Human B cell responses to infection with pathogenic and commensal Neisseria species

by

Nancy Suk Yin So

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Molecular Genetics
University of Toronto

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University of Toronto

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ABSTRACT

The *Neisseria* genus includes pathogens, *Neisseria gonorrhoeae* (*Ngo*) and *Neisseria meningitidis*, as well as commensals. *Ngo*, the cause of gonorrhea, induces massive inflammation but a surprising lack of adaptive immune responses. We have observed that *Ngo* can inhibit both T cell activation and dendritic cell maturation through interaction with the host expressed co-inhibitory receptor carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). Therefore, I wondered whether B cells may also be affected in this manner.

Herein, I examine primary human B cell responses to infection with *Ngo*, as well as the other *Neisseria* species. B cells infected with *Ngo* show no sign of inhibition, regardless of their ability to bind CEACAM1, instead responding to gonococci with robust activation and proliferation. There are distinct subsets of B cells found in the periphery and, intriguingly, the IgM memory B cell subset expand and produce polyreactive IgM in response to gonococcal infection. These cells are innate in function, producing low affinity, polyclonal IgM that is protective against bacterial and fungal dissemination. This effect was broadly specific for *Neisseria sp.*, as B cell infection with all commensal *Neisseria* species examined induced innate B cell responses. Curiously, meningococcal strains avoid inducing the innate B cell responses, making it enticing to hypothesize that its avoidance of such an ancient immune response may
contribute to its ability to cause disease in humans. Finally, I tested whether gonococcal Opa protein binding to CEACAM1 affects primary human B cell activation, and show that no inhibition was observed. This absence of co-inhibitory function of neisserial-bound CEACAM1 may reflect inherent differences between distinctive cell types. Combined, the results in this thesis contribute new insight regarding the poorly characterized human IgM memory B cells, as well as to the function of CEACAM1 in lymphocytes.
Acknowledgments

I’ve learned many things during my time in graduate school, both scientific as well as life lessons, however none of this would have been possible without the help and guidance offered by those around me.

Completing a doctoral degree is challenging, with a myriad of (often frustrating) roadblocks, however I am fortunate enough to have a fantastic mentor, Dr. Scott Gray-Owen, who has always supported me in all of my endeavours. Combined with his incredibly motivational personality, Scott’s unwavering trust in his research team created an environment which not only allowed, but encouraged me to grow my project and make it my own. It is because of Scott and his vision of what science should be: the freedom to ask questions regardless of everything else, that I am the person I am today.

I am indebted to my committee members, Dr. Alberto Martin, Dr. John Brumell and Dr. Alan Cochrane for their guidance on this challenging project. They come from incredibly diverse backgrounds, and were an asset for maintaining perspective during difficult times.

I could not have survived graduate school without the support from past and present members of the Gray-Owen lab. Thank you for sharing your expertise, the stimulating discussions (both science and non-science related), and for listening when things got tough. I am incredibly lucky to have shared a large chunk of my degree with Wendy Dobson-Belaire, Helen Sarantis, Hannah Lee and Michael Brooks, an amazing group of people as well as great scientists.

A special thank you goes to Dr. Paul Sadowski, for taking some of us Gray-Owens under your wing. Your caring nature and natural enthusiasm for science made coming into the lab fun.

The Torontula family provided a much needed distraction from lab work. Initially, ultimate was a way to focus my mind on things other than science. In the end, I gained valuable life experiences and met an incredible group of women, all because of this ‘distraction’. Thank you, Lexi Marsh and Ofer Shai, for encouraging me to eliminate boundaries, a lesson learned on the ultimate field and applied to all areas of life.
I am thankful for my wonderful friends, especially Jooeun Lee, Melissa MacPherson, and Philip Goldbach, who make life in the lab a little better, and life outside of the lab fun.

My family has been a constant source of support during my graduate career, and without them this degree would not have been possible. Thank you to my parents, Paul and Wendy So, and to my sisters, Cheryl and Melissa, for your love and encouragement. My parents made innumerable sacrifices so that my sisters and I could have better lives, and I am grateful to them for this.

I am fortunate enough to have the best feline companions a graduate student could ask for. Kermit and Terra offered their support through loving head-butts and comforting purrs while I struggled through my degree. During stressful times, they are a constant reminder of the important things in life. Finally, I am lucky to have married such a wonderful and understanding husband, Chris Glencross. You have seen me at my best and at my worst, and your unconditional love and support make this degree yours as much as it is mine. Thank you for being there when I needed it most.
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<th>Description</th>
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<tr>
<td>αBCR</td>
<td>B cell receptor cross-linker</td>
</tr>
<tr>
<td>αCEACAM1</td>
<td>CEACAM1 cross-linking antibody</td>
</tr>
<tr>
<td>AID</td>
<td>activation-induced cytidine deaminase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>Btk</td>
<td>Bruton’s tyrosine kinase</td>
</tr>
<tr>
<td>cCpG</td>
<td>control CpG</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>CEACAM</td>
<td>carcinoembryonic antigen-related cell adhesion molecule</td>
</tr>
<tr>
<td>ChoP</td>
<td>phosphorylcholine or phosphocholine</td>
</tr>
<tr>
<td>CR3</td>
<td>complement receptor 3</td>
</tr>
<tr>
<td>CVID</td>
<td>common variable immunodeficiency</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>fHbp</td>
<td>factor H binding protein</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
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<tr>
<td>HSPG</td>
<td>heparin sulfate proteoglycan</td>
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<tr>
<td>iCpG</td>
<td>inhibitory CpG</td>
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<td>interferon alpha</td>
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<td>immunoglobulin</td>
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<td>IL-2</td>
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<td>ITIM</td>
<td>immunoreceptor tyrosine-based inhibition motif</td>
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<td>KLH</td>
<td>keyhole limpet hemocyanin</td>
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<tr>
<td>Lbps</td>
<td>lactoferrin binding proteins</td>
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<td>LOS</td>
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<td>LPS</td>
<td>lipo-polysaccharide</td>
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<tr>
<td>MAMP</td>
<td>microbe-associated molecular pattern</td>
</tr>
<tr>
<td>MDCC</td>
<td>monocyte-derived dendritic cell</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MZ</td>
<td>marginal zone</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide binding oligomerization domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NTHi</td>
<td>nontypable <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>ODN</td>
<td>oligodeoxynucleotide</td>
</tr>
<tr>
<td>OMV</td>
<td>outer membrane vesicle</td>
</tr>
<tr>
<td>Opa</td>
<td>colony opacity-associated protein</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
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<td>phycoerythrin</td>
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<td>toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
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</tr>
<tr>
<td>TT</td>
<td>tetanus toxoid</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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</table>
1 Introduction

1.1 The Neisseria species

The Neisseria are Gram-negative diplococci. They are human-restricted fastidious organisms, and colonization is limited to mucosal surfaces. Only two of the neisserial species are pathogenic: Neisseria gonorrhoeae, colonizer of the urogenital tract and cause of the sexually transmitted infection gonorrhea, and Neisseria meningitidis, colonizer of the nasopharynx and cause of meningococcal disease, including meningitis. Other neisserial species include Neisseria cinerea, Neisseria flavescens, and Neisseria lactamica, all three of which are members of the normal commensal flora within the nasopharynx. While colonization with N. gonorrhoeae (Ngo, gonococci) is considered potentially pathogenic in all cases, individuals are frequently colonized with N. meningitidis (Nme, meningococci) without disease manifestation. The factors that determine whether or not Nme colonization results in meningococcal disease are unknown, however it is clear that the individuals’ immune system is an important determinant. Consistent with this, young infants have the highest risk of developing meningococcal meningitis once maternal antibody titres have waned but before cross-reactive antisera can be developed by exposure to the neisserial species (Goldschneider et al., 1969).

1.1.1 Neisseria gonorrhoeae

With over 62 million new adult cases reported annually (WHO, 2001), gonorrhea is a considerable global health concern. As of 2009, gonorrhea was the second most reported sexually transmitted bacterial infection in Canada, with the rate of infections more than doubling since 1999 (Jayaraman et al., 2010). Disease manifestation differs between men and women, reflecting the primary site of colonization. Experimental urethral challenge of male volunteers demonstrates that symptoms of Ngo infection present within two to five days after infection with as little as 100 bacteria (Schneider et al., 1991). The hallmark of gonorrhea infection in men is the presence of purulent urethral discharge, which contains both neutrophils and the Gram-negative diplococci, however pain during urination is also common. Similarly, women infected with Ngo can experience neutrophil-dominated cervicitis, as the primary site of infection is within the endocervix, although this may or may not be accompanied by vaginal discharge (Gray-Owen et al., 2001). A large proportion of women infected with gonorrhea are either
asymptomatic or present with sub-clinical symptoms (Little and Hartsfield, 1978). This allows for the persistence of *N. gonorrhoeae* within the population, a critical aspect considering that humans are their only natural host. The asymptomatic infections are responsible for the major sequelae associated with *N. gonorrhoeae* infection, since the inflammatory response to established infection can result in damage to the reproductive organs, leading to sterility or disseminated gonococcal disease in both men and women (Gray-Owen et al., 2001). For women, there is an additional risk of pelvic inflammatory disease due to ascending gonococcal infection, which can include fallopian tube scarring leading to increased likelihood of ectopic pregnancies.

Although *N. gonorrhoeae* typically causes infection of the urogenital tract, colonization of other mucosal sites can occur. Infection of the anorectal mucosa is common, with some studies suggesting 40% of women with gonorrhea are also infected at this site. Symptoms of anorectal gonorrhea include proctitis (inflammation of the rectal mucosa), itching, bloody or cloudy discharge from the rectum and pain during bowel movements (Prescott et al., 2005). While the effect of pharyngeal colonization by *Ngo* is unclear, transmission of gonorrhea to men receiving oral sex has been reported (Tice and Rodriguez, 1981), and commensal *Neisseria* in the throat are presumed to be a primary source of antibiotic resistance genes acquired by multi-drug resistant *N. gonorrhoeae*. Gonococcal conjunctivitis is associated with newborns passing through the birth canal of an infected mother. As asymptomatic gonococcal infections are common in women, antibiotic eye drops are routinely given to neonates to prevent corneal scarring and blindness that can occur as a result of untreated gonococcal eye infections.

Although a single dose of antibiotics is sufficient for the treatment of gonorrhea, reinfection even with the same strain of *Ngo* is possible (Fox et al., 1999). Important with respect to this is that both sexual partners receive treatment to prevent a cycle of reinfection by an asymptomatic infection. Increasing rates of antimicrobial resistance has been a problem for the treatment of gonococcal infections, and penicillin, erythromycin and tetracycline are no longer recommended for this indication in Canada (Jayaraman et al., 2010). Recently, fluoroquinolones were no longer recommended for treatment of uncomplicated gonorrhea, also due to antimicrobial resistance, leaving the extended-spectrum cephalosporins as the only recommended drug class (Campos-Outcalt, 2007). Currently, there is no vaccine for *N. gonorrhoeae*. Therefore, an emphasis on understanding how this pathogen interacts with the immune system may aid in the development of new therapies.
1.1.2 Neisseria meningitidis

The rate of \textit{N. meningitidis} asymptomatic carriage has been reported to be about 10.9% of the population, with colonization increasing steadily from birth, peaking at the ages of 15-19, then steadily declining with age (Cartwright et al., 1987). While carriage rates as high as 30% have been reported in some regions, this can increase to 100% during epidemic waves that occur periodically (DeVoe, 1982). Distinct from that of other neisserial species, \textit{Nme} possesses a polysaccharide capsule, which allows for aerosol transmission between individuals (Nelson, 1996), and confers protection against phagocytosis and complement-dependent killing of these bacteria in the bloodstream. The various carbohydrate capsule types have served as a convenient way to differentiate between meningococcal strains. In total, there are 13 serogroups, based on differences between capsular polysaccharides. While 5 serogroups have been the main contributors to disease: A, B, C, Y, and W-135, epidemics of serogroup X have emerged within the past decade. Serogroup B is the primary cause of meningococcal disease in industrialized nations, with a minority of disease caused by serogroup C (van Deuren et al., 2000). Distinctively, serogroup A is most commonly associated with disease in developing nations, as evidenced by the extremely high rate of meningococcal disease incidence, which can be as high as 1% of the population during their frequent epidemics (van Deuren et al., 2000).

The ability of \textit{Nme} to colonize humans asymptotically allows it to persist within the population, however the events which trigger the change from carriage to meningococcal disease are multi-factoral, and include environmental and social factors (De Wals et al., 1981; MacLennan et al., 2006; Stanwell-Smith et al., 1994), immune status of the individual (Ellison et al., 1983; Goldschneider et al., 1969; Stephens et al., 1995; Weller et al., 2008), as well as the infecting strain itself (Caugant et al., 1988). Interestingly, the highest rates of meningococcal disease occurs in young children under the age of two (Booy et al., 2007), which is disproportionate to the relatively low colonization rates within this age group (Gold et al., 1978a).

Meningococcal disease can manifest in a variety of ways, such as sepsis, meningitis, or pneumonia, and less commonly as conjunctivitis or pelvic inflammatory disease. Individuals with disseminated meningococcal disease may initially experience flu-like symptoms, which can rapidly progress to septic shock unless bacterial dissemination in the bloodstream can be
recognized and controlled. Symptoms of disseminated meningococcal disease are general, and include the development of chills, fever, rash, lower back pain or muscle aches. Sepsis can occur rapidly, within hours (van Deuren et al., 2000). Meningitis may occur as a result of disseminated disease, as meningococci tend to drive inflammation after they invade the meninges, the tissues surrounding and protecting the brain (Nassif et al., 2002).

The mortality rate of meningococcal sepsis is high, ranging from 20-80% depending on the study (van Deuren et al., 2000). Disease severity is usually associated with poor clinical outcome. The release of endotoxin through bacterial lysis and/or the constitutive liberation of membrane blebs, which are naturally released during bacterial growth, undoubtedly contributes to pathogenesis (Prins et al., 1998), however uncontrolled dissemination of the meningococci itself likely has a greater contribution to pathogenesis (van Deuren et al., 2000). Meningitis mortality rates range from 3-13% in adults (van de Beek et al., 2006). In children, the overall mortality rate is 4.8%, with an elevated risk of fatality associated with specific age groups: 7.4% for 1-4 year olds and 10% for 10-14 year olds (Theodoridou et al., 2007).

In North America, the current treatment regimen for meningococcal disease includes one of a trio of antibiotics: rifampin, ceftriaxone, and ciprofloxacin. While there is an unusually low rate of antimicrobial resistance development with respect to these three treatment options in the US, rifampin and ciprofloxacin resistant strains have been reported elsewhere globally (Jorgensen et al., 2005). Recently, ciprofloxacin resistant strains were documented in the US (Wu et al., 2009), highlighting the eventual need for new and improved therapeutics.

In light of the potential for the development of antimicrobial resistance, vaccination is being used as a strategy to control, and hopefully one day, eliminate meningococcal disease. Numerous *N. meningitidis* vaccines exist, each of which have their specific strengths and weaknesses. Polysaccharide (PS) vaccines have been successfully used to prevent outbreaks in endemic populations (Flitter et al., 2007; Zombre et al., 2007). High efficacy rates were observed for individuals over the age of 2 (De Wals et al., 2001), however PS vaccines do not protect the most vulnerable population for *N. meningitidis* infection, children under the age of 2 (De Wals et al., 2001; Gold et al., 1975). Triggering T-independent responses, PS vaccines do not elicit immunologic memory and vaccine efficacy wanes dramatically in subsequent years post vaccination (Gold et al., 1978b). Additionally, PS induced antibodies do not affect colonization (Khatami and Pollard, 2010), so herd immunity is
not induced. Also of concern is that immunologic hyporesponsiveness has been observed in connection with PS vaccines, presumably by activation of a substantial proportion of PS-specific B cells without re-establishing a reservoir to replace them (Gold et al., 1975; Granoff and Pollard, 2007).

Protein conjugated PS or ‘conjugate’ Nme vaccines address a lot of the shortcomings of PS vaccines, primarily due to their ability to induce B cell memory through T dependent responses. Conjugate vaccines also eliminate nasopharyngeal carriage, which ultimately reduces transmission and can result in herd immunity (Maiden et al., 2008). However, elimination of asymptomatic Nme carriage could reduce competition amongst colonizers of the pharyngeal niche, and may allow for increased rates of carriage for other opportunistic pathogens such as Streptococcus pneumoniae or Haemophilus influenzae. Waning antibody responses post-vaccination is still a problem, even with conjugate vaccines (Trotter et al., 2004).

Conjugate vaccines have been produced against all serogroups of Nme, with the exception of serogroup B, which is poorly immunogenic. The lack of response to serogroup B is because it contains α2-8 linked N-acetylneuraminic acid polymers, which are also present in a number of fetal tissues including the brain (Finne et al., 1987). Significantly, over 50% of the meningococcal disease in infants under the age of 1 are caused by serogroup B Nme (Baltimore, 2006). Outer membrane vesicles (OMV), which are naturally released during bacterial growth and resemble the membranes from which they are derived, have been successfully used to vaccinate against Nme serogroup B disease during outbreaks (Holst et al., 2005). However, OMV immunization elicits primarily strain-specific responses (Holst et al., 2005; Tappero et al., 1999), and may not affect asymptomatic carriage of Nme (Bjune, 1992; Boslego et al., 1995), thereby limiting its usefulness. Undoubtedly, a greater understanding of how this deadly pathogen interacts with the immune system could lead to improvements in vaccine design.

1.1.3 Commensal Neisseria

The other members of the Neisseria species are commensals, and share the nasopharynx niche along with the pathogenic Nme. Only on rare occasions have commensal Neisseria species been documented to cause disease (Dolter et al., 1998; Orden and Amerigo, 1991; Sinave and Ratzan, 1987; Wertlake and Williams, 1968). While there are many species of commensal Neisseria, including Neisseria cinerea, Neisseria flavescens, and Neisseria lactamica (Nla), the latter has
been the best characterized. Nla carriage rates are highest when children are most susceptible to meningococcal disease (Gold et al., 1978a). It has been suggested that colonization by Nla is protective against meningococcal disease by providing additional competition against colonization by Nme within the same niche. Additionally, carriage of Nla induces the production of antibody that is cross-reactive with multiple serogroups of Nme (Gold et al., 1978a). The commensal Neisseria are unencapsulated, therefore cross-reactive Ig is unlikely to recognize capsular antigens. Undoubtedly, there are many potential antigens that are able to induce cross-reactive Ig. Monoclonal antibodies recognizing LOS from both Nme and Nla suggest that either epitopes found within the LOS contribute in the induction of cross-reactive Ig (Kim et al., 1989). Additionally, a mixture of proteins less than 43 kDa prepared from Nla was used to vaccinate mice and was protective against death caused by Nme infection (Oliver et al., 2002). In an attempt to exploit this relationship to benefit more individuals, N. lactamica-based vaccines are being considered to induce protective immunity against the closely related N. meningitidis, although with varying success (Evans et al., 2011; Gorringe et al., 2009).

1.1.4 Neisserial Infection

All Neisseria sp. are believed to cause infection using similar mechanisms. Colonization occurs when the bacteria come into contact with the apical side of nonciliated mucosal epithelia. Their type IV pilus mediates initial adherence to the cell through interactions with human CD46 (Figure 1.1A) (Kallstrom et al., 1997). Retraction of the pilus (Merz et al., 2000) allows for tight adherence between the pathogen and the host cell, mediated by bacterial colony opacity-associated (Opa) proteins binding to apically expressed carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs, Figure 1.1B) (Chen et al., 1997; Gray-Owen et al., 1997b; Virji et al., 1996). CEACAM engagement by the bacteria triggers engulfment (McCaw et al., 2004), and transcytosis through the epithelial layer (Wang et al., 1998), as opposed to a paracellular route, which would require loosening of the tight junctions between cells (Figure 1.1C). Once released into the sub-epithelial space, the bacteria persist and proliferate using nutrients available in the interstitial fluid, and can interact with a variety of cells, including cells of the immune system (Figure 1.1D,E,F,G).
Figure 1.1  Summary of events leading to infection by *Neisseria* species.

Interaction with mucosal epithelia is initiated by neisserial type IV pili (A), bringing the bacteria close enough to the cell for tight adherence mediated by colony opacity-associated (Opa) proteins and host cell receptors, including carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (B). This allows for the transcytosis of the bacteria across the epithelial barrier (C), allowing for access to the subepithelial space. It is here where the *Neisseria* can interact with a variety of host cells (D, E, G), including lymphocytes (F). Adapted, with permission, from Gray-Owen and Blumberg (Nature Reviews Immunology, Volume 6(6):433-446) ©2006 by Nature Publishing Group.
Complement receptor 3 (CR3) has been suggested to mediate binding and engulfment of gonococci to primary human cervical epithelial cells. CR3 is a phagocytic receptor, its natural function being to engulf bacteria that have been covalently bound by C3b, a component of serum complement. Upon infection of cervical cells, initial association of Ngo is mediated through pilin binding to CR3 (Edwards et al., 2002). This brings the gonococci close to the cell, allowing for additional, likely stabilizing, interactions between porin and CR3 (Edwards et al., 2002), to ultimately result in engulfment of the gonococci (Edwards et al., 2000).

Persistence of the pathogenic Neisseria within the human host, as with all bacteria, requires access to iron for growth, although iron acquisition may not be required for mucosal colonization (Schryvers and Stojiljkovic, 1999). Free iron levels in serum and mucosa are low, reflecting an effective form of ‘nutritional immunity’ used by the host to control the growth of microbes. In the serum, iron is sequestered primarily by the iron transport protein transferrin. In the mucus and at sites of infection, iron is bound by lactoferrin. The pathogenic Neisseria produce receptors that can specifically bind and remove iron from these host proteins. Transferrin binding proteins (Tbps) and lactoferrin binding proteins (Lbps) are produced by both Nme and Ngo (Gray-Owen and Schryvers, 1996), however their usage of the Tbps and Lbps differ. Tbps seem to be more important for establishing gonococcal infection, since human male urethral challenge with gonococci that are deficient for both Tbps and Lbps resulted in no observable disease (Cornelissen et al., 1998). However, gonococci that were deficient for Lbps only, but expressed functional Tbps, were able to cause disease in all volunteers infected with this strain, as evidenced by the production of urethral discharge containing gonococci (Cornelissen et al., 1998). The role of Lbps in the production of gonococcal disease is uncertain, as only 50% of clinical isolates are able to compete for iron bound to lactoferrin (Mickelsen et al., 1982). In contrast, 100% of clinical Nme strains were able to compete for iron bound by lactoferrin (Mickelsen et al., 1982), which suggests that for meningococci, lactoferrin is an important iron source in the airway mucosa (Schryvers and Stojiljkovic, 1999).

1.2 Immune response to Neisseria

1.2.1 Complement

Complement is an important innate mediator of protection from disease caused by the pathogenic Neisseria. 58% of individuals with deficiencies in any of the terminal complement proteins that
form the membrane attack complex have experienced one bout of invasive meningococcal disease, correlating with an 8000-fold increase in risk for infection compared to the general public (Figueroa et al., 1993). The classical complement pathway requires antibody binding to bacteria to initiate complement recruitment. In the alternative pathway, complement component C3b interacts with bacterial surfaces directly following the spontaneous breakdown of C3. Both these pathways converge on the production of C3 convertase, an enzyme that cleaves C3, leading to the formation of C5 convertase. By cleaving C5, the C5 convertase initiates the formation of the pore-forming membrane attack complex, leading to killing of the bacteria due to loss of cellular homeostasis. Other products of these convertases are potent chemoattractants, mediators of inflammation and opsonins.

Complement deposition directly on the bacteria occurs on the lipo-oligosaccharide (LOS) or the capsular polysaccharide. Complement components can also be recruited to IgM or IgG binding to the *Neisseria*. However, IgM is 1000-fold more efficient at fixing complement than IgG (Cooper et al., 1983). While Ig specific for *Ngo* are induced at low levels during infection (Hedges et al., 1999), pre-existing antibodies present in serum of uninfected individuals show cross-reactivity with the pathogenic *Neisseria* (Cohen, 1967; Troncoso et al., 2002). *In vitro* assays show that these ‘natural’ antibodies are able to promote complement-mediated lysis of Gram-negative bacteria (Zhou et al., 2007). The observation that the classical complement pathway is more effective at killing complement susceptible strains of meningococci (Estabrook et al., 1997) indicate that these ‘natural’ antibodies may serve an important protective function.

The pathogenic neisserial species have evolved several mechanisms to subvert complement-mediated killing, highlighting the important role these ubiquitously abundant proteins have in protection from disease. Sialylation of the capsule and LOS can prevent complement deposition on the surface of the bacteria, leading to protection from killing (Estabrook et al., 1997; Gulati et al., 2005; Jarvis and Vedros, 1987). Furthermore, binding of the soluble host protein factor H to bacterial surfaces represents another highly effective mechanism for protection from complement-mediated lysis. Factor H inhibits the alternative complement cascade by preventing the assembly of C3 convertase. It has been reported that all *Nme* strains express a factor H binding protein (fHbp) (Masignani et al., 2003). The gonococci possess two mechanisms for interacting with human factor H. Factor H can be bound by *Ngo* that possess sialylated LOS (Ram et al., 1998b), as well as to porins (Ram et al., 1998a). Although a homolog of the
meningococcal fHbp exists in gonococci, deletion of the fHbp gene did not alter the level of factor H binding or affect serum resistance (Welsch and Ram, 2008). However, some gonococcal isolates have the ability to prevent the assembly of C3 convertase, rendering it resistant to complement-mediated killing by utilizing a mechanism independent of fHbp, involving the binding of another soluble host protein, C4 binding protein (Ram et al., 2001).

1.2.2 TLR and NODs

Recognition of neisserial species through the toll like receptors (TLRs) has been shown to be an important determinant of immune responses generated upon infection. Neisserial LOS, recognized by TLR4 (Pridmore et al., 2001) and the Lip lipoprotein produced by *N. gonorrhoeae*, recognized by the heterodimer TLR2/1 (Fisette et al., 2003), both induce the production of proinflammatory cytokines by cervical epithelial cells. The abundant neisserial outer membrane protein porin is also recognized by TLR2/1 (Massari et al., 2006), and has been shown to induce maturation and upregulation of co-stimulatory molecules on DCs (Singleton et al., 2005), and the activation and proliferation of B cells (Wetzler et al., 1996). In light of this evidence showing that porin is strongly stimulatory, it is not surprising that porin has successfully been used as an adjuvant for antigens that are poorly immunogenic on their own (Chiavolini et al., 2008; Liu et al., 2008; Mackinnon et al., 1999).

The effect of neisserial DNA signalling through TLR9 has been well characterized, and has been shown to induce IFNα production by splenocytes (Magnusson et al., 2007) and plasmacytoid dendritic cells (Dobson-Belaire et al., 2010), as well as the production of proinflammatory cytokines by human PBMC (Mogensen et al., 2006).

Recently, it has been shown that gonococcal OMVs induced the production of inflammatory cytokines by epithelial cells through the delivery of peptidoglycan to nucleotide binding oligomerization domain 1 (NOD1), apparently by fusion of the OMVs with lipid rafts (Kaparakis et al., 2010). Therefore, it is clear that the *Neisseria* species possess a multitude of innate effectors that contribute to the activation of immune responses.

1.2.3 Antibodies

Antibody specific for the Ngo is produced both in local genital secretions (Hedges et al., 1999; McMillan et al., 1979b) and systemically (McMillan et al., 1979a; Miettinen et al., 1989) in
individuals infected with gonorrhea, however the overall antibody responses are weak and its effectiveness in clearing the infection is questionable (Hedges et al., 1999). Epidemiological (Fox et al., 1999) and immunological evidence (Boulton and Gray-Owen, 2002; Lee et al., 2008) suggests that the production of immunologic memory might be suppressed during gonococcal infection. Observations from studies conducted by Plummer et al. suggests that immunologic memory is induced upon Ngo infection, but only with repeated exposure to gonococci, and is only partially protective against recurrent infections (Plummer et al., 1989). The lack of effective immunity induced in response to N. gonorrhoeae explains why individuals can be repeatedly infected by this bacterium.

Meningococcal colonization and/or disease can produce high titre Ig responses (Goldschneider et al., 1969; Pollard et al., 1999). Additionally, colonization by commensal Neisseria species can contribute to the induction of antibodies that are cross-reactive against meningococcal antigens (Gold et al., 1978a; Troncoso et al., 2002). A proportion of antibodies produced by neisserial colonization are T-independent ‘innate’ Ig (Gold et al., 1978a; Troncoso et al., 2002), which have been shown to correlate with protection from meningococcal disease (Goldschneider et al., 1969). The production of this innate Ig increases with age, but is absent in children between the ages of 6 months to 2 years (Goldschneider et al., 1969). Passive immunity imparted from the mother to child wanes during this time, leaving this age group with a disproportionately high risk of developing meningococcal disease upon exposure to the meningococci (Gold et al., 1978a; Trotter et al., 2006). Of interest, is that this period of increased risk of disease coincides with the only time during human development in which the production of innate Ig is compromised. The human innate B cell subset, which are responsible for the production of innate Ig, increase in frequency from the time of birth to 2-3 years of age, at which time adult levels of innate B cells are present within the blood (Weill et al., 2009). Individuals missing the innate B cell subset, either by genetic defects (Carsetti et al., 2005) or removal of the spleen (Kruetzmann et al., 2003), can predispose these individuals to infections with encapsulated bacteria, including meningococcal disease.

1.2.4 Neutrophils

Neutrophils are often thought of as the first line of defense for the host in response to pathogenic insult. Once they are recruited to the site of infection, their anti-microbial arsenal includes the
production of reactive oxygen species, which are highly bactericidal, and the release of granules that contain enzymes and anti-microbial peptides. The hallmark of gonorrhea is a massive urethral or cervical discharge consisting almost exclusively of neutrophils, some of which contain Gram-negative diplococci. The disease manifestations thus appear to result from an over-exuberant response of the neutrophils rather than direct damage caused by the gonococci.

The ability of neutrophils to bind, engulf and ultimately kill gonococci is enhanced by expression of Opa adhesins (Criss et al., 2009; Kupsch et al., 1993; Sarantis and Gray-Owen, unpublished observations). The Opa proteins bind to a variety of CEACAM receptors (Gray-Owen et al., 1997a), however CEACAM3 is the only member of the CEACAM family in which expression is restricted to neutrophils (Nagel et al., 1993). Taking this into consideration, as well as the fact that CEACAM receptors are expressed by a variety of tissues and cells, it has been proposed that CEACAM3 acts as a decoy receptor which aids in the clearance of gonococci during infection (McCaw et al., 2003). Alternatively, gonococci which have turned their Opa expression off may have a survival advantage over Opa⁺ Ngo, as intracellular Opa⁻ gonococci are able to not only survive within neutrophils, but also replicate (Criss et al., 2009). As the Opa adhesin is phase variably expressed (Murphy et al., 1989), the ability to evade neutrophil killing by hiding inside of the very cells meant to eradicate them is likely important for persistence within the host.

1.2.5 Adaptive Immunity

Protection from any infection is mediated by a combination of factors, including the successful production of an adaptive immune response. Considering Ngo is an extracellular pathogen, immunologic protection would likely be mediated by adaptive humoral immunity. Consistent with this, studies done using a cohort of female sex workers in Kenya show that, at least within this population, protection from ascending gonococcal infection correlates with the presence of antibody recognizing Opa adhesins (Plummer et al., 1994). Remarkably, immunity induced upon repeated natural Ngo infection provides incomplete protection against reinfection with the same serovar (Plummer et al., 1989).

While meningococcal disease is usually not fatal in adults, often presenting as pneumonia rather than invasive disease, children under the age of two are especially susceptible to meningococcal meningitis. The incidence of invasive disease in this age group remains high, in part, because of the difficulty in eliciting a PS-specific immune response in this age group (De Wals et al., 2001).
Conjugate vaccines can elicit serum bactericidal antibodies and can successfully protect against meningococcal disease in a serogroup-specific manner, however protection wanes with time (Trotter et al., 2004) making booster immunizations essential to any meningococcal-specific vaccine regimen. While vaccines effective against a variety of meningococcal serogroups are already available, a vaccine that can protect against all meningococcal serogroups with sustained serologic memory in all age groups is the ultimate goal. As such, this next section will focus on the adaptive immune responses generated upon infection with neisserial species.

1.2.5.1 Dendritic Cells

Bridging innate and adaptive immunity, dendritic cells are important sentinels of the immune response. Immature dendritic cells reside within the peripheral tissues and specialize in phagocytosis and collection of antigen at the sites of potential infection. Exposure to microbial-derived products initiates a DC maturation process and promotes their migration to draining lymph nodes. Mature DCs lose expression of phagocytic receptors, correlating with a decreased ability to phagocytose, but increased capacity to present degraded microbial fragments to T cells due to the concomitant upregulation of co-stimulatory markers and surface MHC class II expression. Once matured, DCs are able to prime naive T cells, allowing the initiation of T-dependent adaptive immune responses.

The effect of Ngo infection on DCs has been examined using human monocyte-derived DCs (MDDCs). Upon infection with gonococci, immature DCs appeared to undergo maturation, however infection with gonococci expressing Opa adhesin specific for host receptor CEACAM1 abrogated normal DC maturation (Yu et al., unpublished observations). While the molecular mechanism by which this occurs remains unexplored, it presumably relies on the phosphatase-dependent co-inhibitory signalling effects characterized downstream of Opa-CEACAM1 binding in the CD4+ T cell model of neisserial infection (see section 1.2.5.2.1 T cells and N. gonorrhoeae).

Similarly, infection with Nme has been shown to prevent human MDDC maturation and upregulation of co-stimulatory molecules (Jones et al., 2007). In this case, the ability of meningococci to mediate inhibition of DCs is independent of the co-inhibitory receptor CEACAM1.
1.2.5.2 T cells

T cells are modulators and mediators of co-stimulatory signals which can shape the antibody responses. While these lymphocytes are recruited to the genital tract during infection (Levine et al., 1998), there is a paucity of information regarding how bacteria may effect T cell function. The effect that *N. gonorrhoeae* have on human T cells serves as one of the few paradigms in this regard.

1.2.5.2.1 T cells and *N. gonorrhoeae*

Using purified human CD4$^+$ T cells, it has been established that infection with *Ngo* results in increased cellular activation and proliferation compared to cells left uninfected (Boulton and Gray-Owen, 2002). This is likely due to pattern recognition receptors responding to innate signals provided by the gonococci. The *Neisseria* species produce a number of factors that can interact with cells in different ways, including direct cellular adhesion via T cell-expressed receptor proteins. As described earlier, in section 1.1.4 Neisserial Infection, tight adherence between infecting *Neisseria* and host cells is mediated by Opa adhesins which can bind to CEACAM1 expressed by T cells. The vast majority of neisserial strains isolated from infected individuals bind to CEACAM1 (Virji et al., 1996), suggesting that this interaction is favourable for pathogenesis. Significantly, infection of CD4$^+$ T cells with *Ngo* that can bind CEACAM1 results in the inhibition of activation and proliferation of infected cells without having an effect on cellular viability (Boulton and Gray-Owen, 2002).

CEACAM1 is a transmembrane protein that contains 2 immunoreceptor tyrosine-based inhibition motifs (ITIMs) within its cytoplasmic domain. ITIM-containing receptors, such as FcγRIIB expressed on B cells, are called ‘co-inhibitory’ receptors since their ITIM must be phosphorylated in response to a co-engaged activating receptor before they can initiate an inhibitory cascade (Ravetch and Lanier, 2000). Gonococcal Opa binding to CEACAM1 expressed by CD4$^+$ T cells effectively promotes this prototypical ITIM-mediated effect, as T cell receptor (TCR) engagement in the presence of Opa-expressing *N. gonorrhoeae* causes the accumulation of phosphorylated CEACAM1 (Lee et al., 2008), and subsequent recruitment of SH2-domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 (Boulton and Gray-Owen, 2002), which dephosphorylates the T cell receptor (Lee et al., 2008) so as to reduce the activation and proliferation responses that otherwise occur (Boulton and Gray-Owen, 2002).
Because the gonococci are able to inhibit the ability of T cells to activate during infection, this may prevent the development of immunologic memory (Lee et al., 2008), explaining why repeat infections with the same strain of Ngo can occur (Fox et al., 1999). A potential contributing factor for this delayed development of memory could lie in the observation that neisserial outer membrane vesicles (OMVs) are able to bind CEACAM1 and inhibit human CD4\(^+\) T cell proliferation in a manner reflecting that of the bacteria from which they were liberated (Lee et al., 2007). This suggests that the bacteria may generate a ‘zone of immunosuppression’ due to the increased penetration of these small membrane vesicles away from the site of infection. It also has important consequences for the design of future OMV-based vaccines, since the presence of immune-suppressing Opa proteins are not presumably desirable.

T cell responses have not been a focus of infection models for the other neisserial species. A handful of reports show that mucosal T cell memory recognizing Nme as well as Nla was generated in adults, but are not present in children (Davenport et al., 2003; Morales-Aza et al., 2009; Vaughan et al., 2009). This implies that either the production of memory against these neisserial species are inefficient and requires prolonged colonization and/or that prolonged colonization by other closely related commensal Neisseria species can elicit memory responses which are cross reactive to both Nme and Nla. Whether Opa-CEACAM1 interactions contribute to this delayed development of T cell memory awaits in vivo studies.

1.2.5.3 B cells

B cells produce antibodies, a critical component of the immune response against extracellular pathogens such as N. gonorrhoeae and N. meningitidis. They may also contribute to the persistence of T cell memory due to their antigen presentation capacity (Lanzavecchia, 1985). The three reports discussed here represent the main body of knowledge regarding the effects of neisserial infection on B cell function.

1.2.5.3.1 B cells and N. gonorrhoeae

Only one report has examined the effect of Ngo infection on B cell function, focusing on the effects of Opa-CEACAM1 interaction mediated upon infection. In this report, the authors observed B cell death was induced upon infection with CEACAM1 binding gonococci (Pantelic et al., 2005). Since the majority of clinically isolated Neisseria species bind to CEACAM1 (Virji
et al., 1996), they proposed that this targeted killing of B cells prevents their differentiation into antibody secreting cells and explains the clinical observation of weak Ig responses in gonorrhea infected individuals (Hedges et al., 1999). Of note is that although CEACAM1 was expressed on purified B cells, CEACAM1 over-expression was induced by cytokine treatment prior to gonococcal infection assays (Pantelic et al., 2005). In some of their assays, additional signals from αBCR and CD40 ligand was also added to infection cultures. The artificial stimulation of B cells to induce CEACAM1 over-expression would obviously elicit many other responses, with the potential for activation-induced cell death, making it difficult to separate the specific effect of Ngo infection on B cell function.

There is other evidence to suggest that Opa-CEACAM1 interaction might directly affect B cell responses. Using transfected DT40 chicken B cells as a model, human CEACAM1 was able to inhibit activation events downstream of the BCR (Chen et al., 2001). This inhibition required an intact membrane proximal ITIM (Chen et al., 2001). ITIMs contain tyrosines, which allows for interaction with Src homology 2 (SH-2) domain containing proteins upon phosphorylation. CEACAM1 has been shown to be phosphorylated in response to B cell activation (Lobo et al., 2009), and can recruit SHP-1 and SHP-2, which have been shown to mediate the inhibition of T cells upon infection with Ngo (Chen et al., 2001; Chen et al., 2008; Lee et al., 2008). While signaling downstream of CEACAM1 in B cells remains largely unexplored, it seems plausible that these SHP-1 and SHP-2 dependent effects would parallel those observed in T cells.

Aside from B cell receptor responses, there is a strong body of evidence that certain neisserial factors are able to induce mitogenic responses in murine B cells (Wetzler et al., 1996). In addition to prototypical microbial-associated molecular pattern (MAMP) responses, B cells have also been shown to respond to neisserial porins via through TLR1/2 heterodimer (Massari et al., 2006). In all neisserial species, porin has been shown to be a major component of the outer membrane (Blake and Gotschlich, 1986). It exists as trimers, and allows for the diffusion hydrophilic molecules such as sugars (Olesky et al., 2006). The ability of neisserial porins to act as an adjuvant has been shown in many different systems (Chiavolini et al., 2008; Liu et al., 2008; Mackinnon et al., 1999). In light of this evidence that the neisserial porins can strongly stimulate B cells, and taking into account the potential of inhibitory signaling caused by Opa-CEACAM1 interaction upon infection with neisserial species, it is hard to speculate the cumulative effect of neisserial infection on either resting or pre-activated B cells.
1.2.5.3.2 B cells and *N. meningitidis* and *N. lactamica*

B cell responses to infection with other neisserial species are largely uncharacterized. Vaughan *et al.* suggest that *Nla* induces non-specific proliferation of tonsillar B cells, which depends on binding to either (or both) surface-expressed IgM and IgD by an unidentified proteinaceous factor produced by the bacteria (Vaughan et al., 2010). Their observation that *Nla* is able to bind to IgM and/or IgD is truly novel, and may represent a neisserial version of an IgD binding protein, an activity which has already been described to cause polyclonal B cell activation by both *Moraxella catarrhalis* and *Haemophilus influenzae*, other colonizers of the nasopharynx (Jendholm et al., 2008; Vidakovics et al., 2010). Although they suggest that *Nla* is able to polyclonally activate the naive and innate B cell subsets, the flow cytometry they show does not distinguish between these two subsets as responders, an important point considering that the innate B cell subsets have not been shown to be present in draining lymph nodes such as tonsils without infection. For these reasons, caution should be used when interpreting their conclusions.

In a separate report, Vaughn *et al.* examines the effect of neisserial colonization to induce mucosal memory in the upper respiratory tract (2009). Using palatine tonsils obtained from individuals of various ages, they show that T cell memory specific for *Nla* is present in adults, but absent in children. Since *Nla* colonization is heaviest in children, and decreases steadily with age (Gold et al., 1978a), they conclude that colonization with other neisserial species can induce memory T cells which are cross-reactive to *Nla* antigens. Distinct from this, is that *Nme* does not induce any observable T cell memory responses in children, and weak memory responses in adults. Aside from these conclusions, they also suggest that B cell memory specific for *Nla* is absent in children, however their assay does not consider memory B cells directly, since it is a mixture of total mononuclear cells which were treated with a variety of B cell mitogens, but oddly with the exclusion of *Nla*. Furthermore, they propose that B cells are able to produce polyreactive IgM, in a T-independent manner upon treatment with *Nla* OMVs. Their assay system consisted of total mononuclear cells depleted of T cells, but included other cell types present in the tonsil, including macrophages and dendritic cells which could influence the production of Ig through the production of cytokines or other factors.
1.3 B cell overview

In considering the varying effects that *Neisseria* may have on B cells, it is essential to recognize the diversity of phenotypes and various function of this important cell type. Within humans, a variety of B cell subsets exist, each with distinctive contributions to the generation of the overall immune response. Peripheral B cells are found in the blood, and circulate between lymphoid organs. Only when BCR signalling is induced by antigen recognition will the B cells be retained in the lymphoid organs. This microenvironment promotes interaction between T cells and B cells, allowing for the delivery of T derived co-stimulatory signals which are mandatory to promote B cell expansion and differentiation into either memory or class switched antibody-secreting cells (Allen et al., 1993; Foy et al., 1994; Korthauer et al., 1993). This paradigm of successive signal requirements protects from autoimmunity, while maintaining an incredibly diverse array of receptor specificities, however this is only true for mature naive B cells. Naive B cells are a population of antigenically inexperienced B cells, which have survived selection processes in the bone marrow. Distinctively, naive B cells express both surface IgM and IgD and do not express CD27, a marker of antigen experience (see schematic in Figure 1.2) (Agematsu et al., 2000; Klein et al., 1998).

Antigen experienced B cells are considered to be memory B cells, such that previous exposure to an invading pathogen allowed for the required signals, both spatially and temporally, to induce B cell responses that are protective for the host and detrimental for the pathogen. Under these circumstances, a portion of the activated B cells will differentiate into memory B cells (Foy et al., 1994). These primed cells possess activation thresholds which are lower than that of naive B cells (Good et al., 2009), and do not require signalling through the BCR (Bernasconi et al., 2002) to allow for immediate induction of immune responses upon repeat encounters with the same pathogen. Memory B cells typically express either surface IgG or IgA, as the majority of these cells have undergone class-switch recombination as a result of antigenic stimulation, as well as CD27 (Agematsu et al., 2000). For this reason, these cells will be referred to as switched memory (sw. memory) B cells for the remainder of this thesis.
Figure 1.2 A schematic showing the three B cell subsets present in the human periphery, distinguishable by IgD and CD27 expression, visualized by flow cytometry.

- **Naive (IgD⁺ CD27⁻)**
  - antigenically inexperienced
  - requires two signals for activation and proliferation

- **IgM memory cells (IgD⁻ CD27⁺)**
  - innate function, similar to murine B-1 B cells
  - non-redundant subpopulation
  - produces broadly reactive, low affinity IgM

- **Switched memory cells (IgD⁻ CD27⁺)**
  - have undergone class switch recombination
  - specific, high affinity IgG or IgA
Recently, another subset of B cells were identified within the human periphery, the IgM memory B cells (Klein et al., 1998), and it took another 5 years before the function of these cells was identified (Kruetzmann et al., 2003). These cells express surface IgM, as well as IgD, and CD27. The fact that IgD, which has traditionally been considered a marker of antigenic inexperience and naivety, was co-expressed on a cell along with memory marker CD27 was confounding. The expression of CD27 suggested that these cells originated from germinal centers (Seifert and Kuppers, 2009), which is the reason why they were initially given a “memory” designation. As it is becoming increasingly clear that the IgD+ CD27+ B cells are innate in function, and likely derive from germinal center independent reactions (Weller et al., 2001; Wu et al., 2010), this apparent misnomer can be a source of confusion. Recently, they have been referred to as “natural effector B cells” (Berkowska et al., 2011), however the majority of published literature have described this subset as the IgM memory cells, a naming convention that I will retain for this thesis.

Due to their potential role in innate immune protection and their importance in Neisseria-specific B cell responses revealed during my studies (Chapters 2 and 3), the rest of this section will focus on the known functions of the IgM memory B cell subset in humans, expanding to include discussion with regards to their closest murine analogue, and concluding with a description of the broad and distinctive immunologic roles of the Ig produced by these cells.

1.3.1 Innate B cells in Humans

1.3.1.1 Development

IgM memory B cell development is not well characterized, although some clues suggest that these cells have a development programme that is distinct from that of conventional bone marrow-derived B cells. IgM memory cells from children under the age of two show mutated BCRs (Weller et al., 2008), which requires the expression of activation-induced cytidine deaminase (AID) (Muramatsu et al., 2000). Although the BCRs within the IgM memory subset are mutated, overall the receptor repertoire was highly diverse, and does not resemble switched memory B cell receptors that show signs of clonal expansion (Weller et al., 2008). The observation that AID is present in the IgM memory subset in children, but not in adults, suggests
that somatic hypermutation occurs early in life and that this process occurs independently of a specific immune response (Weller et al., 2008).

A number of pivotal studies have been published which addresses the function of the IgM memory B cells in humans, many of them utilizing cohorts of individuals with immunologic deficiencies. Examination of asplenic individuals, either due to congenital defects or splenectomy, revealed that the spleen is required for the generation and/or maintenance of IgM memory B cells in the periphery (Kruetzmann et al., 2003; Wasserstrom et al., 2008). This is distinct from the development and maintenance of switched memory B cells. Switched memory B cells derive from naive cells originating from the bone marrow, and although the spleen does have a role in the maintenance of switched memory cells, other lymphoid tissues can fulfill this role in the absence of the spleen (Kruetzmann et al., 2003).

The hypothesis that IgM memory B cells require the spleen for development and/or maintenance is reinforced by the finding that the IgM memory B cell subset represents a circulating form of the innate marginal zone (MZ) B cells (Weill et al., 2009). Marginal zone B cells are non-circulating cells which reside within the spleen and are responsible for the rapid induction of T-independent IgM (Guinamard et al., 2000) specifically targeting blood-borne pathogens or antigens. Similar to MZ cells, the IgM memory B cells have a lower threshold of activation than conventional B cells, resulting in the ability to rapidly differentiate into plasma cells upon stimulation (Good et al., 2009; Martin et al., 2001). This allows for their rapid production of innate immunoglobulin in advance of the slower T-dependent responses. MZ B cells are derived from bone marrow progenitors (Pillai and Cariappa, 2009), however this does not preclude special requirements such as the splenic microenvironment or other factors supporting the differentiation and maintenance of the circulating IgM memory subset (Weill et al., 2009). For a summary of the various differentiation schemes of the B cell subsets, see Figure 1.3.

Other similarities exist between the MZ and IgM memory B cells (Weill et al., 2009), including gene expression profiles, which are highly similar between these subsets when compared against other B cell subpopulations (Weller et al., 2004). Experimental polysaccharide vaccination against Streptococcus pneumoniae and Neisseria meningitidis revealed the expansion of a specific μ heavy chain VDJ rearrangement in 50% of IgM memory cells in blood and 58% of splenic marginal zone post-vaccination, whereas only 2.6% of circulating IgM memory cells
were found to express this rearrangement prior to vaccination (Weller et al., 2004). Several of these VDJ rearrangements displayed signs of affinity maturation and were shared between both IgM memory and marginal zone B cell compartments, suggesting that the IgM memory cells may be a circulating form of the splenic marginal zone B cell (Weller et al., 2004).

1.3.1.2 Function

The IgM memory B cells do not require T cell help for either development or generation of immune responses. Individuals with a homozygous mutation in the CD40L gene, which is required for T cell mediated co-stimulation of B cells, are unable to produce switched memory B cells as evidenced by the lack of serum IgG and IgA (Korthauer et al., 1993), but retain normal serum IgM levels and a normal IgM memory subset in the periphery (Weller et al., 2004). Moreover, the IgM memory cells present in these patients show mutated B cell receptor sequences, confirming that receptor editing of these cells occur in the absence of T cell signals (Weller et al., 2004). To further explore the hypothesis that the IgM memory subset functions independently of T cell responses, vaccination using a non-conjugated bacterial polysaccharide, a classical T-independent antigen, was done to assess B cell responses. Vaccination with a PS antigen mediates not only the expansion of IgM memory B cells, but also the production of B cell antigen receptors that showed evidence of subsequent receptor mutation in response to the PS (Weller et al., 2004). Interestingly, it has been well documented that children under the age of two do not respond well to T-independent antigens, such as PS vaccines. The development of the IgM memory B cells occurs in parallel with the switched memory subset, with very little of either subset present at birth but increasing in frequency with age (Weill et al., 2009). The presence of the IgM memory subset in children under the age of two suggests that the inability of these cells to respond to T-independent antigens may lie in the immaturity of other cells which support their activation (Weller et al., 2008).
A) Conventional B cells are derived from bone marrow precursors (i), that develop into circulating mature naive B cells (ii) which, upon antigenic stimulation, can undergo class switch recombination to produce switched memory B cells (iii). B) Although the innate IgM memory B cells (iii) in humans may represent a circulating form of sessile splenic marginal zone B cell (ii), which are also derived from bone marrow precursors (i), it is not known if they arise from marginal zone B cells, or develop from a common progenitor (iv). Alternatively, IgM memory B cells may arise from an entirely different progenitor (v), separate from the marginal zone lineage. In mice, the innate B-1 subset is thought to be self-regenerating, however this has not been conclusively examined for the human IgM memory subset. One of the difficulties in the elucidation of IgM memory B cell development is the significance of the spleen for this subset. It is clear that the splenic niche is important for the maintenance of this subset, however it is unknown if development occurs in this environment or elsewhere. Additionally, the ontogeny of IgM memory B cells remains unclear as to whether they are generated in one wave during childhood and maintained (primarily in the spleen) throughout adulthood, or if they are produced in multiple waves throughout life. BCR classes are indicated by colour, with red = IgM, blue = IgG, green = IgA.
The primary function of the innate IgM memory subset appears to be the prevention of disease caused by a variety of mucosal and systemic pathogens, including bacteria and fungi. The IgM memory B cells mediate protection through the production of low affinity broadly reactive innate IgM, which will be discussed in section 1.3.3 Innate Immunoglobulin. This function is non-redundant, as patients with common variable immunodeficiency (CVID) who are missing this subset of cells are more likely to have recurrent infections with encapsulated bacteria, than CVID patients with an intact IgM memory subset (Carsetti et al., 2005; Kruetzmann et al., 2003). Additionally, decreased levels of IgM memory B cells in HIV-infected patients has been shown to be a predictor of Cryptococcus neoformans induced cryptococcosis (Subramaniam et al., 2009). In the murine model, it has been clearly demonstrated that innate IgM, produced by an analogous innate B cell subset in mice, is able to protect against the dissemination of pathogens (Baumgarth et al., 2000; Briles et al., 1981; Ochsenbein et al., 1999).

Due to differences between the IgM memory subset in humans, and the analogous innate subset in mice, termed B-1, I have focused this section on reports using human cells and cohorts. However to have a deeper understanding of how these innate B cell subsets might function, this next section will focus on the immune function of the murine B-1 B cell subset where more mechanistic studies have been performed.

1.3.2 Innate B cells in Mice

The innate IgM memory B cells are functionally similar to the innate B-1 B cells found in mice, as both are producers of innate immunoglobulin, which are protective against invading pathogens at mucosal sites (Baumgarth et al., 1999; Kruetzmann et al., 2003; Weller et al., 2004). However, differences exist between the human and mouse innate B cell subsets. In humans, the IgM memory subset is found circulating in the blood (Klein et al., 1998). In contrast, the murine B-1 subset is primarily associated with the peritoneum and pleural cavities (Herzenberg and Kantor, 1993), although they can be found in the blood at very low levels (less than 1% of B cells) (Baumgarth, 2011). Other distinctions are related to the innate receptor specificities. It has been well characterized that human IgM memory B cells possess mutated antigen receptors (Weller et al., 2004; Weller et al., 2008), while the majority of antigen receptors for B-1 B cells are unmutated and germ-line encoded (Baumgarth, 2011).
Furthermore, B-1 B cells are somewhat defined by their ability to bind to self antigens (Baumgarth et al., 2005), whereas the rate of autoreactivity is very low in IgM memory cells (Tsuiji et al., 2006). From this, it is clear that the murine B-1 subset is not exactly homologous to the human IgM memory subset, however the B-1 B cells are the best-defined innate subset at the moment for comparison.

1.3.2.1 Development

B-1 B cells have been proposed to originate from the fetal liver, as demonstrated by reconstitution experiments (Herzenberg, 2000; Rosado et al., 2009). Others suggest that a precursor may exist in the bone marrow as well (Dorshkind and Montecino-Rodriguez, 2007). While it is apparent that in humans the spleen is required for the maintenance of IgM memory B cells, the role of the spleen for the maintenance of B-1 B cells is unclear, as some suggest B-1 cells are depleted upon splenectomy (Wardemann et al., 2002), while others have not observed this phenomenon (Kretschmer et al., 2004). While these observations may not be mutually exclusive, it is clear that B-1 B cell development is different from that of conventional B cells. The maintenance of the B-1 B cell population is likely due to self-renewal (Baumgarth, 2011; Lalor et al., 1989), distinct from conventional B cells that are derived from bone marrow progenitors. Of note is that intraperitoneal injection of *Staphylococcus aureus* protein A was not only able to deplete the B-1 subset, likely through BCR-mediated apoptosis, but that this depletion was still apparent sixteen weeks after treatment, whereas the conventional splenic B cell populations had fully recovered over the same time period (Silverman et al., 2000). This not only highlights that the B-1 and conventional B cells are derived differently, but provides additional evidence for the concept of B-1 subset maintenance through self-renewal, since they were not able to recover after mass depletion. Considering the special biology of the innate B-1 B cells providing immediate Ig responses at mucosal surfaces, it is remarkable that pathogenic mechanisms exist which subvert the immune protection provided by these cells.

1.3.2.2 Infection

The murine influenza infection model has been an invaluable tool to assess the function of the innate B cells during infection. Upon intranasal infection with influenza, mice that cannot secrete IgM show reduced survival, but this defect can be partially complemented by the injection of normal serum from uninfected mice (Baumgarth et al., 2000). Keeping in mind that
influenza is not a natural pathogen of mice, this excludes the possibility that the transferred serum contained protective antibodies due to previous influenza exposure. Normal serum from uninfected mice contains naturally occurring IgM, 80% of which is derived from the B-1 subset (Baumgarth et al., 1999). Additionally, all of the IgM found in the respiratory tract in uninfected mice are derived from the B-1 subset (Choi and Baumgarth, 2008). This IgM is usually referred to as ‘natural’ or ‘innate’ IgM, as it exists as part of the pre-immune repertoire.

Upon intranasal infection, greater than 90% of IgM specific for flu in the lungs was derived from the B-1 subset, whereas analogous IgM antibodies found in the serum were primarily derived from conventional B cells (Choi and Baumgarth, 2008). The innate IgM produced in response to infection was highly cross-reactive, since it not only recognized the infecting flu strain but also three other unrelated strains (Baumgarth et al., 2000). This IgM is of immunologic importance, as mice which cannot secrete IgM showed delayed kinetics of viral clearance from their lungs (Baumgarth et al., 2000). Moreover, infection did not affect the level of systemic innate IgM (Baumgarth et al., 1999), emphasizing the localized effects of the B-1 subset.

B-1 B cells secreting flu-specific IgM are shown to accumulate in the local draining lymph nodes upon intranasal infection, however this response was not observed in other uninvolved peripheral lymph nodes (Choi and Baumgarth, 2008). The authors of this report state that there was no observable change in the levels of IgM antibody-secreting cells upon infection in lung tissue, suggesting that the B-1 B cells are not recruited to mucosal tissue (Choi and Baumgarth, 2008). Together, these studies show that upon viral infection of lung mucosa, B-1 B cells are recruited to local draining lymph nodes and are induced to secrete polyreactive innate IgM, some of which cross-reacts with the infecting pathogen. The response is localized, as no change was observed in the systemic innate IgM compartment.

The ability of normal/innate Ig to protect from infection by encapsulated *Streptococcus pneumoniae* was examined using *xid* mice, which are deficient in B-1 B cells but have normal conventional B cell compartments due to a mutation in Bruton’s tyrosine kinase (Btk) (Clarke and Arnold, 1998). The *xid* mice possess lower LD50 scores upon either intravenous or intraperitoneal infection with *S. pneumoniae* compared to mice with an intact B-1 compartment (Briles et al., 1981). Complementation by injection of normal serum from uninfected mice, which contains innate IgM specific for *S. pneumoniae* antigens, protected the *xid* mice from
death (Briles et al., 1981). This protective effect was confirmed to be due to the action of innate IgM, as an IgM hybridoma of a common innate idiotype specific for *S. pneumoniae* antigens were injected into mice prior to infection with live bacteria also protected from death (Briles et al., 1981). Therefore, the innate pre-immune Ig present in serum can be protective against bacterial pathogenesis.

The innate B cell subsets provide immune responses which are temporally distinct from other subsets in response to infection. Using canonical antibody idiotypes to identify either marginal zone or B-1 B cell subsets responding to infection with bacteria, Martin *et al.* describe the non-redundant function of each of these innate subsets (2001). Intravenous injection of *S. pneumoniae* induces responses from both MZ and B-1 subsets, while intraperitoneal injection of the same bacteria induces only responses for the B-1 subset, and failed to activate the splenic MZ B cells (Martin et al., 2001). Therefore, while both subsets are innate in function and primarily respond to T-independent antigens, the B-1 subset clearly functions locally to control dissemination of the pathogen. If this fails, the pathogen can cause systemic disease, at which point the MZ B cells, positioned within the spleen for optimal surveillance of blood-borne antigens, become activated to produce additional innate immunoglobulin as an additional line of defence before the induction of a conventional clonal adaptive immune response.

1.3.3 Innate Immunoglobulin

Two different types of secreted immunoglobulin exist, immune Ig and ‘innate’ or ‘natural’ Ig. Conventional B cells are the producers of immune Ig, which requires exogenous stimulation. This is exemplified by the diminished levels of serum IgG in germ free mice compared to conventionally reared mice (Haury et al., 1997). In contrast, innate Ig is produced without exogenous antigenic stimulation, as serum levels of innate IgM are similar between germ free and conventionally reared mice (Haury et al., 1997). Innate Ig is derived from the innate B cell subsets: the B-1 B cells in mice (Baumgarth et al., 1999; Briles et al., 1981), and the IgM memory B cells in humans (Kruetzmann et al., 2003; Weller et al., 2004). Although antigenic stimulation is not required for the production of innate Ig, innate B cells have been shown to respond to infection by accumulating at the site of infection effectively increasing the local concentration of Ig (Choi and Baumgarth, 2008). The other distinguishing feature of innate Ig is that it is produced in a manner that is completely T-independent (Macpherson et al., 2000;
Weller et al., 2004; Zola et al., 2009). In the next section I will review the function of the innate immunoglobulins, with a focus on their anti-microbial effects.

A defining characteristic of innate Ig responses is that they are low affinity (Notkins, 2004) and broadly non-specific, such that antibodies recognizing a diverse array of epitopes expressed on different microbes, for example, are present in mice which have never been colonized by these organisms (Baumgarth et al., 2000; Haury et al., 1997). Furthermore, innate Ig possess antigen receptors which are polyspecific in nature, enabling one antibody to bind multiple epitopes (Zhou et al., 2007). This polyspecificity undoubtedly aids in the binding of innate IgM to conserved microbial structures. Binding can occur on multiple structures on a microbial surface, which could enhance agglutination, neutralizing effects and limit dissemination (Ehrenstein and Notley, 2010). Notkins has proposed that the antigen binding pockets of these innate Ig are flexible to allow for multiple conformations accommodating the recognition of multiple antigens (2004). Over a third of the polyreactive antibodies possess germ line sequences (Notkins, 2004), which Baumgarth et al. suggests “reflects the innate usage of a series of V genes that evolutionary pressures have selected into the genome to provide an inherent legacy of specificities suitable for protection against pathogen invasion” (2005).

1.3.3.1 Anti-microbial functions

The secreted form of IgM is pentameric, which is able to bind C1q, one of the mediators of the classical pathway of complement activation (Czajkowsky and Shao, 2009). Highly efficient at fixing complement, IgM has 1000-fold greater affinity for C1q than IgG (Cooper et al., 1983). Innate IgM, through the recruitment of complement, has been shown to be able to cause bacterial lysis \textit{in vitro} (Zhou et al., 2007), and prevent dissemination of \textit{S. pneumoniae} \textit{in vivo} (Brown et al., 2002).

While it is clear that innate IgM is able to bind to a variety of microbes (Baumgarth et al., 2000; Briles et al., 1981; Cohen, 1967; Ochsenbein et al., 1999; Troncoso et al., 2002; Zhou et al., 2007), this effectively opsonises them allowing for efficient engulfment by phagocytes, resulting in both bacterial clearance as well as induction of immune responses by the phagocytes themselves. An Fc $\alpha/\mu$ receptor is expressed in antigen presenting cells such as macrophages and B cells, but not on neutrophils or T cells (Shibuya et al., 2000). In B cells, the Fc $\alpha/\mu$ receptor is able to mediate engulfment of IgM opsonized \textit{S. aureus} (Shibuya et al., 2000),
emphasizing the possibility that innate IgM production, in response to pathogenic insult, may in turn increase the ability of B cells to limit dissemination through multiple mechanisms including engulfment and destruction of the pathogen.

Although innate Ig is primarily IgM, innate IgG and IgA have been described (Avrameas, 1991; Cohen and Norins, 1966). The function of innate IgG has been less defined, although local innate IgG has been suggested to limit the intranasal colonization of *Haemophilus influenzae* in a murine model of infection (Zola et al., 2009). Half of the IgA present in the serum (Baumgarth et al., 1999) and in the intestinal lamina propria (Baumgarth et al., 1999; Macpherson et al., 2000), as well as many of the IgA antibody secreting cells present in the respiratory tract (Baumgarth et al., 2005) are derived from innate B cells in mice. The role of innate IgA is similar to innate IgM (Choi and Baumgarth, 2008), such that its production is induced locally upon colonization/infection with bacteria, and not observed systemically (Macpherson et al., 2000). Innate IgA has been shown to protect the intestinal mucosa from *Salmonella typhimurium* invasion of the intestinal epithelium (Wijburg et al., 2006), analogous to the function of innate IgM preventing pathogenic dissemination at mucosal surfaces (Baumgarth et al., 2000; Ochsenbein et al., 1999).

1.4 Thesis objectives

The goal of my thesis is to examine B cell responses to neisserial infection, as these cells have the potential to both directly and indirectly effect immunity to the pathogenic and commensal *Neisseria*. Understanding how these pathogens interact with the cells responsible for producing protective antibody and immunologic memory will also enlighten vaccine designs targeting these pathogens. The *Neisseria* species are human-specific, and bearing in mind the lack of animal models for either gonococcal or meningococcal infection that recapitulates disease pathology as observed in humans, my studies are focused on human B cells purified from peripheral blood.

Others have examined B cell responses to neisserial infection indirectly, using mixed populations of cells (Vaughan et al., 2010; Vaughan et al., 2009). These studies could be considered to reflect a more natural environment to examine B cell responses to infection, as B cell encounters with bacteria or antigen will likely never be in isolation from other cell types. However, since B cell responses to neisserial infection are so poorly understood, use of a mixed cell population makes it difficult to differentiate between effects mediated by B cell interaction with the bacteria
versus effects mediated by other cells present in mixed populations, such as macrophages and dendritic cells, which undoubtedly influence B cell responses.

In terms of immune responses generated during gonococcal infection, the CEACAM1-mediated inhibition of DCs and T cells suggests that B cells may also be inhibited upon infection with \textit{Ngo}. Evidence suggesting that CEACAM1 displays the capacity to inhibit cellular activation upon receptor ligation in chicken B cells supports this hypothesis (Chen et al., 2001). However, treatment with abundantly expressed outer membrane protein PorB results in strong activation and proliferation responses by murine B cells (Wetzler et al., 1996), an effect that is not observed upon treatment with T cells (Simpson et al., 1999).

Because I was initially interested in determining whether gonococcal Opa-CEACAM1 interaction resulted in inhibition of B cell responses, similar to what was observed upon infection of T cells (Boulton and Gray-Owen, 2002; Lee et al., 2008), Chapter 2 of this thesis examines the effect of \textit{Neisseria gonorrhoeae} infection on freshly purified human B cells. My finding that \textit{Ngo} induces a potent activation and proliferative response from the human innate IgM memory B cell subset represents the first report of a pathogen directly inducing cellular responses by these recently recognized population of cells. It is also the first description of the effect of TLR signalling in this subset, as I characterize their TLR responsiveness and show that gonococcal DNA elicits TLR9-dependent innate B cell responses. Since the effect of \textit{Ngo} infection on innate B cell function was so vigorous, Chapter 3 examines the effect of infection with other neisserial species on B cell function. Unexpectedly, the closely related and only other pathogenic \textit{Neisseria} species, \textit{Nme} only induced weak cellular activation, while the commensal \textit{Neisseria} strains elicited strong responses from the IgM memory B cells, including the production of polyclonal IgM. This surprising difference may have implications for understanding immunity in response to infection, as well as vaccine design. Lastly, in Chapter 4, I examine whether the co-inhibitory receptor CEACAM1 has the potential to mediate B cell inhibition in the context of \textit{Ngo} infection by developing an assay system that allows the specific contribution of individual stimuli to be appreciated. I present data showing that in all of the assay conditions examined, \textit{Ngo} infection resulted in robust cellular activation. Taking into consideration the nature of the B cell response to neisserial infection primarily originating from the innate IgM memory B cells, I consider the inherent differences between T cells and B cells
and discuss why during neisserial infection, CEACAM1 functions so differently between these two cell types.

Taken together, this provides a mechanism to explain two apparently disparate clinical observations for individuals infected with *N. gonorrhoeae*: while Ig is induced by infection, pathogen-specific Ig levels are low (Hedges et al., 1999) and re-infections with the same strain can occur (Fox et al., 1999), suggesting an impairment in the development of immunologic memory. Although the innate IgM memory B cells respond to *N. gonorrhoeae* infection with strong proliferation and polyreactive Ig production which includes antibody that recognizes gonococci, dendritic cell maturation and T cell activation is inhibited through Opa-CEACAM1 interactions. This effectively delays or inhibits the induction of T-dependent processes such as the production of high affinity pathogen specific immune Ig and immunologic memory. By itself, the innate Ig provides weak defense against *Ngo* infection, but provides no protection from subsequent gonococcal infections.
2 Vigorous response of human innate IgM memory B cells upon infection by *Neisseria gonorrhoeae*

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Nancy S. Y. So, Mario A. Ostrowski, and Scott D. Gray-Owen

**Author contributions:**

Nancy S. Y. So designed the experiments, collected and analyzed the data, and wrote the paper.

Mario A. Ostrowski provided leukopheresis donor samples.

Scott D. Gray-Owen designed the experiments, analyzed the data, and edited the paper.
2.1 Abstract

*Neisseria gonorrhoeae*, the cause of the sexually transmitted infection gonorrhea, elicits low levels of specific immunoglobulin and re-infection with the same serovar can occur, suggesting that protective immunity is not induced. We considered whether infection might directly influence B cell function. In stark contrast to infection with the prototypical Gram-negative bacteria, *Escherichia coli*, gonococcal infection caused prolonged viability of primary human B cells and elicited both robust activation and vigorous proliferation in the absence of T cells. Upon further analyses, we observed the specific expansion of IgD+ CD27+ B cells in response to gonococcal infection. These cells are reported to have an innate function, conferring protection against a diverse array of microbes by producing low affinity, broadly-reactive IgM without inducing immunologic memory. While *N. gonorrhoeae* infection produced small amounts of gonococcal-specific IgM, IgM specific for irrelevant antigens were also produced, indicative of a polyspecific immunoglobulin response. The B cells effectively bound, engulfed and killed the gonococci, and TLR9-inhibitory CpGs blocked B cell responses suggesting that bacterial degradation allowed innate immune detection within the phagolysosome. This is the first report of a bacterial pathogen having a specific affinity for the human innate subset of B cells, driving their potent activation and polyclonal immunoglobulin response. Combined with the ability of gonococci to inhibit T cell activation, this T-independent response may contribute to both an unfocused immunoglobulin response and the observed absence of immunologic memory to this important human pathogen.

2.2 Introduction

*Neisseria gonorrhoeae* (*Ngo*, gonococci) is a human specific pathogen that causes the sexually transmitted infection gonorrhea and is a major cause of morbidity, with over 62 million new adult infections worldwide in 1999 alone (WHO, 2001). Gonorrhea results from an overwhelming inflammatory response, producing purulent discharge at the site of infection. The majority of women infected with *N. gonorrhoeae* are clinically asymptomatic (Handsfield, 1990). Left untreated, this can progress to serious complications including pelvic inflammatory disease, sterility or ectopic pregnancies due to fallopian tube scarring, or acquired blindness due
to gonococcal conjunctivitis in children born to infected mothers (Edwards and Apicella, 2004). Once infection is detected, gonorrhea can be effectively treated with antibiotics, however antibiotic resistance is increasing (Workowski et al., 2008). Gonococcal co-infection can also increase viral shedding in HIV patients (McClelland et al., 2001), further heightening the urgency to control the spread of this important pathogen.

Despite the overzealous innate immune response that causes symptomatic disease, adaptive immunity against uncomplicated gonococcal infections is surprisingly poor. Gonococci-specific immunoglobulin (Ig) is elicited during infection, but antibody levels are low and there is no evidence of immunologic memory even during infection of rectal tissues containing immune-inductive sites (Hedges et al., 1999). While *N. gonorrhoeae* has a remarkable capacity to vary surface-expressed antigens, which undoubtedly aids in its ability to cause infection and disease (Gray-Owen et al., 2001), re-infection with the same serovar does occur (Fox et al., 1999). Combined, such clinical observations led to early suggestions that *N. gonorrhoeae* can actively suppress the specific memory response in order to promote its survival and persistence within the population (Hedges et al., 1999).

During the course of an infection, the gonococci interact with host cells in the mucosa via their type IV pilus (Kirchner and Meyer, 2005), which docks the bacterium onto the apical side of barrier epithelial cells and then retracts to allow intimate association between integral membrane adhesins and host cellular receptors (Edwards and Apicella, 2004). Of these, neisserial Opa proteins bind to apically-expressed carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs), triggering bacterial uptake and transcytosis through the epithelial layer (McGee et al., 1983; Wang et al., 1998), thus providing the bacteria access to the sub-epithelial compartment where sentinel immune cells are known to reside (Levine et al., 1998; Prakash et al., 2001). Individual gonococci encode up to eleven *opa* alleles (Bhat et al., 1991), the expression of which turns on-and-off at random through the process of phase variation (Murphy et al., 1989). In addition to facilitating entry into tissues, certain Opa variants trigger the co-inhibitory function of CEACAM1, potently inhibiting cellular activation and proliferation by stimulating tyrosine phosphatases that oppose T cell receptor signalling (Boulton and Gray-Owen, 2002; Lee et al., 2008) and epithelial cell innate responses leading to the expression of proinflammatory cytokines (Slevogt et al., 2008). Despite the phase-variable nature of Opa expression, over 95% of clinical neisserial isolates express Opa variants that can bind to
CEACAM1 (Virji et al., 1996), implying a selection for CEACAM1-specific binding function in vivo. Certain Opa variants instead bind basolaterally-expressed heparan sulfate proteoglycan (HSPG) receptors (Chen et al., 1995; van Putten and Paul, 1995) and serum vitronectin and fibronectin (Dehio et al., 1998; van Putten et al., 1998) to facilitate attachment in the submucosa.

While *N. gonorrhoeae* Opa-dependent inhibition of T cell function has been well characterised (Boulton and Gray-Owen, 2002; Lee et al., 2008), the effect of gonococcal infection on B cell function remains relatively unexplored. B cells play an integral role in the prevention of both morbidity and mortality due to infection, primarily (but not limited) to the effective production of Ig in response to invading pathogens. While the activity of B cells are undoubtedly important, especially in the context of extracellular pathogens such as *N. gonorrhoeae*, the specific effect of bacterial infection on overall B cell function is generally not well characterized and remains poorly understood.

We considered that, in addition to suppressing T cell responses (Boulton and Gray-Owen, 2002), gonococcal infection may directly affect B cell function. Taking into account the exquisite host specificity of *N. gonorrhoeae* virulence factors, the intrinsic activation state of human B cell lines and the difference in microbe-associated molecular pattern (MAMP) responses between human and mouse B cells (e.g. TLR4 signalling induces strong responses in murine B cells, but is not detected in human B cells (Bernasconi et al., 2003; Hoshino et al., 1999)), we developed in vitro infection protocols using purified primary human B cells. Unexpectedly, and in stark contrast to parallel infections with the prototypical Gram negative bacteria, *Escherichia coli*, we observed that *N. gonorrhoeae* prolonged B cell viability and elicited a robust cellular activation that led to the polyclonal proliferation and expansion of IgM memory B cells, an innate population of B cells, resulting in the production of immunoglobulin specific for both *Neisseria*-derived and unrelated antigens. These responses are triggered by the effective delivery of CpG-containing DNA to TLR9 within the phagosome after engulfment of the bacteria. This represents the first example of a pathogen specifically targeting the innate IgD⁺ CD27⁺ IgM memory B cell subset in this manner. When combined with the gonococcal ability to suppress T cell activation (Boulton and Gray-Owen, 2002; Gray-Owen and Blumberg, 2006; Lee et al., 2008), this effect may explain the clinical observations of unfocused and non-protective immunoglobulin responses in individuals with uncomplicated gonorrhea infections.
2.3 Results

2.3.1 *N. gonorrhoeae* associates with human peripheral blood B cells.

*N. gonorrhoeae* adheres directly to primary human CD4\(^+\) T cells in an Opa protein-dependent manner (Lee et al., 2008). To establish whether Ngo also interacts with primary human B cells, we exposed freshly purified cells to isogenic strains of gonococci expressing either an Opa variant that is specific for heparan sulfate proteoglycans (Opa\(_{\text{HSPG}}\)), an Opa that is able to bind co-inhibitory receptor CEACAM1 (Opa\(_{\text{CEA}}\)) or no Opa proteins (Opa\(^-\)), and then monitored their association with the B cells over a time course using confocal microscopy. Cellular association with the prototypical Gram-negative bacterium, *Escherichia coli*, was also examined to assess the general propensity of B cells to bind bacteria. To confirm that bacteria were associating with B cells and not a co-purified cell type, the infected cells were stained for B cell receptor (BCR), as depicted in Figure 2.1A. We then quantified the percent of B cells that were associated with at least one bacterium. As illustrated in Figure 2.1B, substantially more B cells are associated with gonococci than *E. coli*, regardless of which neisserial adhesin was expressed. However, even after 44 hours of infection, some B cells did not associate with gonococci, suggesting that binding may be restricted to a specific subpopulation of cells. Moreover, no recruitment of BCR to sites of bacterial attachment was evident, suggesting that this association is independent of this antigen receptor.

While Figure 2.1B quantifies the percent of B cells with at least one bacterium bound, this does not illustrate how many bacteria associate with each cell. We quantified the number of bacteria per cell (Figure 2.1C), and observed that most infected B cells associate with 1 to 5 bacteria per cell. The ability of gonococci to associate with cells is largely Opa-independent (compare Ngo Opa\(^-\)), although Opa expression did have some influence on bacterial association with the lymphocytes in some donors (e.g. Donor 02L).
Figure 2.1  *N. gonorrhoeae* association with primary human B cells.

A) B cells were infected with a MOI of 10 of either texas-red labelled *N. gonorrhoeae* expressing no Opa adhesin (Opa⁺), Opa specific for HSPG (OpaHSPG) or Opa specific for CEACAM receptors (OpaCEA) or texas-red labelled *E. coli* for 6 hours, then visualized by confocal microscopy. DIC, differential interference contrast. Bar length indicates 5 μm. B) B cells were infected as described in *A*) but using unlabelled bacteria that were stained post-infection, and bacterial association was assessed over a time course by confocal microscopy. C) The total number of bacteria associated per cell was quantified 3 hours post infection, considering only cells that have at least one bacteria bound (excluding cells that did not associate with bacteria). Data for two representative donors are shown.
Figure 2.2  *N. gonorrhoeae* infection of primary human B cells promotes viability and does not induce cell death.

A) Viability was examined by comparing forward and side scatter flow cytometric parameters, which directly relates to cellular morphology. The percent of viable cells was obtained by taking the percent of live cells (Live gate) divided by the percent of total cells (All gate). B cells cultured alone include both live and dead cell populations, while B cell cultures treated with staurosporine, a protein kinase inhibitor induces cell death at high concentrations, did not contain viable cells. Representative of 4 independent experiments with different donors. B) The cell populations from A) were analysed for the ability to bind annexin V, marker of cell death. Cells cultured alone (shaded) produce two peaks, indicative of annexin V positive and negative populations within this culture. Cells cultured with staurosporine produce only an annexin V positive peak (solid line). Representative of 2 independent experiments with different donors. B cells were infected with either *Ngo* or *E. coli* for 3 days, and then C) assayed for culture viability by forward vs side scatter, or D) examined for cell death by annexin V staining. Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, $P < 0.05$; **, $P < 0.01$. Data points corresponding to individual donors are represented by the same symbols within each plot.
2.3.2 Gonococcal infection inhibits B cell death and promotes prolonged B cell viability.

Previous work suggested that in the presence of potent stimulation, infection of primary human B cells with Ngo can cause B cell death (Pantelic et al., 2005). This could be explained either by a direct cytotoxic effect of the gonococci or that the bacteria contributes to activation-induced cell death. We explored whether *N. gonorrhoeae* infection affected the viability of normal primary human B cells by monitoring changes in cell morphology and membrane integrity during infection experiments. Loss of membrane integrity is an early event of cell death, and results in a decrease in forward scatter and increase in side scatter, reflecting corresponding changes in cell size and granularity, respectively (Figure 2.2A). Annexin V binding to phosphatidylserine exposed on a depolarized cell membrane is also a well-established method to measure cell viability (Koopman et al., 1994). To confirm the positioning of the live and dead populations within flow cytometry plots, B cell death was induced by treating cells with staurosporine. Comparison between gated populations in Figure 2.2A with annexin V staining profiles of these gates in Figure 2.2B verify the positioning of live and dead cells. For each donor (indicated by symbols), the percent of viable cells are significantly increased upon infection with gonococci compared to uninfected cells, as determined using forward and side scatter (Figure 2.2C). Cells exposed to *E. coli* were indistinguishable from the uninfected controls, confirming that this is not a general response to bacteria. Consistent with this conclusion, the percent of gonococci-infected cells bound by soluble annexin V was lower than cells left uninfected or exposed to *E. coli* (Figure 2.2D). It is pertinent to note that, while Ngo clearly increased viability of the B cells, it did not protect B cells from apoptotic death induced upon treatment with staurosporine (data not shown). When considered together, these data indicate that *N. gonorrhoeae* infection supports the survival of primary human B cells, and that this effect occurs regardless of Opa protein expression.

2.3.3 *N. gonorrhoeae* elicits robust B cell activation.

During flow cytometric analysis, we consistently observed a striking increase in B cell size and granularity upon exposure to *N. gonorrhoeae*, characteristic of a blasting phenotype induced by BCR cross-linking (Figure 2.3A-B). Flow cytometry for one representative donor is shown in
A robust activation response is observed by primary human B cells infected with *N. gonorrhoeae*, but not with *E. coli*.

All B cell cultures were infected for 3 days with either gonococci or *E. coli* at an MOI of 10 prior to cellular activation analysis. A) B cells activated for 3 days with 10 μg/mL of B cell receptor cross-linker (αBCR) displayed a typical blasting phenotype, with an increase in cell size, as reflected in the shifting of the gates live cells along the forward scatter axis. Representative of 2 independent experiments with different donors. B) Blasting cells were observed upon infection with Ngo, but not in uninfected or *E. coli* infected cells. Compare the αBCR activated cells in the live gate with Ngo or *E. coli* infected cells. Representative of at least 6 independent experiments with different donors. C) Quantification of the percent of cells that acquired a blasting phenotype after 3 days of infection with Ngo or *E. coli*. The percent of blasting cells was obtained by taking the percent of blasting cells (Blasting gate) divided by percent of total live cells (Live gate). D) Expression of co-stimulatory receptor and activation marker CD86 on B cells that were infected with either gonococci or *E. coli*. Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, *P* < 0.05; ***, *P* < 0.001. Data points corresponding to individual donors are represented by the same symbols within each plot.
Figure 2.4  
*N. gonorrhoeae* infection, but not *E. coli* infection, induces strong proliferative responses in human B cells.

A) BrdU incorporation was examined in B cells after infection by either *Ngo* or *E. coli* for 3 days.  B) The kinetics of B cell proliferation was measured with purified cells from four different donors, and shows that gonococci can effectively induce B cell proliferative responses for up to 6 days (the longest time point we analyzed). Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, $P < 0.05$; **, $P < 0.01$. Data points corresponding to individual donors are represented by the same symbols within each plot.
Figure 2.3B, displaying robust B cell blasting in response to infection with *Ngo*. This response did not occur in cells infected with *E. coli*. To compare multiple donors, the proportion of blasting cells in each sample was normalized against the percent of total live cells, the gating of which is reflected in Figure 2.3B. As evident in Figure 2.3C, the proportion of blasting cells is significantly higher in *Ngo*-infected, but not *E. coli*-infected cells compared to the uninfected samples. This effect is consistent among all donors examined and occurs regardless of Opa protein expression.

Since blasting is usually indicative of cellular activation, we measured the expression of activation induced co-stimulatory molecule CD86 on cells infected with *N. gonorrhoeae*. Figure 2.3D illustrates that gonococcal infection, but not *E. coli* infection, induces significantly more cells to express CD86 than when cells were left uninfected. Combined, these results demonstrate a potent activation response that is specific to the gonococci.

2.3.4 *N. gonorrhoeae* alone can provide the necessary signals to induce proliferation of B cells in a T-independent manner.

If cells receive the appropriate combination of signals, they will not only become activated, but will also proliferate. In order to ascertain whether simple exposure to *Ngo* was sufficient to promote B cell proliferation, 5-bromo-2-deoxyuridine (BrdU) incorporation was used to observe genome replication. Figure 2.4A illustrates that infection with gonococci promotes significantly increased BrdU incorporation by B cells, compared to uninfected cells. Proliferation of B cells infected with *E. coli* tended to reflect that of uninfected cells, although the extent varied slightly between donors (Figure 2.4B); this again illustrates that B cell proliferation is not a general response to bacterial infection. However, kinetic analysis of infection for all donors shows cellular accumulation of BrdU in response to infection with gonococci, consistent with proliferation as a specific and reproducible response to *Ngo* (Figure 2.4B).

2.3.5 Gonococcal interaction with B cell populations is specific, and favours binding to IgM memory B cells.

Since it was clear that some, but not all B cells associated with the gonococci (Figure 2.1B-C), we considered whether cellular responses to infection correlated with the action of a specific B
Three populations of B cells are found in human peripheral blood, and although gonococci are able to bind to all of them, Ngo association is strongest with IgM memory B cells.

A) The populations of B cells found in human peripheral blood can be differentiated by the combined expression profiles of CD27 and IgD. We show freshly isolated B cells from one representative donor can be separated into the 3 B cell populations found in human peripheral blood: naive (IgD⁺, CD27⁻), IgM memory (IgD⁺, CD27⁺) and switched memory (IgD⁻, CD27⁺). Representative of 4 independent experiments with different donors. B) Expression of IgM, IgG or IgA was examined in uninfected cells by gating on the B cell subpopulations. Solid line – IgG; dotted line – IgA; dashed line – IgM. Representative of 4 independent experiments with different donors. C) B cells were infected with a low MOI of 2 for 3 hours to examine bacterial binding patterns. The percent of bacteria bound to each population of cells was determined by flow cytometric analysis. D) The results in C) are re-graphed to allow for ease of comparison, examining how the different cell populations associate with the different strains of Ngo or E. coli. Bars indicate mean. Stars indicate a comparison between Ngo and E. coli infected cells. **, P < 0.01; ***, P < 0.001. Data points corresponding to individual donors are represented by the same symbols within each plot.
cell subset. In human peripheral blood, naive, IgM memory and switched memory B cells can be differentiated by the combined expression patterns of CD27 and IgD (Weller et al., 2004), as shown in Figure 2.5A. In mature cells in the periphery, CD27 expression typically indicates a memory cell lineage, while IgD is primarily expressed on naive cells only. The majority of freshly purified naive cells (IgD+, CD27−) express surface IgM (Figure 2.5B).

The vast majority of IgD+ CD27+ cells express surface IgM, however IgG- and IgA-expressing cells also exist within this population (Figure 2.5B), suggesting that these cells have recently switched Ig classes (Werner-Favre et al., 2001). Most IgD− CD27+ B cells have undergone Ig class switching to express surface IgG or IgA (Figure 2.5B). While the relative proportion of B cells that represent each subpopulation differed slightly between donors, the average proportion of each subpopulation in freshly purified uninfected peripheral blood mononuclear cell preparations was 57% naive (IgD+ CD27−), 26% IgM memory (IgD+ CD27+), and 11% switched memory (IgD− CD27+) B cells (data not shown).

To identify which populations of B cells were associating with *N. gonorrhoeae*, we prepared live fluorescently labelled Ngo or *E. coli*, and used these to infect purified B cells for 3 hours with a low MOI of 2 bacteria per cell. While *E. coli* binding to all peripheral B cell subsets was very low, gonococci displayed substantial binding to naive, IgM memory and switched memory populations of B cells (Figure 2.5C). Comparable binding by the three isogenic gonococcal strains suggests that this association is not strictly dependent on receptors for either Opa variant, however the ability of the B cell subpopulations to bind different strains of gonococci is not equal (Figure 2.5D). Surprisingly, we observed that the simple expression of Opa adhesin did not translate into higher levels of bacterial binding, as OpaHSPG-expressing *Ngo* associated less with the B cells than did the strain that expresses no adhesin (Opa−; Figure 2.5D). In general, memory cells have a greater affinity for gonococci than do naive cells, but the gonococci had a particular affinity for the IgM memory B cells (Figure 2.5C-D). Strikingly, up to 80% of the IgM memory B cells were associated with the gonococci. While it is possible that some gonococcal recognition could be mediated through the BCR, it is clear that this is not the only mechanism at work, since it is unreasonable to assume this large proportion of B cells are *N. gonorrhoeae* specific in all donors examined. There was little association of any B cell subpopulation with *E. coli*, consistent with the interaction being unique to the gonococci.
Figure 2.6  *N. gonorrhoeae* infection, but not infection with *E. coli*, specifically expands the IgM memory B cell population.

A) B cells were infected for 5 days with either gonococci or *E. coli* at an MOI of 10, then analysed for changes in the populations of peripheral B cells. The percent of IgM memory cells is indicated in each of the contour plots. Representative of 3 independent experiments with different donors. B) Quantification of each B cell population after infection under the same conditions outlined in A). Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. ***, P < 0.01. Data points corresponding to individual donors are represented by the same symbols within each plot.
Figure 2.7  B cell infection with clinical *N. gonorrhoeae* strains also induces proliferation of both IgM and switched memory B cell populations.

A) B cells were infected at an MOI of 10 for each of the indicated bacterial strains for 3 days, then analysed for nuclear proliferation antigen Ki67 within the B cell subpopulations. Gonococcal strains N2061 and N2066 were obtained from male urethra, and were both pilin negative. Strain N2061 does not express Opa adhesin, however N2066 expresses at least one Opa variant. Representative of 3 independent experiments with different donors. B) Quantification of the percent of each B cell subpopulation after infection under the same conditions outlined in A). Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. **, *P* < 0.01; ***, *P* < 0.001. Data points corresponding to individual donors are represented by the same symbols within each plot.
2.3.6 IgM memory B cell population expands in response to infection with *N. gonorrhoeae*.

Considering that B cells proliferate in response to gonococcal infection (Figure 2.4A-B), flow cytometry was used to ascertain whether one or more B cell subsets are specifically induced. After 5 days of infection, the IgD⁺ CD27⁺ IgM memory cell population had clearly expanded in response to gonococci but not to *E. coli* in all donors (Figure 2.6A-B). This increase in the innate IgM memory cells was reflected by a tendency towards a reduction in the other B cell populations. However, in contrast to the increase in IgM memory cells, the apparent changes in naive and switched memory cells were not statistically significant (Figure 2.6B). Therefore, despite variability in the proportion of each of the B cell subpopulations between individuals, there is a consistent expansion of the IgM memory B cell subset in response to gonococci. Moreover, the different levels of bacterial association conferred by OpaCEA and OpaHSPG (Figure 2.5C-D) did not influence B cell expansion (Figure 2.6B).

2.3.7 *N. gonorrhoeae* clinical isolates induce proliferation in both memory B cell populations.

*N. gonorrhoeae* undergo frequent antigenic and phase variation. To confirm that the observed B cell responses were not unique to the prototypical MS11 ‘lab’ strain of *N. gonorrhoeae*, we infected B cells with two unrelated clinical isolates obtained from male urethra. Consistent with their phase variable nature, strains N2061 and N2066 both express multiple colony phenotypes. While strain N2066 expresses Opa proteins detectable by colony morphology and immunoblot analysis, strain N2061 displays a transparent colony phenotype and does not express detectable Opa proteins (data not shown). Flow cytometry was used to monitor the expression of nuclear proliferation antigen Ki67 in all B cell subpopulations three days post infection. All strains of Ngo tested induced robust proliferation by IgD⁺ CD27⁺ IgM memory cells (Figure 2.7A-B). Some IgD⁻ CD27⁺ switched memory cell proliferation was apparent in response to infection by all Ngo strains, but to a much lower extent than in the IgM memory cells (Figure 2.7A-B). This is in clear contrast to infection with *E. coli*, which again induced no proliferation in any B cell subpopulation (Figure 2.7A-B). The potent B cell response thus appears to be an intrinsic feature of *N. gonorrhoeae* rather than being a strain-specific effect.
2.3.8 Polyreactive IgM is produced by B cells infected with *N. gonorrhoeae*.

While naive B cells require antigen receptor signalling for the production of Ig, innate IgM memory B cells have the potential to produce antibody in a T-independent manner, either in the presence and absence of antigen receptor signalling (Bernasconi et al., 2002). For example, CpG DNA has been shown to induce Ig production by the IgM memory B cells, even in the absence of BCR signals (Bernasconi et al., 2002). This prompted us to examine whether immunoglobulin production could be elicited by *N. gonorrhoeae* infection of primary human B cells. As depicted in Figure 2.8A, IgG, IgM and IgA classes of antibody were all induced in significant amounts upon infection with gonococci relative to uninfected cells. This effect was not apparent upon infection with *E. coli*, indicating that it is not a generalized response to bacterial-derived microbe-associated molecular patterns (MAMPs). IgM is the principle class of Ig elicited in response to Ngo. Neither Opa expression nor the relative level of association by the isogenic neisserial strains with B cells (Figure 2.5C-D) correlated with total Ig titres induced or the relative proportion of individual Ig classes (Figure 2.8A). Considering that the study population was not believed to have high risk sexual behaviours and are thereby not uniformly exposed *N. gonorrhoeae*, it is unlikely that this is a result of recall responses.

In order to examine whether gonococcal infection produced Ngo-specific antibody, we prepared ELISA plates coated with the Opa- strain of gonococci. Using this strain to measure Ig responses against all three isogenic Ngo strains eliminates potential skewing of results caused by responses to the strongly immunogenic Opa proteins (Plummer et al., 1994). *N. gonorrhoeae* infection elicited gonococci-specific IgM (Figure 2.8B), whereas no detectable gonococci-specific IgG or IgA were evident (data not shown). Importantly, the baseline amount of Ngo-reactive Ig in *E. coli*-infected samples closely reflected that in uninfected samples, indicating that the observed increase in Ig required exposure to the gonococci.

Next, to assess the specificity of the Ig response, we performed ELISAs to monitor the production of antibodies specific for other, non-neisserial antigens upon *N. gonorrhoeae*-infection of the B cells. For this, we measured Ig specific for a recall antigen, tetanus toxoid (TT), as well as an irrelevant antigen, keyhole limpet hemocyanin (KLH). While little TT-specific Ig was apparent in uninfected samples, significant increases in IgM specific for TT were
Figure 2.8  Primary B cells produce polyclonal IgM, including pathogen specific antibody, in response to infection with *Ngo*, an effect that is not observed with *E. coli* infection.

A) 5 days post infection, cell free supernatants taken from B cell cultures infected with MOI of 10 bacteria per cell were analyzed for total Ig production by ELISA. B, C and D) ELISA plates were coated with either Opa+ gonococci (Ngo), tetanus toxoid (TT), or keyhole limpet hemocyanin (KLH) to examine the antigen specificity of the Ig produced upon infection. After 5 days of infection, supernatants were applied to these plates to examine the production of Ig specific for B) gonococci, to determine if pathogen specific Ig is produced, C) tetanus toxoid, to examine if polyclonal activation of B cells includes activation of switched memory cells, and D) keyhole limpet hemocyanin, to determine if Ig specific for irrelevant antigens is produced. All 3 classes of Ig were tested. All cells from donors examined produced IgM that recognized TT, while 9/10 donors produced IgM specific for KLH and 7/9 donors produced IgM specific for gonococci. Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data points corresponding to individual donors are represented by the same symbols within each plot.
Figure 2.9  TLR9 signalling is induced upon *N. gonorrhoeae* infection of primary human B cells.

A and B) B cells were treated with 2.5 μg/mL of Pam3CSK4, 1 μg/mL of FSL1, 1 μg/mL of LPS, 5 μg/mL of CpG ODN2006, infected with MOI:10 of *N. gonorrhoeae* strain N302, or left untreated for 3 days, before analysis of A) CD86 for cellular activation and B) Ki67 for proliferation were conducted by flow cytometry, differentiating between B cell subpopulations by staining for IgD and CD27.  C) Treatment of B cells with inhibitory CpG (iCpG) decreases proliferation induced upon either infection with gonococci or CpG treatment.  B cells were either left untreated, infected with MOI:10 of *N. gonorrhoeae* strain N302, or treated with 5 μg/mL of CpG ODN2006. iCpG was added to inhibit TLR9 signalling, and in parallel wells, a control for iCpG was added (cCpG).  After 3 days incubation, cells were stained for B cell subpopulations and proliferation by Ki67.  The relative percent of Ki67 positive cells was obtained by taking the ratio between proliferation observed in iCpG treated cells vs cCpG treated cells.  Bars indicate mean.  Stars indicate a comparison between uninfected and infected cells.  *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.  Data points corresponding to individual donors are represented by the same symbols within each plot.
apparent in the culture supernatants of all Ngo-infected cells (Figure 2.8C), and the purified B cells from five out of ten donors produced TT-specific IgG upon exposure to the gonococci (data not shown). Considering that a recall response to TT would be expected to primarily be IgG, this implies that the observed TT-specific IgM observed is not a conventional memory response. TT-specific antibodies were not present in E. coli-infected samples (Figure 2.8C), again indicating that this is not a generalized response to bacteria-derived products. B cells from the majority of donors (90%) also produced IgM reactive with KLH following infection with gonococci but not E. coli (Figure 2.8D) despite the fact that we would not expect the volunteer blood donors to have been exposed to this antigen previously. When combined, these results indicate that N. gonorrhoeae infection of primary B cells results in broad, polyclonal activation and differentiation of IgM-bearing B cells rather than a focused, clonal response to the gonococci.

2.3.9 Memory B cell responses to N. gonorrhoeae infection involve TLR9.

Since all strains of N. gonorrhoeae examined produced robust proliferation in the IgM memory population of B cells, we explored the possibility that gonococci were being detected by innate immune receptors, resulting in downstream proliferation and differentiation of B cells. It has already been established that TLR expression varies between human peripheral B cell subpopulations, such that naive cells express few TLRs, each at very low (to undetectable) levels when they are present, while both IgM memory and switched memory cells express high levels of TLR6, 7, 9, and 10 (Bernasconi et al., 2003). Neisseria species are known to constitutively express outer membrane porins that can be detected by complexes of TLR2 and TLR1 (Massari et al., 2002; Massari et al., 2006), in addition to possessing a lipo-oligosaccharide (LOS) that is recognized by TLR4 (Pridmore et al., 2001). In order to assess functional TLR responses in the B cell subsets, we examined a panel of specific TLR agonists: Pam3CSK4 (a synthetic triacylated lipoprotein that signals through TLR2/1), FSL1 (a synthetic diacylated lipoprotein that signals through TLR2/6), E. coli K12 LPS (TLR4), and CpG ODN2006 (TLR9), for their ability to cause activation and proliferation. Surprisingly, naive cell activation was significantly increased in response to treatment with TLR2/6 agonist FSL1 and TLR9 agonist CpG ODN2006 compared to untreated cells (Figure 2.9A). Naive cells were weakly activated in response to treatment with TLR2/1 agonist Pam3CSK4, however this difference did not reach statistical significance (Figure 2.9A). Memory B cells responded to Pam3CSK4, FSL1 and CpG
ODN2006 with strong and significant increases in cellular activation (Figure 2.9A). Treatment with LPS did not induce any response in any B cell populations (Figure 2.9A-B), confirming previously published reports that TLR4 is not expressed in high enough levels in any human B cell subset to facilitate signalling (Bernasconi et al., 2003; Bourke et al., 2003).

Although naive cells were able to express activation marker upon treatment with some TLR agonists, proliferation was not observed in these cells in response to any of the TLR agonists tested (Figure 2.9B). IgM and switched memory cells underwent strong proliferation responses to CpG ODN2006. While both memory cell populations did tend to show increases in proliferation due to treatment with Pam3CSK4 and FSL1, the difference did not reach statistical significance (Figure 2.9B).

Treatment with all TLR agonists examined resulted in proliferation by both memory subsets to varying degrees, however treatment with TLR9 agonist induced by far the strongest response within both populations (Figure 2.9B). Others have shown that treatment with CpG ODN2006, either alone or in combination with other cytokines, induces IgM antibody only by IgM memory cells, whereas switched memory cells predominately produce IgG and IgA immunoglobulin (Bernasconi et al., 2003). Considering that Ngo infection induces the specific expansion of IgM memory B cells (Figure 2.6B), and that the majority of Ig induced is IgM (Figure 2.8A), we considered whether these effects were mediated through TLR9 signals. A dominant negative CpG-containing oligonucleotide that inhibits TLR9 signaling (iCpG) has been shown to compete with the stimulatory effects of other CpG-containing DNA on both primary murine and human immune cells (Dobson-Belaire et al., 2010; Gursel et al., 2003). We took advantage of this reagent to examine the contribution of TLR9 to the induction of B cell proliferation by gonococcal infection. While the iCpG had little effect on uninfected B cells, this inhibitor effectively reduced the proportion of actively proliferating cells in response to the synthetic CpG-containing oligonucleotide ODN2006 or Ngo by 54.9% and 35.6%, respectively (Figure 2.9C). Thus, TLR9 contributes to B cell responses induced by Ngo infection.
Figure 2.10  B cells are able to engulf and kill whole gonococci.

A) B cells were infected with MOI:10 of indicated strains of *N. gonorrhoeae* for 3 hours, stained for extracellular gonococci first, then permeabilized to stain for total gonococci. Intracellular *Ngo* are single colour stained. DIC, differential interference contrast. Bar length indicates 5 μm. 

B) B cells were infected with the indicated strain of gonococci for 90 minutes, then treated with gentamycin to kill any extracellular bacteria. At 2.5 and 6 hours post infection, cells were washed, lysed, then plated for growth on GC agar. Bars indicate mean. Data points corresponding to individual donors are represented by the same symbols within each plot.
2.3.10 *N. gonorrhoeae* can be engulfed and subsequently killed by primary human B cells.

TLR9, unlike other toll-like receptors, is found within endolysosomes upon stimulation and is not expressed on the cell surface (Latz et al., 2004). While B cells will endocytose soluble antigen (Lanzavecchia, 1985), their ability to engulf intact bacteria is largely unexplored. We considered the possibility that B cells infected with *N. gonorrhoeae* might engulf and kill whole bacteria, effectively delivering bacterial DNA directly to intracellular TLR9. As depicted in Figure 2.10A, primary human B cells are able to engulf whole gonococci, as determined by differential staining of total and extracellular gonococci. In order to determine the fate of the internalized bacteria, a gentamycin protection assay was used to quantify viable intracellular gonococci. This assay takes advantage of the fact that gentamycin does not permeate mammalian membranes, so engulfed bacteria are protected from the antibiotic. B cells were infected with the specified strain of gonococci for 90 minutes before gentamycin addition for 1 hour. Viable intracellular gonococci are observed at this (2.5 hours) point, supporting the microscopic evidence that the bacteria were being internalized. However, when the infection was allowed to persist for an additional 3.5 hours, the vast majority of intracellular gonococci were dead, consistent with effective killing of the bacteria (Figure 2.10B). Taken together, *N. gonorrhoeae* has a high affinity for B cells, which undoubtedly contributes to the ability of these cells to internalize whole gonococci. Once engulfed, the bacteria are effectively killed, delivering a large bolus of bacterial DNA directly to intracellular lysosomal compartments where it could interact with TLR9.

2.4 Discussion

*N. gonorrhoeae* has a curious niche, such that the pathogen seems to penetrate and persist within the subepithelial space (McGee et al., 1983); this site is rich in nutrients, but also patrolled by resident sentinel immune cells (Levine et al., 1998; Prakash et al., 2001) that the bacteria must contend with. B cells accumulate in the endocervical mucosa during both symptomatic and asymptomatic gonococcal infections (Levine et al., 1998), making it likely that infecting *N. gonorrhoeae* come into direct contact with leukocytes including B cells at the site of infection as well as in the draining lymph nodes. When considering this, it is surprising that the antibody
response elicited by Ngo is low compared to other mucosal infections and lacks signs of memory upon subsequent exposure (Hedges et al., 1999). B cells are antigen presenting cells and express many pattern recognition molecules capable of detecting bacterial-derived products (Bernasconi et al., 2003; Kubagawa et al., 2009; Shibuya et al., 2000; Thornton et al., 1994; Wing et al., 2009). Although this suggests that B cells should recognize all bacteria equally, our results unexpectedly indicate that N. gonorrhoeae has a specific tropism for the innate IgM memory subset of human B cells that elicits both their proliferation and a potent but broadly reactive T cell-independent immunoglobulin response.

Infection by a variety of bacteria can cause B cell death (Krocova et al., 2008; Menon et al., 2003), yet we observed that N. gonorrhoeae infection instead supports their viability and promotes the proliferation of primary human B cells, even decreasing cell death that normally occurs during in vitro culture of freshly isolated human B cells. In other cell types, both protection from (Binnicker et al., 2003; Follows et al., 2009) and promotion of (Muller et al., 1999) cell death have been observed upon infection with N. gonorrhoeae. Although the majority of studies examining this effect have focused on epithelial cells, it is becoming clear that infection with Ngo, more often than not, inhibits apoptosis (Follows and Massari, 2010). Considering the effect of Ngo infection on apoptosis in B cells, previous work focused on pre-activated human B cells suggested that gonococci binding to CEACAM1 results in cell death (Pantelic et al., 2005). Our finding that primary human B cells are activated in response to N. gonorrhoeae infection implies that those results may reflect cell death attributable to the overwhelming stimulation applied, which included both gonococci and cytokines, rather than a direct affect of the bacteria itself. While specific conclusions regarding the survival signals elicited by the gonococci cannot be made, we propose that the protection from cell death observed in our assays stem in part from the innate nature of the lymphocytes that are responding. These cells have a lower activation threshold than that of naive cells (Good et al., 2009), suggesting that they have evolved to respond by rapid activation, proliferation and differentiation in response to infection.

It is unreasonable to expect that the large proportion of B cells that we observe respond to N. gonorrhoeae are specific for neisserial antigens, especially considering our observation that it is primarily the IgM memory B cells that vigorously respond to infection by a pathogen that is uncommon in the study population. In general, bacterial products such as polysaccharide
vaccines (Carsetti et al., 2005; Weller et al., 2004) and purified CpG DNA (Bernasconi et al., 2002) have been shown to expand the population of human peripheral blood innate IgM memory B cells. Interestingly, *N. gonorrhoeae* does not express a polysaccharide capsule, confirming that stimulation of this subset does not require antigenic signalling by these structures. As such, this is the first report indicating the direct induction of polyclonal proliferation and IgM production by the IgM memory B cell subpopulation in response to bacteria.

Gonococcal induced B cell proliferation was lower upon treatment with TLR9 antagonist, iCpG (Figure 2.9C), but inhibition was not complete. *N. gonorrhoeae* is a complex organism, with many components that have the potential to interact with host cell receptors and cause immune activation, especially on antigen presenting cells such as B cells. Bacterial products derived specifically from neisserial species can induce cellular responses from immune cells, with neisserial porins and LOS among the best characterized. Neisserial LOS has been shown to signal through TLR4 (Pridmore et al., 2001), leading to cellular activation and cytokine production in a variety of immune cells (Liu et al., 2010; Pridmore et al., 2003; Uronen-Hansson et al., 2004), however we did not observe a TLR4 response in any of the human B cell subsets described herein (Figure 2.9A-B). Neisserial porins are outer membrane proteins that have been shown to strongly induce both activation and proliferation by human and murine B cells through interaction with TLR2 and TLR1 (Massari et al., 2006; Wetzler et al., 1996). Although it has been recently reported that purified porin from *N. lactamica*, a commensal that is closely related to *N. gonorrhoeae*, cannot induce proliferation in primary human B cells (Vaughan et al., 2010), it remains possible that neisserial porins could contribute to the intense proliferation observed upon gonococcal infection, since both switched and unswitched (IgM) memory B cells express sufficient TLR2 and TLR1 to respond to purified FSL1 (Figure 2.9A-B). While expression of TLRs in the IgM memory subset has already been established (Bernasconi et al., 2003), this is the first description of TLR functionality within the human innate B cell population.

Though it has tended to be less of a focus, it is becoming increasingly clear that neisserial DNA signalling through TLR9 also contributes to induction of immune cell responses (Dobson-Belaire et al., 2010; Magnusson et al., 2007; Mogensen et al., 2006). In a murine model of infection, TLR9 deficiency was not only shown to be associated with impaired survival upon infection with *Neisseria meningitidis* (a pathogen that is closely related to *N. gonorrhoeae*), but also with increased bacteraemia and decreased serum bactericidal activity, suggesting that TLR9
recognition of neisserial products is critical for the induction of an effective immune response against neisserial species (Sjolinder et al., 2008). TLR9 signalling in B cells is not inconsequential, as its expression is high compared to other TLRs (Bernasconi et al., 2003). TLR9 is found in intracellular compartments (Latz et al., 2004), suggesting that the specific affinity of \textit{N. gonorrhoeae} for the innate memory B cells contributes to their overwhelming stimulation, while \textit{E. coli} does not. Important in this context has been the demonstration that the delivery of bacterial DNA directly to TLR9-containing compartments is absolutely required for B cell responses, as they are poor at engulfing exogenous DNA (Roberts et al., 2010). B cells are surprisingly effective at engulfing whole gonococci (Figure 2.10A-B), allowing for the delivery of gonococcal DNA to TLR9, while engulfment of \textit{E. coli} was rarely observed (data not shown). This difference may result from the combination of the gonococci’s tropism for B cells increasing the time spent in contact with the cells and the larger size of \textit{E. coli} hindering the engulfment of bound bacteria.

We observe that B cell infection with \textit{N. gonorrhoeae} elicits an impressive production of mainly IgM, which recognizes both immunologically relevant (\textit{Ngo}) and heterologous (TT and KLH) antigens. The production of \textit{Ngo}-specific IgM in response to gonococcal infection in the absence of T cell help would be expected if it was a specific BCR-dependent response, but our observations suggested that \textit{Ngo} recognition by B cells was unlikely due to the BCR alone, so the ELISA assay was designed to maximize the potential of capturing any gonococcal specific Ig produced by using whole, heat killed bacteria containing a vast number of antigenic epitopes as the capture antigen. Even with this strategy, the gonococci induced more IgM specific for TT and KLH, heterologous antigens consisting of single subunit proteins that contain fewer potential epitopes than do the whole bacteria. This both highlights that the T-independent Ig response is broadly polyspecific and makes it intriguing to consider that there may be a selective expansion of heterologous, non-neisserial Ig in response to the gonococci.

While both naive and IgM memory B cells express IgM antigen receptor, it is clear that IgD$^+$ CD27$^+$ IgM memory cells respond to infection with neisserial species, while the naive IgD$^+$ CD27$^-$ cells do not (Figure 2.7A-B). The innate IgM memory cells represent the first line of defence by the adaptive immune response due to their natural production (not requiring antigenic stimulation) of low affinity but polyreactive IgM, the majority of which express germline-encoded receptors that can undergo somatic hypermutation (Weller et al., 2008). Some of this
‘natural’ IgM presumably functions by opsonising encapsulated bacteria (Shi et al., 2005; Zhou et al., 2007) and neutralizing viruses (Choi and Baumgarth, 2008; Ochsenbein et al., 1999). Natural antibody production has been attributed to the IgD⁺ CD27⁺ IgM memory cells (Chen et al., 1998; Kruetzmann et al., 2003). While most IgD⁺ CD27⁺ cells express IgM (Bernasconi et al., 2002; Weller et al., 2004), our observation that IgG- and IgA-expressing cells are also present in this population is reasonable considering that natural IgM, IgG and IgA all occur (Avrameas, 1991; Wijburg et al., 2006) and that class switch recombination by these cells in response to stimulation has been reported (Werner-Favre et al., 2001).

An innate population of B cells with similar characteristics to the human IgM memory B cells exist in mice, and are termed B-1 B cells (Kruetzmann et al., 2003). Functionally, murine B-1 B cells are similar to human IgD⁺ CD27⁺ innate IgM memory cells since they are both considered to be the source of natural antibody (Baumgarth et al., 1999). However, the human innate IgM memory cell population is found in the periphery and represent a circulating form of splenic marginal zone B cells (Weller et al., 2004), whereas similar cells in mice are found primarily associated with peritoneal and pleural cavities (Herzenberg and Kantor, 1993). While there are differences between these two innate populations, they are currently the best defined models we have for comparison. Future work in the field may clarify if they are indeed homologous populations.

Although the human IgM memory B cells have been conclusively identified as a distinct B cell subpopulation relatively recently (Kruetzmann et al., 2003; Weller et al., 2004), it is becoming clear that their immune function is non-redundant in the production of an effective immune response against invading micro-organisms. In the murine model, Baumgarth and colleagues have exquisitely dissected the role of B-1 derived IgM for protection during influenza infection (Baumgarth et al., 2000). In humans, loss of IgM memory B cells correlates with recurrent bacterial pneumonia infections in patients with common variable immunodeficiency (Carsetti et al., 2005; Kruetzmann et al., 2003). Furthermore, low numbers of IgM memory B cells found in the periphery of HIV⁺ patients have been shown to be a predictor of cryptococcosis (Subramaniam et al., 2009). In a similar vein, B cell super-antigen Staphylococcus aureus protein A targets the innate B cells for deletion, leaving a void in the B cell repertoire that remains un-restored fourteen months after the initial treatment (Silverman et al., 2000). Neisseria gonorrhoeae appears to use an alternative approach to prevent the production of a
specific humoral response by suppressing T cell help (Boulton and Gray-Owen, 2002; Lee et al., 2008) while strongly stimulating the innate B cells to produce polyclonal Ig responses, thus lowering the effective concentration of gonococcal-specific antibody. Considering that some of the Ig will bind the bacteria, the benefit of a polyspecific Ig response to the infecting pathogen remains a matter of debate (Montes et al., 2007). However, we have observed that infection with commensal Neisseria species can elicit a similar polyclonal Ig response (unpublished observations), implying that it facilitates colonization rather than being a strict virulence mechanism.

IgG and IgA production by B cells infected with Ngo is significantly higher than the levels produced in response to infection with E. coli (Figure 2.7B). This suggests that either natural IgG and IgA are induced, or that switched memory cells, in addition to the IgM memory cells, are differentiating into antibody secreting cells in response to the gonococci. We consider it plausible that the switched memory cells may benefit from T-independent Neisseria-induced ‘bystander’ activation signals that originate from the innate IgM memory B cells, at least partially explaining the switched memory response. Bernasconi et al. demonstrate bystander activation of switched memory B cells, wherein microenvironments produced upon activation of antigen-specific memory cells supports the activation of non-cognate switched memory cells in a T-dependent manner. They hypothesize that this mechanism of bystander activation functions to maintain long term systemic immunologic memory over the life-time of an individual. Although previously undescribed, the possibility that innate B cells could play a role in the bystander activation of switched memory cells is enticing since it would suggest that the innate B cells could activate polyclonal B cell memory at first sign of infection.
Figure 2.11 Model of how *N. gonorrhoeae* infection effectively prevents the production of a protective immune response in infected individuals.

A) Upon vaccination or infection with microbes that elicit T-dependent B cell responses, activated T cells can provide co-stimulatory signalling to B cells. This is required for clonal expansion of B cells and the generation of a highly-specific Ig response against the antigen/microbe. B) Activation and proliferation of CD4$^+$ T cells is inhibited by infection with *Ngo* through clinically relevant engagement of the co-inhibitory receptor CEACAM1 by the neisserial Opa proteins. This prevents T and B cell collaboration, abrogating the production of protective memory responses. B cells are able to respond to *Ngo* infection in a T-independent manner, mediated partly through direct delivery of neisserial DNA to intracellular TLR9, inducing activation of IgM memory cells to produce a polyclonal IgM response that includes Ig specific for gonococci, but also irrelevant antigens. The overall result is an ineffectual immune response to *N. gonorrhoeae* infection.
Although it is clear that the innate IgM memory B cells are capable of responding to microbes in a T-independent manner, it has been recently suggested that murine innate B cells may possess a form of memory that is distinct from the conventional T-dependent response (Alugupalli et al., 2004). In humans, analysis of female sex workers from Kenya showed the induction of an incomplete, but serotype specific, immunologic memory response following repeated *N. gonorrhoeae* infections (Plummer et al., 1989). Although these women could be slowly acquiring immunity by traditional T-dependent mechanisms, it is tempting to propose the possibility that this memory response results from the repeated *N. gonorrhoeae*-dependent expansion of human innate IgM memory B cells. Further *in vivo* studies will be required to test this postulate.

In this report, we establish that the human specific pathogen *N. gonorrhoeae* elicits a vigorous innate B cell response. Considering that other bacteria, including *E. coli* used in this study and *Listeria monocytogenes* (Menon et al., 2003) and *Francisella tularensis* (Krocova et al., 2008) in previous work, do not elicit a similar response, this appears to be a specific effect of the *Neisseria* rather than being a prototypical immune response. Indeed, it is intriguing to speculate that *Neisseria* may promote the specific expansion of innate B cells to amplify the production of non-specific Ig in a manner that negatively affects specific immunity in the infected individuals. We propose that, in the early stages of infection, circulating innate IgM memory B cells may come into contact with the gonococci, either at the site of infection or in the draining lymph nodes surrounding the urogenital tract. This interaction promotes the engulfment of whole gonococci by the B cells, allowing for the direct detection of bacterial products by innate receptors including TLR9. This results in both a vigorous T-independent proliferation to expand the innate IgM memory B cell population and their production of an acute effector response in the form of low affinity, polyclonal IgM (see model, Figure 2.11). As the infection progresses, attachment to the co-inhibitory receptor CEACAM1 on human T cells by gonococcal Opa proteins prevents the upregulation of co-stimulatory molecules, abrogating the induction of T cell help and clonal T cell expansion (Boulton and Gray-Owen, 2002; Lee et al., 2008). Consequently, the absence of effective T cell help results in a broadly specific and thereby weakly effective humoral response that may assist in clearing the current infection but, in the context of an unguided B cell response, provides no protective memory upon subsequent exposure to *N. gonorrhoeae*. 
2.5 Materials and Methods

2.5.1 Cells

Primary human B cells were obtained by either standard venipuncture or, when large numbers of cells were required, through leukopheresis. All participants gave informed consent in accordance with guidelines for the conduct of clinical research at the University of Toronto and St. Michael’s Hospital, respectively. The protocols used were approved by the University of Toronto and St. Michael’s Hospital institutional review boards (Toronto, Ontario, Canada).

Fresh peripheral mononuclear cells were obtained from Ficoll-Paque Premium (Amersham GE) gradients, according to manufacturer’s specifications. CD19+ B cells were purified using either the EasySep Human CD19 Positive Selection Kit (Stemcell Technologies) or CD19 Microbeads (Miltenyi Biotec). B cell purity consistently exceeded 95%, as measured by a FACScalibur flow cytometer (BD Biosciences) with FlowJo software (Treestar) used for all flow data analysis.

Purified cells were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (CanSera), 4 mM GlutaMAX (Invitrogen) and 50 mM HEPES (Bioshop, Burlington, Canada) at pH 7.4. Cells were cultured at 37°C in 5% CO₂ and humidified air.

2.5.2 Bacterial Strains

Isogenic Neisseria gonorrhoeae strains constitutively expressing no Opa (Opa−, strain N302), the heparan sulfate proteoglycan-specific OpaHSPG (strain N303, expressing Opa50), or CEACAM-specific OpaCEA (strain N309, expressing Opa52) were derived from a pilus-deficient MS11 parent strain (Kupsch et al., 1993), and were graciously provided by Prof. T.F. Meyer (Berlin, Germany). Clinical N. gonorrhoeae strains N2061 and N2066 were isolated from male urethra, and both do not express pilin. Strain N2061 does not express Opa, however N2066 does express at least one Opa adhesin based upon immunoblot analysis. These clinical strains were obtained from a sexually transmitted disease clinic in the district of Pumwani in Nairobi, Kenya, and were a generous gift from Dr. F. A. Plummer (Winnipeg, Canada). Neisseria species were grown from frozen stocks on GC agar (Difco) supplemented with 1% (v/v) IsoVitalex (BBL Microbiology Systems) at 37°C in a 5% CO₂ atmosphere with humidity. A binocular microscope was used for daily selection of gonococcal colony opacity phenotypes for the MS11
strains. Opa protein expression was monitored by immunoblotting using the Opa cross-reactive monoclonal antibody 4B12/C11 (Achtman et al., 1988), graciously provided by Prof. Mark Achtman (Berlin, Germany).

*Escherichia coli* (DH5α) was a gift from Prof. T.F. Meyer (Berlin, Germany). It was grown from frozen stocks in aerated Luria-Burtani broth overnight at 37°C. Liquid cultures were sub-cultured onto Luria-Burtani agar prior to infection assays.

### 2.5.3 Infection of Lymphocytes

Infections using a range of multiplicity of infections (MOIs) for both *N. gonorrhoeae* and *E. coli* were conducted in pilot experiments to assess cellular responses. It was clear that there were dose dependent effects with both bacteria, such that infection with MOI:1 of *Ngo* showed no effect on cellular viability, however viability was strongly increased with an infecting MOI of 100, which was followed by rapid cell death by day 3. *E. coli* infection with an MOI higher than 10 rapidly kills the B cells. Based on these preliminary results, we selected an MOI of 10 for our assay system since it allowed us to compare the effects of gonococcal infection with *E. coli* over time.

Freshly isolated human B cells were infected with a multiplicity of infection (MOI) of 10 bacteria per cell, unless otherwise indicated. Gentamycin (Bioshop, Burlington, Canada) was routinely added to neisserial infection cultures 3 hours post infection to prevent bacterial overgrowth. For *E. coli*, gentamycin was added immediately upon infection to prevent bacterial overgrowth of the B cell cultures.

### 2.5.4 Confocal Microscopy

B cells were infected with the indicated *N. gonorrhoeae* and *E. coli* strains at a MOI of 10 for durations between 1.5 and 44 hours. Acid-washed glass coverslips were coated with mouse monoclonal antibody specific for human CD19 (clone HIB19, eBioscience) to capture B cells for microscopy. Cells were fixed with 4% paraformaldehyde (PFA; Sigma), and gonococci were detected using a polyclonal rabbit anti-gonococcal serum (UTR01) followed by Alexa 488-conjugated secondary antibody (Molecular Probes, Invitrogen) after permeabilization of mammalian cell membranes using 0.4% Triton X-100 (Sigma). *E. coli* was detected using the purified polyclonal goat antibody ab25823 (Abcam), followed by a goat-specific secondary
antibody in a process similar to the gonococcal staining protocol. Where indicated, bacteria were pre-labelled using Texas Red-succinimidy1 ester (Molecular Probes) rather than using antisera. B cells were stained using F(ab\textquotesingle\textquotesingle)\textsubscript{2} of biotinylated goat anti human IgM, IgG and IgA (Jackson ImmunoResearch Laboratories), followed by streptavidin Alexa 488 (Molecular Probes).

To examine if primary human B cells were able to engulf whole bacteria, cells were infected with the indicated \textit{N. gonorrhoeae} strains at a MOI of 10 for 3 hours. Acid washed coverslips coated with anti-human CD19 (clone HIB19, eBioscience) were used to capture B cells prior to fixation. Extracellular gonococci were stained first, using a polyclonal rabbit anti-gonococcal serum (UTR01), followed by anti rabbit IgG Alexa 647 (Molecular Probes). Mammalian cell membranes were permeabilized using 0.4% Triton X-100 (Sigma), allowing for total gonococci to be stained using the polyclonal rabbit anti-gonococcal serum (UTR01), followed by anti-rabbit IgG Alexa 488 (Molecular Probes).

Bacteria and cells were visualized and recorded using a Plan-Apochromat 100x/1.4 Oil DIC objective lens on a Zeiss LSM 510 confocal microscope. All images were obtained at room temperature. Zeiss LSM Image Browser version 4.2.0.121 was used for subsequent image processing. Each infection condition was prepared on duplicate or triplicate coverslips, and at least 60 \textit{Ngo}-infected cells and at least 100 cells for the \textit{E. coli} infection condition were analyzed.

2.5.5 Analysis of B cell responses

All flow cytometric analysis was performed using a BD FACScalibur. B cell subsets were defined using the following antibodies: IgD-PE or IgD-FITC (clone IA6-2, BD Biosciences), CD27-FITC or CD27-APC (clone O323, eBioscience), and Cy5-labelled F(ab\textquotesingle\textquotesingle)\textsubscript{2} fragments of goat and human IgG, IgM, or IgA (Jackson ImmunoResearch Laboratories). CD27 and IgD immunostaining was performed on live cells, whereas all other immunostaining was performed on fixed cells. CompBeads (BD Biosciences) were utilized for fluorescent compensation. All cells were fixed prior to flow analysis.

To identify the B cell subpopulations associating with gonococci or \textit{E. coli}, live bacteria were labelled with Alexa-647 succinimidyl ester (Molecular Probes) and then used to infect purified B
cells at an MOI of 2 bacteria per cell for 3 hours, before treatment with gentamycin. Bacterial association to B cell subpopulations was visualized by flow cytometry.

For viability, activation and proliferation assays, infected cells and uninfected controls were fixed at time points indicated in the figure legends. Where indicated, 16 μM of staurosporine (Sigma) was added to uninfected cells to induce B cell death as a control for viability experiments. Forward and side scatter data and annexin V-PE (BD Biosciences) staining were used to quantify cell death. Cellular activation was quantified using CD86-PE (clone FUN-1, BD Biosciences) and forward vs side scatter data, as activated cells increase in forward scatter compared to cells at rest.

To examine proliferation, B cells were infected overnight with bacteria or were left uninfected, at which point 5-bromo-2-deoxyuridine (BrdU; Sigma) was added to the culture media. BrdU incorporation was quantified using monoclonal mouse anti-BrdU (clone IU-4, Caltag Laboratories, Invitrogen), which was directly conjugated to biotin using Zenon Mouse IgG1 Labelling Kit (Molecular Probes). Streptavidin-APC (Jackson ImmunoResearch Laboratories) was then used to visualize the antibody for detection by flow cytometry. To identify and quantify populations of cells undergoing proliferation at the time of fixation, Ki67-Alexa 647 (clone B56, BD biosciences) was used.

B cell responses to TLR agonists were examined by culturing purified B cells with the following reagents (all from Invivogen): Pam3CSK4 at 2.5μg/mL, FSL1 at 1μg/mL, E. coli K12 LPS at 1μg/mL and CpG ODN2006 at 5μg/mL for 3 days, at which time they were stained for activation marker, CD86-PE (clone FUN-1, BD Biosciences) or nuclear proliferation antigen, Ki67-Alexa 647 (clone B56, BD biosciences) and B cell subpopulation markers CD27 and IgD as described above.

To examine the role of TLR9 signalling in N. gonorrhoeae infection of B cells, inhibitory CpG ODN TTAGGG (iCpG, Invivogen) was used, which functions as a dominant negative TLR9 ligand. B cells were either left untreated/uninfected, treated with CpG ODN2006 (Invivogen) at 0.65 nmoles/mL or infected with N. gonorrhoeae strain N302 Opa´ at a MOI of 10. Cell cultures were then treated with either iCpG or ODN TTAGGG control (cCpG, Invivogen), a control ODN that possess similar sequence to the iCpG but without the ability to inhibit TLR9
signalling, both at 10x molar concentration of stimulating CpG ODN2006 for 3 days, at which time cells were stained for Ki67-Alexa 647 (clone B56, BD biosciences) and B cell subpopulation markers CD27 and IgD as already described.

2.5.6 ELISA

All ELISAs were read at 450 nm using a 1420 Victor³ (PerkinElmer) plate reader, unless otherwise indicated. Total Ig in cell culture supernatant was quantified by IgM, IgG or IgA ELISA kits (Zeptometrix), used according to manufacturer’s instructions.

To monitor antigen-specific immunoglobulin production, 96 well Maxisorp plates (Nunc) were coated with either heat-killed Opa-gonococci (N302), keyhole limpet hemocyanin (KLH; Sigma), or tetanus toxoid (TT; List Biological Labs) resuspended in pH 7.4 phosphate buffered saline (PBS; Wisent). The plates were dried, vacuum sealed into airtight bags, and stored at 4°C until use. 5% bovine serum albumin (BSA; Bioshop, Burlington, Canada) in PBS was used to block wells, and 0.05% Tween 20 (Sigma) in PBS was used as wash buffer. Culture supernatants from uninfected cells or those infected with either gonococci or E. coli were diluted using 1% BSA in 0.05% Tween 20.

For KLH and TT ELISAs, biotinylated F(ab’)2 fragments of goat anti-human IgG, IgM and IgA (Jackson ImmunoResearch Laboratories) were used separately to determine the amount of each class of Ig produced in response to bacterial infection. After incubation with streptavidin horse radish peroxidise (HRP) Ultra (Sigma), 3,3’,5,5’-tetramethylbenzidine (TMB; KPL) was used as the colourmetric reagent for HRP activity.

For N. gonorrhoeae plates, F(ab’)2 fragments of goat anti-human IgG, IgM and IgA directly conjugated to alkaline phosphatase (AP; Jackson ImmunoResearch Laboratories) were used separately to determine the amount of each class of Ig present in culture supernatants. BluePhos (KPL) was used as the developing reagent. These ELISAs were read at 595 nm.

2.5.7 Gentamycin protection assay

To examine the viability of engulfed intracellular gonococci, B cells were infected with a MOI of 50 with the indicated N. gonorrhoeae strains for 1.5 hours. Infection cultures were then treated with and maintained in 100μg/mL of gentamycin (Bioshop, Burlington, Canada) to kill any
extracellular (non-engulfed) gonococci. At 2.5 and 6 hours post-infection, cells were washed and concentrated in PBS containing 1 mM MgCl$_2$ and 0.5 mM CaCl$_2$ using transwells with a 3 μm pore size (Corning). 1% saponin (Sigma) was added to lyse mammalian membranes. Serial dilutions of the lysis mixture were plated on GC agar (Difco) supplemented with 1% (v/v) IsoVitalex (BBL Microbiology Systems) and incubated at 37°C in a 5% CO$_2$ atmosphere with humidity for at least 48 hours before numeration of bacterial colonies.

2.5.8 Statistics

Differences between experimental conditions were determined to be significant when $P < 0.05$ by one-way ANOVA with a Dunnett post-test, calculated by Prism 5.0 (GraphPad).

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One of these things is not like the others: Human innate IgM memory B cell responses to infection with commensal *Neisseria* species are vastly different from infection with pathogenic *Neisseria meningitidis*.

**Author contributions:**

Nancy S. Y. So designed the experiments, collected and analyzed the data, and wrote the chapter.

Mario A. Ostrowski provided leukopheresis donor samples.

Scott D. Gray-Owen designed the experiments, analyzed the data, and edited the chapter.
3.1 Abstract

*Neisseria gonorrhoeae* (*Ngo*, gonococi) and *Neisseria meningitidis* (*Nme*, meningococci) are the only pathogens of the *Neisseria* genus, the rest being commensals, including *Neisseria lactamica* (*Nla*). In the previous Chapter, I described how infection with *Ngo* induced proliferation and differentiation of the human innate IgM memory B cells into antibody secreting cells. The immunoglobulin (Ig) produced was polyreactive and primarily IgM. In humans, IgM memory B cells are responsible for the production of ‘innate’ Ig. Innate Ig is primarily IgM, low affinity, but broadly reactive. It has been shown to be important during the early stages of infection by preventing dissemination of pathogens prior to the development of high-affinity clonal antibody responses. Because the gonococci induced such vigorous responses from the innate B cell subset, we were interested in determining if this effect was common amongst all neisserial species. Unexpectedly, B cell infection with the closely related *Nme* induced poor IgM memory B cell responses, while infection with a number of commensal *Neisseria* strains resulted in robust innate B cell responses, including the production of high levels of polyreactive IgM. The effects of the neisserial commensals on innate B cell responses were, in most cases, stronger than the effects elicited by the gonococci. The *Nla* components inducing the robust IgM memory B cell proliferation were also present within its outer membrane vesicles, blebs of membrane that are naturally released during growth. B cell infection with *Haemophilus influenzae*, an opportunistic pathogen that shares the nasopharyngeal niche with *Nme* and the commensal *Neisseria*, results in IgM memory B cell proliferation and the production of polyreactive IgM. This highlights the distinctive ability of the *Nme* to avoid inducing innate B cell responses, and since this holds true across a variety of meningococcal strains, it is enticing to hypothesize that this inability to induce innate B cell responses may increase the fitness of this pathogen for asymptomatic carriage and/or disease.

3.2 Introduction

The human nasopharynx is heavily colonized with a variety of bacteria, most of which are non-virulent members of the human commensal flora, while others are opportunistic pathogens. One such pathogen is *Neisseria meningitidis* (*Nme*, meningococci), one of the leading causative agents of bacterial meningitis. Meningococcal disease is relatively rare in the general
population, however disproportionately high disease rates occur in children under the age of two, with a second increase in disease incidence observed in teenagers (Booy et al., 2007; Trotter et al., 2006). Colonization with \textit{Nme} increases with age from birth, peaks at 24.5% of 15-19 year olds, then steadily decreases into adulthood (Cartwright et al., 1987). The factors influencing whether individuals develop disease or remain asymptomatic carriers of \textit{Nme} are currently unknown, although elements including virulence of the strain (Caugant et al., 1988), the immune status of the individual (Ellison et al., 1983; Goldschneider et al., 1969; Stephens et al., 1995), and other environmental and social factors (MacLennan et al., 2006; Stanwell-Smith et al., 1994) contribute to the likelihood of developing disease.

The commensal neisserial species that make up the normal flora within the nasopharynx include \textit{Neisseria lactamica} (\textit{Nla}), \textit{Neisseria flavescens} (\textit{Nflav}), and \textit{Neisseria cinerea} (\textit{Ncin}). Although they are very rarely associated with disease, reports of \textit{N. flavescens} (Sinave and Ratzan, 1987; Wertlake and Williams, 1968), \textit{N. cinerea} (Dolter et al., 1998) and \textit{N. lactamica} (Everts et al., 2010; Orden and Amerigo, 1991) causing a variety of complications show that these commensal bacteria are capable of causing disease under some circumstances. 30% of adults are colonized with \textit{Ncin} (Knapp and Hook, 1988), while the carriage rate of \textit{Nflav} has not been examined. The colonization rates of \textit{Nla} has been closely studied, mostly because \textit{Nla} colonization induces the production of immunoglobulin (Ig), that has been shown to cross-react with multiple serogroups of \textit{Nme} (Gold et al., 1978a). Since \textit{Nla} is able to induce this cross-reacting Ig, and the colonization rate for \textit{Nla} is highest during the time period in which individuals are most susceptible to infection by \textit{N. meningitidis}, it has been suggested that \textit{Nla} colonization is protective against meningococcal disease both by preventing colonization by the pathogen and the induction of protective Ig (Evans et al., 2011; Gold et al., 1978a). Consequently, outer membrane blebs constitutively released by \textit{Nla}, containing cross-reactive epitopes, are currently being examined for their ability to induce immune responses against meningococci (Gorringe et al., 2009). More recently, live intranasal colonization with \textit{Nla} has been employed as a vaccination strategy to prevent meningococcal disease (Evans et al., 2011) with little success.

We have previously reported that \textit{Neisseria gonorrhoeae} (\textit{Ngo}, gonococci), the bacteria that causes the sexually transmitted infection gonorrhea, has a tropism for the IgD\(^+\)CD27\(^+\) IgM memory subset of B cells and drives their activation, proliferation and the production of polyreactive Ig (Chapter 2). While this response included a small amount of \textit{Ngo}-specific IgM,
the majority of antibody is irrelevant, implying a broadly specific but ineffective immune response. Such a response is consistent with the appearance of *N. gonorrhoeae*-specific Ig upon active infection (Hedges et al., 1999) but is absent upon secondary challenge suggesting the absence of immunologic memory (Fox et al., 1999).

The human IgM memory B cell subset is uniquely innate in function since they are responsible for the production of polyclonal pre-immune or 'natural/innate' IgM without requiring exogenous antigenic stimulation (Chen et al., 1998; Kruetzmann et al., 2003). They are the first line of defense for the adaptive immune response by providing low affinity but broadly reactive IgM before the induction of high affinity T-dependent Ig (Baumgarth et al., 2000). This innate IgM functions to control the initial stage of infection by preventing pathogenic dissemination (Baumgarth et al., 2000; Briles et al., 1981; Ochsenbein et al., 1999), prior to the induction of antigen specific adaptive immune response. For these reasons, we were interested in examining whether B cell infection by meningococci would induce the proliferation and differentiation of innate IgM memory B cells, as infection with pathogenic gonococci was clearly able to do so (Chapter 2). Additionally, as *Nla* is able to induce the production of Ig that is highly cross-reactive against *Nme* (Gold et al., 1978a), we were interested in examining if *Nla* infection of B cells would result in the production of polyreactive Ig, essentially recapitulating the clinical effects of colonization.

In stark contrast to the effects of gonococcal infection, B cells infected with the *Nme* displayed moderate activation, minimum proliferation and were only weakly induced to produce Ig. This was true for a variety of clinical isolates of *Nme* examined, and was surprising, since the only other pathogenic *Neisseria* species, *Ngo*, were able to elicit these responses. In contrast to *Nme*, B cell infection with *N. lactamica* resulted in strong proliferation responses by both IgM memory and switched memory (B cells that are antigen experienced, and have undergone class switch recombination) B cell subsets, resulting in the production of all three classes of Ig, with the majority of the Ig produced being polyclonal IgM. This effect was not specific for *Nla*, as B cell infection using other commensal neisserial species produced similar results. Co-infection of B cells with *Nla* and *Nme* resembled infection with *Nla* alone, suggesting that the B cells were ignorant of the additional meningococcal-derived MAMPs. The *Nla* factor(s) responsible for the strong innate B cell responses originate from components found in outer membrane vesicles (OMVs) isolated from the respective bacteria, as treatment of B cells with OMVs derived from
Nla or Nme produced B cell responses reflecting that seen upon infection with intact bacteria. The absence of innate B cell responses in response to infection with Nme is unique to this pathogen, since the closely related Neisseria species, as well as another human-specific pathogen colonizer of the nasopharynx, Haemophilus influenzae, clearly induce vigorous innate B cell responses. In light of this, we consider how the meningococci may be avoiding the induction of the innate IgM memory B cell responses, and why it is different from the other neisserial species.

3.3 Results

3.3.1 Divergent human B cell responses to N. gonorrhoeae and N. meningitidis infection

The Neisseria genus includes two pathogenic species: N. meningitidis and N. gonorrhoeae. We have previously described the unique tropism of Ngo for primary human B cells, an interaction that drives the potent activation and proliferation of the innate IgM memory B cell subset (Chapter 2). Herein, we expanded our assessment of Ngo mediated B cell responses to include clinical isolates N2071 and N2075, in addition to the prototypical lab strain MS11. In agreement with our previous observations, B cell infection with all strains of gonococci examined resulted in vigorous IgM memory B cell proliferation (Figure 3.1A). The lack of response elicited upon E. coli infection suggests that the proliferation observed is not a general response of the innate subset to bacterial products.

Based on these observations, we hypothesized that B cell infection with the only other neisserial pathogen, N. meningitidis, would elicit similar responses. B cells infected with either of the closely-related serogroup B ST-32 clonal complex N. meningitidis strains MC58 and H44/76 displayed diminished proliferation responses compared to Ngo infection at the same multiplicity of infection (compare Figure 3.1A vs B). Although memory B cell proliferation was significantly higher in cells infected with MC58, compared to uninfected cells, the response was less pronounced with H44/76 infection. Infection using clinical isolates of serogroup C Nme, n238 and n247, belonging to clonal complex designations ST-8 and ST-11 respectively, showed similar results, that B cells were only mildly responsive to infection (Figure 3.1B). Since the
Figure 3.1  *N. gonorrhoeae*, but not *N. meningitidis*, strongly activates memory B cells.

B cells were infected with the indicated bacteria at a MOI:10 for 3 days, then B cell subpopulations were assayed for the expression of nuclear proliferation antigen Ki67 by flow cytometry. Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data points corresponding to individual donors are represented by the same symbols within each plot.
expression of capsule has been shown to aid in meningococcal immune evasion (Jarvis and Vedros, 1987; Spinosa et al., 2007) and reduce bacterial adhesion to human cells (Unkmeir et al., 2002), we examined the ability of unencapsulated Nme to induce B cell proliferation. Three unencapsulated strains were used, each expressing a different combination of surface antigens. Strain IS-01440 was piliated, and expressed no Opa adhesin, while strain IS-07380 was pilin negative but Opa positive. The MC58 SiaD⁻ strain is a mutant that was engineered with a disruption in the polysialyltransferase-encoding siaD gene, thereby rendering it unable to produce a polysaccharide capsule. Figure 3.1B shows that B cell responses to infection with unencapsulated Nme strains IS-07380, IS-01140 and MC58 SiaD⁻ resemble that of encapsulated Nme. Although MC58 was able to induce some IgM memory B cell proliferation, the remaining meningococcal strains, representing five out of six strains examined, elicited innate B cell responses that were not significantly different from responses induced by E. coli. Therefore, it was clear that the meningococci, regardless of surface antigen expression, were not able to recapitulate the effect of gonococcal infection on the innate subset.

3.3.2 Commensal Neisseria species strongly induce activation and proliferation of memory B cell populations.

The difference in B cell responses elicited upon Ngo versus Nme infection prompted us to determine how B cells would respond to infection with the non-pathogenic commensal Neisseria sp. Based on the clinical evidence that Nla colonization induces the production of Ig that is not only cross-reactive against Nme, but may be protective against meningococcal disease (Gold et al., 1978a; Goldschneider et al., 1969), it is enticing to consider that these Ig may represent a component of a broadly polyspecific innate B cell response generated by Nla. B cells infected with N. lactamica for 3 days displayed a marked ‘blasting’ phenotype (Figure 3.2A). We have previously established that this effect, evident as an increase in cell size and granularity (monitored as forward scatter and side scatter by flow cytometry), is indicative of cellular activation (Chapter 2). To identify which population of B cells were responding to Nla, cells were stained to separate the various B cell subsets by IgD and CD27, while expression of the nuclear proliferation antigen, Ki67, was used to monitor B cells undergoing proliferation at the time of fixation. As shown in Figure 3.2B-C, both the innate IgM and switched memory B cell populations respond to infection by Nla with robust proliferation.
We examined the ability of other commensal *Neisseria* species to elicit these responses, and found that infection with *Nflav* also induced robust memory B cell activation and proliferation (Figure 3.2A-C). *Ncin* was able to induce strong proliferative responses by IgM memory B cells, however the proliferation of switched memory cells was less than that of the other commensals, generally reflecting that induced by pathogenic *Ngo* (Figure 3.2C). This is in contrast to cells infected with *E. coli*, resembling uninfected cells, in that they show no proliferation in any subset (Figure 3.2A-C). These differences clearly highlight that the stimulatory effects induced by the commensal *Neisseria* on the memory B cell populations are not a generalized response to bacterial infection. These results were unexpected, as all three species of commensal *Neisseria* were able to elicit similar, if not stronger IgM memory B cell responses compared to *N. gonorrhoeae*, but B cell infection with *N. meningitidis*, a pathogen which is arguably more similar to the commensal *Neisseria* species than *Ngo* in terms of its anatomical niche and ability to colonize individuals asymptotically, poorly elicits B cell responses.

### 3.3.3 *N. meningitidis* does not inhibit the induction of B cell responses during infection

We were interested in understanding why infection with *Nme* did not induce T–independent B cell responses, while infections with the other neisserial species were able to do so. We hypothesized that *Nme* could potentially inhibit B cell proliferation through the production of a species-specific product and/or engaging an inhibitory receptor expressed by B cells. If this was the case, *Nme* co-infection with *Nla* would result in decreased B cell proliferation, compared to infection with *Nla* alone. To test this, we infected B cells with either *Nme* H44/76 or *N. lactamica* Y92-1009, at MOIs of 5, 10 and 20. Additionally, cells were co-infected with both *Nme* and *Nla*, at MOIs of 5 and 10 of each strain, with a total bacterial load experienced by B cells being either 10 or 20 bacteria per cell, respectively. Figure 3.3 shows that B cells co-infected with equal numbers of *Nme* and *Nla* respond with vigorous proliferation. There is no apparent difference between samples co-infected with *Nme* and *Nla* versus those infected with
Figure 3.2 Commensal *Neisseria* strongly activates both populations of memory B cells.

Primary human B cells were infected at a MOI:10 with the indicated bacteria, then assayed for A) blasting, as an indicator of cellular activation or B-C) proliferation by flow cytometry 3 days post infection. B) B cell subpopulations were examined for expression of Ki67 nuclear proliferation antigen in response to infection by the indicated bacteria. Representative of experiments done with 3 different donors. C) Quantification of Ki67 by each B cell population after infection under the same conditions outlined in B). Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. ***, *P < 0.001. Data points corresponding to individual donors are represented by the same symbols within each plot.
Figure 3.3  *N. lactamica* coinfection with *N. meningitidis* does not result in suppression of memory B cell activation.

B cells were infected with the indicated bacteria at one of 3 MOIs, 5, 10 or 20. For the co-infection samples, a MOI of 5 or 10 of each bacteria was added to the cell culture. After infection for 3 days, expression of nuclear proliferation antigen Ki67 within the B cell subpopulations were assayed by flow cytometry. Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, P < 0.05; **, P < 0.01. Data points corresponding to individual donors are represented by the same symbols within each plot.
*Nla* alone, suggesting that *Nme* is not inhibiting B cell responses. Indeed, we did not observe an increase in B cell proliferation due to the increased bacterial burden on the cells, as co-infection with *Nme* and *Nla* with an MOI of 5 each, resembled *N. lactamica* infection alone at an MOI of 5. Thus, it appears that while *N. lactamica* is able to induce vigorous proliferation of memory B cells, *Nme* avoids inducing B cell responses rather than mediating their inhibition.

3.3.4 IgM memory B cell responses to neisserial-derived OMVs reflect that of infection by live bacteria

Outer membrane vesicles (OMVs) are shed from all Gram-negative bacteria, and physically resemble the bacteria from which they were derived. OMVs are much smaller in size than whole bacteria (Lee et al., 2007), and therefore are able to permeate tissues and be delivered to the draining lymph nodes intact, whereas whole bacteria will most likely encounter lymphocytes at the site of infection/colonization (Bachmann and Jennings, 2010). Additionally, OMVs derived from *N. lactamica* are being examined as vaccine candidates against meningococcal disease (Gorringe et al., 2009). In light of this, we were interested in determining the B cell responses to treatment with OMVs derived from strains that either induce (*Nla* Y92-1009) or do not induce (*Nme* H44/76) memory B cell responses. To this end, purified B cells were exposed to OMV concentrations ranging from 1 to 50 μg/mL for 3 days, at which time the cells were assessed for proliferation within the B cell subsets.

While OMVs derived from *N. lactamica* induced T-independent IgM memory B cell proliferation in a dose dependent manner, the switched memory B cells showed relatively little response to the OMVs when compared to the strong proliferation induced by infection with live *Nla* (Figure 3.4A). The response at 50 μg/mL OMV (the highest concentration tested) does not reach the level of proliferation observed upon infection with live bacteria, suggesting that live *N. lactamica* are better equipped to stimulate the switched memory B cells, through a mechanism that is not apparent within the OMVs. In contrast to this, innate IgM memory cells responded to treatment with *N. lactamica* OMVs in a similar manner to infection with live bacteria, suggesting some of the factor(s) eliciting the strong proliferative responses are found within the outer membrane of the bacteria.
Figure 3.4  B cell responses to OMV treatment are similar to infection with whole bacteria.

Outer membrane vesicles from the indicated bacteria were added at increasing amounts to primary human B cells for 3 days, then expression of Ki67 was assayed within the B cell subpopulations by flow cytometry. In each experiment, B cells were infected with MOI of 10 whole bacteria was included as reference (whole bact.). Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, $P < 0.05$; ***, $P < 0.001$. Data points corresponding to individual donors are represented by the same symbols within each plot.
Figure 3.5  Infection with *Neisseria* species and *E. coli* induces varying levels of B cell activation.

B cells were infected with the indicated bacteria at an MOI of 10, or left uninfected, for 5 days. Total live B cells were analyzed for the expression of activation marker CD86 by flow cytometry. Results from two different donors are shown.
In contrast to the vigorous B cell responses observed upon treatment with the *Nla* OMVs, B cell responses to *Nme* OMVs were muted, and reflected infection with live meningococci for all subsets (Figure 3.4B). While naive and switched memory cells were unresponsive upon exposure to both live *Nme* and meningococcal-derived OMVs, IgM memory B cells displayed a slight dose-dependent response to treatment with *Nme* OMVs, with the highest level of proliferation obtained upon treatment with 50 μg/mL of OMVs, matching the response induced upon infection by live *Nme*.

### 3.3.5 *N. meningitidis* infection of B cells results in moderate levels of cellular activation.

We have previously shown that B cell activation in response to bacteria is not strictly correlated with their proliferation, as exposure to *E. coli* can upregulate expression of activation molecule and co-stimulatory marker CD86 without subsequent cell division (Chapter 2). This is consistent with the idea that innate recognition of MAMPs may not be sufficient to drive cellular proliferation by itself, and in some cases requires a second signal for this to occur. To examine whether meningococcal infection elicited any cellular responses, we examined the level of CD86 expressed by B cells upon exposure to the indicated bacterial strains 5 days post-infection. While all infected B cells expressed more CD86 compared to cells left uninfected, activation was highest in cells infected with *Ngo*, *N. flavescens* and *N. lactamica* (Figure 3.5), consistent with the ability of these to induce the greatest level of proliferation (Figure 3.2). Infection with *Nme* resulted in CD86 expression levels that resembled infection with *E. coli*, suggesting that while the B cells do recognize the presence of the meningococci during infection, the stimulation is not adequate to induce a proliferation response.

### 3.3.6 The human specific opportunistic pathogen *Haemophilus influenzae* can induce T-independent proliferation of IgM memory B cells.

The absence of B cell responses to *Nme* infection prompted us to consider whether this phenotype might be shared by other opportunistic pathogens that reside within the nasopharynx. *H. influenzae* is a Gram-negative, human-restricted bacteria that is a common resident of the nasopharynx yet can cause serious disease when disseminated from this site. While not part of the *Neisseriaceae*, *H. influenzae* and *N. meningitidis* share similar lifestyles and virulence
mechanisms. As such, we exposed purified human B cells to unencapsulated (nontypeable) *H. influenzae* (NTHi) and then monitored their responses. Upon infection with NTHi at an MOI of 10 for 3 days, the IgM memory B cell subset responded by proliferation. In contrast to the commensal *Neisseria* species, but clearly paralleling the effect seen with the pathogenic *Ngo*, the switched memory B cells did not respond to infection with NTHi (Figure 3.6 and Figure 3.1A). The lack of human innate IgM memory cell response to *Nme* is not a characteristic shared by *H. influenzae*.

### 3.3.7 Induction of IgM memory B cell responses correlates with the production of a polyclonal and broadly specific Ig response.

While various forms of cellular activation can induce lymphocyte proliferation, this does not always lead to cellular differentiation and Ig secretion. We examined the ability of bacterial strains used throughout this study to induce Ig production by infecting B cells at an MOI of 10 bacteria per cell for 5 days, at which time the cell-free supernatants were analyzed for the induction of total IgG, IgM and IgA. Varying levels of Ig were produced by B cells upon infection with each bacteria assessed. While Ig from all 3 classes was generally apparent, the major class induced was IgM (Figure 3.7A-C, compare y axis values). Total Ig responses induced by *Ngo* were more similar to commensal *Neisseria* strains than *Nme*, such that all 3 classes of Ig were induced, ranging from 5 to over 230 fold more than that produced by cells left uninfected (Figure 3.7A-C). Therefore, infection with all *Neisseria* strains results in primarily an IgM response.

Infection with NTHi also primarily induced IgM, with a mean ratio of IgM produced being 66 fold higher than cells left uninfected (Figure 3.7A), though moderate amounts of IgG and IgA were produced as well (Figure 3.7B-C). In direct contrast to infection with the *Neisseria* species and NTHi, infection with *E. coli* resulted in very little total antibody produced, consistent with the idea that Ig production is not a general response to bacterial infection.
Figure 3.6  Infection with human specific pathogen *H. influenzae* induces strong T-cell independent proliferation of innate B cells.

B cells were infected with MOI:10 of nontypable *H. influenzae* for 3 days, then proliferation within each B cell subpopulation was examined by flow cytometry. Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. **, *P* < 0.01. Data points corresponding to individual donors are represented by the same symbols within each plot.
Figure 3.7  Infection with *Neisseria* species induces immunoglobulin production.

Cells were infected with the indicated bacteria for 5 days at a MOI:10, at which time cell free supernatants were taken for total Ig analysis by ELISA. Bars indicate mean. Stars indicate a comparison between uninfected and infected cells. *, $P < 0.05$; **, $P < 0.01$. TT = tetanus toxoid. Data points corresponding to individual donors are represented by the same symbols within each plot.
In the previous Chapter, we established that the generation of IgM specific for tetanus toxoid (TT) during neisserial infection correlates with the production of a polyreactive immune response, while the presence of IgG specific for TT represents the re-activation of non-cognate memory B cells (Chapter 2). Therefore, to assess the specificity of the Ig response, we examined cell-free supernatants for the induction of Ig specific for TT in all three classes of antibody. As expected, based upon our previous work, infection with either Ngo or the commensal neisserial species resulted in the strong induction of IgM specific for TT, while infection with Nme did not induce any TT-specific Ig (Figure 3.6D, and data not shown). Infection with NTHi resulted in a moderate induction of TT-specific IgM, at comparable levels to Ngo (Figure 3.7D). No detectable levels of TT-specific IgG or IgA were produced upon infection with any bacteria examined.

Overall, this suggests that N. gonorrhoeae, NTHi, and the commensal Neisseria species we have examined herein, are all able to specifically target the innate IgM memory B cells, eliciting their activation, proliferation and differentiation into antibody secreting cells to produce broadly specific polyreactive Ig responses. Distinct from this, infection of B cells with N. meningitidis avoids inducing these responses, causing limited activation, low levels of proliferation and cellular differentiation, ultimately resulting in the poor induction of T-independent innate Ig responses.
Infection with *N. meningitidis* results in moderate activation of B cells, but low levels of proliferation and poor immunoglobulin response. The absence of innate B cell responses to *N. meningitidis* infection is distinctive as infection using all of the commensal *Neisseria* strains examined, *N. gonorrhoeae*, and non-typable *H. influenzae* results in robust proliferation and production of polyreactive IgM. This natural Ig has been shown to be immunologically relevant during early stages of infection, preventing dissemination of the pathogen prior to the development of T-dependent antigen-specific immune responses. The absence of this antibody may contribute to the ability of *N. meningitidis* to cause disease.
3.4 Discussion

Human IgM memory B cells were conclusively identified in 2003 as a unique subset of B cells with a non-redundant role in protection from infections by encapsulated bacteria (Carsetti et al., 2005; Kruetzmann et al., 2003; Shi et al., 2005). While no truly homologous population exists in mice, the innate B-1 B cells are considered to be similar enough in functionality to allow for comparison (Kruetzmann et al., 2003). IgM memory B cells are responsible for the production of low affinity but broadly reactive IgM, which have a variety of immune functions including complement deposition (Brown et al., 2002; Zhou et al., 2007), opsonization (Shi et al., 2005; Zhou et al., 2007), and preventing pathogenic dissemination (Baumgarth et al., 2000; Ochsenbein et al., 1999). Human IgM memory B cells are distinct from that of conventional memory B cells in a few major ways: 1) they produce polyreactive innate IgM (Chen et al., 1998; Kruetzmann et al., 2003); 2) their antigen receptor has different selection criteria than bona fide memory B cells, favouring common but unrelated microbial motifs (Zhou et al., 2007) such as polysaccharides that commonly make up bacterial capsule; and 3) they are able to respond to a variety of stimulatory signals without T cell help (Bernasconi et al., 2003).

The innate IgM memory B cells increase in frequency from birth, until they reach levels found in adults by 2 years of age (Weill et al., 2009). Although these cells are present during this time of development, they are non-functional, as T-independent immune responses are poor in children under 2 years old (Weller et al., 2008). This correlates exactly with the only period during human development in which the rate of meningococcal disease is disproportionately high (Booy et al., 2007), in comparison to the low rate of *N. menigitidis* colonization (Gold et al., 1978a). An important implication for this is that unconjugated polysaccharide vaccines, which depend on the action of T-independent immune responses, are ineffective in this young and susceptible population (MacDonald et al., 1998).

Innate IgM have a variety of functions (Ehrenstein and Notley, 2010), however most relevant to our study is its ability to interact with microbes. While innate IgM is of low affinity, its pentameric and polyreactive nature confers sufficiently high avidity to allow a single pentamer to promote agglutination, important for protecting against viral infections, and/or opsonin-independent phagocytosis (Subramaniam et al., 2010). Due to its broad reactivity, innate IgM can also facilitate complement deposition via the classical pathway against a wider variety of
epitopes expressed by a potentially higher number of microbes. Indeed, murine models of infection have shown that complement deposition by innate IgM protects from *Streptococcus pneumoniae* sepsis (Brown et al., 2002). Lastly, there is a role for natural IgM in preventing dissemination of a microbe, which is especially important for bacteria colonizing mucosal surfaces (Baumgarth et al., 2000; Ochsenbein et al., 1999) and preventing systemic infections.

Herein, we report that infection of primary human B cells with the human-restricted *Neisseria* and *H. influenzae*, known to colonize mucosal surfaces such as the nasopharynx and urogenital tract, are able to induce proliferation and polyclonal Ig production by the innate IgM memory B cells. Curiously, infection with *N. meningitidis* induced a relatively low level of activation, weak proliferation and poor differentiation into antibody secreting cells by this innate subset. While the reason for this difference remains unknown, our studies suggest that this pathogen avoids inducing strong B cell responses rather than eliciting an inhibitory effect.

The ability to induce IgM memory B cell proliferation and Ig production is not restricted to the *Neisseria* species, since infection with NTHi can also induce strong innate B cell responses. However, infection with *E. coli* did not recapitulate these effects, indicating that the simple presentation of MAMPs to innate B cells is not sufficient to trigger cellular proliferation and differentiation. This is not to suggest that the innate pattern recognition receptors such as TLRs are not able to support IgM memory B cell responses, as we have previously demonstrated that TLR9 partially mediates IgM memory B cell proliferation in response to gonococcal infection (Chapter 2). It is likely a combination of diverse signals elicited by the *Neisseria* and NTHi which results in the induction of innate B cell responses, and we suggest that the meningococci are either missing or down-regulating one or multiple factor present in the other neisserial species, thereby producing signals that are able to induce B cell activation, but not enough to drive proliferation.

### 3.4.1 *Neisseria gonorrhoeae*

*Ngo*, a Gram-negative un-encapsulated diplococcus, produces many known factors that could be recognized by the B cells to induce signaling. The most obvious activating signals to consider in any B cell are those emanating through the B cell receptor. It is unreasonable to consider that the effects observed herein are elicited by BCR-specific binding to epitopes on the bacteria because such a large proportion of the B cells are activated. While the ability of the other neisserial
species to signal through the BCR was not examined extensively in this report, we have used immunofluorescent microscopy to look for an association between fluorescently labeled gonococci and the BCR and observed no appreciable co-localization (Chapter 2).

We have previously established that TLR2/1, TLR 2/6, and TLR9 signaling pathways are functional within the IgM memory B cells, while TLR4 responses were weak at best (Chapter 2), in agreement with other reports that suggest TLR4 expression is low to nonexistent in this memory population (Bernasconi et al., 2003). TLR9, recognizing gonococcal DNA, contributes to the ability of the IgM memory B cells to respond to \textit{Ngo} infection (Chapter 2).

Other potential antigenic determinants the IgM memory cells may be responding to include the lipooligosaccharide (LOS) that make up the outer membranes of \textit{Ngo}, or the proteins that are found within it. However, it is unlikely that neisserial LOS is responsible for the effects induced on the innate cells, since \textit{N. meningitidis} has a similar LOS structure but does not elicit the same response. Moreover, neisserial LOS has been shown to signal through TLR4/MD2 (Pridmore et al., 2001), and we have shown that TLR4 responsiveness is low in these cells (Chapter 2).

Porins make up 60\% of outer membrane proteins (Blake and Gotschlich, 1986). They are antigenically stable (Sandstrom et al., 1982) and highly immunogenic (Plummer et al., 1994; Snapper et al., 1997). Based on the ability of purified gonococcal PorB to induce murine B cell activation and proliferation (Wetzler et al., 1996), we suggest that porins could also induce similar responses in human B cells, since signaling is induced through TLR 2/1 (Massari et al., 2006) which we have shown to be present and responsive to stimulation in the innate B cell population (Chapter 2).

While well-characterized adhesins such as the Opa proteins and pili could presumably affect neisserial adherence to the B cells, we have not observed B cell stimulatory affects correlating with the expression of either antigen above that detected for strains that do not express them.

3.4.2 Commensal \textit{Neisseria} species

The commensal \textit{Neisseria} species examined in this thesis are all non-encapsulated, human-restricted colonizers of the nasopharynx. Colonization by the commensal neisseria is a dynamic process, and can occur for varying periods of time, with multiple or a single strain of a species present. We did not assess our donors for nasopharyngeal colonization by any of the neisserial
species, as an individuals’ colonization history may not be accurately reflected by their current pattern of commensal colonization.

There is evidence that colonization can induce immunologic memory, but may require extended or repeat periods of colonization (Vaughan et al., 2009). This may explain why infection with Nla or Nflav results in such strong switched memory B cell responses, including the production of IgG and IgA. Infection with either commensal may induce recall responses by memory cells that possess cross-reactive antigen receptors for the two species. Alternatively, non-cognate switched memory B cells may be benefiting from bystander activation, produced by the IgM memory B cells during infection. While the ability of innate IgM memory cells to induce bystander activation of non-cognate cells has not been examined, the strong levels of proliferation induced upon infection with all of the commensals examined make this a likely scenario. Additionally, vaccination, colonization, or infection by closely related bacteria could also produce switched memory B cells able to cross-react with the commensal Neisseria strains.

Surprisingly, treatment with Nla live bacteria, but not OMVs, induced significant proliferation from the switched memory B cell subset. It is possible that factors inducing proliferation were either not included in the OMV preparations or are present at diminished levels compared to whole bacteria.

Since most memory cells are class-switched, the IgM was most likely produced as a primary response by the innate IgM memory B cells. Others have described similar results, as a mixture of tonsillar mononuclear cells depleted of T lymphocytes were able to rapidly produce polyreactive IgM upon treatment with 1 mg/mL of Nla OMVs for only two to three days (Vaughan et al., 2009).

The commensal Neisseria we have examined are closely related to the pathogenic Neisseria, sharing many of the same genes and gene products with the pathogenic strains (Marri et al., 2010). By virtue of their polymicrobial niche, horizontal gene transfer between different species of Neisseria can allow for the exchange and accumulation of virulence factors, in addition to other genetic elements, within the commensal genome (Marri et al., 2010). A recent examination of the core neisserial genome revealed that 75% of putative virulence genes are present in at least one commensal Neisseria species examined, with 40% of these genes found in all commensals.
examined (Marri et al., 2010). According to that report, *N. lactamica* is most closely related to the pathogenic *Neisseria* species.

Although we did not examine the ability of the commensals to stimulate through the TLRs in this report, we propose that stimulation through TLR9 by the commensals is likely, if the bacteria are engulfed by the B cells. Delivery of neisserial DNA to intracellular TLR9 requires bacterial engulfment since B cells are poor at taking up exogenous DNA (Roberts et al., 2010). At the same time, DNA could be packaged within OMVs actively shed by the bacteria. B cells can presumably engulf these small particles, representing another mechanism for the direct delivery of DNA to TLR9 (Vidakovics et al., 2010). Future studies should address these possibilities.

Signaling through TLR2/1 by the porins may also contribute to innate B cell responses. *N. lactamica*, and *N. cinerea* each possess one porin, while *N. flavescens* possesses two porins (Derrick et al., 1999). Porins from the *N. flavescens* and *N. cinerea* have not been examined with respect to their ability to induce B cell responses, however purified gonococcal porin can induce murine B cells to proliferate (Wetzler et al., 1996). Although purified porin from *N. lactamica* was unable to induce tonsillar B cell proliferation (Vaughan et al., 2010), inherent differences between tonsillar B cells and B cells found in the periphery, such as tonsillar cells having constant access to antigens, and different subpopulations of B cell present within this organ (Mansson et al., 2006), suggest that the experimental outcome may be different if one were to use peripheral B cells. Additional experiments will be required to examine this hypothesis.

The role of the BCR in propagating signals from commensal *Neisseria* species is unclear. Using blocking antibodies, one group reported that OMVs derived from *N. lactamica* are able to induce tonsillar B cell proliferation through either IgM and/or IgD (Vaughan et al., 2010), however we have observed little co-localization of the BCR with the bacteria by fluorescence microscopy. These disparate results merit further examination.

The commensals express varying numbers of Opa genes, with *N. lactamica* possessing 2 Opas, *N. flavescens* with 1 (Toleman et al., 2001), while the number of Opa genes in *N. cinerea* is currently unknown. The Opas have been shown to be strongly immunogenic (Plummer et al., 1994; Snapper et al., 1997), and the expression of these proteins may contribute to the proliferation responses observed in the IgM memory cells. Paradoxically, we have established that neisserial Opas are able to bind to the co-inhibitory receptor CEACAM1, effectively
inhibiting CD4+ T cell responses (Boulton and Gray-Owen, 2002; Lee et al., 2007). While this receptor has been shown to be transcribed by the IgM memory B cells (Weller et al., 2004), gonococci expressing CEACAM1-binding Opa does not result in inhibition of innate B cell responses (Chapter 2). The contribution of CEACAM1 binding to B cell responses in the context of neisserial infection will be examined in Chapter 4 of this thesis.

3.4.3 Non-typable *Haemophilus influenzae*

*Haemophilus influenzae* (*Hi*), another human-restricted bacterium, is considered to be an opportunistic pathogen, sharing the nasopharyngeal niche with the commensal *Neisseria* species and the meningococci. There are a number of candidate factors produced by NTHi that could be contributing to the induction of stimulatory responses in the IgM memory subset, including some that are homologous to factors found in the *Neisseria* species, including porin, pilin, and LOS, although no one has explicitly assessed their ability to influence B cell responses to our knowledge.

One candidate of particular interest is protein D (PD), a protein that confers the ability to bind IgD. It is expressed by both encapsulated *Hi* and NTHi (Janson et al., 1993), and its gene sequence is highly conserved across the species (Song et al., 1995). While encapsulated *Hi* undoubtedly binds to IgD, the ability of NTHi to bind IgD has been somewhat controversial. Although it has been established that both encapsulated and non-typable strains bind to human IgD (Akkoyunlu et al., 1991; Ruan et al., 1990), one group reported that although PD is expressed by NTHi, they were not able to observe IgD binding (Sasaki and Munson, 1993). More recently, another group described that 50% of *Hi* strains and none of the NTHi strains they examined were able to bind IgD, however binding rates could be affected by the recombinant IgD protein they were using in their assay system, which included murine sequences and was produced by a non-human cell line (Samuelsson et al., 2007).

When an interaction is observed, PD has been shown to bind specifically sites within both the Fab and Fc regions of IgD, but not to the other Ig classes (Ruan et al., 1990). IgM memory B cells express surface IgD, and it is possible that protein D binding to this Ig may induce the B cell activation and proliferation apparent in this study, especially in light of the ability of an IgD BCR to mediate downstream signaling (Lutz et al., 1998). To our knowledge, the capacity of protein D to induce B cell responses has not been explicitly examined.
Another common resident of the human nasopharynx and opportunistic pathogen *Moraxella catarrhalis* expresses MID, an IgD binding protein that can induce both polyclonal B cell activation and the production of polyreactive IgM (Jendholm et al., 2008; Vidakovics et al., 2010). While MID and PD both recognize a similar region within the heavy chain of IgD (Samuelsson et al., 2007; Samuelsson et al., 2006), it is unclear if they share structural similarities. Although the subsets of B cells which respond to MID expressing *M. catarrhalis* have not been examined, infection of purified B cells induce almost mitogenic proliferation, and polyclonal Ig that is primarily IgM (Jendholm et al., 2008), in agreement with our previous work showing that the IgM memory B cells elicit similar responses upon stimulation by Ngo (Chapter 2). An IgD binding protein has not been identified for the *Neisseria* species, however it has been reported that *Nla* can induce B cell proliferation through an interaction with IgD (Vaughan et al., 2010), raising the possibility a neisserial version of an IgD binding protein exists.

Another interesting candidate for mediating IgM memory B cell responses is the phosphorylcholine (ChoP, also called phosphocholine) deposition onto various surface structures of a variety of bacteria, including the commensal and pathogenic *Neisseria* (Serino and Virji, 2000), NTHi (Weiser et al., 1997), *S. pneumoniae* (Brundish and Baddiley, 1968) and *Pseudomonas aeruginosa* (Weiser et al., 1998). The presence of ChoP on bacteria may be important for immune surveillance, as innate Ig specific for ChoP are found in both humans (Briles et al., 1987) and mice (Briles et al., 1981). Remarkably, natural ChoP-specific Ig has been shown to be protective against death caused by *Streptococcus pneumoniae* bacteremia in a murine model of infection (Briles et al., 1981). We hypothesize that innate IgM memory B cells recognizing ChoP expressed by the bacteria examined herein may in part, contribute the proliferation and differentiation we have observed, however we must not rule out the role of additional signals.

Based on these hypotheses, it is apparent that innate B cells may use alternative strategies to recognize and respond to infection with different bacteria. While the mechanism involved in inducing polyreactive Ig may differ between bacterial species, the overall effect is to utilize the natural Ig for the initiation of early immune responses, such as complement deposition and prevention of dissemination.
3.4.4 *Neisseria meningitidis*

*Nme* is a highly adapted opportunistic pathogen, able to cause disseminated disease, but also harmlessly colonize the nasopharynx. Considering that *Nme* expresses many of the same outer membrane proteins that could be responsible for the vigorous induction of proliferation and differentiation of IgM memory B cells, including but not limited to: DNA (Magnusson et al., 2007) and porin (Wetzler et al., 1996), the question that remains is why are B cells unresponsive to infection with meningococci?

Infection using *Nme* strains that express capsule, pilin or Opa show that expression of these factors do not induce B cells responses to infection. Since capsular polysaccharide vaccines produced from meningococci from serogroup C have been shown to be effective in producing an antibody response (Chandramohan et al., 2007), it was surprising that infection with serogroup C meningococci (strains n238 and n247) were not able to induce an immune response from the IgM memory B cells.

There are multiple lines of evidence that suggest the innate IgM memory B cells participate in the production of Ig in response to capsular polysaccharide, a T-independent antigen. Meningococcal disease rates are high in children up until the age of 2, which not only corresponds to the height of *N. lactamica* colonization, but also the age at which the human IgM memory B cells become functional (Weill et al., 2009). The absence of the IgM memory B cell subset in asplenic individuals predisposes them to infections with encapsulated bacteria (Carsetti et al., 2005; Krueztmann et al., 2003; Ram et al., 2010). *SCID* mice that were repopulated with human IgM memory B cells were able to produce IgM in response to both vaccination with heat killed *Streptococcus pneumoniae* and Pneumo23 polysaccharide vaccine (Moens et al., 2008). Our observation that encapsulated *Nme* induces weak responses from innate B cells suggests that *in vivo* colonization with *Nme* may not result in the efficient production of innate Ig by the IgM memory subset, and adds weight to the idea that colonization with commensals such as *N. lactamica*, provide the sole source of cross-reactive innate Ig.

We examined if *Nme* produces a species-specific factor or induces signaling in a receptor leading to an inhibition of B cell responses. An example of a factor that can cause inhibition by simply binding its receptor are the Opa adhesins, the majority of which have been shown to bind the ITIM containing host receptor CEACAM1 (Boulton and Gray-Owen, 2002; Virji et al., 1996).
There have been a handful of proteins that are expressed by \textit{Nme}, and not \textit{Ngo}, including NadA, OpcA, FrpC (Virji, 2009), any of which could have immunomodulatory effects. Alternatively, it could be a factor that has yet to be identified.

We were not able to observe inhibition in the co-infection assay using \textit{Nme} and \textit{N. lactamica}, however this could be a result of overwhelming stimulation caused by the latter. The assay was carried out using equivalent infecting doses of each bacteria, which may mask \textit{Nme} induced suppression. Additional experiments using various ratios of each bacteria should address this issue. It is important to recognize that although the IgM memory B cells do not proliferate or differentiate in response to infection with \textit{Nme}, the cells are able to detect the presence of an infecting bacterium and in turn express activation marker.

On the other hand, \textit{Nme} may be missing, or downregulating an as of yet unidentified factor(s) that are responsible for the strong stimulatory responses observed during infection that are common to the other \textit{Neisseria} species. These absent factors could represent the missing additional signals that would be required to induce cellular responses beyond activation.

Based on the similarities between the meningococci and the commensal \textit{Neisseria}, sharing the ability to colonize the nasopharyngeal niche without causing disease, it was inititally hypothesized that B cell responses to infection with \textit{Nme} would more closely resemble that of the commensals than infection with \textit{Ngo}. However, this was not the case. This brings to light questions regarding the speciation of the meningococci. Based on intrachromosomal rearrangements of the core genomic backbone for \textit{Nme}, \textit{Ngo} and \textit{Nla}, it has been proposed that these three species of \textit{Neisseria} originated from a common unencapsulated ancestor (Schoen et al., 2008). The actual event of speciation may have occurred when \textit{Nme} acquired an insertion sequence that is present in only meningococci and not in any of the other \textit{Neisseria} species, including the ones examined in this thesis (Schoen et al., 2008). The acquisition of its capsular locus, a factor that is required but not sufficient for virulence, occurred sometime later as a horizontal gene transfer event, likely from a member of the \textit{Pasteurellaceae} family, of which \textit{H. influenzae} is a member (Schoen et al., 2008; Schoen et al., 2009). Multiple lines of evidence suggests that \textit{N. meningiditis} speciation occurred relatively recently (Schoen et al., 2009), and reflects the earliest definitive description of meningococcal disease dating from 1805 (Cartwright, 2006). It is enticing to theorize that the reason why \textit{Ngo} and \textit{Nla} are able to elicit
such potent responses from the human innate B cell subset, is because its common ancestor was able to as well. During speciation events for \textit{Nme}, it is possible that loss of the ability to induce innate B cell responses allowed it to be more fit for carriage and/or pathogenesis.

Alternatively, the ability of \textit{Nme} to avoid inducing innate B cell responses may aid in its ability to cause disease. One of the caveats of my experimental design is that I did not have access to asymptomatic carriage strains of meningococci. These carriage isolates are not commonly associated with the acquisition of meningococcal disease. It would be interesting to assess the ability of these carriage meningococcal strains to induce innate B cell responses, and examine if they are able to induce stronger innate B cell responses compared to the poor induction of innate B cell responses due to infection with disease causing meningococcal isolates.

Genome analysis shows that there are few genes that are expressed only by the hypervirulent (mostly associated with disease and rarely associated with asymptomatic carriage) meningococcal strains (Schoen et al., 2008). Therefore, the ability to cause disease is presumably related to a combination of genes altering the fitness of \textit{Nme} towards pathogenesis, rather than single genetic elements that confer virulence (Schoen et al., 2008). In a similar manner, the innate B cell subset likely requires a combination of distinct signals to produce immune responses against complex organisms such as bacteria.

In summary, it appears that many of the colonizers of the nasopharynx are able to activate the IgM memory B cells, to cause a polyclonal Ig response. One commonality amongst the bacteria we have examined is that they are all human restricted, and their ability to activate the production of one of the oldest and most well conserved immune effector proteins, IgM, within a population of B cells that uses mutated but mainly germ-line antigen receptors was not lost on us. Undoubtedly, this is one example of co-evolution between species-specific bacteria and its host.

The induction of polyreactive Ig is a defence mechanism used by the host in an attempt to control the invading pathogen prior to the development of a clonal, T-dependent immune response with lasting memory (Figure 3.8). The intriguing question of how \textit{N. meningitidis} avoids the induction of innate B cell responses likely has to do with the fact that it is highly adapted to the human host.
Future work should be focused on not only the differences between the transcriptome and proteome of *Nme* with respect to the closely related pathogen *Ngo* during infection, but also of the commensal neisserial species since they share a common niche and horizontal gene transfer between species is likely. An analysis of factors present within the outer membranes of each of the neisserial species should be an initial focus, since B cells treated with OMVs derived from *N. lactamica* reveal that the factor(s) responsible for IgM memory B cell responses are present within these outer membrane preparations. This will not only address the questions we have raised herein, but could also potentially reveal new vaccine candidates.

### 3.5 Materials and Methods

#### 3.5.1 Cells

Primary human B cells were obtained by either standard venipuncture or, when large numbers of cells were required, through leukopheresis. All participants gave informed consent in accordance with guidelines for the conduct of clinical research at the University of Toronto and St. Michael’s Hospital, respectively. The protocols used were approved by the University of Toronto and St. Michael’s Hospital institutional review boards (Toronto, Ontario, Canada).

Fresh peripheral mononuclear cells were obtained from Ficoll-Paque Premium (Amersham GE) gradients, according to manufacturer’s specifications. CD19+ B cells were purified using either the EasySep Human CD19 Positive Selection Kit (Stemcell Technologies) or CD19 Microbeads (Miltenyi Biotec). B cell purity consistently exceeded 95%, as measured by a FACSealibur flow cytometer (BD Biosciences) with FlowJo software (Treestar) used for all flow data analysis.

Purified cells were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (CanSera), 4 mM GlutaMAX (Invitrogen) and 50 mM HEPES (Bioshop, Burlington, Canada) at pH 7.4. Cells were cultured at 37°C in 5% CO₂ and humidified air.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Serogroup</th>
<th>Country of Origin</th>
<th>Disease</th>
<th>Strain designation</th>
<th>ST designation</th>
<th>Expression of:</th>
<th>Opa</th>
<th>Pilus</th>
<th>Can this strain bind to CEACAM1?</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC58</td>
<td>B</td>
<td>UK</td>
<td>invasive</td>
<td>B:15:P1.7,16b:</td>
<td>ST-32</td>
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<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ST-32</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H44/76</td>
<td>B</td>
<td>Norway</td>
<td>invasive</td>
<td>B:15:P1.7,16,</td>
<td>ST-32</td>
<td>no</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.7,16, ET5 complex</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>(ST 32)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n238</td>
<td>C</td>
<td>UK</td>
<td>invasive</td>
<td>C: P1.5,2: F1-7:</td>
<td>ST-8</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td>no</td>
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<td></td>
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<tr>
<td>n247</td>
<td>C</td>
<td>Ghana</td>
<td>?</td>
<td>C: P1.5,2: F1-1:</td>
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</table>

Table 3.1 Characteristics and strain information for *N. meningitidis* isolates used in this study.
3.5.2 Bacterial Strains

*Neisseria gonorrhoeae* constitutively expressing no Opa (Opa’, strain N302) was derived from a pilus-deficient MS11 parent strain (Kupsch et al., 1993), and were graciously provided by Prof. T.F. Meyer (Berlin, Germany). Clinical *N. gonorrhoeae* strains N2071 and N2075 were isolated from the female cervix, and both express pilin. Strain N2071 does not express Opa, however N2075 does express at least one Opa adhesin based upon immunoblot analysis. These clinical strains were obtained from a sexually transmitted disease clinic in the district of Pumwani in Nairobi, Kenya, and were a generous gift from Dr. F. A. Plummer (Winnipeg, Canada).

*Neisseria cinerea* (NRL23165) and *Neisseria flavescens* (2380) were gifts from J. S. Kroll (London, UK). *Neisseria lactamica* strains NL0206 and Y92-1009, and *Neisseria meningitidis* strains H44/76 and MC58, were kindly provided by Dr. A. Gorringe (Salisbury, UK). *N. meningitidis* strains n238 and n247 were obtained from Dr. T. Shryver (Calgary, Canada), while strains IS-07380 and IS-01140 were gifts from Raymond Tsang from the Health Agency of Canada (Winnipeg, Canada). Table 3.1 further describes these *N. meningitidis* strains.

A capsule deficient mutant of MC58 (SiaD’) was created by homologous recombination, using the e2690 plasmid with a kanamycin cassette located within a truncated SiaD gene, graciously donated by Dr. T. Shryver (Calgary, Canada). Genotyping was used to confirm the presence of the kanamycin cassette, and absence of capsule expression was verified by dot blot analysis using an anti-meningococcal serogroup B monoclonal antibody NIBSC code 95/750, a gift from Dr. A. Gorringe (Salisbury, UK).

Non-typable *Haemophilus influenzae* (1128 Mee) was originally obtained from a child with otitis media. It was kindly provided by Dr. L. Bakaletz (Columbus, USA). *H. influenzae* was grown from frozen stocks on chocolate agar at 37°C in a 5% CO2 atmosphere with humidity.

*Neisseria* species were grown from frozen stocks on GC agar (Difco) supplemented with 1% (v/v) IsoVitalex (BBL Microbiology Systems) at 37°C in a 5% CO2 atmosphere with humidity. A binocular microscope was used for daily selection of gonococcal colony opacity phenotypes for the MS11 strain. Gonococcal Opa protein expression was monitored by immunoblotting using the Opa cross-reactive monoclonal antibody 4B12/C11 (Achtman et al., 1988), graciously provided by Prof. Mark Achtman (Berlin, Germany).
*Escherichia coli* (DH5α) was a gift from Prof. T.F. Meyer (Berlin, Germany). Both were grown from frozen stocks in aerated Luria-Burtani broth overnight at 37°C. Liquid cultures were sub-cultured onto LB agar prior to infection assays.

3.5.3 Preparation of OMVs

*Nme* and *Nla* vaccine OMVs derived from *Nme* H44/76 and *Nla* Y92-1009 were generous gifts from Dr. A. Gorringe (Salisbury, UK). Bacterial strains were cultured as previously described (Finney et al., 2008). The OMVs were prepared in 0.2 M glycine pH 8.0, with 3.0% sucrose, filter sterilized and stored at -80°C.

3.5.4 Infection of B Lymphocytes

Freshly isolated human B cells were infected with a multiplicity of infection (MOI) of 10 bacteria per cell, unless otherwise indicated. Gentamycin (Bioshop, Burlington, Canada) was routinely added to infection cultures 3 hours post infection to prevent bacterial overgrowth. For *E. coli*, gentamycin was added immediately upon infection to prevent bacterial overgrowth of the B cell cultures.

3.5.5 Treatment of B Lymphocytes with OMVs

Freshly isolated human B cells were treated with OMVs from either *N. meningitidis* H44/76, or *N. lactamica* Y92-1009 at concentrations ranging from 1-50 μg/mL for 3 days.

3.5.6 Analysis of B cell proliferation responses

B cells were infected with bacteria or treated with OMVs for 3 days, at which time cellular proliferation within each B cell subset was assessed by flow cytometry. B cell subsets were defined using the following antibodies: IgD-PE (clone IA6-2, BD Biosciences) and CD27-FITC (clone O323, eBioscience). To identify and quantify populations of cells undergoing proliferation at the time point in question, Ki67-Alexa 647 (clone B56, BD biosciences) was used. CD27 and IgD immunostaining was performed on live cells, whereas Ki67 immunostaining was performed on fixed cells that had been permeabilized. CompBeads (BD
Biosciences) were utilized for fluorescent compensation. All cells were fixed prior to flow analysis. All flow cytometric analysis was performed using a BD FACScalibur.

3.5.7 ELISA

All ELISAs were read at 450 nm using a 1420 Victor³ (PerkinElmer) plate reader. Total Ig in cell culture supernatant was quantified by IgM, IgG or IgA ELISA kits (Zeptometrix), used according to manufacturer’s instructions.

To monitor antigen-specific immunoglobulin production, 96 well Maxisorp plates (Nunc) were coated with tetanus toxoid (TT; List Biological Labs) and resuspended in pH 7.4 phosphate buffered saline (PBS; Wisent). The plates were dried, vacuum sealed into airtight bags, and stored at 4°C until use. 5% bovine serum albumin (BSA; Bioshop, Burlington, Canada) in PBS was used to block wells, and 0.05% Tween 20 (Sigma) in PBS was used as wash buffer. Cell free culture supernatants from uninfected cells or those infected with Neisseria species, H. influenzae, or E. coli for 5 days were diluted using 1% BSA in 0.05% Tween 20.

Biotinylated F(ab’)₂ fragments of goat anti-human IgG, IgM and IgA (Jackson ImmunoResearch Laboratories) were used separately to determine the amount of each class of Ig produced in response to bacterial infection. After incubation with streptavidin horse radish peroxidise (HRP) Ultra (Sigma), 3,3',5,5'-tetramethylbenzidine (TMB; KPL) was used as the colourmetric reagent for HRP activity.

3.5.8 Statistics

Differences between experimental conditions were determined to be significant when \( P < 0.05 \) by one-way ANOVA with a Dunnett post-test, calculated by Prism 5.0 (GraphPad).

3.6 Acknowledgements

We thank our volunteer blood donors; without them our study would not have been possible. We are grateful to Dr. Kay Johswich for providing information regarding the clinical N. meningitidis strains used in this report.
4 Influence of CEACAM1 on B cell responses to *Neisseria gonorrhoeae*.

Nancy S. Y. So, Mario A. Ostrowski, and Scott D. Gray-Owen

**Author contributions:**

Nancy S. Y. So designed the experiments, collected and analyzed the data, and wrote the chapter.

Mario A. Ostrowski provided leukopheresis donor samples.

Scott D. Gray-Owen designed the experiments, analyzed the data, and edited the chapter.
4.1 Abstract

*Neisseria gonorrhoeae* (*Ngo*, gonococci), the bacteria that causes the sexually transmitted infection gonorrhea, expresses an adhesin, Opa, that binds to human carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a receptor that is expressed on a variety of host cells, including immune cells. CEACAM1 is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing co-inhibitory receptor that has been shown to possess the capacity to inhibit primary human CD4+ T cell activation and proliferation upon infection with gonococci able to bind this receptor. While primary human B cells express CEACAM1, infection with CEACAM1-binding gonococci does not appear to inhibit cellular responses. As a co-inhibitory receptor, CEACAM1 affects the activation threshold of a cell, a set point that is determined by the appropriate recruitment of downstream effectors and the persistence of ITIM-dependent inhibitory signalling. Therefore, we conducted a careful examination of whether CEACAM1 had the potential to inhibit B cell responses to *N. gonorrhoeae* infection in an assay system that would allow us to control CEACAM1 engagement. Regardless of the conditions used, CEACAM1 engagement did not affect the B cell responses to gonococci, since infection consistently caused strong cellular activation. In combination with our previous observations, these results establish that in the context of neisserial infection, the effect of CEACAM1 on T cell and B cell responses differs, revealing its co-inhibitory function in T cells but not in B cells exposed to CEACAM1-binding gonococci. In this respect, we suggest that the ability of CEACAM1 to cause cellular inhibition during neisserial infection is dependent on cell type.

4.2 Introduction

Gonorrhea is spread primarily through sexual contact, although vertical transmission from mother to newborn, while passing through the birth canal, can also occur (Edwards and Apicella, 2004). *Neisseria gonorrhoeae* (*Ngo*, gonococci), the pathogen that causes gonorrhea, induces significant morbidity in infected patients, including tremendous inflammation at the site of infection. Undoubtedly, there are a multitude of neisserial factors which could induce responses from immune cells, however only a handful have been well characterised. Microbial-associated molecular pattern (MAMP) containing molecules, including DNA (Dobson-Belaire et al., 2010;
Magnusson et al., 2007), lipoooligosaccharides (Pridmore et al., 2001), peptidoglycan (Kaparakis et al., 2010), and lipoprotein (Fisette et al., 2003) are shared with other bacteria. More specific to the *Neisseria* species, the neisserial porins have been shown to mediate B cell proliferation (Wetzler et al., 1996), while the Opa adhesins can promoted neutrophil oxidative burst and degranulation killing responses (Sarantis and Gray-Owen, 2007).

Although pathogen-specific antibody is induced upon gonococcal infection, immunoglobulin (Ig) levels are low compared to other infections (Hedges et al., 1999) and there is no evidence of immunologic memory to protect from subsequent gonorrhea infections (Fox et al., 1999). While the induction of immunity to *Ngo* has been described in a cohort of female sex workers in Kenya, protection from *Ngo* infections was directly proportional to the length of time spent as a sex worker, and was observed for some, but not all, serovars (Plummer et al., 1989), indicating that the generation of protective immunity is inefficient and/or requires prolonged exposure to the pathogen. Therefore, despite the fact that the innate immune response is clearly functional during gonorrhea infection, there is an apparent absence of effective adaptive immunity induced in response to infections with *Ngo*.

*N. gonorrhoeae* colonization begins with attachment to host cells through its pilus, which anchors the bacteria to the cell (Kirchner and Meyer, 2005), allowing for tight adherence between the neisserial Opa adhesin and host receptors (Wang et al., 1998). The Opa adhesins are integral outer membrane proteins and although its expression is phase variable (Murphy et al., 1989), evidence from male urethral challenge studies demonstrate that there is a selection for Opa expression during infection (Jerse et al., 1994; Swanson et al., 1988). While a few Opa adhesins have been shown to bind heparin sulphate proteoglycan (HSPG) receptors (Chen et al., 1995), over 95% of Opa variants from clinical *Neisseria* strains adhere to carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) (Virji et al., 1996), suggesting that gonococcal Opa-CEACAM1 interaction may confer a competitive advantage for establishing an infection.

CEACAM1 is expressed in many cell types, including a wide variety of epithelia and endothelia (Prall et al., 1996). Most relevant with respect to this thesis is the role of CEACAM1 as a receptor for pathogenic *N. gonorrhoeae* and its expression on cells of the immune system, including T cells, B cells, neutrophils, and monocytes (Kammerer et al., 1998; Yu et al., 2006).
The immuno-regulatory function of CEACAM1 has been best characterized with respect to T cell signalling. In T cells, CEACAM1 expression is activation-induced (Boulton and Gray-Owen, 2002; Kammerer et al., 1998). It is a co-inhibitory receptor that contains within its cytoplasmic domain two immunoreceptor tyrosine based inhibition motifs (ITIMs), capable of inhibiting T cell receptor signalling (Chen et al., 2008; Kammerer et al., 1998). Moreover, *Ngo* are able to mediate direct inhibition of primary human CD4+ T cell activation and proliferation simply through Opa binding to CEACAM1 (Boulton and Gray-Owen, 2002). T cell help, through the expression of activation and co-stimulatory markers, is not only required to generate high affinity Ig through the mechanism of affinity maturation, but is also necessary for the development of immunologic memory (Lee et al., 2003) which is either altered or absent in individuals with uncomplicated gonorrhea infections (Fox et al., 1999). It has been suggested that this inhibition of T cells, which by extension leads to an inhibition of T cell dependent responses, could explain lack of immunologic memory observed in clinical gonorrhea infections (Lee et al., 2008).

The signalling capabilities of CEACAM1 in the context of B cells are poorly understood. CEACAM1 expression in B cells is less clear, as one group has suggested that, similar to T cells, CEACAM1 expression requires activation (Lobo et al., 2009), whereas others have shown CEACAM1 expression in cells at rest (Greicius et al., 2003; Kammerer et al., 2001; Pantelic et al., 2005; Singer et al., 2002), albeit at varying levels. CEACAM1 is a co-inhibitory receptor, and as such, requires a concomitant activation signal in order to mediate inhibitory pathways (Lee et al., 2008). Studies using DT40 chicken B cells show that the membrane proximal ITIM can mediate inhibition of B cell receptor (BCR) induced signalling upon co-ligation to the BCR (Chen et al., 2001). Treatment of B cells with BCR cross-linker (αBCR) induces phosphorylation of CEACAM1 (Lobo et al., 2009), similar to TCR induced phosphorylation of CEACAM1 in T cells (Chen et al., 2008). Moreover, CEACAM1-mediated inhibition of B cells, as well as T cells, require signalling pathways involving Src homology 2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 (Chen et al., 2001; Chen et al., 2008; Lee et al., 2008). Combined, these studies imply that CEACAM1 may function as an inhibitory receptor in B cells, in a manner similar to T cells.

Only two studies to date have examined the effect of *N. gonorrhoeae* infection on human B cells. Pantelic *et al.*, using prestimulated human B cells to induce the expression of CEACAM1, show
that B cell death is induced upon infection with CEACAM1-binding gonococci (2005). Conversely, we have previously reported that primary human B cells infected with gonococci results in strong activation, proliferation and Ig production, with no evidence of cellular inhibition even when cells were infected with CEACAM1 binding Ngo (Chapter 2). One difference between the two reports was that in the former study, CEACAM1 expression in B cells was induced by cytokines, while in our study, B cells were naturally expressing CEACAM1, likely at lower levels than the stimulated B cells. Lower levels of CEACAM1 expression, especially in the absence of BCR-mediated activation to theoretically potentiate CEACAM1 phosphorylation, could affect the inhibitory function of CEACAM1 in our previously reported assay system.

In light of this, we were interested in examining if CEACAM1 is able to inhibit the activation of B cells, specifically in response to infection with \textit{N. gonorrhoeae}, through the careful design of an assay system that would allow us to isolate the respective activation and inhibition signals required for CEACAM1 function as an inhibitory receptor. The work presented in this Chapter was initiated at the start of our studies and was completed before our discovery of the different B cell subsets having distinct responses to gonococcal infection. For that reason, we assessed total B cell activation in response to our various assay conditions, and did not examine activation within each of the subsets. In Chapter 2, we demonstrate that Ngo infection of B cells specifically induces the proliferation of mainly the IgM memory subset. Due to the large proportion of this subset within freshly purified peripheral B cells (Chapter 2), an effect of CEACAM1-mediated inhibition within this subset may be noticeable in the total B cell population. However, we show that in B cells, CEACAM1 ligation in the context of Ngo infection, in a variety of assay conditions, did not inhibit cellular activation. Therefore, the ability of CEACAM1 to mediate inhibition of cellular responses during neisserial infection in primary lymphocytes depends on multiple factors, including fundamental differences between T cells and B cells.
4.3 Results

4.3.1 CEACAM1 expression is inducible in primary human B cells, and confers increased \textit{N. gonorrhoeae} association.

We have previously observed that although resting primary human CD4$^+$ T lymphocytes express CEACAM1, its expression is inducible upon cellular activation (Boulton and Gray-Owen, 2002). CEACAM1 expression in human B cells may also be activation induced (Kammerer et al., 1998; Pantelic et al., 2005), however the induction of CEACAM1 was previously observed upon stimulation of a mixed population of peripheral blood mononuclear cells (PBMCs), implying that factors from non-B cells were required for CEACAM1 upregulation. Additionally, both groups used IL-2 to stimulate PBMCs. While peripheral blood B cells can respond to IL-2 (Ichikawa et al., 1999), IL-2 is primarily considered an activator of T cells. For those reasons, we were interested in examining whether direct stimulation of the B cell receptor could upregulate CEACAM1 expression in purified B cells. Using whole cell lysates, we confirm that freshly purified peripheral blood B cells express CEACAM1 and that treatment with B cell receptor cross-linker (\(\alpha\)BCR) upregulates CEACAM1 expression (Figure 4.1A).

We have previously shown that CD4$^+$ T cells expressing CEACAM1 are bound by gonococci expressing Opa adhesin specific for this receptor (Lee et al., 2008), however whether differential expression of CEACAM1 affects cellular association of CEACAM1-binding \textit{Ngo} to lymphocytes has not been tested. B cells from two different donors were either left untreated or treated with \(\alpha\)BCR to induce CEACAM1, then infected with each of three isogenic strains of gonococci: \textit{Ngo Op}_{\text{ACEA}}, expressing the Opa$_{52}$ adhesin that binds to CEACAM1 (Gray-Owen et al., 1997b), \textit{Ngo Op}_{\text{HSPG}}, a control strain of \textit{Ngo} expressing the Opa$_{50}$ adhesin which recognizes heparin sulphate proteoglycans, but not CEACAM1 (Chen et al., 1995; Gray-Owen et al., 1997b), and \textit{Ngo Op} -, another control strain of \textit{Ngo} expressing no Opa adhesin. Upon examination of bacterial association, there was a variable effect, apparently due to both CEACAM1 dependent and independent factors (Figure 4.1B).

The ability of \textit{Ngo Op}_{\text{ACEA}} to bind to CEACAM1 on B cells was confirmed by fluorescent confocal microscopy. Upon infection with \textit{Ngo Op}_{\text{ACEA}}, CEACAM1 was observed co-localize with the gonococci (Figure 4.1C). Visualization of fluorescently labelled CEACAM1 in untreated B cells was weak (data not shown), most likely due to the low levels of receptor
expression. CEACAM1 expression is therefore inducible in purified primary human B cells, and may affect gonococcal attachment.

4.3.2 Culture conditions were optimized to assess the inhibitory effect of CEACAM1 in the context of *N. gonorrhoeae* infection.

Considering the described similarities of CEACAM1 signalling between T cells and B cells, it is surprising that B cells infected with CEACAM1 binding gonococci show robust activation and proliferation (Chapter 2), while T cell activation is effectively inhibited by Opa-CEACAM1 interactions. Therefore, we were interested in examining if CEACAM1 could inhibit gonococcal induced B cell activation under conditions that should favour inhibitory signalling of this receptor.

Assay conditions were designed to reflect conditions during initial infection with *Ngo*, with a low multiplicity of infection (MOI) per cell, while also providing the B cell activation signals necessary for CEACAM1 to become phosphorylated and signal as a co-inhibitory receptor. Therefore, we included stimulation by αBCR to facilitate CEACAM1 signalling. CD40 ligand (CD40L) is a co-stimulatory molecule that is upregulated on both B cells and T cells after cellular activation, although its expression is much higher in T cells (Grammer et al., 1995). *In vitro* stimulation of CD40 receptor on B cells by CD40L can influence proliferation and differentiation of human peripheral blood derived B cells (Grammer et al., 1995; Neron et al., 2005). CD40L was included in our assay conditions because it provides another level of cellular stimulation. For the infection, we used a low MOI of Opa- gonococci, allowing for the delivery of bacterial derived MAMPs, providing additional activating signals for B cells without CEACAM1 engagement. This allowed us to control the level of CEACAM1 ligation through the addition of CEA DAKO, a polyclonal antisera that has been shown to inhibit primary T cell activation and proliferation through CEACAM1 cross-linking (Boulton and Gray-Owen, 2002).

Combining multiple stimulatory signals together may cause a synergistic effect on B cell activation, and since we were interested in examining inhibition of activation, standard assay conditions needed to be carefully optimized so that either co-inhibitory or co-stimulatory effects could be detected. αBCR, CD40L and *Ngo* were added in various amounts to cause an increase in cellular activation compared to cells at rest, but also ensuring that B cells were not maximally stimulated, as observed by treating cells with excess αBCR and *Ngo*, in the presence of CD40L.
Figure 4.1  Activation induced expression of CEACAM1 increases *Ngo* association to primary B cells.

A) Cell lysates from B cells that were left untreated, or treated with 1 μg/mL of αBCR for 2 days. 5.2x10⁵ cells were loaded per lane. Representative of 3 experiments with different donors.

B) Untreated or αBCR (1 μg/mL for 2 days) treated cells were infected at a MOI:10 with the indicated *Ngo* strains for 4 hours prior to immobilization onto coverslips. 

C) Cells were prestimulated with 1 μg/mL αBCR for 2 days before infection with MOI of 1 *Ngo* OpaCEA for 4 hours before fixation onto coverslips. Analysis for B-C was done by confocal fluorescent microscopy.
Figure 4.2  Optimization of culture conditions for assays to examine the inhibitory effect of CEACAM1 in human B cells.

A) B cells were either treated with CD40L at 0.1 μg/mL or left untreated. Cells that were treated with CD40L also received increasing amounts of αBCR.  B) B cells were either left untreated and uninfected, or treated with CD40L at 0.1 μg/mL and Ngo at an MOI of 1, then increasing amounts of αBCR were added. For both A and B, cells were assessed for a blasting phenotype by flow cytometry after 5 days in culture. Representative of two experiments done with different donors.  C) Primary human B cells were left untreated, or stimulated with 0.1 μg/mL of αBCR, 0.1 μg/mL CD40L and MOI:1 of Opa− Ngo (designated 3x), MOI:1 of Opa− Ngo (designated Opa−), or 10 μg/mL of αBCR for 2 days, then cells were lysed and immunoblotted for CEACAM1 expression. 6x10^5 cells were loaded per lane. Representative of experiments done with 4 different donors.
Initially, we treated B cells with 0.1 μg/mL of CD40L, as treatment with less CD40L resulted in inconsistent levels of cellular activation, as measured by the expression of activation marker CD86 (data not shown). We then treated B cells with increasing levels of αBCR, at 0, 0.1 and 0.3 μg/ml. As shown in Figure 4.2A, treatment with 0.1 μg/mL of CD40L without any αBCR resulted in an increase in cellular activation. There was a noticeable dose-dependent effect upon the addition of αBCR, however we decided to use the lower amount of 0.1 μg/mL as this resulted in a small increase in activation, implying that further stimulation would still be evident.

We have already observed that B cells infected with an MOI of 10 gonococci per cell results in overwhelming activation and proliferation (Chapter 2), so we used an MOI of 1 in our assay. While we did examine the effect of MOI of 0.5 gonococci per cell, there was no consistent effect on CD86 expression (data not shown). B cells were then treated with 0.1 μg/ml of CD40L, with an MOI of 1 \(Ngo\) Opaq, and increasing amounts of αBCR. Figure 4.2B shows that while treatment with 0.1 μg/mL and 0.3 μg/mL of αBCR gave similar levels of activation, the cells were still able to respond to activating signals beyond that observed for our final assay conditions: 0.1 μg/mL CD40L, 0.1 μg/mL αBCR and MOI of 1 \(Ngo\) Opaq (which will be referred to as our standard assay conditions). As such, effects of CEACAM1 engagement should be evident regardless of whether they are activating or inhibitory.

Finally, we assessed the ability of the cells to express CEACAM1 under our standard assay conditions. As shown in Figure 4.2C, uninfected and untreated B cells express a small amount of CEACAM1, whereas treatment of cells under our standard assay conditions induced the expression of CEACAM1, although at lower levels than treatment with αBCR at 10 μg/mL (our positive control). This result further supports our observation that CEACAM1 expression is induced by B cell activation. Interestingly, infection with the low MOI of 1 gonococci in the absence of other stimuli did not increase CEACAM1 expression relative to uninfected (Figure 4.2C).
Figure 4.3  Soluble stimulation of B cells does not affect Ngo induced cellular activation.

A) Schematic of infection conditions, indicating that all 4 components of the assay were added as soluble factors. Antibody indicates either CEACAM1 cross-linking Ig (αCEACAM1) or control control.  
B) Cells were cultured under the conditions outlined in A) for 5 days, then assayed for expression of co-stimulatory marker CD86 by flow cytometry.  Shaded histograms indicate isotype staining.  No Ig = no antibody added.  Control Ig = normal Ig added.  αCEACAM1 = CEA DAKO added.  Representative of experiments done with 3 different donors.
Figure 4.4  CEACAM1 capping on B cells does not affect Ngo induced cellular activation.

A) Schematic showing assay conditions, indicating that CEACAM1 cross-linking antibody (or isotype control) was immobilized to promote cell surface capping of CEACAM1. B) Cells were cultured as indicated in A) for 5 days, then cellular expression of co-stimulatory marker CD86 was assessed by flow cytometry. Shaded histograms indicate isotype staining. No Ig = no antibody added. Control Ig = normal Ig added. αCEACAM1 = CEA DAKO added. Representative of experiments done with 2 different donors.
4.3.3 CEACAM1 ligation by polyclonal antisera.

Our initial analysis of the inhibitory ability of CEACAM1 employed only soluble signals, as illustrated in Figure 4.3A. CEACAM1 cross-linking was induced by adding CEA DAKO, a polyclonal antisera that has been shown to mediate inhibition of T cell activation and proliferation (Boulton and Gray-Owen, 2002). The B cells were left untreated and uninfected, or treated with our standard assay conditions supplemented with either no additional Ig, the addition of 10 μg/mL of normal rabbit immunoglobulin (Ig) control for effects due to the addition of Ig, or the addition of 10 μg/mL of CEA DAKO. Cells that were left untreated and uninfected expressed low levels of CD86, while cells treated under standard assay conditions strongly induced CD86 activation marker expression regardless of the presence of CEA DAKO (Figure 4.3B).

4.3.4 Polarized CEACAM1 engagement does not inhibit cellular activation.

The addition of soluble anti-CEACAM1 antibody to induce cross-linking may elicit different signals than the ligation of CEACAM1 due to a larger multivalent particle, such as bacteria. It has been shown in the context of BCR signalling, that although soluble bivalent stimulation with a BCR specific antibody can induce phosphorylation of the antigen receptor, larger multivalent complexes induce stronger and more prolonged activating signals, and correlates with the creation of polarized BCR caps (Thyagarajan et al., 2003). Consistent with this, polarized membrane bound signals, such as the ones that could be elicited by bacteria or large multivalent antigens, are more efficient at inducing cellular responses than soluble signals, since the threshold of antigen required to induce cellular responses is lower (Batista et al., 2001). These concepts could be applied to CEACAM1 signalling within the B cell, as CEACAM1 signalling may be enhanced if it was physically concentrated so as to allow downstream inhibitor effectors to accumulate.

To induce capping of CEACAM1, we immobilized either CEA DAKO or normal Ig to the bottom of tissue culture wells (Figure 4.4A). The rest of the standardized assay components were added in soluble form to the purified B cells first, and then cultured in the presence of either immobilized Ig (CEA DAKO or normal Ig) or untreated wells. B cells treated under our
standard assay conditions responded with a strong induction of activation marker, and no difference was observed when cells were cultured in the presence of either immobilized CEA DAKO or normal Ig antibody (Figure 4.4B).

4.3.5 CEACAM1 and BCR induced capping does not affect B cell activation.

In activated peripheral blood T cells, CEACAM1 is observed to colocalize to microdomains containing T cell receptor (Chen and Shively, 2004). Additionally, CEACAM1 overexpressed in Jurkat T cell lines are found within lipid rafts (Chen and Shively, 2004), suggesting that CEACAM1 is not evenly dispersed on the surface of the cell but is segregated into specific microdomains. The BCR has been shown to exist within microclusters after antigen encounter (Depoil et al., 2008), and interestingly, co-ligation of the BCR with ITIM containing receptor FcγRIIB has been shown to negatively affect the stability of BCR microclusters (Sohn et al., 2008) thereby inhibiting BCR mediated activation signals. Therefore, we altered our assay to examine the effect of co-localizing the BCR with CEACAM1 by immobilizing both αBCR, and either CEA DAKO or normal Ig.

In case overall cellular responses were sensitive to the ratio of stimulatory signal (αBCR) to potential inhibitory (CEA DAKO) signal, we examined a range from 0 to 10 μg/mL of immobilized αBCR concentrations, along with our standard assay conditions which contained 10 μg/mL of either CEA DAKO or normal Ig (Figure 4.5A). B cells cultured under standard assay conditions using 0.1 μg/mL of immobilized αBCR with no further additional of antibody strongly expressed CD86 (Figure 4.5B). Upon treatment with increasing amounts of αBCR, no difference in cellular activation was observed, illustrating that under these culture conditions, CEACAM1 does not induce or inhibit B cell activation (Figure 4.5B).

4.3.6 Immobilized isogenic strains of *N. gonorrhoeae*, including a strain that binds to CEACAM1, robustly activates B cells.

In T cells, unless CEACAM1 is held at the cell surface through binding either antibody or gonococci, the receptor will be recycled from the surface back into intracellular stores (Lee et al., 2008). This suggests that the ability of CEACAM1 to mediate inhibitory signalling may be due to the gonococci holding this receptor at the cell surface, allowing for ITIM phosphorylation and
the recruitment of phosphatases which ultimately shuts down TCR signalling (Lee et al., 2008).

Engulfment of whole gonococci has not been observed in T cells (Lee et al., 2008), whereas Ngo are clearly engulfed by B cells (Chapter 2).

To examine if the ability of CEACAM1 to function as an inhibitory receptor required sequestration at the cell surface, we cultured B cells with immobilized gonococci expressing either: OpaCEA, which allows the Ngo to bind CEACAM1, OpaHSPG, which is a control strain of Ngo which expresses Opa adhesin, but cannot bind to CEACAM1, and Opa−, a strain expressing no Opa adhesin (Figure 4.6A). After 3 days, the cells were assessed for CD86 expression. No difference was observed between B cells cultured with any of the three strains of gonococci, as activation was strongly induced, even when cells were cultured with immobilized CEACAM-binding gonococci (Figure 4.6B).
Figure 4.5 Localization of activation and co-inhibitory signaling on B cells does not affect Ngo induced activation.

A) Schematic of cell culture conditions, indicating that both $\alpha$BCR and $\alpha$CEACAM1 (or control Ig) were immobilized to encourage co-localization of stimulatory and co-inhibitory signalling. B) Cells were cultured as in A) for 3 days, then expression of co-stimulatory marker CD86 was examined by flow cytometry. No Ig = no antibody added. Control Ig = normal Ig added. $\alpha$CEACAM1 = CEA DAKO added. Representative of experiments done with 2 different donors.
Figure 4.6 Immobilization of whole Ngo strongly activated B cells.

A) Schematic of culture conditions, indicating that heat-killed whole bacteria were used to coat the bottom of culture wells. B) Cells were cultured as in A) for 3 days, then assayed for expression of co-stimulatory marker CD86 by flow cytometry. Representative of experiments done with 2 different donors.
4.4 Discussion

While CEACAM1 has been extensively studied in the context of T cells, CEACAM1 in B cells has been less defined. Some have suggested that CEACAM1 is only expressed upon cellular activation (Lobo et al., 2009; Pantelic et al., 2005), whereas others observe that CEACAM1 is expressed in resting B cells (Greicius et al., 2003; Kammerer et al., 2001; Singer et al., 2002). In our assay system, CEACAM1 is clearly expressed in uninfected and unstimulated cells, however CEACAM1 expression is upregulated upon B cell activation through the BCR. This is an important effect to consider since 95% of clinically isolated Neisseria species use CEACAM1 as a pathogenic receptor (Virji et al., 1996), and we have shown that induction of CEACAM1 can affect the association of CEACAM1-binding gonococci to B cells. Remarkably, infection with a low MOI of gonococci did not induce CEACAM1 expression which suggests that specific activation signals may induce CEACAM1 expression on B cells.

The physiological role of inducing CEACAM1 upon activation is not currently known. As a cell adhesion molecule, both cis- (Hunter et al., 1996) and trans- (Sundberg and Obrink, 2002) homophilic (CEACAM1 to CEACAM1) and trans-heterophilic (CEACAM1 to other CEACAM family members) have been described (Oikawa et al., 1992). It is interesting to note that CEACAM1 transcript levels are higher in human innate IgM memory B cells than compared to the other B cell subsets (Weller et al., 2004). IgM memory B cells are a subset of B cells found in the periphery, along with naive and switched memory B cell subpopulations. The IgM memory B cells are the immediate responders to invading pathogens, by producing low affinity, polyclonal IgM to prevent microbial dissemination in the days before high affinity, antigen specific Ig is produced (Baumgarth et al., 2000; Ochsenbein et al., 1999). In light of this, CEACAM1 may function as a cell adhesion molecule in these B cell populations, aiding the recruitment of IgM memory cells to the sites of infection or mediating cell-to-cell contacts between these and other immune cells. The role of CEACAM1 as a cell adhesion molecule with respect to cellular migration or inter-cellular association in the context of B cells remains to be studied.

The immunomodulatory effects of CEACAM1 have been described in a variety of cell types, including dendritic cells (Kammerer et al., 2001), monocytes (Yu et al., 2006), neutrophils (Singer et al., 2005), and has been very well characterized in T cells. Although CEACAM1
engagement on T cells has been reported to induce stimulatory signals (Donda et al., 2000; Kammerer et al., 1998), the majority of reports show an inhibitory effect for CEACAM1 (Boulton and Gray-Owen, 2002; Chen and Shively, 2004; Chen et al., 2008; Kammerer et al., 1998; Lee et al., 2007; Lee et al., 2008; Nakajima et al., 2002). Previous work in our lab has established that *N. gonorrhoeae* Opa protein binding to CEACAM1 on CD4+ T cells suppresses their activation and proliferation relative to infection with gonococci that cannot bind to this co-inhibitory receptor (Boulton and Gray-Owen, 2002). This inhibitory effect is mediated by the phosphorylation of CEACAM1 upon receptor binding by either antibody or *Ngo*, which recruits tyrosine phosphatases SHP-1 and SHP-2 to the signalosome allowing them to dephosphorylate the T cell receptor, ultimately terminating activation responses (Lee et al., 2008).

CEACAM1 function in B cells has been less studied, and therefore, less defined. One study showed that CEACAM1 has a stimulatory effect on murine B cells, by combining soluble antibody specific for CEACAM1 with αIgM-coated sepharose beads to induce proliferation and IgM production (Greicius et al., 2003). In contrast, another group examined the function of the ITIM motifs present in the cytoplasmic domain of CEACAM1 fused to the transmembrane and extracellular portions of FcγRIIB in DT40 chicken B cells. In this case, the membrane proximal ITIM motif was required for inhibition of activation induced calcium flux, and required SHP-1 and SHP-2 to mediate these inhibitory responses (Chen et al., 2001) in a manner that resembles CEACAM1 mediated inhibition in T cells. Others have examined the role of CEACAM1 in human B cell lines, and shown that it negatively affects BCR signalling through association with SHP-1 (Lobo et al., 2009). Finally, Pantelic *et al.* reported that B cells activated to express CEACAM1 were killed upon infection with gonococci that could specifically bind CEACAM1 (2005).

Based on this body of literature, as well as the ability of CEACAM1 to function as a co-inhibitory receptor in the context of T cell infection with *Ngo*, we initially hypothesized that infection of B cells would result in CEACAM1-dependent inhibition. Instead of artificially stimulating B cells to induce CEACAM1 expression and adding extraneous stimulatory factors to the infection assay (Pantelic *et al.*, 2005), we specifically designed our infection assay conditions to include non-stimulated freshly purified B cells that expressed low levels of endogenous CEACAM1, and then infected these with a low MOI of *Ngo* (Chapter 2). Surprisingly, infection with CEACAM1 binding gonococci did not result in inhibition of B cell
responses, but induced overwhelming B cell activation and proliferation (Chapter 2). One major difference in the assay systems exists between these studies. The CEACAM1 expression level on B cells is likely higher in the study by Pantelic et al. (2005) compared to the untreated B cells used in our previous study (Chapter 2). Significantly, CEACAM1 expression is elevated from basal resting levels in the majority of studies examining the function of this receptor in immune cells, either by pre-stimulation of cells (Boulton and Gray-Owen, 2002; Chen and Shively, 2004; Chen et al., 2008; Lee et al., 2008; Lobo et al., 2009; Pantelic et al., 2005), or by transfection of CEACAM1 into cells (Chen and Shively, 2004; Chen et al., 2004; Chen et al., 2001). Overexpression or upregulation of CEACAM1 could abrogate the requirement of receptor recruitment to the T cell or B cell receptor signalosome, which has been proposed to be necessary for the inhibition of cellular responses (Gray-Owen and Blumberg, 2006; Lee et al., 2008). In our previous study, it is possible that due to the low CEACAM1 expression level and/or lack of BCR signalling, CEACAM1 was not recruited to the BCR signalosome.

Therefore, we decided to examine whether or not CEACAM1 is able to function as a co-inhibitory receptor in B cells, specifically in the context of infection with N. gonorrhoeae. Our assays were based on assays that were previously carried out in another study (Boulton and Gray-Owen, 2002), but were specifically adapted for B cells. We established an assay system that allowed us to alter the activation state of the primary B cells by varying individual stimulatory versus inhibitory effects. A variety of assay conditions were examined, all of which included B cell stimulatory (BCR) and costimulatory (CD40L) signals in combination with a low MOI of N. gonorrhoeae expressing no Opa adhesin. The concentrations of αBCR, CD40L and infectious dose of gonococci were chosen to stimulate B cell activation, but not provide overwhelming signals to B cells. In order to control for this, we ensured that the level of cellular activation obtained with our standard assay conditions was between that apparent with our negative and positive controls. We were not able to observe any B cell inhibition upon CEACAM1 cross-linking by soluble CEA DAKO, immobilized CEA DAKO or immobilized CEA DAKO with αBCR. While it is possible that CEACAM1 may not function as an inhibitory receptor in B cells, evidence suggests that the cytoplasmic ITIMs should be able to recruit SHP-1 and SHP-2 phosphatases to CEACAM1 during BCR mediated activation (Chen et al., 2001; Lobo et al., 2009). It is enticing to consider that in human B cells, the combination of
costimulatory molecule and MAMP signalling provides a synergistic signals that CEACAM1 cannot suppress, although this will require further experimentation to confirm.

Gonococci are not engulfed by T cells, allowing for prolonged propagation of inhibitory signalling by engaged CEACAM1 which accumulates on the cell surface (Lee et al., 2008). In contrast, gonococcal infection of B cells results in engulfment of the bacteria through a mechanism that has yet to be identified (Chapter 2). This concept of sequestering signals at the cellular membrane as a way of prolonging and intensifying cellular responses has been described for the BCR (Thyagarajan et al., 2003). Similarly, membrane bound antigens are able to induce B cell activation at lower concentrations compared to soluble antigens (Batista et al., 2001), suggesting that the immobilization of a whole bacteria on the surface of a cell effectively creates a small host-pathogen synapse between B cell membrane components and ligands expressed by gonococci (Lee et al., 2008). As such, CEACAM1 might be expected to function in a similar manner, by magnifying inhibitory signals through the maintenance of CEACAM1 at the membrane by binding gonococci. We examined this directly by culturing B cells with gonococci immobilized on the walls of a plastic well. A strong induction of cellular activation was observed upon culturing with all strains of *Ngo*, and there was no difference between cells cultured with gonococci that could bind CEACAM1 and those that could not. Despite immobilizing whole gonococci to prevent bacterial engulfment, it is possible that B cells were able to bind and engulf fragments of gonococci resulting in cellular activation. This effect may mask any CEACAM1-mediated inhibition if the resulting stimulatory effects overcome the CEACAM1 inhibitory signals. This possibility is difficult to discount considering the dynamic nature of two cells probing each other.

A question that remains is why CD4+ T cells are inhibited upon infection with CEACAM1-binding gonococci, while B cells are not. The answer must presumably stem from the inherent differences between T cells and B cells. One difference between T cells and B cells is the expression of pattern recognition receptors (PRRs) present on the two cell types, which certainly contributes to the overall activation threshold of the cell during infection with a bacterial pathogen like *N. gonorrhoeae*. The more PRRs responding during infection with *Ngo*, the more likely the cell will undergo cellular activation. Components of neisserial species have been shown to activate TLR4 (Pridmore et al., 2001), TLR2/1 (Fisette et al., 2003; Massari et al., 2006; Wyllie et al., 2000) and TLR9 (Bernasconi et al., 2003; Mogensen et al., 2006) pathways...
in a variety of cell types. We have shown that although purified human peripheral blood B cells are not responsive to TLR4 ligand, treatment with TLR2/1 and TLR9 agonists induced both activation and proliferation (Chapter 2). In general, primary human T cells produce less TLR transcript than B cells (Hornung et al., 2002), suggesting that protein levels of TLRs are also reduced in T cells compared to B cells. Moreover, while T cells have been shown to express TLR1, 2, 4 and 9 (Babu et al., 2006), TLR signalling is commonly associated with modulating TCR signals, as opposed to directly inducing cellular responses (Kabelitz, 2007). This is in contrast to TLR signalling in B cells, which can result in proliferation, differentiation and cytokine production independent of the BCR (Bernasconi et al., 2003; Bernasconi et al., 2002).

Liberated by Gram negative bacteria during natural growth, OMVs resemble the membranes of the bacteria they were derived from (Pettit and Judd, 1992). Recently, it has been shown that N. gonorrhoeae OMVs are able gain entry into non-phagocytic epithelial cells via lipid rafts, and effectively induce NOD1 signalling (Kaparakis et al., 2010). Both T cells (Petterson et al., 2011b) and B cells (Petterson et al., 2011a) express NOD1, suggesting that both cell types may be able to respond to Ngo-released OMVs, that are constantly shed by the gonococci during growth. However, NOD derived signals have been shown to act in synergy with TLR agonists (Herskovits et al., 2007; Traub et al., 2004), suggesting that B cell responses to gonococci, a bacteria which has been shown to possess a multitude of factors that can activate innate receptors including NODs and TLRs, may be stronger than T cell responses to gonococci. Recently, Sasawatri et al. had identified a proton-coupled histidine and oligopeptide cotransporter, SLC15A4, found in late endosomes/lysosomes and is one of potentially many transporters of NOD1 ligands from lysosomes to the cytosol, where they are recognized by NOD1 (2011). SLC14A4 is expressed in a variety of cell types including antigen presenting cells such as dendritic cells, macrophages and B cells (Sasawatari et al., 2011), but its expression has not been described for T cells. Considering gonococci are engulfed and killed by B cells (Chapter 2), but not T cells (Lee et al., 2008; Youssef et al., 2009), it is possible that degradation products such as gonococcal peptidoglycan could be transported into the cytoplasm by SLC15A4, or a similar mechanism, to activate NOD1. Interestingly, SLC15A4 has been implicated in the sensing of CpG DNA by TLR9 (Sasawatari et al., 2011), a signalling pathway that is utilized by both plasmacytoid dendritic cells (Dobson-Belaire et al., 2010) and B cells (Chapter 2) to respond to infection with Ngo.
In the context of primary human CD4⁺ T cells, CEACAM1 ligation by antibody or \textit{N. gonorrhoeae} elicits strong inhibition of both activation and proliferation. CEACAM1 is activation induced in both T cell and B cells, the expression of this co-inhibitory receptor in T cells is greater than in B cells. In terms of responses to pathogenic insult, the difference between the two lymphocyte populations is inherent within the respective lineages. B cells are antigen presenting cells, and possess many pattern recognition receptors, which can act synergistically, to aid them in the identification and induction of immune responses against potential invading microbes. Many of these pattern recognition receptors are either absent, or expressed in lower levels, in T cells. This difference in activation potential could affect the overall activation state of the cell, since the inhibition due to CEACAM1 in B cells is clearly not enough to counter the strongly stimulatory signals delivered by MAMP receptors.
We have shown that Ngo infection of B cells results in the production of polyclonal IgM, some of which recognizes the gonococci (Chapter 2). B cells, but not T cells, have been shown to express an Fc α/μ receptor, which not only binds to IgM opsonised Staphylococcus aureus, but also mediates its internalization (Shibuya et al., 2000). Fc receptor mediated engulfment of Ngo could effectively deliver gonococcal products to intracellular pattern recognition receptors such as the aforementioned TLRs and NODs, resulting in B cell activation. Overall, it is evident that B cells are able to respond to gonococcal infection through multiple mechanisms, many of which may not be present in T cells.

Another distinction between T cells and B cells is the level of CEACAM1 expressed. Prestimulated T cells express vast quantities of CEACAM, as observed by immunoblotting and flow cytometry (Boulton and Gray-Owen, 2002). D14HD11, the monoclonal antibody used for detection of CEACAM1 by flow cytometry in that study, is not sensitive enough to detect CEACAM1 induced upon pretreatment of B cells with αBCR at induction levels used within this study (data not shown), even though analysis by immunoblot clearly shows that CEACAM1 is induced upon activation. While we did not conduct a direct comparison between CEACAM1 expression levels between activated T cells and B cells, the flow cytometric results suggest that CEACAM1 expression on B cells may be less than that of T cells. The lower level of CEACAM1 expressed in B cells compared to expression levels in T cells, combined with the various stimulatory pathways elicited upon infection with N. gonorrhoeae may result in no observable cellular inhibition (Figure 4.7).

4.5 Materials and Methods

4.5.1 Primary Cells

Primary human B cells were obtained by either standard venipuncture or, when large numbers of cells were required, through leukopheresis. All participants gave informed consent in accordance with guidelines for the conduct of clinical research at the University of Toronto and St. Michael’s Hospital, respectively. The protocols used were approved by the University of Toronto and St. Michael’s Hospital institutional review boards (Toronto, Ontario, Canada).
Fresh peripheral mononuclear cells were obtained from Ficoll-Paque Premium (Amersham GE) gradients, according to manufacturer's specifications. CD19+ B cells were purified using either the EasySep Human CD19 Positive Selection Kit (Stemcell Technologies) or CD19 Microbeads (Miltenyi Biotec). B cell purity consistently exceeded 95%, as measured by a FACScalibur flow cytometer (BD Biosciences) with FlowJo software (Treestar) used for all flow data analysis.

Purified cells were maintained in RPMI 1640 medium (Invitrogen), supplemented with 10% heat-inactivated fetal bovine serum (CanSera), 4 mM GlutaMAX (Invitrogen) and 50 mM HEPES (Bioshop, Burlington, Canada) at pH 7.4. Cells were cultured at 37°C in 5% CO₂ and humidified air.

4.5.2 Bacterial Strains

Isogenic *Neisseria gonorrhoeae* strains constitutively expressing no Opa (Opa−, strain N302), the heparan sulfate proteoglycan-specific OpaHSPG (strain N303, expressing Opa₅₀), or CEACAM-specific OpaCEA (strain N309, expressing Opa₅₂) were derived from a pilus-deficient MS11 parent strain (Kupsch et al., 1993), and were graciously provided by Prof. T.F. Meyer (Berlin, Germany).

*Neisseria* species were grown from frozen stocks on GC agar (Difco) supplemented with 1% (v/v) IsoVitalex (BBL Microbiology Systems) at 37°C in a 5% CO₂ atmosphere with humidity. A binocular microscope was used for daily selection of gonococcal colony opacity phenotypes for the MS11 strains. Opa protein expression was monitored by immunoblotting using the Opa cross-reactive monoclonal antibody 4B12/C11 (Achtman et al., 1988), graciously provided by Prof. Mark Achtman (Berlin, Germany).

4.5.3 Optimization assays

B cells were treated with 0.1 μg/mL of human rCD40L (R&D Systems Inc.) and 0, 0.1 of 0.3 μg/mL of F(ab′)₂ goat anti-human IgG, IgA, IgM to induce B cell receptor cross-linking (αBCR, Jackson ImmunResearch Laboratories), with a multiplicity of infection (MOI) of 1 bacteria per cell of Ngo that does not express Opa adhesin (Ngo Opa−) as indicated. Cultures were incubated at 37°C in 5% CO₂ and humidified air for 5 days (unless otherwise indicated), at which time cells were fixed with 4% paraformaldehyde (PFA, Sigma) and assessed for activation marker by flow cytometry as outlined below.
4.5.4 Immobilization of gonococci

The indicated strains of *Ngo* were immobilized onto UV sterilized maxisorp 96 well plates (Nunc) using the following method. *Ngo* were grown as described, resuspended in PBS pH 7.4 (Wisent) and treated with 1 mg/mL of gentamycin (Bioshop, Burlington, Canada) for at least 2 hours at 37°C. 2x10⁸ bacteria were added to each well and were allowed to dry completely. Wells were washed at least three times with PBS pH 7.4 (Wisent) before addition of primary B cells.

4.5.5 Confocal Microscopy

To assess *Ngo* association with B cells expressing high levels of CEACAM1, cells were either pretreated with 1 μg/mL of αBCR for 2 days before infection, to induce CEACAM1 expression, or left untreated. They were then infected with the indicated *N. gonorrhoeae* strains at a MOI of 10 for 4 hours. Acid-washed glass coverslips were coated with mouse monoclonal antibody specific for human CD19 (clone HIB19, eBioscience) to capture B cells for microscopy. Cells were fixed with 4% PFA (Sigma). Mammalian cell membranes were permeabilized using 0.4% Triton X-100 (Sigma), allowing for total gonococci to be stained using the polyclonal rabbit anti-gonococcal serum (UTR01), followed by anti-rabbit IgG Alexa 647 (Molecular Probes). CEACAM1 was visualized after permeabilization using the murine monoclonal antibody T84.1, a generous gift from Dr. J. Shively, followed by anti-mouse IgG Alexa 488 (Molecular Probes).

Bacteria and cells were visualized and recorded using a Plan-Apochromat 100x/1.4 Oil DIC objective lens on a Zeiss LSM 510 confocal microscope. All images were obtained at room temperature. Zeiss LSM Image Browser version 4.2.0.121 was used for subsequent image processing. Each infection condition was prepared on duplicate or triplicate coverslips, and at least 60 *Ngo*-infected cells per infection condition were analyzed.

4.5.6 Immunoblotting

To visualize CEACAM1 protein expression, primary B cells or B cell lines were either treated with a combination of either with 1 μg/mL or 10 μg/mL of αBCR (Jackson ImmunoResearch Laboratories), MOI 1 of *Ngo* Opa⁻, or MOI 1 of *Ngo* Opa⁻ + rCD40L at 0.1 μg/mL (R&D Systems Inc.) + 0.1 μg/μL αBCR (Jackson ImmunoResearch Laboratories) or left untreated for the indicated number of days. Cells were then enumerated, and an equal number of cells were
lysed with SDS-PAGE sample buffer and boiled. Immunoblot analysis for total CEACAM1 expression used murine monoclonal D14HD11 (Abcam). Tubulin (Sigma) was used to show equivalent protein loading between lanes. Anti-mouse horseradish peroxidise (HRP) secondary antibody (Jackson Immunoresearch) was used to visualize the bands corresponding to CEACAM1 and tubulin.

4.5.7 Flow Cytometry

All flow cytometric analysis was performed using a BD FACScalibur. To assess B cell activation in response to treatment or infection as outlined in figure legends, primary B cells were fixed, then stained for activation marker CD86-PE (clone FUN-1, BD Biosciences).

B cell lines were allowed to reach confluency, at which point they were fixed and stained for surface CEACAM1 expression by Gran-10 (Abcam). Isotype control MOPC-31c (BD Biosciences) was used as a staining control, followed by anti-mouse IgG APC (BD Biosciences) for all samples.
5 Thesis summary and Future Perspectives

While CEACAM1-mediated inhibition of T cells may delay or prevent the induction of immunologic memory (Lee et al., 2008), in agreement with clinical observations that recurrent bouts of gonorrhea are not uncommon (Fox et al., 1999), we were interested in examining why small amounts of Ig are still produced during gonococcal infection (Hedges et al., 1999), upon gonococcal infection. Potential stimulatory signals from the gonococci, mediated through neisserial factors such as MAMPs and porin, must integrate with the potential inhibitory signals mediated through Opa-CEACAM1 interactions. The initial goal of this thesis was to elucidate how the combination of signals would affect gonococcal-induced humoral responses. Unexpectedly, the co-inhibitory effect of CEACAM1 evident in other cell types is not apparent in primary human B cells. Instead, my results establish that *N. gonorrhoeae* has a potent agonistic effect on human B cells, and reveals a potentially important role for a poorly characterized subset of innate B cells found only in humans, the circulating IgM memory B cells. Moreover, by examining the effects of the other *Neisseria* species on IgM memory B cell responses, I demonstrate that the commensal *Neisseria sp.* elicit a similar effect on the innate like B cells, whereas the pathogenic *N. meningitidis* is unexpectedly distinctive in that it elicits a relatively muted effect. This difference was not expected due to the many similarities that exist between the pathogenic and commensal *Neisseria*.

5.1 Infection by *N. gonorrhoeae* mediates IgM memory B cell activation and polyclonal Ig production

Prior to the start of this study, there was evidence to suggest that during gonococcal infection, Opa proteins binding to CEACAM1 expressed on immune cells would mediate the inhibition of cellular responses (Boulton and Gray-Owen, 2002; Lee et al., 2008; Yu et al., unpublished observations), however there was also evidence suggesting that gonococcal infection of B cells may induce cellular activation (Wetzler et al., 1996). Using freshly purified primary human B cells, I observed no inhibition of cellular activation or proliferation upon infection with gonococci, regardless of their ability to bind CEACAM1. Instead, the innate IgM memory B cells responded strongly to gonococcal infection, by proliferation and polyclonal IgM
production, including IgM specific for Ngo. The specific response of this B cell subset was unexpected and, significantly, this represents the first report of bacteria directly initiating cellular responses by the IgM memory B cells. A number of studies have established that these innate B cells are protective against bacterial and fungal infections (Carsetti et al., 2005; Kruetzmann et al., 2003; Subramaniam et al., 2009), however no one has assessed their response to direct infection with a pathogen until now.

After ruling out the possibility of Ngo causing this strong stimulation through the BCR, I examined the role of TLRs in mediating these responses. While TLR expression within the B cell subsets have been previously reported through the assessment of relative transcript levels (Bernasconi et al., 2003), I show that examining TLR functionality through the treatment of cells with specified agonists not only increases the sensitivity for detection of TLRs expressed, it also establishes that the TLRs expressed within the subsets are functional. I observed that TLRs 1, 2, 6, and 9 are expressed and functional in all B cell subsets, and confirmed that human B cells are unresponsive to stimulation with TLR4 agonists. Previous reports have shown that TLR expression in the naive subset is low to undetectable (Bernasconi et al., 2003), however my observations suggest that even at these low levels, they are still able to propagate signals resulting in cellular activation but not proliferation. These observations fit with the idea that naive cells possess higher activation thresholds than memory B cells (Good et al., 2009), and that they require BCR stimulation for proliferation to occur (Bernasconi et al., 2002). Furthermore, this is the first report of TLR functionality within the IgM memory subset, and my observation that the IgM memory B cells are more responsive to treatment with TLR agonists compared to the switched memory subset supports their proposed function in the generation of immune responses to pathogenic infection: to provide a rapid response to invading microbes before the induction of conventional B cell responses.

Because of the strong responses to TLR9 stimulation I observed by the IgM memory B cells, and evidence which suggested that CpG DNA treatment of this subset produced only IgM class antibody (Bernasconi et al., 2003), I examined the ability of Ngo to stimulate the innate B cells through TLR9. Not only did I show that Ngo mediates B cell proliferation through TLR9, I observed B cells engulfing whole gonococci, effectively delivering a large amount of neisserial DNA directly to the intracellular TLR9 for signalling. TLR9 has long been thought of as an important mediator of B cell responses, due to its high levels of expression in this cell type,
however the ability of B cells to engulf, and subsequently kill whole bacteria remains controversial in the field. Human B cells are inefficient at internalizing exogenous DNA (Roberts et al., 2010), therefore the engulfment of gonococci is an effective mechanism for the delivery of DNA to TLR9. Others have shown that primary human B cells are able to engulf and kill whole bacteria (Vidakovics et al., 2010), suggesting that this underappreciated ability of B cells may also aid in the direct clearance of bacteria during infection.

Importantly, if I had not used freshly purified B cells to examine the effect of gonococcal infection, I would not have been able to identify the innate B cells as responders to infection by Ngo. Peripheral blood mononuclear cells that have been previously frozen and thawed for B cell purification show drastically decreased levels of viable IgM memory B cells (my observations). Use of a murine model might not have resulted in these findings either, due to inherent differences between the human IgM memory and murine B-1 innate B cell populations.

The work that I present in Chapter 2 illustrates that multiple factors contribute to the weak induction of gonococcal-specific antibody during infection (Hedges et al., 1999), and the lack of immunologic memory (Fox et al., 1999) observed in gonorrhea infections. Considering that the majority of clinical nesseriral species bind to CEACAM1 (Virji et al., 1996), the ability of neisserial Opa adhesin mediating inhibition of dendritic cell maturation (Yu et al., unpublished observations) and T cell activation (Boulton and Gray-Owen, 2002; Lee et al., 2008) diminishes the likelihood of inducing protective memory responses during gonococcal infection. However, the production of IgM specific for Ngo has been observed in local secretions during gonococcal disease (Hedges et al., 1999; McMillan et al., 1979b), and could potentially originate from the IgM memory B cell subset. Overall this results in a weakly effective T-independent immune response that is inefficient at clearing the infection and does not protect from subsequent infections.

5.2 *N. meningitidis* and *N. gonorrhoeae* mediate different responses by IgM memory B cells.

Since Ngo infection of B cells resulted in such strong responses from the innate B cell subset, I was interested in determining if this effect was specific for gonococci or if all *Neisseria* species
elicited these responses. Unexpectedly, B cell infection with the only other pathogenic *Neisseria, Nme*, resulted in moderate cellular activation but no T-independent proliferation and little Ig. Moreover, B cell infection with commensal *Neisseria* species induced IgM memory cell responses that were similar to infection with *Ngo*: robust proliferation and the production of polyclonal Ig. The perceived inability of *Nme* to induce IgM memory B cell responses was not due to its pathogenic ability, as B cell infection with *Haemophilus influenzae*, which shares this niche with the *Neisseria* species and is also an opportunistic pathogen, induces proliferation of the innate subset and the production of polyreactive Ig.

The question of why *Nme* does not induce T-independent responses by the IgM memory B cells was examined in two ways. First, I differentiated between *Nme* actively suppressing cellular activation versus simple inability of the meningococci to stimulate the B cells by utilizing a co-infection assay with both *Nme* and *Nla*. No inhibition of the *Nla*-induced activation of human B cells was observed in cultures containing both *Nme* and *Nla*, with B cell responses to co-infection resembling cultures in which *Nla* alone was added. Second, treatment of B cells with outer membrane vesicles (OMVs) derived from *Nme* or *Nla* recapitulated cellular responses observed upon infection with live bacteria. Together, this suggests that *Nme* is likely not suppressing an inherent ability to induce IgM memory B cell responses, but is instead either missing or downregulating one or more B cell stimulatory factors that are common to the other *Neisseria* species.

These results offer insight into how the highly adapted human specific pathogen *Nme* interacts with the host immune system, and it is enticing to speculate that the ability of meningococci to avoid the induction of IgM memory cell responses could represent a form of immune evasion. The IgM memory B cells are the source of innate Ig in humans (Weller et al., 2004). The function of innate Ig is to offer early protection from invading microbes in the form of low affinity, highly-cross reactive Ig (Notkins, 2004), primarily IgM (Moens et al., 2008), in the time period prior to the development of T-dependent, high affinity clonal antibody responses. Innate IgM functions locally, to prevent pathogenic dissemination during the early stages of infection (Baumgarth et al., 2000; Briles et al., 1981; Martin et al., 2001; Ochsenbein et al., 1999), a function that is critically important for host defense against pathogens like *Nme*, which are capable of disseminated disease. Moreover, innate IgM is also able to mediate complement dependent lysis of Gram negative bacteria (Zhou et al., 2007), including the meningococci.
(Estabrook et al., 1997), and can cause agglutination of the infecting pathogen by the nature of its polyclonal antigen receptor and pentameric structure (Ehrenstein and Notley, 2010). Furthermore, the innate Ig are clearly protective against disease caused by encapsulated bacteria, as individuals missing the innate B cell subset are more susceptible to these infections (Kruetzmann et al., 2003; Weller et al., 2004), and the only period during human development in which the innate B cell subset is non-functional (Weller et al., 2008) corresponds to the time when we are most susceptible to meningococcal disease: as children under the age of 2 (Booy et al., 2007).

5.3 CEACAM1 does not inhibit B cell responses during gonococcal infection.

For the last data chapter of my thesis, I was interested in examining if CEACAM1 is able to function as an inhibitor of B cell responses during infection with Ngo. My work in the Gray-Owen lab was initially focused on this question, in light of the evidence that CEACAM1, through one of its immunoreceptor tyrosine based inhibition motifs (ITIMs), is able to inhibit BCR signalling (Chen et al., 2001), in a manner similar to CEACAM1 mediated inhibition of T cell receptor signalling (Boulton and Gray-Owen, 2002). In Chapters 2 and 3, I did not observe any inhibition of cellular responses despite infection with neisserial species that have been demonstrated to bind to CEACAM1, even when using the Opa\textsubscript{CEA} strain of Ngo (in Chapter 2) which has previously been shown to inhibit T cell responses (Boulton and Gray-Owen, 2002). The B cells used in the assays were freshly purified and not subject to other stimulatory signals, to induce additional CEACAM1 expression. Therefore, I designed assays to specifically examine if CEACAM1 ligation, specifically in the context of gonococcal infection, is able to inhibit B cell responses to the pathogen. The assays were adapted from experiments designed to observe CEACAM1 mediated inhibition in T cells (Boulton and Gray-Owen, 2002), and contained BCR stimulation (\(\alpha\text{BCR}\)), co-stimulatory signals (CD40L), Ngo expressing no Opa adhesin and either \(\alpha\text{CEACAM1}\) antibody or isotype control antibody, allowing me to control the level of CEACAM1 binding. Regardless of the assay configurations used, I did not observe inhibition of B cell responses to gonococcal infection. This result suggests that CEACAM1 may not act as an inhibitory receptor in B cells. The data presented in Chapters 2 and 3 show that the
majority of B cell responses to neisserial infection are derived from the memory subset. Memory B cells naturally possess lower activation thresholds, compared to naive B cells (Good et al., 2009). B cells also have a different role in the generation of immune responses against invading microbes, compared to T cells. B cells are antigen presenting cells, and express a variety of innate receptors including TLRs, Fc receptors and NODs to aid in the detection of microbes, resulting in cellular activation that does not require BCR signalling (Bernasconi et al., 2003; Bernasconi et al., 2002; Shibuya et al., 2000). In contrast, TLRs expressed by T cells are primarily modulators of TCR signalling, and are not adept at directly inducing cellular responses on their own (Kabelitz, 2007). I, along with others (Vidakovics et al., 2010), have shown that B cells are able to both engulf and kill whole bacteria, functions that T cells lack (Lee et al., 2008; Youssef et al., 2009). Considering the vigorous, almost mitogenic B cell responses to infection with all strains of gonococci examined, and taking into consideration the role of B cells as APCs, I conclude that in the context of Ngo infection, CEACAM1 does not function as an inhibitor of B cell responses due to overwhelmingly stimulatory signals.

5.4 Summary

The research presented within this thesis not only provides novel insight into how the Neisseria species of bacteria interact with the immune system, but also contributes to the expanding body of work describing the function and relevance of the human innate IgM memory subset and further distinguishes them from the innate B cell subsets present in rodents.

During N. gonorrhoeae infection, DC maturation (Yu et al., unpublished observations) and T cell activation (Boulton and Gray-Owen, 2002; Lee et al., 2008) is inhibited by Opa adhesin binding to CEACAM1. While this may preclude the production of protective immunity, since this requires T cell activation (Foy et al., 1994), B cells are able to respond to gonococcal infection in a T-independent manner, through the production of low affinity, cross-reactive IgM that is localized to the site of infection (Figure 5.1). While this innate IgM is presumed to function by preventing dissemination of gonococci from the site of infection, it can also mediate bacterial killing through complement-mediated pathways (Estabrook et al., 1997; Zhou et al., 2007). The low rate of disseminated gonococcal disease may be, in part, due to the ability of these IgM memory B cells to rapidly produce innate Ig. However, innate Ig is not able to clear colonization
(Khatami and Pollard, 2010), so the gonorrhea persists until treatment with antibiotics. The ability of the meningococci to avoid the induction of innate Ig may contribute to its ability for colonization and/or cause disease. Although this hypothesis has yet to be directly tested, individuals who are unable to produce innate Ig are at risk of meningococcal disease (Booy et al., 2007). Consequently, identification of the factor(s) responsible for the induction of innate B cell responses may impact *Nme* vaccine design in the future.

My work significantly expands our knowledge of how the human IgM memory B cells respond to infection. While innate B cells in mice are able to respond to an invading pathogen by accumulating at the site of infection, which effectively increases the local concentration of innate Ig, the innate B cells do not proliferate and innate Ig is not induced by infection (Choi and Baumgarth, 2008). This is clearly not the case in humans, at least in the case of *N. gonorrhoeae*. The IgM memory B cells respond to infection by the *Neisseria* species, by vigorous proliferation and differentiation into antibody secreting cells. These results are a reminder that murine models, while they are valuable research tools, are only models and relevant findings should be confirmed in humans whenever possible.

The role of innate IgM has been vastly underappreciated, so much so that many immunological assays designed to assess the protective capability of Ig omit assessment of IgM altogether, since the current dogma dictates that immune IgG and IgA are the important classes for protection both systemically and locally. The emerging perception is that the innate Ig produced by the IgM memory B cell subset may be vitally important for preventing pathogenic dissemination during the initial stages of infection, before the T-dependent adaptive immune response is activated. This protective function of the innate Ig may determine whether or not disease occurs. My work demonstrates that the innate IgM memory subset is able to specifically respond to infection by expansion and the induction of innate Ig. Armed with this knowledge, taking an approach to incorporate the activation of both broadly specific innate and high affinity immune Ig may support vaccination programs and improve vaccine efficacy, especially for susceptible populations and during outbreak conditions.
Infection with CEACAM1 binding gonococci inhibits DC maturation and T cell activation, effectively preventing the production of immunologic memory. In contrast to this, innate IgM memory B cells respond to infection by Ngo with proliferation and the production of immunoglobulin, including polyreactive IgM, a small proportion of which recognizes the gonococci. This weak production of Ngo-specific IgM, combined with the lack of T-dependent immune responses results in an ineffective adaptive immune response against \textit{N. gonorrhoeae} infection.
Furthermore, my findings bring into question the current definition of what an innate or ‘natural’ antibody is. While it is undeniable that the production of innate Ig does not require exogenous antigenic stimulation (Haury et al., 1997), I show that the innate B cells are clearly able to respond to pathogenic infection and produce innate Ig which is polyreactive but also contains pathogen-specific Ig. The current dogma is that any antibody produced in response to colonization or infection is not innate, even though the Ig is derived from the innate B cell subset, produced in a T-independent manner and is associated with mucosal tissues (Macpherson et al., 2000). Although this may be thought of as an exercise in semantics, the ability of the human innate IgM memory B cells to produce innate Ig in response to infection has never been directly assessed until now, and updating the definition of what classifies as innate Ig will reflect and acknowledge the broad functionality of the human IgM memory B cell subset.

5.5 Future Perspectives

5.5.1 The nature of the innate Ig and T-independent IgG and IgA produced in response to Ngo infection

One of the unique properties that has been described of the innate Ig derived from mice is that they are polyclonal, in that one antibody clone can potentially bind to multiple, structurally diverse epitopes (Zhou et al., 2007). This distinctive quality of innate Ig, combined with the pentameric organization of secreted IgM undoubtedly improves its ability to cause agglutination between individual microbes, and enhances binding to microbial surfaces through multiple epitopes (Ehrenstein and Notley, 2010). Whether this is also true for innate Ig produced in humans remains unclear. In my assays, Ngo infection of B cells results in the production of polyreactive Ig, since only a portion of the IgM produced recognized the gonococci and the IgG and IgA were not specific for Ngo at all. It would be interesting to determine if a single antibody clone is able to bind multiple epitopes, either expressed by the gonococci, other neisserial species, or unrelated antigens, as this would further define and differentiate the distinct roles between the human innate Ig and conventional immune Ig.

In Chapter 2, I observed that Ngo infection of purified B cells induced the production of all three classes of immunoglobulin. Although the majority of the response was IgM, which I attributed
to the activation and differentiation of the innate subset, a question that remains is how and from what subset the IgG and IgA induced upon gonococal infection were derived. One hypothesis is that the T-independent production of IgG and IgA suggests an innate origin. Innate Ig in all three classes has been described (Avrameas, 1991; Cohen and Norins, 1966). There is direct evidence that the human innate B cell subset are able to produce Ig classes besides IgM in a T-independent manner, as adoptive transfer studies using SCID mice repopulated with human IgM memory B cells showed that both IgM and IgG were produced in response to vaccination with either PS vaccine or heat-killed bacteria (Moens et al., 2008). Specifically, a portion of the IgG2 class may be derived from the innate repertoire (Baumgarth et al., 2000). The production of innate IgG2 early during an infection may act to opsonise the invading microbe, and target it for clearance by neutrophils expressing FcγRIIa, and Fc receptor with a high affinity for this subclass. Significantly, individuals possessing an FcγRIIa variant that binds IgG2 poorly are more susceptible to pneumococcal and meningococcal disease (Bredius et al., 1994; Platonov et al., 1998; Yee et al., 2000). Another hypothesis is that the IgG and IgA were produced as a result of bystander activation of non-cognate switched memory B cells. Bystander activation of the switched memory subset has been suggested as one of the mechanisms for the maintenance of serologic memory over the lifetime of an individual (Bernasconi et al., 2002). If this were the case, the bystander signals in my assays likely originate from the IgM memory B cells upon interaction with Ngo. The implications of a study such as this one emphasizes the potentially diverse functions of the human innate B cell subset, possibly having a role in bridging innate and adaptive memory responses, and may influence vaccine innovations.

5.5.2 Further characterization of the interaction between Neisseria species and B cells

I, along with others (Vidakovics et al., 2010), have observed the ability of primary human B cells to engulf whole bacteria during in vitro infection. However the mechanisms involved in the engulfment of these bacteria have not been well characterized, especially if it occurs independently of the BCR, as is the case for neisserial engulfment. My preliminary observations suggest that B cell engulfment of gonococci occurs without the accumulation of actin, reminiscent of CEACAM1 mediated engulfment of Ngo in cervical epithelial cells (McCaw et al., 2004). Furthermore, Fc α/μ receptor expressed by B cells has been shown to mediate engulfment of IgM opsonized S. aureus (Shibuya et al., 2000). The cytoplasmic domain of this
Fc α/μ receptor does not contain any tyrosines (Shibuya et al., 2000), which suggests that its mechanism of engulfment is distinct from that of Fc γ receptors containing immunoreceptor tyrosine-based activation motif (ITAM) sequences (resulting in actin remodelling upon receptor engagement), although the authors of this report did not assess actin recruitment. Taking into account that Ngo infection can induce IgM, engulfment through the Fc α/μ receptor may contribute to the ability of B cells to take up gonococci. Generally, although B cells are antigen-presenting cells, they are considered to engulf primarily soluble antigens such as toxins or proteins rather than whole, intact bacteria. The often unappreciated ability of B cells to engulf microbes may impact the kinetics of pathogen clearance at the site of infection, and it is plausible that it can aid in the generation of B cell mediated immune responses.

One of the main outcomes of Chapter 2 is that gonococcal detection is mediated in part by TLR9. However, while treatment of the infection culture with TLR9 antagonist effectively suppressed cellular proliferation in response to Ngo infection, some B cell response persisted. Whether this resulted from incomplete blocking by the antagonist and/or simultaneous stimulation of other innate receptors remains to be explored. If the latter occurs, it would be interesting to examine which host/bacterial factors are involved in the induction of proliferation responses in the innate B cell subset. It is likely that PorB will be able to induce IgM memory B cell proliferation, as it is strongly mitogenic for murine B cells (Wetzler et al., 1996). Moreover, in Chapter 4, I show that H. influenzae is able to induce innate B cell proliferation and polyreactive Ig production. Others have observed that primary human B cell infection with M. catarrhalis induces the production of polyreactive Ig (Vidakovics et al., 2010), which I propose may arise from an innate subset. Both these human-specific opportunistic pathogens produce proteins that are able to bind IgD (Ruan et al., 1990; Samuelsson et al., 2007; Samuelsson et al., 2006), and only M. catarrhalis strains that bind IgD are able to induce B cell proliferation and IgM production (Vidakovics et al., 2010). Additionally, N. lactamica has been described to bind IgM and/or IgD, promoting the proliferation of certain B cell subsets (Vaughan et al., 2010). Therefore, while we observed no evidence of B cell receptor involvement, it may be worthwhile to re-examine whether Ngo is able to bind IgD, especially since IgD is surface expressed by the IgM memory B cells, and IgD may possess signalling capabilities similar to an IgM BCR (Lutz et al., 1998).
5.5.3 The role of anti-phosphorylcholine innate antibodies in humans

In mice, there is one particularly well studied innate derived IgM idiotype specific for phosphorylcholine (also called phosphocholine) (Masmoudi et al., 1990). Phosphorylcholine (ChoP) is fixed to the surface structures of a variety of bacteria, most of them colonizers of mucosal surfaces, including the commensal and pathogenic Neisseria species (Serino and Virji, 2000), S. pneumoniae (Brundish and Baddiley, 1968), H. influenzae (Weiser et al., 1997), as well as Pseudomonas aeruginosa (Weiser et al., 1998). Expression of ChoP is phase variable on H. influenzae (Weiser et al., 1997), P. aeruginosa and pathogenic Neisseria species (Weiser et al., 1998), as it imparts both functions in pathogenesis as well as eliciting host immune responses. ChoP has been shown to mediate adherence to (Serino and Virji, 2000), and delay clearance from host tissues (Pang et al., 2008). H. influenzae expressing ChoP induce less proinflammatory cytokines from macrophages (West-Barnette et al., 2006) and are less susceptible to anti-microbial peptides (Lysenko et al., 2000). Immune recognition of ChoP expressed by these microbes has been described. Using B-1 deficient mice, innate IgM expressing an idioytic antigen binding domain for ChoP was shown to be more effective in protecting against intravenous infection of S. pneumoniae, compared to anti-capsular antibody (Briles et al., 1981). Similar results were obtained when human innate Ig specific for ChoP was transferred into mice prior to infection (Goldenberg et al., 2004), and alludes to the idea that ChoP specific innate Ig may be important for immune protection in humans. Therefore, it would be interesting to examine the receptor specificities of the polyreactive Ig produced by the IgM memory B cells in response to infection with NTHi, gonococci and the commensal Neisseria strains. Desirable features of the ChoP epitope is that it is expressed on a wide variety of disease causing pathogens, including NTHi, for which there is currently no vaccine, and that the innate Ig produced in response to ChoP are highly effective in local mucosal secretions, suggesting that an oral ‘vaccine’ of commensal bacteria carrying ChoP epitopes may confer protection from pathogenic dissemination. Others have examined the ability of ChoP containing vaccines to protect from infection (Tanaka et al., 2007; Trolle et al., 2000), however it is difficult to tease apart the relative contributions from the innate versus immune adaptive responses from this work. A study such as the one I propose here may clarify the role of ChoP binding Ig in humans, ChoP-specific innate Ig post-vaccination (using animal models), and possibly provide insight into the value of eliciting innate Ig responses in conjunction with current vaccination protocols.
5.5.4 Transcriptome and Proteomic analysis of *Neisseria* species during infection

Although I observed that *Ngo* and commensal *Neisseria* species induced proliferation and Ig secretion, this was not a general response to bacterial infection by the IgM memory B cells, since infection with other Gram-negative bacteria such as *E. coli* and *N. meningitidis* did not induce similar responses. It is clear that multiple neisserial factors contribute to the vigorous response by the innate B cells, however only a few candidates, as discussed in Chapter 3, have been characterized. Of interest is that while these candidate factors may contribute to innate B cell proliferation and differentiation, all of them are also present in *Nme*, but are clearly not able to induce similar responses by the IgM memory B cells when expressed within the context of the meningococci. Transcriptome and/or proteomic analysis of the gene products present during infection may reveal the factors which are differentially expressed between the meningococci, and the other neisserial species. This could potentially lead to the identification of these bacterial factors that could induce innate B cell responses, and may influence the design of therapeutics for meningococcal disease.

5.5.5 The function of CEACAM1 in B cells

The majority of the assays herein utilize freshly purified resting B cells, to minimize the bias of skewing the relative proportions of the B cell subsets by prestimulation prior to infection. However, the expression level of CEACAM1 would presumably affect the cellular responses to infection with CEACAM1-binding gonococci. Although B cells were stimulated to induce additional CEACAM1 expression prior to gonococcal infection in selected experiments for Chapter 4, there was no observable inhibition of cellular activation. As discussed in that chapter, cells over-expressing CEACAM1 are utilized in the majority of studies by others aimed at examining the signalling capabilities of CEACAM1, however the inhibitory function of CEACAM1 may not have been appreciated without such studies. Since I have already described the effect of gonococcal infection on B cells expressing endogenous levels of CEACAM1, it would be interesting to examine the effect of CEACAM1 overexpression on B cell responses to *Ngo* infection. The results of such a study could address whether or not this co-inhibitory receptor is able to function such a manner in these antigen presenting cells, or if it is simply expressed by B cells to promote bacterial adherence leading to cellular activation and other responses. Preliminary results from assays in which human B cell line C1R, naturally expressing
abundant levels of CEACAM1, were infected with *Ngo* suggests that there is a defect in the killing of gonococci able to bind CEACAM1, an affect that was not apparent with gonococci that do not bind CEACAM1 (Figure 5.2). This result represents an intriguing starting point to begin assessing the signalling capacities and function of CEACAM1 in B cells during *Neisseria* infection.

Throughout this thesis, there are multiple lines of evidence which alludes to CEACAM1 not functioning as an inhibitory receptor in B cells that express endogenous levels of this receptor during neisserial infection, even though it has been shown to be capable of inhibiting BCR mediated signals in other more contrived assay systems (Chen et al., 2001). Transcriptome analysis of resting IgM memory B cells, as well as their splenic counterparts the marginal zone B cells, demonstrates that CEACAM1 is preferentially expressed by the innate subsets, compared with the other B cell subpopulations (Weller et al., 2004). Histological assessment of human lymph nodes illustrates that CEACAM1 is highly expressed by cells within germinal centers (Lobo et al., 2009), sites that are rich in B cells that are undergoing proliferation, class switch recombination, affinity maturation and differentiation into memory cells. It seems counter-intuitive that the expression of an inhibitory receptor is localized within a structure that promotes such robust cellular activation, although it may function to limit proliferation within the follicle or as a cell adhesion molecule at these sites. A role for CEACAM1 in cell adhesion has not been described for lymphocytes. Intriguingly, the relatively high expression of this receptor on resting IgM memory B cells (Weller et al., 2004), combined with the observation that in mice, the B-1 B cells are recruited to the site of infection, and are maintained there for at least 10 days after pathogenic challenge (Choi and Baumgarth, 2008) suggests that a cell adhesion function for CEACAM1 may be possible, and merits further examination. Transgenic mice have been developed which do not express CEACAM1, and effort has been made to construct a mouse that expressed only human CEACAM1 (without the murine version). These tools will undoubtedly aid in determining the function of CEACAM1 in these important immune cells, including during *in vivo* infection.
Figure 5.2 The human B cell line C1R expresses CEACAM1, and infection with CEACAM1 binding gonococci results in increased engulfment, but also a noticeable delay in the killing of intracellular bacteria.

A) Cell lysate for C1R was immunoblotted for CEACAM1 expression. 1x10^6 cells loaded per lane. Tubulin was used as a loading control. B) Flow cytometry of surface expression of CEACAM1 in the C1R human B cell line. C-D) C1R cells were infected at a MOI:50 with each of the indicated Ngo strains for 90 minutes prior to 1 hour treatment with gentamycin. C) Microscopy showing CEACAM1 expression and bacterial binding for Ngo Opa^HSPG and Ngo Opa^CEA, and engulfment for Ngo Opa^CEA (white arrow) 90 minutes post infection. Red indicates extracellular gonococcal staining. Blue indicates total gonococcal staining, post permeabilization. Green indicates CEACAM1. White bars indicate 5 μm. White arrow highlights intracellular gonococci. D) After gentamycin treatment to kill extracellular Ngo, intracellular colony forming units were examined by dilution plating.
5.5.6 Conclusion

The work presented in this thesis reveals novel insights into the immune responses elicited by the *Neisseria* species, as well as the biology of the recently described human IgM memory subset. Future studies will aid in delineating the contribution of the effects described in this thesis in infection, immune protection and the immunopathology associated with neisserial and other infectious disease.


