THE ROLE OF THE ITAM-CONTAINING CEACAM3 RECEPTOR IN THE NEUTROPHIL RESPONSE TO INFECTION BY NEISSERIA GONORRHOEAE

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Bacterial species of the genus *Neisseria* include pathogens that are responsible for diseases of humans including bacterial meningitis (*Neisseria meningitidis*) and the sexually transmitted disease gonorrhea (*Neisseria gonorrhoeae*). These diseases are often characterized by a massive influx and activation of neutrophils, white blood cells involved in the early/innate immune response to pathogens, at the infection site. *Neisseria spp.* bind to and activate neutrophils via their Opacity-associated (Opa) outer membrane proteins, which interact with some members of the human carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family. One of these CEACAMs, CEACAM3 (CD66d), is unique in its restriction to neutrophils and its expression of a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM; YxxL/Ix₆₋₈YxxL/I). In the course of my thesis work, I have shown that this motif is critically dependent for the activation of the neutrophil response to *Neisseria*, through the coupling of neisserial binding to activation of the tyrosine kinase, Syk, which initiates downstream signaling responsible for the antimicrobial responses of neutrophils. These data contribute to the knowledge of how seemingly unrelated receptors of neutrophils (such as the IgG-binding Fcγ receptors, the fungal receptor Dectin-1, and the bacterial-binding CEACAM3) converge functionally on the presence of the ITAM.
ACKNOWLEDGEMENTS

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATRA</td>
<td>all-trans retinoic acid</td>
</tr>
<tr>
<td>ASGP-R</td>
<td>asialoglycoprotein receptor</td>
</tr>
<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BPI</td>
<td>bactericidal permeability-increasing protein</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase activation and recruitment domain</td>
</tr>
<tr>
<td>CEACAM</td>
<td>carcinoembryonic antigen-related cellular adhesion molecule</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>DAP10</td>
<td>DNAX-activating protein of 10 kDa</td>
</tr>
<tr>
<td>DAP12</td>
<td>DNAX-activating protein of 12 kDa</td>
</tr>
<tr>
<td>DC</td>
<td>dendritic cell</td>
</tr>
<tr>
<td>DFP</td>
<td>diisopropyl fluorophosphate</td>
</tr>
<tr>
<td>DHR</td>
<td>dihydrorhodamine</td>
</tr>
<tr>
<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>eIgG</td>
<td>opsonized sheep erythrocyte</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>F-actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor (receptor for Fc portion of immunoglobulin)</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>fMLP</td>
<td>f-Met-Leu-Phe</td>
</tr>
<tr>
<td>GEF</td>
<td>guanidine nucleotide exchange factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>HBP</td>
<td>heparin-binding protein</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HNP</td>
<td>human neutrophil peptide</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulfate proteoglycan</td>
</tr>
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</table>
IF immunofluorescence
Ig immunoglobulin
IP immunoprecipitation
ITAM immunoreceptor tyrosine-based activation motif
ITAM$_i$ inhibitory ITAM
ITIM immunoreceptor tyrosine-based inhibition motif
LOS lipooligosaccharide
LPS lipopolysaccharide
MAC membrane attack complex
MAPK mitogen-activated protein kinase
MHV mouse hepatitis virus
MOI multiplicity of infection
MPO myeloperoxidase
MPRO mouse promyelocyte
NADPH nicotinamide adenine dinucleotide phosphate
NET neutrophil extracellular trap
Ngo *Neisseria gonorrhoeae*
NK natural killer (cell)
NLR Nod-like receptor
Opa opacity protein
P5 protein 5
PAMP pathogen-associated molecular pattern
PBS phosphate-buffered saline
PFA paraformaldehyde
PGN peptidoglycan
PI-3K phosphatidylinositol 3-kinase
PI(3,4,5)P$_3$ phosphatidylinositol-3,4,5-triphosphate
PI(3)P phosphatidylinositol 3-phosphate
PLC$\gamma$ phospholipase C-gamma
PMA 12-phorbol-13-myristate acetate
PMN polymorphonuclear neutrophil
PRR pattern recognition receptor
PSG pregnancy-specific glycoprotein
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>sLe&lt;sup&gt;x&lt;/sup&gt;</td>
<td>sialylated Lewis X</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane</td>
</tr>
<tr>
<td>Uspa1</td>
<td>ubiquitous surface protein 1</td>
</tr>
<tr>
<td>VAMP-2</td>
<td>vesicle-associated membrane protein 2</td>
</tr>
</tbody>
</table>
CHAPTER 1.
INTRODUCTION
1.1 General Characteristics of the Family Neisseriaceae

Neisserial species are characterized by their unique cellular morphology (diplococci that are attached at a flattened edge), their negative reactivity to the Gram stain, and positive reaction in the oxidase test. These organisms are fastidious, being restricted to their human host, and can grow both aerobically or anaerobically (using nitrite as a terminal electron acceptor) (Rest et al., 1994). Neisserial species typically colonize mucosal surfaces of the respiratory (N. lactamica, N. meningitidis) or the urogenital/anorectal (N. gonorrhoeae) tracts of humans. While the presence of N. gonorrhoeae in the human genital tract is always considered pathogenic, the nasopharynx is typically colonized by commensal species, including family members N. lactamica, N. cinerea, N. flavescens, and N. sicca. Indeed, even the pathogen N. meningitidis (the causative agent of meningococcal meningitis) can asymptptomatically colonize the nasopharynx of healthy individuals (up to 30% of a population at a given time; Caugant et al., 1994). There is little known about what mediates the “switch” from asymptomatic colonization of the nasopharynx to meningococcal disease, that is, infection of the lungs, blood, and meninges.

1.2 Neisseria gonorrhoeae

Neisseria gonorrhoeae (the 'gonococcus') is the causative agent of the sexually transmitted disease gonorrhea, an infection which is typified by a prominent infiltration of immune cells, primarily neutrophils, to the infected site. Infection involves the colonization of the urogenital or anorectal mucosa, and can be followed by ascension to sterile sites such as the urethra (in males, leading to urethritis) or the endocervix (in females, resulting in cervicitis), followed by intracellular passage of the mucosal epithelium. Infections can be transmitted through close sexual contact, as well as from mother to child during childbirth. Complications of gonococcal infection include female infertility as a result of fallopian tube scarring, ectopic pregnancy, and blinding of neonates born to infected mothers. While readily treatable with antibiotics such as penicillin and sulfonamide, infection with N. gonorrhoeae is still prevalent, with an estimated 60 million new cases a year, worldwide (Gerbase et al., 1998). Importantly, infection often comes without symptoms, particularly in females, thus facilitating transmission of N. gonorrhoeae. Furthermore, it has been shown that gonococcal infection increases transmission of other sexually transmitted diseases.
including HIV (human immunodeficiency virus) (Chen et al., 2003). There is no vaccine currently available for *N. gonorrhoeae*.

### 1.3 Colonization of the Host

Because of the restriction of *Neisseria* spp. to their human host, most studies of *N. gonorrhoeae* have been accomplished with the use of immortalized tissue culture cell models. In some cases, however, insight into processes involved during *in vivo* infection have been gained through the use of primary tissues, as well as experimental intraurethral infection of male volunteers. The methods of attachment and invasion of human tissues and some discussion of *in vivo* infection models will be addressed below.

### 1.4 Adhesins of the Pathogenic *Neisseria*

Neisserial species possess a number of adhesins that promote attachment to and invasion of host cells. These include the neisserial pilus, which is thought to be responsible for the initial attachment to host cells, and is absolutely required for *in vivo* infection; neisserial lipooligosaccharide, which can bind receptors on urethral epithelial cells; porins, which allow invasion of epithelial cells and modulate functions of immune cells; and the neisserial Opacity proteins, which allow binding to various cell types, including the mucosal epithelium and immune cells. These will be described in more detail below.

#### 1.4.1 Pilus

In general, clinical isolates from both localized and invasive disease are piliated (Plant and Jonsson, 2003), suggesting an importance for pilus expression during *in vivo* infection. The neisserial pilus is composed of the major pilin subunit PilE. It has been shown to bind to the CD46 protein, which is found on virtually all nucleated cells in humans (Kallstrom et al., 1997), although it has been suggested that other receptors may also exist (Kirchner et al., 2005). The role of the pilus in infection involves the promotion of initial attachment to cells and microcolony formation, while retraction of the pilus is required for invasion into host cells (Merz and So, 2000). The neisserial pilus has also been shown to mediate binding to cervical epithelial cells via one of the cellular receptors for complement, CR3, in a process involving deposition of the serum complement component iC3b and gonococcal porin.
expression (Edwards et al., 2002). Like other gonococcal proteins, including Opa (see below), the neisserial pilus is thought to contribute to evasion of host adaptive immune responses via intragenic recombination of silent pilin gene fragments into the expressed gene, thus generating a mosaic of proteins that are less likely to be recognized by protective antibodies due to constant immunologic selection for new variants (Swanson et al., 1986).

1.4.2 Lipooligosaccharide (LOS)

*Neisseria* spp. do not express typical “endotoxin” or lipopolysaccharide (LPS) in their outer membranes. Instead, these organisms produce lipooligosaccharide (LOS) molecules characterized by a lack of repeating O-antigen side chains. LOS has been shown to interact with the asialoglycoprotein receptor (ASGP-R) in studies with primary male urethral epithelial cells (Harvey et al., 2001). Neisserial LOS moieties can be sialylated in vivo via a gonococcal sialytransferase, leading to serum resistance, potentially through the masking of epitopes that would otherwise be sensed by the innate immune system (van Putten et al., 1995).

1.4.3 Porin

Neisserial porins are the most abundant proteins in the outer membranes of these bacteria. These proteins are present as trimeric integral outer membrane proteins, creating a pore for the exchange of ions between the bacterium and its environment (Massari et al., 2003). Gonococcal strains can express one of two porin types, PorB1A and PorB1B. PorB1A has been shown to allow invasion of host cells (van Putten et al., 1998; Kuhlewein et al., 2006). Neisserial porins have also been shown to inhibit certain functions of neutrophils, including actin polymerization, degranulation, phago-lysosome fusion, and phagocytosis (Bjerknes et al., 1995). Interestingly, gonococcal porins have also been shown to play a role in promoting serum resistance through the binding and sequestration of factor H or C4b-binding protein of the complement system (Massari et al., 2003). Porins are often considered vaccine candidates because of their antigenicity, however their antigenic variability is the basis for serotyping of strains and as such may not constitute a broad enough vaccine target (Hook et al., 1984).
1.4.4 Opacity (Opa) Proteins

Neisserial Opacity (Opa) proteins have been shown to be important adhesins for interactions with human cells. Indeed, the use of Opa as a bacterial adhesin was first noted in human neutrophils, where it was shown that Opa expression made *N. gonorrhoeae* sensitive to phagocytosis and killing by these cells (Virji and Hecke, 1986; Fischer and Rest, 1988). Opa proteins are integral outer membrane proteins, ranging from 30 to 35 kDa in molecular weight, with eight transmembrane-spanning regions resulting in the expression of four extracellular loops. *Neisseria* genomes harbor multiple (separate) opa genes, which can be divided into two groups based on protein binding to either i) heparan sulfate proteoglycan (HSPG)-containing receptors or ii) members of the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family (see below). The expression of neisserial Opa proteins is phase-dependent and can be turned on and off randomly via a frameshifting mechanism involving insertions or deletions of repetitive DNA sequences coding for the leader peptide (Stern et al., 1986). In the MS11 strain of *N. gonorrhoeae*, there are 11 opa genes, thus, in theory, any one bacterium can express from 0-11 Opas, such that in a population there is a tremendous amount of heterogeneity in Opa expression, and therefore in binding to HSPG and CEACAM receptors on human cells. This plays a role in determining tissue tropism for a population of bacteria, as these host receptors are differentially expressed on human tissues. It is important to note that the majority of neisserial isolates collected from patients are Opa positive (Swanson et al., 1988; Jerse et al., 1994), and of these, the vast majority are capable of binding to CEACAM1 (Virji et al., 1996). As noted above for the neisserial pilin genes, there is also a great degree of variability in the sequences of opa genes due to reassortment between extracellular loops, once again allowing for the evasion of host immune responses, and producing difficulty for vaccine production strategies (Aho et al., 1991).

1.5 Interactions with the genital mucosal epithelium

A number of studies involving intraurethral inoculation of male volunteers with *N. gonorrhoeae* have provided insight into the mechanisms and requirements for *in vivo* infection by this organism. Infection of these individuals leads to an inflammatory response and urethritis typical of that observed during a natural infection, however occurring in a more rapid fashion likely due to the dose and route of infection. These seminal studies
showed a predominance of Opa-positive organisms in urine and urethral swab specimens, even when non-opaque (Opa-) organisms were used for the initial inoculation. Furthermore, using genetic mutants of *N. gonorrhoeae* lacking genes for pilus biogenesis or iron acquisition proteins, it was shown that these proteins are critically required for *in vivo* infection (Cohen and Cannon, 1999). Due to the potential for complications during gonococcal infection of females, studies involving *N. gonorrhoeae* and the female genital tract mucosa have involved primary organ cultures from women with naturally acquired gonococcal infections. As with males, there is a predominance of Opa-positive colonies among isolates, except i) during menses (James and Swanson, 1978) and ii) when the infection has reached the fallopian tubes (even if the cervix is populated with Opa-expressing organisms) (Draper *et al.*, 1980). Ultrastructural studies with a human fallopian tube organ culture model demonstrated that attachment of gonococci to fallopian tube mucosa was pilus-dependent, and involved gonococcal adherence to the microvilli of non-ciliated cells (Stephens *et al.*, 1982). Interestingly, while some damage of the epithelium was observed upon infection, gonococci were shown to transverse the epithelium intracellularly. This intracellular passage of the epithelium (i.e. through the cells, not between them) was also noted during primary mucosal cell observation (Ward and Watt, 1972), and more directly, by Wang and others (Wang *et al.*, 1998), who used a polarized T84 epithelial cell model to show that gonococci used to apically infect an intact monolayer could be collected basolaterally without a change in trans-epithelial resistance, and with ultra-structural observation of bacteria within the cells themselves. Upon entry into the sub-epithelial space, gonococci can cause extensive local disease, particularly in females, including salpingitis (with a potential for fallopian tube scarring, presumably from unrestricted neutrophil recruitment and activation) and pelvic inflammatory disease. Alternatively, gonococci can also enter the blood vessels and cause disseminated disease such as arthritis and septicemia.

**1.6 General Characteristics of the Carcinoembryonic Antigen-Related Cellular Adhesion Molecules (CEACAMs)**

The carcinoembryonic antigen (CEA) gene family is divided into two subgroups: the membrane-bound carcinoembryonic antigen-related cellular adhesion molecules
(CEACAMs) and the secreted pregnancy specific glycoproteins (PSGs). In humans, these genes cluster on the long arm of chromosome 19 (Gray-Owen and Blumberg, 2006). While the majority of human CEA family members are human-restricted, one can find homologues of CEACAM1 in other mammals, including Mus musculus (Ceacam1 and the closely-related Ceacam2) and Rattus norvegicus (Ceacam1) (Robbins et al., 1991). In general, the structures of CEACAMs encompass an N-terminal immunoglobulin (Ig) variable-like domain, followed by between zero and six Ig C2-type constant-like domains, all of which show a great amount of glycosylation (up to 50% of the total mass of the protein) (Hammarstrom, 1999). These proteins are bound to the cell membrane by either a glycosylphosphatidylinositol (GPI) moiety or a proteinaceous transmembrane region (Figure 1.1). Of note, three transmembrane-anchored CEACAMs of humans, CEACAMs -1, -3, and -4, harbour amino acid motifs in their cytoplasmic domains that are/can be involved in phosphotyrosine-based signaling cascades: CEACAM1 contains two immunoreceptor tyrosine-based inhibition motifs (ITIMs), while CEACAMs -3 and -4 each contain an immunoreceptor tyrosine-based activation motif (ITAM) (these motifs will be described in more detail later). Of the various CEACAMs of humans, CEACAM1 has the broadest distribution, including stomach, small intestine, colon, pancreas, liver, gall bladder, kidney, urinary bladder, prostate, cervix, endometrium, granulocytes, natural killer cells, T and B lymphocytes, dendritic cells, and monocytes/macrophages (Hammarstrom, 1999).

CEACAM6 is also fairly broadly expressed (including epithelial cells and granulocytes), as is CEACAM5 (colonic epithelium, stomach, cervix, prostate), while CEACAM3 is restricted to granulocytes (Nagel et al., 1993). In these tissues, CEACAMs have been shown to perform a variety of functions, including the promotion of cellular adhesion, the promotion and prevention of tumorigenesis (depending on the tissue and particular CEACAM in question), roles in leukocyte recruitment, and, most importantly in the context of my thesis work, the ability to serve as viral and bacterial receptors on host cells. These functions will be described in greater detail below.

1.6.1 CEACAMs as Adhesion Molecules

A number of CEACAMs have been shown to promote cellular adhesion through trans-homophilic (CEACAMS -1, -5, and -6) and trans-heterophilic (CEACAMs -1, -5, and -6, and CEACAM8) intercellular interactions, which are mediated by the N-terminal Ig-variable like
Figure 1.1. The carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family.
The domain structure and predicted glycosylation pattern of the main isoforms encoded by the CEA gene family are shown, as well as tissues in which the various isoforms are expressed. Four of these CEACAMs, CEACAM1, CEACAM3, CEACAM5, and CEACAM6, have been shown to act as receptors for Opa proteins of *Neisseria*, as well as Protein 5 of *Haemophilus influenzae*, and the Uspa1 protein of *Moraxella catarrhalis*. Binding to these microbial proteins is mediated by amino acid residues in the amino-terminal IgV-like domain. Note that some transmembrane- and cytoplasmic-domain containing CEACAMs contain tyrosine-based amino acid motifs in their cytoplasmic domains: CEACAM1 contains two immunoreceptor tyrosine-based inhibition motifs (ITIMs), while CEACAM3 and CEACAM4 both express an immunoreceptor tyrosine-based activation motif (ITAM). Figure adapted from Gray-Owen and Blumberg, 2006 (Nature Reviews Immunology, 6(6):433-46); used with permission.
domain (Benchimol et al., 1989; Oikawa et al., 1991; Yamanaka et al., 1996). This function could play a role in metastasis through contact-dependent spreading (Hammarstrom, 1999). There is also evidence that CEACAM5 ligation of CEACAM1 contributes to early tumor formation in the colon, through an induction of apoptosis in CEACAM1-expressing cells (Nittka et al., 2008). It is interesting to note that specific expression of CEACAMs in mouse embryos (Obrink, 1997), as well as the intercellular localization of CEACAM5 in the developing intestine (Benchimol et al., 1989), suggests roles for these proteins in the formation of tissue architecture during development, which could be attributed to their adhesion functions.

1.6.2 CEACAMs in Tumors

The name CEA is derived from 'carcinoembryonic antigen', which is the former name for CEACAM5. CEACAM5 is widely used as a tumor marker, as its expression is greatly increased in sera of patients with various tumor types including those of the colon, lung and breast, among others (Hammarstrom, 1999). The adhesive activities and specific GPI anchors of CEACAM5 and CEACAM6 have been shown to be required for their roles as transformation factors (Obrink, 1997). In contrast, CEACAM1 tends to act as a tumor suppressor (Gray-Owen and Blumberg, 2006). CEACAM1 expression is downregulated in number of cancers, including hepatomas, colon, prostate, endometrium, and breast. Studies with Ceacam1−/− mice have shown a propensity for these animals to develop increased tumor burdens in an azoxymethane-induced cancer model (Leung et al., 2006). Interestingly, over-expression of CEACAM1 in human tumor cell lines where CEACAM1 has been downregulated shows a reversal of tumorigenic properties in an ITIM-dependent fashion (Kunath et al., 1995). CEACAM1 has also been shown to play a role in angiogenesis, as evidenced by defects in neovascularization, vascular remodeling and vessel stabilization in Ceacam1−/− mice (Horst et al., 2006), which could contribute to its effects on tumor growth in vivo. Seemingly opposite to this, the expression of CEACAM1 in DU145 prostate cancer cells specifically blocked the growth of endothelial cells, arguing for an inhibition of angiogenesis (and thus a suppressive role) in this model (Volpert et al., 2002). It is known that CEACAM1 can be over-expressed in some cancers such as metastatic melanoma, as
well as during some stages of cancers where it was previously downregulated (Gray-Owen and Blumberg, 2006). Interestingly, it has also been shown that homophilic interactions between CEACAM1 molecules as well as heterophilic interactions between CEACAM1 and CEACAM5 inhibit the capacity of natural killer cells to kill tumor cells (Markel et al., 2004) (Stern et al., 2005). Thus, CEACAM1 expression by a tumor cell reduces its capacity to grow yet protects it from cell-mediated immunity.

1.6.3 CEACAM1 interactions with the Insulin Receptor
CEACAM1 has been shown to be involved in the receptor-mediated endocytosis and degradation of insulin (Najjar, 2002). This process involves phosphorylation by the insulin receptor tyrosine kinase on Ser503 and Tyr488 in the cytoplasmic domain of CEACAM1 (Najjar et al., 1995), and recruitment of the tyrosine phosphatase SHP-1 (Dubois et al., 2006). Importantly, the expression of a liver-specific dominant-negative allele of Ceacam1 (S503A) in a transgenic mouse model resulted in impaired insulin clearance and hyperinsulemia, confirming a role for this protein in insulin receptor function in vivo (Poy et al., 2002).

1.6.4 CEACAMs and Leukocyte Recruitment
CEACAMs are considered leukocyte activation antigens due to their granular localization in neutrophils. Studies of neutrophil granules have shown that CEACAM1 and CEACAM8 (also known as CD67) are found in specific granules, while CEACAM6 is found in primary granules (the significance of the various neutrophil granule subtypes will be described later) (Ducker and Skubitz, 1992; Kuroki et al., 1995). The granular localization of CEACAM3 has not been well studied, but it is thought that this protein is present on the neutrophil surface in resting cells. In human neutrophils, CEACAMs are heavily glycosylated and sialylated. Stimulation of neutrophils causes granular exocytosis, bringing these proteins to the neutrophil surface and allowing their binding to activated endothelia expressing E-selectin, which serves as a receptor for the sialylated Lewis-X (sLe^X) motifs on CEACAM molecules (Kuijpers et al., 1992). Furthermore, sLe^X has been shown to mediate the binding of both CEACAM1 and the CD11b integrin (which also expresses sLe^X) to DC-SIGN, a marker of
immature dendritic cells (van Gisbergen et al., 2005). This interaction may play a role in a neutrophil-stimulated maturation of these cells at the site of infection.

1.6.5 CEACAMs as Microbial Receptors

In the mouse, Ceacam1 is used by the coronavirus, MHV (Mouse Hepatitis Virus) for entry into host cells (Dveksler et al., 1993; Nedellec et al., 1994). In humans, CEACAMs promote the binding and/or entry of various bacterial species into host tissues (Figure 1.2). Certain strains of Escherichia coli and some Salmonella spp. can bind to CEACAMs -1, -5, and -6 through interactions of their type 1 fimbriae with mannose-containing carbohydrate chains on CEACAMs (Leusch et al., 1991; Sauter et al., 1993), while diffusely adhering E. coli strains instead bind CEACAMs via protein-protein interactions mediated by the Afa/Dr family of adhesins (Guignot et al., 2000). It is also known that CEACAMs-1, -3, -5, and -6 (and not CEACAMs -4, -7, and -8) (Virji et al., 1996; Gray-Owen et al., 1997a; Schmitter et al., 2007b) mediate the binding and cellular entry of a number of human-specific bacterial pathogens through protein-protein interactions. Specifically, Opa proteins of N. meningitidis and N. gonorrhoeae have been shown to interact with the unglycosylated CFG-face of the N-terminal domain of these CEACAMs (Virji et al., 1999). Interestingly, Haemophilus influenzae and Moraxella catarrhalis also use CEACAMs as host receptors using completely unrelated bacteria adhesins, namely the outer membrane protein 5 (P5) (Hill et al., 2001) and ubiquitous surface protein A1 (UspA1) (Hill and Virji, 2003), respectively. When considering the differential expression of CEACAMs on various host tissues (see above), this interaction can affect the tropism of these various pathogens for a number of cell types. Also, bacterial infection of a number of cell types, including epithelial and endothelial cells, elicits the NF-κB-dependent increase of CEACAMs on the cell surface, which further promotes colonization, thus creating a positive feedback loop (Muenzner et al., 2001; Muenzner et al., 2000).

1.6.6 The Role of CEACAM1 in Immune Cell Function

CEACAM1 is expressed on a number of hematopoietic cells including neutrophils, dendritic cells, monocytes/macrophages, and lymphocytes. A number of the effects that have been documented for CEACAM1 in these various cell types are a result of the tyrosine-based
Figure 1.2. **CEACAM receptor binding by the pathogenic *Neisseria***.

Initial adherence (a) to epithelial cells is mediated by interactions between the neisserial type IV pilus and receptors on host cells. This is followed by tight adherence (b), mediated by neisserial Opa proteins that bind CEACAMs on epithelia. This interaction results in the transcytosis (c) of the epithelial monolayer, allowing the bacteria to enter the subepithelial space. The transversal of the epithelium can result in a number of outcomes, including sepsis (d), as well as interactions with immune cells including dendritic cells (DCs) (e), lymphocytes (f), and polymorphonuclear neutrophils (g), where CEACAM family members are differentially expressed. Figure adapted from Gray-Owen and Blumberg, 2006 (Nature Reviews Immunology, 6(6):433-46); used with permission.
amino acid motifs in its cytoplasmic domain. In T lymphocytes, it was shown that ligation of CEACAM1 by the Opa proteins of Neisseria resulted in the inhibition of T cell activation and proliferation (Boulton and Gray-Owen, 2002). Intriguingly, the same effect is also seen with outer membrane ‘blebs’ shed naturally from the surface of Neisseria, and thus can occur distant from sites in which whole bacteria are present (Lee et al., 2007). Further study of the molecular mechanisms involved in this inhibitory process have revealed that the ITIM-dependent recruitment of protein phosphatases to CEACAM1 serves to desphosphorylate the CD3ζ chain of the activated T cell receptor (TCR), and presumably lead to a further arrest of downstream components of TCR signaling (Lee et al., 2008; Chen et al., 2008). Curiously, other groups argue that CEACAM1 phosphorylation serves to promote TCR signaling (Kammerer et al., 1998), which may reflect different physiological outcomes based on intensities of signal induction, physical characteristics of ligand, or cell state. Consistent with this, ligation of CEACAM1 on human monocytes (Yu et al., 2006) and B lymphocytes (So and Gray-Owen, unpublished) promotes survival of these immune cells, and CEACAM1 ligation results in a positive stimulation of B cell receptor-induced proliferation of murine B cells (Greicius et al., 2003).

1.6.7 The Specific Role of CEACAM3 as a Host Receptor for N. gonorrhoeae

While the number of physiological functions attributed to the CEA family is vast, one human-restricted CEACAM, CEACAM3, does not appear to perform any of these functions. Instead, it only acts as a receptor for the Gram-negative human-restricted pathogens N. gonorrhoeae, N. meningitidis, M. catarrhalis, and H. influenzae (Schmitter et al., 2004). This peculiar observation has been explained by ourselves and others as a function of co-evolution between pathogen and host. Because CEACAMs -1, -5, and -6 are useful for colonization as well as immune evasion, and thus contribute greatly to the pathogenicity of these organisms, while interactions with CEACAM3 are generally deleterious to the bound bacterium (via its destruction by the neutrophil killing responses), we have speculated that CEACAM3 represents a ‘molecular mimic’ that serves to neutralize CEACAM-utilizing bacterial pathogens. In support of this argument, a study conducted in our laboratory demonstrated that while the majority of clinical isolates obtained from persons infected with N. gonorrhoeae were capable of binding to CEACAMs -1, -5 and -6, only a small percentage of strains had the ability to bind CEACAM3, suggesting that the expression of
Opa proteins capable of binding to this receptor promotes clearance in vivo (Wong and Gray-Owen, unpublished). The mechanism of CEACAM3-mediated functions in response to N. gonorrhoeae depends greatly on the presence of its cytoplasmic ITAM. This will be elaborated upon in the final section of this chapter.

1.7 The Innate and Adaptive Immune Responses to Infection

The immune response to infection is often described as a dichotomy between the early ('innate') and progressive ('adaptive') responses. In 1989, Charles Janeway described a specific arsenal of 'pattern recognition receptors' (PRRs) that served to recognize non-self general antigens of pathogenic organisms (pathogen-associated molecular patterns; PAMPs), such as bacterial peptidoglycan (PGN) and lipopolysaccharide (LPS), as well as foreign nucleic acids (e.g. bacterial CpG DNA and viral single and double stranded RNA). Because PRRs are germline-encoded and expressed on a number of cells of the innate immune system, such as neutrophils, macrophages and natural killer cells, they are available to rapidly respond to an infection. This concept was in clear contrast to the adaptive immune response, which was known to involve the random assembly of genomic DNA and somatic hypermutation events that resulted in the enormous diversity of T cell and B cell receptor specificities. This allows the recognition of virtually any antigen; however, due to the small number of antigen-specific cells present at any given time, adaptive immunity requires days for the clonal proliferation of the 'right' cells to create a population of cells that are capable of fighting the infection. The adaptive immune response is also responsible for the production of memory, such that future infections with the same pathogens can be rapidly cleared. These days it is clear that these two separate immune responses are not as distinct as once believed, as components of each feed into the other and guide courses of action.

1.8 Receptors of the Innate Immune System

The innate immune system is comprised of a number of receptors responsible for sensing pathogens. The classic PRRs include members of the Toll-like receptor (TLR) and Nod-like receptor (NLR) families. These receptors recognize a variety of intracellular or extracellular bacterial products, which when ligated couple their activation to NF-κB activation (via TIR-
based MyD88-dependent and –independent pathways in TLRs) or NF-κB and MAPK activation (via RIP2 and CARD9, respectively, in the NLRs NOD1 and NOD2) (Ishii et al., 2008). A number of receptors which can still be considered ‘innate’ but are more specific (i.e. not recognizing patterns per se) include members of the C-type lectin family (such as the beta-glucan receptor Dectin-1), receptors for complement (the integrins CR1 and CR3), and the receptors for the Fc portion of immunoglobulin (FcRs). As discussed above, we believe that CEACAM3 also falls into this category. The receptors of the innate system act to initiate immune responses by acting as sensors (TLRs, NLRs), or through the binding and engulfment of pathogens via phagocytes such as macrophages and neutrophils (CRs, FcRs, Dectin-1). Some receptors perform both of these functions (for instance, certain Fcγ receptors and Dectin-1 are both phagocytic and initiate transcription of genes for inflammatory mediators) (Song et al., 2002; Fernandez et al., 2002). Furthermore, a number of these receptors have been shown to associate and synergize functionally (Hu et al., 2007).

1.9 Opsonization: The Role of Complement and the Fc Receptors

In contrast to specific ligand-receptor interactions in which a given receptor recognizes a microbial product, the mammalian immune system also makes use of opsonins, which can coat a foreign particle, thus providing the ligand (the opsonin itself) that can then allow binding to host receptors. Opsonins include complement proteins (which are recognized by complement receptors) and soluble immunoglobulin (which are recognized by the Fc receptors). The complement cascade can be initiated by pathogen binding by IgG, IgM, or mannose-binding lectin, or via the constitutive alternative pathway, both of which result in the assembly of a C3 convertase on the microbial surface that acts to cleave the C3 protein to form the C3b opsonin, which coats the cell and allows recognition by the type 1 and type 3 phagocytic complement receptors, CR1 (CD35) and CR3 (CD11b/CD18) (Tosi, 2005). Activation of the C5 convertase then initiates a pathway that culminates in the assembly of a membrane attack complex (MAC) on target cells, allowing killing via the lysis of target membranes. Receptors for the Fc portion of IgG (the Fcγ receptors) facilitate the binding and phagocytosis of IgG-opsonized particles, resulting in the activation of microbicidal functions of antigen presenting cells such as macrophages and neutrophils. Human neutrophils express two phagocytic Fcγ receptors, FcγRIIA (CD32; a single polypeptide
containing an ITAM) and FcγRIII (CD16; which associates with the ITAM-containing Fcγ chain), as well as the non-phagocytic ITIM-containing FcγRIIB (Cassel et al., 1993). Interestingly, synergism between the two complement receptors CR1 and CR3 has been noted to occur with Fcγ receptors (Jongstra-Bilen et al., 2003).

1.10 The Players of the Innate System: Roles of Specific Cell Types

1.10.1 Epithelia

Epithelial cells can be considered cells of the innate immune system due to their barrier function, their ability to produce and secrete anti-microbial compounds such as β-defensins and cathelicidin (LL-37), and their capacity to sense pathogens (e.g. via TLRs and NLRs) (Fritz et al., 2008). Activation of these as well as other key pattern recognition receptors leads to NF-κB –dependent and –independent cytokine and chemokine production and release, including IL-6, 1L-8, MCP-1, MIP-1β, TNF-α, GM-CSF and G-CSF, which serve to recruit neutrophils, monocytes, and other immune cells to infection sites (Wira et al., 2005). Also involved in the response at the epithelial barrier are mucosal 'innate' B lymphocytes, as well as sampling by tissue-resident dendritic cells (Iwasaki, 2007).

1.10.2 The Mucosal Epithelium of the Genito-urinary Tract

Because *N. gonorrhoeae* infects its human host via the genital tract mucosa, some discussion of this epithelium is of interest here. In females, the vaginal and ectocervical epithelium consists of stratified squamous epithelial cells, while the endocervix, endometrium and fallopian tubes are lined by columnar epithelial cells overlying basal stromal fibroblasts (Wira et al., 2005), typical of type II and type I mucosal surfaces, respectively (Iwasaki, 2007). Cells of the female reproductive tract have been shown to express TLRs 1-6 and MD2 (Darville et al., 2003; Pioli, 2004), although the presence of the LPS-responsive molecules TLR4 and MD2 has been debated in the vaginal and ectocervical mucosa (Fichorova et al., 2002). This may suggest the involvement of intracellular sensors of infection such as members of the NLR family, and may reflect a general rule of intracellular PRRs in non-sterile sites (Philpott and Girardin, 2004). Indeed, Fichorova et
al. (2002) noted NF-κB activation and IL-8 production in response to gonococcal infection of reproductive tissues, even in the absence of TLR4. Importantly, in vivo studies of both male volunteers and the analysis of isolates from women with gonococcal infection have shown the production of neutrophil-recruiting chemokines (such as IL-8 and IL-6), and a correlation between the amounts of these chemokines with the number of infiltrating neutrophils. In light of this, it is interesting to note that recently a new T cell subtype has been identified, known as Th17, which can be produced from naïve CD4+ T cells via IL-6 and TGF-β stimulation (Nurieva et al., 2008; Zhou et al., 2007). These cells produce the neutrophil chemoattractant IL-17 upon activation. It would be of interest to determine the role of these cells in the production of IL-17 in diseases involving prominent neutrophil infiltration such as gonorrhea.

1.10.3 Endothelial Cell Activation and the Leukocyte Recruitment Cascade

One of the first cell types at an infection site is the polymorphonuclear neutrophil (PMN), which can be distinguished from other leukocytes by its characteristic segmented nuclear morphology and possession of multiple intracellular granule subtypes. PMNs are recruited to infection sites from the blood through a process termed chemotaxis, in which the cells respond to “danger” cues produced by infected epithelial cells, such as a chemotactic gradient of chemokines or other inflammatory mediators (Figure 1.3). A number of chemokines have been shown to recruit neutrophils to infected tissues, including IL-8 (or KC and MIP-2 in the mouse), TNF-α, IL-6, GM-CSF, and TGF-β, among others (Pober and Sessa, 2007). In response to an infection in the tissues, endothelial cells lining the postcapillary venules can become activated, and begin to express proteins such as E-selectin and P-selectin, which can bind a number of glycosylated proteins on PMNs including L-selectin, PSGL-1, as well as sialylated Lewis X-containing proteins including CEACAMs (discussed above) (Ley et al., 2007). This causes PMNs in the blood to slow and loosely interact with activated endothelial cells, in a process called ‘rolling’. This is then followed by a firm adhesion (‘arrest’), promoted by newly-expressed β-integrins LFA-1 and Mac-1 (CD11b/CD18) on the neutrophil surface, in response to stimulation by HSPG-presented chemokines on endothelial cells. These β-integrins can bind to ICAM-1 on activated endothelia. Arrest is followed by transmigration (intercellular passage of endothelium), which involves interactions with junction proteins such as PECAM1 and CD99, as well as
Figure 1.3. The leukocyte recruitment cascade.
Leukocytes are recruited into the tissues from the blood through a process that begins with the rolling of the cells on endothelia, mediated by interactions between L-selectin on PMNs, which interacts with P- and E-selectin on endothelial cells. Once PMNs begin rolling, they become further activated and start to express β-integrins on their surface, which can bind endothelial ICAM-1, resulting in firm adherence, or arrest. PMNs can then transmigrate between endothelial cells, or transcellularly, and gain access into the tissues, where they migrate, or chemotax, towards infected sites. Figure adapted from Ley et al, 2007 (Nature Reviews Immunology, 7(9):678-689); with permission.
PMN release of degradative enzymes to allow passage of the basement membrane, such as gelatinase (Delclaux et al., 1996). There is also evidence of direct transcellular passage of the epithelium (Ley et al., 2007). As the infection continues, endothelial cells begin to express monocyte-recruiting markers on their surfaces including VCAM1 and ICAM-1, thus initiating another leg of the immune response and resolving the early PMN infiltration into the tissues (Munro et al., 1989).

### 1.10.4 Neutrophils at the Site of Infection

At infection sites, neutrophils play important antimicrobial roles through both oxidative and non-oxidative killing mechanisms. These include the oxidative burst response, which involves an increased consumption of molecular oxygen that is converted to superoxide anion (O$_2^-$) via the activation-induced assembly of the multimeric NADPH oxidase complex (Figure 1.4). Superoxide anion can then be converted to other reactive oxygen intermediates through the action of the enzymes superoxide dismutase (SOD), which converts O$_2^-$ to H$_2$O$_2$, and myeloperoxidase (MPO), which converts H$_2$O$_2$ to the highly bactericidal HOCl. Neutrophils also contain multiple cytoplasmic granules (the primary, or azurophilic granules; the secondary, or specific granules; the tertiary, or gelatinase granules; and the secretory granules) that contain preformed proteins ready to be released upon cellular insult, both extracellularly (in response to an extracellular pathogen) or intracellularly into a formed phagosome (in response to an internalized pathogen) (Figure 1.5). This process of granule release is called 'degranulation', and generally follows a hierarchy, in which certain granules are released more readily than others, in the order (from easiest to degranulate, to most difficult): secretory>gelatinase>specific>azurophilic. This hierarchy has been explained by the density of vesicle-associated membrane protein (VAMP-2) in the granule membrane, which is greatest in the secretory granules and least in the azurophilic granules (Brumell et al., 1995). Azurophilic granules are thought to harbor the majority of the antimicrobial products of PMNs, including lysozyme, the α-defensins, cathelicidin (LL-37), and bactericidal permeability-increasing proteins (BPIs) (Brinkmann and Zychlinsky, 2007). Secretory, gelatinase and specific granules are known to be stores of adhesion molecules (including CD11b/CD18, chemokine receptors and some CEACAMs) (Lominadze et al., 2005). After activation, neutrophils typically undergo apoptosis, and are
Figure 1.4. Production of reactive oxygen species by the neutrophil oxidative burst.

Assembly of the multi-molecular NADPH oxidase on the cellular or phagosomal membrane following cellular activation allows the transfer of an electron from NADPH to molecular oxygen, resulting in the production of superoxide anion (O$_2^-$). This can be further reduced to other reactive oxygen species by the action of enzymes, such as superoxide dismutase (SOD), which catalyzes the production of hydrogen peroxide (H$_2$O$_2$) from O$_2^-$, and myeloperoxidase (MPO), a primary granule protein that catalyzes the production of the highly bactericidal hypochlorous acid (HOCl) from H$_2$O$_2$. Figure adapted from Dahlgren and Karlsson, 1999 (Journal of Immunological Methods, 232, 3-14), with permission.
**Figure 1.5. Neutrophil granulopoiesis and characterization of granule subsets.**

Production of neutrophil granules and granule proteins is intimately related to the given stage of maturation of neutrophil precursors. Abbreviations: MB, myeloblast; PM, promyelocyte; MC, myelocyte; MM, metamyelocyte; BC, band cell; PMN, mature segmented neutrophil. Azurophilic granules are produced first, followed by specific granules, gelatinase granules, and secretory vesicles. Some key characteristic granule proteins are also shown. It has been suggested that sorting of proteins into granules reflects the 'time' (i.e. during the neutrophil differentiation program) that a given protein is produced (the 'targeting-by-timing' hypothesis). Figure adapted from Faurschou and Borregaard, 2003 (Microbes and Infection, 5, 1317-1327), with permission.
removed by macrophages, in an effort to resolve inflammation to prevent continued tissue
damage (Serhan and Savill, 2005). It is thought that neutrophil death is also responsible for
the production of NETs, or neutrophil extracellular traps, which are essentially nuclear
material released by PMNs, complexed with granule proteins, that serve to capture and kill
extracellular microbes (Brinkmann and Zychlinsky, 2007).

1.10.5 Neutrophils and the Recruitment of Effector Cells
It has long been appreciated that certain PMN granule proteins have chemotactic ability,
including LL-37, cathepsin G, human neutrophil peptides (HNPs) 1-3, and heparin-binding
protein (HBP) (Agerberth et al., 2000; Chertov et al., 1997; Territo et al., 1989). It has
recently begun to be accepted that neutrophils can produce and release a variety of
chemokines as well, including IL-8, GRO-α/β and certain PMN-recruiting CXC chemokines,
which serve to create a positive feedback loop in response to infection. PMNs have also
been shown to release chemokines serving to recruit Th1 lymphocytes (IP-10, MIG, I-TAC)
and other cell types that contribute to the intiation of adaptive immunity, such as monocytes
and immature dendritic cells (DCs) (Scapini et al., 2000) (Table 1.1).

1.10.6 APCs and the Adaptive Immune Response
The adaptive immune response to infection is initiated either directly in the infected tissue
via secondary lymphoid structures (Iwasaki, 2007), or via draining lymph in the secondary
lymphoid organs (Junt et al., 2008). In both of these settings, antigen presenting cells
(APCs; macrophages and dendritic cells) can capture pathogens and present digested
peptides on their surface to immature lymphocytes (B cells and T cells), which can recognize
the MHC:peptide complex via their respective receptors (B cell receptor (BCR) or T cell
receptor (TCR)). Lymphocytes that are stimulated via this interaction as well as a second
'co-stimulatory' signal (provided by DCs, macrophages, or B cells in the case of T-
lymphocytes; or provided by the T cell for the B lymphocyte) can then proliferate, resulting
in the production of specific antibodies (via B cells), the production of inflammatory
mediators (via T helper cells), or the capacity for direct killing of infected cells (via cytotoxic
T cells) (Medzhitov, 2007). This process also allows for the creation of memory cells, which
<table>
<thead>
<tr>
<th>Chemokine class/Receptor bound</th>
<th>Target cells</th>
<th>Chemokine</th>
<th>Stimulus shown to result in production</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-X-C (ELR)/CXCR1,2</td>
<td>neutrophils</td>
<td>IL-8/MIP-2/KC</td>
<td>LPS, TNF-α, fMLP, GM-CSF, eIgG</td>
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<td></td>
<td></td>
<td>GRO-α</td>
<td>LPS, TNF-α, fMLP, eIgG</td>
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<td></td>
<td></td>
<td>GRO-β</td>
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<tr>
<td>C-X-C (non-ELR)/CXCR3</td>
<td>Th1 lymphocytes</td>
<td>IP-10</td>
<td>IFN-γ+LPS, IFN-γ+TNF-α</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MIG</td>
<td>IFN-γ+LPS, IFN-γ+TNF-α</td>
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<tr>
<td></td>
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<td>I-TAC</td>
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<tr>
<td>C-C/CCR1,5,8</td>
<td>eosinophils, basophils, monocytes, immature DCs, NK cells, T lymphocytes</td>
<td>MIP-1α</td>
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<td>MIP-1β</td>
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**Table 1.1. Chemokine production by neutrophils.**

Neutrophils produce chemokines of two classes, C-X-C or C-C (classification based on the number of amino acid residues between the first two cysteines in their peptide sequence). C-X-C chemokines are further classified based on the presence of a tripeptide motif, glutamic acid-leucine-arginine (ELR), in the amino-terminus. Due to cell-specific expression of the various chemokine receptors, the target cell population is different for various chemokine types. Also shown are stimuli that have been shown to stimulate the production of indicated chemokines by neutrophils (eIgG indicates an IgG-opsonized particle). Adapted from Scapini et al., 2000 (Immunological Reviews, 177; 195-203); with permission.
provide protection from subsequent re-infections by the same pathogen. APCs can also
direct the immune response by the profile of cytokines that they produce; for instance,
dendritic cells can stimulate Th1 (IL-12-dependent) or Th2 (IL-10-dependent) responses
(Pulendran et al., 2008). While PMNs are phagocytic, they are not generally considered
antigen presenting cells because they do not present peptides to immature lymphocytes. It
has been suggested, however, that macrophages that have ingested apoptotic PMNs can
somehow present peptides digested by the PMNs themselves. Interestingly, neutrophils
have been shown to express both MHC class II protein and co-stimulatory molecules
(Sandilands et al., 2003; Radsak et al., 2000; Fanger et al., 1997), and a percentage of
human PMNs were shown to express fully rearranged TCRα and TCRβ chains (Puellmann et
al., 2006). While the physiological relevance of these data is still unclear, both these
examples do show how immunological techniques such as staining for lineage-restricted
receptors have significantly increased our understanding of the complexity of the immune
cell repertoire, not just in PMNs but in the plethora of subtypes of other immune cells that
continue to be discovered. The discovery of 'adaptive' immune receptors on 'innate'
immune cells and vice versa is just another example of how these two 'arms' of the immune
system are becoming more and more inter-dependent, and also argues for more in vivo
approaches to the study of infection as 'pure' cell populations of one type are obviously not
always the case in nature.

1.11 Tyrosine-Based Signals in Receptors of the Immune System

The coupling of extracellular signals to cellular responses in the immune system often
involves molecular signaling cascades based on phosphorylation on serine, threonine, and
tyrosine amino acid residues. Two common but opposing tyrosine-based amino acid motifs
found in immunoreceptors are the immunoreceptor tyrosine-based activation motif (ITAM;
YxxL/Ix₆₋₈YxxL/I) (Reth, 1989) and the immunoreceptor tyrosine-based inhibition motif
(ITIM; V/L/Iₓ₆₋₈YxxL/V) (Daeron et al., 1995). ITAMs can be found singly or in multiples in
a number of diverse proteins, including those of the immunoglobulin superfamily (T cell
receptor, B cell receptor, FcRγ chain) and the C-type lectin family (Dectin-1, Mincle) (Table
1.2). Furthermore, a number of proteins lacking an ITAM, including integrins, PSGL-1, the
chemokine receptor CXCR4, and plexins, can undergo ITAM-dependent signaling events
<table>
<thead>
<tr>
<th>Protein</th>
<th>ITAM Sequence</th>
<th>Expression</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAP12</td>
<td>YQELQGQRSDVYSDL</td>
<td>Myeloid and NK cells</td>
<td>Couples signals to NKG2D-S, KIR-2D, Ly49, TREM1,2,3</td>
</tr>
<tr>
<td>FeRγ</td>
<td>YTGLSTRNQETYETL</td>
<td>Broad hematopoietic</td>
<td>Couples signals to FcεR, FcαR, FcγR, OSCAR, PIR-A, Dectin-2, GPVI, TCR</td>
</tr>
<tr>
<td>Igα, Igβ</td>
<td>YEGLNLDDCSMYEDI</td>
<td>B cells</td>
<td>Antigen recognition, B cell activation</td>
</tr>
<tr>
<td>CD3ζ</td>
<td>YQGLSTATKDTYDAL</td>
<td>T cells</td>
<td>MHC-antigen binding, T cell activation</td>
</tr>
<tr>
<td>Moesin</td>
<td>YLKIAQDLEMYGVNYFSI</td>
<td>Broad (hematopoietic, epithelial, endothelial)</td>
<td>Actin-binding, couples to PSGL-1, receptor for P-selectin</td>
</tr>
<tr>
<td>Dectin-1</td>
<td>YHPDLENLDEGDYTQL</td>
<td>Myeloid cells</td>
<td>Fungal recognition, phagocytosis, inflammatory responses</td>
</tr>
<tr>
<td>FcγRIIA</td>
<td>YMTLNPRAPTDKNIYTLT</td>
<td>Myeloid cells</td>
<td>Antibody recognition, inflammatory responses, phagocytosis, degranulation</td>
</tr>
<tr>
<td>CEACAM3</td>
<td>YEELKHDTNIYCRM</td>
<td>Neutrophils</td>
<td>Bacterial recognition, inflammatory responses, phagocytosis, degranulation</td>
</tr>
</tbody>
</table>

**Table 1.2. ITAM-containing proteins used for signaling in cells.**

Shown are examples of ITAM-containing proteins, the amino acid sequence of their ITAM, and the cell types in which they are expressed. Also shown are cellular functions that are initiated upon ITAM phosphorylation via activation by the respective ligand. Adapted from Abram and Lowell, 2007 (Science Signaling, 377:re2), with permission.
through associations with ITAM-containing adaptor proteins adaptor (such as the Fc receptor γ-chain (FcRγ) or DNAX-activating protein of 12 kDa (DAP12) and DNAX-activating protein of 10 kDa (DAP10)) (Abram and Lowell, 2007). Ligation of ITAM-containing receptors or a receptor coupling with an ITAM-containing adaptor results in the initiation of cellular signaling events. These include cellular activation and proliferation (e.g. BCR and TCR ligation in lymphocytes), phagocytosis and release of inflammatory mediators (e.g. FcγR or Dectin-1 ligation in macrophages and neutrophils), as well as cell migration and adhesion phenotypes (e.g. integrin signaling in myeloid cells). The beauty of this system involves the ability of the ITAM to link the specific ligand-binding functions of receptors with no intrinsic signaling capacity to a conserved pathway of downstream signaling and cellular activation. The paradigm of ITAM signaling is described below.

1.12 Model of ITAM Signaling

The activation of an ITAM-containing receptor begins with the aggregation of multiple receptors through binding of multivalent ligand (also, see inhibitory ITAMs, below). This signals for the phosphorylation of the ITAM tyrosines by Src family kinases. The doubly phosphorylated ITAM can then act as a docking site for the paired SH2 domains of the Syk family of kinases (Syk in myeloid cells and B lymphocytes; ZAP-70 in T lymphocytes) (Indik et al., 1995; Cooney et al., 2001). This docking results in Syk phosphorylation, which is a starting point for a number of downstream effector pathways (Figure 1.6). One such pathway involves the activation of the guanidine nucleotide exchange factor (GEF), Vav, which is responsible for the GTP-loading and activation of the Rho family GTPases, Rac and Cdc42 (Bustelo, 2000). These proteins interact to promote actin remodeling and thus phagocytosis of a captured particle, such as a bacterial pathogen. Syk activation also results in the activation of the enzyme phospholipase C-γ (PLCγ), which is responsible for creating an increase in intracellular calcium levels, and thus the promotion of degranulation. A number of other molecules have also been shown to mediate signaling downstream of ITAMs, including SLP76, which is involved in PMN activation downstream of FcγR receptors (Koretzky et al., 2006). SLP76 is an SH2-domain containing adaptor protein that serves to bind Vav, Nck and Itk via its N-terminal tyrosines, as well PLCγ and Gads via its proline-rich
Figure 1.6. Paradigm of ITAM signaling.

(A) Signaling of diverse ITAM-containing proteins converges on the recruitment and activation of Syk family kinases. Adapted with permission from Abram and Lowell, 2007 (Science Signaling, 377:re2). (B) Signaling downstream of Syk. Syk activation results in the initiation of multiple downstream signaling pathways, including the activation of transcription factors, F-actin rearrangements, and phospholipid and calcium signaling. Adapted with permission from Nimmerjahn and Ravetch, 2008 (Nature Reviews Immunology, 8, 34-47).
domain (Bezman and Koretzky, 2007). Other pathways downstream of Syk activation include those that result in the stimulation of transcription, via the MAPK pathway (through the activation of Erk, Jnk, and p38), or NF-κB, resulting in the production of inflammatory mediators such as chemokines and cytokines (Vines et al., 2001).

1.13 ITIM Signaling: Shutting off ITAM Responses

In contrast to ITAM-mediated activation signals, a number of receptors contain cytoplasmic ITIMs, which elicit inhibitory signals. Examples of ITIM-containing receptors include CEACAM1, FcγRIIB, and a number of C-type lectins including Ly49 family members and NKG2A/B (Daeron et al., 2008). An interesting point about these proteins is that they often have ITAM-containing counterparts, for instance FcγRIIB and FcγRIIA, thus the same ligand can activate both receptors. Conversely, ITIM-containing proteins can inhibit signals from unrelated proteins (such as CEACAM1 and the CD3ζ chain of the TCR—see above). ITIM inhibitory signaling is the result of SH2 domain-containing phosphatase recruitment to ITIM phosphotyrosines. These phosphatases (including the tyrosine phosphatases SHP-1 and SHP-2, and the lipid phosphatases SHIP-1 and SHIP-2) desphosphorylate ITAMs and downstream signaling molecules, thus inhibiting activation cascades initiated by ITAM-containing receptors (Barrow and Trowsdale, 2006). In this regard, ITIM-containing receptors are often considered regulators of immune responses.

1.14 Signaling Downstream of the CEACAM3 ITAM

As described above, the CEA family receptor CEACAM3 contains an ITAM in its cytoplasmic domain. Over the past decade, a great deal of research has served to elucidate signaling events that occur upon CEACAM3 binding and activation by neisserial Opa proteins. Because of the restriction of CEACAM3 to the PMN, a cell type that is difficult to genetically manipulate, and which also co-expresses the Opa-binding CEACAM1 and CEACAM6, the majority of these studies have been conducted using transfected epithelial cell models. These studies have shown that bacterial binding to CEACAM3 results in the F-actin-dependent engulfment of bound bacteria (Billker et al., 2002), coincident with the deposition of the signaling lipid PIP₃ at the plasma membrane (Booth et al., 2003). These
structures are similar to those seen during FcγR-mediated phagocytosis, previously shown to involve two members of the Rho family of GTPases, Rac and Cdc42 (Caron and Hall, 1998). Studies using dominant-negative forms of Rac and Cdc42 have shown that these proteins are also involved in CEACAM3-mediated phagocytosis (Billker et al., 2002). CEACAM3 activation has been suggested to result in the GTP-loading of Rac1, through binding of the guanidine nucleotide exchange factor Vav directly to the CEACAM3 ITAM via its SH2 domain (Schmitter et al., 2007a; Schmitter et al., 2004). The SH2 domain of PLCγ has also been shown to co-localize with CEACAM3-bound gonococci in transfected epithelial cells (McCaw et al., 2003). An interesting point regarding the docking of these SH2 domain-containing proteins directly to CEACAM3 involves the capacity of the cytoplasmic tyrosine kinase Syk to activate these proteins downstream of its own binding to a phosphorylated ITAM. Thus, it would be interesting to determine if both Syk-dependent and –independent processes contribute to the activation of Vav and PLCγ in the neutrophil. Bacterial engulfment by CEACAM3 is partly dependent on the phosphorylation of CEACAM3’s ITAM in a Src-family kinase-dependent fashion, although phosphorylation-independent uptake is also seen in the transfected epithelial cell model (McCaw et al., 2003). In transfected epithelial cells, the internalized bacteria are enclosed in a membrane-bound phagosome, which gradually acidifies and acquires markers of late endosomes/early endosomes (e.g. LAMP-1), as well as an accumulation of PI3P (Booth et al., 2003).

1.15 ITAM Signaling: Beyond the Paradigm

1.15.1 Transmembrane-Based Associations

One characteristic of certain ITAM-containing proteins is their ability to associate with transmembrane (TM) regions of other proteins via charged amino acid residues. For instance, a conserved arginine residue in the TM region of NKG2D serves to permit the association of this receptor with aspartic acid residues found in the TM domain of DAP10 (Garrity et al., 2005), while all receptors that associate with FcRγ do so via a critical TM arginine residue (Feng et al., 2006). Similarly, the TCRα chain associates with CD3δε via a critical lysine, while all receptors known to assemble with DAP12 do so via TM lysine or aspartic acid residues (Call and Wucherpfennig, 2007). The TM regions of CEACAM1 and
CEACAM3 contain a number of amino acid residues that could allow binding to TM regions of other receptors, which is an interesting point considering a previous suggestion that CEACAMs could exist as a complex on the neutrophil surface (Singer et al., 2002). In this hypothetical situation, CEACAM3 could transduce activation signals induced by binding of not only CEACAM3, but CEACAM1 and potentially CEACAM6 as well. However, such associations have not been experimentally explored.

1.15.2 Co-operative Signaling with other Immunoreceptors

An area of recent interest in immunoreceptor signaling has been the co-operation of signaling by diverse receptor types. One of the main areas of focus in this regard is synergies between ITAM-containing receptors and members of the Toll-like receptor (TLR) family. It has been shown that while antibody-mediated crosslinking of TREM-1 (which signals via DAP12) causes the production of TNF by monocytes and IL-8 by PMNs, when this experiment is done in the presence of LPS, a 25-fold increase in cytokine secretion is seen (Bouchon et al., 2000), suggesting synergistic effects with TLR4. Similarly, the β-glucan receptor Dectin-1 has been shown to synergize with TLR2 to cause an increased induction of cytokines in response to fungal particles (Gantner et al., 2003). This collaboration is dependent on both Syk- and MyD88-dependent signaling pathways, and the simultaneous stimulation of both receptors (Dennehy et al., 2008). It is interesting to note that a bacterium such as N. gonorrhoeae should be able to present multiple PAMPs to PMNs at once, and could therefore result in synergistic signaling.

1.15.3 Recently Characterized Downstream Signaling Events

Part of the explanation for the aforementioned synergistic signaling involves recently characterized signaling events that appear to be shared by TLRs, NLRs, and ITAM-containing receptors that signal via the Syk family of kinases. CARD9, a caspase recruitment domain (CARD)-containing protein, has recently been shown to contribute to Dectin-1-mediated myeloid cell activation, cytokine production, and anti-fungal immunity through the activation of NF-κB (Gross et al., 2006). CARD9 has also been shown to signal downstream of other ITAM-containing proteins including DAP12 and FcRγ, and importantly, has also been implicated in TLR-induced activation of dendritic cells (Hara et
al., 2007). Similarly, Nod1/Nod2 and and TCR-based signals converge on the serine/threonine kinase Rip2 (Kobayashi et al., 2002). Interestingly, both CARD9 and Rip2 activation ultimately results in NF-κB activation via Bcl10 and Malt1 (Gross et al., 2006). These converging signaling cascades likely produce distinct effects in cells where multiple receptors are stimulated by the same agonist, which is a likely scenario when considering a stimulus as complex as an intact bacterial pathogen.

1.15.4 Inhibitory ITAMs

Studies involving IgA, which mediates its effects on cell signaling by binding to the Fcγ-associated FcαRI receptor, showed that this molecule could initiate activating or inhibitory signals based on whether it was soluble (serum IgA) or multimeric (via aggregation with antigen) (Pasquier et al., 2005). These studies gave rise to the concept of the 'inhibitory ITAM' (ITAMI) (Pinheiro da Silva et al., 2008) where weak activation of an ITAM-containing receptor allows recruitment of phosphatases and thus promotes inhibitory signals, whereas strong phosphorylation overcomes phosphatase recruitment via the docking of Syk family kinases, and thus the initiation of downstream activation signals. This concept has been extended to other molecules as well, including DAP12, in which it was reported that macrophages from DAP12 knockout mice curiously expressed higher concentrations of proinflammatory cytokines in response to TLR ligands (Hamerman et al., 2005). Similarly, TREM-2 deficient mice (TREM-2 signals via DAP12) are also hyperresponsive to LPS and other TLR ligands (Hamerman et al., 2006). Such results are supported by evidence that ITAMs of, for instance, the FcεRI β-chain (Kimura et al., 1997) and FcγRIIA (Ganesan et al., 2003) have been shown to associate with phosphatases, and may reflect a further level of regulation of these tyrosine-based signals.

1.16 Thesis Objective

The purpose of my thesis work has been to characterize how CEACAM3 carries out its functions in response to infection by N. gonorrhoeae in its natural environment, the neutrophil. In Chapter 2, I use a transfected epithelial model (HeLa-CEACAM3), as well as an inhibitor-based approach in human neutrophils to study signaling events that occur upon CEACAM3 stimulation by Opa-expressing bacteria. In Chapters 3 and 4, I use
humanized murine models to study the role of human CEACAM receptors in the neutrophil response to *N. gonorrhoeae* infection. In Chapter 5, I present a summary of my data as well as discuss topics for future study.
CHAPTER 2.
THE SPECIFIC INNATE IMMUNE RECEPTOR CEACAM3 TRIGGERS NEUTROPHIL BACTERICIDAL ACTIVITIES VIA A SYK KINASE-DEPENDENT PATHWAY

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Helen Sarantis and Scott Gray-Owen designed the experiments, analyzed research, and wrote the paper.

Helen Sarantis contributed all figures.
2.1 Abstract

The human-restricted pathogens *Neisseria gonorrhoeae, Neisseria meningitidis, Haemophilus influenzae* and *Moraxella catarrhalis* colonize host tissues via carcinoembryonic antigen-related cellular adhesion molecules (CEACAMS). One such receptor, CEACAM3, acts in a host-protective manner by orchestrating the capture and engulfment of invasive bacteria by human neutrophils. Herein, we show that bacterial binding to CEACAM3 causes recruitment of the cytoplasmic tyrosine kinase Syk, resulting in the phosphorylation of both CEACAM3 and Syk. This interaction is specific for the immunoreceptor tyrosine-based activation motif (ITAM) in the CEACAM3 cytoplasmic domain. While dispensable for the phagocytic uptake of single bacteria by CEACAM3, Syk is necessary for internalization when cargo size increases or when the density of CEACAM-binding ligand on the cargo surface is below a critical threshold. Moreover, Syk engagement is required for an effective bacterial killing response, including the neutrophil oxidative burst and degranulation functions in response to *Neisseria gonorrhoeae*. These data reveal CEACAM3 as a specific innate immune receptor that mediates the opsonin-independent clearance of CEACAM-binding bacteria via Syk, a molecular trigger for functional immunoreceptor responses of both the adaptive (TCR, BCR, FcR) and innate (Dectin-1, CEACAM3) immune systems.

2.2 Introduction

The hallmark of infection by the human-restricted pathogen *Neisseria gonorrhoeae* (Ngo, gonococcus) is the appearance of a urethral or cervical exudate consisting of polymorphonuclear neutrophils (PMNs) associated with Gram-negative diplococci (Rest and Shafer, 1989). This interaction is opsonin-independent, and depends on bacterial expression of the colony opacity-associated (Opa) proteins, which allow PMNs to effectively recognize, engulf and kill the pathogen (Kupsch et al., 1993, Virji et al., 1986). While some Opa variants bind to cell surface-expressed heparan sulfate proteoglycans (HSPGs) (van Putten et al., 1995, Freissler et al., 2000), the majority bind members of the CEACAM (carcinoembryonic antigen-related cellular adhesion molecule) family (Gray-Owen et al., 1997a). Four different CEACAM receptors (CEACAM1, CEACAM3, CEACAM5 and CEACAM6) can each, independently, allow gonococci expressing CEACAM-specific Opa
proteins to associate with various human cell types. CEACAM binding allows neisserial adherence to and trans-cellular transcytosis across polarized epithelia (Wang et al., 1998), attachment to immune-activated endothelia (Muenzner et al., 2000), and inhibition of immune cell responses (Boulton and Gray-Owen, 2002). Recently, it has been proposed that the neutrophil-restricted CEACAM3 receptor functions in the innate immune response to \textit{Neisseria gonorrhoeae} and various other Gram-negative human-restricted bacterial pathogens (McCaw et al., 2003, Schmitter et al., 2004). Such a role for CEACAM3 reflects its neutrophil-restricted expression, as PMNs are professional phagocytes that can rapidly recruit to infection sites to destroy bacterial and fungal pathogens using a combination of antimicrobial functions, including both the production of toxic reactive oxygen species (ROS) and release of deleterious soluble mediators from intracellular granules (Hampton et al., 1998, Faurschou et al., 2003).

The cytoplasmic tyrosine kinase Syk and its T-cell counterpart ZAP-70 play a key role in host immunity as has been shown by their absolute requirement for signaling downstream of receptors central to the adaptive (T- and B-cell receptors (DeFranco and Law, 1995); the Fc\( \gamma \)-receptors for the Fc portion of IgG (McCaw et al, 2003; Strzelecka et al, 1997)) and innate (the C-type lectin receptor for \( \beta \)-glucan, Dectin-1 (Brown, 2006)) immune responses. Syk and ZAP-70 are activated upon docking to phosphorylated tyrosine residues within a characteristic amino acid sequence termed the immunoreceptor tyrosine-based activation motif (ITAM; YxxL/I(x)\(_7-12\)YxxL/I) via paired, amino-terminal SH2 domains (Futterer et al., 1998). In the case of the phagocytic Fc\( \gamma \) receptors, ensuing signaling triggers the neutrophil functions of phagocytosis and oxidative burst (Kiefer et al., 1998). Similar to the aforementioned immunoreceptors, CEACAM3 contains an ITAM within its cytoplasmic domain (Chen et al., 2001, McCaw et al., 2003), and CEACAM3 engagement by Opa\textsubscript{CEA}-expressing gonococci leads to a Src family kinase-dependent phosphorylation of both tyrosines within the CEACAM3 ITAM (McCaw et al., 2003, Schmitter et al., 2004), Rac GTPase-dependent assembly of F-actin into a phagocytic cup (McCaw et al., 2003, Billker et al., 2002, Schmitter et al., 2004), and phosphatidylinositol 3-kinase activation (Booth et al., 2003). This process ultimately allows the opsonin-independent phagocytosis of not only \textit{Neisseria} species, but bacteria of the \textit{Haemophilus} and \textit{Moraxella} genera as well (Schmitter et al., 2004). Using a transfected chicken B cell model (DT40), Chen \textit{et al} (2001) showed that CEACAM3 engagement by Opa\textsubscript{CEA}-expressing gonococci stimulated both bacterial
phagocytosis and an intracellular calcium flux. Both responses were shown to be dependent on the CEACAM3 ITAM, Syk, and phospholipase C-gamma (PLCγ) (Chen et al., 2001), however CEACAM3-dependent engulfment of bacteria in other cell types does not require Syk (McCaw et al., 2003, Billker et al., 2002, Schmitter et al., 2004).

In this work, we sought to confirm that the cytoplasmic domain of CEACAM3 is an authentic ITAM, and to ascertain how this contributes to the neutrophil response to Neisseria gonorrhoeae. We demonstrate that bacterial binding promotes Syk recruitment to CEACAM3 in an ITAM-dependent manner. We show that Syk function is dispensable for the engulfment of single bacteria, but is required for entry when particle size is increased, or when the density of CEACAM-binding ligand on the particle surface is decreased. Furthermore, regardless of particle size, Syk directs the PMN CEACAM3-dependent oxidative burst response, primary and secondary granule release, and bacterial killing. This work underlies the critical importance of CEACAM3-directed signaling pathways in the innate immune response against Neisseria gonorrhoeae, and further highlights the role of Syk as an essential molecular trigger in bacterial (CEACAM3) and fungal (Dectin-1) clearance by neutrophils.

2.3 Results

2.3.1 Syk Kinase is Recruited to CEACAM3-Bound Gonococci During Infection of HeLa cells

In order to study neisserial Opa protein interactions with CEACAM3 in the absence of other neutrophil-expressed CEACAM receptors, we have used transfected epithelial cells expressing recombinant CEACAM3 (McCaw et al., 2003). In these cells, OpaCEA binding causes CEACAM3 phosphorylation and extensive re-organization of actin into phagocytic cups that ultimately enclose the bacteria (Billker et al., 2002, McCaw et al., 2003, Schmitter et al., 2004). Although it has been reported that Syk expression extends to epithelial cells (Renedo et al., 2001), we could not detect Syk protein in our HeLa cell lines (data not shown). Consequently, we transfected cDNA encoding an EGFP-tagged Syk into HeLa-
Figure 2.1. Syk associates with activated CEACAM3 in HeLa cells.
(A) Co-localization of Syk-EGFP with OpacEA-expressing gonococci during HeLa-CEACAM3 infection. HeLa-CEACAM3 (i and ii) or HeLa-CEACAM1 (iii) were transfected with Syk-EGFP and infected with OpacEA-expressing gonococci (i and iii) or OpahSPG-expressing gonococci (ii) before staining for immunofluorescence microscopy. Insets within Ai show higher magnification of bacteria enclosed in the white box. (B) The percentage of adherent OpacCEA-gonococci co-localizing with Syk or F-actin per cell after indicated times at 37°C was also quantified. Total bacteria adhering per cell was similar for all time points (data not shown). Student's t-tests were performed for relevant samples, with p values < 0.01 indicated by an asterisk (*). Statistical analyses were conducted as described in Experimental Procedures. (C) Syk co-immunoprecipitates with CEACAM3 in OpacEA-gonococci-infected HeLa cells. HeLa-CEACAM3 were infected with Opaa or OpacEA-expressing gonococci at an MOI of 50 at 37°C. Samples were lysed at indicated time-points and immunoprecipitated with anti-CEACAM antibody (Dako), followed by resolution with SDS-PAGE and immunoblotting for Syk with the 4D10 antibody (top panel) or total CEACAM3 with the D14HD11 antibody (bottom panel) to demonstrate equal loading of samples.
CEACAM3 to assess whether Syk associated with CEACAM3 upon bacterial binding. In uninfected cells, Syk-EGFP displayed even staining throughout the cytoplasm (data not shown). We observed dramatic colocalization of Syk with OpaCEA-expressing gonococci during infection of HeLa-CEACAM3 cells (Fig. 2.1A). Such colocalization was not observed upon infection of HeLa-CEACAM3 with gonococci expressing a heparan-sulfate specific Opa that does not engage CEACAM3, or when HeLa-CEACAM1 cells were infected with OpaCEA-expressing bacteria. Gonococci were never seen to colocalize with EGFP alone (data not shown). While Syk-associated bacteria often co-localized with densities of F-actin at early time points, this was not the case later in the infection, where we observed that bacteria remained associated with Syk even after F-actin rich cups had dispersed (Fig. 2.1B). These data mirror Syk/F-actin associations in other receptor systems including FcγR (Strzelecka et al., 1997), Dectin-1 (Underhill et al., 2005), and Complement Receptor 3 (CR3) (Shi et al., 2006). To confirm that Syk was associating with activated CEACAM3, we immunoprecipitated CEACAM3 from lysates of Syk-EGFP-transfected HeLa-CEACAM3 cells that had been infected with OpaCEA-expressing gonococci. We observed that Syk co-immunoprecipitated with CEACAM3 when cells were infected with OpaCEA-expressing gonococci specifically, whereas only basal levels of association were seen with Opa-gonococci (Fig. 2.1C). Normal rabbit serum did not immunoprecipitate CEACAM3 or Syk (data not shown). In agreement with our microscopy-based colocalization data, Syk association with immunoprecipitated CEACAM3 was maximal during the first 30 minutes of infection.

2.3.2 Syk is Phosphorylated upon Infection of Human Neutrophils with OpaCEA-expressing Gonococci

To assess if Syk associated with CEACAM3 in its natural cell type, the human neutrophil, we used primary neutrophils isolated from healthy human blood donors. Again, Syk specifically co-immunoprecipitated with CEACAM3 upon infection with OpaCEA-expressing gonococci, and not when Opa-gonococci were used (Fig. 2.2A). Syk association with CEACAM3 was transient, being maximal at either 2 or 5 minutes post infection (depending on the experiment), in a significantly more rapid manner than in HeLa cells, reflecting the
Figure 2.2. Syk is phosphorylated during infection by Opa\textsuperscript{CEA}-expressing gonococci in human neutrophils.

(A) Syk co-immunoprecipitates with CEACAM\textsubscript{3} in human neutrophils infected with Opa\textsuperscript{CEA}-gonococci. DFP-treated neutrophils were infected with Opa\textsuperscript{−} or Opa\textsuperscript{CEA}-expressing gonococci at an MOI of 10 for indicated times, followed by lysis and immunoprecipitation of samples with anti-CEACAM IgG (Dako). Immunoprecipitates were resolved by SDS-PAGE and probed for Syk (top panel) or total CEACAM\textsubscript{3} (bottom panel). (B) Syk is phosphorylated during Opa\textsuperscript{CEA}-gonococci infection of human neutrophils. PMN were infected with Opa\textsuperscript{−} or Opa\textsuperscript{CEA}-gonococci at an MOI of 10 at 37°C. Post-infection, cells were spun down, re-suspended in boiling lysis buffer, followed by resolution by SDS-PAGE and immunoblotting for phospho-Syk (top panel) or total Syk (bottom panel) to demonstrate equal loading of samples (i). Pre-treatment of neutrophils with the Src family kinase inhibitor PP2 at 20 μM abolished Syk phosphorylation in response to Opa\textsuperscript{CEA}-expressing gonococci (ii).
accelerated nature of CEACAM3 tyrosine phosphorylation in human neutrophils (McCaw et al., 2003). To investigate whether Syk was activated upon recruitment to CEACAM3 in response to gonococcal infection, we took advantage of an antibody directed against a phosphorylated form of Syk. We observed that Syk was phosphorylated during infection of PMNs with OpaCEA-expressing gonococci, and not when Opa- bacteria were used (Fig. 2.2Bi). Syk no longer became phosphorylated when human neutrophils were treated with the Src family kinase-specific inhibitor PP2 before infection with OpaCEA-expressing gonococci (Fig. 2.2Bii), suggesting that Syk recruitment and activation are downstream of CEACAM3 ITAM phosphorylation.

2.3.3 Efficient Docking of Syk to CEACAM3 Requires an Intact ITAM

We sought to determine whether one or both of the CEACAM3 ITAM tyrosines were required for Syk recruitment. To accomplish this, we co-transfected wild-type or various ITAM mutated-CEACAM3 alleles (Fig. 2.3) along with Syk-EGFP into HeLa cells. By immunofluorescence microscopy, we observed that colocalization of Syk and OpaCEA-expressing gonococci was most evident when HeLa cells expressed full length CEACAM3 (L) (54.6% of cells). Appreciable colocalization was also observed when the membrane distal tyrosine (Y241) was mutated to phenylalanine (CEACAM3-Y241F) (27.0% of cells), suggesting that Syk could be recruited to CEACAM3 without tandem phosphorylated tyrosine residues, albeit with lower efficiency. Syk recruitment to bound gonococci was never evident in cells expressing CEACAM3-YYFF (both ITAM tyrosine residues mutated to phenylalanines) or the CEACAM3-IC1 natural splice variant (lacking the ITAM and most of the cytoplasmic domain; Fig. 2.3), and OpaCEA-expressing gonococci did not bind HeLa cells appreciably in the absence of CEACAM transfection (data not shown).

2.3.4 Syk is Dispensable for Gonococcal Internalization by CEACAM3

Syk is absolutely required for phagocytosis of IgG-opsonized particles by the phagocytic Fcγ receptors on macrophages and neutrophils (Crowley et al., 1997, Kiefer et al., 1998). While HeLa-CEACAM3 effectively engulf OpaCEA-expressing gonococci in the absence of Syk (McCaw et al., 2003, Billker et al., 2002, Schmitter et al., 2004), we sought to determine whether an exogenous Syk-EGFP could facilitate internalization. We observed that the
Figure 2.3. Maximal association of Syk with CEACAM3 requires an intact ITAM.
HeLa cells were seeded onto acid-treated glass coverslips in 24-well plates, co-transfected the following day with CEACAM3 constructs shown schematically (i-v) in conjunction with the Syk-EGFP plasmid, and infected the following day with OpaCEA-gonococci at an MOI of 15 for 20 minutes at 37°C, followed by processing for microscopic analysis. Numbers indicated in parentheses are average values for the percentage of bacteria per cell showing co-localization with Syk-EGFP, taken from a representative experiment. Student’s t-tests were performed for each mutant in comparison with full-length CEACAM3 (CEACAM3-L, top panel), and p values < 0.01 are indicated by an asterisk (*) beside parentheses. Statistical analyses were conducted as described in Experimental Procedures.
presence of Syk did not significantly alter bacterial binding, the total number of bacteria engulfed, nor did it increase the rate of gonococcal uptake (Fig. 2.4A). We next assessed whether Syk facilitated gonococcal uptake by primary human PMNs by testing the effect of the Syk-specific inhibitor piceatannol. To ensure that the inhibitor was active, we assessed entry of IgG-opsonized sheep erythrocytes (eIgG), and confirmed that entry decreased by 50% upon Syk inhibition (Fig. 2.4Bi). Piceatannol treatment did not affect gonococcal engulfment by PMNs, which proceeded at a high rate despite the presence of the inhibitor (Fig. 2.4Bii). These data suggest that neisserial internalization by CEACAM3 can occur independently of Syk.

2.3.5 CEACAM3-mediated Phagocytosis Requires Syk

Because Syk’s role in phagocytosis has been characterized for particles of considerably larger size than *Neisseria* (IgG-coated erythrocytes or polystyrene beads), and one potential role for Syk has been in phagosomal closure, which is critically size-dependent (Crowley et al., 1997), we wondered if we could detect a role for Syk in CEACAM3-mediated engulfment using larger particles. To accomplish this, we linked anti-CEACAM IgG to carboxylated beads of different sizes (1, 2.4, and 5.6 µm diameter). These beads bound specifically to HeLa-CEACAM3, while beads coated with normal rabbit serum did not bind appreciably (Fig. 2.5A, lower panel). Analyzing bead internalization by HeLa-CEACAM3, we observed that while smaller beads, which reflect the size of *N. gonorrhoeae*, were internalized in the absence of Syk, the 5.6 µM beads were only internalized when Syk was present (Fig. 2.5A). This indicates that particle size determines whether or not Syk is required for successful CEACAM3-mediated engulfment. Interestingly, Syk-mediated internalization of 5.6 µm beads was phosphatidylinositol 3-kinase (PI3K)-dependent, since entry was blocked when cells were pre-incubated with the PI3K inhibitor LY294002 (Fig. 2.5B).

Surprisingly, 1 µm bead entry in the absence of Syk did not fully recapitulate Neisserial uptake (compare Fig. 2.5A (~40% of 1 µm beads internalized) versus Fig. 2.4A (~85% gonococci internalized at 30 min)). To assess whether this difference was a consequence of antibody versus Opa ligation of CEACAM3, or due to a difference in valency of these two agonists (while Opa is highly expressed on the neisserial surface, antibody bound to carboxylated beads does not preferentially have the F(ab’)2 region exposed), we employed a
Figure 2.4. CEACAM3 does not require Syk for neisserial internalization

(A) HeLa-CEACAM3 cells were transfected in 24-well tissue culture plates with or without Syk-EGFP, and infected the following day with OpaCEA-gonococci at an MOI of 15. Fixed cells were processed for staining to allow visualization of entry via indirect immunofluorescence as described previously (McCaw et al., 2003). (B) A similar experiment was performed in human neutrophils with the Syk inhibitor piceatannol. Piceatannol activity was confirmed by assessing FcγR-mediated internalization of eIgG at an MOI of 5 (i). CEACAM3-mediated phagocytosis was studied as in (A), with an MOI of 10, and the addition of piceatannol to assess the Syk dependence of entry (ii). Student’s t-tests were performed for inhibitor-treated samples in comparison with untreated/carrier (denoted as “-“ Piceatannol), and p values < 0.01 are indicated by an asterisk (*). Statistical analyses were conducted as described in Experimental Procedures.
Figure 2.5. CEACAM3 requires Syk for phagocytosis of specific particles

(A) Syk is required for CEACAM3-mediated phagocytosis of particles larger than a critical threshold. HeLa-CEACAM3 were either left untransfected or transfected in 24-well tissue culture plates with cDNA encoding Syk-EGFP, incubated the following day with anti-CEACAM IgG-coated polystyrene beads of indicated diameters, and processed to quantify engulfment via indirect immunofluorescence. In the top panel is the average number of intracellular beads per cell (shown as a percentage), and average beads bound per cell is shown in the lower panel.

(B) Syk-mediated phagocytosis of large particles requires PI3K. HeLa-CEACAM3 cells were pre-treated with LY294002 before infection with 5.6 µM anti-CEACAM IgG-coated carboxylated beads. Internalization was quantified as in (A). Student’s t-tests were performed for relevant samples in (A) and (B), and p values < 0.01 are indicated by an asterisk (*). Statistical analyses were conducted as described in Experimental Procedures. (C) Syk is required for CEACAM3-mediated phagocytosis of particles bearing CEACAM3-binding ligands at a density below that of a critical threshold. Mixtures of rabbit anti-CEACAM and control IgG were used to saturate Pansorbin cells in varying ratios (for instance, in a 10% anti-CEACAM sample, the total IgG label would be composed of 10% anti-CEACAM and 90% isotype). These Pansorbin particles were then incubated with HeLa-CEACAM3 that were transfected with Syk-EGFP cDNA (ii) or left untransfected (i). Visualization of engulfment was accomplished as in (A). The percentage of intracellular bacteria per cell was graphed to emphasize differences at low surface ligand density (iii). Student’s t-tests were performed for relevant samples: one asterisk (*) denotes p < 0.06 and two asterisks (**) denote p < 0.01. As above, statistical analyses were conducted as described in Experimental Procedures.
strategy using PFA-fixed *Staphylococcus aureus* (Pansorbin). Pansorbin is similarly sized to *Neisseria*, and expresses Protein A in large quantities on its surface, allowing high-density binding of antibody in an orientation appropriate for our studies. We labeled these particles to saturation, varying the ratios of anti-CEACAM and isotype IgG on the surface anywhere from 100% anti-CEACAM to 100% isotype. Using our labeled Pansorbin array, we observed that the density of CEACAM-specific antibodies had little effect on binding to CEACAM3-expressing cells (Fig. 2.5Ci). Internalization, however, clearly decreased as the proportion of anti-CEACAM IgG was reduced, suggesting a critical degree of receptor occupancy is important for particle internalization by CEACAM3. Importantly, Syk expression enhanced the engulfment of Pansorbin possessing lower surface densities of anti-CEACAM IgG (equal to or less than 40%) (Fig. 2.5Cii, 2.5Ciii). We believe these data explain why gonococci do not require Syk for entry, and demonstrate that Syk substantially increases the efficacy of particle internalization when CEACAM3-binding ligand surface density is below a critical threshold.

### 2.3.6 Syk is Required for Neutrophil Killing Functions

Because Syk was not required for gonococcal engulfment, but was still recruited to gonococci upon CEACAM3 binding, we wondered whether this interaction contributed to other downstream effects. We decided to investigate Syk’s role in PMN function by first analyzing primary and secondary granule release. We used cytochalasin B to shunt the intra-phagosomal degranulation response to gonococci towards the PMN cell surface (Bjerknes et al., 1995) allowing the appearance of the primary granule protein CEACAM6 (Ducker et al., 1992, Kuroki et al., 1995) and secondary granule protein CEACAM8 (Ducker and Skubitz, 1992) to be assessed by flow cytometry. As shown previously, degranulation in response to eIgG was Syk-dependent, whereas PMA-induced degranulation was still apparent in the presence of piceatannol (Fig. 2.6A). We observed an enhanced degranulation of both granular markers when gonococci bound to PMN CEACAM receptors (compare Opa- and Opa<sub>CEA</sub>, Fig 2.6A). When Syk was inhibited, this degranulation response was significantly retarded, indicating that Syk was required for PMN degranulation in response to Opa<sub>CEA</sub>-expressing *Neisseria gonorrhoeae*. PMNs respond to Opa<sub>CEA</sub>-expressing gonococci by triggering an oxidative burst response (Gray-Owen et al., 1997b). To ascertain whether this response also requires Syk, we used the cell-permeable DCFH-DA,
A  

i) Primary Granules

- DMSO
- 50 μM Piceatannol

Opa
- 480
- 311

Opa_CEA
- 965
- 413

elG
- 1271
- 407

PMA
- 1341
- 979

ii) Secondary Granules

- DMSO
- 50 μM Piceatannol

Opa
- 16.2
- 5.9

Opa_CEA
- 25.0
- 9.34

elG
- 23.4
- 8.33

PMA
- 26.5
- 22.2

B  

Oxidative Burst

- DMSO
- 50 μM Piceatannol

Opa
- 2.63
- 2.42

Opa_CEA
- 3.78
- 2.82

elG
- 3.57
- 2.13

PMA
- 83.6
- 61.4

C  

i) Invasion:

- Intracellular Bacteria
- Total Bacteria

ii) Bacterial Survival:

- Time (min)

- % Viable Bacteria per Well

** Significant difference
Figure 2.6. Syk is required for PMN killing functions in response to *Neisseria gonorrhoeae*.

(A) Syk directs the degranulation of both primary and secondary human neutrophil granules in response to OpaCEA-gonococci. Human neutrophils (10⁷/ml) were treated with 5 μM cytochalasin B to redirect degranulation to the cell surface, followed by DMSO or piceatannol (50 μM). Agonists were added to 3 x 10⁶ cells (Ngo: MOI of 10; eIgG: 5 particles per cell; PMA: 1 μg/ml) and infections proceeded at 37°C for 10 minutes. Post-infection, cells were washed and fixed, and samples were split, with one-half stained for the primary granule marker CEACAM6 (CD66c; panel i), and the other-half stained for the secondary granule marker CEACAM8 (CD67; panel ii). Samples are shown as black lines with the geometric mean of the FL-2 signal from a gated sample of 10,000 cells indicated, and un-stimulated cells (Primary FL-2: 310; Secondary FL-2: 8.23) as grey lines.

(B) Syk is Required for the PMN Oxidative Burst Response to OpaCEA-gonococci. Human neutrophils (10⁷/ml) were treated with DMSO or piceatannol (50 μM), and 1.5 x 10⁶ were treated with DCFH-DA for 15 minutes at 37°C, followed by addition of agonists as described in (A). Infections proceeded at 37°C. Shown is the fluorescence signal evident at 60 minutes post-infection, indicated as the geometric mean of the FL-1 signal from a gated sample of 10,000 cells, with test samples as black lines and unstimulated (FL-1: 2.57) cells as grey lines.

(C) Syk is required for PMN Killing of OpaCEA-expressing bacteria. Human neutrophils were treated with DMSO or piceatannol (50 μM) and infected with Opa- or OpaCEA-expressing *E. coli* strain JM103 at an MOI of 15 in 24-well tissue culture plates (5 x 10⁵ cells per well). Bacteria were allowed to interact with the neutrophils for 30 minutes, and either washed and fixed for immunofluorescence microscopy (i) or treated with gentamicin to kill extracellular bacteria following indicated times at 37°C, before treatment with 0.4% saponin in HBSS to release viable bacteria for dilution plating. Colony forming units (cfu) were counted the following day (ii). Student’s t-tests were performed for relevant samples in (C). p values < 0.05 are indicated by an asterisk (*), while p values of <0.01 are indicated by two asterisks (**). Statistical analyses were conducted as described in Experimental Procedures.
which is modified inside the cell to a compound that fluoresces upon oxidation during the burst response (Jakubowski and Bartosz, 2000). As expected, we observed that Opa\textsubscript{CEA}-expressing gonococci promoted a neutrophil oxidative burst similar to that apparent in response to opsonized RBCs, whereas the PMNs did not respond to Opa\textsuperscript{'} gonococci (Fig. 2.6B; compare mean fluorescence intensities). While the oxidative burst detected in response to Opa\textsubscript{CEA} or opsonized RBC are small relative to that apparent in response to PMA, the latter causes a generalized activation of the phagocytic oxidase whereas there is only a targeted release of reactive oxygen intermediates into the \textit{Neisseria}-containing phagosome (Naids & Rest, 1991). Kinetic studies indicated that this response is maximal at 60 minutes post-infection (data not shown). The Opa\textsubscript{CEA}- and IgG-dependent oxidative bursts were completely abolished when Syk was inhibited with piceatannol, while the PMA-induced oxidative burst, which bypasses Syk, was not blocked by this inhibitor (Fig. 2.6B). Syk is, therefore, required for the CEACAM-dependent oxidative burst response to Opa\textsubscript{CEA}-expressing \textit{Neisseria gonorrhoeae}.

Our data to this point suggested that while Syk does not affect gonococcal engulfment by PMNs, it is critical for triggering neutrophil bactericidal activities, suggesting that piceatannol should increase the proportion of viable bacteria inside PMN. \textit{Neisseria spp.} are extremely susceptible to neutrophil granule proteins such as cathepsin G, which can be released during the detergent-mediated PMN lysis required to free intracellular bacteria for plating (Shafer \textit{et al.}, 1986). We thus employed the recombinant \textit{Escherichia coli} strain JM103 that either lacked (Opa\textsuperscript{'}), containing an empty expression vector) or expressed Opa\textsubscript{CEA} (Gray-Owen \textit{et al.}, 1997b) for our survival studies, since \textit{E. coli} are inherently more resistant than \textit{N. gonorrhoeae} to PMN killing (Belland \textit{et al.}, 1992). Using immunofluorescence microscopy, we observed that Opa\textsubscript{CEA}-expressing \textit{E. coli} bound and were engulfed by PMN better than Opa\textsuperscript{'} bacteria (Fig. 2.6Ci). Furthermore, we observed a dramatic increase in the survival of intracellular Opa\textsubscript{CEA}-expressing JM103 when Syk function was inhibited (Fig. 2.6Cii). Piceatannol treatment did not have any apparent effect on bacterial survival in PMN-free samples (data not shown). Taken together, these data demonstrate Syk’s role as a key mediator of CEACAM3-dependent bacterial killing by human neutrophils.
2.4 Discussion

In this study, we sought to characterize the process by which the innate immune receptor CEACAM3 interacts with CEACAM-binding pathogens in the context of the human neutrophil. We observed that ligation of CEACAM3 by *Neisseria gonorrhoeae* caused Syk kinase recruitment to CEACAM3 in an ITAM-dependent fashion. Syk was not required for CEACAM3-mediated gonococcal engulfment, but was essential for the internalization of particles that were either i) expressing a low valency of CEACAM3-specific ligand or ii) of a size larger than *Neisseria*, the latter of which would reflect neutrophil association with bacterial aggregates. Moreover, Syk orchestrated the neutrophil killing functions of oxidative burst and degranulation. These data suggest that Syk functions as the molecular trigger in controlling CEACAM3-mediated internalization and killing of *Neisseria gonorrhoeae* by human neutrophils.

Analogous to other ITAM-containing receptors, Syk associated specifically with CEACAM3 upon receptor ligation via OpaCEA-expressing gonococci, doing so in a phosphotyrosine-dependent manner. Such association was not observed upon ligation of the immunotyrosine-based inhibition motif (ITIM)-containing CEACAM1 receptor, suggesting that the critically spaced phosphotyrosines of the CEACAM3 ITAM were required for engagement of Syk’s paired SH2 domains. Accordingly, the full-length CEACAM3-L construct allowed maximal engagement of Syk, while ITAM mutants allowed marginal Syk recruitment. Interestingly, we noted that mutation of the membrane-distal (Y241) tyrosine still allowed significant recruitment of Syk, with about half as many gonococci associated with Syk compared to that seen with the full-length receptor. This is in accordance with experiments by Chen and coworkers (Chen et al., 2001), who used a CEACAM3-transfected chicken B cell (DT40) model to show that the membrane-proximal (Y230) tyrosine was more important for Syk-mediated calcium flux in response to OpaCEA-expressing gonococci. That a single ITAM tyrosine can recruit Syk via one of its SH2 domains is not surprising, as it has been shown in other systems (Kim et al., 2001, Herre et al., 2004). It is also clear, however, that both tyrosines are required for full engagement of Syk’s paired SH2 domains.

We observed that Syk was not required for and did not enhance CEACAM3-mediated phagocytosis of individual *Neisseria gonorrhoeae* in HeLa cells or in human neutrophils. Internalization of gonococci by HeLa-CEACAM3 in the absence of Syk is remarkably
efficient, with the majority (~90%) of bacteria internalized after 20 minutes at 37°C. To validate this finding in the natural context, we looked at Syk involvement in phagocytosis by human neutrophils. Because of the lack of a CEACAM3-expressing neutrophil/pre-neutrophil cell line, and an inability to genetically manipulate primary human neutrophils due to their limited viability when extracted from fresh blood, we used the Syk-specific inhibitor piceatannol. In contrast to studies performed using large particles in the Fcγ receptor system (Crowley et al., 1997, Kiefer et al., 1998), we observed that inhibition of Syk had no effect on gonococcal internalization even in neutrophils.

Our results also contrast the results of Chen et al (2001), who observed a requirement for Syk in the CEACAM3-mediated engulfment of gonococci by the chicken B cell line, DT40. This may have been due to a general defect in phagocytosis of the Syk−/− DT40-CEACAM3 cell line. More likely, however, are the effects of inherent differences between the engineered chicken B cell line and the more relevant human PMNs. CEACAM3 thus displays activity reminiscent of the ITAM-containing Dectin-1, which internalizes zymosan independently of Syk, yet requires Syk for other PMN functions (Herre et al., 2004, Underhill et al., 2005).

Using various sizes of anti-CEACAM IgG-coated beads, we observed a size-dependence for Syk in the CEACAM3-mediated phagocytosis of cargo. Our results with the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 suggest that the Syk-dependent uptake of large particles via CEACAM3 occurs in a PI3K-dependent manner. This is a logical result, as both Syk and PI3K play a role in phagosomal closure during phagocytosis, shown by the strikingly comparable phagocytic defects (an apparent inability to enclose the bound particle into a phagosome due to insufficient pseudopod extension) in both PI3K-inhibited (Araki et al., 1996, Cox et al., 1996, Cox et al., 1999) and Syk-deficient cells (Crowley et al., 1997, Kiefer et al., 1998). CEACAM3’s ability to mediate the engulfment of large particles may be especially relevant because of the potential for Opa proteins to cause bacterial aggregation, as well as the capacity of N. gonorrhoeae to grow in biofilms (Greiner et al., 2005).

Upon comparing phagocytic efficiencies between our anti-CEACAM IgG-coated bead array and gonococci, we noted that the 1 μm beads did not fully re-capitulate the incredible
efficacy with which OpaCEF-expressing bacteria were internalized, which was surprising since gonococci are of a similar size. We speculated this difference might be explained by a decreased density of exposed CEACAM-binding ligand (i.e. the F(ab’)2 portion of the antibodies) on the bead surface, relative to Opa on gonococci. To address this, we used an array of IgG-saturated Protein A-expressing *Staphylococcus aureus* (Pansorbin) containing varying densities of anti-CEACAM IgG, and found that the efficacy of uptake and requirement for Syk depended upon ligand density. To our knowledge, this is the first indication that Syk can amplify responses to low valency binding. In light of these results, we hypothesize that Syk may act to amplify ITAM-dependent signals required to engulf particles bound to CEACAM3. Syk may allow an increased recruitment of signaling proteins to itself, when bound to the CEACAM3 ITAM. Alternatively, Syk may amplify signaling by modulating the phosphorylation state of CEACAM3 and its downstream effectors. Indeed, we have observed that the presence of Syk in HeLa-CEACAM3 allows a prolongation and enhancement of CEACAM3 phosphorylation in response to gonococcal infection (Sarantis and Gray-Owen, unpublished data). Regardless, our data clearly indicates that ligand density is less important for particle binding than for internalization, and suggests that Syk is not necessary for the engulfment of *Neisseria* because of the high density of Opa on the bacterial surface.

It is known that the intracellular granules of neutrophils are important reservoirs of pre-formed proteins with proteolytic and bactericidal (azurophilic granules) and cell-adhesion (specific granules) capabilities (Faurschou and Borregaard, 2003). We observed that mobilization of both primary and secondary granules in response to CEACAM3-dependent signals was Syk-dependent. It has been shown that PMN killing of *Neisseria gonorrhoeae* is mediated by azurophilic granule proteins, specifically the cationic antimicrobial protein cathepsin G (Shafer *et al.*, 1986) and neutrophil elastase (Rest and Shafer, 1989). While a link between Syk and degranulation has not been previously established, we have observed that CEACAM3-dependent degranulation and oxidative burst both require Syk. These processes are intimately linked; specifically, flavocytochrome b558 is restricted to secondary granules, and must be incorporated into either the plasma or phagosomal membrane to assemble a functional NADPH oxidase (Jesaitis *et al.*, 1990). It is also likely that the CEACAM3-mediated activation of the small GTPase Rac (Schmitter *et al.*, 2004) contributes to the PMN killing response, as small G-proteins are critically involved in neutrophil
degranulation (Barrowman et al., 1986). Unlike Syk, which clearly represents a point of divergence between bacterial internalization and killing pathways, Rac may act as a point of convergence that contributes to both responses via distinct effectors.

In this study, we have observed that the tyrosine kinase Syk governs the opsonin-independent response of human neutrophils to *Neisseria gonorrhoeae*, acting specifically through the innate immune receptor CEACAM3. Our results highlight the critical importance of CEACAM3 in the clearance of *Neisseria gonorrhoeae*, and may extend to pathogens of the *Haemophilus* and *Moraxella* genera that colonize human tissues via CEACAM family receptors. Our work also re-emphasizes the importance of Syk as a key player in the functional responses of seemingly unrelated ITAM-containing receptors of the adaptive (FcR, BCR, TCR) and innate (Dectin-1, CEACAM3) immune systems.

2.5 Methods

2.5.1 Reagents and Antibodies

All reagents were from Sigma (Oakville, ON) unless otherwise indicated. The diisopropyl fluorophosphate (DFP) was from BioShop (Burlington, ON). Pansorbin and PP2 were from Calbiochem (La Jolla, CA). The anti-gonococcal polyclonal rabbit antibody (UTR01) was described previously (McCaw et al., 2003). The rabbit anti-CEACAM polyclonal and normal rabbit serum were from Dako (Mississauga, ON). The CEACAM-specific mouse monoclonal D14HD11 and anti-CEACAM6 (9A6) were from Genovac GmbH (Freiburg, Germany). The mouse anti-CEACAM8 monoclonal (80H3) was from Immunotech (Mississauga, ON). Sheep erythrocytes and rabbit anti-SRBC IgG were from Cappel (Cochraneville, PA). Fluorescent conjugates were from Jackson ImmunoResearch Laboratories (Mississauga, ON), except for Texas Red-phalloidin, which was from Molecular Probes (Eugene, OR). The Syk monoclonal antibody was from Upstate (Charlottesville, VA) and the anti-phospho Syk antibody was from Cell Signaling Technology (Pickering, ON). The Syk-EGFP construct was generously provided by Dr. Alan Schreiber (University of Pennsylvania, Philadelphia, PA). CEACAM3 constructs in pCEP4 were created by Dr. A. Popp and kindly provided by Prof. T. F. Meyer (Max-Planck-Institut fuer Infektionsbiologie, Berlin, Germany).
2.5.2 Cell Lines and Bacterial Strains
The stably transfected HeLa-CEACAM1 and HeLa-CEACAM3 cell lines have been described previously (Berling et al., 1990, Nagel et al., 1993), and were maintained in RPMI-1640 with L-glutamine and 10% fetal bovine serum (FBS; HyClone; Logan, UT), in 5% carbon dioxide (CO₂)-containing humidified air at 37°C. Transfections were done with FuGENE 6 (Roche; Laval, PQ) according to manufacturer instructions. The Opa⁻ (N302), OpaHSPG (N303), and OpaCEA (N313) neisserial strains and Opa-expressing E. coli were kindly provided by Dr. T. F. Meyer (Max-Planck-Institut fuer Infektionsbiologie, Berlin, Germany), and have been described previously (Kupsch et al., 1993, Gray-Owen et al., 1997b).

2.5.3 Bacterial Colocalization Studies using Immunofluorescence Microscopy
HeLa cells were seeded at ~50% confluence into 24-well plates containing 12 mm HCl-treated coverslips, transfected 18 hours later, and infected 18 hours thereafter. Prior to infection, cells were washed three times with RPMI-1640 without FBS. Gonococci were centrifuged onto cells for 5 minutes at 63 g, at a multiplicity of infection (MOI) of 15, followed by a 5-minute deceleration. Cells were then washed two times with RPMI-1640 to remove non-adherent bacteria, and shifted to 37°C for indicated times. Post-infection, cells were washed 3 times with Phosphate-Buffered Saline (PBS) supplemented with 0.5 mM MgCl₂ and 1 mM CaCl₂ (PBS/Mg/Ca), and fixed using 3.7% paraformaldehyde (PFA). Cells were permeabilized using 0.4% Triton X-100, and stained and observed as described previously (McCaw et al., 2003).

2.5.4 Primary Neutrophil Isolation
PMNs were isolated from citrated whole blood taken from healthy volunteers by venipuncture using Ficoll-Paque Plus (Amersham Biosciences; Buckinghamshire, England). Contaminating erythrocytes were removed by dextran sedimentation and hypotonic shock, as described previously (McCaw et al., 2003). Neutrophils were thereafter maintained at 10⁷ cells/ml in Hanks Buffered Salt Solution (HBSS; Invitrogen; Burlington, ON) supplemented with 10 mM Hepes (Sigma), adjusted to a pH of 7.4.
2.5.5 Immunoprecipitations and Western Blot

For biochemical analyses of infected HeLa cells, cells were seeded into 6-well plates, transfected 18 hours later, and infected 18 hours post-transfection with an MOI of 50. Post-infection, cells were washed two times with PBS-pervanadate buffer (1 mM EDTA, 1 mM PMSF, 1 µg/mL each aprotinin, leupeptin, and pepstatin A, 1 mM NaF, 100 µM Na₃VO₄, 10 mM H₂O₂, and 50 µg/mL soybean trypsin inhibitor in PBS), and lysed with radioimmunoprecipitation (RIPA) buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/mL each aprotinin, leupeptin, and pepstatin A, 1 mM NaF, 100 µM Na₃VO₄, 10 mM H₂O₂, and 50 µg/mL soybean trypsin inhibitor). Following micro-centrifugation at 16,000 g for 15 minutes, lysates were immunoprecipitated with anti-CEACAM antibody (CEA-DAKO) for 2 h, incubated with Protein A-sepharose for 1 h, washed two times with RIPA, and boiled for 5 minutes.

Immunoprecipitated samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences), the membrane was blocked with 5% skim milk in TBS with 0.05% Tween for 30 minutes at room temperature, incubated with appropriate primary and HRP-conjugated secondary antibodies, and chemiluminescent detection performed using ECL+ (Amersham Biosciences). Infections in neutrophils were conducted in 1.5 ml tubes, using 10⁷ PMN and an MOI of 10. Pre-infection, cells were treated with DFP at 1 mM for 15 minutes to prevent proteolytic degradation of Syk. Immunoprecipitation and immunoblotting were as described for HeLa samples.

2.5.6 Whole Cell Phosphorylation Assays

Human neutrophils were treated with 20 µM PP2 or solvent (DMSO) for 20 minutes at 37°C at a concentration of 10⁷ cells/ml. PMN (10⁶ total) were then infected with gonococci in 1.5 ml tubes at an MOI of 10. Infections were stopped on ice, and cells were lysed in boiling SDS sample buffer and boiled for a further 10 minutes. Samples were resolved and immunoblotted as described above.
2.5.7 Polystyrene Bead and Pansorbin Labeling

Carboxylated polystyrene beads (Bangs Laboratories, Fishers, IN) were activated using water-soluble carbodiimide for 15 minutes in 100 mM MES buffer (pH 6), washed in 0.2 M borate buffer (pH 8) and allowed to bind antibody to saturation for 4 hours at room temperature. Reactions were stopped with 0.1 M methanolamine and 1% BSA. Pansorbin cells were incubated in saturating concentrations of antibody in PBS overnight with rotation at 4°C, followed by washing by sequential pelleting and resuspension to remove unbound antibody.

2.5.8 DCFH-DA Oxidative Burst Assay

Human neutrophils (10^7/ml) were treated with piceatannol (50 µM) or DMSO for 20 minutes at 37°C. 150 µl of cells were treated with DCFH-DA by adding 5 µl of a 100 µM solution and incubating for 15 minutes at 37°C. Infections were initiated by adding 350 µl of test stimuli (MOI of 10 for bacteria, 5 particles per cell for IgG-opsonized sheep erythrocytes (eIgG), or PMA at 1 µg/ml) and reactions proceeded at 37°C. Samples were analyzed by flow cytometry using a FACSCalibur with CellQuest software (Becton Dickinson, San Diego, CA), gathering the FL-1 signal from a gated sample of 10,000 cells.

2.5.9 Neutrophil Degranulation Assay

Human neutrophils (10^7/ml) were treated with cytochalasin B at 5 µM for 5 minutes at 37°C to direct degranulation to the plasma membrane (Bjerknes et al., 1995). Drug treatments were conducted as described above, and infections were initiated by adding 300 µl cells to 200 µl of test stimuli before incubation at 37°C for 10 min. Infections were stopped on ice and cells were pelleted by centrifugation at 2,400 g for 3 min at 4°C. The cell pellet was washed in HBSS and fixed in 1% PFA. Samples were split, staining half with 2.5 µg of 9A6 anti-CEACAM6 (primary granule marker), and the other half with 1 µg of 80H3 anti-CEACAM8/CD66b (secondary granule marker), followed by an RPE-conjugated secondary, in a total volume of 50 µl. Flow cytometry was conducted as above, gathering the FL-2 signal from a gated sample of 10,000 cells.
2.5.10 Bacterial Survival Assay in Human Neutrophils

PMN were treated with inhibitors as described above, and seeded \(5 \times 10^5\) onto FBS-treated coverslips placed in 24-well plates that had been washed twice with PBS. Cells were spun onto coverslips at 253 \(g\), and bacteria were added to an MOI of 15 followed by centrifugation to promote interaction between the bacteria and PMN. Plates were then shifted to 37\(^\circ\)C for 30 min to allow bacterial invasion, after which one plate was washed and fixed for immunofluorescence microscopy to allow bacterial invasion to be assessed. Gentamicin (150 \(\mu g/ml\)) was added to other plates to kill extracellular bacteria, and cells were shifted to 37\(^\circ\)C for indicated times. Post-infection, cells were washed with HBSS to remove gentamicin and lysed before dilution plating.

2.5.11 Statistical Analyses

Immunofluorescence experiments and plating assays were conducted with triplicate samples, and standard deviations were calculated from averages between three individual coverslips (25 cells counted per coverslip) from a single representative experiment. All results depicted in this study are representative of those obtained from at least 3 independent experiments, each conducted on separate days. Where indicated, a two-tailed Student’s t-test assuming equal variance was used to compare the significance of differences observed, taking into account all cells from each condition of a particular experiment. \(p\) values of < 0.05 were deemed significant, however specific limitations are listed separately for each figure.
CHAPTER 3.
THE ITAM-CONTAINING CEACAM3 RECEPTOR GOVERNS NEUTROPHIL RESPONSES TO PATHOGENIC NEISSERIA

Helen Sarantis and Scott D. Gray-Owen

**Author Contributions:**

Helen Sarantis and Scott Gray-Owen designed the experiments, analyzed research, and wrote the paper.

Helen Sarantis contributed all figures.
3.1 Abstract
Symptomatic infection with the bacterial pathogen *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease, gonorrhea, involves massive infiltration of polymorphonuclear neutrophils (PMNs) to the infection site. Neisserial species interact directly with these immune cells in an opsonin-independent manner, via interactions between bacterial Opa proteins and members of the human carcinoembryonic antigen related-cellular adhesion molecule (CEACAM) family. In this work, we use a genetic approach to assess the specific contribution of individual human CEACAM receptors in the PMN response to *Neisseria*. We show that all Opa-binding CEACAMs of PMNs (CEACAM1, CEACAM3 and CEACAM6) can capture and engulf *N. gonorrhoeae*. In striking contrast to this, however, we show that only the immunoreceptor tyrosine-based activation motif (ITAM)-containing CEACAM3 receptor signals for the neutrophil oxidative burst and degranulation functions in response to neisserial infection, in a manner dependent on the tyrosine kinase, Syk. This work shows that previously observed CEACAM-dependent bactericidal responses of neutrophils are instead CEACAM3-dependent, and solidifies the role of this receptor in the innate immune response to CEACAM-binding bacterial pathogens.

3.2 Introduction
Diseases caused by pathogenic neisserial species such as *Neisseria meningitidis* (causative agent of bacterial meningitis) and *Neisseria gonorrhoeae* (causative agent of the sexually transmitted disease, gonorrhea) are often characterized by a prominent influx of polymorphonuclear neutrophils (PMNs) to the infection site. Neutrophils are considered one of the first lines of defense against invading microbes, through their rapid recruitment to infected tissues where they perform antimicrobial functions including phagocytosis, degranulation, and mobilization of the oxidative burst (Brinkmann and Zychlinsky, 2007). While neutrophils express a variety of receptors capable of binding opsonins such as serum complement components or immunoglobulin, the interaction between neisserial species and PMNs is opsonin-independent (Swanson et al., 1974). Instead, these microorganisms bind to and activate neutrophils via their Opacity-associated (Opa) outer membrane proteins, some of which bind members of the CEACAM (carcinoembryonic antigen-related cellular adhesion molecule) family (Gray-Owen et al., 1997a; Virji et al., 1996).
To date, four CEACAMs have been shown to bind neisserial Opa proteins, namely CEACAM1, CEACAM3, CEACAM5, and CEACAM6 (Bos et al., 1998; Chen et al., 1997; Gray-Owen et al., 1997a). Human neutrophils express three of these CEACAMs (CEACAM1, CEACAM3, and CEACAM6), as well as CEACAM4 and CEACAM8, which do not bind Opa (Schmitter et al., 2007b; Gray-Owen et al., 1997b). It has long been established that Opa-mediated binding of *Neisseria* to PMNs results in bacterial killing through the binding and internalization of these pathogens, followed by mounting of the neutrophil oxidative burst and degranulation responses (Virji and Heckels, 1986; Rest et al., 1982). It is not clear, however, which specific CEACAMs are involved in these responses. All three Opa-binding CEACAMs of PMNs have been shown to mediate bacterial engulfment by transfected epithelial cell models (McCaw et al., 2004). However, reflective of their different cytoplasmic signaling motifs, studies of these receptors separately in a number of cell types, including lymphocytes (Boulton and Gray-Owen, 2002), epithelial cells (McCaw et al., 2004), and endothelial cells (Muenzner et al., 2001), have shown that they can elicit distinct cellular responses. Ligation of CEACAM1, which contains an immunoreceptor tyrosine-based inhibition motif (ITIM; V/L/Ix7YxL/V) (Daeron et al., 1995), results in the recruitment of phosphatases that can suppress tyrosine phosphorylation-based signaling cascades (Chen et al., 2008; Lee et al., 2008). In contrast, ligation of CEACAM3, which contains an activating sequence, the immunoreceptor tyrosine-based activation motif (ITAM; Yxx(L/I)x6-8YxxL/I) (Reth, 1989), results in kinase recruitment and the propagation of signaling (Schmitter et al., 2004; McCaw et al., 2003). Unexpectedly, bacteria that bind CEACAM1 and not CEACAM3 can elicit the typical activating response of neutrophils (Gray-Owen et al., 1997b; Belland et al., 1992). It is, therefore, of interest to determine how ligation of each different CEACAM contributes to the observed responses of PMNs to neisserial infection.

In this work, we have used a genetic approach to study the roles of CEACAM1, CEACAM3 and CEACAM6 in the neutrophil. We show that all three CEACAM receptors can bind and engulf *N. gonorrhoeae*, yet only the ITAM-containing CEACAM3 receptor is capable of triggering the oxidative burst and degranulation responses of PMNs. This receptor, in the absence of other Opa-binding CEACAMs, initiates ITAM-dependent signaling, leading to the phosphorylation of the key immune response protein Syk, and the guanidino nucleotide

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exchange factor, Vav. Interestingly, CEACAM1, which we show can be phosphorylated in PMNs, and has been shown to be inhibitory in other systems (Boulton and Gray-Owen, 2002), does not suppress CEACAM3 function in PMNs. Surprisingly, we observed that CEACAM3 is capable of transducing signals resulting from CEACAM1 ligation. Our work shows that while CEACAMs play an important role in the binding and internalization of Neisseria species by PMNs, the previously well-described CEACAM-dependent bactericidal responses of PMNs can be solely attributed to the CEACAM3 receptor.

3.3 Results

3.3.1 CEACAMs -1, -3, and -6 all contribute to bacterial uptake in neutrophils

In order to delineate the individual functions of CEACAMs -1, -3 and -6 in the neutrophil, we used a ‘knock-in’ approach where we introduced individual human CEACAMs into mouse promyelocytic cells (MPRO) (Tsai and Collins, 1993). Rather than being immortalized by an oncogenic event, the normal differentiation of MPRO cells is arrested by the introduction of a dominant-negative retinoic acid receptor alpha (RARα), a defect which can be overcome with supra-physiological doses of retinoic acid. The MPRO model allowed us to circumvent two issues: one, since the cells are murine, they contain no Opa-binding CEACAMs, and Opa-dependent effects could be attributed to introduced human CEACAMs (Figure 3.1B); and two, since the cells are promyelocytes, we could create stable cell lines for propagation in culture, which could be differentiated in vitro to mature, functional neutrophils (Lian et al., 2001; Lawson et al., 1998).

We introduced cDNAs encoding human CEACAM1-L, CEACAM3-L and CEACAM6, as well as the empty vector (puro) into MPRO cells using retroviral transduction, to create monoclonal, stable cell lines. All three human CEACAMs were expressed in much the same manner as in human neutrophils, with CEACAMs -1 and -6 in larger quantities than CEACAM3 (Figure 3.1A). Flow cytometric analysis showed that the individual CEACAMs were surface-expressed (data not shown). All three CEACAM-expressing cell lines were capable of binding N. gonorrhoeae in a manner reflecting their expression level, such that MPRO-CEACAM3 PMNs tended to bind fewer bacteria, on average, than did MPRO cells.
Figure 3.1. Human CEACAM expression in murine neutrophils.
(A) Human CEACAMs are expressed in MPRO neutrophils. 1. MPRO-puro (transfected with empty vector); 2. MPRO-CEACAM1L; 3. MPRO-CEACAM3L; and 4. MPRO-CEACAM6. Cells were differentiated for 72 hours, pelleted, and lysed in RIPA. Human CEACAMs were immunoprecipitated with a rabbit polyclonal anti-CEA antibody, and detected after SDS-PAGE with a mouse monoclonal against all Opa-binding CEACAMs (D14HD11). (B) Murine neutrophils do not bind *N. gonorrhoeae*, and human neutrophils bind *N. gonorrhoeae* in an OpaCEA-dependent manner. Bone marrow neutrophils from wild-type FVB mice were infected with *N. gonorrhoeae* either lacking (Opa-) or expressing an Opa adhesin that binds CEACAMs (OpaCEA). Neither strain was able to bind efficiently to murine neutrophils. The same experiment was done with human neutrophils, and it was observed that binding to these cells occurred in an Opa-dependent manner. (C) All human CEACAMs are capable of binding and internalizing Opa-expressing *N. gonorrhoeae*. Differentiated MPRO cells were infected on glass coverslips with *N. gonorrhoeae* expressing OpaCEA for 30 minutes at 37°C at an MOI of 25, followed by fixation with paraformaldehyde. Intra- and extra-cellular bacteria were differentially stained as described previously (McCaw *et al.*, 2003), and quantified via immunofluorescence microscopy. (D) F-actin dynamics differ during bacterial internalization by CEACAM1, CEACAM3 and CEACAM6. Cells were infected as in (C), except for a shorter duration (5 minutes at 37°C) and with an MOI of 50. MPRO-CEACAM3L (and to a lesser extent, MPRO-CEACAM6) neutrophils internalized *N. gonorrhoeae* with a prominent re-organization of F-actin at sites of bacterial attachment.
expressing CEACAM1 and CEACAM6 (Figure 3.1C, top panel). Despite this, each CEACAM-expressing cell line effectively engulfed Opa-expressing *N. gonorrhoeae* (Figure 3.1C, bottom panel). The mechanisms of uptake appeared to differ in terms of F-actin dynamics: engulfment of *N. gonorrhoeae* by CEACAM1 occurred largely without actin remodeling, while CEACAM3 and CEACAM6 (albeit less so) mediated engulfment that often coincided with the appearance of F-actin-rich structures (Figure 3.1D).

### 3.3.2 CEACAM3 is Distinct in its Ability to Elicit Neutrophil Oxidative Burst and Degranulation Responses

Once we had established that the human CEACAMs were functional in the murine background, we sought to study the contribution of the different CEACAMs to other neutrophil functions. Human neutrophils respond to stimulation with Opa-expressing *N. gonorrhoeae* by triggering an increased consumption of oxygen, resulting in the production of oxygen radicals in the cell (the ‘oxidative burst’ response; Figure 3.2A), as well as by initiating the release of neutrophil granule components to the cell surface or into the newly formed phagosome (a process known as ‘degranulation’; Figure 3.2B). Strikingly, the MPRO-CEACAM3 neutrophils mirrored the response seen in human neutrophils (Figure 3.2C and 3.2D). Importantly, neither CEACAM1 nor CEACAM6 could contribute to either of these neutrophil functions, even though both receptors were fully capable of binding and internalizing the bacteria (Figure 3.1C). These data are strong evidence that CEACAM3 is solely responsible for the human neutrophil response to *N. gonorrhoeae*.

### 3.3.3 Mounting of the Neutrophil Oxidative Burst and Degranulation Functions, but not Internalization, is Dependent on the CEACAM3 ITAM

CEACAM3 is both neutrophil-restricted, and harbors an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain, an amino acid motif shared by many important receptors in the innate and adaptive immune systems (Reth, 1989) (Chen and Gotschlich, 1996). We suspected that the ITAM was responsible for the functions we observed in the MPRO-CEACAM3 neutrophils. To test this hypothesis, we constructed cell lines expressing CEACAM3 proteins with either one (MPRO-CEACAM3-Y230F or MPRO-
Figure 3.2. **CEACAM3 governs the CEACAM-dependent responses of PMNs to *N. gonorrhoeae* infection.**

(A) The CEACAM-dependent oxidative burst response in human neutrophils. Human neutrophils were treated with the oxidative burst reagent DHR-123, and then infected with Opa- *N. gonorrhoeae* (dashed line), OpaCEA-expressing *N. gonorrhoeae* (which bind CEACAMs; black line), PMA (gray line), or left uninfected (gray histogram). Bacterial multiplicity of infection was 10. After 60 minutes at 37°C, cells were fixed and the FL-1 signal was collected from a gated sample of 10,000 cells by flow cytometry to monitor the oxidative burst response. (B) The CEACAM-dependent degranulation response in human neutrophils. Human neutrophils were infected as in (A). Post-fixation, cells were stained with an antibody to the granule protein CEACAM6 (9A6) followed by a PE-conjugated secondary, to assess degranulation by flow cytometry (FL-2 channel). Histograms are labeled as in (A). (C) CEACAM3-dependent oxidative burst response to *N. gonorrhoeae* infection in murine neutrophils. Cells were treated with DHR-123, infected, and analyzed as in (A). While all differentiated MPRO cell lines are capable of mounting the oxidative burst to PMA treatment, only MPRO-CEACAM3 cells can carry out this response to *N. gonorrhoeae* infection, in an OpaCEA-dependent manner. (D) CEACAM3-dependent degranulation response to *N. gonorrhoeae* infection of murine neutrophils. Cells were infected and analyzed as in (B), except in murine neutrophil degranulation experiments we monitored the surface expression of the granule marker CD11b using a PE-conjugated rat anti-mouse CD11b antibody. Again, only MPRO-CEACAM3 neutrophils were capable of degranulating in response to *N. gonorrhoeae* infection, while all cells could degranulate in response to PMA treatment. In all panels, an arrow denotes a CEACAM-dependent (A and B) or CEACAM3-dependent (C and D) event.
CEACAM3-Y241F) or both (MPRO-CEACAM3-Y230F/Y241F) ITAM tyrosines mutated to phenylalanine, or the natural splice variant of CEACAM3 lacking the ITAM and most of the cytoplasmic domain (MPRO-CEACAM3-IC1). The various CEACAM3 mutant proteins were expressed in the MPRO cells (Figure 3.3A) and were surface expressed as assessed by flow cytometric analysis (data not shown). Curiously, an intact ITAM motif was not required for bacterial internalization (Figure 3.3B). This is in contrast with previous data (McCaw et al., 2003; Schmitter et al., 2004), and may reflect a more robust internalization process by PMNs, which unlike transfected epithelial models, are professional phagocytes. These data are in agreement with the ITAM-independent entry by CEACAM1 and CEACAM6 shown in Figure 3.1C. Importantly, we show that mutation of the CEACAM3 ITAM motif, even of just one of the two phosphorylatable tyrosines, is sufficient to eliminate the capacity of neutrophils to mount the oxidative burst (Figure 3.3C) and degranulation response in response to *N. gonorrhoeae* (Figure 3.3D). Thus, while internalization can occur in the absence of an ITAM, these bactericidal responses of neutrophils are strictly ITAM-dependent.

### 3.3.4 CEACAM3 activation couples to a highly conserved tyrosine phosphorylation-based signaling cascade involving the tyrosine kinase, Syk

Because of the distinct functional effects observed in MPRO-CEACAM3 neutrophils, we were interested in studying the signaling events that were involved. To this end, we infected the various MPRO neutrophils with Opa-expressing *N. gonorrhoeae* and assessed tyrosine phosphorylation of the individual CEACAMs. We show that both CEACAM1 and CEACAM3 are phosphorylated on tyrosine in response to *N. gonorrhoeae* infection (Figure 3.4A), while CEACAM6 was not (data not shown). Neither CEACAM1 nor CEACAM3 were phosphorylated in response to infection by *N. gonorrhoeae* lacking Opa expression (data not shown). We have shown previously that infection of human neutrophils by Opa-expressing *N. gonorrhoeae* leads to the phosphorylation of the tyrosine kinase, Syk, and that a pharmacological inhibitor of Syk prevents human neutrophil oxidative burst and degranulation responses to *N. gonorrhoeae* (Sarantis and Gray-Owen, 2007). We suspected that CEACAM3 was engaging Syk to activate these PMN bactericidal responses, as Syk family kinases have been shown to initiate signaling cascades required for PMN functions such as the oxidative burst (Kiefer *et al.*, 1998), through binding to phosphorylated ITAMs
Figure 3.3. The CEACAM3-dependent neutrophil response to *N. gonorrhoeae* is ITAM-dependent.

(A) CEACAM3 mutant alleles are expressed in MPRO cells. Cells were lysed and CEACAMs were immunoprecipitated as in Figure 3.1A. The tested cell lines are as follows: 1. MPRO-puro; 2. MPRO-CEACAM3-L; 3. MPRO-CEACAM3-Y230F (the first ITAM tyrosine mutated to phenylalanine); 4. MPRO-CEACAM3-Y241F (the second ITAM tyrosine mutated to phenylalanine); 5. MPRO-CEACAM3-IC1 (a natural short version of CEACAM3 lacking the ITAM altogether); 6. MPRO-CEACAM3-Y230/241F (both ITAM tyrosines mutated to phenylalanine). (B) Internalization of *N. gonorrhoeae* by CEACAM3 proceeds in an ITAM-independent manner in murine neutrophils. Cells were infected and analyzed as in Figure 3.1C. CEACAM3-L, CEACAM3-IC1, and CEACAM3-Y230/241F are all capable of internalizing *N. gonorrhoeae* (approximately 80% of bound bacteria are internalized after 30 minutes of infection at 37°C). (C) The CEACAM3-dependent oxidative burst response to *N. gonorrhoeae* is ITAM-dependent. Cells were infected and analyzed as in Figure 3.2C. Mutation of either ITAM tyrosine is sufficient to abolish the oxidative burst response to *N. gonorrhoeae*. Gray histogram: Opa-N. gonorrhoeae; black line, OpaCEA-expressing N. gonorrhoeae; gray line, PMA. (D) The CEACAM3-dependent degranulation response to *N. gonorrhoeae* is ITAM-dependent. Cells were infected and analyzed as in Figure 3.2D. Mutation of either ITAM tyrosine is sufficient to abolish the neutrophil degranulation response to *N. gonorrhoeae*. Histograms labeled as in (C).
Figure 3.4. CEACAM3-specific Syk and Vav activation in response to \textit{N. gonorrhoeae} infection of murine neutrophils.

(A) CEACAM1 and CEACAM3 are tyrosine phosphorylated in response to Opa\textsubscript{CEA}-expressing \textit{N. gonorrhoeae} infection of murine neutrophils. MPRO-puro, MPRO-CEACAM1 and MPRO-CEACAM3 cells that had been differentiated to neutrophils were infected with Opa-expressing \textit{N. gonorrhoeae} for 0, 2, 5 or 10 minutes at 37\textdegree C, and then lysed, followed by immunoprecipitation of phosphotyrosine-containing proteins. SDS-PAGE-resolved samples were then probed for CEACAM, to assess CEACAM phosphorylation. Both CEACAM1 and CEACAM3 were phosphorylated in response to neisserial infection. (B) CEACAM3 Activation Specifically Couples to Syk Activation in Neutrophils. Cells were infected and lysed as in (A), followed by resolution of whole cell lysates by SDS-PAGE and probing for activated forms of Syk (pSyk) and one of its downstream effectors, Vav (pVav). (C) Activation of Vav downstream of CEACAM3 is Syk-Dependent. Cells were pre-treated with the Syk-specific inhibitor, piceatannol, or vehicle (DMSO), and infected at 37\textdegree C for indicated times. Following infection, cells were lysed and whole cell lysates were resolved and probed as in (B).
via its paired SH2 domains (Indik et al., 1995). Probing of lysates from CEACAM-expressing MPRO neutrophils infected with *N. gonorrhoeae* showed that Syk was phosphorylated, as was its downstream effector, the guanidine nucleotide exchange factor, Vav, only in the presence of CEACAM3 (Figure 3.4B). Phosphorylation of both these effectors was sensitive to pharmacological inhibition of Src-family kinases, or Syk kinase itself (Figure 3.4C, and data not shown). These data show that CEACAM3 acts similarly to a number of ITAM-containing receptors that couple to an ancient, conserved signaling network, while maintaining the ability to uniquely respond to a specific pathogen (Abram and Lowell, 2007).

### 3.3.5 CEACAM3 Function is not Inhibited by CEACAM1 or CEACAM6

While we have shown that MPRO-CEACAM3 neutrophils function remarkably similarly to human neutrophils, we were curious as to how the introduction of other CEACAMs into the MPRO-CEACAM3 cells would affect their function. Specifically, CEACAM1 has been shown to lead to the inhibition of ITAM-containing receptor function through its phosphorylation and subsequent recruitment of inhibitory phosphatases (Lee et al., 2008; Boulton and Gray-Owen, 2002). Surprisingly, we observed that co-expression of CEACAM1 or CEACAM6 with CEACAM3 did not affect CEACAM3 function in neutrophils. Specifically, MPRO-CEACAM1+CEACAM3 and MPRO-CEACAM3+CEACAM6 neutrophils were capable of mounting both oxidative burst and degranulation responses to *N. gonorrhoeae*, indistinguishable from those seen in MPRO-CEACAM3 neutrophils (Figures 3.5A and 3.5B). Co-expression of a CEACAM1 protein lacking the inhibitory ITIM sequence (CEACAM1-S) with CEACAM3 functioned similarly to co-expression of CEACAM1-L (data not shown). This implies that the mechanisms of inhibition by CEACAM1 are receptor- and/or cell type-specific, and the lack of inhibition of CEACAM3 by CEACAM1 ligation as seen here may reflect the special environment of the neutrophil.

### 3.3.6 CEACAM3 can Transduce Activation Signals from other CEACAMs

Finally, we were interested to determine the outcome of gonococcal interactions with PMNs that did not involve binding to CEACAM3. It is known that hundreds of Opa variants exist in *N. gonorrhoeae* species, the majority of which bind CEACAM1, while only a select few bind CEACAM3, an observation we have attributed to selective pressures against CEACAM3.
Figure 3.5. Co-expression of CEACAM1 or CEACAM6 with CEACAM3 does not inhibit its function in murine neutrophils.

(A) We introduced CEACAM1 or CEACAM6 into MPRO-CEACAM3 cells to create two double-CEACAM-expressing cell lines, MPRO-CEACAM1+CEACAM3 and MPRO-CEACAM3+CEACAM6. Cells were differentiated for 72 hours and then treated with DHR-123 and infected as described in Figures 3.2C and 3.3C. The introduction of CEACAM1L or CEACAM6 into MPRO-CEACAM3 did not affect CEACAM3 function in response to *N. gonorrhoeae* infection. Histograms: uninfected (gray histogram), OpaCEA+expressing *N. gonorrhoeae* (black line), PMA-treated (gray line).

(B) Introduction of CEACAM1L or CEACAM6 into MPRO-CEACAM3 does not inhibit degranulation in response to *N. gonorrhoeae* infection. Cells were infected and fixed as in (A), followed by staining for the intracellular granule marker CD11b. Histograms are labeled as in (A)
binding *in vivo* (Virji et al., 1996) (Wong and Gray-Owen, unpublished). Importantly, Opa proteins that bind CEACAM3 always bind CEACAM1, while numerous Opa proteins exist that bind CEACAM1 and not CEACAM3 (Bos et al., 1998; Chen et al., 1997; Gray-Owen et al., 1997b). We took advantage of a non-CEACAM3-binding Opa protein of the *N. gonorrhoeae* MS11 strain, Opa59, to determine if binding to CEACAM1 alone could result in evasion of the CEACAM3 killing response. Consistent with this model, at a low multiplicity of infection we noted that the degranulation of MPRO-CEACAM1+CEACAM3 neutrophils was not stimulated by *N. gonorrhoeae* expressing the CEACAM1-specific Opa59 variant, but was observed when the cells were infected with an isogenic strain that instead expressed the CEACAM1- and CEACAM3-bispecific Opa57 (OpaCEA) (Figure 3.6A). Unexpectedly, however, when a higher multiplicity of infection was used, the MPRO-CEACAM1+CEACAM3 cells began to degranulate in response to CEACAM1 binding (Figure 3.6B). This required CEACAM3, as MPRO-CEACAM1 neutrophils did not degranulate in response to either gonococcal strain (Figure 3.2B and data not shown). These data suggest that CEACAM3 can transduce signals from other CEACAMs, albeit less effectively than through direct CEACAM3 engagement. This lends credence to a previous suggestion that CEACAMs may exist as a complex on the activated PMN surface (Singer et al., 2002).

### 3.4 Discussion

A great deal of research has sought to characterize molecular mechanisms of CEACAM functions in response to *N. gonorrhoeae* infection of host cells (Billker et al., 2002; McCaw et al., 2004; McCaw et al., 2003; Schmitter et al., 2004; Schmitter et al., 2007). The majority of this work has been conducted in transfected epithelial cell lines in an effort to study individual CEACAM functions in the absence of other Opa-binding CEACAMs expressed by PMNs (Schmitter et al., 2007b; McCaw et al., 2004; Billker et al., 2002). The use of epithelial cell models, however helpful, does not permit the study of PMN functions such as oxidative burst and degranulation. Furthermore, epithelial cells lack signaling proteins that are critical for CEACAM3 functions, such as the tyrosine kinase Syk (Sarantis and Gray-Owen, 2007). As such, we felt that studying these receptors in the natural context of the neutrophil was critical to understand its authentic function. With respect to the murine pre-neutrophil model, while mice express murine CEACAM1 (Ceacam1 and
Figure 3.6. A CEACAM1-specific Opa protein evades the CEACAM3-dependent killing response at low multiplicity of infection, but couples to CEACAM3 signaling at high multiplicity of infection.

We used a CEACAM1-specific Opa variant (Opa<sub>59</sub>) to determine if bacteria could evade the CEACAM3-dependent neutrophil response. Differentiated MPRO-puro, MPRO-CEACAM3 and MPRO-CEACAM1+CEACAM3 cells were infected with Opa<sub>59</sub>-expressing <i>N. gonorrhoeae</i> (dashed line), OpaCEA-expressing <i>N. gonorrhoeae</i> (black line), PMA-treated (gray line), or left untreated (gray histogram). Cells were then fixed, stained for surface appearance of the intracellular granule marker CD11b, and then analyzed via flow cytometry for FL-2 signal. While only CEACAM3-binding bacteria could stimulate degranulation in MPRO-CEACAM3 and MPRO-CEACAM1+CEACAM3 neutrophils at low MOI (thus, Opa<sub>59</sub> could ‘evade’ the CEACAM3 immune response in this situation) (A), at high MOI, bacterial binding through CEACAM1 was sufficient to mediate a signal through CEACAM3 (B). This did not occur with MPRO-CEACAM3 cells, which are incapable of binding Opa<sub>59</sub>. 
Ceacam2) (Robitaille et al., 1999), neither bind neisserial Opa proteins, allowing for a clean background to introduce Opa-binding human CEACAM receptors. MPRO cells were created by transducing bone marrow cells ex vivo with a dominant negative form of the retinoic acid receptor (RARα), a neutrophil differentiation factor, which resulted in cells arrested at the promyelocyte stage of neutrophil differentiation (Tsai and Collins, 1993). Addition of supraphysiological concentrations of retinoic acid is sufficient to overcome the arrest, likely stimulating endogenous RARα proteins that are not associated with the dominant negative form, allowing the cells to proceed along the normal PMN differentiation pathway. These cells are, therefore, completely normal PMNs once differentiation is complete, not transformed or defective as are leukemic cell lines. Indeed, it has been shown that HL-60 cells (Breitman et al., 1980) lack the ability to undergo chemotaxis in response to f-Met-Leu-Phe (fMLP) (Sirak et al., 1990), and both HL-60 and NB4 (Lanotte et al., 1991) mis-sort secondary granule proteins such as CD11b/CD18 (Gregoire et al., 1998; Le Cabec et al., 1997), while these and other functions are normal in MPRO-differentiated PMNs (Gaines et al., 2005; Lian et al., 2001; Lawson et al., 1998).

One of the major impetuses for studying the various PMN CEACAMs in isolation was the capacity of both CEACAM1 and CEACAM3 to transduce phosphotyrosine-based signals (Skubitz et al., 1995). To our knowledge, this is the first study that has looked at the interaction between these two ‘opposing’ receptors in detail. We suspect that the lack of inhibitory effects of CEACAM1 on CEACAM3 signals in neutrophils can be attributed to the special characteristics of the PMN environment. For instance, it has been shown that an invariant cysteine residue present in the catalytic motif of tyrosine phosphatases (proteins recruited to phosphorylated ITIMs that serve to dephosphorylate ITAMs and downstream effectors) is accessible to oxidation, and this has been proposed as a way in which PMNs prolong tyrosine phosphorylation to carry out their functions (Tonks, 2005). This might explain why CEACAM1 binding does not cause dephosphorylation of CEACAM3 in PMNs. Consistent with this premise, co-expression of CEACAM1 and CEACAM3 in epithelial cells leads to a loss of CEACAM3 phosphorylation in response to N. gonorrhoeae infection (Sarantis and Gray-Owen, unpublished data), suggesting that CEACAM1-dependent phosphatases dephosphorylate CEACAM3 in this context. This contrast underlies the importance of studying these receptors in their natural cellular environment.
An unexpected result emanating from this work was the capacity for a CEACAM1-binding Opa protein to elicit CEACAM3-dependent PMN activation. Whether this is a CEACAM-specific co-operative response (as suggested by Singer et al., 2002) or a result of a non-specific clustering of receptors on PMNs, such as through presence in similar membrane micro-environments, remains to be determined. One characteristic of certain ITAM-containing proteins is their ability to associate with transmembrane regions of other proteins via charged amino acid residues (Call and Wucherpfennig, 2007). Visual inspection of the transmembrane regions of CEACAM1 and CEACAM3 reveals the presence of a number of amino acid residues that could be involved in this type of binding. Co-operative signaling could also be the result of shared signaling pathways, as has been shown by the intersection between Dectin-1 and TLR signaling cascades (Gross et al., 2006). With respect to the lack of activating signals to CEACAM1-binding bacteria at low multiplicity of infection (Fig. 6A), it is of interest that there is variability in PMN responses to gonococci bearing different Opa variants (Gray-Owen et al., 1997b; Belland et al., 1992). It it has also been observed in some clinical cases of gonorrhea that some gonococci in urethral smears are not associated with PMNs (King et al., 1978). It is interesting to note that the ability of different Opa variants to activate PMNs had been speculated to be a product of the Opa proteins themselves, as a result of variable sequence conferring different affinities for a single receptor (Belland et al., 1992). In light of our data, we suspect these variabilities may reflect the specific outcome of binding to CEACAM3 rather than direct binding to CEACAM1 or CEACAM6.

While the number of physiological functions attributed to the CEA family is vast, ranging from cell adhesion and insulin signaling to angiogenesis, the only known role for CEACAM3 in human tissues is as a receptor for the Gram-negative, human-restricted pathogens *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Moraxella catarrhalis*, and *Haemophilus influenzae* (Schmitter et al., 2004). This peculiar observation has been explained by ourselves and others as a function of co-evolution between pathogen and host. Because CEACAMs -1, -5, and -6 are useful for colonization (Muenzner et al., 2000; Wang et al., 1998) as well as immune evasion (Boulton and Gray-Owen, 2002), while CEACAM3 is generally deleterious for bacteria, we have speculated that CEACAM3 represents a 'molecular mimic' that serves to neutralize CEACAM-utilizing bacterial pathogens. The data in this work confirm the role of CEACAM3 as an immunoreceptor in neutrophils, and
clearly implicate the acquisition of its ITAM as a central strategy in which this receptor can mediate bacterial clearance. We also provide evidence for the evasion of the PMN response by bacteria which cannot bind CEACAM3, which may explain previously described patterns of PMN activation by Opa variants.

3.5 Methods

3.5.1 Reagents and Antibodies
All reagents were from Sigma (Oakville, ON) unless otherwise indicated. The diisopropyl fluorophosphate (DFP) was from BioShop (Burlington, ON). PP2 was from Calbiochem (La Jolla, CA). The anti-gonococcal polyclonal rabbit antibody (UTR01) was described previously (McCaw et al., 2003). The rabbit anti-CEACAM polyclonal and normal rabbit serum were from Dako (Mississauga, ON). The CEACAM-specific mouse monoclonal D14HD11 and the CEACAM6-specific monoclonal 9A6 were from Genovac GmbH (Freiburg, Germany. Fluorescent conjugates were from Jackson ImmunoResearch Laboratories (Mississauga, ON), except for Texas Red-phalloidin, which was from Molecular Probes (Eugene, OR). The anti-phospho Syk and anti-phospho Vav antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The rat anti-mouse CD11b antibody was from BD Biosciences (Mississauga, ON).

3.5.2 MPRO Cell Culture and Differentiation to Neutrophils
The MPRO cells were purchased from the American Type Culture Collection, and were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 2-5% conditioned HM-5 supernatant, 10% horse serum (Invitrogen) and 1% Glutamax supplement (Invitrogen). The HM-5 GM-CSF producing-cell line was maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (Hyclone; Logan, UT), and was a gift from Dr. Steven Collins (Fred Hutchinson Cancer Research Center, Seattle, WA). GM-CSF-conditioned media was prepared as described in Lawson et al, 1998. MPRO differentiation was induced with 10 µM all-trans retinoic acid (ATRA; Sigma) in normal growth medium for roughly 72 hours, after which cells were washed and
maintained in Hank’s balanced salt solution (HBSS; Invitrogen; Burlington, ON) supplemented with 10 mM HEPES (Sigma), adjusted to a pH of 7.4), or Medium 199 (Invitrogen).

### 3.5.3 Recombinant DNA Constructs and Establishment of Human CEACAM-expressing MPRO Cells

Plasmids containing CEACAM1 and CEACAM6 in pRC/CMV were provided by Dr. Wolfgang Zimmermann (Munchen, Germany), and plasmids containing CEACAM3 WT, short (IC1), and mutant cDNAs in pCEP4 were created by Dr. A. Popp and kindly provided by Prof. T. F. Meyer (Max-Planck-Institut fuer Infektionsbiologie, Berlin, Germany). cDNAs were amplified (5’ primers contained a Kozak sequence, GCC ACC ATG, for protein expression) via polymerase chain reaction, and subcloned into pMSCVpuro (all cDNAs; Clontech Laboratories, Inc.) or pMSCVblast (a recombinant pMSCVpuro in which the puromycin resistance gene had been swapped for a blastocidin resistance gene from pCDNA6 (Invitrogen)) (CEACAM1 and CEACAM6 cDNAs only, for creation of MPRO-CEACAM1+CEACAM3 and MPRO-CEACAM3+CEACAM6 cell lines). These constructs were introduced into un-differentiated MPRO cells using a pantropic retroviral expression system (Clontech Laboratories, Inc.) as per manufacturer instructions. Briefly, the packaging cell line, GP-293, was co-transfected with the respective pMSCV vector containing CEACAM cDNA as well as pVSV-G, using FuGene 6 (Roche), as per manufacturer instructions. After 48 hours, virus-containing supernatants were collected and concentrated by ultra-centrifugation as described by (Zhou et al., 2005) at 120,000 x g for 2 hours at 4°C, and then un-differentiated MPRO cells were infected with the VSV-G-pseudotyped virus by centrifuging the cells with the concentrated virus preparation for 2 hours at 3,000 x g at room temperature. The infected MPRO cells were left overnight at 37°C, and selected the following day with 10 µg/ml puromycin (Sigma) (for pMSCVpuro-containing virus) or 10 µg/ml blastocidin (Invitrogen) (for pMSCVblast-containing virus). Single drug-resistant cells were then cloned to create monoclonal, stable cell lines, which were differentiated to PMN using ATRA (see above) for use in experiments.
3.5.4 Bacterial Strains

The Opa− (N302), Opa59 (N306), and OpaCEA (N313) neisserial strains were kindly provided by Dr. T. F. Meyer (Max-Planck-Institut fuer Infektionsbiologie, Berlin, Germany), and have been described previously (Kupsch et al., 1993, Gray-Owen et al., 1997b).

3.5.5 Immunofluorescence Microscopy

5 x 10^5 MPRO PMN were centrifuged onto FBS-coated coverslips (that had been washed 2 times with HBSS) at 63 x g for 10 minutes. Cells were then infected at a multiplicity of infection of 25 in a volume of 500 µl, and re-centrifuged for 5 minutes with no brake to facilitate bacterial association with cells. The cells were then shifted to 37°C for 5 minutes (for F-actin co-localization studies) or 30 minutes (for binding and internalization studies). Post-infection, the cells were washed once with HBSS, and fixed using 3.7% paraformaldehyde. Cells were permeabilized using 0.4% Triton X-100, and stained and observed as described previously (McCaw et al., 2003).

3.5.6 Immunoprecipitation and Western Blot

For assessment of CEACAM tyrosine phosphorylation, prior to infection, MPRO PMN were treated with DFP at 1 mM for 15 minutes to prevent proteolytic degradation. Cells (5x10^6 per sample) were then infected with *N. gonorrhoeae* at a multiplicity of infection of 10. Infections were stopped by centrifugation at 2,400 g for 3 min at 4°C, and pellets were resuspended in 50 µl of PBS-pervanadate buffer (1 mM EDTA, 1 mM PMSF, 1 µg/mL each aprotinin, leupeptin, and pepstatin A, 1 mM NaF, 100 µM Na_3V_2O_4, 10 mM H_2O_2, and 50 µg/mL soybean trypsin inhibitor in PBS), and then lysed with 50 µl of radioimmunoprecipitation (RIPA) buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/mL each aprotinin, leupeptin, and pepstatin A, 1 mM NaF, 100 µM Na_3V_2O_4, 10 mM H_2O_2, and 50 µg/mL soybean trypsin inhibitor) containing 2% SDS, and left on ice for 15 minutes. Lysates were then topped up with 900 µl of RIPA and rotated for 30 minutes at 4°C. Following micro-centrifugation at 16,000 g for 15 minutes, lysates were immunoprecipitated with anti-phosphotyrosine antibody (4G10) for 2 h, incubated with Protein A-sepharose for 1 h, washed two times with RIPA, and boiled for 5 minutes. Immunoprecipitated samples were separated by SDS-PAGE and transferred to a
nitrocellulose membrane (Amersham Biosciences), the membrane was blocked with 5% milk in TBS with 0.05% Tween for 30 minutes at room temperature, incubated with appropriate primary and HRP-conjugated secondary antibodies, and chemiluminescent detection performed using ECL+ (Amersham Biosciences) (or ECL for anti-tubulin blots-Perkin Elmer). For assessment of CEACAM expression in MPRO cells, 5 x 10^6 cells were pelleted and resuspended in 1 ml of RIPA, and the protocol proceeded as described above, using a rabbit polyclonal to CEA (CEA-DAKO) for immunoprecipitation.

3.5.7 Primary Neutrophil Isolation
PMNs were isolated from citrated whole blood taken from healthy volunteers by venipuncture using Ficoll-Paque Plus (Amersham Biosciences; Buckinghamshire, England). Contaminating erythrocytes were removed by dextran sedimentation and hypotonic shock, as described previously (McCaw et al., 2003).

3.5.8 Whole Cell Phosphorylation Assays
10^6 MPRO PMN per sample were treated with 20 µM PP2, 50 µM piceatannol, or solvent (DMSO), as indicated, for 30 minutes at 37°C at a concentration of 10^7 cells/ml. PMN were then infected with N. gonorrhoeae at a multiplicity of infection of 10, in 250 µl of HBSS. Infections were stopped by centrifugation at 2,400 g for 3 min at 4°C, lysed in boiling SDS sample buffer, and boiled for a further 10 minutes. Samples were resolved and immunoblotted as described above.

3.5.9 Oxidative Burst Assay
10^6 MPRO PMN per sample were treated with 2 µM of dihydrorhodamine-123 (DHR-123; Sigma) for 20 minutes at 37°C at a concentration of 10^7 cells/ml prior to agonist treatment. Samples were then treated with agonists (N. gonorrhoeae at a multiplicity of infection of 10, unless otherwise indicated, or PMA at 1 µg/ml) in 500 µl of HBSS for 60 minutes at 37°C. Infections were stopped by centrifugation at 2,400 g for 3 min at room temperature, and cell pellets were fixed in 1% PFA prior to analysis by flow cytometry using a FACS-Calibur
with CellQuest software (Becton Dickinson, San Diego, CA), gathering the FL-1 signal from a gated sample of 10,000 cells.

3.5.10 Degranulation Assay

10^6 MPRO PMN per sample were treated with agonists as described above in 500 µl Medium 199 (Invitrogen; Burlington, ON) for 30 minutes at 37°C. Infections were stopped by centrifugation at 2,400 g for 3 min at room temperature. Cell pellets were fixed in 1% PFA, and stained with 1.25 µg of PE-conjugated rat anti-mouse CD11b in a total volume of 50 µl. Flow cytometry was conducted as above, gathering the FL-2 signal from a gated sample of 10,000 cells.
CHAPTER 4. TRANSGENIC MOUSE NEUTROPHILS EXPRESSING HUMAN CEACAMS REPLICATE THE RESPONSES OF HUMAN NEUTROPHILS TO NEISSERIAL INFECTION

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Author contributions:

Helen Sarantis and Scott Gray-Owen designed the experiments, analyzed research, and wrote the paper.

Carlos Chan, Clifford Stanners, and Michael Glogauer provided animals.

Helen Sarantis contributed all figures.
4.1 Abstract

Infection with the bacterium *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease, gonorrhea, commonly involves infiltration of polymorphonuclear neutrophils (PMNs) to the infected genital tract mucosa. *Neisserial* species can directly bind these immune cells via bacterial Opa protein interactions with members of the human carcinoembryonic antigen related-cellular adhesion molecule (CEACAM) family. In this work, we use a CEACAM-expressing transgenic mouse model, CEABAC, to study the role of human CEACAMs in the neutrophil response to *neisserial* infection. We show that PMNs from wild-type littermates are unable to bind *N. gonorrhoeae*, while CEABAC PMNs can bind, engulf, and mount the oxidative burst and degranulation responses upon infection with Opa-expressing *N. gonorrhoeae*. We have shown previously that of the Opa-binding CEACAMs of human neutrophils (CEACAM1, CEACAM3, and CEACAM6), the immunotyrosine-based activation motif (ITAM)-containing CEACAM3 is responsible for neutrophil-specific responses to *Neisseria gonorrhoeae*. In confirmation of this, we show that CEABAC neutrophils phosphorylate a number of proteins on tyrosine in response to infection with Opa-expressing *N. gonorrhoeae*, including the ITAM-binding Syk tyrosine kinase and its downstream effector, Vav. Vav is known to be a guanidinium exchange factor for Rho family GTPases, including Rac, which has been implicated in a number of neutrophil functions. We bred CEABAC mice with mice lacking the Rac isoforms of neutrophils, Rac1 or Rac2, to determine the role of these proteins in CEACAM-dependent responses. We show that genetic ablation of either Rac1 or Rac2 in PMNs is not sufficient to abolish the functions of phagocytosis, oxidative burst, and degranulation, suggesting either a redundancy in function, or the involvement of another GTPase. Finally, we show that CEABAC neutrophils, but not neutrophils from wild-type littermates, can produce the pro-inflammatory chemokines MIP-2 and MIP-1α in response to neisserial infection, providing an avenue for the progression of the immune response to *N. gonorrhoeae in vivo*.

4.2 Introduction

Infection with the bacterial pathogen, *Neisseria gonorrhoeae*, the causative agent of the sexually transmitted disease, gonorrhea, often coincides with the appearance of polymorphonuclear neutrophils (PMNs) at the site of infection. PMNs are known to be
important mediators of immune responses through their recruitment to infected tissues from the blood, where they perform a number of antimicrobial functions including the phagocytosis and subsequent killing of ingested pathogens through the activation of the oxidative burst and degranulation responses (Brinkmann and Zychlinsky, 2007). PMNs in urethral smears from individuals infected with *N. gonorrhoeae* are often decorated with intracellular- or extracellular-associated bacteria, an association that has been shown to be opsonin-independent (Swanson *et al.*, 1974). Rather, interactions between proteins on the bacteria themselves (members of the Opacity-associated, or Opa outer membrane protein family) have been shown to bind to certain human carcinoembryonic cellular adhesion molecules (CEACAMs) on PMNs (Chen and Gotschlich, 1996; Virji *et al.*, 1996; Gray-Owen *et al.*, 1997a). Specifically, Opa proteins of *N. gonorrhoeae* have been shown to bind CEACAMs -1, -3, -5 and -6, of which CEACAMs -1, -3 and -6 are expressed on human PMNs (Gray-Owen *et al.*, 1997a).

Infection by neisserial species is restricted to the human host. In our previous work, we have exploited the lack of tropism of *Neisseria* for murine tissues by introducing human CEACAMs into a murine neutrophil model to study individual CEACAM functions. In that study, we showed that all three Opa-binding human CEACAMs of PMNs (CEACAM1, CEACAM3, and CEACAM6) were capable of binding and internalizing *N. gonorrhoeae*, while only the ITAM-containing CEACAM3 receptor was required for the initiation of the neutrophil oxidative burst and degranulation responses displayed by human PMNs (Chapter 3). Recently, another human CEACAM-expressing murine model, the CEABAC transgenic mouse, has been generated. These mice were engineered to express a bacterial artificial chromosome (BAC) containing complete genes of human CEACAM3, CEACAM5, CEACAM6, and CEACAM7 (Chan and Stanners, 2004). Neutrophils from these animals are predicted to express human CEACAM3 and CEACAM6. Importantly, while CEACAM3 contains an immunoreceptor tyrosine-based activation motif (ITAM; YxxL/I(x)6-8YxxL/I) (Reth, 1989) in its cytoplasmic domain, CEACAM6 is linked to the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor, and thus cannot initiate intracellular signaling in response to activation. Indeed, in our previous work we have seen that triggering of CEACAM6 alone does not result in the tyrosine phosphorylation of key signaling proteins in response to infection with *N. gonorrhoeae* (Chapter 3). Importantly, the other Opa-binding CEACAM capable of initiating tyrosine-based signals in the neutrophil, CEACAM1 (which
contains an immunoreceptor tyrosine-based inhibition motif, or ITIM; V/L/IxYxxL/V (Daeron et al., 1995) was not introduced into the CEABAC animals. Thus, signals in response to infection of CEABAC PMNs by Opa-binding \textit{N. gonorrhoeae} can be attributed to CEACAM3 activation.

In this work, we exploit neutrophils from CEABAC mice to further explore the role of human CEACAMs in the neutrophil response to \textit{N. gonorrhoeae} infection. We show that CEABAC neutrophils behave similarly to human neutrophils, binding and internalizing \textit{N. gonorrhoeae} in an Opa-dependent manner, as well as stimulating the oxidative burst and degranulation responses. These results confirm our previous data that the presence of human CEACAMs is sufficient to change the tropism for murine cells such that they are able to bind \textit{N. gonorrhoeae}, and that the presence of CEACAM3 is sufficient to allow murine neutrophils to acquire the ability to mimic the human neutrophil response to these pathogens. In addition, we took advantage of mice knocked out for the Rho family GTPases Rac1 and Rac2 to clarify the role of these proteins downstream of CEACAM3. Rac has been implicated in CEACAM-dependent responses of human neutrophils, specifically the function of phagocytosis (Schmitter et al., 2004), and is known to be a component of the NADPH oxidase of neutrophils (Dinauer, 2003). We show that while Rac2 appears to contribute to the oxidative burst response of CEABAC neutrophils, neither Rac1 nor Rac2 are essential for this or other responses. Finally, we show that infection of CEABAC neutrophils by \textit{N. gonorrhoeae} induces the PMN-dependent secretion of the chemokines MIP-2 and MIP-1α. These data implicate CEACAMs in neutrophil capture and elimination of \textit{N. gonorrhoeae}, as well as the propagation and orchestration of the ensuing immune by stimulating the production of pro-inflammatory mediators.

\textbf{4.3 Results}

\textbf{4.3.1 CEABAC Neutrophils Express Human CEACAM3 and CEACAM6, and Bind \textit{N. gonorrhoeae} in an Opa-dependent Manner}

CEABAC transgenic mice were engineered to express a bacterial artificial chromosome (BAC) containing 187 kb of the human CEA gene cluster containing complete CEACAM3,
CEACAM5, CEACAM6, and CEACAM7 genes (Chan and Stanners, 2004). Studies of CEACAM expression in CEABAC tissues showed that the spatiotemporal expression patterns of CEACAM5, CEACAM6 and CEACAM7 were remarkably similar to that of humans (Chan and Stanners, 2004). This initial analysis did not establish the presence of CEACAM3 in CEABAC mice, since CEACAM expression by neutrophils was not considered. Therefore, we used purified populations of bone marrow neutrophils from CEABAC mice to assess CEACAM protein expression. Immunoblot of lysates from CEABAC neutrophils with a CEACAM-specific monoclonal antibody showed the presence of a ~35 kDa protein, the size of human CEACAM3 (Figure 4.1A), as well as a ~100 kDa protein corresponding to CEACAM6 (Figure 4.1.B). Flow cytometric analysis with antibodies specific for CEACAM3 and CEACAM6 showed that both proteins were surface expressed in CEABAC neutrophils (Figure 4.1C). *N. gonorrhoeae* is a human-specific pathogen, and does not bind murine cells (Chapter 3). Infection of CEABAC neutrophils with Opa-expressing *N. gonorrhoeae* resulted in bacterial binding and engulfment (Figure 4.1Dii), while bacteria that do not express Opa proteins were not recognized (Figure 4.1Di). Similarly, neutrophils from wild-type littermates did not bind *N. gonorrhoeae* (data not shown), illustrating the importance of CEACAM and Opa in neisserial interactions with host cells.

### 4.3.2 CEABAC Neutrophils Mount Oxidative Burst and Degranulation Responses to *Neisseria gonorrhoeae*

CEABAC neutrophils express two of three Opa-binding CEACAMs (CEACAM3 and CEACAM6) of human neutrophils. Based on our previous data that showed CEACAM3 was responsible for the responses of PMNs to *N. gonorrhoeae* (Chapter 3), we suspected that CEABAC neutrophils would behave similarly to human neutrophils because of their expression of CEACAM3. Indeed, we observed that CEABAC neutrophils mounted the oxidative burst (Figure 4.2A) and exocytosed intracellular granules (Figure 4.2B) in response to infection with Opa-expressing *N. gonorrhoeae*. Wild-type neutrophils were not able to mount either of these responses when infected with *N. gonorrhoeae*, but were able to respond to stimulation with the chemical agonist phorbol myristate acetate (PMA).
Figure 4.1. Human CEACAM expression in CEABAC neutrophils.

(A) CEACAM3 and (B) CEACAM6 are expressed in CEABAC neutrophils. Neutrophils \(5 \times 10^6\) from CEABAC mice and wild-type littermates were spun down and lysed in RIPA. Human CEACAMs were immunoprecipitated with a rabbit polyclonal anti-CEA antibody (Dako), and detected after SDS-PAGE with a mouse monoclonal against all Opa-binding CEACAMs (D14HD11). Expression of mouse Ceacam1 (used as a loading control) was assayed by probing whole cell lysates from \(10^6\) cells with the antibody CC1. (C) CEACAM3 and CEACAM6 are surface-expressed on CEABAC PMNs. \(10^6\) CEABAC PMNs or PMNs from wild-type littermates were spun down and fixed in 1% PFA in HBSS. Cells were then stained using the CEACAM3/CEACAM5-specific antibody, COL-1, the CEACAM6-specific antibody, 9A6, or an IgG\(_\text{2A}\) isotype, followed by a PE-conjugated secondary, to assess surface expression by flow cytometry (FL-2 channel). (D) Expression of human CEACAMs is sufficient to change tropism of murine neutrophils for \textit{N. gonorrhoeae}. Neutrophils from CEABAC mice were infected on FBS-coated coverslips with i) Opa\(^{-}\) (do not bind CEACAM) or ii) Opa\(^{\text{CEA}\text{-}}\)expressing \textit{N. gonorrhoeae} (bind CEACAMs -1, -3, -5 and -6) at an MOI of 25, for 30 minutes at 37°C. Cells were then washed and fixed with 3.7% PFA, and stained for intra- and extracellular bacteria as described previously (McCaw et al, 2003).
Figure 4.2. CEABAC neutrophils, but not those from wild-type mice, mount the oxidative burst and degranulate in response to infection by Opa-expressing *N. gonorrhoeae*.

(A) CEACAM-dependent oxidative burst responses in murine neutrophils. Neutrophils (10⁶) from wild-type or CEABAC mice were treated with the oxidative burst reagent DHR-123, and then infected with Opa⁺ (gray histogram) or OpACEA-expressing *N. gonorrhoeae* (black line) at an MOI of 10, or treated with PMA (gray line). After 60 minutes at 37°C, cells were fixed and the FL-1 signal was collected from a gated sample of 10,000 cells by flow cytometry to monitor the oxidative burst response. (B) CEACAM-dependent degranulation response in CEABAC neutrophils. Neutrophils (10⁶) from wild-type or CEABAC mice were infected with Opa⁺ *N. gonorrhoeae* (dotted line), OpACEA-expressing *N. gonorrhoeae* (black line), PMA (gray line), or left untreated (gray histogram). Bacterial multiplicity of infection was 10. After 30 minutes at 37°C, cells were fixed, and then stained with 2.5 µg of a FITC-conjugated anti-CD63 antibody. The FL-1 signal was collected from a gated sample of 10,000 cells by flow cytometry to monitor degranulation.
4.3.3 Infection of CEABAC Neutrophils with N. gonorrhoeae Results in a Tyrosine Phosphorylation Cascade Involving Syk and Vav

Infection of human neutrophils with N. gonorrhoeae results in the phosphorylation of multiple proteins on tyrosine, including Src family kinases (Hauck et al., 1998), Vav (Schmitter et al., 2007a), Syk (Chapter 2) and CEACAM3 itself (McCaw et al., 2003). CEACAM1 also encodes a tyrosine-based amino acid motif in its cytoplasmic domain that is phosphorylated upon neisserial binding (Boulton and Gray-Owen, 2002; Lee et al., 2008). We have shown previously that CEACAM3, in the absence of CEACAM1, can initiate tyrosine phosphorylation-dependent signaling in response to neisserial infection (Chapter 3). This finding is confirmed in CEABAC neutrophils, which undergo potent tyrosine phosphorylation in response to infection with Opa-expressing N. gonorrhoeae (Figure 4.3Ai). Importantly, neisserial infection also results in the phosphorylation of Syk (Figure 4.3Aii) and its downstream effector, Vav (Figure 4.3B), a pathway that we (Chapter 2 and 3), and others (Kiefer et al., 1998; Crowley et al., 1997), have shown to be important for the functions of oxidative burst and degranulation.

4.3.4 Genetic Ablation of Rac1 or Rac2 in PMN affects, but does not eliminate, the PMN Oxidative Burst Response

Rac has been shown to be important for neutrophil function through associations with F-actin, leading to phagocytosis and chemotaxis, and through its association with the neutrophil NADPH oxidase. Three isoforms of Rac exist, two of which are expressed in neutrophils (Rac1 and Rac2). These isoforms are highly homologous (92% identity) (Dinauer, 2003), but specific deletion of each isoform in mice has shown that they perform non-redundant functions in neutrophils. Specifically, Rac2 is thought to be involved in bacterial phagocytosis (Koh et al., 2005), oxidative burst (Glogauer et al., 2003), primary granule release (Abdel-Latif et al., 2004), and actin polymerization (Gu et al., 2003), while Rac1 controls directional navigation during chemotaxis (Sun et al., 2004). We bred mice that were knocked out in Rac2 (Rac2<sup>−/−</sup>) or conditionally knocked out in Rac1 in cells of the granulocyte/monocyte lineage (Rac1<sup>cre−/−</sup>) with CEABAC mice, and studied the phenotypes of neutrophils from their progeny with respect to the involvement of these Rac isoforms.
Figure 4.3. CEACAM-Specific Tyrosine Phosphorylation and Syk and Vav Activation in Response to N. gonorrhoeae Infection of CEABAC Neutrophils.

(A) Multiple proteins are phosphorylated on tyrosine in response to OpaCEA-expressing N. gonorrhoeae infection of CEABAC neutrophils. 1 x 10^6 PMN from CEABAC mice or wild-type littermates were infected with Opa- (CEABAC PMNs only) or OpaCEA-expressing N. gonorrhoeae at a multiplicity of infection of 25, for 2, 5, or 10 minutes at 37°C. Cells were then spun down and lysed, and samples were resolved by SDS-PAGE, and probed for phosphotyrosine-containing proteins (i) or an activated form of Syk (pSyk; ii). Mouse Ceacam1 protein is shown as a loading control (iii).

(B) CEACAM binding by N. gonorrhoeae results in Vav Activation in CEABAC Neutrophils. 10^6 cells were infected and lysed as in (A), followed by resolution of whole cell lysates by SDS-PAGE and probing for an activated form of Vav (pVav; i). β-tubulin is shown as a loading control (ii).
in CEACAM-specific functions. We observed that specific deletion of either isoform did not completely inhibit the neutrophil functions of oxidative burst, secondary granule exocytosis, and phagocytosis (Figure 4.4). Rac2 appeared to affect the magnitude of the CEACAM-dependent oxidative burst response (Figure 4.4A), while neither Rac isoform appeared to be necessary for degranulation in response to neisserial infection (Figure 4.4B). Interestingly, phagocytosis of *N. gonorrhoeae* was unaffected in Rac-deficient neutrophils (Figure 4.4C), which is in contrast to published data obtained using antisense-mediated protein knockdown (Schmitter *et al.*, 2004).

4.3.5 CEABAC PMNs Produce the Pro-Inflammatory MIP-2 and MIP-1α chemokines in response to Infection by Opa-expressing *N. gonorrhoeae*

A function of neutrophils that has been of heightened interest recently is their capacity to produce and release soluble mediators of inflammation, including chemokines (Scapini *et al.*, 2000). We were interested to see whether neutrophils could produce these chemokines in response to neisserial infection, and if this function was CEACAM-dependent. Strikingly, we observed that CEABAC neutrophils produce both the neutrophil-recruiting MIP-2, as well as the monocyte/T lymphocyte/DC/NK-recruiting MIP-1α in response to neisserial infection (Figure 4.5). These responses are not seen in PMNs from wild-type littermates, showing that they are CEACAM-specific. The release of these chemokines is rapid (can be seen an hour after infection), suggesting release from intracellular stores. This data provides evidence for a previously unrecognized effect of bacterial binding to CEACAMs driving the initiation and progression of the immune response *in vivo*.

4.4 Discussion

In this work, we have used a human CEACAM-expressing transgenic mouse model to study the contribution of CEACAMs in the neutrophil response to neisserial infection. Previously, we used a mouse pre-neutrophil cell line, MPRO (mouse promyelocyte), which was genetically arrested at an immature stage of neutrophil differentiation and thus could be propagated in cell culture and differentiated *in vitro* to neutrophils (Tsai and Collins, 1993), to assess the specific roles of the Opa-binding CEACAMs of PMNs. We showed that while
Figure 4.4. Genetic Ablation of Rac2 Affects, but does not Inhibit, the CEACAM-dependent oxidative burst response.

We crossed CEABAC mice with mice knocked out for Rac1 (Rac1<sup>−/−</sup>; conditional knockout) or Rac2 (Rac2<sup>−/−</sup>) to generate CEABAC<sup>+</sup>Rac1<sup>−/−</sup> and CEABAC<sup>+</sup>Rac2<sup>−/−</sup> mice. PMNs were isolated from these mice and their wild-type littermates (CEABAC<sup>+</sup>Rac1<sup>+/−</sup> or CEABAC<sup>+</sup>Rac2<sup>+/−</sup>) to assess the role of Rac in the CEACAM-dependent responses to <i>N. gonorrhoeae</i> infection. (A) While genetic ablation of Rac1 did not appear to affect any function tested, loss of Rac2 dampened the CEACAM-dependent oxidative burst response to <i>N. gonorrhoeae</i>. Cells were treated with Opa<sup>−</sup> (gray histogram) or Opa<sub>CEA</sub>-expressing (black line) <i>N. gonorrhoeae</i> at an MOI of 10, or treated with PMA (gray line). Experiment was done as described for Figure 4.2A. Arrow denotes dampening of CEACAM-dependent oxidative burst response in Rac2<sup>−/−</sup> cells. (B) Degranulation and (C) phagocytosis are unaffected by loss of either Rac1 or Rac2. In (B), cells are treated with Opa<sup>−</sup> (dotted line) or Opa<sub>CEA</sub>-expressing (black line) <i>N. gonorrhoeae</i>, PMA (gray line), or left untreated (gray histogram). Experiment was done as described for Figure 4.2B. (C). Cells (CEABAC<sup>+</sup>Rac2<sup>−+/−</sup> or CEABAC<sup>+</sup>Rac2<sup>−/−</sup>) were infected with Opa<sup>−</sup> or Opa<sub>CEA</sub>-expressing <i>N. gonorrhoeae</i> at an MOI of 25, infected at 37°C for 30 minutes, and fixed, followed by staining for intra- and extracellular bacteria as described in Methods. Cells were analyzed by IF microscopy for bound (black bars) and intracellular (white bars) bacteria.
Figure 4.5. CEABAC neutrophils release the pro-inflammatory chemokines MIP-2 and MIP-1α in response to infection with Opa-expressing *N. gonorrhoeae*.

We infected neutrophils from CEABAC mice and their wild-type littermates with Opa<sup>+</sup>, Opacea (binds all Opa-binding CEACAMs), and Opaccm1 (binds CEACAM1 only, which is not present in the CEABAC mice) -expressing *N. gonorrhoeae* at multiplicities of infection of 1, 10, and 25. Unstimulated cells and PMA-treated cells were used as negative and positive controls, respectively. Legend is as follows: 1- unstimulated; 2- Opa<sup>+</sup> Ngo, MOI 1; 3- Opaccm1 Ngo, MOI 1; 4- Opacea Ngo, MOI 1; 5- Opa<sup>+</sup> Ngo, MOI 10; 6- Opaccm1 Ngo, MOI 10; 7- Opacea Ngo, MOI 10; 8- Opa<sup>+</sup> Ngo, MOI 25; 9- Opaccm1 Ngo, MOI 25; 10- Opacea Ngo, MOI 25; 11-PMA. While wild-type and CEABAC PMNs produced similar amounts of MIP-1α and MIP-2 in response to PMA stimulation, only CEABAC PMNs could produce these chemokines in response to neisserial infection, in a dose-dependent manner (see CEABAC PMN, bars 4, 7, and 10).
all three of these CEACAMs (CEACAM1, CEACAM3, and CEACAM6) were capable of binding and internalizing \textit{N. gonorrhoeae}, only CEACAM3 was capable of initiating the PMN oxidative burst and degranulation responses (Chapter 3). We show here that normal bone marrow neutrophils from transgenic mice, termed CEABAC, that were engineered to express the complete genes of CEACAM3, CEACAM5, CEACAM6, and CEACAM7 (Chan and Stanners, 2004), behave remarkably similar to both human and MPRO-CEACAM3 murine neutrophils. This work both solidifies our results that CEACAM3 transduces signals downstream of Opa binding in PMNs, and further expands our knowledge of CEACAMs of PMNs with respect to the role of the GTPases Rac1 and Rac2, as well as the function of CEACAMs in the production of inflammatory mediators by neutrophils.

The coupling of extracellular signals to cellular responses in the immune system often involves molecular signaling cascades based on phosphorylation on tyrosine. In receptors without intrinsic signaling capacity, the presence of a phosphorylatable tyrosine-containing amino acid motif, the ITAM (immunoreceptor tyrosine-based activation motif; YxxL/Ix_{6-8}YxxL/I) (Reth, 1989) is sufficient to allow the propagation of tyrosine-based signals in response to receptor aggregation. We think that the acquisition of the ITAM by CEACAM3 has separated this receptor functionally from other Opa-binding CEACAMs on PMNs. Importantly, ITAMs can be found singly or in multiples in a number of diverse receptors, including those of the immunoglobulin superfamily (T cell receptor, B cell receptor, FcRγ chain) and the C-type lectin family (Dectin-1, Mincle). While the ligands for these receptors differ, the resulting signaling cascades are similar amongst these proteins, through the convergence of ITAM phosphorylation on the recruitment of the tyrosine kinase Syk (Abram and Lowell, 2007). Indeed, we see that Syk and its downstream effector, Vav, are phosphorylated in response to \textit{N. gonorrhoeae} infection of CEACAM3-expressing cells (Chapter 2; Chapter 3; Chapter 4).

With respect to the role of Rac in the CEACAM-dependent responses of neutrophils, it is interesting that genetic deletion of Rac2 did not fully abolish the neutrophil oxidative burst and phagocytosis responses, as these have been implicated as Rac2-dependent processes previously (Glogauer et al., 2003; Schmitter et al., 2004; Koh et al., 2005). CEACAM3-dependent entry has been shown to be F-actin dependent, and bacterial binding correlates with the formation of F-actin-rich phagocytic cups at sites of bacterial attachment (McCaw
It is possible that the lack of a defect in neisserial uptake by CEABAC\(^+\)Rac2\(^{-/-}\) neutrophils is attributable to residual uptake by CEACAM6, which we have shown to be phagocytic in neutrophils (Chapter 3) and which internalizes \textit{N. gonorrhoeae} in an F-actin independent manner (McCaw \textit{et al.}, 2004). Alternatively, the lack of a requirement of Rac2 or F-actin in the CEACAM-mediated uptake by PMNs may reflect \textit{N. gonorrhoeae} as a cargo. We have shown previously that the presence of Syk, which is required for phagocytosis by the Fc receptors (Kiefer \textit{et al.}, 1998), is not required for neisserial internalization, something we attributed to this bacterium’s small size and the high density of CEACAM-binding ligand (Opa) on its surface (Chapter 2). This may also explain the discrepancy seen in the requirement for Rac in phagocytosis. With respect to the role of Rac2 in the oxidative burst response, while Rac1 deletion in PMNs does not affect the oxidative burst response to fMLP, deletion of both Rac1 and Rac2 affects the oxidative burst more so than Rac2 deletion alone (Gu \textit{et al.}, 2003; Glogauer \textit{et al.}, 2003), suggesting some redundancy in function.

One of the most interesting results from this study involved the observation that CEACAM binding by Opa-expressing \textit{N. gonorrhoeae} resulted in chemokine release from PMNs. The release of the neutrophil-recruiting MIP-2 chemokine may explain the massive accumulation of neutrophils during gonorrhea. This infiltration has been thought to contribute to the permanent sequelae of \textit{N. gonorrhoeae} infection, including fallopian tube scarring, which is a cause of infertility in females. Interestingly, we also saw the release of MIP-1\(\alpha\) in response to neisserial infection, which is particularly exciting because of the capacity for this chemokine to recruit a broader spectrum of cell types, including eosinophils, basophils, monocytes, immature dendritic cells, NK cells, and T lymphocytes (Scapini \textit{et al.}, 2000). A problem with the study of chemokine release from neutrophils is the capacity for a minute amount of monocyte contamination to skew results, because monocytes have the capacity to produce a significantly larger amount of these chemokines than neutrophils. Importantly, with respect to leukocytes, CEACAM3 and CEACAM6 are only expressed in neutrophils, thus providing a clean background for this type of study. Therefore, this data suggests that the initiation of the immune response at the level of the neutrophil may contribute to the propagation and guidance of ensuing responses.
One of the major impediments to the study of *N. gonorrhoeae* infection has been the lack of an animal model of infection. This can be attributed to the intimate association between these organisms and their host. A number of host proteins required by *N. gonorrhoeae* for infection are lacking outside of primates, including (and likely not limited to) Opa-binding members of the CEA family, receptors for the neisserial pilus, as well as receptors for the iron transport protein transferrin (Cohen and Cannon, 1999). Transient colonization of the murine genital tract has been accomplished without these human proteins (Jerse, 1999) with the use of estrogen-treated female mice that have been treated with antibiotics to reduce their normal commensal flora. This study represents the first description of mice expressing a humanized neutrophil that phenocopies the human PMN response to *Neisseria gonorrhoeae*. Considering the central role played by these cells in the innate immune response, this bodes well for future studies aimed at understanding the immunopathology of infection by this important pathogen.

### 4.5 Methods

#### 4.5.1 Reagents and Antibodies

All reagents were from Sigma (Oakville, ON) unless otherwise indicated. The diisopropyl fluorophosphate (DFP) was from BioShop (Burlington, ON). The anti-gonococcal polyclonal rabbit antibody (UTR01) was described previously (McCaw *et al.*, 2003). The rabbit anti-CEACAM polyclonal and normal rabbit serum were from Dako (Mississauga, ON). The CEACAM-specific mouse monoclonal D14HD11, the CEACAM3/CEACAM5-specific mouse monoclonal COL-1, and the CEACAM6-specific mouse monoclonal 9A6 were from Genovac GmbH (Freiburg, Germany). The antibody to mouse Ceacam1 (CC1) was generously provided by Dr. Kathryn V. Holmes (University of Colorado Health Sciences Center, CO). Fluorescent conjugates were from Jackson ImmunoResearch Laboratories (Mississauga, ON), except for Texas Red-phalloidin, which was from Molecular Probes (Eugene, OR). The anti-phospho Syk and anti-phospho Vav antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The rat anti-mouse CD11b antibody was from BD Biosciences (Mississauga, ON).
4.5.2 Animals

All procedures were carried out in accordance with the Guide for the Humane Use and Care of Laboratory Animals and were approved by the University of Toronto Animal Care Committee. CEABAC mice expressing two copies of the BAC containing complete human CEACAM3, CEACAM5, CEACAM6, and CEACAM7 genes (Chan and Stanners, 2004) were generously provided by Dr. Clifford P. Stanners (McGill Cancer Centre, PQ). Mice conditionally knocked out for Rac1 in the granulocyte/monocyte lineage ($\text{Rac1}^{-/-}$; Glogauer et al., 2003) and Rac2-deficient mice ($\text{Rac2}^{-/-}$; Roberts et al., 1999) were provided by Dr. M Glogauer (University of Toronto, ON). Breedings to generate CEABAC mice with Rac1 or Rac2 knocked out in PMN involved the breeding of CEABAC transgenic male animals that were heterozygous for Rac1 or Rac2 ($\text{CEABAC}^{+}\text{Rac1}^{+/-}$ or $\text{CEABAC}^{+}\text{Rac2}^{+/-}$) with females that were CEABAC negative and homozygous deleted for Rac1 and Rac2 ($\text{CEABAC}^{-}\text{Rac1}^{-/-}$ or $\text{CEABAC}^{-}\text{Rac2}^{-/-}$). Genotyping for CEABAC, Rac1, Rac2, and LysM was carried out as described previously (Chan and Stanners, 2004; Sun et al., 2004).

4.5.3 Neutrophil Preparations

8 to 10-week old mice were killed by CO$_2$ inhalation. Femurs and tibias were removed and bone marrow was isolated and separated on a discontinuous Percoll gradient (80%/65%/55%). Neutrophils were recovered at the 80%/65% interface. After hypotonic lysis to remove red blood cells, neutrophils were kept at $10^7$/ml in Hanks balanced salt solution (HBSS), which was prepared as described previously (Sarantis and Gray-Owen, 2007).

4.5.4 Bacterial Strains

The Opa$^-$ (N302) and Opa$^{CEA}$ (N313) $N.~gonorrhoeae$ MS11 strains were kindly provided by Dr. T. F. Meyer (Max-Planck-Institut fuer Infektionsbiologie, Berlin, Germany), and have been described previously (Kupsch et al., 1993, Gray-Owen et al., 1997b).
4.5.5 Immunofluorescence Microscopy

5 x 10^5 neutrophils were centrifuged onto FBS-coated coverslips (that had been washed 2 times with HBSS) at 63 g for 10 minutes. Cells were then infected at a multiplicity of infection of 25 (for phagocytosis experiments) or 50 (for F-actin co-localization studies) in a volume of 500 µl, and re-centrifuged for 5 minutes with no brake to facilitate bacterial association with cells. The cells were then shifted to 37°C for 5 minutes (for F-actin co-localization studies) or 30 minutes (for binding and phagocytosis studies). Post-infection, the cells were washed once with HBSS, and fixed using 3.7% paraformaldehyde. Cells were permeabilized using 0.4% Triton X-100, and stained and observed as described previously (McCaw et al., 2003).

4.5.6 Immunoprecipitation and Western Blot

For assessment of CEACAM tyrosine phosphorylation, prior to infection, neutrophils (10^7/ml) were treated with DFP at 1 mM for 15 minutes to prevent proteolytic degradation. Cells (5x10^6 per sample) were then infected with N. gonorrhoeae at a multiplicity of infection of 10. Infections were stopped by centrifugation at 2,400 g for 3 min at 4°C, and pellets were resuspended in 50 µl of PBS-pervanadate buffer (1 mM EDTA, 1 mM PMSF, 1 µg/mL each aprotinin, leupeptin, and pepstatin A, 1 mM NaF, 100 µM Na$_3$VO$_4$, 10 mM H$_2$O$_2$, and 50 µg/mL soybean trypsin inhibitor in PBS), and then lysed with 50 µl of radioimmunoprecipitation (RIPA) buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 µg/mL each aprotinin, leupeptin, and pepstatin A, 1 mM NaF, 100 µM Na$_3$VO$_4$, 10 mM H$_2$O$_2$, and 50 µg/mL soybean trypsin inhibitor) containing 2% SDS, and left on ice for 15 minutes. Lysates were then topped up with 900 µl of RIPA and rotated for 30 minutes at 4°C. Following micro-centrifugation at 16,000 g for 15 minutes, lysates were immunoprecipitated with anti-phosphotyrosine antibody (4G10) for 2 h, incubated with Protein A-sepharose for 1 h, washed two times with RIPA, and boiled for 5 minutes. Immunoprecipitated samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences), the membrane was blocked with 5% milk in TBS with 0.05% Tween for 30 minutes at room temperature, incubated with appropriate primary and HRP-conjugated secondary antibodies, and chemiluminescent detection performed using ECL+ (Amersham Biosciences) (or ECL for anti-tubulin blots-Perkin Elmer). For assessment of CEACAM expression in CEABAC neutrophils, 5 x 10^6 cells
were pelleted and resuspended in 1 ml of RIPA, and the protocol proceeded as described above, using a rabbit polyclonal to CEA (CEA-DAKO) for immunoprecipitation. Mouse Ceacam1 expression was assessed in whole cell lysates from 10⁶ cells, using the CC1 antibody.

4.5.7 Whole Cell Phosphorylation Assays
10⁶ neutrophils per sample were infected with *N. gonorrhoeae* at a multiplicity of infection of 10, in 250 µl of HBSS. Infections were stopped by centrifugation at 2,400 g for 3 min at 4°C, lysed in boiling SDS sample buffer, and boiled for a further 10 minutes. Samples were resolved and immunoblotted as described above.

4.5.8 Oxidative Burst Assay
10⁶ neutrophils per sample were treated with 2 µM of dihydrorhodamine-123 (DHR-123; Sigma) for 20 minutes at 37°C at a concentration of 10⁷ cells/ml prior to agonist treatment. Samples were then treated with agonists (*N. gonorrhoeae* at a multiplicity of infection of 10, unless otherwise indicated, or PMA at 1 µg/ml) in 500 µl of HBSS for 60 minutes at 37°C. Infections were stopped by centrifugation at 2,400 g for 3 min at room temperature, and cell pellets were fixed in 1% PFA prior to analysis by flow cytometry using a FACSCalibur with CellQuest software (Becton Dickinson, San Diego, CA), gathering the FL-1 signal from a gated sample of 10,000 cells.

4.5.9 Degranulation Assay
10⁶ neutrophils per sample were treated with agonists as described above in 500 µl Medium 199 (Invitrogen; Burlington, ON) for 30 minutes at 37°C. Infections were stopped by centrifugation at 2,400 g for 3 min at room temperature. Cell pellets were fixed in 1% PFA, and stained with 1.25 µg of PE-conjugated rat anti-mouse CD11b in a total volume of 50 µl. Flow cytometry was conducted as above, gathering the FL-2 signal from a gated sample of 10,000 cells.
4.5.10 Chemokine Measurements

To assess MIP-2 and MIP-1α production by neutrophils, 10⁶ cells were infected with *N. gonorrhoeae* at various multiplicities of infection, or with PMA, in a total volume of 100 µl. After 1 hr incubation at 37°C, reactions were stopped by centrifugation at 2,400 g for 3 min at room temperature, and supernatants were collected and frozen at -80°C until ELISAs were performed. Quantitative measurements of MIP-2 and MIP-1α in culture supernatants were made by applying 50 µl of supernatant to the mouse CXCL2/MIP-2 and mouse CCL3/MIP-1 alpha Quantikine ELISA kits from R&D Systems (Minneapolis, MN), as per manufacturer instructions.
CHAPTER 5. SUMMARY AND FUTURE DIRECTIONS
5.1 Summary of Thesis Work

Just over a decade ago, four independent groups identified a group of proteins that served as cellular receptors for the neisserial Opa proteins (Virji et al., 1996; Gray-Owen et al., 1997a; Chen and Gotschlich, 1996; Bos et al., 1998). Since then, there has been a great deal of study on how these proteins, CEACAM1, CEACAM3, CEACAM5, and CEACAM6, mediate host cell responses to neisserial infection. A prominent conclusion emanating from these studies has been that the outcomes of interactions with these various receptors differs based on the specific receptor engaged, as well as the cellular environment in which the receptor is activated. These observations have been the impetus for my work with the human neutrophil-restricted receptor for Opa, CEACAM3.

My first area of study involved the characterization of the role of the tyrosine kinase, Syk, in the activation cascade downstream of CEACAM3 (Chapter 2). We were interested in the involvement of this protein because of the presence of the CEACAM3 ITAM, which we knew was phosphorylated on tyrosine upon stimulation with Opa-expressing bacteria (McCaw et al., 2003). Syk family kinases had been well-documented as initiators of cellular signaling downstream of phosphorylated ITAMs (Indik et al., 1995), and the generation of mice lacking Syk in myeloid cells (Kiefer et al., 1998) showed the importance of Syk in a number of neutrophil functions, including oxidative burst and phagocytosis. The results from our work showed that, indeed, Syk was recruited to CEACAM3 and activated upon neisserial stimulation, and that this interaction was important for the neutrophil killing functions of oxidative burst and degranulation. Interestingly, Syk did not affect the phagocytosis of *N. gonorrhoeae* by CEACAM3, something we attributed to the size of the bacteria and to the density of the CEACAM-binding ligand (Opa) on the bacterial surface. This particular result highlights how cargo differences (consider particles as different as opsonized sheep red blood cells versus a bacterium) can affect the results of a given experiment. They also show that CEACAM3 functions differ in their requirements for binding partners: both the work in Chapter 2 and the data in Figures 3.1 and 3.3 show that while the ITAM governs the neutrophil-specific functions of CEACAM3, phagocytosis is distinctly separate with respect to the signals required (Figure 5.1).
A. Epithelial cells:

B. Neutrophils:
Figure 5.1. Signaling events that occur upon CEACAM3 ligation by neisserial Opa proteins.

(a) Signaling pathways as elucidated in transfected epithelial cell lines. Binding of Opa-expressing *N. gonorrhoeae* to CEACAM3 results in Src-family kinase-dependent ITAM phosphorylation. This results in the recruitment and activation of Syk (large particles only, Chapter 2) and Vav, which can dock to either the ITAM directly (Schmitter et al., 2007), or can be activated downstream of Syk (Chapter 3). Vav activation results in the GTP-loading of Rac, which results in F-actin assembly, and the creation of the phagocytic cup. PLC-γ has also been shown to bind phosphorylated CEACAM3, which may influence actin dynamics. Class I PI 3-kinases are thought to be activated upon neisserial binding to CEACAM3, as accumulation of the signaling lipid PIP3 occurs at sites of bacterial entry, likely contributing to lipid remodeling at the forming pseudopods. Once inside the cell, the *Neisseria*-containing phagosome matures, in a process involving the creation and deposition of PI3P by class III PI 3-kinases, leading to an acidified, degradative lysosome that results in bacterial killing. (b) Signaling pathways as elucidated in neutrophil models. Binding of Opa-expressing *N. gonorrhoeae* to CEACAM3 results in Src-family kinase-dependent ITAM phosphorylation. This results in the recruitment and phosphorylation of Syk, which then leads to phosphorylation of Vav. If results in epithelial cells are recapitulated in neutrophils, presumably some level of Vav recruitment directly to the CEACAM3 ITAM also will occur. This signaling results in the mounting of the neutrophil oxidative burst and degranulation responses, which culminates in bacterial killing. While ITAM phosphorylation is seen upon neisserial binding to CEACAM3 in the neutrophil, mutation of the ITAM sequence in the two critical tyrosines does not affect entry (Chapter 3), thus leaving the mechanism of neisserial phagocytosis by the neutrophil in question. The Syk-dependent cascades are therefore necessary for activation of the neutrophil bactericidal response, but not bacterial uptake.
It is obvious that if we didn’t look at Syk function in the human neutrophil, we never would have realized what it was doing in the context of CEACAM3. The inability of epithelial cells to perform basic neutrophil functions such as oxidative burst and degranulation was the driving force for the rest of my thesis work (Chapters 3 and 4). The work that I present in Chapter 3 is important because it brings the role of CEACAM3 as an immunoreceptor to the forefront of CEACAM-dependent neutrophil responses to Neisseria. Since the discovery that these proteins served as bacterial receptors, a number of groups have studied interactions of individual CEACAMs with CEACAM-binding pathogens in various cell types. To my knowledge, this is the first time that the human CEACAMs of neutrophils, CEACAM1, CEACAM3, and CEACAM6 have been studied in this cell type in isolation. The importance of CEACAM3 for neutrophil oxidative burst and degranulation in response to neisserial Opa proteins shows that these responses, which have been observed for decades, can be attributed not only to this tiny receptor, but specifically to its ITAM. During the course of my thesis work, a number of ITAM-containing receptors have been brought to the forefront of study in microbiology and immunology. While ligand specificities differ for these various receptors, the fact that their signaling converges on the recruitment and activation of Syk-family kinases (Abram and Lowell, 2007) is phenomenal.

As a confirmation of my work in Chapter 3, and in an effort to expand our knowledge of CEACAM function in the neutrophil, I have also studied neutrophils from mice that were made transgenic for four human CEACAMs, CEACAM3, CEACAM5, CEACAM6 and CEACAM7 (Chan and Stanners, 2004) (Chapter 4). As we had seen previously, the acquisition of human CEACAMs by murine neutrophils was sufficient for the acquisition of tropism for these cells by human-restricted CEACAM-binding pathogens. In addition to providing new insights regarding neisserial phagocytosis and chemokine responses, the data in this chapter are encouraging for the development of murine models of infection to CEACAM-binding organisms.

5.2 Implications for Future Work

The data from my thesis work as well as the work of others (Schmitter et al., 2007a; Schmitter et al., 2004), and the recent interest in ITAM-containing receptors, raises a
number of interesting questions with respect to CEACAM3 function in response to *N. gonorrhoeae* infection. These are described below.

5.2.1 The Mechanisms of Bacterial entry via CEACAMs on Neutrophils

While the study of bacterial entry via the various Opa-binding CEACAMs has been studied greatly in the context of transfected epithelial cell lines, it would be of interest to continue these studies in the neutrophil. It is interesting that in our murine neutrophil model (Chapter 3), phagocytosis of *N. gonorrhoeae* by CEACAM3 and CEACAM6 was accompanied with F-actin assembly at sites of bacterial binding. How does this occur?

Stefanova and others (Stefanova et al., 1991) have shown that GPI-anchored proteins can interact with tyrosine kinases such as Lck. It would be interesting to see if the inhibition of tyrosine phosphorylation affected phagocytosis by CEACAM6 in our neutrophil model. The role of Vav, through its recruitment to the CEACAM3 ITAM and its activation of Rac, has been implicated in CEACAM3-mediated phagocytosis (Schmitter et al., 2007a), but how does this explain the entry we see in murine neutrophils expressing a CEACAM3 protein that lacks the ITAM? Could the mechanisms of entry via these various receptors be conserved, and are there other binding partners involved? This is an exciting point of study for the future.

5.2.2 CEACAM3 Associations with other Receptors in the Neutrophil

One of the most interesting results emanating from the work in Chapter 3 involved the capacity of CEACAM3 to transduce signals resulting from the binding of CEACAM1. It is important to determine if this result reflects a non-specific clustering of CEACAM3 in the vicinity of CEACAM1, or if this is a result of a specific interaction between CEACAMs on neutrophils. Visual inspection of transmembrane sequences of CEACAM1 and CEACAM3 reveals charged amino acid residues which could act as points of interaction between these proteins. It would be interesting to determine if such interactions do exist, and if they can be extended to other CEACAMs, or other transmembrane proteins of neutrophils.
5.2.3 Characterization of Co-operative Signals in Response to CEACAM3 Ligation

In light of recent observations that suggest functional interplay of signals emanating from diverse receptors (e.g. TLRs and Dectin-1), it would be interesting to further characterize signals downstream of CEACAM3 activation. CARD9 activation has been shown to link Syk activation via Dectin-1 to NF-κB activation (Gross et al., 2006), and has also been implicated downstream of DAP12, FcRγ, and certain TLRs (Hara et al., 2007). It would be of interest to determine signals further downstream of Syk with respect to CEACAM3 activation. Novel interactions could give an indication for co-operative signaling that could occur by aggregation of multiple immunoreceptors by *N. gonorrhoeae*.

5.2.4 Inhibitory CEACAM3 Signals?

An important concept that has emerged in the ITAM literature recently is the concept of how valency can affect signals downstream of ITAM-containing receptor ligation. The paradigm of 'inhibitory ITAM' signaling involves the distinction between a mild versus robust phosphorylation of ITAM containing-receptors, resulting in either the recruitment of SH2-domain containing phosphatases on singly-phosphorylated ITAMs ('mild' activation) versus fully phosphorylated ITAMs that are sufficient to recruit Syk and initiate cellular activation. It is interesting to note that I have observed the recruitment of the lipid phosphatase, SHIP-1, in response to gonococcal infection of HeLa-CEACAM3 cells that were transfected with a construct expressing a GFP-tagged SHIP-1. It would be of some interest to determine the effect of valency in outcomes of CEACAM3 ligation, using monoclonal antibodies that could be cross-linked with increasing concentrations of a secondary antibody, or a pansorbin array as was used in Chapter 2.

5.2.5 In vivo infection of a Humanized Transgenic Mouse Model

One of my main interests during the work with the CEABAC neutrophils was the capacity to test for CEACAM-dependent functions during *in vivo* infection. For the most part, the colonization of these animals with *N. gonorrhoeae* has been unsuccessful to date, likely due to their not being humanized 'enough' for infection by human-restricted organisms. It might be of interest to use the established murine model of infection (Jerse, 1999) in
CEABAC mice, in conjunction with neutrophil depletion (Daley et al., 2008) and re-establishment with neutrophils from wild-type littermates to determine the role of CEACAMs on neutrophils during in vivo infection. Introduction of other human elements required for neisserial infection (such as the pilus receptor, or the human transferrin receptor) will likely be required to produce a fully functional animal model for neisserial infection in the future.


