collected in triplicate by Kasturi Banerjee, Stony Brook University. Bacterial counts were normalized to percent of vehicle control at that time point. Error bars show standard deviation. Data were analyzed by two-way ANOVA and each treatment was compared to 0 hours. P-value <0.05 are shown as comparisons on the plot.

KPNA-14 increases phagocytosis and serum killing of *K. pneumoniae* MMC38.

MAbs were provided to Stony Brook University to test the hypothesis that anti-CPS mAbs increase complement-mediated killing of *K. pneumoniae*. Murine macrophage-like cell line J774A.1 was incubated with bacteria pre-opsonized with mAb and human serum and then intracellular bacteria were counted. Compared to the vehicle control, PBS, or a control mAb rSTAU-228 IgG1, both rKPNA-14 IgG1 and rKPNA-14 IgG2 significantly increased phagocytic uptake of *K. pneumoniae* strain MMC38 by J774A.1 cells (**Figure 16**). Since this system incorporates phagocytes and serum, isolated serum killing assays were performed using MMC38 and rKPNA-14. After 2 hours, there was significantly increased killing by human serum when bacteria were opsonized with rKPNA-14 as IgG1 or IgG2 (**Figure 17**). Thus, the *wzi50* strain reactive mAb, KPNA-14, induces opsonophagocytosis and complement mediated killing of *K. pneumoniae* MMC38. These data suggest the potential for KPNA-14 to have effects upon the pathogenesis of *K. pneumoniae* using an *in vivo* model of infection.

Discussion

The capsular polysaccharide of *K. pneumoniae* is an important virulence factor and a potential target for vaccination and therapeutic antibodies. Vaccines to CPS have reduced global disease burdens significantly by decreasing infections with *H. influenzae*, *N. meningitidis*, and *S. pneumoniae* (14). Since CPS vaccines have been so successful for other organisms, much has been deliberated about CPS vaccination for *K. pneumoniae*. A *K. pneumoniae* K1 vaccine was tested in a small Phase 1 trial with successful induction of antibody responses generating sera that were capable of passively protecting mice (170). No follow-up to that study has been

published and it is not known through what antibacterial functions the generated antibodies protected. In this work, we sought to build upon a series of studies demonstrating the efficacy of murine mAbs at protecting mice from *K. pneumoniae* challenges by the enhancement of antibody-dependent complement-mediated killing. We used human hybridoma technology to generate naturally occurring human monoclonal antibodies specific for *K. pneumoniae*. These mAbs are in the process of being further evaluated.

Intriguingly, we were not able to distinguish serum reactivity of a specific donor from background pooled human serum. This phenomenon of high general population sero-positivity to bacterial antigens is surprisingly common (personal communications). In some instances, it may be more challenging to find a true negative control serum than to find a donor with a high level of antibodies against the targets of interest. This obstacle is a major contrast to viral pathogens, where sero-positivity is a correlate of protection and can be used as a metric with which to select donors likely to have affinity-matured, highly potent antibodies to the organism (171). A significant difference between viral and bacterial pathogens is that many bacterial organisms are, or are closely related to, human commensal bacteria. There is evidence from studies of inflammatory bowel disease that IgG are frequently induced to intestinal commensals during states of acute or chronic inflammation in both diseased and healthy humans (172, 173). The generation of antibodies to commensals has likely been naturally selected for through evolutionary pressures as increasingly important roles for anti-commensal immunoglobulins are regularly being discovered during microbiome research.

In the intestine, IgA generated in Peyer's patches are reactive to numerous unique antigens and the IgA coat commensal bacteria and the secreting B cells can be isolated systemically (174). These IgA modulate the host immune reaction to the microbiota and alter the

physiology of the commensal organisms. IgA to the commensal fungus and opportunistic pathogen *Candida albicans* bind to fungal hyphae and as a result, the more pathogenic hyphal *Candida* are less fit in the intestine (175). In that system, IgA are pushing *C. albicans* towards a commensal lifestyle instead of a pathogenic phenotype. IgA are typically treated as the primary immunoglobulin effector of mucosal surfaces, but this dogma is not always true. Recent work has found that systemic IgGs to intestinal *E. coli* are secreted into breastmilk by the neonatal Fc receptor, FcRn . These secreted IgG protect neonatal mouse pups from systemic or intestinal challenge by *E. coli*, challenging the dogma that IgA are the only important mucosal antibody (152). These data support the hypothesis that there may be evolutionary benefits to generating systemic immune responses to commensal organisms. *K. pneumoniae* is a commensal organism in an estimated 23% of people and is found in the intestine and nares (168, 169). The microbiome varies across time, so a larger portion of people may be exposed to *K. pneumoniae* over their lifetimes, resulting in the generation of specific antibodies (176). Microbiome exposure and variability may explain why we were unable to differentiate between human donor serum and pooled human sera yet were able to identify four unique antibodies to *K. pneumoniae* from human donor PBMCs.

We identified three distinct patterns of binding to the clinical isolates we tested. KPNA-5 exclusively bound to 33576 WT, suggesting specificity for a unique antigen, possibly to the product of *wzy* or an antigen downstream of this synthesis pathway, since it does not bind to 33576*Δ*. KPNA-13 and KPNA-16 bound broadly to all *wzi154* strains, including the capsule deficient strain 33576*Δ*. These mAbs could be worth further pursuing since they may target *K. pneumoniae* proteins. However, since CPS is a nearly ubiquitous *K. pneumoniae* virulence factor, these mAbs may have minimal binding to clinically relevant isolates due to CPS

obscuring the proteins to which they could bind. Similar steric inhibition of antibody binding by CPS or LPS has been reported for *A. baumannii* and *E. coli* (11, 47). KPNA-14 is unique in that it binds to MMC38, the sole *wzi50* isolate in the tested panel. Isolates with *wzi50* are more frequently carbapenem and fluoroquinolone resistant and may be more deadly than other isolates (177). No previous mAbs have been published that bind to *wzi50*. Further experimentation to more definitively determine the antigens of all four KPNA mAbs is necessary but can be done in parallel to functional characterization to better understand how naturally occurring mAbs to *K. pneumoniae* modulate the host-pathogen interface.

K. pneumoniae has been reported as sensitive to complement-mediated killing that is enhanced after opsonization with antibody (75, 162). We hypothesized that KPNA-14 would enhance complement-mediated killing of MMC38, although the killing may be limited to strains of identical *wzi50* CPS. Collaborators at Stony Brook University tested this hypothesis and upon analyzing the data they provided, we determined that there is a statistically significant increase in serum-mediated bacterial killing when *K. pneumoniae* is treated with KPNA-14, however the experimental design lacked heat-inactivated serum controls for additional comparison. This experiment makes it apparent that naturally occurring human anti-CPS antibodies to *K. pneumoniae* do enhance complement-mediated antibacterial activity. It was also important to assess the interactions of KPNA mAbs with mammalian phagocytic cell lines. Murine macrophage-like RAW264.7 or J774A.1 cells or human differentiated HL-60 acute myeloid leukemia cells may exhibit increased phagocytosis and killing of mAb opsonized *K. pneumoniae*. Collaborators at Stony Brook University again co-incubated KPNA-14 with MMC38 to opsonize the bacteria and then added human serum as a complement source followed by co-incubation with J774A.1 macrophage-like cells. By enumeration of phagocytosed bacterial counts, we

determined that KPNA-14 increased phagocytosis of *K. pneumoniae*. These data once again are greatly informative to the understanding of the human immune response to *K. pneumoniae*. Naturally elicited antibodies engage in both phagocyte-dependent and complement-mediated killing of *K. pneumoniae*. Presumably, the remaining untested antibodies that bind to protein in ELISA but may not bind to the surface of intact bacteria will not exhibit enhanced opsonophagocytic killing nor increased complement-mediated killing.

Immune dynamics are complex though, and non-surface exposed antigens may be present during infection because of bacterial lysis or antigen display by host antigen presenting cells. Presentation of non-surface exposed antigens could mean that non-opsonizing antibodies have effects upon the immune response, possibly increasing generalized inflammation that enhances antibacterial immune effector functions independently of antibody binding to intact bacteria. Such activity can only be determined by *in vivo* modeling of *K. pneumoniae* infection. Murine intratracheal inoculations are often used to model *K. pneumoniae* pulmonary infections. Mice can be prophylactically (pre-inoculation) or therapeutically (post-inoculation) given mAb, or the inoculating bacteria can be pre-opsonized with mAb prior to inoculation. Mice can be monitored for bacterial burdens in various organs and for alterations of inflammatory markers such as Ifnγ, IL1-β, IL-6, or TNFα. These techniques are likely to detect any protective phenotypes exhibited by a mAb, but they are likely not sensitive enough to detect subtler modulation of inflammation, such as that potentially caused by a non-opsonizing mAb. In those instances, it may be necessary to perform broad host and pathogen RNA sequencing to assess the differential expression of a broader panel of inflammatory host genes and bacterial stress response genes. A similar approach was taken by Rollenske *et al*. to evaluate the effect of monoclonal IgA upon *E. coli* in a murine intestinal colonization model and noted distinct changes in both host and pathogen gene

expression during mAb treatment (52). These additional studies will significantly contribute to the broad understanding of how *K. pneumoniae* mAbs alter bacterial pathogenesis in humans.

CHAPTER IV

IV. A novel cellular impedance assay to rapidly screen antibodies to *Bordetella pertussis* toxin.

Introduction

Bordetella pertussis is a Gram-negative aerobic bacterium that causes whooping cough, a highly contagious acute respiratory disease, predominantly in children. Although the burden of pertussis disease has drastically decreased since the introduction of pertussis vaccination in the 1940s, there has been a resurgence in recent years due to the epidemic of vaccine denialism and the switch from whole cell to acellular vaccines in many countries (178, 179). Additionally, the most severe pertussis disease occurs in neonates and infants who are unable to be vaccinated due to their young age and immature immune systems. The re-emergence of pertussis has increased disease incidence and has fueled efforts to generate pertussis therapeutics, focusing on monoclonal antibodies to pertussis toxin.

Pertussis toxin is one of the primary effectors of pertussis disease. Two cases of pertussis toxin deficient *B. pertussis* infections have been reported, both were significantly less severe than other similar cases (180, 181). Pertussis toxin has many diverse functions in a mammalian host, many related to modulation of inflammatory pathways. Pertussis toxin limits lymphocyte extravasation and inhibits T-cell and B-cell function, suppressing antibody responses. Through the stimulation of innate immune cells to produce CXCL1, CXCL2, and IL17A, pertussis toxin decreases the recruitment of neutrophils and decreases phagocytosis of bacteria (182). Pertussis toxin also is a modulator of G protein coupled receptors (GPCR) leading to a wide variety of poorly characterized downstream effects, including pulmonary edema and possibly the

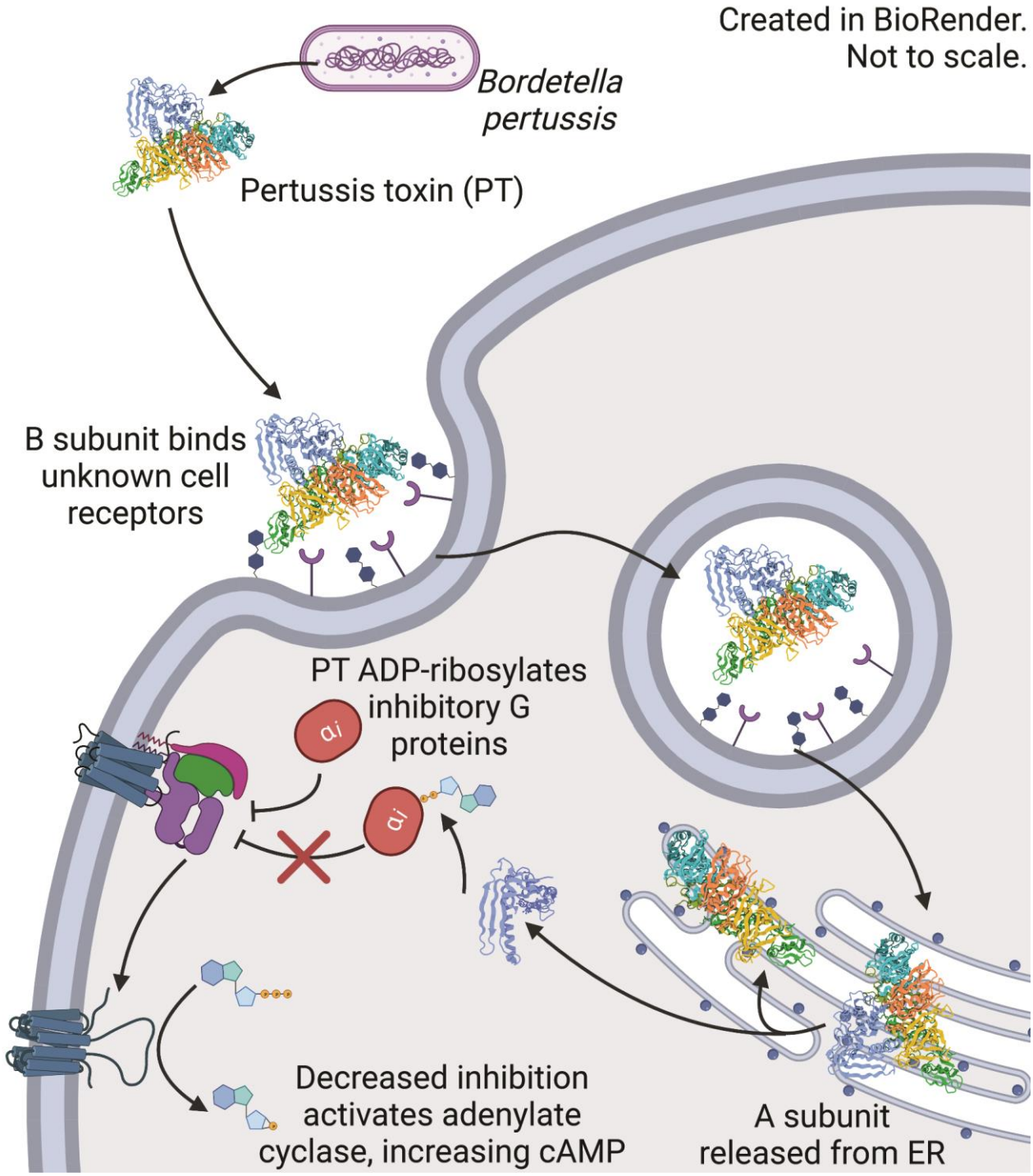

Figure 18: Diagram of pertussis toxin mechanism. Pertussis toxin is secreted by *B. pertussis* and binds to unknown receptors via the B subunit of the toxin. The toxin is endocytosed and trafficked through the Golgi apparatus to the endoplasmic reticulum where the A subunit is released. The A subunit then ADP-ribosylates GTP binding proteins, predominantly the α_i GPCR component. This relieves GPCRs from inhibition, increasing adenylate cyclase activity resulting in increased cAMP generation. How exactly this causes the pathophysiology of pertussis diseases is poorly understood.

pathognomonic cough (183, 184). Pertussis toxin is an AB type toxin. It is comprised of five binding (B) subunits and one enzymatically active (A) subunit. The B subunit is responsible for the adhesion to and trafficking into host cells. The A subunit catalyzes the transfer of ADP-ribose to the carboxyl terminus of several GTP binding proteins, primarily the inhibitory α_i subunit of G_i. This addition of ADP prevents the inhibitory GTP binding protein from associating with its cognate receptor, increasing GPCR activity and increasing cyclic adenosine monophosphate production (cAMP) (185). This process is shown graphically in **Figure 18**. Inhibition of pertussis toxin can significantly decrease the severity of pertussis disease and may protect against symptom development.

The first antibodies to inhibit pertussis toxin were described in 1984 by Sato *et al*. from BALB/c mice immunized with deactivated pertussis toxin. Two mAbs, 1B7 and 3F10, bound to the A subunit and one mAb, 1H2, bound the B subunit. 1B7 was neutralizing and showed therapeutic effects *in vitro* (186). Additional mAbs specific for pertussis toxin have been discovered and assessed, but the murine IgG2a 1B7 has consistently neutralized the toxin more effectively than any other described mAb (187-192). One of the key assays used to assess pertussis toxin potency and neutralization of the toxin is the Chinese hamster ovary (CHO) cell clustering assay, first described by Hewlett *et al*. as a technique to test for active pertussis toxin contamination in deactivated toxin for vaccinations. This assay uses adherent CHO K1 cells and exposes them to pertussis toxin, which induces the cells to clump (193). The exact mechanism leading to this phenotype is not fully understood. Pertussis toxin is trafficked into the cells and the A subunit ADP-ribosylates a 41-kDa target protein (194). The downstream effects of pertussis toxin trafficking and how it results in cell clumping are not understood. It is apparent that CHO cell clumping depends significantly upon the B subunit of the toxin, but the A subunit

does contribute (195). Therefore, the CHO cell clumping assay can be used to assess neutralization of pertussis toxin by antibodies to both the B subunit and the A subunit. An important caveat is that because the B subunit contributes more to the clumping phenotype, antibodies to the B subunit may appear more potent by CHO cell clumping assay than A subunit binding antibodies. The CHO cell clumping assay also has some significant obstacles that have been well-discussed in articles using the assay. The assay uses microscopy to evaluate the degree of clumping. The degree of clumping must be accurately rated by a skilled microscopist who

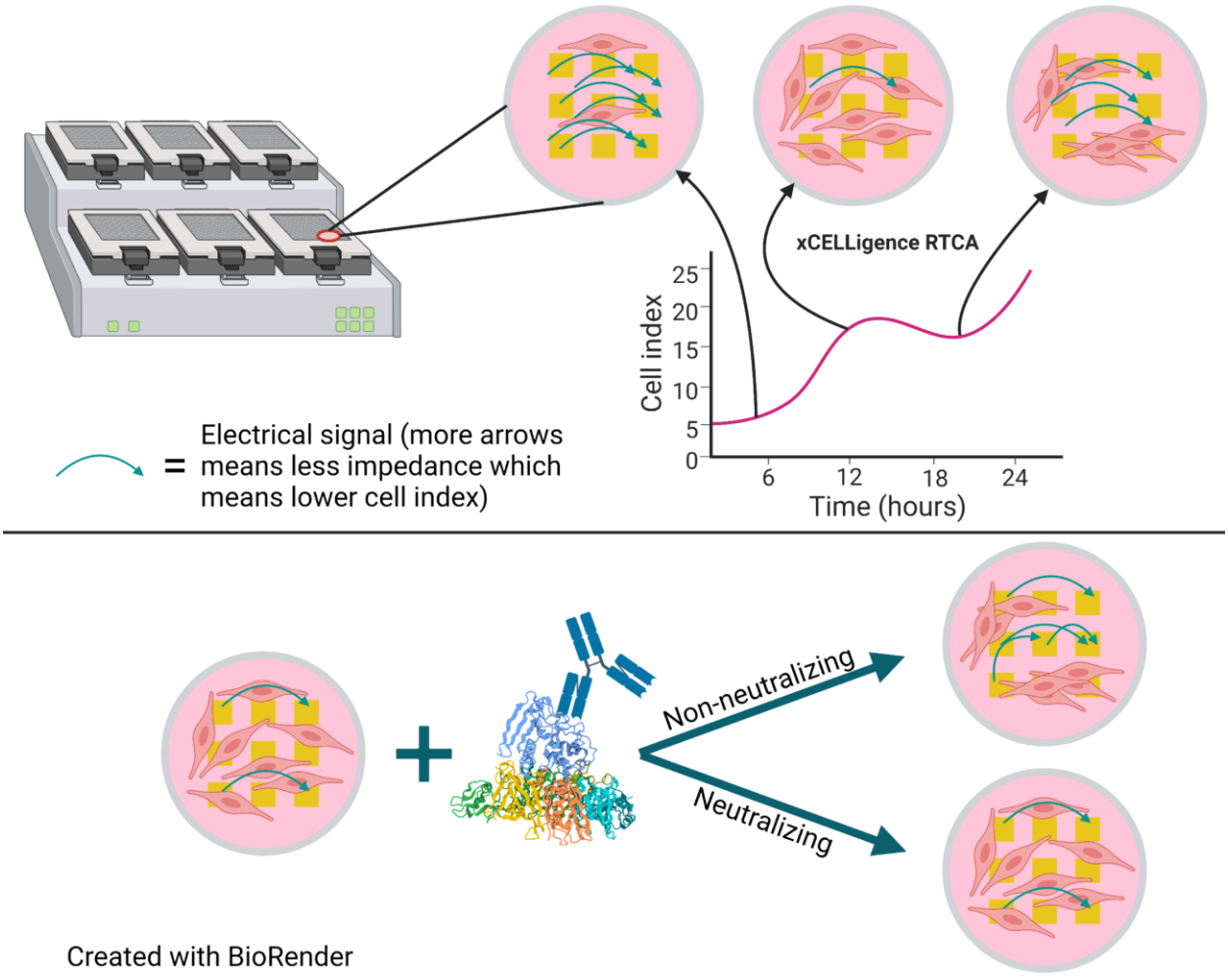

Figure 19: Diagram of xCELLigence CHO clumping assay. CHO K1 cells are allowed to grow in 96-well plates with electrodes coating the bottom of each well. The xCELLigence measures the impedance between electrodes, which increases as cells cover electrodes as they grow. This is referred to as cell index. When pertussis toxin is added, CHO K1 cells clump, exposing electrodes and decreasing the impedance and thus the cell index.

must remain consistent across plates and time to enable accurate comparisons. The time and effort necessary to image and assess cell monolayers by microscope greatly limits the ability to scale this assay and minimizes the ability to consistently quantify pertussis toxin neutralization.

To address these problems, we utilized real-time cell analysis (RTCA) on the Agilent xCELLigence platform to update the CHO cell clumping assay for the 21st century. The xCELLigence system uses 96- or 384-well electrode-coated plates to assess the electrical impedance of cell monolayers (196). As electrodes on the plate are covered by cells, the impedance or resistance increases and thus the impedance correlates with cell growth and can be interpreted as a cell index (**Figure 19**). These plates can be run in large quantities and be monitored continuously over many hours. The xCELLigence platform enables significant improvements to the CHO cell clumping assay to assess pertussis toxin neutralization and we describe use of this assay to probe the neutralizing functions of human monoclonal antibodies isolated against pertussis toxin.

Methods

Generation of human monoclonal antibodies. Human PBMC samples were collected as part of a clinical trial being conducted by Novartis/Sanofi/GlaxoSmithKline. Five participants were immunized with 4 µg of genetically inactivated pertussis toxin, 4 µg of filamentous hemagglutinin, 8 µg of pertactin, 2 Lf (limit of flocculation units) of diphtheria toxoid, and 5 Lf of tetanus toxoid. Five participants were immunized with 8 µg of chemically inactivated pertussis toxin, 8 µg of filamentous hemagglutinin, 2.5 µg of pertactin, 2.5 Lf of diphtheria toxoid, and 5 Lf of tetanus toxoid. PBMCs were collected and processed from whole blood 30

days post-vaccination. Monoclonal antibodies were generated using human hybridoma technology as previously published (115). Thirty-one human mAbs to pertussis toxin were generated; 26 of 31 were obtained from participants who received the genetically inactivated toxin. These mAbs were produced by hybridoma culture and purified from hybridoma supernatants using fast protein liquid chromatography (FPLC) with Protein G affinity columns. Unless otherwise noted, antibodies used in this study were purified from hybridoma supernatants. A selection of antibody transcripts were sequenced by 5' random amplification of cDNA ends and cloned into expression vectors by Twist Biosciences. These mAbs were expressed in ExpiCHO cells and purified on MAbSelect affinity columns by FPLC.

CHO K1 cell culture. ATCC CCL-61 CHO K1 cells were stored in liquid nitrogen until use. Cells were cultured in Ham's F12 Kaighn's Medium supplemented with 10% heat inactivated fetal bovine serum (F12K10). Cells were maintained in 25 or 75 cm^2 flasks between 3.0 x 10^4 cells/mL and 2.5 x10^6 cells/mL. To passage or prepare for xCELLigence, cell monolayers were released from tissue culture flasks with Trypsin-EDTA and then counted by Trypan blue exclusion.

xCELLigence CHO clumping assay. The culture time, cell count, and pertussis concentration were optimized for this assay. The optimized protocol is included here; tested variations are discussed in figures and results. After verifying CHO K1 cells were >80% confluent and viable, 50 µL of F12K10 was added to all wells and the background of each well was measured on an xCELLigence instrument. Then 50 µL per well of CHO K1 cells at 2.5 x 10^5 cells/mL were added to each well and allowed to settle and adhere at room temperature for 30 minutes. Plates

were then placed on the xCELLigence instrument and incubated for 24 hours at 37°C 5% CO_2. The next day, mAbs were serially diluted in F12K10 and mixed 1:1 with 8 ng/mL of pertussis toxin in F12K10 (for a final concentration of 4 ng/mL). MAbs were allowed to bind pertussis toxin at 37°C 5% CO_2 for 90 minutes. Medium was aspirated carefully from CHO K1 cells on xCELLigence plates and then replaced with 100 µL of mAb treated pertussis toxin. Plates were returned to the xCELLigence and monitored for 24-48 hours.

Results

Optimization of xCELLigence CHO K1 clumping assay.

To determine if the xCELLigence platform could be used to replace microscopy in the pertussis toxin CHO K1 clumping assay, we optimized pertussis toxin concentration, culture time, and cell densities. To determine optimal timing, CHO K1 cells were seeded onto xCELLigence plates at a uniform density of 25,000 cells/well and allowed to grow for 4, 8, 24, or 32 hours. The medium was then replaced with medium containing 4, 8, or 16 ng/mL of pertussis toxin or a vehicle control. After pertussis toxin was administered, cells were monitored for approximately 18 hours. The resulting RTCA curves were analyzed to select the optimal culture time of 24 hours (**Figure 20**). This time was selected because there was the maximum difference between the pertussis toxin and no pertussis toxin conditions and the highest consistency between replicates. At longer times the cells began to die before toxin was administered and at shorter incubation times the cells were not at the appropriate density for the assay to function optimally. To optimize cell densities, CHO K1 cells were seeded onto xCELLigence plates at multiple densities for 24 hours. The medium was replaced after 24 hours

with medium containing 4, 8, or 16 ng/mL of pertussis toxin or the vehicle control and the cells were monitored for about 18 hours (**Figure 21**). The 12,500 cell/well condition generated the maximum differentiation between no pertussis toxin and pertussis toxin treated conditions and was consistent between replicates. The 4 ng/mL pertussis toxin concentration was selected as it exhibited the maximum clumping phenotype while minimizing reagent use.

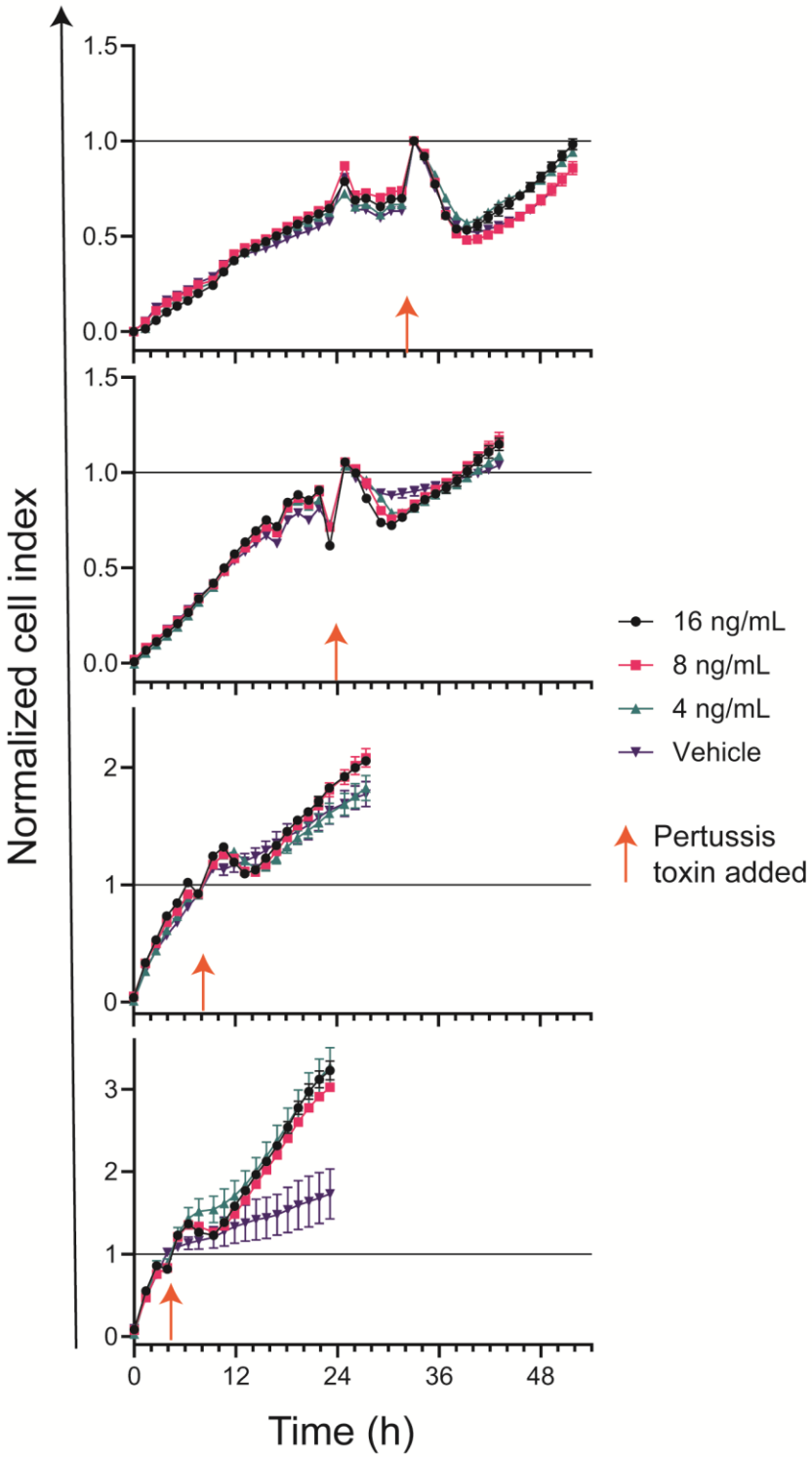

Figure 20: Time optimization of xCELLigence CHO clumping assay. Cell monolayers in xCELLigence plates must be optimally confluent with enough cells to measure clumping. 25,000 cells per well were added to xCELLigence plates and allowed to grow for 4, 8, 24, and 32 hours. Cells were then treated with multiple concentrations of pertussis toxin at the time indicated with an orange arrow and the ability to induce clumping was monitored for an additional ~18 hours. Cell indices were normalized to 1 hour after toxin administration to correct for any disruption of the layer by pipetting. The optimal time of 24 hours was selected.

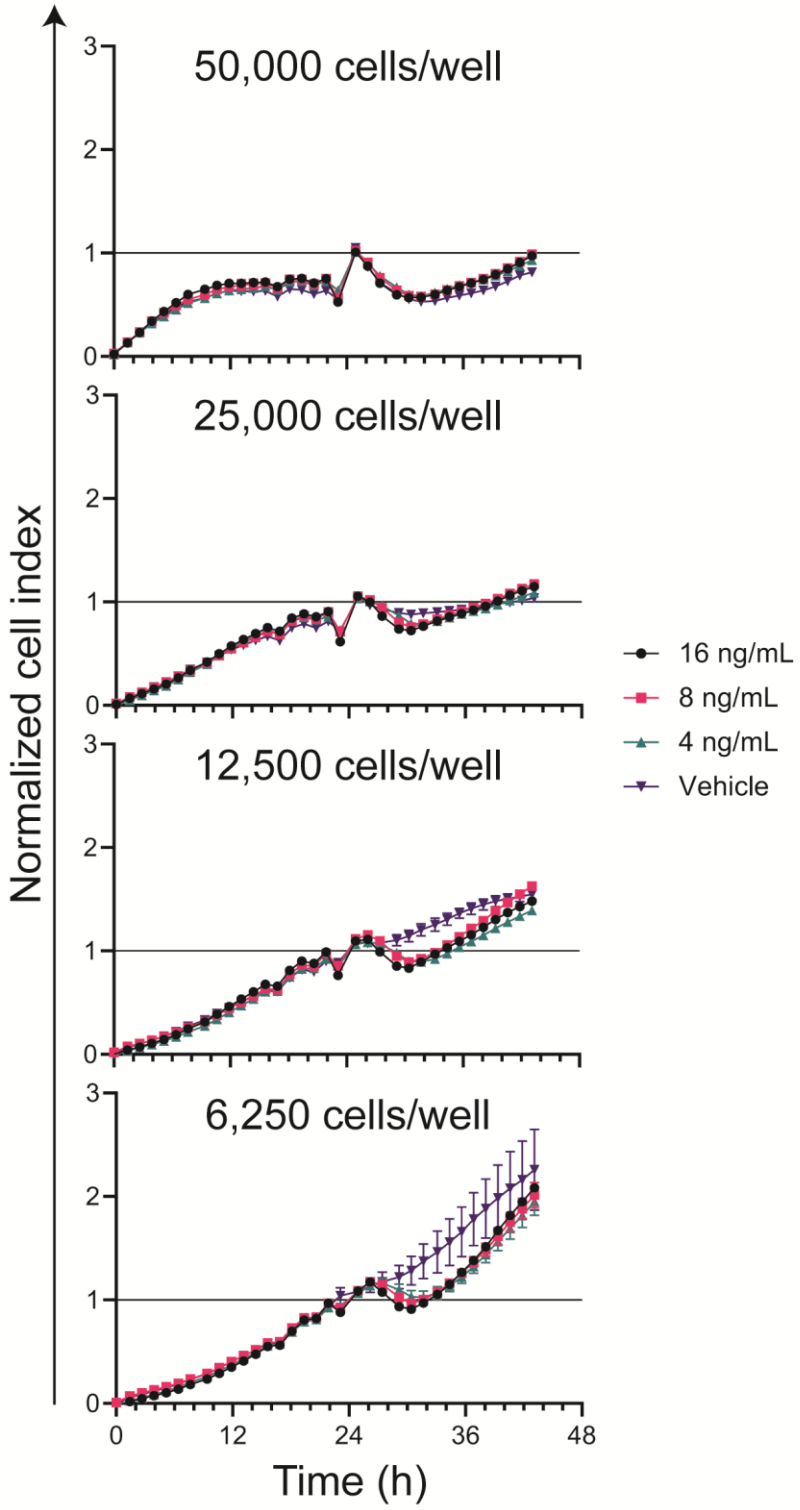

Figure 21: Cell count optimization of xCELLigence CHO clumping assay. To further optimize the xCELLigence assay, various cell densities were optimized. Wells were seeded at various densities per well and allowed to grow for 24 hours before the addition of multiple concentrations of pertussis toxin. The cell index was monitored for an additional ~18 hours. Cell indices were normalized to 1 hour after toxin administration to correct for any disruption of the cell monolayer by pipetting. The optimal cell density of 12,500 cells/well and optimal toxin concentration of 4 ng/mL were chosen.

Testing neutralization of pertussis toxin by mAb.

Having demonstrated that the xCELLigence platform could be used to perform the CHO K1 pertussis toxin clumping assay, we next sought to test whether the assay could detect neutralization of pertussis toxin by mAb. For consistency with the microscopic variation of the assay, mAb and pertussis toxin were preincubated together for 90 minutes. This incubation was performed in the CHO K1 medium and done at 37°C 5% CO_2 to allow the medium to reach homeostatic pH prior to addition to cell layers. Thirty-six antibodies were tested for their capacity to neutralize pertussis toxin but only five representative mAbs are shown here for simplicity. The CHO K1 xCELLigence plate medium was replaced with the preincubated mAb and pertussis toxin medium and monitored for ~18 hours. We observed stark differences between neutralizing mAbs and non-neutralizing mAbs. As previously demonstrated, when no pertussis toxin is added to CHO K1 cells, the cells continue to expand over the course of the assay. A non-neutralizing antibody, PERT-223, is unable to prevent the clumping of CHO K1 cells. Neutralizing mAbs 1B7, rPERT-169, PERT-182, and rPERT-221 are each able to neutralize pertussis toxin to varying degrees (**Figure 22**). To more accurately compare mAbs to each other, data from 8 hours after toxin administration were selected because this time point exhibited maximum clumping in non-neutralizing mAb conditions. By analyzing mAb at a single time point, dose-response curves were generated by comparing mAb treated cell indices with no pertussis toxin cell indices (**Figure 23**). Since pertussis toxin does not kill CHO K1 cells, the minimum percentage of the no toxin controls is about 40%. Comparing these curves, it is evident that rPERT-221 and rPERT-169 are the most potent neutralizing mAbs of pertussis toxin, while PERT-182 and 1B7 exhibit intermediate neutralizing potency phenotypes.

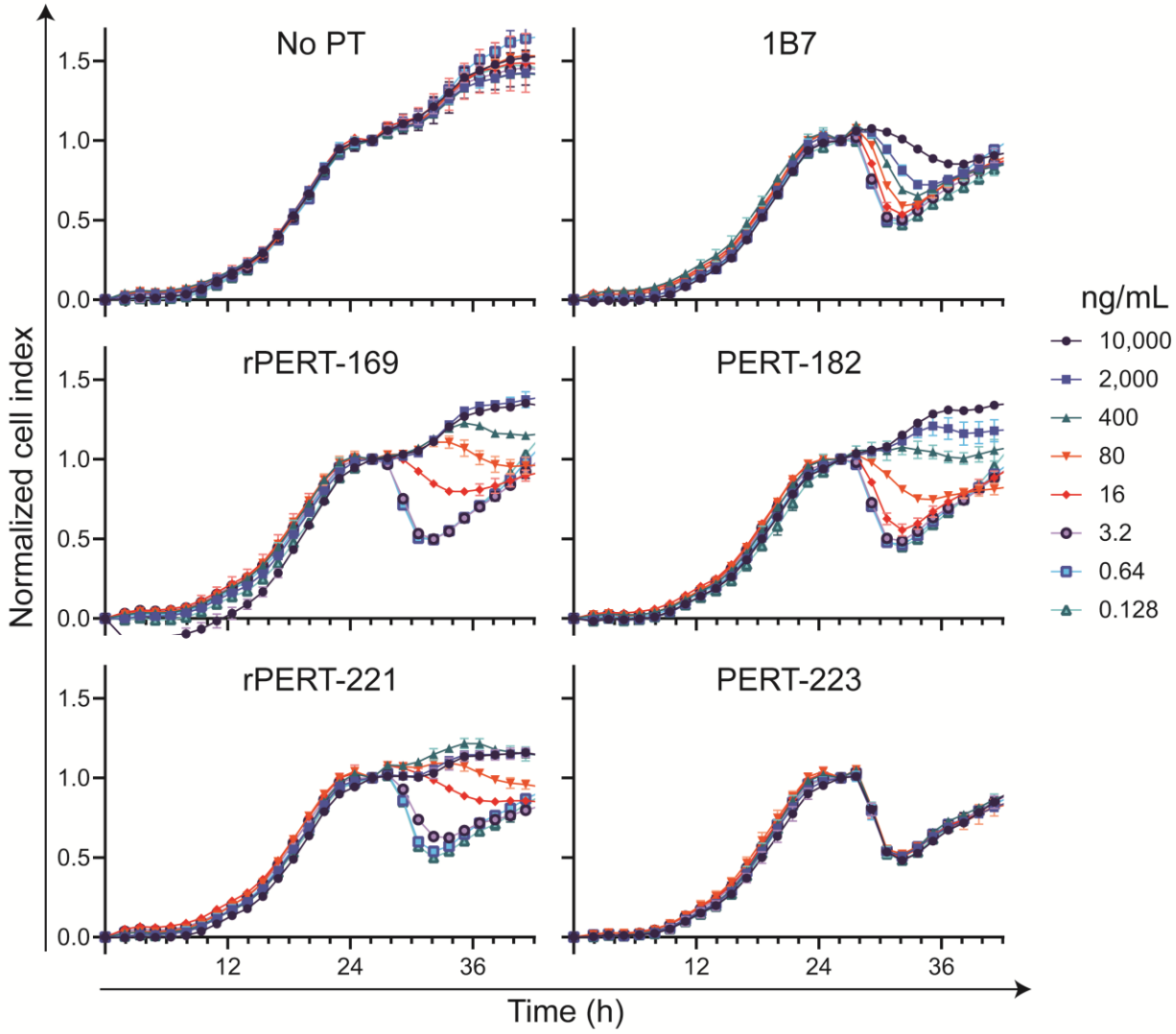

Figure 22: xCELLigence RTCA plots of pertussis toxin neutralization. xCELLigence plates were prepared as optimized and pertussis toxin was preincubated with mAb that had been serially diluted. Preopsonized mAb was added to the CHO K1 monolayers after 24 hours of culture and then the cell index was monitored for ~18 hours. Cell indices were normalized to 1 hour after toxin administration to correct for any disruption of the cell monolayer by pipetting. Points show the arithmetic mean of duplicate data, error bars show the standard deviation, every 5th data point is plotted to simplify the visual appearance of the graphs. Data were collected in two independent experiments.

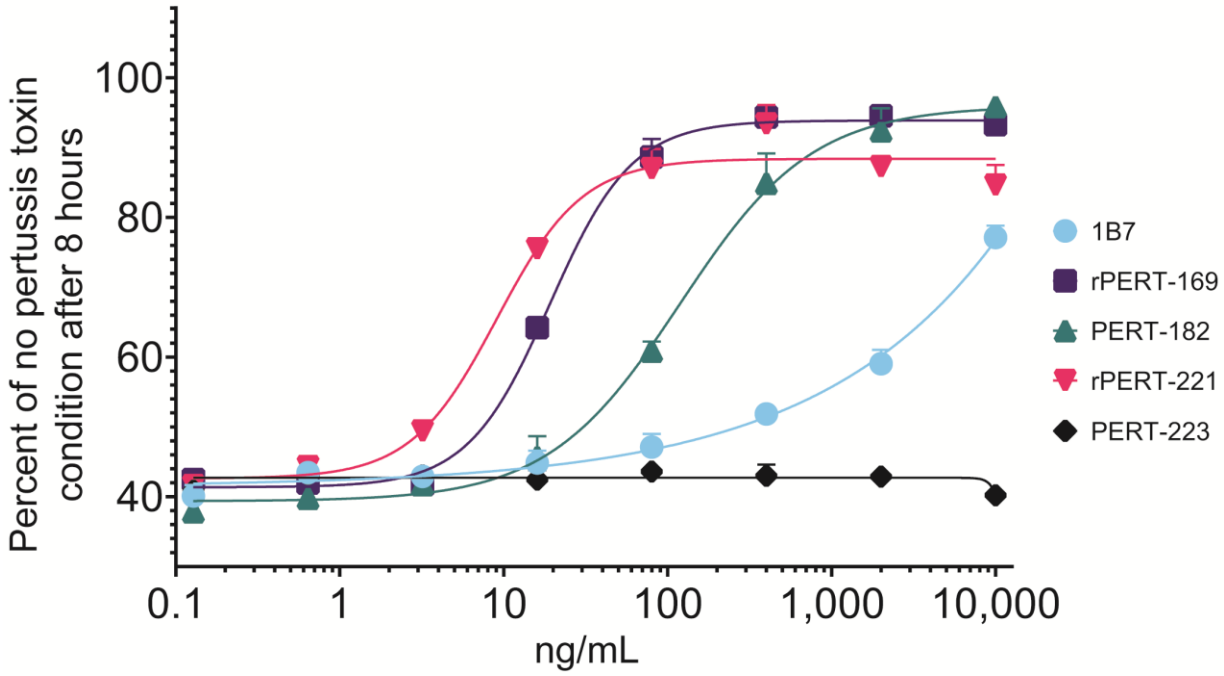

Figure 23: xCELLigence dose-response curves of pertussis toxin neutralization. By analyzing data at a single time point, 8 hours after pertussis toxin administration, dose-response curves can be generated. Normalized cell indices are compared with the no pertussis toxin condition at the same time point to determine the percent of no toxin controls. Because the CHO K1 cells do not die, the lowest percentage calculated is about 40% of the no pertussis toxin condition. Data were collected in two independent experiments. Points represent the arithmetic mean of duplicates, error bars show standard deviations, curves show the four-parameter logistic regression.

Discussion

Whooping cough, or pertussis disease, is reemerging across the globe and causes significant morbidity and surprisingly high mortality among high-risk patients, such as premature neonates and infants, who cannot be vaccinated. *B. pertussis* has a number of virulence factors that are involved in its pathogenesis, but pertussis toxin is thought to be the primary effector of disease. Pertussis toxin is a complex of a single A subunit and a B subunit composed of five components. The B subunit is responsible for adhesion to and trafficking within host cells. The A subunit conjugates ADP-ribose to host G-proteins, primarily the α_i inhibitory G-protein. This

binding blocks the association of G-proteins with their cognate GPCRs and interferes with regulation of GPCR activity (195). Altered GPCR activity leads to several major effects that are thought to result in pertussis disease. Because both subunits of the toxin exhibit distinct functions, pertussis toxin function can be inhibited by blocking the interaction of either subunit with their receptors or substrates. Numerous murine mAbs have been described that are capable of inhibiting pertussis toxin. One murine mAb, 1B7 has been pursued as a potentially therapeutic mAb to prevent the development of whooping cough in susceptible or unvaccinated individuals.

While 1B7 and other mAbs have been discovered using relatively simple assays to screen for binding to pertussis toxin, it would be optimal to screen for functionally active mAbs that inhibit pertussis toxin activity through neutralization. The established assay to assess pertussis toxin neutralization is cumbersome and difficult to perform with rigor and reproducibility because it relies upon microscopic evaluation and scoring of CHO K1 cell clumping in 96-well plates. xCELLigence RTCA provides a powerful tool that may be able to replace the microscopy-based CHO K1 cell clumping assay. By increasing the throughput and reproducibility as well as the objectivity of this assay, RTCA can supercharge the search for pertussis neutralizing mAbs.

In this chapter, the xCELLigence CHO K1 clumping assay was optimized and used to assess the capability of a panel of mAbs to inhibit pertussis toxin function. I determined that the xCELLigence assay detects pertussis toxin-induced clumping of cell monolayers. More importantly, the resolution of the xCELLigence assay was great enough that accurate dose-response curves could be generated to compare mAbs to the reference control 1B7. Several mAbs, such as PERT-221 and PERT-169, were found to neutralize pertussis toxin more potently

than 1B7. These data strongly support the use of xCELLigence as a next-generation tool to identify toxin neutralizing mAbs from a variety of different cell sources.

There are some limitations of this assay, although they are almost all shared with the microscopy-based pertussis toxin neutralization assays. First, there are two subunits of pertussis toxin and both subunits may be inhibited. Binding of mAb to the B subunit may block association of the toxin with its mammalian cell receptors or it may prevent the toxin from being correctly trafficked from an endosome into the Golgi apparatus then to the endoplasmic reticulum. Notably, binding of the B subunit does not directly inhibit the toxic activity of the A subunit. MAb may also bind to the A subunit, and by doing so can inhibit its enzymatic activity, fully inactivating the toxin. Antibody binding of the A subunit may also alter the intracellular trafficking of pertussis toxin. All these mechanisms have been reported for humanized forms of the murine mAbs 1B7, which is A subunit specific, and 11E6, which is B subunit specific (197). There is some thought that A subunit inhibition may have superior *in vivo* efficacy; however comprehensive head-to-head comparisons of A and B subunit mAbs using *in vivo* models have not been completed. Another limitation of the CHO cell clumping assay is that there is evidence that the clumping phenotype relies more upon the B subunit than on the A subunit (195). The larger role of the B subunit in the CHO cell clumping assay could mean that screening for functional toxin inhibition may be most effective at identifying B subunit binding antibodies. My findings that 1B7 is less potent than several mAbs that bind to the B subunit supports the idea that the CHO cell clumping assay depends more heavily on the B subunit. All these caveats need to be considered, but do not preclude the use of the xCELLigence CHO K1 cell clumping assay as a functional pertussis toxin neutralization screening tool.

In conclusion, xCELLigence RTCA is a powerful tool that enables quantitative, objective, and high throughput functional screening of antibodies or other inhibitors of pertussis toxin. Here I demonstrated that xCELLigence can detect pertussis toxin activity on CHO K1 cell monolayers. I also used a previously generated panel of pertussis toxin antibodies and determined that the xCELLigence assay I described detects toxin neutralization in a quantifiable, reproducible manner. Using this assay, I tested thirty-six human monoclonal antibodies and observed toxin neutralization activity more potent than that exhibited by the reference antibody 1B7. Further study is necessary to fully understand the intricacies of why these mAbs differ from 1B7. Additional screening for even more potent mAbs or for additional A subunit mAbs can be done using the xCELLigence assay described here.

CHAPTER V

V. Conclusions and future directions

Summary and conclusions

Gram-negative bacterial diseases are a significant problem facing modern medicine and society. Bacteria including *A. baumannii, E. coli, K. pneumoniae,* and *P. aeruginosa* are opportunistic pathogens that frequently exhibit significant antimicrobial resistance. Many modern medical innovations depend upon the ability to intentionally disrupt antimicrobial functions. For example, surgeries disrupt physical barriers to bacterial infections and cancer therapies suppress the ability of the immune system to prevent and clear infections. These interventions are only possible because antibiotics allow for the treatment or prevention of bacterial infection. The pandemic of antimicrobial resistance threatens our ability to perform some of the foundational interventions of modern medical care. There is an urgent need to better understand the interactions between the human immune system and these Gram-negative pathogens to inform generation of vaccines, therapies, and treatment strategies. Since most species of bacteria exhibit unique determinants of pathogenesis, each organism has its own pattern of interactions with the human immune system. The humoral immune system, particularly the antibody response, is exceptionally useful for evaluating the host-pathogen interface because antibodies have multiple uses as powerful tools to understand biology, guide therapeutic development, and inform vaccine design and potentially as intrinsically active effector molecules.

In my thesis work, I evaluate three classes of antibacterial antibodies to three different Gram-negative bacterial pathogens and begin to describe how antibodies alter bacterial pathogenesis. In Chapter 1, I review the antibacterial antibody classes I evaluate in subsequent chapters and describe how those antibody types have been studied so far. In Chapter 2, I evaluate antibodies to outer membrane proteins and identify mechanisms through which they alter *E. coli* host-pathogen interactions. Chapter 3 describes antibodies generated to *K. pneumoniae,* one of which targets capsular polysaccharide and increases complement-mediated killing of the bacteria This chapter also highlights important future studies to determine the functional mechanisms of anti-CPS antibodies. Finally, Chapter 4 defines a novel assay to probe the impacts of antitoxin antibodies that neutralize pertussis toxin from the reemerging pathogen *B. pertussis*.

Antibodies to outer membrane proteins (OMPs) are highly interesting as tools and drugs because they can inform knowledge about basic OMP functions. OMPs serve as critical mediators of bacterial interactions with the outside environment, and I hypothesized that mAbs may inhibit these OMP-dependent functions. I was unable to identify human antibodies to *A. baumannii* siderophore receptors, but in the process of validating antigen purity, I was able to identify and isolate four unique, naturally occurring antibodies that bound to OmpA of *E. coli*. Using recombinant partial rOmpA$_{Cterm}$ and natural *ompA* sequence variability, I determined that ECOL-11 bound to the extracellular loops of the N-terminal porin domain of OmpA. Binding of ECOL-11 to *E. coli* was visible by immunofluorescence of the laboratory adapted commensal K12 MG1655 strain of *E. coli*, but less prominent with the clinical uropathogenic isolate UTI89. My initial hypothesis was that OmpA expression levels would explain this differential binding, however that hypothesis was proven incorrect by demonstrating the inverse difference in mRNA transcript levels and no differences between protein level expression assessed by Western blot.

I next evaluated the capsular thickness of K12 compared to UTI89 and showed that UTI89 produces a thicker polysaccharide capsule layer that I propose is responsible for the decreased binding to UTI89 by immunofluorescence that we observed. Antibodies can have a variety of functions due to interactions with the host immune system. Antibody-dependent cellular phagocytosis (ADCP or opsonophagocytic killing) is one important host reaction to antibody bound to a pathogen. I assessed the ability of ECOL-11 to enhance ADCP and found that ECOL-11 decreased adhesion to and phagocytosis by RAW 264.7 macrophage-like cells. Because this observation persists when FcγR binding deficient LALA-PG and when single binding site Fab were used, I concluded that this decrease was due to ECOL-11 blocking the natural adhesion functions of OmpA that have previously been reported. Finally, since host-pathogen interactions are of higher complexity than what can currently be modeled *in vitro*, I assessed the capacity of mAbs to *E. coli* to alter organ bacterial burdens using a murine model of lethal sepsis infection. I did not detect any significant changes in bacterial burdens with anti-OmpA mAb prophylaxis. By studying mAbs to OMPs, I confirmed that humans generate somatically hypermutated and class-switched antibodies to *E. coli* even in the absence of known infection. Furthermore, I supported evidence that OmpA serves as an important adhesion to eukaryotic cells that can be inhibited.

Perhaps the biggest success story of antibacterial antibodies comes from the results seen by vaccination using capsular polysaccharide to protect from *N. meningitidis*, *H. influenzae*, or *S. pneumoniae*. These vaccines have reduced the morbidity and mortality of these infections immensely since their implementation. Capsular polysaccharides are a key virulence factor for *K. pneumoniae*, an organism that frequently is carbapenem resistant. To better understand the contribution of anti-CPS antibodies to the human immune response to *K. pneumoniae*, I

generated four antibodies that bound to methanol-fixed whole bacteria. These mAbs were unique and exhibited three binding patterns against the small strain panel I tested. Two mAbs bound to a wide variety of isolates, suggesting specificity for a conserved motif, possibly a protein. One mAb bound a WT isolate but not an isogenic *wzy* deletion strain, indicating it may be specific for Wzy or for a product of the Wzy polysaccharide polymerase function. A final mAb, KPNA-14 only bound to a single isolate, but further work by our collaborators indicated that it was CPS specific and exhibits significant alterations of *K. pneumoniae* pathogenesis *in vitro* and *in vivo*. It is especially intriguing that we were able to generate potently binding mAbs to *K. pneumoniae* from a donor without a known history of *K. pneumoniae* infection. These data are the foundation of additional studies to further delineate the functions human anti-CPS mAbs have during *K. pneumoniae* disease.

Anti-toxin antibodies are historically the class of antibodies that have been used to benefit human health for the longest. The earliest known organism targeted therapeutics were anti-sera from immunized animals that neutralized the toxins of bacteria including *Corynebacterium diphtheriae* or *Clostridium tetani*. A major obstacle to enhancing anti-toxin therapies using monoclonal antibodies has been the difficulty of identifying neutralizing mAbs. Antitoxin antibodies are important to understand for *B. pertussis* because pertussis toxin-mediated Whooping cough has been resurgent in recent decades. To generate a rigorous and reproducible toxin neutralizing assay, I adapted a CHO cell clumping assay that had been piloted once previously by Gabriela Alvarado. Using real-time cell analysis on an xCELLigence instrument, I determined that it was possible to quantitatively monitor CHO cell clumping in response to pertussis toxin. Critically, I showed that human mAbs to pertussis toxin, previously identified by binding to pertussis toxin, could be assessed using this assay and the degree of

neutralization directly compared between mAbs at numerous concentrations and time points. The novel xCELLigence format I developed is superior to previous assays because it removes the subjective, highly trained observer and greatly decreases the labor required by the microscopy-based assay.

Future directions

Donor Identification

One of the most challenging aspects of studying antibacterial antibodies is identifying which donors to select for further study. For viral pathogens, there is longstanding precedent of using serum neutralization or binding titers to triage donors and select those with the highest titers of antibody. However, because bacteria are far more ubiquitous than most viruses and have significantly more antigens presented on their surfaces, screening serum for antibacterial antibody function is problematic. When a single antigen is used, serum screening distinguishes specific donors (21, 23, 109, 165); however, when using whole bacteria or enriched pools of antigens such as extracted outer membranes, serum screening of donors is challenging. We were unable to distinguish serum from pooled humans from any specific donors for any of the bacterial targets we evaluated, yet were able to generate somatically-mutated, class-switched IgG to *E. coli* and *K. pneumoniae*. There are a variety of possible explanations for the general reactivity of humans to bacteria. Perhaps most interestingly given the current trends in microbiology, is that many bacterial pathogens can also be commensal organisms or are closely related to members of the microbiota (168, 176, 198).

Since serum screening is not always useful for selecting donors of interest for bacterial targets, it may be beneficial to explore alternative sources of PBMCs that may be enriched for B cells secreting antibodies specific for bacteria. Human tonsillar tissue is an easily accessible tissue and is densely populated with B cells, which comprise >60% of all lymphocytes in that tissue (199). We have begun to explore the possibility of isolating mAbs from tonsillar B cells. Early evidence suggests that bacteria-specific antibodies are common in tonsillar B cell pools, though the breadth of species specificity is unknown. Even if antibacterial antibody-secreting B cells are infrequently found in tonsils, it is relatively easy to get very large B cell pools from tonsils, which can increase the probability of finding low frequency bacteria-specific B cells. Challenges remain in generating techniques to rationally recover antibacterial antibody sequences from B cells. Specifically, it is difficult to determine which bacterial antigens to target, and some bacterial antigens are difficult to produce. These obstacles impair antigen sorting of B cells, which many currently established high-throughput antibody sequencing workflows depend upon. However, developing antigen independent antibody sequence recovery techniques is an incredibly important continuing direction and significant new efforts are being conducted with this goal in mind.

Role of the microbiome

I identified antibodies to two Gram-negative pathogens, *E. coli* and *K. pneumoniae* from human donors with no known histories of infection with either organism. Both of these organisms are members of the commensal intestinal microbiome and exposure to these organisms from the microbiome could explain why somatically-mutated antibodies were present

in samples from these patients. Incredible efforts are being channeled into microbiome research to parse apart the interactions between the microbiome and human health. There are studies that identify specific pro-inflammatory and anti-inflammatory functions of antibodies specific for commensal microbes (174, 175). Most studies use polyclonal antibodies, although there has been limited work with monoclonal antibodies (52). The body of literature discussing interactions of the microbiome with the host immune system is growing rapidly, but largely focuses upon broad questions and obtaining an overarching understanding of these interactions. There are incredible opportunities to drastically increase our understanding of immune development and regulation by studying the specific interactions of organisms within the microbiome with specific components of the immune system, such as antibodies. By greatly expanding the repertoire of well-characterized antibacterial monoclonal antibodies, there are many questions that can be answered regarding the implications of antibody binding upon the homeostatic relationships between host and microbiome. Does antigen specificity impact the roles an antibody takes? Antibodies are often presented as a uniform entity that is proinflammatory or anti-inflammatory in the context of the microbiome, however there may be much more nuance to the antibody-microbiome interaction. Monoclonal antibodies with distinct antigen specificities can be used to answer these questions. Since commensal organisms often are consistently present, the host is likely intermittently exposed as mucosal barriers are disrupted (172). It would be interesting to follow the development, either practically with a time series, or computationally by evaluation of large sequencing panels to determine probable evolutionary pathways, of an antibody to a common bacterial target, such as *E. coli* OmpA. Since the exposure to antigen is likely intermittent over a long period, the selective course of antibody maturation may differ from both a “one-hit” infection like Ebola virus and a chronic infection like HIV. Studying the generation

and maturation of antibacterial antibodies could add additional details to our understanding of B cell development dynamics.

Antibody engineering

Over the course of my thesis, I discussed a variety of antibodies to several Gram-negative bacteria that bind and act through unique mechanisms. A significant dogma in the field of antiviral antibodies is that neutralization is sufficient for an antibody to prevent or minimize disease. Neutralization is a potent effector function for bacterial antitoxin antibodies, which can intrinsically inhibit toxin function through the action of binding, as I demonstrated for antibodies to pertussis toxin in Chapter IV. Neutralization is not, however, a function that is likely to have significant impacts upon bacterial pathogenesis for antibodies that bind polysaccharides nor outer membrane proteins. Two main pathways of antibacterial function are through opsonophagocytic killing and complement-mediated killing (200-202). The former depends on antibody coating the organism and interacting with Fc receptors on host phagocytic cells. Ravetch *et al.* have evaluated Fcγ receptors to elucidate the roles that antibody binding to FcγR has during infections. Their work has identified specific changes to Fc regions, primarily differential glycosylation, that significantly alter mAb-FcγR interactions. Sialyation of the Fc domain increases Type II FcγR binding and has anti-inflammatory effects while fucosylation of the Fc domain preferentially increases Type 1 FcγR binding and has proinflammatory effects (203). Altering Fc glycosylation increased the antitoxin efficacy of mAbs to *Bacillus anthracis* lethal toxin and improved efficacy of SARS-CoV-2 therapeutic mAbs (204-206). Alterations that

enhance FcγR binding could greatly increase the antibacterial efficacy of mAbs by increasing opsonophagocytic killing.

Complement mediated killing is also dependent upon Fc interactions. The classical complement pathway is initiated by antibody binding to an organism. C1q then associates with the antibody to initiate the cascade that culminates in the formation of the membrane attack complex (1). C1q is most effectively fixed by IgM pentamers and hexamers, although IgG3 can also efficiently fix complement. Antibody engineering has also generated hexameric IgG that more effectively fix C1q than monomeric IgG (207). Since these two host-dependent effector functions are highly important for control of bacterial infections, there is the potential for antibody engineering to greatly enhance the therapeutic or prophylactic potential of antibacterial mAbs. The bispecific antibody MEDI-3902 to *Pseudomonas aeruginosa* PcrV and Psl antigens (106) is an excellent example of how the study of monoclonal antibody functions and antibody engineering can be combined to rationally design therapeutic antibodies that are optimally therapeutically effective. Historically, IgG have been the only antibodies targeted for therapeutic development because of the difficulties of producing the other isotypes in a manner and volume compatible with pharmaceutical-grade production. Antibody engineering may increase the ability to develop IgM or IgA antibodies that may be more effective at antibacterial effector functions, such as increased complement fixation by pentameric and hexameric IgM.

Antibody engineering also is a powerful tool for investigating the mechanisms through which antibodies act upon bacteria. I was able to demonstrate the importance of antibody engineering in Chapter II using FcγR variant antibodies to determine that the decreased phagocytosis by RAW264.7 cells was not due to the antibody Fc domain binding to FcγR on the cells. The LALA-PG mutations (L234A, L235A, P329G) eliminate FcγR binding while

preserving the overall structural integrity and antigen specificity of a mAb. As a result of decreased FcγR binding, LALA-PG variant mAbs are highly useful to understand the contribution of FcγR binding to the observed functions of the wild-type mAb. A very large number of antibody variants have been described (122). One of particular relevance to antibacterial mAbs is the K322A variation. K322 is important for the association of IgG with C1q to trigger the classical complement pathway (208). Mutating the mAb sequence to change this residue to an alanine largely eliminates antibody-dependent complement fixation. Although I did not test K332A variant antibodies for altered activity to *K. pneumoniae*, I hypothesize that this variation would eliminate the serum killing we observed and possible minimize the phagocytosis increase we detected by decreasing pro-phagocytic C3b deposition upon the bacterial surface.

Animal models

A major limitation of my work and important future direction for any study of antibacterial antibodies is the difficulty of developing *in vivo* models of infection that accurately represent the pathogenesis and physiology of bacterial infection in a human. For my work, we designed a highly stringent, lethal model of disseminated septic *E. coli* exposure. However, bacterial burdens decline over the course of the model, suggesting that infection is not established (data not shown). Mice also rapidly succumb to systemic symptoms of septic shock after administration of Gram-negative bacteria, like *E. coli*. Neither of these observations are consistent with human bacterial infections that require medical intervention. While other models of bacterial infection exist, they are often highly specific to a particular disease state, such as

urinary tract infection or osteomyelitis and are technically more challenging to perform. Site-specific models combine with the relatively poorly studied dynamics of human antibodies in murine models to make antibody therapeutic models of infection even more complex. Halpern *et al.* radiolabeled human IgM mAbs and monitored their organ distribution and found that the majority of mAb is found in the liver after 4 hours (209). Interestingly, in my studies to establish the *E. coli* septic model we used in Chapter II, bacterial burdens were highest and most consistent in the liver. However, the distribution of IgG appears to differ from IgM and different organs likely have differential ability to recruit immune effector cells that could change the dynamics of antibody-bacteria-host interactions (210). Our model was designed for general applicability, and we did not detect any significant changes in bacterial burdens. Our model does not eliminate the possibility that alternative models may detect important physiologic interactions with mAb treated *E. coli* infections. Significant future work could be done to optimize murine models of both *E. coli* infection and antibacterial antibody treatment.

Systemic inoculation with Gram-negative bacteria usually results in the development of septic shock in a mouse and septic shock is often lethal, independent of the organism used. I am particularly interested in using TLR4-deficient mice to investigate systemic bacterial infections, as these mice are unable to respond through the traditional LPS response pathway. A few potential strains include C3H/HeJ mice which have a spontaneous mutation in the TLR4 gene, B6.B10ScN-Tlr4$^{lps\text{-}del}$/JthJ mice which have a 74,723-nucleotide deletion that completely eliminates the TLR4 gene, or B6(Cg)-Tlr4$^{tm1.2Karp}$/J mice which have been engineered to lack exon 3 of the TLR4 gene. These mice are less susceptible to LPS-induced septic shock, and it may be possible to more effectively establish Gram-negative bacterial infections in these mice to accurately model the disease course in humans.

Strains and growth conditions

As I progressed through this thesis work, I repeatedly encountered instances in which there were strain-to-strain differences in binding, aggregation, phagocytosis, and complement killing. In many cases there were also differences within the same strain grown under different conditions. These observations are not surprising, the variability of bacterial physiology is colossal. It does, however, emphasize the immense importance of taking significant time to determine the optimal strain, medium, and growth conditions with which to culture an organism prior to initiating an antibody characterization effort. It is tempting to approach all *E. coli* infections as just that, an *E. coli* infection. This universal grouping is not an accurate representation of *E. coli* disease though, as meningitis causing *E. coli* experience significantly different selective pressures than those that cause urinary tract infections or those that persist on spinach and cause diarrheal disease (142, 211). These selective pressures alter the antigens that an *E. coli* is presenting to the host immune system and the infection site changes the host-dependent effector functions that are relevant to that disease (212). There is also significant genetic variability among isolates of a single bacterial species that may drastically alter the pathogenesis and the impact of mAb upon that strain (213-215). Determining the specific disease, the strains of interest, and how to replicate the *in vivo* nuances of that disease using *in vitro* culture systems are essential to maximizing the potential to generate and study antibodies that are physiologically relevant and can answer fundamental biological questions about the pathogenesis of a bacterial disease.

Concluding thoughts

Over the course of my thesis, I studied antibodies to several bacterial pathogens that bind to different antigenic classes. I identified specific functions that these antibodies had, such as altering opsonophagocytic activity and inducing aggregation. I have also tested the impact of antibodies upon numerous other possible antibacterial functions and was not able to develop effective assays due to the lack of controls or did not identify significant phenotypes associated with mAb binding to bacteria. I have not discussed these incomplete and unsuccessful assays in this document. The sheer breadth of possible antibacterial functions is exceptionally daunting when approached in an unbiased manner, as I did. I also discussed several other obstacles to the identification of antibacterial antibodies in the future directions. None of these obstacles are new, but to this day, the barriers to interrogating the interactions between antibodies and bacteria remain a significant impediment to our understanding of the antibody-mediated host-bacterial interface. New technologies and approaches continue to advance the field and many of these novel techniques were adapted in my work, and more are being adapted by my colleagues. By reemphasizing the challenges of antibacterial antibody development in the context of our unique approaches and skills, my work has helped identify critical frameworks necessary for success going forward with antibacterial antibody research. Even with the complications I encountered, I successfully identified and characterized multiple antibodies to unique Gram-negative bacteria. More skillfully strategized plans can continue to improve upon my successes and failures.

First and foremost, the desired functional outcome of an antibody-bacterial interaction must be defined at the onset, and all screening assays should be executed with this in mind. The approach we are currently taking to identify antibodies that induce complement-dependent

killing of non-typeable *H. influenzae* is an excellent example of rational screen design. The pertussis toxin neutralization assay I developed and described in Chapter 4 is another example of an approach that uses functional screens to identify antibodies of a defined class. Reporter bacterial strains are another powerful tool that should be used to identify antibodies that will help understand basic bacterial biology. A strain of *A. baumannii* that produces luminescence or fluorescence upon de-repression by the ferric uptake regulator (Fur) could enable the detection of human antibodies that alter bacterial metal metabolism. By defining the desired functional outcome early, less tailored downstream steps can be used to increase the throughput and rapidity of antibacterial antibody development.

Second, processing large numbers of B cells should take priority over identification of the ideal donor who has recovered from infection. In this thesis work, I studied unique, somatically-mutated, class-switched antibodies from donors with no known history of infection with the bacterial target of those antibodies (*E. coli* or *K. pneumoniae*). I also generated data suggesting the general human population has a high basic level of sero-reactivity to bacteria. However, antibodies to bacteria that are matured and act in highly functional manners are rare and isolating these mAbs requires studying as many antibodies as possible. Since normal human donors possess B cells that secrete antibacterial antibodies, normal human PBMCs, or PBMCs from patients with other infections, could be used for antibacterial antibody isolation. Using B cells from normal donors also addresses another concern of antibacterial antibody research, that most bacterial pathogens are opportunistic microbes that only cause disease in the presence of stressors that induce immunocompromised states. In other words, patients infected with bacterial pathogens may not generate the desirable highly potent antibodies necessary to better understand bacterial biology. Without doing in depth comparisons of healthy donors B cells and B cells from

donors who recovered from bacterial infections, it may be best to evaluate pre-existing antibacterial antibodies that emerge through life in the absence of infection.

Third, in addition to defining the desired functional outcome, it would be best to define the disease state of interest beyond the broad category of Gram-negative bacterial infection. Choosing to study a specific subset of Gram-negative bacterial disease, such as uropathogenic *E. coli*, greatly simplifies the strain and animal model selection to subsequently study the influence of antibody upon disease or critical virulence factors. Selecting the disease state also helps to guide the functional assay selection. Since adhesion to urothelial cells is a critical step for the establishment of urinary tract colonization and infection, antibodies that inhibit adhesion will likely induce more interesting phenotypes in models relevant for uropathogenesis than in models of meningitis. Essentially, when evaluating antibacterial antibodies, it is necessary to greatly increase the resolution of how we define the disease to rationally advance the field.

Overall, despite new techniques and the use of human hybridoma technology to approach the study of Gram-negative bacterial antibodies, we still recapitulated many of the barriers to antibacterial mAb research that have previously been reported. In doing so however, I have helped develop strategies to avoid and overcome the encountered difficulties. Despite the complications I faced, I identified antibodies with specific activities that alter Gram-negative bacterial biology and increased our understanding of the role OmpA has in interactions with host phagocytes. My thesis work also emphasizes the importance of tailoring antibacterial approaches to each pathogenic organism, as bacterial diversity means antibodies to OMPs, to CPS, or to toxins will be highly efficacious for some species and impractical for others. With this knowledge in hand, continued study of antibacterial antibodies will slowly but surely develop into a critical component of microbiology and modern medicine.

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