The role of C-Reactive Protein in immune responses to *Neisseria meningitidis*

by

Rosalyn Alice Casey

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Faculty of Health and Medical Sciences
University of Surrey

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Immunity to *Neisseria meningitidis* (*N. meningitidis*) involves both innate and antibody-mediated mechanisms. The acute-phase serum protein C-reactive protein (CRP) helps to protect the host from several bacterial pathogens, often by binding to surface phosphorylcholine (PC). Pathogenic *Neisseria* species can exhibit phase-variable PC modification on type 4 pili of class 1 and 2. This investigation demonstrates that CRP can bind to piliated meningococci in a calcium-dependent manner that is concentration-dependent, of low affinity and specific for PC. Separate experiments confirmed that CRP can bind to both live and paraformaldehyde-fixed meningococci, suggesting that CRP has a role in the immune response to meningococci.

CRP binding to PC on commensal species of *Neisseria* is known to increase serum bactericidal activity by initiating the classical complement cascade. In these investigations, CRP-opsonisation did not increase serum killing of *N. meningitidis*. CRP binding did, however, significantly increase phagocytic uptake of fixed meningococci by macrophages and neutrophils (and increased uptake by dendritic cells was also indicated). This CRP-mediated uptake was attributed to recruitment of the cellular receptor for CRP, FcyR, as blocking of these receptors with human IgG abrogated the increased uptake by macrophages in a concentration-dependent manner. In contrast, macrophage uptake of live meningococci was not increased by CRP-opsonisation and preliminary investigations detected no increase in intracellular killing of CRP-opsonised meningococci, indicating that live meningococci may evade CRP-mediated phagocyte detection.

The absence of a direct killing effect of CRP led to the hypothesis that CRP provides an indirect advantage to the host by modulation of inflammation and antigen presentation by phagocytes. CRP-opsonised *N. meningitidis* induced greater macrophage HLA-DR expression, IL-10 and IL-1β secretion compared to non-opsonised *N. meningitidis* in the majority of human donors. These studies indicate that CRP may increase antigen presentation and therefore accelerate the development of acquired immunity to meningococcal infection, whilst limiting the inflammatory damage to the host via increased IL-10 production.
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<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>APR</td>
<td>Acute phase response</td>
</tr>
<tr>
<td>AU</td>
<td>Arbitrary units</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain-heart infusion broth</td>
</tr>
<tr>
<td>BIC</td>
<td>Carbonate/bicarbonate buffer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>Complement</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees centigrade</td>
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<tr>
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<td>Columbia blood agar</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
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<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Enzyme-linked immunosorbant assay</td>
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<tr>
<td>Fab</td>
<td>Antibody binding fragment</td>
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<td>Divalent antigen binding fragment</td>
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<td>FACS</td>
<td>Fluorescence activated cell scanning</td>
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<td>FcγR</td>
<td>Receptor for Fc region of Immunoglobulin G</td>
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<td>FACS staining buffer</td>
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<tr>
<td>g</td>
<td>Gravity</td>
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<td>GM-CSF</td>
<td>Granulocyte/monocyte colony stimulating factor</td>
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<td>HLA-DR</td>
<td>Human leukocyte antigen</td>
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<td>Interferon gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Kd</td>
<td>Dissociation constant</td>
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<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
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<td>Late complement component deficiency</td>
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<td>Lipopolysaccharide</td>
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<tr>
<td>LOS</td>
<td>Lipoooligosaccharide</td>
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<td>MAC</td>
<td>Membrane attack complex</td>
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<td>Mannose-binding lectin</td>
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<td>mCRP</td>
<td>Modified CRP</td>
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<td>Milligram</td>
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<td>Multiplicity of Infection</td>
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<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphorylcholine</td>
</tr>
<tr>
<td>pg</td>
<td>Picogram</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium Iodide</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum amyloid P</td>
</tr>
<tr>
<td>SBA</td>
<td>Serum bactericidal assay</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium didecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SPA</td>
<td>Surfactant protein-A2</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
</tbody>
</table>
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CHAPTER ONE

GENERAL INTRODUCTION
The *Neisseriaceae* family of gram negative diplococci are mostly harmless commensals of mucosal tracts. There are, however, two pathogenic species; *Neisseria gonorrhoea* (*N. gonorrhoea*) and *Neisseria meningitidis* (*N. meningitidis*, or the meningococcus) which are capable of causing devastating local and systemic infections. *N. meningitidis* is an etiological agent of arthritis, pericarditis, metastatic sepsis, conjunctivitis, endophthalmitis, urethritis, urithritis and infant vulvovaginitis. The two most common diseases caused by *N. meningitidis*, however, are purulent meningitis and severe sepsis. Meningococcal disease is a global problem, causing around 1.2 million cases of disseminated disease and 135,000 deaths each year (Anonymous, 2006). Mortality rates can be high, depending on the clinical presentation of infection which ranges from meningitis (with mortality rates of 4-6%) to sepsis and septic shock (with mortality rates of up to 40%) (Emonts et al., 2003). This exclusively human pathogen can either cause virulent disease or live as a harmless commensal within different hosts. It is estimated that 10% of adults worldwide are asymptomatic carriers of *N. meningitidis*, which colonises the nasopharynx. In 25% of individuals, carriage is of months in duration, in another third of cases, carriage lasts from days to weeks and in the remaining 40% of cases, carriage is only transient (Tzeng and Stephens, 2000). The bacterium is transmitted between hosts by aerosol or contact with infected secretions.

*N. meningitidis* strains have historically been classified by serological typing methods based on capsule characteristics (serogroup), major outer membrane proteins (serosubtype and serotype) and lipooligosaccaride structures (immunotype). Thirteen serogroups have been identified with the five major groups being A, B, C, W135 and Y. Serogroup A isolates cause cerebrospinal meningitis in most countries, with certain genetically defined clones being associated with epidemics in Sub-Saharan Africa and Russia (Achtman, 1994). Serogroup B strains cause epidemics and local outbreaks mostly in the western world. In the past 50 years there have been serogroup B outbreaks in Norway, Cuba, Brazil, Chile and New Zealand (Borrow et al., 2006). Serogroup C clones cause some epidemics but are mostly associated with local outbreaks of disease in the US, Canada and Europe (Jackson et al., 1995). Serogroup W135 was particularly associated with a major worldwide outbreak of meningococcal disease following the Hajj pilgrimage to Mecca in 2000 and 2001.
Serogroup Y disease emerged in the mid-1990s and has caused increased rates of disease in the United States and Israel (Stephens, 2007). The serological typing techniques used to classify epidemic populations of bacteria have proven insufficient for tracking *Neisseria* strains over time due to the poor resolving power of the techniques and the high frequency of phase and/or antigenic variation of surface structures displayed by *N. meningitidis*. Modern genotyping methods such as multi locus sequence typing (MLST) developed from the proteomic method of multi locus electrophoresis typing are now widely used to type epidemic strains and clonal complexes (Enright and Spratt, 1999).

1.2 The pathogenesis of *N. meningitidis* infection

Meningococcal pathogenesis depends on three factors: the reservoir of human hosts (i.e. human transmission and carriage rates), the susceptibility of the host and the virulence of the particular strain. Once present in the nasopharynx of a susceptible host, virulent meningococci are capable of attaching to epithelial cells, entering the cells by endocytosis and accumulating within phagocytic vacuoles (Stephens et al., 1983). Epithelial cells damaged by smoking, previous infection or environmental damage also provide a route for meningococci to invade the upper respiratory epithelium. From here, meningococci are released into inter-epithelial spaces beneath epithelial cell tight junctions (Tzeng et al., 2000). Upon entry into the bloodstream, the meningococcus must overcome humoral and phagocytic host defences in order to cause meningitis or acute sepsis. The multiplication of meningococci within the blood and at secondary sites of infection such as the meninges, pericardium and joints causes the systemic release of pro-inflammatory cytokines such as Interleukin -1 (IL-1), IL-6, and tumour necrosis factor alpha (TNF-α), which increase inflammation and recruit leucocytes to the site of infection. The lipopolysaccharide (LPS) of the bacterial membrane is a potent mediator of cytokine release which mediates many of the symptoms of meningococcal disease including cellular apoptosis, coagulation of blood vessels and ischemia (Brandtzaeg et al., 1992). Recently, however, several non-LPS components of *N. meningitidis* have been shown to influence inflammation. One study, in which wild-type and LPS-deficient *N. meningitidis* were incubated with peripheral blood mononuclear cells demonstrated that non-LPS components were responsible for
a substantial part of TNFα and IL-1β production and virtually all the interferon gamma (IFNγ) production by the cells (Sprong et al., 2001).

1.2.1 The pathogenesis of meningococcal meningitis

The human meninges which surrounds the brain and spinal chord consists of the dura mater and the leptomeninges; made up of the arachnoid mater, pia mater and membranous trabeculae that traverse the CSF-filled subarachnoid space (see figure 1.1) (Feurer and Weller, 1991).

Despite decades of study, it is still unclear how *N. meningitidis* penetrates the blood-brain barrier to infect the meninges. One popular theory is that the choroid plexus (the part of the brain that produces cerebrospinal fluid (CSF)) forms a point of weakness which is permeabilised by the action of TNF-α and IL-1 to allow the meningococcus to gain entry to the CSF (Brandtzaeg et al., 1992). It is clear, however, that certain isolates are more adept at penetrating the blood-brain barrier than others. A recent study using humanised CD46-transgenic mice and LPS variants of *N. meningitidis* strain NMB, found wild-type meningococci in the CSF of all infected mice, whereas different LPS variants had different abilities to cross into the CSF (Plant et al., 2006). In order to progress to the brain, meningococci need to traverse a monolayer of tight junction-expressing epithelial or endothelial cells. This can be achieved by breaking through the tight junctions between cells or by a trans-cellular route through the cells. Evidence for *N. meningitidis* taking the trans-cellular route comes from *in vivo* observations of meningococci localised within the endothelial cells of the brain of an infected
patient and from observing in vitro that *N. meningitidis* can traverse tight junction forming monolayers of endothelial cells without altering the organisation of tight junctions (Nassif et al., 2002).

Interaction of *N. meningitidis* with cells of the human meninges has been modelled in vitro by culturing cells from benign tumours of the meninges (meningioma cells) (Hardy et al., 2000). This group highlighted pili as the major ligand for facilitating adherence to the human cells, with pilin variation modulating the interactions. The authors detected no internalisation of the meningococci by the meningioma cells, which is consistent with the apparent impenetrability of the leptomeninges to *N. meningitidis* in vivo (Hardy et al., 2000). Inflammatory responses to meningococcal infection in the same model included high levels of the pro-inflammatory cytokines IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1), and lower amounts of RANTES ("regulated upon activation, normal T cell expressed, and secreted") and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fowler et al., 2004). The pathology of meningococcal meningitis is, therefore, clearly influenced by a plethora of pro-inflammatory signals. A recent study demonstrated that although *N. meningitidis* was incapable of invading meningeal cells, the infection caused death of the cells by necrosis; thus the bacterium causes tissue damage without the need for intracellular access (Fowler et al., 2006). These recent investigations may have underlined the mechanisms of meningeal infection but the route of entry from the bloodstream to the brain still remains unclear.

1.3 Age-related incidence of meningococcal carriage and infection

Meningococcal disease incidence is highest in infants (<2 years of age) followed by a second peak in young adults (Rosenstein et al., 1999). The reasons for these age-related peaks in disease incidence are complex and not simply correlated with carriage rates, as despite up to 40% of the adult population carrying *N. meningitidis*, meningococcal disease in the over 25s is very rare (Robinson et al., 2002). High disease incidence in the very young is thought to be due to the absence of protective antibody. Neonates are protected passively by maternal antibody but as antibody titres wane, susceptibility to meningococcal infection increases (Goldschneider et al., 1969). The immature immune systems of infants are incapable of eliciting effective T-cell memory responses. A study by Pollard and Levin found that even when serogroup B-specific IgG antibodies are
produced in infants, they are of much lower avidity than those produced in adults, which may partly explain the absence of serum bactericidal activity in these infants (Pollard and Levin, 2000). Thus a window of opportunity for meningococcal infection exists between infancy and the time when memory lymphocytes are generated after asymptomatic colonisation, often with the closely related commensal species *N. lactamica*.

The reasons behind the second peak in meningococcal disease in adolescents are relatively poorly understood. Rates of meningococcal carriage increase throughout childhood (Cartwright et al., 1987) and so risks of transmission are obviously higher in adolescents than younger children, but this is not the only cause. Several environmental factors are thought to be involved such as cramped living conditions and changing social behaviours, highlighted by the peak incidence of meningococcal disease in new army recruits and university students. A recent population-based study of 15-19 year olds published in the British Medical Journal reported that the factors that increased the likelihood of disease were previous illnesses, being a student and participating in intimate kissing with multiple partners (Tully et al., 2006).

1.4 Meningococcal virulence factors

*N. meningitidis* possesses an arsenal of virulence factors including capsular polysaccharide, outer membrane proteins and LPS. Several of these surface structures are subject to frequent alterations allowing adaptation to the various micro-environments within the host. Variability is achieved through large-scale exchange and mutation of DNA and phase variation. Phase variation occurs when slipped-strand mis-pairing of DNA strands causes a change in the length of simple sequence repeats. This variation can alter the transcriptional reading frame and if the repeat is present within a promoter sequence, it will affect the transcription of that open reading frame (ORF). The meningococcus is also capable of responding to environmental changes directly through transcriptional regulation. Indeed the *crgA* gene encodes a transcriptional regulator involved in cross-talk between the meningococcus and human epithelial cells (Deghmane et al., 2000). This capacity to adapt to microenvironments is key to *N. meningitidis*’s success in evading immune detection. For example, the LPS of serogroup B and C strains can be sialylated, which inhibits detection of the bacterium by the host immune system by
decreasing complement and antibody interactions with molecules of the bacterial outer membrane (Vogel and Frosch, 1999). Sialylation of LPS also limits detection of the meningococcus by receptor-ligand interactions of carcinoembryonic antigen-related cell adhesion molecule (CEACAM) receptors with opacity proteins (Virji et al., 1996; McNeil and Virji, 1997). Sialylation of LPS can be switched on or off by phase variation of the gene IgtA (Jennings et al., 1995). A schematic of several of these membrane-bound virulence factors is provided in figure 1.2. It is, however, beyond the scope of this introduction to cover all virulence factors of the meningococcus. Attention is therefore focused on one factor of particular interest; the pilius.

Figure 1.2 Schematic representation of *N. meningitidis* (reproduced from Corbett et al., 2004)

Schematic figure showing the major surface adhesins of *N. meningitidis*: pili, opacity proteins and LPS, all important in host cell attachment.

1.4.1 The meningococcal pilus

Pili are hair-like structures present in many bacterial species with several functions including motility, genetic exchange, protein secretion and host cell attachment. Pili have historically been classified on the basis of their morphology and ability to agglutinate erythrocytes in the presence or absence of D-mannose (Duguid et al., 1966). Both pathogenic and commensal species of *Neisseria* can
express type IV pili which facilitate the initial attachment of meningococci to respiratory epithelial cells (Soto and Hultgren, 1999). The core structure of the meningococcal pilus is composed of a single repeated subunit of 145-160 amino acids encoded by the *pilE* locus. The *pilC1* and *pilC2* genes are also essential for piliation and PilC has been confirmed as the major pilus adhesin as piliated *pilC*-mutants cannot adhere to epithelial cells (Nassif et al., 1994). Additional genes essential for pilus biogenesis include *pilT*, *pilG* and *pilQ*. PilT is a cytoplasmic nucleotide-binding protein which is required for intimate attachment to epithelial cells following pilus-mediated adhesion by retracting the pilus fibre (Pujol et al., 1999). PilG is thought to be a pilus-assembly protein due to its homology with a *Pseudomonas aeruginosa* gene of that function (Tonjum et al., 1995). PilQ shares homology with secretin proteins of other type IV piliated species, and reduces pilus expression when mutated (Tonjum et al., 1998).

![Figure 1.3 Immunofluorescent microscope image of piliated *N. meningitidis*](http://www.necker.fr/u570/images/pilli-xn.jpg) Xavier Nassif.

Red staining shows encapsulated diplococcus with green-stained pili extruding from the surface.

The clinical importance of pili is highlighted by their ubiquity amongst meningococcal isolates from invasive disease (Stephens et al., 1985). The pilus is thought to be essential for host cell binding in encapsulated strains (Tzeng et al., 2000). More recently, however, work on Chinese hamster ovary (CHO) and COS cell lines (an African green monkey kidney cell line) which do not express pilus receptors has shown that capsulate bacteria are capable of adhesion to host cells via opacity (Opa) proteins in the absence of pili, if the host cell Opa receptor density is sufficiently high (Bradley et al., 2005). The complement regulatory protein membrane cofactor protein (MCP or CD46) has been
postulated as the pilus receptor for Neisseria (Kallstrom et al., 1997). This has led to the development of a humanised mouse model expressing CD46 (Johansson et al., 2003). No studies to date, however, have confirmed a direct interaction between CD46 and the PilC adhesin. Indeed, it has been demonstrated in gonococci that the level of CD46 expression does not correlate with the level of pilus-mediated bacterial adhesion to epithelial cells (Tobiason and Seifert, 2001). Other studies also suggest that a CD46-independent form of PilC binding occurs between Neisseria and epithelial cells. A recent study at the Max Planck institute in Berlin supported the hypothesis of a pilus-specific receptor and identified it as an epithelial cell surface protein (Kirchner and Meyer, 2005). This receptor has yet to be characterised, thus the identity of the true pilus receptor or receptors still requires confirmation.

1.5 Models of N. meningitidis infection

Animal models are extremely useful for vaccine and antibacterial drug development but an animal model has been very difficult to develop for meningococcal disease due to a number of key characteristics of the infection. Firstly, the only natural host for N. meningitidis is humans, due to the specific range of surface proteins that interact with the host, including interaction of pili with human CD46 (the putative pilus receptor) and Opa and Opc with human CD66 (carcinoembryonic antigen-related cell adhesion molecule 1 or CEACAM-1). Survival within the host also depends on iron uptake systems which are specialised to bind the human iron transport proteins such as transferrin and lactoferrin. A good system would also include nasal carriage. Even if these issues could be overcome, a further difficulty remains in the complex pathogenesis of infection from nasal carriage towards septicaemia and/or meningitis.

Current rodent models include the mouse intraperitoneal challenge model, infant rat intraperitoneal infection model, intranasal infection models and the new humanized mouse model (Gorringe et al., 2005b). Intraperitoneal mouse models require an exogenous iron source to reach high enough infection rates to assess active and passive immunisation. They therefore do not represent the pathogenesis of the disease, but are useful in modelling the massive septicaemia observed in invasive meningococcal disease. Rat intraperitoneal models do not require exogenous iron but are limited by short duration of
bacteraemia and cannot model active protection. Rodent intranasal models have been developed in an attempt to mimic the human course of pathogenesis. Although high doses of meningococci co-administered with human iron-binding proteins result in nasal colonisation and a progression to bacteraemia, this route of infection is via pneumonia, which does not occur in humans (Mackinnon et al., 1993). Perhaps the most promising rodent model is the humanised mouse model. Mice expressing the human CD46 molecule are susceptible to meningococcal disease without the need for an exogenous iron source. Intranasal challenge with piliated meningococci has been shown to progress to a human-like disease, with bacteria crossing the blood-brain barrier of transgenic CD46 mice (Johansson et al., 2003, Gorringe et al., 2005b). Although the data on CD46 as the pilus receptor has been criticised (see section 1.4.1), the humanised mouse model presents a useful tool for studying meningococcal pathogenesis and for vaccine development. A recent study incorporating bioluminescent imaging of CD46 transgenic mice has, for example, illustrated the temporal dynamics of disease progression through the body of the mouse during meningococcal infection (Sjölinder, 2007).

Several human ex vivo models are utilised to study meningococcal pathogenesis and immune responses including the use of palatine tonsils after tonsillectomy and fresh brain tissue and cultured meningioma cells after extraction of brain tumours (Quiding-Jarbrink et al., 1995; Hardy et al., 2000). Whole blood infection assays and peripheral blood-derived leucocytes are commonly used in vitro as well as cell lines of monocyte and granulocyte-like cells to investigate immune cell responses to whole N. meningitidis and to various components of the meningococcal cell (Hauck et al., 1997; Ison et al., 1999).
1.6 The immune response to \textit{N. meningitidis}

Like all pathogens, the meningococcus strives to avoid detection and destruction by the immune system in order to proliferate and cause disease. The first line of defence to newly acquired and previously unencountered pathogens is provided by the cells and products of innate immunity.

1.6.1 Innate immunity to \textit{N. meningitidis}

Macrophages and immature dendritic cells (DCs) are the sentinels of immune surveillance at mucosal surfaces where the meningococcus first invades. Upon dissemination into the bloodstream the major phagocytic cells encountered will be the granulocytes, most numerous being the neutrophils. Phagocytic cells can destroy the bacteria ingested within them, but some can also present immunogenic fragments on their surfaces, stimulating the induction of an adaptive immune response. Such cells are known as antigen presenting cells (APC) and include the macrophages and DCs. DCs are phagocytic in their immature state and when a bacterium is encountered by an immature DC, it will be phagocytosed and activate the DC. Activated DC start to differentiate and migrate from the tissue through the blood to draining lymph nodes where the matured phenotype can present their processed antigens to T cells. The profile of co-stimulatory molecule expression and cytokine secretion by the APC determines the type of T cell response induced. DCs are unique amongst APCs in their ability to stimulate naïve T cells and determine their differentiation into effector or memory cells (Banchereau \textit{et al.}, 2000).

Several soluble and membrane-bound receptors function as pattern recognition receptors (PRR) by recognising molecular motifs common to multiple pathogens which are known as pathogen-related molecular patterns (PAMPS). This innate immune recognition strategy enables many different pathogens to be identified by the same receptor and is, therefore, more general than antibody interactions. Membrane bound PRR which recognise meningococci include the LPS receptor CD14, toll-like receptor 4 (TLR-4), and scavenger receptors. Soluble PRR such as mannose-binding lectin (MBL) and several complement proteins act as opsonins once bound to PAMPs and activate cells \textit{via} binding to their membrane-bound receptors. MBL binds to opacity-proteins and porins on the surface of \textit{N. meningitidis} (Estabrook \textit{et al.}, 2004) and MBL binding has been shown to activate complement-mediated killing, phagocytic uptake and killing.
and to increase the production of inflammatory cytokines by phagocytes in response to meningococci (Jack et al., 2001a; Jack et al., 2001b; Jack et al., 2005).

1.6.2 Specific immunity to \( N. \) meningitidis

Specific immunity to the meningococcus is initiated when meningococcal antigens are processed by APCs and presented via MHC molecules to the T cell-receptor. This interaction, combined with signalling from co-stimulatory molecules stimulates clonal proliferation of naïve T lymphocytes specific for meningococcal antigens which differentiate into effector or memory cells. Effector T cells can either be CD8\(^+\) (with cytotoxic killing activity) or CD4\(^+\) T helper cells (which secrete specific cytokines to B cells). Upon activation via co-stimulatory molecules and cytokines from antigen-specific T helper cells, naïve B cells specific for that antigen are clonally selected and proliferate and differentiate into memory B cells and effector B cells. Effector B cells are also known as plasma cells, which can produce large amounts of the meningococcal antigen-specific antibody. Memory B cells are long lived and can respond with much greater efficiency than naïve B cells to future exposure to the same antigen presented by T cells. This is because they are present at higher numbers in lymph organs, and have undergone affinity maturation and class switching to provide high-affinity IgG molecules in response to the antigen. Meningococcal-specific antibodies can aid destruction of the bacteria by activating the classical complement cascade and opsonising bacteria for increased uptake by phagocytes.

In addition to antibodies specific to the meningococcus, antibodies generated against closely related commensal species can be protective against meningococcal disease (Li et al., 2006). The gold-standard method for testing the antimicrobial activity of specific antibodies is the serum bactericidal assay (SBA). The SBA measures antibody-dependent complement-mediated killing of bacteria \textit{in vitro} after incubation of bacteria with titrated antibody and baby rabbit or human complement (Borrow et al., 2005). Antibodies can be active for SBA if the antibody-antigen complex fully activates the complement cascade to form the membrane attack complex (MAC) on the surface of the bacteria, which leads to bacterial death by lysis (see figure 1.4) (Goldschneider et al., 1969). The absence of bactericidal antibody, however, does not always correlate with susceptibility (Toropainen et al., 2005), thus other mechanisms of antibody
protection such as opsonisation and killing by phagocytic cells have recently been considered of increased importance. This is particularly relevant with respect to serogroup B isolates which experience greater in vitro killing by opsonophagocytosis than serogroups A, C, Y, or W135, despite equivalent amounts of C3 binding to serogroup B and serogroup Y strains (Ross et al., 1987). The opsonophagocytosis assay (OPA) can, therefore, be an accurate correlate of protection for anti-meningococcal antibodies (Lehmann et al., 1997).

Mucosal immunity is another important defence to meningococcal infection and the first humoral defence encountered following colonisation. Asymptomatic carriers of N. meningitidis have increased concentrations of IgA in their saliva (Robinson et al., 2002). Although this does not prevent further colonisation, it may prevent invasion of the epithelial cells, possibly by antibody blockade of meningococcal ligands used during invasion (Griffiss JM, 1995). However, IgA bound to the meningococcus can block complement-mediated lysis and opsonophagocytosis via IgM and IgG. This is because surface bound IgA can be cleaved by meningococcal Ig1A protease at the hinge region into F(ab)_2 fragments that have no Fc portion for cell signalling or complement activation (Jarvis and Griffiss, 1991). This antibody blockade of meningococcal targets means that meningococcal-specific IgA can actually increase susceptibility to meningococcal disease (Griffiss, 1975; Griffiss and Bertram, 1977; Griffiss and Goroff, 1983). Thus the role of IgA in protection from meningococcal infection is not yet clearly understood.
1.7 Host genetic factors of meningococcal disease

The entrance of \textit{N. meningitidis} into the blood stream activates the complement cascades and inflammatory response. Several host genetic polymorphisms have been identified which are involved in the differences seen in susceptibility, severity and outcome of meningococcal disease between individual patients (Emonts \textit{et al.}, 2003).

1.7.1 Complement deficiencies

The classical, alternative and lectin complement cascades can all be involved in meningococcal disease and are summarised schematically in figure 1.4. C3 is a key component of all three complement cascades and is associated with meningococcal disease susceptibility and severity. C3 is cleaved in all three pathways into C3a (a strong chemoattractant and proinflammatory molecule alongside C5a) and C3b (an opsonin and component of the C5 convertase, capable of cleaving C5 into C5a and C5b). C5b begins the formation of the membrane attack complex (MAC) C5b6789n, which forms pores in the membrane of the bacterium causing death by lysis. Thus C3 is critical for permitting the late events in the complement pathway which result in the formation of the MAC and bacterial death. In addition, deficiency in C3 results in individuals with a reduced capacity to opsonise and phagocytose bacteria, thereby increasing their susceptibility to meningococcal and bacterial disease in general (Janeway \textit{et al.}, 2001). A similar outcome is observed for individuals with “late complement component deficiency” (LCCD). This disease is characterised by recurrent Gram-negative infections due to the failure of MAC assembly. However, although disease incidence is higher, the severity of disease is significantly lower compared to the total patient population, reflecting the adverse effects of complement activation on the host during meningococcal infections (Emonts \textit{et al.}, 2003).
Figure 1.4 Complement pathways
The classical, alternative and lectin pathways all generate C3 convertases which cleave C3 into C3b which is used in the formation of the C5 convertase allowing the first parts of the membrane attack complex (MAC) to associate with the bacterial membrane (C5b, 6.7 & 8). Polymerisation of C9 in the bacterial membrane then completes the MAC and triggers cell lysis.
Polymorphisms in receptors for IgG (FcyR's) have also been
demonstrated to affect disease severity in LCCD individuals. A study of 15
LCCD patients and families found the FcyRIIA-R/R131 and FcyRIIB-NA2/2
phenotypes to be over-represented in LCCD patients who experienced
meningococcal infection (Fijen et al., 2000).

Properdin acts in the alternative complement pathway to stabilise the
alternative C3 convertase C3bBb. Three types of properdin defect have been
identified to date; with properdin being either absent from patient plasma (type
1), in low but detectable levels (type 2) or replaced by a normal concentration of
a dysfunctional variant of the protein (type 3). All three deficiencies are
correlated with increased susceptibility to meningococcal infection (Emonts et
al., 2003) presumably because of the decreased efficiency of activation of
complement-mediated killing of invading meningococci by the alternative
complement pathway.

Mannose binding Lectin (MBL) is an acute phase protein which forms
multimers and binds carbohydrates and LPS on bacterial surfaces, causing the
activation of MBL-associated serine proteases MASP1 and MASP2 which in turn
activate the C4 component of the lectin pathway. Three allelic variants of MBL
have been described in exon 1, with heterozygotes for the variations displaying
10% of the normal serum concentrations of MBL, and homozygotes retaining
only 1% of the normal serum level of MBL (Hibberd et al., 2001). Children
displaying the variant alleles appear to show associations with meningococcal
infection, with homozygosity for the variant alleles associated with slightly less
severe disease than heterozygotes or wild-type genotypes. MBL and the lectin
pathway, therefore, provide an important degree of protection from invading
meningococci, whilst contributing to the negative side-effects of inflammation.
The study reporting this phenomenon could not, however, prove that the
association was statistically significant (Hibberd et al., 1999).
1.7.2 Innate immune system deficiencies

Defects in innate pattern recognition receptors such as toll-like receptors (TLR) and lipopolysaccharide-binding protein (LBP) predispose individuals to meningococcal disease. A common polymorphism of TLR4 known as the Asp299Gly polymorphism results in hypo-responsiveness to LPS because the extracellular domain of the receptor on respiratory epithelial cells is rendered non-functional. However, the polymorphism itself is not linked to increased susceptibility or severity of meningococcal disease (Read et al., 2001). LBP binds bacterial LPS forming a complex which binds TLR4 via CD14. Cys98Gly and Pro436Leu polymorphisms in LBP have been shown to correlate with poor outcomes of bacterial sepsis in men, presumably because of the amplification of inflammatory stimuli initiated by certain types of LBP (Hubacek et al., 2001).

Recently, genetic polymorphisms in another pattern-recognition molecule; Surfactant protein-A2 (SPA) have been shown to be associated with increased susceptibility to meningococcal disease. SPA in the respiratory tract binds to microbial carbohydrates and activates inflammatory and phagocytic defences. A single nucleotide polymorphism which substitutes glutamine with lysine at residue 223 in the carbohydrate domain of SPA has been shown to increase both the susceptibility to meningococcal disease and the mortality of the disease (Jack et al., 2006). Thus, many pattern recognition receptors provide important protection from the meningococcus in the healthy host.

1.7.3 Specific immune system deficiencies

Three families of Fcγ receptors (I, II & III) are responsible for IgG-mediated signalling in a number of cell types and are thought to be highly significant in host defence against meningococcal infection. FcγRIIa located on mononuclear leucocytes and macrophages interacts with IgG2 and IgG3 in man. A single point mutation has been identified at amino acid position 131 in exon 4, with the 131R allotype (arginine) showing lower binding efficiency than the 131H allotype (histidine). This may lead to speculation that individuals homozygous for the 131R allele may be more susceptible to meningococcal disease, but association studies between the FcγRIIa 131 polymorphism and meningococcal disease have so far proved inconclusive due to the limitations of small scale retrospective studies (Emonts et al., 2003).
1.7.4 Cytokine deficiencies

TNFα is central to activation of the inflammatory response and levels are raised in all patients with meningococcal disease. TNFα activates inflammatory mediators such as IFNγ, IL-6 and IL-8. Its many effector functions include stimulating the up-regulation of expression of adhesion molecules on leucocytes (such as ICAM-1) which allow cells to adhere to receptors (such as integrins) on the vascular epithelium and eventually cross by diapedesis to reach sites of inflammation in the tissues. Polymorphisms in the TNFα genes seem to affect meningococcal sepsis but the relative importance of the known polymorphisms remains unclear, partly due to age and genetic differences in the groups studied (Emonts et al., 2003). Polymorphisms in IL-1, IL-6 and IL-10 have also been associated with altered susceptibility and severity of meningococcal disease (Emonts et al., 2003) with non-survivors of meningococcal disease having significantly higher levels of the pro-inflammatory IL-6 than survivors. Families producing high levels of the anti-inflammatory cytokine IL-10 also have a 20-fold higher risk of fatal meningococcal disease than the normal population (Kornelisse et al., 1996; Westendorp et al., 1997). Thus, survival from meningococcal disease depends on a delicate balance between too little pro-inflammatory signalling to promote bacterial clearance and too much inflammatory signalling leading to systemic tissue damage.

1.8 Meningococcal vaccine development

Serogroup B disease is now the most common form of meningococcal disease in the United States and Europe (Mariagrazia Pizza et al., 2000) and there is no broadly cross-protective vaccine available against all strains. Successful polysaccharide and polysaccharide-conjugate vaccines have been developed for serogroups A, C, Y and W-135 as both single and multivalent vaccines. The serogroup B capsule, however, contains (α2-8)-linked polysialic acid which is poorly immunogenic even when coupled to carrier proteins because it is identical to structures on the human neural cell adhesion molecule, N-CAM (Finne et al., 1987). Additionally, the generation of antibodies cross-reactive with host molecules would not be desirable for vaccine safety. Alternative serogroup B vaccines are under development using specific outer-membrane proteins (OMP) and entire outer-membrane vesicles (OMV) which are membrane blebs, naturally shed from the surface of the meningococcus.
Individual OMPs such as PorA can be highly immunogenic but are subject to antigenic variation and each type provides protection against a limited range of strains (Morley and Pollard, 2001). Specific OMPs can be useful, however, in targeting a vaccine against specific clonal complexes which cause epidemics of disease. An example of a valuable narrow-range OMV vaccine is the MeNZB vaccine developed in New Zealand against the epidemic clone causing the majority of disease in that country. MeNZB is specific for group B meningococci subtype P1.7b,4, and is now routinely administered to New Zealand residents aged 6 weeks to 19 years (O’Hallahan et al., 2005). Previous to this, a similar approach was also successful in Cuba (Sierra et al., 1991) and has been used in several other countries where clonal epidemics have been experienced.

In less geographically remote areas where meningococcal disease is multiclonal, such as much of Europe and the USA, a more broad-range vaccine is needed to provide immunity against the full range of strains that may be encountered. This means finding an immune target or panel of targets present in the whole meningococcal population. Thus the search continues for cross-reactive protective antigens in the meningococcus. A classical route to vaccine development is the use of attenuated mutants. The \textit{phoP} mutant of a serogroup B \textit{N. meningitidis} strain was generated by allele exchange at the University of Surrey (Newcombe et al., 2004). PhoP is part of a two-component regulatory system which, when inactivated, leads to avirulence in pathogenic organisms including \textit{Salmonella} species (Miller et al., 1989). The \textit{phoP} knock-out mutant in \textit{N. meningitidis} was avirulent in a mouse intraperitoneal challenge model and provided cross-reactive bactericidal activity against a range of meningococcal strains from different serogroups and serotypes (Newcombe et al., 2004). This data suggests that the \textit{phoP} mutant shows potential as a live attenuated vaccine, as well as being a useful tool for investigating cross-reactive immunogenic antigens.

Several other routes have been taken in pursuit of a broadly cross-protective serogroup B vaccine. The discovery of de-N-acetyl sialic acid residues in meningococcal LPS that do not cross-react with host tissues has renewed interest in targeting a bactericidal antibody response to the serogroup B capsule (Moe et al., 2005). OMV from \textit{N. lactamica} have also been shown to be cross-protective against \textit{N. meningitidis} infection in mice, without targeting the hyper-variable PorA antigen (as this is absent in \textit{N. lactamica}). There is, however, no
bactericidal antibody activity in response to *N. lactamica* OMV and the mechanism of protection of the vaccine is unconfirmed (Gorringe *et al*., 2005a). The process of "Reverse Vaccinology" has been utilised to identify surface exposed targets common to all disease-causing isolates following the publication of the complete meningococcal genome sequence. This approach has enabled the development of a vaccine formulation against 5 such targets which has produced promising pre-clinical data (Giuliani *et al*., 2006). A further technique for vaccine development has been to load OMV with over-expressed recombinant meningococcal antigens such as factor H-binding protein, which generates a much stronger antibody response as part of an OMV vaccine than as a protein-based vaccine (Koeberling *et al*., 2007).

### 1.9 C-Reactive Protein and the acute phase response

The acute phase response describes the non-specific physiological and biochemical responses of higher eukaryotes to infection, inflammation and tissue damage. The response is characterised by the rapid upregulation of synthesis of certain proteins (such as plasma lectins) and down regulation in the production of other proteins (such as albumin and transferrin) within liver hepatocytes as a result of cytokine release from the source of pathology. The two proteins showing the most significant range of synthesis during the acute phase response are C-Reactive Protein (CRP) and the related protein serum amyloid A (SAA). Other acute phase reactants include proteinase inhibitors, complement and transport proteins (Pepys and Baltz, 1983). CRP is the major acute phase protein in humans and was discovered and named in 1930 because of its ability to react with the cell wall C-polysaccharide of *Streptococcus pneumoniae* (*S. pneumoniae*) (Tillet, Jr., 1930). CRP is part of a phylogenetically ancient family of pentameric proteins known as "pentraxins" with homologous proteins present in species as remote as the 70 million year old horseshoe crab, *Limulus polyphemus*. 


The CRP pentamer measures 102Å in diameter with a 30Å central pore. The structure has been determined by X-ray crystallography at 3 Å resolution and confirmed as five noncovalently associated protomers arranged around the central pore as shown in figure 1.5 (Emsley *et al.*, 1994). Each protomer is around 36Å in diameter and consists of 206 amino acids folded into two antiparallel β sheets with “flattened jellyroll topology” (Volanakis, 2001). The pentameric structure displays a five-fold symmetry axis with the “top” face of the ring designated as the “effector face” and the underside known as the “recognition face” (Shrive *et al.*, 1996). An unusual cleft on the effector face is formed by the positioning of a long α helix folded against one of the two β sheets and by parts of the amino and carboxyl termini of the promoter. This narrow cleft is believed to house the C1q-binding site for complement activation (Agrawal *et al.*, 2001) and the Fcy receptor binding site for phagocytic cell recruitment (Marnell *et al.*, 1995). The recognition face contains two Ca$^{2+}$ ions attached to side chains and main chain carbonyls on each protomer which both participate in ligand binding (Volanakis, 2001).

The principal ligand for CRP is phosphorylcholine (PC). Crystallographic analysis of CRP-PC complexes has shown that two oxygens of the phosphate group directly associate with the two Ca$^{2+}$ bound to CRP, while the choline group rests within the hydrophobic pocket (Thompson *et al.*, 1999). Site-directed
mutagenesis has confirmed the importance of the hydrophobic pocket for PC binding (Agrawal et al., 1992). The affinity of CRP for PC is moderate (in the micromolar range) but multipotent attachment of CRP to surfaces containing a high density of PC groups makes the avidity very strong.

1.1 The synthesis of CRP

Primary synthesis of CRP occurs in liver hepatocytes as part of the acute phase response. CRP is also expressed and secreted in the respiratory tract by epithelial cells (Gould and Weiser, 2001) and by peripheral blood mononuclear cells (Haider et al., 2006). CRP synthesis in the liver is stimulated by factors including IL-6, IL-1, and corticosteroids (Du Clos TW and Mold C, 2004). Blood concentrations of CRP can rise from resting levels of less than 1 μg/ml to greater than 1000 μg/ml at the height of an acute phase response. The protein is highly stable and has a half-life of 19 hours (Vigushin et al., 1993). Monomers of CRP are produced and assembled as a pentamer within the endoplasmic reticulum (ER) of hepatocytes. In the resting state, CRP is retained within the ER, bound to carboxylesterases (Macintyre et al., 1994). When stimulated by certain cytokines, binding decreases and the transit time from the ER to secretion is greatly reduced (Macintyre et al., 1985). This enables high levels of CRP to rapidly appear in serum during the acute phase response. Indeed, CRP levels rise and fall more dramatically than any other acute phase marker, making it particularly useful for following clinical disease course and response to therapy.

The human CRP gene is found on the long arm of chromosome 1q23.3. No genetic deficiencies in CRP have been identified and only fleetingly rare single nucleotide polymorphisms have been discovered (Crawford et al., 2006). Genetic determinants of baseline CRP level regulation have, however, been identified due to variations in the number of dinucleotide repeats in the intronic region of the CRP gene (Robey and Liu, 1981). Although no association has been observed between this polymorphism and human disease, two additional polymorphisms have recently been discovered which do correlate with disease susceptibility and severity. One mutation occurs in the 3' untranslated region and the other is found in exon 2. Both these polymorphisms were found to be associated with lower basal levels of serum CRP and disease in a cohort of families suffering from systemic lupus erythematosus (SLE) a systemic autoimmune disease (Russell et al., 2004). This finding supports the hypothesis
that CRP is important in limiting inflammatory pathologies as reduced levels of CRP can fail to remove potentially immunogenic material from the tissues, resulting in autoimmune inflammatory damage.

1.12 The CRP receptors

The interaction of CRP with Fcy Receptors (FcyR) was first reported in 1977 (Mortensen and Duszkiewicz, 1977). FcyRI was first shown to bind to CRP by transfecting COS cells with FcyRI and knocking out FcyRI on mouse cells and measuring CRP binding to these cells (Crowell et al., 1991; Marnell et al., 1995). These findings were then supported by data from surface plasmon resonance experiments, which determined that the affinity of CRP for FcyRI was greater than the receptor’s affinity for IgG (Bodman-Smith et al., 2002b), thereby confirming FcyRI as a cellular receptor for CRP.

Further studies with transfected COS cells identified FcyRIIa as a second CRP receptor (Bharadwaj et al., 2001). FcyRIIa exists in two allelic forms; with arginine at position 131 (R131) or histidine at this position (H131). CRP binding to FcyRIIa on neutrophils and monocytes was shown to increase phagocytosis of CRP-opsonised particles, with CRP preferentially binding to the homozygous R131 phenotype (Stein et al., 2000a). Binding of CRP to FcyRIIa was confirmed by binding and phagocytosis of CRP-opsonised PC-labelled sheep red blood cells by FcyRIIa transfected COS cells and the subsequent inhibition of these events by F(ab’)2 anti-CRP antibodies (Bodman-Smith et al., 2004). Individuals who are homozygous for R131 are statistically more susceptible to bacterial infections (including infection by N. meningitidis) possibly because, although this polymorphism shows higher affinity for CRP, IgG2 binding is of lower affinity than in the H131 genotype (Bredius et al., 1994). The two polymorphisms exhibit no difference, however, in IL8 production or NADPH oxidase activity upon phagocytosis of CRP-opsonised S. pneumoniae (Rodriguez et al., 2004), suggesting a role for other CRP receptors such as FcyRI in these effects.

Site-directed mutagenesis has identified individual residues that are important for binding of CRP to FcyRI, FcyRIIa and the C1q complement component. Thr173 and Asn186 are vital for CRP binding to FcyRIIa. Lys114 and Leu176 are needed for binding to FcyRI but not FcyRIIa. Additionally, alterations at Lys 114, Asp169, Thr 173, Tyr 175 and Leu 176 altered CRP binding to C1q. These data indicate that the three binding sites are overlapping
on the CRP surface, lending further evidence to support the hypothesis that FcγR binds to the effector face of the protein (Bang et al., 2005). It is notable that CRP does not bind the highly pro-inflammatory FcγRIII expressed on neutrophils, inflammatory macrophages, mast cells and natural killer (NK) cells (Du Clos TW et al., 2004). This observation lends support for a role for CRP as an immune-modulator rather than an inflammation activator.

1.13 The functions of CRP

CRP recognises pathogens and damaged or apoptotic cells and facilitates their removal by recruiting the complement cascade and phagocytic cells. It has also been postulated as a key player in inflammatory diseases such as arthritis and atherosclerosis. CRP binds to PC residues with highest affinity, which is a moiety almost universally expressed by eukaryotic cells (Harnett and Harnett, 1999). PC is displayed on the teichoic acids of S. pneumoniae (Brundish and Baddiley, 1968), on the LPS of Haemophilus influenzae and Pseudomonas aeruginosa (Gillespie et al., 1996), on the surface of N. meningitidis and N. gonorrhoeae (Kolberg et al., 1997) and on Proteus morganii and Aspergillus fumigatus (Jensen et al., 1986). CRP has been shown to bind to most of these species (excepting pathogenic Neisseria before the completion of this thesis) and has been shown to increase host protection (Du Clos and Mold, 2001). CRP can also bind to the lipophosphoglycan of the intracellular parasite Leishmania donovani (L. donovani) (Culley et al., 1996) which appears to use CRP to increase access to its intracellular niche, without the host benefiting from increased killing of the pathogen (Bodman-Smith et al., 2002a).

1.13.1 CRP-mediated clearance of apoptotic and tumorigenic cells

In human cells, PC is found in the outer membrane leaflet as the polar head-group of lecithin and sphingomyelin. CRP associates with damaged and necrotic cell membranes, but not healthy cells (Kushner and Kaplan, 1961). The reason for this exclusivity is that cell damage or apoptosis causes an exchange of phospholipids between the inner and outer membrane leaflets. This "flip-flop" results in the enrichment of the outer leaflet with phosphatidylethanolamine and phosphatidylerine which are normally present within the inner leaflet. These phospholipids are then susceptible to hydrolysis by human secretory phospholipase A₂, generating lysophospholipids such as lysolecithin which allows
binding of CRP to the PC polar headgroup on the cell surface (Hack et al., 1997). In this context, CRP binding of apoptotic cells and cell debris leads to increased phagocytic uptake to clear potentially pro-inflammatory stimuli from the blood and tissues. CRP may, however, have a less beneficial role in the response to tumours as CRP can bind to FcγR on myeloma tumour cells and protect these cells from chemotherapy drug-induced apoptosis (Yang et al., 2007). Thus CRP binding to host cells can be either protective or deleterious depending on the nature of the receptors involved in the interaction.

1.13.2 CRP-mediated activation of complement

When human CRP is aggregated or bound to macro-molecular ligands of microorganisms or autologous ligands of host cells, it is recognised by C1q. This causes the potent activation of the classical complement pathway by activating the complement protein C3, and can result in formation of MAC C5-C9 as described in figure 1.4 (Volanakis, 1982). CRP can, however, inhibit complement activation via the alternative complement pathway by binding to the complement regulatory protein, factor H (Mold C et al., 1986). Factor H inhibits the alternative pathway amplification loop, inhibiting the formation of the C3 convertase which is needed for the formation of the alternative pathway C5 convertase. Subsequently, the formation of the MAC via the alternative pathway is inhibited. It has been postulated that CRP deposited at sites of injury and inflammation may limit tissue damage by its interaction with factor H. In vivo evidence to support this role has come from observations of CRP colocalised to myocardial infarction lesions, and the discovery of complexes of CRP and C3/C4 split products in the circulation of patients suffering from sepsis (Wolbink et al., 1996; Lagrand et al., 1997).

There has been much interest during the last two decades in the various structural forms of CRP and changes in biological activity associated with the different forms. The pentameric disc shape of native CRP is now known to disassemble into individual monomers upon contact with negatively charged lipid monolayers and by treatment with heat, acid, urea or by immobilising onto polystyrene surfaces (Kresl et al., 1998; Wu et al., 2003). This modified CRP (mCRP) has newly exposed antigenic epitopes and causes greater activation of platelets, granulocytes and monocytes than native CRP in vitro (Potempa et al., 1988). mCRP also shows an increased ability to bind to complement
regulatory proteins \textit{in vitro} (Biro et al., 2007). The \textit{in vivo} relevance of this finding is, however, under scrutiny as although imuno-histochemical staining has labelled mCRP in vascular tissues, native CRP is highly stable and requires harsh denaturing conditions to be separated into protomers and there is no compelling evidence that denatured mCRP can persist \textit{in vivo} (Diehl et al., 2000; Pepys et al., 2003).

### 1.13.3 CRP protection from \textit{S. pneumoniae} infection

The interaction between CRP and \textit{S. pneumoniae} has received much attention since the initial discovery of CRP, with particular interest in the protective effect of CRP against streptococcal infection in mouse models. CRP is not an acute phase protein in mice, and human CRP (either administered passively or generated by mice transgenic for human CRP) protects mice from lethal \textit{S. pneumoniae} infection (Mold et al., 1981; Szalai et al., 1995). It was initially supposed that protection was provided by two of the known actions of CRP; complement activation and increased opsonophagocytosis via FcyR recruitment. The latter mechanism was discounted after observations that CRP protected FcyR deficient mice from streptococcal infection equally as well as wild-type mice (Mold et al., 2002). Subsequent attention turned to CRP's role in activating complement. A CRP mutant that can bind PC but not C1q was shown to protect mice from \textit{S. pneumoniae} infection equally as well as wild type CRP, thus CRP does not need to bind complement to afford protection (although a complete complement system is needed) (Suresh et al., 2006). In addition, CRP mutants that are incapable of binding to \textit{S. pneumoniae} were shown to be equally effective as wild type CRP in protecting mice from \textit{S. pneumoniae} infection (Suresh et al., 2007). This led to the conclusion that CRP may influence the cells of the immune system independently of binding to bacteria and may therefore be of use in loading antigen presenting cells to enhance treatment of bacterial infections (Suresh et al., 2007). These studies also revealed that although human CRP activates human complement by binding C1q, human CRP is incapable of binding to mouse C1q which limits the usefulness of the mouse model in studying CRP's involvement in antibacterial protection.

CRP has recently been shown to increase the acquired immune response to \textit{S. pneumoniae} as CRP-opsonisation increases uptake of streptococci into
DC. This enhanced uptake results in an increased memory IgG response to streptococcal antigens and protection from virulent streptococci after transfer of CRP-opsonised streptococci-pulsed DC into recipient mice (Thomas-Rudolph et al., 2007). All these effects were absent in mice deficient in Fcγ chain expression and were, therefore, attributed to uptake via Fcγ receptors (Thomas-Rudolph et al., 2007). The use of CRP opsonization to enhance the effectiveness of *S. pneumoniae* vaccination is therefore being investigated (Du Clos TW et al., 2004).

1.13.4 CRP-induced inflammatory cytokine production

Binding of CRP to Fcγ receptors on neutrophils and monocytes leads to the production of cytokines including the pro-inflammatory cytokines IL-1β, IL-6 and TNFα. The production response of these cytokines is dose-dependent on the level of CRP, with a maximal response seen at around 50μg/ml. (Ballou and Lozanski, 1992). In alveolar macrophages, CRP also causes the release of IL-1β and TNFα (Galve-de Rochemonteix et al., 1993). These two observations support the role of CRP in enhancing the human inflammatory response at tissue sites of inflammation. However, other experimental data suggests that CRP may also be involved in down-regulation of the inflammatory response. For example, human peripheral blood mononuclear cells (PBMCs) stimulated by CRP produced higher quantities of the anti-inflammatory cytokine IL-1-receptor agonist (IL-1RA) than the pro-inflammatory cytokine IL-1 (Tilg et al., 1993). Although the cytokines produced as a response to CRP in monocytes and macrophages are similar to those induced by aggregated IgG, the fact that CRP does not bind to the highly pro-inflammatory FcγRIII means a more modest inflammatory response is seen from CRP (Du Clos TW et al., 2004). It is the moderately acute-phase levels of CRP that are most effective in inducing cytokine production, and this effect is synergistic with the response to LPS and other microbial products. As higher levels of CRP induce lower levels of macrophage inflammatory cytokine secretion than moderate levels, CRP can have both pro- and anti-inflammatory effects in different settings (Bodman-Smith, unpublished., Nabata et al., 2007).
1.14 The principal ligand for CRP is expressed on the pili of *N. meningitidis*

The expression of PC by bacteria appears to be a critical factor in the colonisation of the human respiratory tract, but can also enhance complement-mediated killing in human serum (Weiser *et al.*, 1998b). No reports have been published (previous to this investigation) on the binding of CRP to PC on the pili of pathogenic species of *Neisseria*. However, binding has been demonstrated between CRP and PC decoration found on the LPS of commensal species such as *N. lactamica* and *N. subflava* (Serino and Virji, 2000). Unlike commensal species, pathogenic *Neisseria* do not express PC on their LPS, but the epitope has been detected on both class I and class II pili of *N. meningitidis* and *N. gonorrhoeae* (Weiser *et al.*, 1998a). Interestingly, although some commensal *Neisseria* posses pili homologous to class II pili of pathogenic strains, these commensal pili do not react with anti-PC antibody (Serino *et al.*, 2000).

The absence of PC on the LPS of pathogenic *Neisseria* species may protect from bactericidal activity triggered by anti-PC antibodies. PC decoration on the pili of pathogenic *Neisseria* has been shown to be phase variable, much like the expression of the pili themselves (Weiser *et al.*, 1998a), which may be a useful way of hiding an important virulence factor from immune detection. PC is expressed on the surface of the pilus by action of the pilin phosphorylcholine transference A gene (*pptA*) which was discovered by insertional inactivation of candidate ORFs and western analysis with anti-PC antibody (Warren and Jennings, 2003). Thus despite the absence of any published data on CRP binding to *N. meningitidis* at the onset of this study, much evidence was available to suggest that CRP may be able to interact with PC on the surface of the meningococcus.
1.15 Hypothesis

The hypothesis of this thesis is that CRP is capable of binding to PC-expressing *N. meningitidis* and that this binding will have immunological consequences in the way that cells and proteins of innate immunity respond to a meningococcal infection.

1.16 Objectives

1. To discover if CRP is able to bind to the PC moiety displayed on the pilus of *N. meningitidis* and to characterise the specificity and kinetics of this binding.

2. To explore the effect of CRP binding to *N. meningitidis* on the innate immune response to meningococcal infection. This will involve targeting the defence mechanisms known to be involved in CRP protection from other organisms, namely serum killing, phagocytosis by and activation of professional phagocytes and the consequent production of inflammatory cytokines to further progress the response to infection.

3. To ascertain from the results of the above investigations whether CRP binding to *N. meningitidis* will be of net benefit to the pathogen or to the host.
CHAPTER TWO

MATERIALS AND METHODS
2.1 Materials

2.1.1 Bacterial strains

*S. pneumoniae* strain R36A was used for assay development as a positive control for CRP binding. R36A is a non-encapsulated variant of virulent *S. pneumoniae* capsular type 2 (D39) (Avery et al., 1944). Piliated serogroup B *N. meningitidis* strain C311 and a natural non-piliated mutant of the same strain (C311p-) were kindly donated by Professor Mumtaz Virji at the university of Bristol. An isolate of *N. meningitidis* H44/76 containing a Green Fluorescence Protein (GFP)-expressing plasmid "pEG2" was gratefully received from Dr Myron Christodoulides, University of Southampton (Christodoulides et al., 2000). This plasmid is a hybrid shuttle vector containing the red-shifted gfp (rs-gfp) gene under the control of a porA promoter and has been modified by replacing the ampicillin resistance cassette (bla) with an erythromycin resistance cassette (ermC), resulting in "pEG2-Ery" (M. Christodoulides unpublished). The mouse-virulent serogroup C *N. meningitidis* strain L91543 and a phoP knockout mutant of this strain were received from Jane Newcombe, University of Surrey. The phoP knockout was generated by allele exchange (Newcombe et al., 2004). All bacterial cultures were stored in Mueller Hinton broth (MHB) supplemented with 15% glycerol at -80°C.

2.1.2 Bacterial culture media

- Mueller Hinton broth was prepared from powder stocks as per manufacturer's instructions (Oxoid, UK).
- Brain heart infusion agar was prepared from powder stocks as per manufacturer's instructions (Oxoid, UK).
- Columbia blood agar (CBA) was prepared from powder stocks as per manufacturer's instructions (Oxoid, UK) and supplemented with 5% defibrinated horse blood (Tissue culture systems, UK).

2.1.3 Cell lines

The human monocyte-like cell lines THP-1 and U937 were purchased from the European Collection of Cell Cultures (ECACC, UK) and confirmed as mycoplamsa free by a commercial ELISA-based Mycoplasma detection kit (Roche, Germany).
2.1.4 Buffering solutions
Buffering solutions were prepared and autoclaved wherever possible. All chemicals were purchased from Sigma-Aldrich UK unless stated otherwise.

Bicarbonate buffer (0.1M)
0.2M NaC$_2$O$_3$ was slowly added to 0.2M NaHCO$_3$ until a pH of 9 was reached.

Citrate-phosphate buffer
257ml of 0.2M dibasic sodium phosphate was added to 243ml 0.1M citric acid and 500ml double distilled water. pH was adjusted to 5.0 where necessary.

FACS buffer
Phosphate-buffered saline (PBS) was prepared from tablets and supplemented with 1% bovine serum albumin and 0.05% sodium azide. 1% normal human serum was added immediately before use.

SDS-PAGE resolving buffer (4X)
1.5M Tris-HCl was adjusted to pH 8.8 before adding 0.4% weight/volume sodium dodecyl sulphate (SDS). This was a 4X concentration stock.

SDS-PAGE stacking buffer (4X)
0.5M Tris-HCl was adjusted to pH 6.8 before adding 0.4% weight/volume SDS. This was a 4X concentration stock.

SDS-PAGE protein tank buffer (10X)
A solution containing 0.5M Tris and 0.55M glycine was supplemented with 1% weight/volume SDS (pH 8.3). The solution was diluted 1 in 10 with double distilled water before use in the protein gel tank.

SDS-PAGE sample loading buffer (2X)
A stock of 2X loading buffer was prepared by adding 5ml of 4X stacking buffer to 2ml glycerol, 1.8ml of 10% SDS, 1.2% sterile distilled water and 10mg of bromophenol blue. The stock was supplemented with 10% 2-mercaptoethanol immediately before use.
12% SDS-PAGE resolving gel
6ml of 30% acrylamide/bis solution (37.5:1 ratio, Biorad, UK) was added to
3.75ml of 4X resolving buffer, 200μl 10%SDS, 50μl 10% ammonium persulphate,
4ml sterile distilled water and 10μl tetramethylethylenediamine (TEMED).

4% SDS-PAGE stacking gel
1.625ml acrylamide/bis solution was added to 2.5ml of 4X stacking buffer, 100μl
10% SDS, 50μl 10% ammonium persulphate, 5.275ml sterile distilled water and
3μl TEMED.

Western transfer buffer
A solution of 25mM Tris and 0.2M glycine was prepared with 80% distilled water
and 20% methanol.

2.1.5 Antibodies
The anti-pilin antibody SM1 was generated in rabbits using highly purified
pili of *N. gonorrhoeae* MS211ms (Jonsson et al.,1991) and has been shown to
bind to a highly conserved region within the 5' end of class 1 PilE of
*N. meningitidis*, with the minimum epitope identified as the linear peptide EYYLN
(Virji et al.,1989;Jonsson et al.,1991). Anti-CRP was purchased as goat anti-
human CRP affinity purified Immunoglobulin (Biogenesis, Poole, UK). Anti-
phosphorylcholine antibody was purchased as mouse IgA TEPC15 (Sigma-
Aldrich, UK). Anti-serogroup B and anti-serogroup C meningococcal monoclonal
antibodies were purchased from NIBSC, UK. Anti-*S. pneumoniae* antibody was
purchased from Biogenesis, UK as polyclonal rabbit anti-*S. pneumoniae*.
Antibodies were controlled for isotype during Fluorescence Activated cell
Scanning (FACS) analysis (mouse IgG1 (Sigma-Aldrich,UK), mouse IgA (BD
biosciences, UK) and ChromaPure goat IgG F(ab')2 fragment (Jackson
ImmunoResearch, USA)). Secondary antibodies for FACS analysis were
fluorescein isothiocyanate (FITC)-conjugated from Sigma-Aldrich, UK (anti-
mouse IgA-FITC, anti-mouse IgG-FITC, anti-goat IgG-FITC and anti-rabbit IgG-
FITC). Secondary antibodies for Western blot analysis were peroxidase-
conjugated (anti-mouse IgG, anti-mouse IgA and anti-goat IgG-peroxidase
conjugates, all from Sigma-Aldrich, UK). For the high sensitivity CRP ELISA,
antibodies were purchased from Dako Cytomation, Denmark (polyclonal rabbit anti-human CRP and peroxidise-conjugated polyclonal rabbit anti-human CRP).

For FACS analysis of activation marker expression on macrophages, antibodies were directly conjugated to FITC or Phycoerythrin (PE). Anti-CD11b, ICAM-1 and CD14, were all from Sigma-Aldrich, UK. Anti-CD11c, CD64 and HLA-DR were all from BD Biosciences, UK. Isotype controls were from BD Biosciences, UK and Serotec, UK. For double-staining FACS analysis, CD11b, ICAM-1 and CD14 antibodies were FITC-conjugated whereas CD11c, CD64 and HLA-DR antibodies were PE-conjugated.

For TNFα ELISAs, mouse anti-human TNFα and biotinylated mouse anti-human TNFα were purchased from BD Biosciences, UK. All antibodies were titrated to determine concentrations for optimal activity before use in assays.
2.2 Methods

2.2.1 Bacterial culture and growth curve experiments

Bacteria were sub-cultured from frozen stocks onto Columbia blood agar (CBA) with 5% horse blood and incubated overnight. Agar plates were supplemented with antibiotics for the selection of mutant strains (10µg/ml erythromycin for GFP-expressing strains, 100µg/ml kanamycin for the phoP mutant of *N. meningitidis*). Plates were used to inoculate warm broth (MHB for *N. meningitidis* and brain-heart infusion (BHI) broth for *S. pneumoniae*) to a known optical density at 595nm or 600nm (usually OD 0.1 for *S. pneumoniae* and OD 0.02 for *N. meningitidis*). For experiments using the serogroup C strain L91543 and the phoP mutant, MHB was supplemented with 20mM MgCl₂. Cultures were shaken at 35°C for *N. meningitidis* and 37°C for *S. pneumoniae* and aliquots of culture were taken at regular intervals to measure optical density and to serially dilute cultures in PBS to allow enumeration of colonies formed from bacteria in 10µl drops plated in quadruplicate onto CBA.

Initial experiments revealed that when *N. meningitidis* strain C311 was grown for 4 hours from a starting optical density at 595nm of 0.02 and cultures were serially diluted in PBS and plated out overnight on CBA, a bacterial density of 1 X 10⁸ colony forming units/ml (cfu/ml) was achieved when the bacterial suspension was of an optical density of 1 at 595nm.

For most experiments with fixed *N. meningitidis*, cultures were grown in 20mls of MHB in small tissue culture flasks from an optical density at 595nm of 0.02 for 4 hours at 35°C with shaking at 125rpm before transferring to 50ml centrifuge tubes and spinning at 1000 x g for 15 minutes, measuring the optical density and fixing overnight in 4% paraformaldehyde. Fixed cultures were then washed twice in PBS before use in assays.

2.2.2 Construction of a Green Fluorescence Protein-expressing *N. meningitidis* strain C311

The GFP-containing plasmid pEG2-Ery was harvested from strain H44/76 using a standard kit, according to the manufacturer’s instructions (QIAprep miniprep, Qiagen, UK). The plasmid was then transformed into strain C311 as follows. Strain C311 was subcultured from frozen stocks onto a CBA plate and incubated overnight. 20 bacterial colonies were suspended in 1ml MHB supplemented with 10mM MgCl₂. 500µl of this suspension was transferred to a
sterile 1.5ml tube with 500ng of plasmid and incubated at 35°C for one hour. The remaining 500μl of bacterial suspension was incubated alongside as a negative control. 4.5mls of MHB containing 10mM MgCl₂ was added to each bacterial suspension and the cultures were incubated with gentle shaking for 4.5 hours at 35°C. 500μL aliquots were plated onto CBA plates supplemented with 10μg/ml erythromycin and the plates were incubated at 35°C for up to 48 hours. Transformed cultures grew on the selective agar and separate colonies were subcultured onto CBA supplemented with erythromycin and frozen down as stock cultures. GFP expression was confirmed by fluorescence microscopy.

2.2.3 Human cell culture

Primary cell cultures and monocyctic cell lines were maintained in complete RPMI media containing 100units/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine and 10% heat inactivated foetal bovine serum (all from Invitrogen, UK).

2.2.3.1 Culture of monocyctic cell lines

The monocyte-like cell lines THP-1 and U937 were maintained in complete RPMI media. For differentiation into adherent macrophage-like morphology, THP-1 cells were seeded at 4×10⁵/ml (400μl/well) in 8-well plastic chamber slides (Nunc, UK) in media containing 10nM phorbol myristate acetate (PMA, Sigma-Aldrich, UK) for 72 hours prior to experiments. Similarly, U937 cells were seeded at 2×10⁵/ml and were differentiated with 10nM PMA for 72 hours and 100nM Vitamin D₃ and 100nM retinoic acid for 24 hours (both Sigma-Aldrich, UK).

2.2.3.2 Culture of macrophages and dendritic cells from peripheral blood

Mononuclear cells were extracted from healthy consenting volunteer blood in accordance with Local Research Ethics Committee (LREC) guidelines (LREC 04/Q1909/19RSCH) and separated using Histopaque 1077 as per the manufacturer’s instructions (Sigma-Aldrich, UK). Peripheral blood mononuclear cells (PBMCs) were seeded at 3×10⁶/ml in 8 well plastic chamber slides (400μl/well) or 6 well plates (3ml/well) in RPMI without serum and positively selected for by plastic adherence after 2 hours culture at 37°C in serum-free media. Approximately 10% of the cells adhered after vigorous washing. Adherent
cells were washed and cultured in complete RPMI (with 10% heat-inactivated foetal calf serum). Monocytes were cultured for 7 days with one change of media at day 3-4 to allow differentiation into macrophages. Monocytes were differentiated into dendritic cells by the addition of 1000 units/ml Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF, Peprotech, UK) and 100 units/ml human IL-4 (Perotech, UK) on the day of seeding and 2 and 4 days after seeding. Dendritic cells were used as immature day 5 cells. Morphology was examined by light-microscopy.

2.2.3.3 Culture of granulocytes from peripheral blood

Granulocytes were extracted from healthy consenting volunteer blood using Polymorphoprep as per the manufacturer’s instructions (Axis-Shield, Norway). Granulocytes were seeded at 2×10⁷/ml in chamber slides in RPMI without serum and positively selected for by plastic adherence after 20 minutes incubation at 37°C.

2.2.4 Preparation of human CRP

Purified human CRP (>97%) was purchased from The Binding Site, UK and further purified by affinity column chromatography based on the method detailed by Volanakis (Volanakis et al., 1978). A purifying column was prepared by suspending 3g of activated CH sepharose 4B (GE healthcare, UK) in 15ml 1mM HCl and the gel was allowed to swell for fifteen minutes at room temperature. Beads were washed 4 times with ice-cold 1mM HCl by centrifugation at 10°C. P-amino phosphorylcholine (Sigma-Aldrich, UK) was dissolved in 10ml coupling buffer (0.1M NaHCO₃, pH8 + 0.5M NaCl₂) and added to 5mls of sepharose gel and rotated overnight at 4°C. Excess ligand was removed with 4 changes of coupling buffer and any remaining active groups on the beads were blocked with 0.1M Tris-HCl at room temperature. The gel was washed with 3 cycles of 50ml 0.1M sodium acetate pH4 + 0.5M NaCl₂ followed by 50ml 0.1M Tris-HCl pH8 + 0.5M NaCl₂. The gel was equilibrated with 10 bed volumes of Tris-buffered saline (TBS) and incubated with human CRP in TBS + 0.5mM CaCl₂ overnight at 4°C with rotation. The gel was gently poured into a column and 1ml fractions collected whilst adding TBS + 0.5mM CaCl₂ until the absorbance of fractions at 280nm reached 0. The bound CRP was then eluted in 0.5ml fractions with TBS + 10mM EDTA until the absorbance of fractions at
280nm reached 0. The most concentrated fractions were pooled and concentration established by absorbance at 280nm using the specific extinction coefficient for pure CRP of 1.75 for 1mg/ml (Nelson et al., 1991). The purified CRP was then dialysed for 12 hours at a time, twice in TBS and 3 times in phosphate-buffered saline (PBS, Sigma-Aldrich, UK) to remove traces of TBS and sodium azide and purity was confirmed by SDS-PAGE under reducing conditions.

Recombinant human CRP, sourced from Calbiochem, UK was also used in non-cellular assays. Recombinant CRP was produced in *Escherichia coli* as a non-glycosylated polypeptide chain of molecular mass 115kDa with the same amino acid sequence as native human CRP as cited by Woo et al. (Woo et al., 1985). The recombinant protein was purified at Calbiochem, UK, using phosphorylcholine affinity chromatography and was determined pure by SDS-PAGE analysis as described in section 2.2.6. The product was reported by the manufacturers to contain only 9ng/ml LPS as determined by the limulus amoebocyte lysate assay.

### 2.2.4.1 Conjugation of alkaline phosphatase to recombinant CRP

CRP was conjugated to alkaline phosphatase (AP) using gluteraldehyde in a standard one-stage procedure (Avrameas, 1969). 2000 units of alkaline phosphatase (Sigma-Aldrich, UK) was spun at 15000 \( \times g \) for 6 minutes, resuspended in 1ml PBS and dialysed for 24 hours in PBS at 4°C. 0.8mg of recombinant CRP was then added to the AP with a final concentration of 0.2% gluteraldehyde (Sigma-Aldrich, UK) and rolled in darkness for 2 hours at room temperature. The AP-conjugated CRP was then dialysed for 48 hours in 4 changes of PBS before adding 1% BSA and 0.02% NaN\(_3\) (Sigma-Aldrich, UK). The stock was then filter-sterilized through a 0.2μM filter and stored at 4°C.

### 2.2.5 Preparation of bacterial samples for Western blot analysis

*N. meningitidis* cultures were grown for 4 hours and spun down as per section 2.2.1. Cells were resuspended to an OD\(_{595}\) of 1 in PBS with 0.5mM CaCl\(_2\) or PBS with 0.5mM CaCl\(_2\) and 10mM EDTA. Cell suspensions were opsonised with 50μg/ml recombinant CRP (or mock opsonised with PBS alone) for 30 minutes. Non-bound CRP was washed off with PBS during 3 spins of 2 minutes.
at 4000 x g. To increase the sensitivity of detection, samples were concentrated to 1/5th of their original volumes in PBS.

### 2.2.6 SDS-PAGE and Western blot of samples

Cell suspensions were prepared as per 2.2.5. Human CRP was further purified as per section 2.2.4 and diluted in PBS. Samples were mixed with equal volumes of SDS-PAGE sample loading buffer and boiled for 5 minutes before loading onto a 12% polyacrylamide gel with 5% stacking gel alongside pre-stained protein standard molecular weight markers (BioRad, UK). The gel was run at 80mV in SDS-PAGE tank buffer and blotted overnight onto polyvinylidene fluoride membrane (PVDF, Roche) soaked in Western transfer buffer. Replicate gels were stained for 1 hour with coammassie stain (0.1% (w/v) coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid) and washed in destaining solution (50% (v/v) methanol in water with 10% (v/v) acetic acid) to ensure equal loading of wells.

### 2.2.7 Immunoprobing of Western blots

Western blots prepared as per section 2.2.6 were divided into sections and blocked for 1 hour in Western blocking buffer (PBS, 0.05% Tween 20, 2% BSA). Sections were then placed inside universal tubes and rolled for 1 hour at room temperature with primary antibodies in diluting buffer (PBS, 0.05% Tween 20, 1% BSA). Primary antibodies were specific for class 1 pili (SM1 antibody, gift from Professor Mumtaz Virji University of Bristol), phosphorylcholine (TEPC-15, mouse IgA, Sigma-Aldrich, UK) and human CRP (goat anti-human CRP IgG, Biogenesis, UK). Blot sections were washed for 30 minutes with three changes of diluting buffer then rolled for 1 hour at room temperature with peroxidase-conjugated secondary antibodies (anti-mouse IgA A4789, anti-mouse IgG A2304 and anti-goat IgG A4174, all Sigma-Aldrich, UK). Blot sections were washed for 30 minutes with three changes of diluting buffer then soaked in Luminal solution (Roche, UK) and developed onto BioMax light film (Kodak, UK). Films were dried then overlaid over blots to allow the position of pre-stained molecular weight markers to be traced onto the film.
2.2.7.1 Immunoprobing of phosphorylcholine expression in *N. meningitidis*

Cultures of piliated C311 and the non-piliated mutant C311p- were grown in MHB, washed and resuspended in sample buffer every 2 hours over a 10 hour time-course. Cell lysates were subjected to SDS-PAGE and Western blotting as per sections 2.2.5 and 2.2.6 and probed with anti-PC antibody (TEPC-15, Sigma-Aldrich, UK) as per section 2.2.7. The blots were photographed using a Gene Genius bioimaging system and densitometry of bands was performed using GeneTools software (Synoptics, UK).

2.2.8 High sensitivity ELISA for the measurement of CRP

Nunc maxisorp ELISA plates were coated, 50μl/well with 0.5μg/ml polyclonal rabbit anti-human CRP in 0.1M bicarbonate buffer and incubated overnight at 4°C. Plates were washed with PBS-Tween (PBS-T,0.05%) and blocked with 2% BSA in PBS for 1 hour at 37°C. Plates were washed with PBS-T and DAKO CRP standards were diluted from 100mg/ml to 100ng/ml, 30 ng/ml, 10 ng/ml, 3 ng/ml, 1 ng/ml, 0.3 ng/ml, 0.1 ng/ml, 0.03 ng/ml, and 0.01 ng/ml in PBS-T-BSA (0.2%). Control and test samples were added in triplicate at 50μl/well and the plates were incubated for 1 hour at 37°C. Plates were washed with PBS-T and 50μl/well peroxidase-conjugated rabbit anti-human CRP was added at 1μg/ml in PBS-BSA (0.2%) for 1 hour at 37°C. Plates were washed again with PBS-T followed by a wash with citrate-phosphate buffer (0.05M, pH5). Substrate was prepared by adding 1 tablet of TMB substrate (3,3',5,5'-tetramethylbenzidine, Sigma-Aldrich, UK) per 10ml 0.05M citrate-phosphate buffer, pH5 + 2μl 30% H₂O₂. TMB substrate was added, 50μl/well and plates incubated at room temperature for 10 minutes. The reaction was halted with 12.5μl/well 2M H₂SO₄ and plates read at 450nM on a Biotek, USA ELISA plate reader.

2.2.8.1 Preparation of supernatant samples for high-sensitivity CRP ELISA

*N. meningitidis* and *S. pneumoniae* cultures were prepared as per section 2.2.1 and grown for 4 or 6 hours respectively. Cells were spun for 10 minutes at 1000 × g and resuspended in PBS with 0.5mM CaCl₂ to an optical density of 10 at 595nm for *Neisseria* and an optical density of 2 at 600nm for Streptococci. Bacteria were opsonised in a final concentration of 50μg/ml purified human CRP (either in PBS with 0.5mM CaCl₂ alone, or with 10mM EDTA or 1mg/ml soluble...
phosphorylcholine-calcium salt) for 30 minutes at 37°C. Bacteria were spun at 6000 \( \times \) g for 3 minutes and supernatants reserved. Pellets were washed twice in PBS and spun at 6000 \( \times \) g for 3 minutes, reserving supernatants and pooling each sample's supernatants from the 3 spins. Pooled supernatants were filter sterilised and diluted for measuring CRP concentrations in an in-house high sensitivity CRP ELISA as per section 2.2.8.

2.2.8.2 Preparation of eluted samples for high sensitivity CRP ELISA

* N. meningitidis* cultures were prepared as per section 2.2.1 and grown for 4 hours in MHB with 100\( \mu \)g/ml CRP or in plain MHB. Bacteria were washed with three spins at 1000 \( \times \)g for 4 minutes and resuspended in PBS. After the third spin, cultures were resuspended in PBS+10mM EDTA and left to elute for 10 minutes. Cultures were spun a fourth time and the supernatant collected and filter sterilised. Eluents were then serially diluted and assayed for CRP concentration using the high sensitivity CRP ELISA as per section 2.2.8.

2.2.9 Microtitre plate-based assay to measure CRP binding to *N. meningitidis*

Bacteria were grown and fixed as per section 2.2.1 then spun at 4000 \( \times \)g for 6 minutes and resuspended in RPMI at an optical density of 5 at 595nm. The method used to determine CRP binding to *N. meningitidis* was based on a method developed for *L. donovani* (Culley et al., 2000). Briefly, 50\( \mu \)l aliquots of (washed) fixed bacterial culture were dispensed onto 96 well Nunc maxisorp ELISA plates alongside RPMI-only controls. Plates were dried overnight in an incubator at 35°C and the dried wells were blocked with 1% bovine serum albumin (Sigma-Aldrich, UK) in PBS for 1 hour. Wells were washed with PBS and 25\( \mu \)l of treatments were added to each well in triplicate. Treatments were prepared in PBS and included PBS only, 10mM EDTA, and phosphorylcholine-calcium salt at a range of concentrations (Sigma-Aldrich, UK). AP-conjugated CRP (CRP-AP) was prepared as per section 2.2.4.1. CRP-AP was diluted to 20\( \mu \)g/ml in PBS + 1mM CaCl\(_2\) and added in 25\( \mu \)l volumes to wells (final concentrations of 10\( \mu \)g/ml CRP and 0.5mM CaCl\(_2\)). Plates were incubated in the dark for 2 hours then washed 3 times with PBS and once with 0.1M bicarbonate buffer pH9.6. Plates were developed with 50\( \mu \)L/well pnitrophenyl phosphate (pNPP, Sigma-Aldrich, UK) at 1mg/ml in 0.1M bicarbonate buffer pH 9.6
containing 2mM MgCl₂. Plates were incubated in the dark for a further hour and read at 405/490nm on a microplate reader (Biotek, USA).

For time-course binding experiments, the standard binding assay was performed on cultures harvested between 2 and 10 hours of incubation. For saturation experiments, the assay was carried out with a range of concentrations of CRP-AP. Competitive inhibition assays used the original concentration of CRP-AP with added phosphorylcholine-calcium salt (Sigma-Aldrich, UK) diluted in PBS at a range of concentrations.

Dose-response experiments, to find the optimal density of bacteria to detect CRP-AP binding, utilised bacteria in a range of doubling dilutions from an optical density of 10 down to an optical density of 0.3 at 595nm.

2.2.10 Immunoglobulin G depletion of human plasma

Laboratory volunteers were used according to University ethical guidelines (Local Research Ethics Committee 04/Q1909/19RSCH). 5 x 9ml Vacutainer tubes with EDTA (Greiner Bio-one, UK) of peripheral blood were taken from each volunteer. Blood was transferred to 50ml centrifuge tubes and spun at 2000 x g for 10 minutes and the plasma layer collected with a Pasteur pipette. Plasma was snap-frozen in a boiling dry ice waterbath in cryovial tubes and stored at -80°C. On the assay day, plasma was gently defrosted on ice. In the cold room, a 10ml syringe was filled with ice cold Hanks balanced salt solution (HBSS) and connected drop-to-drop (to avoid air bubbles) to a 1ml protein-G sepharose column (GE Biosciences, UK). The column was equilibrated with 10mls HBSS at a rate of 1ml per minute then 1ml of plasma was added to the column at a rate of 1ml per minute. The column was incubated on ice for 5 minutes then plasma run through the column into a pre-weighed tube using 2ml ice cold HBSS. The tube was weighed to estimate the dilution factor of the original sample. The column was washed with 10ml HBSS then 5ml elution buffer (0.1M glycine-HCl) and fractions were collected for analysis. The column was then washed with 10ml HBSS and stored in HBSS+20% ethanol. IgG-depleted plasma was filter sterilised and stored on ice until use. Purity was confirmed retrospectively by running IgG-depleted plasma on a 12% SDS-PAGE gel and comparing to the original sample and to the eluted IgG fractions.
2.2.11 Adapted serum bactericidal assay

The piliated \textit{N. meningitidis} strain C311 and the non-piliated mutant C311p- were grown and spun as per section 2.2.1 then resuspended in bactericidal buffer (HBSS + 0.5mM CaCl$_2$ + 1% BSA) to an optical density of 0.1 at 595nm. Cultures were diluted 1 in 2000 in bactericidal buffer with a final concentration of 50\mu g/ml CRP or bactericidal buffer alone. Bacteria were incubated with or without CRP for 15 minutes at 35°C then equal volumes of bacteria were added to a 1 in 5 dilution of IgG-depleted or complete human plasma (final dilution 1 in 10) in duplicate for each sample. Inoculum was serially diluted and plated out onto CBA for enumeration and plates were incubated at 35°C for 30 minutes. After incubation with plasma, 2 \times 10\mu l aliquots of each sample were dispensed onto CBA plates tilted at 45° to allow the droplets to spread down the length of the plate. CBA plates were incubated at 35°C overnight and colonies counted the following morning.

2.2.12 Activation of macrophages by CRP-opsonised bacteria

To investigate the effect of CRP-opsonised bacteria on the expression of markers of activation by macrophages, bacteria were prepared as per section 2.2.1 then adjusted to an optical density at 595nm of 4 or 0.2 before opsonising with either 100\mu g/ ml CRP, 50\mu g/ml CRP, or PBS alone for 30 minutes. THP-1 and U937 cell lines were differentiated into macrophages and peripheral blood-derived macrophages were prepared as per section 2.2.3 in small flasks, 10mls per flask at a density of 2-4 \times 10^5 cells/ml. On the assay day, media was aspirated, cells gently washed with serum-free media and media replaced with 5mls serum-free media. For negative controls, 500\mu l PBS was added to the flask. For positive controls, final concentrations of 100 units/ml Interferon gamma (IFN\gamma, R&D Systems, UK) and 1\mu g/ml lipopolysaccharide (LPS, Sigma-Aldrich, UK) were added to the cells. For CRP treatment, 500\mu l of CRP at 100\mu g/ml or 50\mu g/ml CRP with 5\mu g/ml polymixin B was added to cells (final CRP concentrations of 10 & 5 \mu g/ml respectively). For bacterial treatments, 500\mu l of the PBS-opsonsied meningococci was added to cells. For CRP-opsonised bacterial treatment, 500\mu l of the CRP-opsonised meningococci was added to the cells. All cells were incubated for 24 hours before staining for FACS analysis.
2.2.13 Fluorescence activated cell scanning (FACS) of bacteria and phagocytic cells

Bacterial and mammalian cells were prepared for FACS analyses as per sections 2.2.13.1-4. Bacteria and single-labelled cell lines were examined using a Becton Dickinson FACscan flow cytometer (BD, UK) and data were analysed using WinMDI software (http://facs.scripps.edu). All other cells were examined on the BD FACScanto flow cytometer using FACSDiva software (BD, UK). The cell populations were gated by size and granularity to exclude debris and at least 10,000 cells were counted in any test.

2.2.13.1 Detection of CRP binding to bacteria by FACS

*S. pneumoniae* or *N. meningitidis* C311p- and C311p+ cultures were prepared as per section 2.2.1. Cultures were adjusted to $5 \times 10^6$ CFU/ml in PBS + 0.5mM CaCl$_2$ (approximate optical density of 0.05 at 595nm where previous inoculum counts revealed that an OD$_{595}$ of 1 corresponds to $1 \times 10^8$ CFU/ml for *N. meningitidis* C311). Purified CRP was added at 50pg/ml and tubes were incubated for 30 minutes at 37°C then washed twice with cold PBS and 2 minute 15000 $\times$ g spins and resuspended in FACS buffer. Primary antibodies were first titrated to determine optimal activity concentrations and ultimately diluted to 0.5µg/ml in FACS buffer (mouse IgA anti-phosphorylcholine (TEPC), goat IgG anti-human CRP, mouse IgG anti- *S. pneumoniae* or mouse IgG anti-serogroup B *Neisseria meningitidis*) and incubated on ice with cells for 1 hour. Cells were washed twice with cold PBS for 2 minutes spinning at 15000 $\times$ g and resuspended in FACS buffer. Tubes were incubated with FITC-conjugated secondary antibodies (anti-mouse IgA, anti-mouse IgG and anti-goat IgG respectively) for 1 hour on ice. Cells were washed twice with cold PBS for 2 minutes at 15000 $\times$ g then fixed in 4% paraformaldehyde before examining on the BD FACScan flow cytometer as per section 2.2.13.

2.2.13.2 Detecting association of GFP-expressing *N. meningitidis* with human phagocytes using FACS

The GFP-expressing *N. meningitidis* strain C311 was grown and fixed as per section 2.2.1. Bacteria were then washed, and resuspended in PBS with 0.5mM CaCl$_2$ to an optical density of 4 at 595nm before opsonising with either 100µg/ml purified human CRP in PBS with 0.5mM CaCl$_2$ or plain PBS with
0.5mM CaCl₂ for 30 minutes at 37°C. Macrophages (either PMA-differentiated THP-1 cell line or peripheral blood derived 7 day old macrophages) were collected from plastic surfaces by gentle scraping before washing in serum-free media and resuspending in serum free media to a density of approximately 5 × 10⁵/ml. 100μl of opsonised bacteria was added to each ml of cells in universal tubes which were incubated at 37°C for 2 hours. Tubes were then spun at 250 × g for 5 minutes and washed in PBS 3 times before fixing in 2% paraformaldehyde for at least 30 minutes before examining on the flow cytometer as per section 2.2.13.

2.2.13.3. Investigating the expression of activation markers on macrophages by single and double-labelled FACS

Macrophages were prepared and incubated with controls or bacteria as per section 2.2.12. After 24 hours incubation, supernatants were collected and stored at -80°C and cells were washed in HBSS without calcium or magnesium, trypsinised (with 0.05%Trpsin-EDTA, Invitrogen, UK) and harvested in FACS buffer (PBS + 2% bovine serum albumin + 0.05% NaN₃). Cells were washed in FACS buffer and resuspended in 1ml FACS buffer with 1% normal human serum to block Fc receptors. For single staining, cells were resuspended in 40μl aliquots of FSB with the following concentrations of antibody:

<table>
<thead>
<tr>
<th>Antibody</th>
<th>(µg of Ab)</th>
<th>Isotype controls</th>
<th>(µg of Ab)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>0.5</td>
<td>IgG1 - FITC</td>
<td>0.5</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.5</td>
<td>IgG1-PE</td>
<td>0.125</td>
</tr>
<tr>
<td>FcyR1 (CD64)</td>
<td>0.125</td>
<td>IgG2a-PE</td>
<td>0.03</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Amounts of antibody used in FACS staining

For double staining experiments, each aliquot of cells was incubated with both a PE-conjugated and a FITC-conjugated specific antibody. For double-staining FACS analysis, the FACScanto was pre-calibrated using BD calibration beads coated with the same concentrations of antibodies used in the staining procedure. This allowed for compensation for any possible leaching between red and green fluorescence channels.

For both single and double-stained cells, plates were incubated on ice in the dark for 1 hour before washing 3 times with FACS buffer and resuspending in 2% paraformaldehyde after the final wash. Fixed cells were analysed as per section 2.2.13.
2.2.14 Confocal fluorescence microscopy

Human granulocytes and macrophages were prepared from volunteers and adhered to 8-well plastic chamber slides (Nunc, UK) as detailed in section 2.2.3.2-3. Cells were treated with bacteria with and without CRP as per sections 2.2.14.1 – 2.2.14.4. Slides were either fixed in 4% paraformaldehyde after the experiment and mounted in vectashield soft-set mounting media with propidium iodide (PI) or stained with the lipophilic dye FM6-46 before fixing and mounting in vectashield soft-set mounting media without PI. In all cases, coverslips were applied and sealed with clear nail polish before examining on the Zeiss LSM5.1 confocal fluorescent microscope on at least ×400 magnification. The microscope was set up to read Cy2 and CY3 channels to allow both red and green fluorescence to be detected simultaneously. Images were scanned and saved for analysis using LSM image browser (www.zeiss.com). For each chamber slide well examined, at least 10 randomly selected fields of view were recorded.

2.2.14.1 Investigating the effect of CRP-opsonisation on association of meningococci with human phagocytes using immunolabelled bacteria

Macrophages were prepared and adhered to chamber slides as detailed in section 2.2.3.2. *S. pneumoniae* R36 A and *N. meningitidis* C311 p- and C311 p+ cultures from overnight plates were suspended at 8×10⁷ cfu/ml in PBS and opsonised for 30 minutes at 35°C with 50μg/ml CRP in PBS containing 0.5mM CaCl₂, or 50μg/ml CRP in PBS with 0.5mM CaCl₂ and 10mM EDTA or CRP-free control (PBS containing 0.5mM CaCl₂ alone). Cultures were spun at 1000 x g for 10 minutes, washed once in PBS and resuspended to the original volume in RPMI. 40μl of bacterial suspension was added to each of 6 wells per chamber slide and the slides were incubated at 35°C for 2 hours then gently washed with warm PBS before fixing overnight with 4% paraformaldehyde. Fixed chamber slides were gently washed 3 times with PBS and cells were permeabilised with 0.1% TRITON X-100 (Sigma-Aldrich, UK) for 5 minutes. Fc receptors were blocked by incubating with 1% normal human serum (Sigma-Aldrich, UK) for 20 minutes. Slides were washed and incubated with mouse anti-meningococcal antibody at a 1 in 100 dilution in PBS with 1% normal human serum for 1 hour. Slides were washed thoroughly then incubated with goat f(ab')₂ anti mouse IgG-FITC at a 1 in 100 dilution in PBS with 1% normal human serum.
for 1 hour. Slides were washed thoroughly then mounted in soft-set mounting media with PI and examined as per section 2.2.14.

2.2.14.2 Investigating the effect of CRP-opsonisation on association of meningococci with human phagocytes Using GFP-expressing meningococci

GFP-expressing bacteria detailed in section 2.2.2 were prepared as per section 2.2.1 then fixed overnight in 4% paraformaldehyde, washed and resuspended in PBS containing 10mM CaCl₂ to an optical density at 595nm of 4.00. Bacteria were then treated for 30 minutes with equal volumes of either pooled normal human serum, 50µg/ml purified CRP or PBS. Treated bacteria were added in 40µl volumes, in duplicate, to a final volume of 400µl/well of differentiated monocytic cell lines or peripheral blood derived macrophages and incubated for 2 hours at 37°C +5% CO₂ to allow phagocytosis to occur. For the granulocyte uptake assays, phagocytosis was allowed to occur for 20 minutes only. The wells were washed 4 times with PBS, fixed with 1% paraformaldehyde then prepared for confocal microscopy as per section 2.2.14. At least 100 cells from 10 randomly selected fields of view were analysed for enumeration of phagocytes and co-localised bacteria. The presence of internalised bacteria was confirmed by staining macrophages post-infection with a lipophilic membrane dye (FM 4-64FX, Molecular Probes, UK) as per the manufacturers instructions and mounting in vectashield soft-set mounting media without PI. This allowed a z-stack of images to be taken through the depth of the cell.

2.2.14.3 IgG blocking of Fcγ receptors

The method detailed in section 2.2.14.2 was modified to investigate the effect of blocking Fcγ receptors on CRP-mediated uptake by macrophages. Before adding opsonised or unopsonised bacteria to macrophages adhered to chamber slides, macrophages were first treated for 30 minutes with human IgG (Sigma-Aldrich, UK) at 100µg/ml, 10µg/ml, 1µg/ml or 0µg/ml in serum-free RPMI. Non-bound IgG was then washed off and slides incubated with bacteria and examined as per the section 2.2.14.
2.2.14.4 Association of live meningococci with macrophages examined by confocal fluorescence microscopy

The method detailed in section 2.2.14.2 was modified to allow detection of uptake of live meningococci by macrophages. Macrophages were cultured on chamber slides as usual but on the assay day, all procedures were carried out in the \textit{Neisseria} class 1 safety cabinet. Bacteria were opsonised with PBS alone or a final concentration of 100\mu g/ml CRP before infecting macrophages at a 1 in 10 dilution for 2 hours. Chamber slides were washed 4 times with PBS then fixed overnight in 4\% paraformaldehyde before examining on the microscope the following day as per section 2.2.14.

2.2.15 Association of live meningococci with macrophages assayed by gentamicin exclusion

Peripheral blood and cell line-derived macrophages were prepared as per section 2.2.3.1-2 and cultured in 24-well plates. Meningococci were prepared as per section 2.2.1 and resuspended in PBS + 0.5mM CaCl\textsubscript{2} to an optical density of 4 at 595nm. Bacteria were opsonised for 30 minutes with either 100\mu g/ml CRP, 25\% heat inactivated normal human serum or PBS alone then added to macrophages at a 1 in 10 dilution. Macrophages were allowed to phagocytose for 2.5 hours before washing and either lysing with 1\% saponin in PBS to enumerate adherent and intracellular bacteria or incubating with 200\mu g/ml gentamicin for 1 hour before lysing with saponin to enumerate intracellular organisms only. Saponin treated macrophage lysates were serially diluted in PBS alongside bacterial inocula and dispensed onto CBA plates to count the numbers of intracellular and adherent bacteria.

2.2.16 Measuring the effect of CRP on intracellular killing by time-kill assay

Macrophages in tissue culture plates were infected with CRP-opsonised and non-opsonised meningococci as per section 2.2.15. At regular intervals, duplicate wells were washed and incubated with gentamicin for one hour and lysed with saponin for enumeration of intracellular bacteria as per 2.2.15. This procedure was repeated at regular intervals throughout a time-course.
2.2.17 Quantification of macropage-secreted TNFα by ELISA

An in-house TNFα ELISA was optimised to quantify TNFα production in cell culture supernatants. 96-well ELISA plates were coated with 50μl/well 0.5μg/ml anti-human TNFα (BD Biosciences, UK) in 0.1mM Na2HPO4, pH 9. Plates were incubated overnight at 4°C then washed four times with PBS-T (0.05%) before blocking for 1 hour with 2% BSA in PBS-T. Plates were washed a further three times before incubating with either control standards or test samples. Control standard TNFα (NIBSC, UK) was serially diluted in PBS-T-BSA (0.2%) from 20ng/ml down to 9pg/ml. Test and control samples were incubated in triplicate, 50μl/well at 4°C overnight then washed four times with PBS-T and incubated at room temperature for 1 hour with 50μl/well biotin-conjugated anti-human TNFα (BD Biosciences, UK) diluted to 1μg/ml in PBS-T-BSA (0.2%). Plates were washed five times with PBS-T then incubated for 30 minutes with 50μl/well of avidin-HRP (BD Biosciences, UK) at 2μg/ml in PBS-T-BSA (0.2%). Plates were washed 5 times with PBS-T followed by 3 washes with citrate-phosphate buffer (pH5). Plates were developed by adding 50μl/ well TMB substrate dissolved in citrate-phosphate buffer (pH 5) with 60 parts per million H2O2 as per the manufacturer's instructions. The reaction was stopped by adding 12.5μl of 2mM H2SO4 per well. Plates were read at 450nM using a Biotek microplate reader. The standard dilutions were used to construct a calibration curve of TNFα concentration which was used to calculate corresponding TNFα concentrations for test samples on each plate.

2.2.18 Quantification of inflammatory cytokines by Cytometric Bead Array

A BD biosciences Human Inflammation Cytometric Bead Array kit was used as per the manufacturer's instructions to measure IL-1β, IL-6, IL-8, IL-12p80, IL-8 and TNFα concentrations from cell culture supernatants. In principal, the cytometric bead array consists of 6 populations of beads with distinct fluorescence intensities that are coated with capture antibodies for the 6 inflammatory cytokines listed. This allows simultaneous quantification of cytokines from cell culture supernatants after incubating with test and control samples and a PE-conjugated secondary antibody. The in-house TNFα assay results were used to choose appropriate dilutions of samples to use in the assay. Cytometric bead arrays were run on the BD FACScalibur with Dr Mark Bodman-Smith at St Georges Hospital, University of London.
2.3 Statistical analyses

Data are expressed as arithmetic means ± standard deviation (SD) or standard error of the mean (SE). Means were compared using Students t-test for paired data. Analysis of variance was used to measure coefficients of variation for 3 or more groups of variables using the 1-way ANOVA, correcting with Tukey post test for multiple comparisons. All data was analysed using GraphPad Prism 4 software. For all analyses, P values of less than 0.05 were considered statistically significant.
CHAPTER THREE

INVESTIGATING THE ABILITY OF C-REACTIVE PROTEIN TO BIND TO *NEISSERIA MENINGITIDIS*
3.1 Rationale and aims

The principal ligands for CRP are phosphorylcholine (PC) and other phosphorylated carbohydrates which are found on the surface of a variety of eukaryotic and prokaryotic cells (Gillespie et al., 1996; Jansen et al., 1999; Culley et al., 2000; Chang et al., 2002). PC has been identified on the pilus of pathogenic strains of Neisseria (Weiser et al., 1998a), although there was no published evidence of CRP binding to this ligand before the onset of this investigation. It was, therefore, considered important to determine whether CRP was capable of binding to this target at physiologically relevant concentrations.

The first aim of this chapter was to develop a suitable method to detect CRP binding to piliated meningococci and to investigate the specificity and affinity of this interaction. The piliated serogroup B meningococcal strain C311 was utilised for these studies because it was a clinical isolate that had previously been shown to express PC (Weiser et al., 1998a). The C311 strain was also valuable because of the availability of a non-piliated spontaneous mutant that could be used as a negative control in binding experiments.

The second aim of this chapter was to investigate if CRP bound to live as well as fixed meningococci as these two events may have very different downstream immunological effects. The final aim of this chapter was to examine CRP binding to other meningococcal strains. A second serogroup B strain H44/76 was tested for CRP-binding ability as was a virulent Serogroup C strain L91543 and a phoP knockout mutant made from this strain, which shows reduced virulence in mouse models of meningococcal infection (Newcombe et al., 2004). These last two strains were tested to investigate whether differences in the transcriptome of strains resulted in differences in CRP binding.
3.2 Results

Initial development of CRP-binding assays utilised S. pneumoniae because this gram positive organism has been well characterised in its ability to bind CRP and can be manipulated using standard containment level 2 conditions. Following this, CRP-binding to N. meningitidis was investigated using several methodologies and the specificity and affinity of the interaction was explored. Several additional strains of N. meningitidis were also tested for CRP binding ability. Following the characterisation of CRP-binding to fixed meningococci, binding to live organisms was investigated using a high-sensitivity CRP-ELISA.

3.2.1 Development of a fluorescence activated cell scanning (FACS) assay to demonstrate CRP binding to S. pneumoniae

To capture S. pneumoniae cultures at an actively dividing growth phase, growth curve experiments were performed as described in 2.2.1. Figure 3.1 shows a representative example of a growth curve in which the 6 hour time-point was chosen as an appropriate mid-log phase time point to harvest S. pneumoniae.

Figure 3.1 Growth curve of S. pneumoniae R36A
S. pneumoniae R36A was used to inoculate BHI broth. Cultures were shaken and aliquots taken at 2 hour time points for serial dilution in PBS and plating out onto Columbia blood agar for enumeration. Data are from one representative experiment.
FACS analysis of CRP-opsonised *S. pneumoniae* cultures was performed as described in 2.2.1. Bacteria were opsonised with human CRP, washed and probed with anti-CRP antibody and FITC-conjugated secondary antibody as described in 2.2.13.1. Figure 3.2 demonstrates the results of the proof of principle experiments using *S. pneumoniae*, with a clear right shift in fluorescence of the population of cells opsonised with CRP (geometric mean 277.7) when compared to cells not opsonised with CRP (geometric mean 8.3).

![Figure 3.2](image)

Figure 3.2 Representative histogram showing relative fluorescence of anti-CRP probed *S. pneumoniae*

*S. pneumoniae* strain R36A was probed with anti-CRP antibody and FITC-conjugated secondary antibody after opsonising with CRP (black) or mock-opsonising with PBS (Red).
3.2.2 Development of a FACS method to investigate CRP binding to *N. meningitidis*

Following the detection of CRP bound to the surface of *S. pneumoniae* by FACS analysis, the method was optimised for use with Paraformaldehyde-fixed *N. meningitidis*. Initially, the cell surface structures of capsule and pili were targeted to act as positive controls for the assay. All antibodies were titrated before use in the assay and compared to isotype-matched non-specific controls. Figure 3.3 demonstrates that probing with the anti-capsular antibody gave a clear right-shift of the cell population fluorescence compared to the negative control (which was probed with non-specific isotype-matched antibody, geometric means 404.2 and 12.5 respectively). Probing with the anti-pili antibody, however, did not result in an increase in fluorescence as compared to the isotype control (geometric means 11.2 and 12.5 respectively). This indicated that the anti-pili antibody was unable to bind to the meningococci in this assay (the activity of the antibody had previously been confirmed by Western blot analysis of denatured meningococcal whole cell lysates, see figure 3.16).

![Figure 3.3 Representative histogram showing relative fluorescence of anti-pili and anti-capsule probed meningococci](image)

*N. meningitidis* C311 was stained with anti-meningococcal antibody (green), anti-pilin antibody (red) or isotype control antibody (black). Data are from one representative experiment.
The lack of staining of *N. meningitidis* strain C311 with the anti-pili antibody could be a result of pili being lost during the many washing steps of the assay. To investigate this, meningococci were probed with anti-PC antibody. Figure 3.4 shows the effect of staining with anti-PC antibody with the geometric mean of fluorescence recorded as 5.7 in the isotype control and 6.7 in the PC-stained population. This indicated that the PC antibody was unable to bind to the cells in these assay conditions. The activity of the anti-PC and anti-pilin antibodies had previously been confirmed by Western blot analysis, as shown in figure 3.16, but SDS-PAGE gels present antigen in a denatured form which may be different from the native form of protein presented on the bacterial surface. For this reason it could not be confirmed whether pili and PC were being lost in the washing steps or if the antibodies were unable to label their targets in their native states on the surface of the bacteria.

![Figure 3.4 Representative histogram showing relative fluorescence of anti-PC stained meningococci](image)

*Figure 3.4 Representative histogram showing relative fluorescence of anti-PC stained meningococci*

*N. meningitidis* C311 was probed with anti-PC antibody (red) or isotype control antibody (black) and FITC-conjugated secondary antibody. Data are from one representative experiment.

CRP binding to PC is a calcium-dependent process. Thus to examine CRP binding to the meningococcus, *N. meningitidis* strain C311 was opsonised with CRP in the presence of calcium, or calcium with EDTA before washing and probing with anti-CRP antibodies and FITC-conjugated secondary antibodies. Figure 3.5 reveals that the cells probed with anti-CRP antibody did not show increased fluorescence compared to isotype controls (geometric mean 8.84), either in the presence of calcium (geometric mean 5.39) or in a calcium-depleted solution (geometric mean 6.15 or 6.84 with isotype control antibody). The relative fluorescence of all cell populations was less than 10 suggesting
that CRP does not bind to the piliated *N. meningitidis* strain in the conditions of this FACS assay.

**Figure 3.5 Histogram showing relative fluorescence of anti-CRP probed cells**

*N. meningitidis* C311 was opsonised with CRP in the presence of calcium and probed with anti-CRP antibody (red) or isotype control antibody (black). Control opsonisation was in the presence of EDTA followed by anti-CRP antibody (blue) or isotype control antibody (green). Data are from one representative experiment.
3.2.3 CRP binds to piliated meningococci in a microtitre plate-based assay

In order to control for the possible loss of pili during washing steps, a new strategy was devised to investigate CRP binding to the meningococcus. Large numbers of meningococci grown in broth to specific time-points were fixed and immobilised on the base of a microtitre plate and probed with AP-conjugated recombinant human CRP (CRP-AP) in a binding assay detailed in section 2.2.9. CRP binding to *N. meningitidis* strain C311 was investigated over a 10 hour time-course of growth. As can be seen in figure 3.6, CRP binding was observed in the presence of calcium after 2 hours and peaked at 4 hours of growth. Binding then rapidly decreased to negligible levels by 8 hours and remained low up to the final 10 hour time-point. The maximum CRP binding at the 4 hour time-point corresponded with the end of lag phase/early log phase in the growth curve of *N. meningitidis* C311 in MHB (see figure 3.6). At six hours, growth of *N. meningitidis* was still exponential but CRP binding was almost halved. No CRP binding was observed in the absence of calcium, supporting the hypothesis that CRP binds to the meningococcus by the classical calcium-dependent binding via the ligand binding face of the molecule.

![Figure 3.6 CRP binding to piliated *N. meningitidis* over a time-course of growth](image)

*N. meningitidis* C311 (Nm) was grown in MHB, dried onto microtitre plates and probed with CRP-AP in the presence of calcium (red squares) or EDTA (blue triangles). Plates were washed thoroughly and bound CRP-AP was detected by adding pNPP substrate and measuring the absorbance at 405nm with a reference wavelength of 490nm. The dashed green line represents the growth curve of the cultures used as measured by optical density. Data are expressed as mean of 4 replicate experiments +/- standard deviation.
To establish the concentration of bacteria which should be used in the CRP binding assay, 4 hour old cultures of *N. meningitidis* were serially diluted from an optical density of 10 down to 0.3 at 595nm before drying onto plates and performing the standard CRP binding assay. Figure 3.7 reveals that a bacterial suspension of optical density 5 at 595nm gave the greatest differential between CRP binding in the presence and absence of calcium and accordingly, this concentration of bacteria was used for all further experiments.

![Graph showing CRP binding vs density of bacteria](image)

**Figure 3.7 Dose response of density of bacterial suspension vs CRP binding**

*N. meningitidis* C311 was serially diluted from an optical density of 10 at 595nm down to an optical density of 0.3 before drying onto a microtitre plate and probing with CRP-AP in the presence (red line) or absence (blue line) of available calcium.
3.2.4 CRP binding to *N. meningitidis* requires the presence of pili and calcium

As optimum CRP binding to piliated *N. meningitidis* was observed after 4 hours growth in MHB, the adapted ELISA was repeated at this time-point and compared to CRP binding to a spontaneous non-piliated mutant of the same strain (C311p-). Figure 3.8 demonstrates that negligible CRP binding of either strain was observed in the absence of calcium, whereas in the presence of calcium, the piliated strain bound significantly more CRP than the non-piliated mutant (*p* = 0.011). The piliated strain also bound significantly more CRP in the presence of calcium compared to binding in the absence of available calcium (*p* = 0.002) and there was no significant difference in the binding of CRP to non-piliated C311p- in the presence or absence of available calcium (*p* = 0.181).

![Figure 3.8 CRP binding to piliated and non-piliated *N. meningitidis* in the presence or absence of calcium](image)

The piliated strain C311 (red) and non-piliated mutant C311p- (blue) were dried onto microtitre plates and incubated with CRP-AP in the presence of calcium (calcium rich) or EDTA (calcium depleted). Data are expressed as the mean absorbance of six replicate experiments + standard error of the mean. (*' denotes a *p* value < 0.05).
In order to confirm that CRP binding to the piliated strain was dependent on the presence of calcium rather than the absence of EDTA, CRP was incubated with the meningococcus with zero added CaCl$_2$ or increasing concentrations of CaCl$_2$ up to the standard 0.5mM concentration (Figure 3.9). CRP binding increased with calcium concentration and was significantly higher in the standard 0.5mM CaCl$_2$ compared to the both zero and 0.005mM CaCl$_2$ (p=0.029 and p=0.031 respectively).

**Figure 3.9 Titration of calcium for CRP binding to N.meningitidis**
Strain C311 was dried onto microtitre plates and probed with CRP-AP at a range of calcium concentrations or without added calcium. Data are expressed as the mean absorbance of 3 replicate experiments plus standard deviation (* denotes p value <0.05).
3.2.5 CRP binding to *N. meningitidis* correlates with phosphorylcholine expression

To investigate whether the differences seen in CRP binding at different time-points (figure 3.6) were due to changes in PC expression, Western blots of whole cell lysates of *N. meningitidis* grown from 2 to 10 hours were probed with anti-PC antibody. In these experiments, PC was not expressed in the non-piliated strain at any point during the time-course, whereas PC was detected in the piliated strain at all time-points tested. Figure 3.10 displays the results of densitometry analysis of the bands from one representative experiment. PC expression in the piliated strain peaked at 4 hours of culture growth; the time point that also gave maximum CRP binding. PC expression dropped over two and a half times between 4 and 6 hours of growth and remained low for the remaining time-points, correlating with CRP binding data.

![Figure 3.10](image_url)

*Figure 3.10 PC expression by piliated *N. meningitidis* with time*

*N. meningitidis* C311 (red squares) and non piliated C311p- (green triangles) were grown in broth culture and harvested at 2 hour intervals. Whole cell lysates were subjected to SDS-PAGE and Western blot and probed for PC expression. The blot was captured digitally and densitometry performed to quantify relative expression of PC. Data are from one representative experiment. CRP binding data from figure 3.6 for the piliated strain C311 is plotted in grey for comparison to PC expression.
3.2.6 CRP binding to *N. meningitidis* is inhibited by free phosphorylcholine

Further to the discovery that CRP binding correlates with PC expression in the meningococcus, it was decided to investigate the specificity of binding by adding increasing concentrations of soluble PC to the CRP binding assay detailed in section 2.2.9. Figure 3.11 demonstrates that PC concentrations below 1μM did not inhibit binding of CRP to piliated meningococci, but concentration-dependent inhibition was demonstrated above 5μM. 50% inhibition was seen at a PC concentration of 20μM and the inhibitory effect was saturated at concentrations of PC above 100μM. This provides further evidence that binding between CRP and the meningococcus is specific for PC.

![Figure 3.11 PC inhibition of CRP-binding to piliated *N. meningitidis*](image)

*N. meningitidis* C311 were dried on to microtitre plates and incubated with CRP-AP containing soluble PC at a range of concentrations. Data are expressed as mean percentage of inhibition of absorbance of 3 replicate experiments +/- standard deviation.
3.2.7 CRP binding to *N. meningitidis* is concentration dependent and of low affinity

The affinity of binding between CRP and piliated *N. meningitidis* was investigated by saturation binding experiments as described in section 2.2.9. CRP-AP was added at increasing concentrations to microtitre plates coated with a constant number of meningococci. The binding of CRP to the wells is expressed as a percentage of the value recorded for 10μg/ml CRP-AP to overcome inter-plate/day variation of the assay. As can be seen from figure 3.12, CRP binding to *N. meningitidis* was concentration-dependent and began to plateau at 20μg/ml, whereas CRP concentrations below 1μg/ml did not result in detectable binding to immobilised meningococci.

Figure 3.12 Saturation of CRP binding to piliated *N. meningitidis*
Meningococci were dried onto microtitre plates and incubated with CRP-AP at a range of concentrations. Data are expressed as mean percentage of maximum absorbance of 3 replicate experiments +/- standard deviation.

Scatchard analysis is a method of linearising saturation binding data by plotting specific binding/free ligand concentration on the Y axis vs. specific binding on the X axis. This gives a straight line with the following equations

- Y intercept = Bmax/Kd
- X intercept = Bmax
- Slope = 1/Kd

Where Bmax is the point where all receptors are saturated with ligand and Kd is the dissociation constant, a measure of affinity of binding between a receptor and ligand in terms of how easy it is to separate the receptor-ligand complex.
Scatchard analysis is a good way to visualise saturation binding data but the most accurate way to determine binding constants is by fitting a hyperbola directly to the non-linearised data and using graphical programmes to calculate the constants from this line. Figure 3.13 displays the results of Scatchard analysis and non-linear regression of the saturation data giving a dissociation constant $K_d$ of $2.18 \times 10^{-4}$ M.

![Graph showing binding curve and Scatchard plot](image)

Figure 3.13 Binding curve and Scatchard plot of CRP binding to piliated *N. meningitidis*

Data from saturation binding experiments in figure 2.13 were utilised for examining the affinity of binding of CRP for *N. meningitidis*. Non-linear regression analysis was performed using GraphPad Prism 4.1 Mean values of triplicate data from one representative experiment are shown.
3.2.8 CRP binding to other important strains of meningococci

A second serogroup B strain of *N. meningitidis*, strain H44/76 was examined for its ability to bind CRP over a 10 hour time course of growth using the standard assay as described in section 2.2.9. Figure 3.14 clearly demonstrates that CRP is able to bind to the meningococcal strain H44/76 in the presence of available calcium. Maximum CRP-binding was seen at 4 hours of growth, as was the case for strain C311.

![Figure 3.14 Time-course of CRP binding to *N. meningitidis* strain H44/76](image)

*N. meningitidis* strain H44/76 (Nm) was grown in MHB, dried onto microtitre plates and probed with CRP-AP in the presence of calcium (red squares) or EDTA (blue triangles). The dashed green line denotes the growth curve of the cultures used as measured by optical density at 595nm. Data are expressed as mean of 3 replicate experiments +/- standard deviation.

The virulent serogroup C strain of *N. meningitidis* L91543 and the *phoP* knockout mutant were both examined for CRP-binding ability during a 10 hour time course. Figure 3.15 shows that both strains bound CRP at all time-points in their growth curves and the *phoP* mutant bound significantly more CRP at the 8 hour time point compared to the wild type strain at this time-point (p=0.02). When EDTA was added to the assay, binding of CRP to both strains was negligible. Growth of the *phoP* mutant is known to be magnesium-dependent, but even in magnesium supplemented broth, the mutant strain did not grow as fast as the wild type and reached its maximum optical density at 8 hours,
compared to the wild type which continued to grow to a higher optical density after 10 hours of incubation.

Figure 3.15 Time course of CRP binding to *N. meningitidis* L91543 wild type and *phoP* mutant

*N. meningitidis* of strain L91543 and the *phoP* knock-out mutant of the same strain (Nm) were both grown in MHB supplemented with 10μg/ml MgCl₂. During 10 hour time-course, cultures were collected and measured for optical density (dashed lines, orange wild type and green *phoP*) and dried onto plates for CRP-binding assay (red squares wild type, blue triangles *phoP*). The grey dashed line shows the maximum CRP binding of either strain in the presence of EDTA. Data are expressed as mean of 3 replicate experiments +/- standard deviation.

3.2.9 Development of assays to detect CRP binding to live meningococci

All CRP-binding results detailed in section 3.2.2-3.2.8 utilised PFA-fixed meningococci to reduce the variability of assays (as bacteria are fixed at a chosen time-point of growth) and to enable experiments to be performed outside the category 2* containment laboratory. It was, however, considered important to investigate the capacity of CRP to bind to live meningococci as this interaction may result in different down-stream immunological effects. Accordingly, assays were developed to investigate CRP binding to live meningococci.
3.2.9.1 Investigation of CRP binding to live *N. meningitidis* by Western blot analysis

Piliated and non piliated live meningococci were opsonised with purified CRP in the category 2* facility and subjected to Western blot analysis as detailed in sections 2.2.5 – 2.2.7. Figure 3.6 displays a representative Western blot of non-piliated and piliated strains of C311 probed separately with anti-PC antibody (columns B and C) and anti-pili antibody (columns D and E). Both antibodies bound to the piliated strain only. Non-piliated and piliated strains were opsonised with CRP in the presence of EDTA and probed with anti-CRP antibody which did not bind in these conditions (columns G and H). Non-piliated and piliated strains were also opsonised with CRP in the presence of calcium which resulted in minimal binding of the anti-CRP antibody to the non-piliated strain but greater binding to the piliated strain (columns I and J). All CRP-positive bands were in the correct position for the 23kD relative molecular mass of the CRP subunit.

**Figure 3.16.** Representative Western blot analysis of CRP opsonised live *N. meningitidis*

Non-piliated and piliated meningococci were opsonised with CRP in the presence of EDTA (columns G&H respectively) or calcium chloride (columns I&J) run in an SDS-PAGE gel, blotted onto PVDF membrane and probed with anti-CRP antibody. A CRP control was included for comparison (column K) alongside molecular weight markers (A &F). Separate blots were probed with anti-pili antibody (columns D&E) and anti-PC antibody (columns B&C).
3.2.9.2 Use of a high sensitivity CRP enzyme-linked immunosorbant assay (ELISA) to demonstrate CRP binding to live bacteria

Western blot analysis suggested that CRP was capable of binding to the live meningococcus. Consequently, a more sensitive method was developed to measure CRP binding to live meningococci. Initially, *S. pneumoniae* was utilised as a positive control organism to examine the suitability of the method to detect CRP binding to bacteria.

*S. pneumoniae* was incubated with CRP in the presence of calcium alone or with EDTA or soluble PC (to act as a competitive inhibitor of binding). The amount of CRP bound to the bacteria was then calculated indirectly by measuring how much CRP was left in supernatants by high sensitivity CRP-ELISA, as described in section 2.2.8. Figure 3.17 demonstrates that significantly less CRP was measured in culture supernatants of *S. pneumoniae* incubated with CRP in the presence of calcium (2.83± 0.99 μg/ml) as compared to the bacteria incubated with CRP in the presence of soluble PC (6.75 ± 1.97 μg/ml, p= 0.042). More CRP was present in the supernatants of streptococci in the presence of the calcium-chelating agent EDTA (5.38 ±2.24 μg/ml) than in supernatants with calcium, although this difference was not statistically significant (p= 0.162).

![Figure 3.17 Measurement of residual CRP post-incubation with live S. pneumoniae](image)

Streptococci of strain R36A were incubated with CRP in the presence of calcium (red) or EDTA (blue) or soluble phosphorylcholine (green). Bacteria were spun and the supernatants collected and pooled with washing supernatants before measuring the concentration of CRP in the supernatants by high-sensitivity CRP ELISA. Data are expressed as the mean of 4 replicate experiments plus standard deviation. (*) denotes p<0.05.
Following the successful detection of CRP binding to the pneumococcus by high sensitivity CRP ELISA, the method was adapted to measure CRP binding to *N. meningitidis*. It can be seen in figure 3.18 that although the mean CRP concentration in the supernatant after incubation with *N. meningitidis* in the presence of calcium was lower than for bacteria incubated in the presence of EDTA or PC, the difference was much smaller than that seen for *S. pneumoniae* and was not statistically significant. This may, in part, be due to the large variation seen in the assay as evidenced by the spread of the error bars.

![Graph](Image)

**Figure 3.18 Measurement of residual CRP post-incubation with live *N. meningitidis***

Non-piliated (C311p-) and piliated (C311p+) meningococci were incubated with CRP in the presence of calcium (red) or EDTA (blue) or soluble phosphorylcholine (green). Bacteria were spun and the supernatants collected and pooled with washing supernatants before examining concentration of CRP in the supernatants by high-sensitivity CRP ELISA. Data are expressed as the mean of 4 replicate experiments plus standard error.

Estimating CRP binding to bacteria by measuring the concentrations of unbound CRP in the supernatant is an indirect way of measuring CRP binding. It was, therefore, decided to measure the quantity of CRP bound to the meningococcus by eluting the bound CRP from the surface of the cells with EDTA after washing off any non-bound protein. CRP was then measured by the standard high-sensitivity ELISA. Figure 3.19 demonstrates that although some CRP was detected on the surface of the non-piliated strain of *N. meningitidis* (2.25 ± 0.28 µg/ml), the piliated strain C311 bound significantly more CRP (5.85 ± 1.19 µg/ml, *p* = 0.03).
Figure 3.19 Analysis of CRP binding to *N. meningitidis* as measured by high sensitivity ELISA of CRP eluted from the surface of the bacteria

Non-piliated (red) and piliated (blue) meningococci were incubated with CRP in the presence of calcium. Bacteria were spun and washed 3 times then CRP bound to the surface of the bacteria was eluted with EDTA. The CRP concentration in the eluent was measured by high-sensitivity CRP ELISA. Data are expressed as the mean of 3 replicate experiments plus standard deviation (* denotes p<0.05).
3.3 Discussion

The aim of this chapter was to identify and characterise binding between CRP and *N. meningitidis*. CRP was shown to bind to pillated *N. meningitidis* in a calcium-dependent manner that was of low affinity and specific for PC. CRP was shown to bind to several virulent strains of paraformaldehyde-fixed meningococci and also to live organisms.

3.3.1 Characterisation of CRP binding to *N. meningitidis* strain C311

Initial experiments to investigate CRP binding to the surface of the meningococcus utilised fluorescence activated cell scanning (FACS) methodology to detect fluorescently labelled anti-CRP antibodies on the surface of the bacteria. The method was developed using *S. pneumoniae* as a positive control organism because it is well established that CRP binds to the PC of the pneumococcus. Fluorescent anti-CRP antibodies could be detected bound to the surface of the pneumococcus after opsonisation with CRP. Subsequently, the method was optimised for use with meningococci. The meningococcal capsule could be clearly labelled, however, the pilus was not detectable using the SM1 anti-class II pilin antibody and PC was also undetectable on the surface of the bacteria using an anti-PC antibody. Anti-CRP antibodies were not detected on the meningococcal surface following opsonisation with CRP.

The absence of staining with anti-pili and anti-PC antibodies could be due to either a lack of antibody binding or a lack of target structures on the surface of the organism during the experiment. The anti-PC and anti-pilin antibodies have both been used extensively in the literature to label denatured proteins in SDS-PAGE gels and blots, but their binding ability for native pili and PC has not been established. Pili (and consequently PC on the pili) may not have been present on the surface of the bacterium when passing through the flow cytometer as pili are very long and brittle hair-like structures, easily shed through mechanical shearing and could be lost in the staining process. The protocol included 8 spins at 15,000 × *g* which could shear the pilus from the cell surface and any CRP bound to the pilus would be lost during washing and not detected in the FACS analysis. Slower spin speeds were investigated, but the number of spins needed to adequately wash cells meant that labelling of pili was still not possible (data not shown). Because of the difficulty in detecting the
pilus in this way, it was decided to develop less aggressive methods to visualise CRP binding to the meningococcus.

A microtitre plate-based method was developed with large numbers of fixed bacteria immobilised on the surface of the plates. This assay revealed that CRP bound to the piliated strain C311 but not to the non-piliated mutant of the same strain, suggesting that the pilus is a crucial factor in CRP binding. The non-piliated mutant used in these assays was a natural mutant, making it possible that the failure of this isolate to bind CRP might be due to other uncharacterised changes to the surface of the bacterium. This possibility is, however, highly unlikely in view of the fact that the pilus has been confirmed as the structure expressing PC on \textit{N. meningitidis} by Western blot analysis of purified pili from C311 and other piliated strains (Weiser \textit{et al}., 1998a). In addition, a panel of 64 pathogenic \textit{Neisseria} strains were screened for the presence of the PC epitope and only the pilin subunits of class I and class II piliated strains were positive for PC expression (Serino and Virji, 2000). Thus it is implied that the pilus is critical for CRP binding to \textit{N. meningitidis}. Further confirmation of this would be possible by examining CRP binding to meningococcal mutants with deliberately inactivated pilin genes.

CRP did not bind to the piliated strain C311 in the presence of EDTA (which chelates calcium and therefore prevents calcium-dependent binding). To ensure that CRP binding to the meningococcus was due to the presence of available calcium rather than the absence of EDTA, calcium-free media was used for binding assays and calcium was titrated back in. These experiments revealed a dose-response for CRP binding with increasing concentrations of calcium, confirming that CRP binds to its ligand via the classical calcium-dependent mechanism reported for its principal ligand; PC. Subsequently, PC expression was measured in the piliated strain throughout a growth-curve experiment. In these assays, PC expression correlated with the level of CRP binding over a 10 hour time-course of bacterial growth, strongly suggesting PC as the target for CRP on the meningococcus. Both PC expression and CRP binding peaked at 4 hours, coinciding with the early log phase of growth but decreased thereafter, dropping to negligible levels in the late log phase of growth. The factors involved in control of PC modification of pili during cell growth are currently unclear, but may play a significant role in the interaction of \textit{N. meningitidis} with the immune system, as growth rates of the meningococcus
may vary in different tissues within the host. Also, different capacities for PC expression may make strains better adapted for different environments. Indeed, Weiser et al. tested 31 isolates from blood, cerebro-spinal fluid and throat cultures and found that PC expression was lower in the *N. meningitidis* isolates taken from blood compared to isolates from other sites (Weiser et al., 1998a). Final confirmation that CRP binding was PC specific was provided by competition experiments in which the addition of soluble PC inhibited CRP binding to meningococci in a dose-dependent fashion, with 50% inhibition observed at 20 μM PC.

Several studies have highlighted the increase in circulating CRP levels during infectious disease, with CRP levels in bacteraemic patients reported as 10-100 μg/ml compared to less than 10 μg/ml in non-infected controls (Gill et al., 1981; McCabe and Remington, 1984). The saturation binding experiments performed in this investigation revealed that CRP binding to *N. meningitidis* was concentration-dependent, with negligible binding occurring below 1 μg/ml and the beginning of a plateau at 100 μg/ml, well within the clinical range expected during a bacterial infection.

Saturation binding experiments were used to estimate the binding affinity of CRP for *N. meningitidis* (dissociation constant \((K_d) = 2.18 \times 10^{-4} \text{M}\)). This binding affinity was low when compared to the affinity of CRP for PC itself (estimated as \(1.8 \times 10^{-7} \text{M}\) by capillary electrophoresis (Heegaard and Robey, 1993)) and for other organisms such as *L. donovani*, which has a \(K_d\) of \(10^{-11} \text{M}\) (Culley et al., 1996). *L. donovani*, however, is an intracellular parasite which utilises the opsonic properties of CRP to increase uptake into human macrophages (Culley et al., 1996) without increasing activation or killing ability of the phagocytes (Bodman-Smith et al., 2002a). In contrast, *N. meningitidis* is an extracellular pathogen and would not be expected to benefit from increased uptake into macrophages where, classically, phagocytosis results in clearance of the organism from the host. For this reason a lower affinity of binding is not unexpected for the meningococcus.

### 3.3.2 CRP binds to other important strains of *N. meningitidis*

It was considered important to determine if CRP could bind to other virulent strains of meningococci of serogroup B. The clinical isolate H44/76 was examined for its ability to bind CRP and was shown to bind in a similar pattern
to strain C311 throughout a 10 hour time-course of bacterial growth. PC expression was not measured throughout this time-course but it could be hypothesised that PC expression would correlate with CRP binding as seen for strain C311. It would be interesting to investigate a wider range of piliated serogroup B strains to see if the same binding pattern occurs for each, or if inter-strain variation causes differences in CRP binding throughout the growth-curve of the organism.

The availability of a virulent serogroup C strain of *N. meningitidis* L91534 and a *phoP* knockout mutant of the strain which shows reduced virulence in mice (Newcombe *et al.*, 2004) provided the opportunity to investigate if CRP binding could vary in strains of differing virulence. CRP was able to bind to both of these strains throughout the 10 hour time-course and in the second half of the time-course, the *phoP* mutant bound more CRP than the wild type with significantly more CRP bound by the mutant at the 8 hour time-point. The difference in CRP binding of wild type and *phoP* mutant at the 8 hour time-point is particularly interesting because it could mean that CRP binding is associated with reduced virulence in meningococci, although this speculation would of course need to be investigated further with many more strains.

The observed difference between CRP binding to the L91534 wild type and the *phoP* mutant could be due to the difference in individual growth rates, or due to differences in their expression profiles. Several genes involved in biogenesis of the pilus are down-regulated in the *phoP* mutant compared to the wild type when grown on blood agar for four hours (Newcombe *et al.*, 2005) and the pilin phosphorylcholine transferase A (*pptA*) gene was also downregulated by a 0.4 fold-change in the *phoP* mutant (JohnJoe McFadden, personal communication). The time-course assay in these studies utilised very different experimental conditions to those used in the microarray study. If, however, a down-regulation in pilus expression did occur in the *phoP* mutant during our assay, it would mean that the increased CRP binding would be due to increased PC expression, possibly by upregulation of the *pptA* gene, which was not observed in the studies by Newcombe *et al*. To investigate such a theory would require Western blot analysis of PC and pili expression throughout the time-course as well as quantitative PCR analysis of the *pptA* and pili genes.
3.3.3 CRP binds to live meningococci as well as fixed organism

One study of CRP binding to *S. pneumoniae* and other bacterial pathogens found that CRP bound dead streptococci significantly better than live streptococci (De Beaufort *et al.*, 1997). It was, therefore, considered important to examine if CRP could bind live meningococci as well as PFA-fixed meningococci. Due to the constraints of working with the biological hazard of live meningococci, a Western blot immuno-detection method was developed to examine CRP binding to live meningococci. This method confirmed the presence of pili and PC on the C311p+ strain and their absence in the C311p- strain. Minimal binding of CRP was indicated in the non-piliated strain but much greater CRP binding was seen for the piliated strain in the presence of calcium. Neither strain was shown to bind CRP in the presence of EDTA, indicating that binding was calcium-dependent. The small amounts of CRP detected on the non piliated strain could either be non-specific binding or small amounts of CRP binding to a non pilus-associated site on the surface of the cells.

The Western blot method was useful in confirming the piliated and PC-decorated phenotype of the C311p+ strain and for indicating CRP binding to this strain but the result was not quantitative or highly sensitive. Attempts to reduce non-specific binding by increasing the number of washes resulted in a loss of anti-CRP antibody binding in all conditions (data not shown). Subsequently, a quantitative assay was developed to determine CRP binding to live meningococci. A high sensitivity CRP ELISA was used to calculate CRP bound to bacteria indirectly by measuring residual CRP in the supernatants from several washing steps after incubation of CRP with bacteria. This method was validated using the positive control organism (*S. pneumoniae*) where the amount of CRP present in the cell washing supernatants from streptococci incubated with CRP in the presence of calcium was significantly lower than in supernatants from bacteria incubated with CRP in the presence of soluble PC. Following this proof of principle experiment, the method was used with piliated and non-piliated meningococci of strain C311. In these experiments, less CRP was detected in the supernatant of cells opsonised with CRP in the presence of calcium than either the EDTA or PC conditions, but these differences were not statistically significant due to considerable inter-assay variation. As the non-piliated strain does not express PC (as confirmed by FACS and Western blot analysis) it would not be expected to show differences in the amount of residual...
CRP in the cell washing supernatants whether cells were opsonised with CRP in the presence of calcium alone, or calcium with EDTA or PC. The amount of CRP left in supernatants did, however, show variation but this was smaller than the variation between assays.

Estimating CRP bound to cells indirectly by measuring CRP concentration of supernatants is clearly not a robust technique. CRP could be lost from supernatants by sticking to the sides of tubes, or by disturbing the pellets during the several washing steps. A more direct method was subsequently developed by washing opsonised cells thoroughly then eluting bound CRP with EDTA and measuring eluted CRP with the CRP ELISA. This method detected significantly more CRP bound to the piliated strain than the non-piliated strain of *N. meningitidis*, providing further evidence that CRP can bind to live as well as dead meningococci.

CRP binding of live meningococci may have differing and additional effects to binding of dead meningococci. CRP binding to dead organisms may have downstream effects on antigen processing and presentation as well as on the clearance of bacterial debris from the body, but binding of CRP to live meningococci may also affect how phagocytes recognise and destroy the infectious organisms. The binding experiments using live *N. meningitidis* provide evidence that CRP can bind to the live meningococcus, although it was not possible to determine the relative affinity of binding between live and dead meningococci as different methods were used to measure binding in each state.

### 3.3.4 Conclusions

In conclusion, the development and optimisation of several methods to examine CRP binding to the meningococcus resulted in the development of a robust and quantitative microtitre plate-based method which demonstrated that CRP can bind to piliated, PC expressing *N. meningitidis* in a calcium-dependent manner that is specific for PC. Further investigation into the kinetics of binding revealed that CRP binding to the meningococcus was of relatively low affinity and that CRP could also bind to live organisms as well as PFA fixed unviable bacteria. This exciting discovery opens up many questions as to the biological consequences of this binding in the human response to meningococcal infection and those questions are the focus of the following chapters of this thesis.
CHAPTER FOUR

THE EFFECT OF CRP ON INNATE IMMUNE DEFENCES AGAINST *NEISSERIA MENINGITIDIS*: SERUM KILLING AND MACROPHAGE OPSONIC PHAGOCYTOSIS
4.1 Rationale and aims

Following the discovery and characterisation of CRP binding to piliated meningococci, the focus of the investigation moved to the immunological consequences of CRP-opsonisation. These studies utilised in vitro models of important host defence mechanisms of the innate immune system; namely bactericidal serum killing and clearance of bacteria by macrophages.

Serum killing by complement activation is a defence mechanism characteristic of innate immunity and can also be initiated by specific antibody opsonisation. CRP is a soluble pattern recognition receptor which is capable of activating the classical complement cascade in human serum (Kaplan and Volanakis, 1974; Siegel et al., 1974; Claus et al., 1977; Gaboriaud et al., 2003). CRP-dependent complement killing has been characterised for several species closely related to N. meningitidis including H. influenzae and commensal species like N. lactamica (Weiser et al., 1998b; Serino and Virji, 2002). CRP-mediated serum killing of commensal Neisseria species directly correlates with expression levels of PC (Serino et al., 2002). It was, therefore, considered important to examine if PC on the pili of N. meningitidis resulted in increased CRP-mediated serum killing of this pathogenic species.

The second aim of these studies was to investigate the effect of CRP-opsonisation on the recognition and killing of meningococci by macrophages. Macrophages are important professional phagocytes that act as sentinels in the tissues to detect and destroy invading pathogens. The recruitment of macrophages to a site of infection enables defences of both innate and specific immunity to be activated as macrophages can phagocytose and kill the pathogen, secrete immuno-modulatory cytokines, and also present antigen to T lymphocytes in order to initiate adaptive immunity. In this chapter, the effect of CRP-opsonisation on recognition and killing of meningococci by macrophages is investigated in vitro using both human macrophage-like cell lines and monocyte-derived macrophages from the peripheral blood of volunteers.
4.2 Results

To investigate the effect of CRP binding on the killing of meningococci by human serum, a modified serum-bactericidal assay was performed with and without pre-opsonisation of the bacteria with CRP. The effect of CRP-opsonisation on association of dead and live meningococci with macrophages was also investigated using macrophages differentiated from 2 monocyte-like cell lines; THP-1 & U937 as well as peripheral blood mononuclear cell (PBMC)-derived macrophages from healthy volunteers. The involvement of Fcγ receptors was investigated by blocking receptors with human IgG before infecting macrophages with non-opsonised and CRP-opsonised meningococci. The effect of CRP-opsonisation on adherence and invasion of macrophages by meningococci was also investigated using PBMC-derived macrophages.

4.2.1 CRP-opsonisation does not increase serum killing of \textit{N. meningitidis}

\textit{N. meningitidis} strain C311 and the non-piliated mutant C311p- were prepared as per section 2.2.1 and subjected to a CRP serum-bactericidal assay as detailed in section 2.2.11 using complete or IgG-depleted plasma from one donor as described in section 2.2.10. Titration experiments revealed a final plasma dilution of 1 in 10 to be the highest concentration of IgG-depleted plasma that did not cause bacterial lysis in the absence of CRP (data not shown). This concentration was, therefore, used in serum-bactericidal assays. Figure 4.1 demonstrates that despite inoculating with identical numbers of each meningococcal strain, fewer non-piliated bacteria survived than piliated bacteria in complete plasma, IgG depleted plasma and heat inactivated plasma (used as a negative control for bactericidal killing). This pattern occurred whether the bacteria were unopsonised or CRP-opsonised, although the difference was only statistically significant in heat-inactivated plasma (p=0.004). Pre-opsonisation with CRP had no effect on the bactericidal activity of complete plasma for either the non-piliated or piliated strain as comparable numbers of bacteria survived in the plasma with and without CRP. Survival of all bacteria was greater in IgG-depleted plasma and more comparable to the heat-inactivated control, but there was no effect of CRP-opsonisation on bactericidal activity in either the non-piliated or the piliated strain.
Complete IgG-depleted Heat inactivated

Figure 4.1 Effect of CRP-opsonisation on the survival of non-piliated and piliated meningococci in complete human plasma

Non-piliated (red) and piliated (green) \textit{N. meningitidis} strain C311 were opsonised with purified human CRP (checks) or PBS alone (plain) before incubating with complete human plasma, IgG-depleted plasma or heat inactivated plasma. Aliquots of culture were plated onto CBA agar to allow enumeration of surviving bacteria. Data are expressed as means of 3 replicate experiments + standard deviation.

4.2.2 Investigating macrophage association with CRP-opsonised \textit{N. meningitidis} using immunofluorescence

THP-1-derived macrophages were adhered to chamber slides as per section 2.2.3.1 and infected with paraformaldehyde-fixed CRP-opsonised or unopsonised meningococci to investigate the effect of CRP-opsonisation on association of meningococci with macrophages. After infecting, washing and fixing, slides were immuno-labelled with anti-meningococcal capsule antibody and FITC-conjugated secondary antibody (as per section 2.2.14.1) and examined by confocal fluorescence microscopy (as per section 2.2.14). Initial experiments produced high amounts of background staining with the FITC-conjugated secondary antibody even in the absence of bacteria. Blocking with heat inactivated normal human plasma before immuno-labelling was carried out to decrease this non-specific staining. However, as figure 4.2 demonstrates, the non-specific background staining remained high and the effect of CRP-opsonisation on association with the macrophages could not be determined.
4.2.3 CRP-opsonisation increases association of Green Fluorescent Protein (GFP)-expressing *N. meningitidis* with cell line-derived macrophages

Dr Myron Christodoulides at the University if Southampton kindly provided an isolate of *N. meningitidis* strain H44/76 containing a plasmid for the expression of Green Fluorescence Protein (GFP). This plasmid was transformed into strain C311 (as described in section 2.2.2). GFP-expressing strain C311 could now be visualised directly on the confocal fluorescence microscope after incubating with macrophages on chamber slides. This method was used to investigate the effect of CRP-opsonisation on association of the meningococcus with macrophages as per section 2.2.14.2. Figure 4.3 displays a representative confocal fluorescence microscope image of THP-1-derived macrophages incubated with non-opsonised, heat inactivated serum-opsonised and CRP-opsonised GFP-expressing *N. meningitidis*. These images demonstrate that fewer non-opsonised bacteria than CRP-opsonised bacteria were associated with macrophages and that heat inactivated serum resulted in an even greater association of bacteria with macrophages.
THP-1-derived macrophages were incubated with GFP-expressing *N. meningitidis* opsonised with heat-inactivated normal human serum (a), purified human CRP (b) or PBS only (c). Macrophages were stained red with propidium iodide. Images are from one representative experiment.

Uptake experiments were replicated at least 3 times with macrophages differentiated from the THP-1 and U937 cell lines. For each experiment, at least 100 macrophages in 10 randomly selected fields of view were analysed. Figure 4.4 shows that a significantly higher percentage of THP-1-derived macrophages co-localised with CRP-opsonised bacteria compared to non-opsonised bacteria (*p*=0.045). A significantly higher average number of bacteria per cell was calculated for CRP-opsonised meningococci compared to non-opsonised meningococci in this cell line (*p*=0.014). Using U937 cells, a similar pattern was seen with CRP-opsonisation resulting in a higher percentage of macrophages associating with bacteria and a higher average number of cell-associated bacteria per macrophage, but these differences were not statistically significant (*p*=0.48 and *p*=0.11 respectively).
Figure 4.4 Association of GFP-expressing meningococcal strain C311 with cell line-derived macrophages

Enumeration of CRP-opsonised bacteria (red) and unopsonised bacteria (blue) co-localised with THP-1- and U937-derived macrophages. The percentage of macrophages associated with bacteria was calculated (a) as well as the mean number of adherent or intracellular bacteria per macrophage (b). Data are expressed as means of at least 3 replicate experiments + standard deviation. (* denotes a p value < 0.05, "nsd" denotes no statistical difference).

The serogroup B meningococcal strain H44/76 was also used for uptake assays as detailed in section 2.2.14.2. Figure 4.5 reveals that a significantly higher percentage of THP-1-derived macrophages were associated with CRP-opsonised bacteria compared to non-opsonised bacteria (p=0.032) and that a significantly higher level of association (bacteria per cell) was seen for CRP-opsonised meningococci in this cell line (p =0.03). U937 macrophages also demonstrated an increased percentage of macrophages associated with H44/76 bacteria and mean number of bacteria per macrophage when meningococci were opsonised with CRP, but these differences were not statistically significant (p=0.42 and p=0.11 respectively).
4.2.4 CRP-opsonisation increases association of GFP-expressing \textit{N. meningitidis} with human peripheral blood-derived macrophages

Following the discovery that CRP-opsonisation increased the association of two GFP expressing strains of \textit{N. meningitidis} with THP-1-derived macrophages, the method detailed in section 2.2.14.2 was used to examine the effect of CRP-opsonisation on the association of paraformaldehyde-fixed meningococci with PBMC-derived macrophages from volunteers. Figure 4.6 displays representative fluorescence microscopy images of PBMC-derived macrophages incubated with bacteria that were pre-opsonised with CRP or PBS only. These images demonstrate that a higher number of CRP-opsonised meningococci were associated with the macrophages as compared to non-opsonised bacteria. Figure 4.7 shows that in 4 replicate experiments with separate donors, CRP-opsonisation significantly increased the number of macrophages associated with meningococci (p = 0.032) and significantly increased the mean number of cell-associated bacteria per macrophage (p=0.013) compared to PBS-opsonised meningococci.
Figure 4.6 Confocal fluorescence microscopy images of *N. meningitidis* incubated with PBMC-derived macrophages

Representative images of PBMC-derived macrophages incubated with *N. meningitidis* opsonised with PBS only (a) or purified human CRP (b). Macrophages were stained red with propidium iodide.

Figure 4.7 Association of GFP-expressing meningococci strain C311 with PBMC-derived macrophages

Heat inactivated serum–opsonised bacteria (red), CRP-opsonised bacteria (blue) and unopsonised bacteria (green) were incubated with PBMC-derived macrophages. The percentage of macrophages associated with bacteria was calculated (a) as well as the mean number of adherent or intracellular bacteria per macrophage (b). Data are expressed as means of at 4 replicate experiments ± standard deviation. (* denotes a p value < 0.05).
Propidium iodide staining allowed the percentage of macrophages associated with meningococci to be enumerated but did not permit differentiation between cell-associated and intracellular bacteria. Therefore, in separate experiments, macrophage membranes were stained with the lipophilic dye FM646 instead of propidium iodide in order to allow Z-stacking through the depth of the cell to reveal the presence of intracellular bacteria (as described in 2.2.14). Figure 4.8 displays the results of one such experiment with several bacteria clearly positioned within the cell walls of the macrophage.

![Image](image.jpg)

**Figure 4.8 Internalised *N. meningitidis* within macrophages**

Representative confocal fluorescent microscope image of PBMC-derived macrophages incubated with CRP-opsonised GFP-expressing meningococci. Macrophage membranes were stained with a fluorescent lipophilic dye post infection to enable z stacking through the macrophage to reveal internalised bacteria. The arrow points to an example of meningococci within the macrophage cell wall.
4.2.5 Non-adherent macrophages exhibit a small increase in association with meningococci when opsonised with CRP

Macrophages adhered to microscope slides may show different phagocytic activity to non-adhered macrophages in a liquid suspension. It was considered prudent, therefore, to investigate the effect of CRP-opsonisation on the association of paraformaldehyde-fixed meningococci with PBMC-derived macrophages that were removed from tissue-culture plastic surfaces with gentle scraping before incubating with bacteria in suspension as detailed in section 2.2.13.2. These assays revealed that although more CRP-opsonised meningococci were taken up by the macrophages compared with PBS-opsonised meningococci, the increase in association caused by CRP-opsonisation was not statistically significant (p=0.093, figure 4.9).

Figure 4.9 Effect of CRP-opsonisation on association of GFP-expressing meningococci with macrophages in suspension
PBMC-derived macrophages were infected with CRP-opsonised (green) and PBS mock-opsonised (blue) GFP-expressing meningococci (GFP-Nm) or left uninfected (red). Association of GFP-expressing bacteria with macrophages was measured by FACS analysis of macrophages on the FL-1 channel. Data are expressed as the mean percentage of cells positive for bacteria from 4 replicate experiments + standard deviation.
4.2.6 Blocking of Fcγ receptors inhibits CRP-mediated association of meningococci with macrophages

Following the discovery that CRP-opsonisation increased the association of meningococci with macrophages adhered to chamber slides, it was decided to explore the mechanism of this increased association. CRP is known to increase uptake of opsonised cells by recruiting Fcγ receptors on the surface of phagocytes (Mortensen and Duszkiewicz, 1977; Bodman-Smith et al., 2002b), therefore the involvement of Fcγ receptors in CRP-mediated association of paraformaldehyde-fixed meningococci with macrophages was investigated. PBMC-derived macrophages were pre-incubated with a range of concentrations of human IgG. Residual IgG was washed off before allowing phagocytosis of opsonised meningococci to take place. Slides were then examined by fluorescence microscopy as described in section 2.2.14.3.

Figure 4.10 demonstrates that PBS-opsonised meningococci were associated with the same percentage of macrophages regardless of the concentration of IgG the macrophages were pre-incubated with. There was also no statistical difference in the mean number of bacteria per macrophage for PBS-opsonised meningococci incubated with macrophages at the different IgG concentrations (see figure 4.10b). CRP-opsonised meningococci were co-localised with a significantly higher percentage of macrophages and resulted in a higher average number of cell-associated bacteria per macrophage than PBS-opsonised meningococci when macrophages were not pre-incubated with IgG (figure 4.10 a & b, p= 0.043 & p= 0.023 respectively).

When macrophages were not pre-incubated with IgG, a higher percentage of macrophages were co-localised with CRP-opsonised meningococci compared to macrophages that had been pre-incubated with 0.1%, 1%, or 10% human IgG (p=0.112, p=0.049 and p=0.055 respectively). Additionally, when macrophages were not pre-incubated with IgG, the average number of cell-associated bacteria per macrophage was higher for CRP-opsonised meningococci when compared to macrophages that had been pre-incubated with 0.1%, 1%, or 10% human IgG (p=0.021, p=0.090 and p=0.013 respectively). Thus blocking of Fcγ receptors significantly reduced CRP-mediated association of meningococci with macrophages.
IgG concentration

Figure 4.10 IgG blocking of CRP-mediated association of *N. meningitidis* with PBMC-derived macrophages

PBMC-derived macrophages were incubated with a range of concentrations of human IgG before washing and incubating with GFP expressing *N. meningitidis* strain C311 opsonised with CRP (red) or mock-opsonised with PBS (blue). The percent of macrophages that were found co-localised with bacteria (a&c) were calculated alongside the mean number of cell-associated bacteria per macrophage (b&d). Data are expressed as mean values from 7 replicate experiments + standard error. (* denotes p<0.05).

4.2.7 CRP-opsonisation does not increase association of live GFP-expressing meningococci with THP-1-derived macrophages

Following the discovery that paraformaldehyde-fixed meningococci can bind CRP and that this binding causes an increase in association of the bacteria with monocytic cell line- and PBMC-derived macrophages and that live meningococci can also bind CRP, it was considered important to investigate the effect of CRP-opsonisation on the association of live meningococci with macrophages.

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Live meningococci were opsonised with CRP and incubated with THP-1-derived macrophages as detailed in section 2.2.14.4. Figure 4.11 reveals that the percentage of macrophages associated with CRP-opsonised live meningococci was not different from the percentage of macrophages associated with PBS-opsonised meningococci (p=0.345). The mean number of cell-associated bacteria per macrophage was also unaffected by opsonisation with CRP compared to PBS alone (p=0.520). As expected, the percentage of macrophages associated with bacteria and the mean number of bacteria per macrophage for heat-inactivated serum-opsonised meningococci was greater than results for CRP-opsonised meningococci, although this difference was not statistically significant (p=0.090 and p=0.105 respectively).

Figure 4.11 Association of live GFP-expressing meningococci with THP-1-derived macrophages
Macrophages on chamber slides were incubated with GFP-expressing meningococci that had been pre-opsonised with heat-inactivated human serum (red), CRP (blue), or mock-opsonised with PBS alone (green). Slides were visualised on the confocal fluorescence microscope to allow enumeration of the percentage of macrophages colocalised with meningococci (a) and to calculate the mean number of cell-associated bacteria per macrophage (b). Data are presented as the mean of 4 replicate experiments + standard deviation.
4.2.8 CRP-opsonisation does not increase adhesion to or invasion of THP-1-derived macrophages by live *N. meningitidis*

Confocal fluorescent microscopy of macrophages incubated with live meningococci revealed that CRP-opsonisation did not increase the association of macrophages with the bacteria. Working with live meningococci enabled the use of gentamicin exclusion assays to enumerate the number of viable intracellular bacteria as well as total numbers of cell-associated bacteria after incubating macrophages with CRP-opsonised meningococci as detailed in section 2.2.15.

It is evident from figure 4.12 that CRP-opsonisation did not increase the total number of bacteria both inside and adhered to macrophages or the number of true intracellular bacteria. It is notable, however, that even heat-inactivated serum did not increase adherence or phagocytosis of the bacteria in this assay, and overall numbers of bacteria recovered from the assay were extremely low.

![Figure 4.12 Adherence to and invasion of cell line-derived macrophages by CRP-opsonised meningococci](image)

**Figure 4.12 Adherence to and invasion of cell line-derived macrophages by CRP-opsonised meningococci**

THP-1-derived macrophages were infected with live meningococci (Nm) that had been pre-opsonised with heat inactivated serum (red), CRP (blue) or mock-opsonised with PBS (green). Macrophages were washed and either lysed immediately to enumerate both intracellular and bound extracellular meningococci (a) or macrophages were incubated with gentamicin for 1 hour before lysing to enumerate intracellular meningococci only (b). Data are expressed as the mean values from 3 replicate experiments + standard deviation.
4.2.9 CRP-opsonisation does not increase adhesion to or invasion of PBMC-derived macrophages by live *N. meningitidis*

Following the observation that THP-1-derived macrophages demonstrated poor association with serum-opsonised meningococci, the assays detailed in section 2.2.15 were repeated using PBMC-derived macrophages to overcome any peculiarities of the cell line. Figure 4.13 reveals that although the number of adherent and intracellular bacteria per macrophage was slightly higher overall in PBMC-derived macrophages compared to THP-1-derived macrophages, there was still no difference between the numbers of CRP-opsonised, PBS-opsonised, or heat-inactivated serum-opsonised meningococci adhered to or inside macrophages (a). The average number of intracellular CRP-opsonised meningococci per cell was actually lower than the average number of PBS-opsonised meningococci per cell, but this difference was not statistically significant (p=0.243).

![Figure 4.13 Adherence to and invasion of PBMC-derived macrophages by CRP-opsonised meningococci](image)

**Figure 4.13 Adherence to and invasion of PBMC-derived macrophages by CRP-opsonised meningococci**

PBMC-derived macrophages were infected with live meningococci (Nm) that had been pre-opsonised with heat-inactivated serum (red), CRP (blue) or mock-opsonised with PBS (green). Macrophages were washed and either lysed immediately to enumerate both intracellular and bound extracellular meningococci (a) or macrophages were incubated with gentamicin for 1 hour before lysing to enumerate intracellular meningococci only (b). Data are expressed as the mean values from 3 replicate experiments + standard deviation.
4.2.10 CRP-opsonisation has no effect on intracellular killing of *N. meningitidis* within macrophages

To investigate whether CRP binding affects intracellular killing of live *N. meningitidis* by macrophages, a time-course assay of intracellular killing was performed on CRP-opsonised and non-opsonised meningococci. Equal numbers of live bacteria were incubated with macrophages for 3 hours before enumerating intracellular bacteria at this time point and 2 and 5 hours later as detailed in section 2.2.16. Figure 4.14 demonstrates that although an increased number of CRP-opsonised meningococci were found to be viable inside the macrophages at the 3 hour time-point, this difference was not statistically significant and the numbers of intracellular bacteria were comparable for CRP-opsonised and PBS-opsonised meningococci by the 5 hour time point. All intracellular bacteria were destroyed 8 hours after infection.

![Figure 4.14](image)

**Figure 4.14 Time-course analysis of macrophages infected with CRP-opsonised meningococci**

CRP-opsonised (red) and unopsonised (blue) *N. meningitidis* (Nm) were incubated with PBMC-derived macrophages in 6-well plates. At regular intervals, gentamicin was added to replicate wells to kill extracellular bacteria. Intracellular bacteria were enumerated by lysing the cells and plating the lysate onto agar plates. Data are presented as mean values for 3 replicate experiments ± standard deviation.
4.3 Discussion

The aim of this chapter was to uncover the effect of CRP-binding on several innate immune defences to meningococcal infection. This was achieved by firstly investigating the effect of CRP-opsonisation on serum-killing of meningococci, followed by an exploration of the effect of CRP-opsonisation on the phagocytosis of dead and live *N. meningitidis* by macrophages.

4.3.1 *N. meningitidis* does not exhibit increased serum killing in the presence of CRP

CRP binding to commensal species of *Neisseria* has been reported to increase serum killing via activation of the complement cascade (Serino *et al.*, 2002). To investigate whether CRP had a similar effect on pathogenic species, CRP-opsonised and non-opsonised meningococci were incubated with human plasma as a complement source. CRP-opsonisation had no effect on serum killing of pathogenic meningococci in either IgG-depleted plasma or complete plasma (which is likely to contain antibodies capable of binding to the meningococcus).

The difference in the effect of CRP binding on serum killing seen between pathogenic and commensal *Neisseria* may be due to differences in the way that PC is exposed on the different species. Commensal *Neisseria* have been shown to incorporate choline from growth media in order to produce PC on their LPS, which requires the expression of 4 genes (*LicA-D*) that are present in *N. lactamica* but are absent from the meningococcal genome (Serino and Virji, 2000). Pathogenic *Neisseria*, conversely, only express PC on class 1 and 2 pili but never on their LOS and expression is dependent on the gene *pptA* (Weiser *et al.*, 1998a; Warren and Jennings, 2003). In the closely related species *H. influenzae*, the PC moiety is incorporated into the LPS at different sites (on a chain extension from heptose III or heptose I of the LPS) and the differences in location have been shown to affect the level of detection by CRP and anti-PC antibodies in different strains and variants (Lysenko *et al.*, 2000). The difference in location of PC between pathogenic and commensal *Neisseria* could, therefore, explain the difference in serum-killing effects of CRP. It could be speculated that PC accessible to CRP on the LPS of commensal species of *Neisseria* is a prime site for formation of the membrane attack complex (MAC), resulting in the formation of pores in the surface of the bacterium. In contrast, CRP binding to
PC on the pilus of pathogenic *Neisseria* may be sufficient to initiate the classical complement cascade, but may not result in pore formation if the site on the pilus is remote from the bacterial cell wall or if the pilus is shed. Thus the site of the PC moiety on *N. meningitidis* would appear much better suited to the pathogenic nature of the meningococcus if it enables the species to escape serum killing via CRP and possibly, naturally occurring anti-PC antibodies.

Serum killing of both piliated and non-piliated meningococcal isolates was greater in complete plasma than IgG depleted plasma, probably due to the presence of natural and acquired anti-meningococcal antibodies that catalyse complement activation by binding to the C1q component of the classical complement cascade. Unexpectedly, however, the non-piliated mutant showed poorer survival than the piliated strain in all 3 types of plasma despite wells being inoculated with the same number of bacteria as the piliated strain. The reasons for the inferior survival of the non-piliated strain are unknown. Growth rates are comparable between the 2 strains and incubation with plasma was for 30 minutes only, making it unlikely that the piliated strain replicated faster than the non-piliated mutant. The difference in survival could be due to the absence or presence of the pilus itself, which could have been investigated by removing pilli with vigorous centrifugation before incubating with plasma but as this was not the main focus of the investigation, the phenomenon was not studied further.
4.3.2 CRP-opsonisation increases association of meningococci with macrophages

Immuno-fluorescent probing methods to visualise the uptake of meningococci using anti-meningococcal antibodies were marred by high background staining. Consequently, assays were developed using GFP-expressing meningococci that could be imaged on the fluorescent microscope without additional staining. These experiments demonstrated that CRP-opsonised *N. meningitidis* were more readily taken up by macrophages than unopsonised bacteria. This was the case for THP-1- and PBMC-derived macrophages and resulted in increases in the percent of macrophages associated with bacteria and the mean number of bacteria per macrophage. Due to the limits of the experimental procedure used, bacteria could not be confirmed as truly "intracellular", thus phagocytic index was not stated in these experiments. Membrane staining of macrophages and z-stacking through the depth of the cell did, however, confirm the intracellular location of many meningococci.

U937-derived macrophages did not exhibit increased association with CRP-opsonised meningococci, possibly because of differences in the state of activation or expression of cell surface receptors. CRP is known to bind to Fcy receptors FcyRI and FcyRII on both human and mouse leucocytes (Zeller et al., 1989; Marnell et al., 1995; Stein et al., 2000a; Stein et al., 2000b; Bodman-Smith et al., 2002b; Bodman-Smith et al., 2003; Bodman-Smith et al., 2004). U937 and THP-1 cell lines were selected for uptake experiments as they have both been reported to express FcyRI and FcyRII receptors (Looney et al., 1986; Fleit and Kobasiuk, 1991; Kiener et al., 1993), but phenotypic differences obviously exist between different cell lines. For example, THP-1 monocytes have been shown to constitutively express up to four times more FcyRI than U937 cells, which may have an impact on CRP-mediated association with bacteria (Fleit and Kobasiuk, 1991). In addition, the expression profile of known or putative NFkB target genes in response to LPS stimulation in U937 cells is much less similar than THP-1 cells to PBMC-derived macrophages (Sharif et al., 2007). Thus phenotypic differences between the two cell lines may be the reason why U937-derived macrophages showed no increase in association with meningococci after CRP-opsonisation. PBMC-derived macrophages from several donors provide the most relevant *in vitro* model for examining macrophage uptake and these cells
demonstrated a significant increase in association with meningococci following opsonisation with CRP.

The effect of CRP-opsonisation on the association of *N. meningitidis* with macrophages removed from tissue culture plastic and suspended in media was investigated to discover if association was greater when allowed to occur in 3 dimensions. In these experiments, 33.78 ± 13.47 percent of non-adhered macrophages were positive for PBS-opsonised bacteria compared to 39.68 ± 12.76 percent of non-adhered macrophages positive for CRP-opsonised meningococci, but this difference was not statistically significant. Background bacterial co-localisation in non-adhered macrophages was higher than in adherent macrophages and may have made the increase caused by CRP much harder to detect. A positive control of heat-inactivated serum-opsonisation would confirm this speculation but due to limited cell numbers available, this control was not included in these assays. One other possible explanation is that the cell scraping could dislodge many of the Fcγ receptors that CRP may bind to for augmenting uptake. Staining of Fcγ receptors on the cells could have confirmed this. In future experiments, less damaging methods of detachment could be trialled to ensure cell surface receptors were available before incubation with bacteria.

CRP has been shown to increase uptake of opsonised particles and cells via FcγRI, and FcγRIIa (Bodman-Smith *et al.*, 2002b; Bodman-Smith *et al.*, 2003; Bodman-Smith *et al.*, 2004). Following the discovery that CRP increased macrophage association with fixed meningococci, the involvement of Fcγ receptors in this association was investigated by blocking receptors with IgG. Purified human IgG was incubated with macrophages at a range of concentrations in order to block all available types of Fcγ receptor (although it follows that FcγRI receptors would have been preferentially blocked due to their higher affinity for monomeric IgG compared to FcγRII). In these experiments, the mean percent of macrophages associated with CRP-opsonised meningococci decreased as the concentration of IgG that the macrophages had been incubated with increased, indicating that as Fcγ receptors are blocked, the opsonising effect of CRP diminishes. This suggests that CRP is indeed recruiting Fcγ receptors to facilitate the increased association of fixed *N. meningitidis* with macrophages.
It would be interesting, in further studies, to investigate the involvement of each individual Fcγ receptor type in CRP-mediated uptake of meningococci. This could be achieved by antibody blockade of receptors using anti-FcγRI and anti-FcγRIIa antibodies. Although these antibodies are commercially available, they are produced as whole IgG molecules which have Fc portions that would bind to all types of Fc receptor, not just the one targeted with the Fab2 region of the antibody. To overcome this, large quantities of anti-Fcγ receptor antibody would be needed in order to pepsin-digest the Fc portion and purify the remaining Fab2 portions. An alternative method of inactivating individual Fcγ receptor types to examine the relative involvement of each sub-class in CRP-mediated uptake of meningococci would be to transfect macrophages with small interfering RNA (siRNA) specific for each subclass to inactivate the receptors one by one.

To investigate the involvement of FcγRIIa in CRP-mediated phagocytosis of *N. meningitidis* would have been particularly interesting because of the differences in biological activity of different alleles of this receptor. CRP has been shown to preferentially bind to the allelic form of the receptor that has arginine at position 131 (FcγRIIa R131) rather than the form that has histidine at this position (FcγRIIa H131) (Stein et al., 2000a). Individuals who are homozygous for FcγRIIa R131 have increased susceptibility to bacterial infections including streptococcal and meningococcal disease (Bredius et al., 1994; Rodriguez et al., 1999; Yee et al., 2000). It was not known which alleles were present in the donors used for experiments with PBMC-derived macrophages and haplotyping of donors may give interesting information about the expression profile of donors and how this may have affected CRP-mediated uptake.

The effect of CRP-opsonisation on macrophage phagocytosis of *N. meningitidis* was investigated in a complement-free environment. Several complement components act as opsonins to coat pathogens and initiate phagocytosis via complement receptors. Complement was deliberately excluded from uptake experiments so that effect of CRP alone could be measured but it is possible that CRP may cooperate with other opsonins to facilitate increased uptake of meningococci, as it has been shown to interact synergistically with other pattern recognition receptors in streptococcal infection (Ng et al., 2007). This phenomenon could be investigated in the future by adding in individual complement proteins and examining uptake of CRP-opsonised meningococci.
4.3.3 CRP-opsonisation does not increase association of live meningococci with macrophages

Uptake assays clearly demonstrated that CRP-opsonisation increases association of paraformaldehyde-fixed *N. meningitidis* with macrophages. This pattern was not reproducible in live meningococci which showed no significant increase in association with macrophages when opsonised with CRP as measured by fluorescent microscopy or viable counting of intracellular and macrophage-adhered bacteria.

It is tempting to compare association of live and fixed meningococci with PBMC-derived macrophages, but such a comparison is purely speculative as experiments were carried out on separate days and on several different donors. Unopsonised meningococci were actually associated with a higher percentage of macrophages when bacteria were live instead of dead (mean of 30.0 ± 3.05 and 21.3 ± 3.13 respectively). Whereas CRP-opsonisation caused the percentage of macrophages associated with dead meningococci to more than double, the mean number of macrophages associated with live meningococci did not change. When comparing the percentage of macrophages associated with meningococci opsonised with the positive control of heat-inactivated serum to unopsonised meningococci in the live and dead uptake assays, serum-opsonisation more than tripled the percent of macrophages associated with dead bacteria whereas the association of macrophages with live meningococci increased by just 7.5%. These findings suggest that live meningococci may be able to reduce the opsonic properties of both serum and CRP in some way that dead meningococci are incapable of doing.

Surveying the literature has not yielded any relevant information on the relative uptake rates of live and dead meningococci by macrophages, but it has been reported that another professional phagocyte, the dendritic cell exhibits lower uptake of live meningococci compared to dead meningococci in the presence of 10% foetal calf serum (Uronen-Hansson *et al.*, 2004). The cause of this decreased uptake of live meningococci is unknown, but could be due to changes in expression of surface molecules on the live bacterium in response to co-culture with macrophages. Studies on phagocytosis of the yeast *Cryptococcus neoformans* by macrophages have shown that live yeast can
inhibit phagocytosis by macrophages, which is thought to be mediated by the active release of LPS or other anti-phagocytic proteins (Kozel and Mastroianni, 1976; Luberto et al., 2003; Luo et al., 2006). Live meningococci could possibly employ similar defences to avoid phagocytosis by macrophages that the fixed meningococci are incapable of utilising.

CRP-opsonisation did not increase the number of live intracellular bacteria or macrophage-adhered bacteria when investigated by viable counting experiments. In these assays, even serum opsonisation did not significantly increase the number of adhered or intracellular meningococci per cell, supporting the hypothesis that macrophage phagocytosis is less efficient for live meningococci than dead meningococci. The C311 strain used in these experiments is a virulent, encapsulated clinical isolate. It has previously been shown that encapsulation protects live serogroup B meningococci from phagocytosis, as non-encapsulated mutants show increased adherence to macrophages compared to wild type encapsulated strains (Read et al., 1996). The authors of this work postulated that the decreased adherence of the encapsulated strain was due to a combination of negative charge, steric hindrance, masking of adhesin ligands and a decrease in complement deposition. In the experiments detailed in this chapter, paraformaldehyde fixation of meningococci may have reduced the bacteria's defences to opsonisation and phagocytosis, and this may explain why the CRP- and serum-opsonisation resulted in an increase in macrophage association with dead meningococci that was absent in live meningococci.

To complete the investigation into the effect of CRP-opsonisation on phagocytosis of live meningococci, time-course assays of intracellular survival were performed on CRP-opsonised and non-opsonised meningococci. In these assays, no significant difference in the macrophage association or killing rate of intracellular bacteria was observed, providing further evidence that in this in vitro model of macrophage phagocytosis, CRP is not able to increase phagocytosis or cell killing of the meningococcus.
4.3.4 Conclusions

CRP opsonisation can be a help or a hindrance to fighting an infection depending on the nature of the pathogen. CRP binding helps the intracellular parasite *L. donovani* to gain access to macrophages without increasing killing of the organism (Bodman-Smith *et al.*, 2002a) but has been shown to increase uptake of live *S. pneumoniae* into human phagocytes and to protect mice from fatal infection (Mold *et al.*, 1981; De Beaufort *et al.*, 1997). The studies detailed in this chapter revealed that CRP binding increased the association of fixed meningococci with macrophages and indicated Fcy receptors as crucial components of this increased association. Although unviable bacteria are of less danger to the host, phagocytic uptake of dead bacteria may be important in generating specific immune responses to surface antigens of viable meningococci. The investigations using live *N. meningitidis* did not uncover an increase in serum killing, macrophage association or intracellular killing of live CRP-bound meningococci, but it is still plausible that CRP binding to *N. meningitidis* may protect the host from meningococcal infection by less direct methods such as increasing the activation state of macrophages. CRP-mediated macrophage activation can result in changes in surface marker expression and increased inflammatory cytokine responses to the bacteria as is seen with CRP-opsonisation of *S. pneumoniae* (Mold and Du Clos, 2006). CRP-opsonisation could, therefore increase the activation of macrophages in response to meningococci and this theory is investigated in chapter 6.
CHAPTER FIVE

THE EFFECT OF CRP ON UPTAKE OF *NEISSERIA MENINGITIDIS* BY NEUTROPHILS AND DENDRITIC CELLS
5.1 Rationale and aims

Following the observation that CRP-opsonisation of paraformaldehyde-fixed *N. meningitidis* caused an increase in association with macrophages that was inhibited by blocking Fcy receptors, it was decided to investigate the effect of CRP-opsonisation on the association of meningococci with other phagocytic cells known to express Fcy receptors; namely neutrophils and dendritic cells.

Granulocytes (or polymorphonuclear leucocytes) are the most numerous leucocytes in the blood stream, with neutrophils being the most common type of granulocyte. Neutrophils are short-lived but hugely active immune cells which are responsible for the clearance of bacteria through non-opsonic and opsonic phagocytosis. Neutrophils provide an important defence against meningococcal infection, especially in complement-deficient individuals (Ross *et al.*, 1987; Schlesinger *et al.*, 1994). They can destroy engulfed bacteria within phagolysosomes *via* the production of reactive oxygen and nitrogen species (ROS & RNS) and a variety of oxygen-independent enzymes and microbial peptides (Borregaard and Cowland, 1997; Hampton *et al.*, 1998). Neutrophils are known to constitutively express FcyRII and when activated by proinflammatory signals such as IFNγ, can also express FcyRI (Hoffmeyer *et al.*, 1997). The effect of CRP-opsonisation on the association of meningococci with neutrophils was, therefore, investigated.

Neutrophils are highly efficient professional phagocytes but are classically short lived and do not directly present antigen to activate the specific arm of the immune system. Antigen presentation is not usually achieved until apoptotic neutrophils are phagocytosed, typically by macrophages. Dendritic cells, on the other hand, are highly specialised antigen presenting cells which form a link between innate and adaptive immunity, phagocytising pathogens in their immature state and maturing en-route to immune tissues to present processed antigens to T cells (Palucka and Banchereau, 1999). Dendritic cells are the only antigen presenting cell capable of presenting to naïve T cells and so can initiate primary immune responses to previously unencountered pathogens. Dendritic cells can be targeted for effective vaccine delivery and inflammatory dendritic cell responses to *N. meningitidis* have been shown to be dependent on phagocytosis of the bacterium (Uronen-Hansson *et al.*, 2004). If CRP could increase the uptake of meningococci into dendritic cells, this could affect how efficiently meningococcal antigens are processed and presented and would, therefore,
modulate the specific immune response to meningococcal infection. Thus it was considered important to discover if CRP could increase association of *N. meningitidis* with immature dendritic cells.

### 5.2 Results

CRP-opsonised and non-opsonised GFP-expressing paraformaldehyde-fixed meningococci were incubated with human peripheral blood neutrophils and PBMC-derived dendritic cells from volunteers which were imaged on the confocal fluorescent microscope. Dendritic cells were also detached from plastic surfaces before incubating with GFP-expressing meningococci in suspension and association of meningococci with these cells was measured by FACS. Comparisons of phagocytosis were carried out by paired Student’s t-test.

#### 5.2.1 CRP-opsonisation increases the association of *N. meningitidis* with neutrophils

Granulocytes were separated from volunteer blood as detailed in section 2.2.3.3 and adhered to plastic chamber slides. The separation method was approved for purity of cultures by giemsa-wright staining of cells which revealed large cells with multi-lobed nuclei. As less than 3% of granulocytes are eosinophils or basophils, and these cell types are poorly phagocytic, the cells in this assay are now referred to as neutrophils. Cells were incubated with CRP-opsonised or PBS-opsonised GFP-expressing meningococci and slides were washed and examined on the confocal fluorescent microscope as per section 2.2.14.2. Figure 5.1 displays a representative fluorescent microscope image of CRP-opsonised GFP-expressing meningococci colocalised with neutrophils and non-opsonised meningococci more sparsely associated with the neutrophils.
Figure 5.1 Fluorescence microscopy images of CRP-opsonised meningococci colocalised with neutrophils

CRP-opsonised (a) and non-opsonised meningococci (b) were incubated with peripheral-blood derived neutrophils in chamber slides and imaged on the confocal fluorescent microscope. Nuclei are stained red with propidium iodide (PI). Images are from one representative experiment.

The neutrophil uptake assay was repeated in 3 donors, enumerating cell-associated bacteria from 10 randomly selected fields of view in each experiment. Figure 5.2 reveals that significantly more neutrophils were associated with CRP-opsonised meningococci compared to non-opsonised meningococci (p=0.048) and that the average number of cell associated bacteria per neutrophil was also significantly higher for CRP-opsonised meningococci (p=0.013).
Figure 5.2 Association of *N. meningitidis* with neutrophils

GFP-expressing *N. meningitidis* C311 were grown, fixed in PFA, washed and opsonised with CRP (red) or incubated with PBS (blue) before incubating with neutrophils adhered to plastic chamber slides. Slides were washed, fixed, PI stained and the percentage of neutrophils associated with bacteria (a) and the mean number of adherent or intracellular bacteria per neutrophil (b) were enumerated using the confocal fluorescence microscope. Data are expressed as means of 3 replicate experiments + standard deviation (* denotes a p value < 0.05).

5.2.2 CRP-opsonisation can increase Association of meningococci with PBMC-derived dendritic cells

Monocytes were separated from whole human blood as per section 2.2.3.2 and adhered to plastic chamber slides. Monocytes were differentiated into an early dendritic cell phenotype with IL-4 and GM-CSF. Cells were incubated with non-opsonised or CRP-opsonised meningococci for 2 hours before washing and examining on the confocal fluorescent microscope as per section 2.2.14.2. Figure 5.3 displays a representative confocal fluorescent microscope image of CRP-opsonised GFP-expressing meningococci colocalised with dendritic cells and non-opsonised meningococci more sparsely associated with the dendritic cells. These monocyte-derived dendritic cells appeared morphologically very different from the monocyte-derived macrophages seen in chapter 4 when examined by light microscopy and confocal fluorescent microscopy.
Figure 5.3 Fluorescence microscopy images of meningococci colocalised with PBMC-derived dendritic cells

CRP-opsonised (a) and non-opsonised (b) meningococci were incubated with PBMC-derived dendritic cells on chamber slides and imaged on the confocal fluorescent microscope. Cells were stained red with PI. Images are from one representative experiment.

The dendritic cell uptake assay was repeated in three donors and cell-associated bacteria were enumerated from 10 randomly selected fields of view in each experiment. Figure 5.4 displays the results of this analysis with a higher percentage of dendritic cells associating with meningococci opsonised with CRP compared to non-opsonised bacteria. The low number of donors meant that this difference was not statistically significant by t-test (p=0.08).

Figure 5.4 Association of N. meningitidis with PBMC-derived dendritic cells

GFP-expressing C311 were grown, fixed in PFA, washed and opsonised with CRP (red) or incubated with PBS (blue) before incubating with dendritic cells (DC) adhered to plastic chamber slides. Slides were washed, fixed, PI stained and the percentage of dendritic cells associated with bacteria was enumerated. Data are expressed as means of 3 replicate experiments + standard deviation.
5.2.3 CRP-opsonisation may increase Association of meningococci with human PBMC-derived dendritic cells released into suspension

To investigate whether dendritic cells released from plastic surfaces showed any difference in association with CRP-opsonised meningococci, dendritic cells were differentiated in 6 well tissue culture plates as per section 2.2.3.2 and released from the plate by gentle scraping. Cells were washed then incubated with CRP-opsonised and non-opsonised GFP-expressing meningococci and 10,000 cells gated by size and granularity were analysed by FACS. Figure 5.5 displays the results of a representative experiment with association with GFP-expressing meningococci detected by a right shift in FL-1 fluorescence in the gated dendritic cell population. This figure shows that although 13.7% of dendritic cells were in the positive region for green fluorescence, and therefore positive for association with the GFP-expressing non-opsonised bacteria, 17.3% of dendritic cells were positive for bacteria when meningococci were pre-opsonised with CRP.

![Figure 5.5 Dendritic cell association with CRP-opsonised GFP-expressing N. meningitidis in suspension](image)

Representative dot plots showing relative fluorescence (FITC-A, X axis) vs. size (FSC-H, Y axis) of dendritic cells removed from a plastic surface, following incubation with PBS alone (a), with non-opsonised GFP-meningococci (b) or with CRP-opsonised GFP-meningococci (c).

It was intended to repeat the dendritic cell FACS uptake assay several more times but this was not possible due to time constraints, thus figure 5.5 serves as a proof of principal for the suitability of the method and as preliminary data to suggest that CRP may increase uptake of meningococci into dendritic cells in suspension.
5.3 Discussion

The aim of this chapter was to discover the effect of CRP-binding on the association of paraformaldehyde-fixed meningococci with important non-macrophage phagocytes. The phagocytes of interest were the highly phagocytic but short lived neutrophils of the blood and the highly specialised tissue-resident dendritic cells which link innate and adaptive immunity.

5.3.1 CRP-opsonisation increases the association of \textit{N. meningitidis} with neutrophils

Although antibody-mediated serum killing of \textit{N. meningitidis} has long been held as the primary defence against meningococcal disease, the role of phagocytosis, and opsonophagocytosis by neutrophils in particular, has more recently been highlighted as having great importance, particularly with respect to serogroup B disease (Estabrook \textit{et al.}, 1992; Bredius \textit{et al.}, 1994; Schlesinger \textit{et al.}, 1994). Neutrophils may be crucial for clearance of meningococci as low counts in sepsis patients are consistent with poor prognosis for recovery (Gedde-Dahl \textit{et al.}, 1990). Activation of neutrophils, however, is also central to the vascular damage and multi-organ failure seen in extreme cases of meningococcal sepsis (Sotto \textit{et al.}, 1976). Thus neutrophils can both resolve meningococcal infection and contribute to the pathology of sepsis.

CRP-opsonisation of piliated \textit{N. meningitidis} resulted in a small but significant increase in association with human neutrophils. This could increase the efficiency with which bacteria are cleared from the site of infection, but because bacterial killing was not measured in this assay, it is not known if CRP-mediated uptake could help increase neutrophil killing of the meningococci. Increased phagocytosis does not always correlate with increased killing of \textit{N. meningitidis} by neutrophils (Heyderman \textit{et al.}, 1999). Thus the intriguing possibility arises that meningococci could, under certain conditions, use neutrophils as a refuge during infection, although this theory requires much further study, including time-kill assays and measurement of reactive oxygen and nitrogen species production by neutrophils in response to non-opsonised and CRP-opsonised \textit{N. meningitidis}. Encapsulated \textit{N. meningitidis} is capable of survival and replication within THP-1- and U937-derived macrophages in contrast to non-capsulated mutants which do not replicate within these cells.
(Spinosa et al., 2007), but survival of meningococci within neutrophils has not been confirmed in the literature. The closely related species *N. gonorrhoeae* has been shown to resist intracellular neutrophil killing and to actively replicate within neutrophils, even delaying apoptosis to facilitate a longer time-frame for replication inside the cells (Simons et al., 2005; Simons et al., 2006). To investigate whether *N. meningitidis* is capable of such activity would be of great interest to meningococcal research.

The CRP-mediated increase in association with meningococci was much smaller for neutrophils than the increase seen in macrophages (see section 4.2.4). This difference is likely to be due to the much shorter incubation time used for neutrophils but may also be influenced by the scarcity of FcyRI receptors on neutrophils, which constitutively express FcyRII and FcyRIII, but require activation for the upregulation of FcyRI expression. Additionally, CRP binding to FcyRIIa has been shown to be dependent upon clustering of CRP molecules upon the surface of particles such as erythrocytes or *S. pneumoniae* (Bodman-Smith et al., 2004). It is likely, therefore, that scantily opsonised particles such as *N. meningitidis* may show poor uptake via the FcyRIIa of neutrophils, and this could be investigated with further assays using specific antibody blockade of FcyRI and FcyRIIa.

Priming of neutrophils *in vitro* with proinflammatory signals such as LPS has been shown to increase the uptake of meningococci (Heyderman et al., 1999). Here, neutrophils were not primed, because the assay was designed to investigate the effect of CRP on meningococcal uptake by resting neutrophils and accordingly, cells were incubated with meningococci for only 20 minutes, immediately after separation from blood. Activated neutrophils may show a greater uptake of meningococci and the influence of CRP may be more, or indeed, less significant. The assays using resting neutrophils were designed to model the action of neutrophils encountering *N. meningitidis* early in the infection process. The use of activated neutrophils would better model the response of neutrophils summoned to the site of inflammation by signals such as LPS and TNFα later in the infection process. Clearly, such a model is of interest in uncovering the role of CRP in the response to meningococcal infection and should be included in further investigations.

The closely related pentaxtrin Serum amyloid P (SAP) is CRP’s counterpart in the mouse and has been shown to bind to *N. meningitidis in vitro*
Interestingly, rather than acting as an opsonin to increase phagocyte uptake of the organism, SAP binding of *N. meningitidis* decreases phagocytosis by neutrophils, enhancing virulence of the organism in the bloodstream. It is an interesting difference between CRP and SAP that human neutrophils display increased uptake of *N. meningitidis* after opsonisation with CRP but as these two proteins bind to different receptors, it is very possible that one could activate while the other down-regulates phagocytosis.

It has been shown for *S. pneumoniae*, that CRP-opsonisation not only increases uptake of bacteria into neutrophils, but also increases IL-8 production and oxidative burst (Rodriguez et al., 2004). The investigation into the effect of CRP-opsonisation on neutrophil response to meningococci was a preliminary exploration of association of the cells with bacteria, but could be complemented in the future by a more thorough analysis of neutrophil activation and bactericidal activity. This would include exploring the effect of CRP-opsonisation on meningococcal intracellular survival within neutrophils, on respiratory burst activation, cytokine secretion and integrin expression as well as defining the types of FcγR utilised in neutrophil phagocytosis of CRP-opsonised meningococci.

### 5.3.2 CRP-opsonisation can increase association of *N. meningitidis* with dendritic cells

Dendritic cells link the innate and specific immune systems by operating antimicrobial surveillance in non-immune sites, phagocytosing pathogens and maturing en-route to lymphoid organs where they can present antigen to naïve T cells. Dendritic cells both initiate and guide adaptive immunity as the cytokines produced by antigen presenting dendritic cells, such as IL-12 or IL-10 and the costimulatory molecules expressed direct the development of the T cell response into a T helper type 1, T helper type 2 or regulatory T cell response. The dendritic cell uptake assays detailed in this chapter indicate that CRP may be able to increase the uptake of *N. meningitidis* by immature dendritic cells, which may have many downstream consequences.

CRP-opsonisation has recently been shown to increase uptake of *S. pneumoniae* by dendritic cells (Thomas-Rudolph et al., 2007). That study demonstrated that the CRP-mediated uptake was facilitated by Fcγ receptors and led to increased antibody responses and protection when loaded dendritic
cells were transferred into mice that were subsequently infected with virulent streptococci (Thomas-Rudolph et al., 2007). It would be interesting to discover if the CRP-mediated increase in association of *N. meningitidis* with dendritic cells indicated here has similar effects on activation of the cells. Phagocytic uptake of encapsulated meningococci by dendritic cells is classically very low, with non-encapsulated strains showing greater adherence and uptake and reduced intracellular survival (Kolb-Maurer et al., 2001; Kolb-Maurer et al., 2003). The capsule is clearly important in preventing dendritic cell detection, but phase variation of encapsulated strains during a meningococcal infection mean the capsule will not always be expressed. Several components of the outer membrane of *N. meningitidis* have been shown to activate and increase antigen presentation in dendritic cells, usually by upregulating the expression of MHC class II and the T cell costimulatory molecule CD86 on the dendritic cell surface. This has led to the proposal of including components such as *Neisseria* adhesion molecules, very small proteoliposomes, PorA and modified meningococcal LPS as adjuvants in vaccines (Steeghs et al., 2006; Bracho et al., 2006; Mazzon et al., 2007; Venier et al., 2007). It would be interesting to discover if opsonisation with CRP augments or diminishes the expression of these activation molecules on the dendritic cell surface in response to meningococci.

A recent study using human monocyte-derived dendritic cells investigated the effect of CRP on the differentiation, maturation and functional activities of dendritic cells. The study revealed that CRP (unbound to any carrier at a concentration of 10μg/ml) inhibited the expression of maturation markers and antigen presentation markers and inhibited endocytosis (Zhang et al., 2006). This inhibition of dendritic cell activity is very different from the response of dendritic cells to meningococci, making the prospect of discovering the effect of CRP-opsonised meningococci all the more attractive.

Further investigation into the effect of CRP-opsonisation on activation of dendritic cells will be carried out within the immunology group at the University of Surrey by Dr Jessica Jenson, who was a collaborator on the DC uptake assays included in this thesis. Following confirmatory assays of the positive effect of CRP-opsonisation on uptake by dendritic cells, Dr Jenson will investigate activation markers such as CD86 and MHC-II and cytokine production to examine the potential for T cell activation of CRP-meningococcal-primed
dendritic cells. Subsequent work will include the co-culture of CRP-opsonised meningococci-loaded dendritic cells with T cells to examine the T cell response. In the preliminary assays described here, purity of dendritic cells was confirmed by visual inspection under the light microscope and by CD11c expression in FACS procedures. Future experiments will also confirm purity of dendritic cell cultures by FACS measurement of DC markers such as CD11c, CD80, CD83 and CD40.

5.3.3 Conclusions

CRP-opsonisation of bacteria can increase uptake of the pathogen by Fcy receptor-expressing cells including neutrophils and dendritic cells. Preliminary investigations into the effect of CRP-opsonisation on uptake of meningococci indicate that CRP can increase the association of the meningococcus with these cell types. The increased association with neutrophils may be important for increasing clearance of meningococci from the blood stream and at sites of inflammation where neutrophils are trafficked, but the effect of CRP-opsonised meningococci on neutrophil activation and intracellular killing has not been investigated. CRP-mediated dendritic cell uptake results in increased antigen presentation to other CRP-binding pathogens and may have similar effects with meningococci. Further investigation is needed to explore these possible consequences but the fact that CRP appears to increase the association of N. meningitidis with these two important phagocytes is a promising indication of further roles for CRP in the response to meningococcal infection.
CHAPTER SIX

THE EFFECT OF CRP ON THE ACTIVATION OF MACROPHAGES BY 
NEISSERIA MENINGITIDIS
6.1 Rationale and aims

The increased phagocytic association of CRP-opsonised meningococci with macrophages was demonstrated in chapter 4. Cross-linking of cell surface receptors initiated by phagocytosis transmits signals within the macrophage, affecting the response of that cell in terms of oxidative burst, granule release, cytokine release and the expression of important cell surface receptors. The effect of the CRP-mediated increase in uptake of meningococci on activation of macrophages was, therefore, investigated by measuring macrophage surface markers of activation and secreted inflammatory cytokines.

Cell surface markers of macrophage activation include receptors which, when up-regulated, may increase the efficiency of the macrophage response to infection. Such receptors include complement receptors (which bind and take up complement-coated particles), adhesion molecules (which facilitate migration of leucocytes to sites of inflammation and secondary immune organs), major histocompatibility complex (MHC) molecules (which aid the presentation of antigen to T cells), receptors that recognise pathogen-related molecular patterns and Fc receptors (which bind immunoglobulin-coated particles and pathogens).

In order to quantify a selection of these markers, FACS analysis was performed on macrophages after 24 hours incubation with CRP-opsonised and non-opsonised meningococci.

Inflammatory cytokines are pivotal to macrophage communication with other cells during infection or tissue damage. Macrophages can produce a range of pro- and anti-inflammatory cytokines upon contact with bacteria and other activating signals (such as exogenous cytokines). These cytokines can activate or inhibit the action of neighbouring macrophages and lymphocytes, but can also damage host tissues locally and systemically. In the experiments detailed in this chapter, cell culture supernatants were reserved after incubating macrophages with CRP-opsonised and non-opsonised meningococci in order to measure the levels of a range of pro- and anti-inflammatory cytokines secreted by the macrophages. Peak cytokine production following LPS challenge can occur between 1 and 48 hours after stimulation, depending on the individual cytokine and the concentration of LPS. Ideally, time course experiments would have been performed to discover the time-point of peak production of each cytokine tested, but as cells were being used for FACS staining experiments after 24 hours incubation, this was not possible. In addition, collaborators at St Georges
Hospital, University of London have demonstrated that 24 hours is an appropriate time-point for monokine secretion analysis for PBMC-derived macrophages. In these experiments, the 24 hour time-point proved suitable for TNFα, IL-1β, IL-6 and IL-10 but was less suitable for IL-8 and IL-12p70 for reasons that will be discussed later.

6.2 Results

Macrophages were incubated with CRP-opsonised meningococci and appropriate controls in order to quantify the expression of cell surface markers of activation and to measure the production of inflammatory cytokines by the cells. These assays were originally performed using a multiplicity of infection (MOI) of 200 bacteria per macrophage and a final CRP concentration of 5μg/ml. In these experiments, the high number of bacteria evoked a very large macrophage response and any modulatory activity of CRP was not detectable. Consequently, a series of experiments were performed using a much lower MOI of 10:1 and a final CRP concentration of 10μg/ml, which was more representative of moderate acute-phase levels of CRP. All data were analysed by repeated measures ANOVA with Tukey multiple comparison post test. P values of less than 0.05 were considered statistically significant.

6.2.1 The effect of CRP-opsonised N. meningitidis at a high multiplicity of infection on activation marker expression in cell line-derived macrophages

THP-1- and U937-derived macrophages were incubated with controls and test treatments for 24 hours as described in section 2.2.12. The MOI was estimated as 200 bacteria per cell by plating out inoculum onto CBA agar before paraformaldehyde-fixing and counting sacrificed wells of macrophages in a haemocytometer. Macrophage surface markers were stained as detailed in section 2.2.13.2-3 and cells were examined on the BD FACScan as detailed in section 2.2.13. For all activation markers, 5μg/ml CRP alone had no statistically significant effect on either the percentage of cells positive for activation markers or for the mean expression of surface marker per macrophage. CD14 detection was minimal for both cell lines and so CD14 cell line data has been excluded from this analysis.
6.2.1.1 Activation marker expression of THP-1-derived macrophages

85.4 ± 4.2 % of THP-1-derived macrophages expressed CD11b in the resting state (negative control) and over 77.2 ± 5.9% of positive control and CRP-treated cells expressed CD11b (figure 6.1a). The percentage of cells treated with non-opsonised \textit{N. meningitidis} or CRP-opsonised \textit{N. meningitidis} that were positive for CD11b was significantly lower than negative control cells (p<0.001 for both). There was a small decrease in the percentage of cells expressing CD11b when bacteria were opsonised with CRP compared to non-opsonised bacteria, but this was not statistically significant. The average expression of CD11b per cell, shown as mean fluorescence intensity (MFI) is displayed in figure 6.1b. This graph indicates that the positive control, non-opsonised meningococci and CRP-opsonised meningococci all reduced the mean CD11b expression compared to the negative control, but the differences were not statistically significant.

Figure 6.1 Activation marker expression by THP-1-derived macrophages incubated with CRP-opsonised \textit{N. meningitidis}

THP-1-derived macrophages were incubated with PBS (Neg), IFNy & LPS (Pos), CRP, \textit{N. meningitidis} mock-opsonised with PBS (Nm) or \textit{N. meningitidis} pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD11b (red), CD11c (blue), ICAM-1 (green) and CD64 (orange). Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means + standard deviation of 4 replicate experiments.
Over 90% of all THP-1-derived macrophages were positive for CD11c regardless of treatment (figure 6.1a). All treatments caused an increase in the average CD11c expression per cell (figure 6.1b) compared to the negative control, but these increases were not statistically significant. The percentage of THP-1-derived macrophages expressing ICAM-1 was high in all conditions (figure 6.1a) but the average expression of ICAM-1 per cell varied with treatments (figure 6.1b). ICAM-1 expression in the positive control was significantly higher than the negative control (p<0.05). The increases in average ICAM-1 expression for macrophages incubated with CRP, non-opsonised meningococci and CRP-opsonised meningococci were not, however, statistically significant compared to the negative control. The percentage of THP-1-derived macrophages positive for CD64 expression was unaffected by the positive control and CRP treatments, but was significantly reduced by incubation with non-opsonised and CRP-opsonised meningococci (figure 6.1a, p<0.001 and p<0.01 respectively). Although CRP-opsonised meningococci appear to have caused less of a reduction in CD64 expression than non-opsonised meningococci, the difference was not statistically significant. Measuring the average CD64 expression per macrophage (figure 6.1b) revealed a reduction in expression caused by non-opsonised and CRP-opsonised meningococci compared to the negative control (both p<0.05).

In summary: the significant differences in THP-1-derived macrophage marker expression in response to meningococci at a high MOI were a reduction in CD11b and a reduction in CD64 expression.

6.2.1.2 Activation marker expression of U937-derived macrophages

Over 87% of U937-derived macrophages were positive for CD11b expression after every test and control treatment (figure 6.2a). The average level of CD11b expression per macrophage for untreated cells was significantly higher than the positive control cells and cells incubated with non-opsonised and CRP-opsonised meningococci (p<0.01, p<0.001 and p<0.001 respectively). There was no significant difference between the average CD11b expression per cell for macrophages incubated with non-opsonised and CRP-opsonised meningococci. A high percentage of cells were positive for CD11c in all treatments (>70% figure
6.2a) with no significant differences in mean expression per cell between any of the control or test treatments (figure 6.2b).

![Activation marker expression by U937-derived macrophages incubated with CRP-opsonised N. meningitidis](image)

**Figure 6.2 Activation marker expression by U937-derived macrophages incubated with CRP-opsonised N. meningitidis**

U937-derived macrophages were incubated with PBS (Neg), IFNy & LPS (Pos), CRP, *N. meningitidis* mock-opsonised with PBS (Nm) or *N. meningitidis* pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD11b (red), CD11c (blue), ICAM-1 (green) and CD-64 (orange). Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means + standard deviation of 4 replicate experiments.

Over 90% of U937-derived macrophages from all control and test treatments were positive for ICAM-1 expression (figure 6.2a). The mean expression of ICAM-1 per cell was significantly higher in positive control cells compared to negative control cells (figure 6.2b, p<0.05). ICAM-1 expression was non-significantly increased by CRP, non-opsonised and CRP-opsonised meningococci treatment of cells. The percentage of U937-derived macrophages positive for CD64 was greater than 73% for all control and test treatments of cells. The positive control treatment increased the average CD64 expression per cell compared to the negative control, but this increase was not statistically significant and there was no effect on average CD64 expression per cell for any of the remaining treatments compared to the negative control.

In summary: the significant difference in U937-derived macrophage marker expression in response to meningococci at a high MOI was a moderate reduction in CD11b expression.
6.2.2 The effect of CRP-opsonised *N. meningitidis* at a high multiplicity of infection on activation marker expression in PBMC-derived macrophages

69.7 ± 10.1% of resting PBMC-derived macrophages were positive for CD11b expression and levels did not change with the positive control or CRP treatments (figure 6.3a). Treatment with non-opsonised and CRP-opsonised *N. meningitidis*, however, caused a significant decrease in the percentage of cells positive for CD11b compared to the untreated cells (p<0.005 for both). When comparing the average CD11b expression per cell between treatments, the only significant change in expression occurred after incubation of macrophages with unopsonised and CRP-opsonised meningococci (figure 6.3b, p<0.01 for both). Greater than 92% of all PBMC-derived macrophages were positive for CD11c expression and the average level of CD11c expression per cell did not change significantly with any of the treatments.

**Figure 6.3 Activation marker expression by PBMC-derived macrophages incubated with CRP-opsonised *N. meningitidis***

PBMC-derived macrophages were incubated with PBS (Neg), IFNy & LPS (Pos), CRP, *N. meningitidis* mock-opsonised with PBS (Nm) or *N. meningitidis* pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD11b (red), CD11c (blue) and ICAM-1 (green). Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means + standard deviation of 8 replicate experiments from separate donors.

86.3 ± 1.8% of resting PBMC-derived macrophages were positive for ICAM-1 expression and this value increased non-significantly to 96.6 ± 0.2% for positive control treated cells and no other treatments caused a significant change in expression compared to the negative control (figure 6.3a). Comparing the average ICAM-1 expression per cell, however, revealed that positive control treated cells expressed significantly more ICAM-1 than resting macrophages.
There was no significant difference between ICAM-1 expression in resting PBMC-derived macrophages incubated with any of the other treatments. No difference was observed in ICAM-1 expression in macrophages incubated with non-opsonised meningococci compared to macrophages incubated with CRP-opsonised meningococci.

Less than 50% of PBMC-derived macrophages from any treatment were positive for CD14 expression and there was no significant difference between the level of CD14 expression after any treatment compared to the negative control (figure 6.4a). Comparing the average expression of CD14 per cell, the positive control treatment caused an increase in CD14 expression that was not statistically significant (figure 6.4b).

**Figure 6.4 Activation marker expression by PBMC-derived macrophages incubated with CRP-opsonised N. meningitidis**

PBMC-derived macrophages were incubated with PBS (Neg), IFNy & LPS (Pos), CRP, N. meningitidis mock-opsonised with PBS (Nm) or N. meningitidis pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD14 (turquoise), CD64 (orange) and HLA-DR (purple). Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means + standard deviation of 8 replicate experiments from separate donors.

The percentage of PBMC-derived macrophages expressing CD64 in the resting state was approximately 60%. This level of expression increased with the positive control treatment but the difference was not statistically significant. Incubation of macrophages with non-opsonised and CRP-opsonised meningococci caused a non-significant decrease in the percentage of cells positive for CD64 expression (figure 6.4a). Incubation of macrophages with meningococci caused a significant decrease in CD64 expression compared to the negative control (figure 6.4b, p<0.05).
As PBMC-derived macrophages are a more biologically relevant model than cell lines, it was decided to extend the panel of markers measured to include antigen presentation markers. As expected of professional antigen presenting cells, over 73% of all PBMC-derived macrophages were positive for HLA-DR expression (figure 6.4a). The positive control appeared to increase the average level of HLA-DR expression per cell, but this increase was not statistically significant. Incubation with non-opsonised and CRP-opsonised meningococci had no effect on the level of HLA-DR expression per cell (figure 6.4b).

In summary: the significant differences in PBMC-derived macrophage marker expression in response to meningococci at a high MOI were a reduction in CD11b and a reduction in CD64 expression.

6.2.3 The effect of CRP-opsonised *N. meningitidis* at a low multiplicity of infection on activation marker expression in macrophages

Macrophage activation marker expression was altered by incubation with meningococci, but there was no difference in the response to CRP-opsonised meningococci compared to non-opsonised meningococci at the high MOI of 200 bacteria per macrophage, possibly due to macrophages being overwhelmed by high numbers of bacteria. The assays were, therefore, repeated using a MOI of 10 bacteria per macrophage and an increased opsonising concentration of 100μg/ml CRP (final concentration of 10μg/ml to increase the CRP concentration to levels more related to moderate acute-phase levels) as detailed in sections 2.2.12 and 2.2.13. A new cohort of PBMC donors was recruited and a new batch of early-passage THP-1 cells was purchased from the European cell culture collection. U937 cells were not included in these experiments because of the poor responses observed in marker activation and bacterial association. The final concentration of 10μg/ml CRP had no effect on activation marker expression when non-aggregated CRP alone was added to the macrophages.
6.2.3.1 Activation marker expression of THP-1-derived macrophages (low MOI)

81.3 ± 0.9% of resting THP-1-derived macrophages were positive for CD11b expression. This figure was reduced (non-significantly) after positive control treatments and incubation with non-opsonised and CRP-opsonised meningococci (figure 6.5a). The mean CD11b expression per cell decreased (non-significantly) with the positive control treatment but was significantly lower than resting macrophage expression levels when macrophages were incubated with non-opsonised and CRP-opsonised meningococci (figure 6.5b, p<0.01 and p<0.001 respectively). CD11c was highly expressed in all conditions and did not vary significantly in average expression per cell, as was observed in the higher MOI experiments (figure 6.5a and b).

![Figure 6.5 Activation marker expression by THP-1-derived macrophages incubated with CRP-opsonised N. meningitidis at a low multiplicity of infection](image)

**Figure 6.5 Activation marker expression by THP-1-derived macrophages incubated with CRP-opsonised N. meningitidis at a low multiplicity of infection**

THP-1-derived macrophages were incubated with PBS (Neg), IFNy & LPS (Pos), CRP, *N. meningitidis* mock-opsonised with PBS (Nm) or *N. meningitidis* pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD11b (red), CD11c (blue) and ICAM-1 (green). Bacterial multiplicity of infection was 10:1. Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means + standard deviation of 5 replicate experiments.

The percentage of THP-1-derived macrophages positive for ICAM-1 expression was significantly increased in the positive control (p<0.05) and non-significantly increased by incubation with non-opsonised and CRP-opsonised meningococci (figure 6.5a). Comparing the mean ICAM-1 expression per macrophage revealed that the positive control, non-opsonised and CRP-opsonised meningococci all significantly increased ICAM-1 expression (figure
6.5b, p<0.001, P<0.01 and p<0.01 respectively). No difference was observed in the response to non-opsonised as compared with CRP-opsonised meningococci.

Figure 6.6 Activation marker expression by THP-1-derived macrophages incubated with CRP-opsonised \textit{N. meningitidis} at a low multiplicity of infection

THP-1-derived macrophages were incubated with PBS (Neg), IFNy & LPS (Pos), CRP, \textit{N. meningitidis} mock-opsonised with PBS (Nm) or \textit{N. meningitidis} pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD64 (orange) and HLA-DR (purple). Bacterial multiplicity of infection was 10:1. Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means ± standard deviation of 5 replicate experiments.

40.1 ± 4.7% of resting THP-1-derived macrophages and 32.4 ± 8.0% of cells treated with LPS and IFNy were positive for CD64 expression. Incubation of macrophages with non-opsonised and CRP-opsonised meningococci resulted in a significant reduction in the percent of cells expressing CD64 to 6.7 ± 1.8% and 4.8 ± 1.3 % respectively (figure 6.6a both p<0.001). Incubation of macrophages with unopsonised and CRP-opsonised meningococci also caused a significant reduction in the mean CD64 expression per cell (figure 6.6b, both p<0.001). The positive control was the only treatment to significantly increase the percentage of THP-1-derived macrophages positive for HLA-DR expression and the mean HLA-DR expression per macrophage (figure 6.6a and b). Neither non-opsonised nor CRP-opsonised meningococci had any effect on HLA-DR expression at this lower MOI.

In summary: the significant differences in THP-1-derived macrophage marker expression in response to meningococci at a low MOI were a reduction in CD11b and CD64 expression, and an increase in ICAM-1 expression.
6.2.3.2 Activation marker expression of PBMC-derived macrophages (low MOI)

As observed previously using a higher MOI and lower concentration of CRP, both CD11b and CD11c expression were high in resting PBMC-derived macrophages and unchanged by any of the treatments (figure 6.7a & b). Although the percentage of PBMC-derived macrophages positive for ICAM-1 expression was not significantly increased by the positive control treatment or incubation with non-opsonised and CRP-opsonised meningococci, the mean expression of ICAM-1 per macrophage was significantly increased by these three treatments (p<0.001 for all).

Figure 6.7 Activation marker expression by PBMC-derived macrophages incubated with CRP-opsonised *N. meningitidis* at a low multiplicity of infection

PBMC-derived macrophages were incubated with PBS (Neg), IFNy & LPS (Pos), CRP, *N. meningitidis* mock-opsonised with PBS (Nm) or *N. meningitidis* pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD11b (red), CD11c (blue) and ICAM-1 (green). Bacterial multiplicity of infection was 10:1. Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means + standard deviation of 8 replicate experiments.

The percentage of macrophages expressing CD14 was low in resting PBMC-derived macrophages and unchanged by any of the treatments (figure 6.8a). Although incubation with non-opsonised and CRP-opsonised meningococci caused a slight increase in the mean expression of CD-14 per cell, these increases were not statistically significant (figure 6.8b). The percentage of PBMC-derived macrophages positive for CD64 expression was not affected by
the positive control treatment, but was significantly decreased by incubation with both non-opsonised and CRP-opsonised meningococci (both p<0.05).

![Graph showing activation marker expression by PBMC-derived macrophages incubated with CRP-opsonised N. meningitidis.](image)

**Figure 6.8 Activation marker expression by PBMC-derived macrophages incubated with CRP-opsonised N. meningitidis at a low multiplicity of infection**

PBMC-derived macrophages were incubated with PBS (Neg), IFNγ & LPS (Pos), CRP, *N. meningitidis* mock-opsonised with PBS (Nm) or *N. meningitidis* pre-opsonised with CRP (Nm+CRP) for 24 hours before staining with fluorescent antibodies specific for CD14 (turquoise), CD64 (orange) and HLA-DR (purple). Bacterial multiplicity of infection was 10:1. Data are presented as percentage of cells positive for each marker (a) or mean fluorescence intensity of each marker (b) as means + standard deviation of 8 replicate experiments.

The mean expression of CD64 per cell was unchanged by the positive control treatment but significantly reduced by incubation with both non-opsonised and CRP-opsonised meningococci (both p<0.01). The percentage of PBMC-derived macrophages positive for HLA-DR expression was not significantly changed by any of the treatments, despite appearing to increase with positive control treatments (figure 6.8a). Also, no treatments significantly changed the mean HLA-DR expression per cell (figure 6.8b).

In summary: the significant difference in PBMC-derived macrophage marker expression in response to meningococci at a low MOI was a reduction in CD64 expression.
Although HLA-DR expression did not significantly increase in response to meningococci, figures 6.8a and b indicated that HLA-DR expression by PBMC-derived macrophages was greater after incubation with CRP-opsonised meningococci compared to non-opsonised meningococci in terms of both the percentage of cells positive for HLA-DR expression and the mean HLA-DR expression per cell. However, the large inter-donor variation makes this difference statistically non-significant. Individual donor differences between HLA-DR expression on macrophages incubated with CRP-opsonised compared to non-opsonised meningococci were therefore examined. Figure 6.9 shows that 5 out of 8 donors had a higher percentage of cells positive for HLA-DR expression and mean HLA-DR expression per cell upon incubation with the CRP-opsonised meningococci as compared with macrophages incubated with non-opsonised meningococci. This trend was not, however, statistically significant by paired Students t-test (p=0.255 and p=0.350 respectively).

**Figure 6.9 HLA-DR expression by PBMC-derived macrophages incubated with CRP-opsonised *N. meningitidis***

The percentage of cells positive for HLA-DR expression (a) and mean HLA-DR expression per cell (b) on macrophages derived from 8 separate donors after incubation with CRP-opsonised *N. meningitidis* or non-opsonised *N. meningitidis*. 
6.2.4 The effect of CRP-opsonised meningococci on inflammatory cytokine production by macrophages infected at a high multiplicity of infection

Cell culture supernatants from all experiments detailed in sections 6.2.1-6.2.2 were reserved. TNFα levels in aliquots of the supernatants were measured by in-house ELISA to allow an estimation of the dilutions required for a cytometric bead array (data not shown). Supernatants were then used neat and at a 1 in 50 dilution in a cytometric bead array, measuring a panel of 6 inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNFα) as detailed in sections 2.2.17 and 2.2.18. IL-12p70 concentrations were extremely low for all test samples, probably because IL-12 production typically peaks within 2 hours of inflammatory stimulation and so this data has been excluded from the analysis. IL-8 concentrations were often higher than the maximum detection limit of the assay at the dilutions used, and so IL-8 has also been excluded from analysis.

6.2.4.1 The effect of CRP-opsonised meningococci at high MOI on inflammatory cytokine production by THP-1-derived macrophages

Resting THP-1-derived macrophages produced low but detectable levels of TNFα. The positive control, 5μg/ml CRP and non-opsonised and CRP-opsonised meningococci all induced a TNFα response of over 2500pg/ml by the macrophages, but these increases were not statistically significant by ANOVA (figure 6.10a). Resting macrophages produced low levels of IL-10, with little response observed after incubating cells with the positive or CRP controls. Incubation of macrophages with non-opsonised and CRP-opsonised meningococci produced a higher IL-10 response, but this increase was not statistically significant (figure 6.10b).

Resting THP-1-derived macrophages secreted little IL-6 and levels were significantly increased in the positive control (figure 6.10 c, p<0.05). CRP did not cause an increase in IL-6 production, but non-opsonised and CRP-opsonised meningococci both caused a non-significant increase, with the most IL-6 secreted in response to non-opsonised meningococci. Resting THP-1-derived macrophages produced little IL-1β. This was increased non-significantly by the positive control treatment and CRP-opsonised meningococci (P>0.05, figure 6.10d ). IL-1β secretion by macrophages incubated with non-opsonised meningococci was significantly higher than in unstimulated cells (p<0.05).
In summary: the statistically significant change in cytokine production by THP-1-derived macrophages in response to meningococci at a high MOI was an increase in IL-1β secretion.

![Graph showing cytokine production by THP-1-derived macrophages](image)

**Figure 6.10** Inflammatory cytokine production by THP-1-derived macrophages in response to CRP-opsonised *N. meningitidis* at a high multiplicity of infection

THP-1-derived macrophages were incubated for 24 hours with PBS alone (red) IFNγ & LPS (blue), 5μg/ml CRP (green) non-opsonised *N. meningitidis* (orange) or CRP-opsonised *N. meningitidis* (yellow) at an MOI of 200:1 before removing cell culture supernatants and measuring TNFα (a), IL-10 (b), IL-6 (c) and IL-1β (d) concentrations by cytometric bead array.

Data are represented as means of 4 replicate experiments + standard deviation.
6.2.4.2 The effect of CRP-opsonised meningococci at high MOI on inflammatory cytokine production by U937-derived macrophages

Resting U937-derived macrophages secreted low levels of TNFα. Treatment with CRP or LPS & IFNγ increased the TNFα production (statistically non-significant). The increase in TNFα production caused by incubation with non-opsonised or CRP-opsonised meningococci was, however, statistically significant (figure 6.11a, p<0.01 for both) but there was no difference in the response to non-opsonised compared to CRP-opsonised meningococci.

![Figure 6.11](image)

**Figure 6.11** Inflammatory cytokine production by U937-derived macrophages in response to CRP-opsonised *N. meningitidis* at a high multiplicity of infection

U937-derived macrophages were incubated for 24 hours with PBS alone (red), IFNγ & LPS (blue), 5µg/ml CRP (green) non-opsonised *N. meningitidis* (orange) or CRP-opsonised *N. meningitidis* (yellow) at an MOI of 200:1 before removing cell culture supernatants and measuring TNFα (a), IL-10 (b), IL-6 (c) and IL-1β (d) concentrations by cytometric bead array. Data are represented as means of 4 replicate experiments ± standard deviation.
Resting U937-derived macrophages produced little IL-10, with no change observed after incubating cells with the positive or CRP controls. Incubation of macrophages with non-opsonised and CRP-opsonised meningococci resulted in the greatest IL-10 secretion, but this increase was statistically non-significant (figure 6.11b).

Resting U937-derived macrophages secreted very small amounts of IL-6, and there was little increase in response to CRP alone. Positive control treatments did increase IL-6 production, as did incubation of macrophages with non-opsonised and CRP-opsonised meningococci (not statistically significant, figure 6.11c). CRP-opsonised meningococci could be capable of inducing greater IL-6 production than non-opsonised meningococci, but due to inter-assay variation, it was not possible to statistically validate this observation.

IL-1β production by resting U937-derived macrophages was very low and increased only slightly with positive control and CRP treatments (figure 6.11d, not statistically significant). Incubation of macrophages with both non-opsonised and CRP-opsonised meningococci caused a significant increase in IL-1β production (p<0.05). Although CRP-opsonised meningococci appeared to induce more IL-1β production in U937-derived macrophages than non-opsonised meningococci, this difference was not statistically significant.

In summary: the two cytokines to be significantly up-regulated by incubation of U937-derived macrophages with N. meningitidis at a high MOI were TNFα and IL-1β.

6.2.4.3 The effect of CRP-opsonised meningococci at high MOI on inflammatory cytokine production by PBMC-derived macrophages

TNFα secretion by resting and CRP-treated PBMC-derived macrophages was very low but was increased (non-significantly) by the positive control, non-opsonised and CRP-opsonised meningococci treatments (figure 6.12a). IL-10 production was low in resting PBMC-derived macrophages and increased non-significantly with positive control treatment (figure 6.12b). Incubation of PBMC-derived macrophages with non-opsonised and CRP-opsonised meningococci significantly increased IL-10 production (both p<0.05).
Low but detectable levels of IL-6 were secreted by resting PBMC-derived macrophages. Incubation of macrophages with positive control treatment and non-opsonised and CRP-opsonised meningococci increased IL-6 production, with the increases caused by the meningococci statistically significant (figure 6.12c, p<0.05). IL-1β production increased significantly from very low levels in resting macrophages to higher levels in the positive control (figure 6.12d p<0.05). Incubation of macrophages with non-opsonised and CRP-opsonised meningococci caused a smaller but still significant increase in IL-1β secretion (p<0.05).

In summary: Incubation of PBMC-derived macrophages with meningococci at a high MOI resulted in an increase in the secretion of IL-10, IL-6 and IL-1β.
6.2.5 The effect of CRP-opsonised meningococci on inflammatory cytokine production by macrophages infected at a low multiplicity of infection

In order to circumvent any overloading of macrophages with bacteria and to test more physiologically relevant concentrations of CRP (reflecting low acute-phase levels of CRP that would be present in the bloodstream and tissues during infection): inflammatory cytokines were measured in cell culture supernatants from macrophages incubated with CRP-opsonised *N. meningitidis* at a lower MOI (10 bacteria per macrophage) and higher final concentration of CRP (10μg/ml).

6.2.5.1 The effect of CRP-opsonised meningococci at low MOI on inflammatory cytokine production by THP-1-derived macrophages

TNFα secretion by resting THP-1-derived macrophages was low in resting cells and was significantly increased in the positive control (figure 6.13a, p<0.001). Increasing the CRP concentration from 5μg/ml to 10μg/ml CRP had no effect on TNFα production in response to the CRP control. Non-opsonised and CRP-opsonised meningococci both stimulated a statistically significant increase in TNFα production (p<0.01 and p<0.001 respectively). IL-10 secretion was very low in resting macrophages and increased with the positive control and with non-opsonised and CRP-opsonised meningococci (figure 6.13 b, p<0.05, p<0.01 and p<0.01 respectively).

IL-6 secretion was low in resting THP-1-derived macrophages and was significantly increased by the positive control, non-opsonised and CRP-opsonised meningococci treatments (figure 6.13c, p<0.001, p<0.05 and p<0.05 respectively). IL-6 secretion by macrophages was unchanged by incubation with 10μg/ml CRP. IL-1β production was at low but detectable levels in resting THP-1-derived macrophages and was increased by the positive control treatment, and unaffected by incubation with 10μg/ml CRP (figure 6.13d, p<0.05 and p>0.05 respectively). Incubation of macrophages with non-opsonised and CRP-opsonised meningococci caused a significant increase in IL-1β secretion (p<0.05).

In summary: the cytokines to be significantly up-regulated by incubation of THP-1-derived macrophages with meningococci at the lower MOI were TNFα, IL-10 and IL-1β.
Figure 6.13 Inflammatory cytokine production by THP-1-derived macrophages in response to CRP-opsonised N. meningitidis at a low multiplicity of infection
THP-1-derived macrophages were incubated for 24 hours with PBS alone (red) IFNγ & LPS (blue), 10µg/ml CRP (green) non-opsonised N. meningitidis (orange) or CRP-opsonised N. meningitidis (yellow) at an MOI of 10:1 before removing cell culture supernatants and measuring TNFα (a), IL-10 (b), IL-6 (c) and IL-1β (d) concentrations by cytometric bead array. Data are represented as means of 3 replicate experiments + standard deviation.

6.2.5.2 The effect of CRP-opsonised meningococci at a low MOI on inflammatory cytokine production by PBMC-derived macrophages

TNFα secretion in resting PBMC-derived macrophages was very low and was increased by positive control treatment and incubation with non-opsonised and CRP-opsonised meningococci at a low MOI (figure 6.14a, p>0.05, p<0.01 and p<0.01 respectively). There was no increase in TNFα production caused by incubation of macrophages with 10µg/ml CRP alone. IL-10 production was low in resting PBMC-derived macrophages with a small (non significant) increase caused by the positive control. IL-10 secretion was significantly increased by incubation with non-opsonised and CRP-opsonised meningococci (figure 6.14b, p<0.001 and p<0.001 respectively).
Figure 6.14 Inflammatory cytokine production by PBMC-derived macrophages in response to CRP-opsonised N. meningitidis at a low multiplicity of infection

PBMC-derived macrophages were incubated for 24 hours with PBS alone (red) IFNy & LPS (blue), 10μg/ml CRP (green) non-opsonised N. meningitidis (orange) or CRP-opsonised N. meningitidis (yellow) at an MOI of 10:1 before removing cell culture supernatants and measuring TNFα (a), IL-10 (b), IL-6 (c) and IL-1β (d) concentrations by cytometric bead array. Data are represented as means of 5 replicate experiments + standard deviation.

Low but detectable IL-6 secretion by resting donor-derived macrophages was non-significantly increased by the positive control treatment and significantly increased by incubation of macrophages with non-opsonised and CRP-opsonised N. meningitidis at a low MOI (figure 6.14c, p>0.05, P<0.05 and p<0.05 respectively). CRP at 10μg/ml did not cause any change in IL-6 production. Low IL-1β production by resting macrophages was increased by positive control treatments and incubation with non-opsonised and CRP-opsonised meningococci, with the increase in response to CRP-opsonised meningococci compared to the negative control being statistically significant (figure 6.14d, p<0.01).
In summary: Incubation of PBMC-derived macrophages with meningococci at a low MOI resulted in statistically significant increases in TNFα, IL-6 and IL-10 production and incubation with CRP-opsonised meningococci caused a statistically significant increase in IL-1β.

Closer scrutiny of the data for IL-10 and IL-1β secretion revealed that although there was no significant difference in the response to CRP-opsonised compared to non-opsonised meningococci as measured by ANOVA, 4 out of 5 donors produced more IL-10 in response to CRP-opsonised than non-opsonised meningococci (figure 6.15a) and 5 out of 5 donors produced more IL-1β in response to CRP-opsonised compared to non-opsonised meningococci (figure 6.15b). This trend was not, however, sufficient to provide statistical significance by paired Students t-test (p=0.151 and p=0.123 respectively).

![Figure 6.15 IL-10 and IL-1β production by PBMC-derived macrophages incubated with CRP-opsonised meningococci](image)

Cell culture supernatants from macrophages from 5 separate donors incubated with non-opsonised or CRP-opsonised meningococci were measured by cytometric bead array for IL-10 (a) and IL-1β production (b).
6.3 Discussion

The aim of this chapter was to explore the effects of CRP-opsonised *N. meningitidis* on the activation of human macrophages. This was achieved by investigating the expression of surface markers of activation and inflammatory cytokine production by human monocytic cell line-derived and PBMC-derived macrophages. Activation responses were examined after 24 hours incubation of macrophages with CRP-opsonised meningococci at both a high MOI (200 bacteria per macrophage, to model a high inflammatory stimulus) and a lower MOI (10 bacteria per macrophage). In high MOI experiments, a final CRP concentration of 5µg/ml was used but this was increased to 10µg/ml in low MOI experiments to better represent moderately raised acute-phase levels.

6.3.1 The effect of CRP-opsonisation on surface marker expression by macrophages in response to *N. meningitidis*

THP-1-, U937- and PBMC-derived macrophages were incubated with PBS alone, IFNγ and LPS, CRP alone, unopsonised *N. meningitidis* and CRP-opsonised *N. meningitidis*. Activation markers expressed on the surface of the macrophages were measured by FACS.

6.3.1.1 CD11b and CD11c expression

CD11b was expressed by resting THP-1-, U937- and PBMC-derived macrophages. Expression was significantly reduced by incubation with *N. meningitidis* with or without CRP-opsonisation in all three cell types during the high multiplicity of infection assays. At the lower multiplicity of infection, however, a lower percentage of THP-1-derived resting macrophages were positive for CD11b expression and the reduction in expression caused by *N. meningitidis* was no longer statistically significant. CD11c was expressed in a high percentage of all types of resting macrophage during both low and high MOI experiments and was unchanged by any of the treatments.

Both CD11b and CD11c co-operate individually with CD18 to form receptors of the β2 integrin family: complement receptor 3 (CR3, CD11b/CD18) and complement receptor 4 (CR4, CD11c/CD18). These receptors facilitate phagocytosis of particles that are opsonised with iC3b, bind to ICAM-1 on cells to facilitate diapedesis (Diamond *et al.*, 1991), and bind to bacterial LPS (Wright *et al.*, 1989). The results presented here demonstrate that *N. meningitidis* at a MOI
of 10 to 1 does not affect expression of these complement receptors. This finding is supported by the work of Mukhopadhyay et al. who demonstrated that mouse peritoneal macrophages incubated overnight with whole killed *N. meningitidis* at a MOI of 20:1 (twice the MOI used in the low MOI experiments presented here) had no effect on CR3 expression (Mukhopadhyay et al., 2004). In contrast to this, at the high MOI used here, CD11b expression was down-regulated by *N. meningitidis* with or without CRP-opsonisation. This could be as a result of the recruitment and internalisation of complement receptors, but this seems unlikely because these experiments were performed in a complement-free system to mimic the conditions of the uptake experiments of chapter 4. Had complement been present, a different pattern of complement receptor expression may have been observed. It is possible that meningococci down-regulate macrophage CD11b expression in order to reduce the adhesiveness of the macrophage by reducing binding to integrins on cells lining the vascular walls. This hypothesis could be tested in the future by examining adhesion of macrophages (post exposure to meningococci) with epithelial cell monolayers.

CD11b in resting THP-1-derived macrophages was lower in the low MOI experiments than the high MOI experiments. High MOI experiments were performed using THP-1 cells kindly donated by Dr Fiona Green at the University of Surrey who brought the cell line with her from the University of Oxford. These cells were of unknown passage number and in the early activation marker experiments, the stained cells were examined using the BD FACScan flow cytometer. The low MOI experiments were performed several months later using THP-1 cells purchased from the ECCAC and used between passage numbers 5 and 15. The stained cells were then examined on the newly purchased BD FACScanto flow cytometer, which may also explain the difference in MFI readings seen for low MOI and high MOI treated THP-1-derived macrophages. These differences mean that the two sets of data must be compared with caution. CD11b expression in PBMC-derived macrophages during the low MOI experiments was also lower than observed during the high MOI experiments, possibly due to differences in the cohort of volunteers used as both sets of experiments were analysed on the same BD FACScanto. In these low MOI PBMC-derived macrophage experiments, incubation with meningococci did not cause a change in the expression of CD11b.
CD11c expression is also a useful marker for myeloid cells. A high percentage of CD11c positive cells indicates a high purity of PBMC-derived macrophages, as contaminating lymphocytes are CD11c negative. In some experiments, the percentage of cells positive for CD11c dropped to below 90%, creating the possibility that some of the activation markers and cytokines produced may be due to contaminating lymphocytes. By using positive selection of monocytes with CD14 beads, purity of the resultant macrophage population would be increased.

6.3.1.2 CD14 expression

CD14 expression was very low in macrophages differentiated from both cell lines and so these cells were not included in the analysis. Approximately 30% of PBMC-derived macrophages were positive for CD14, but no difference was seen in expression in response to any of the treatments at either the high or low MOI. CD14 is a monocyte marker which is down-regulated upon differentiation of monocytes into macrophages, thus the low level of CD14 expression by macrophages in these assays was not unexpected. Meningococcal LPS binds to LPS-binding protein which can then bind to CD14, which lacks an intracellular signalling domain and therefore recruits toll-like receptor 4 (TLR4) and myeloid differentiation protein 2 (MD-2) for intracellular signal transduction. This can result in monocyte and macrophage production of pro-inflammatory cytokines such as IL-6 and IL-1β. In the experimental system presented here, CD14 was poorly detected on macrophages. It would be more relevant, however, to investigate CD14 expression by monocytes in response to CRP-opsonised meningococci in future experiments because this interaction could occur in the blood phase of meningococcal infection where monocytes will be encountered by the bacteria alongside high acute-phase levels of CRP.
6.3.1.3 ICAM-1 expression

The level of ICAM-1 expression in THP-1- and PBMC-derived macrophages was increased by the positive control treatment and by non-opsonised and CRP-opsonised meningococci at a low MOI. There was no difference between the response to non-opsonised and CRP-opsonised bacteria at the high MOI and expression was not significantly increased. Adhesion molecules, such as ICAM-1 play important roles during inflammation and injury by trafficking leucocytes to sites of inflammation and immune organs. ICAM-1 has been identified as an important host molecule on epithelial cells for the attachment of both N. gonorrhoea and N. meningitidis and is up-regulated on these cells by contact with the bacteria (Jarvis et al., 1999: Robinson et al., 2004).

ICAM-1 expression is low in resting monocytes and macrophages but can be upregulated by TNFα and IL-1, which may have been induced in the macrophages in these experiments in response to the LPS and bacterial treatments. ICAM-1 associates with integrins during inter-cellular signal transduction for T cell activation and leucocyte recruitment. ICAM-1 is important for extravasation and diapedesis between endothelial cells to traffic phagocytes to appropriate tissues. The up or down regulation of ICAM-1 expression can, therefore, change the nature of the inflammatory response (Schleimer and Bochner, 1998). Up-regulation of macrophage ICAM-1 by other meningitis-causing pathogens is thought to increase leucocyte recruitment and thereby increase the inflammation of the disease (Al Numani et al., 2003). The meningococcal-induced upregulation of human macrophage ICAM-1 demonstrated here indicates a similar response in meningococcal disease which may lead to increased homing of macrophages as antigen presenting cells to lymph nodes or as inflammatory mediators to tissues.

6.3.1.4 CD64 expression

CD64 expression in THP-1- and PBMC-derived macrophages was significantly down-regulated by incubation with non-opsonised and CRP-opsonised meningococci at both low and high MOI. In either cell type, however, there was no difference between the response to non-opsonised and CRP-opsonised meningococci. CD64 is the receptor for the Fc portion of IgG (also known as FcyRI) which binds monomeric IgG with the highest affinity of all Fcy receptors. FcyRI has been demonstrated to bind free CRP with a high affinity.
when transfected into COS-7 cells, showing increased affinity of binding (30 fold) when the cells are co-transfected with the gamma chain on the same plasmid, presumably due to the increase in surface stability of the receptor molecules (Bodman-Smith et al., 2002b: Röcker et al., 2007).

The demonstration that *N. meningitidis* can down regulate FcγRI on the surface of macrophages has important implications in the immune response to meningococci. The intracellular pathogen *Mycobacterium tuberculosis* causes a down regulation in IFNγ-mediated CD64 expression in macrophages, facilitating the inhibition of innate and adaptive immune response to the pathogen (Nagabhushanam et al., 2003). *N. meningitidis* could employ a similar strategy to down-regulate the immune response, but no role for CRP was observed in this interaction as the expression response to non-opsonised and CRP-opsonised meningococci was identical. Alternatively, FcγRIIa instead of FcγRI could be interacting with CRP-opsonised particles and this marker should be explored in future experiments.

### 6.3.1.5 HLA-DR expression

HLA-DR expression in resting THP-1-derived macrophages was significantly up-regulated by the positive control treatment but was not affected by incubation with meningococci with or without CRP at either low or high MOI. PBMC-derived macrophages showed a much higher percentage of cells positive for HLA-DR expression in the resting state compared to THP-1-derived macrophages and in the first cohort of volunteers, no increase in expression was seen in response to the positive control treatment or incubation with meningococci at a high MOI. The PBMC-derived macrophages from the second cohort of volunteers had resting expression levels of HLA-DR expression lower than the previous cohort, which was not significantly increased by positive control treatment or incubation with meningococci at the low MOI.

HLA-DR is a component of the MHC-II complex of proteins and is upregulated by IFNγ in monocytes and macrophages (Seljelid and Eskeland, 1993). In these experiments, less than half of the THP-1 cells were positive for the marker and MFI was low even with the positive control treatment. This is not unexpected as the pre-monocyte THP-1 cell is differentiated into a more macrophage-like morphology by PMA treatment, but the phenotype has not been reported to include antigen presentation capability in the literature.
Closer scrutiny of the PBMC-derived macrophage data revealed that, for the majority of donors, HLA-DR expression after incubation of macrophages with CRP-opsonised meningococci was greater than expression following incubation with non-opsonised meningococci at the low MOI. This indicates that CRP could induce an increased expression of HLA-DR by PBMC-derived macrophages, leading to enhanced antigen presentation and initiation of humoral immunity. Inter-donor variation is a significant impediment to obtaining statistically robust data from these assays. Using more donors or controlling for age, sex or recent infections could improve this, particularly if donors were genotyped for factors known to be important in response to meningococcal infection (e.g. FcγRIIa haplotypes).

Recent work using proteoliposomes of *N. meningitidis* has revealed that these membrane vesicles can up-regulate MHC-II expression on both macrophages and dendritic cells as well as up regulating MHC-I, CD40, CD80, and CD86 and can increase the production of cytokines important in T cell activation (Rodriguez *et al.*, 2005). This was not observed in the experiments using whole paraformaldehyde-fixed organisms, however, as no change in macrophage HLA-DR expression was seen in response to meningococci. This data was, however, supported by studies by Mukhopadhyay *et al.* who observed that overnight incubation of mouse peritoneal macrophages with whole killed *N. meningitidis* at an MOI of 20 bacteria per macrophage had no effect on MHC-II expression and actually prevented the IFNγ-Induced MHC-II expression seen in the absence of meningococci. (Mukhopadhyay *et al.*, 2004). These mouse studies also indicate that innate activation of macrophages by *N. meningitidis* is distinct from cytokine-induced activation by IFNγ and that both methods of activation result in specific changes to the surface phenotype of the macrophage (Mukhopadhyay *et al.*, 2004). The human data presented here supports the hypothesis that macrophage surface markers induced by incubation with meningococci form a distinct phenotype from LPS and IFNγ treated cells.
6.3.2 The effect of CRP-opsonisation on macrophage cytokine secretion in response to \textit{N. meningitidis}

Inflammatory cytokines were measured in cell culture supernatants from activation marker experiments using both cell-line- and PBMC-derived macrophages. TNF\(\alpha\), IL-10, IL-6 and IL-1\(\beta\) were all induced by positive control treatments and by non-opsonised and CRP-opsonised \textit{N. meningitidis}, but no differences were seen in the response to CRP-opsonised meningococci as compared to non-opsonised meningococci. Overall secretion of pro-inflammatory cytokines was very high in response to meningococci, this coupled with the lack of definitive CRP-opsonisation response in macrophage surface marker expression suggests that a high MOI could initiate an inflammatory response large enough to mask any modulatory effect of CRP-opsonisation. Experiments were, therefore, repeated with a lower MOI and a final CRP concentration more comparable to moderate acute phase levels at 10\(\mu\)g/ml. The variability of these assays was less pronounced in the control samples than test samples and was probably a result of variation in the batch cultures of \textit{N. meningitidis}.

6.3.2.1 TNF\(\alpha\) secretion

Meningococci are known to induce the secretion of high levels of proinflammatory cytokines such as TNF\(\alpha\) by host cells during infection (Waage \textit{et al.}, 1987; van Deuren \textit{et al.}, 1995; Brandtzaeg \textit{et al.}, 2001). TNF\(\alpha\) has pleiotropic systemic effects in the inflammatory response when produced at high levels and is particularly important as an early activator of other cytokines (such as IL-1\(\beta\) and IL-6). TNF\(\alpha\) also stimulates the liver to produce acute phase proteins and the hypothalamus to produce fever-inducing prostaglandins. TNF\(\alpha\) secretion by THP-1-derived and PBMC-derived macrophages was increased non-significantly by incubation with non-opsonised and CRP-opsonised meningococci at the high MOI and increased significantly in U937-derived macrophages compared to resting cells. In the lower MOI experiments, both meningococcal treatments caused a significant increase in TNF\(\alpha\) secretion in both THP-1- and PBMC-derived macrophages. Although the lower MOI of meningococci induced a lower level of secretion of this proinflammatory cytokine, the response was less variable between donors. In THP-1-derived macrophages the TNF\(\alpha\) response to CRP-opsonised bacteria was greater than for non-opsonised bacteria at the low
MOI, but the difference was not statistically significant and did not occur in PBMC-derived macrophages.

High plasma concentrations of TNFα and IL-1β are associated with high disease severity and mortality in meningococcal disease, because these pro-inflammatory cytokines induce shock and disseminated intravascular coagulation (Waage et al., 1987; Waage et al., 1987; van Deuren et al., 1995). A delicate balance exists between the pathology of high TNFα levels and poor defence of low TNFα levels and individuals who present a low production capacity for TNFα combined with a high production capacity for the anti-inflammatory cytokine IL-10 or poor regulation of the IL-1 system have a higher risk of invasive meningococcal disease (Westendorp et al., 1995). Both multiplicities of infection tested here induced high levels of TNFα, greater than or comparable to those induced by LPS and IFNγ treatment. This is consistent with the view of *N. meningitidis* as a potent stimulator of pro-inflammatory signals in the human host.

We might expect CRP to decrease the inflammatory TNFα response to *N. meningitidis* as it has been shown to decrease LPS-induced TNFα production by human alveolar macrophages (Casals et al., 2003). However, this was not apparent from the data detailed in this chapter, possibly due to differences between alveolar macrophages and PBMC-derived macrophages, or more probably, due to the very high levels of CRP used in the alveolar macrophage study (125 or 250µg/ml). Testing a range of higher CRP concentrations to determine whether CRP is capable of immunomodulating the TNFα response of macrophages to meningococci should, therefore, be the goal of future experiments. CRP at concentrations of either 5 or 10 µg/ml had no statistically significant effect on TNFα secretion by macrophages. This finding is not concurrent with recent literature and will be discussed in section 6.3.2.5.

### 6.3.2.2 IL-6 secretion

The host inflammatory response to *N. meningitidis* is known to include the secretion of high levels of IL-6 and non-survivors of meningococcal disease have higher IL-6 levels than survivors (Vermont et al., 2006). IL-6 is a pluripotent cytokine that enhances lymphocyte maturation and alongside TNFα and IL-1β, stimulates liver hepatocytes to produce acute phase proteins such as CRP, fibrinogen and complement components as well as activating the hypothalamus.
to induce fever (Heinrich et al., 1990). In the studies detailed in this chapter, non-opsonised and CRP-opsonised meningococci caused a significant increase in IL-6 production at a high MOI in all cell types. At the low MOI, non-opsonised and CRP-opsonised meningococci resulted in an increase in IL-6 secretion that was not statistically significant in any cell type. This could indicate that higher numbers of meningococci will stimulate a greater pro-inflammatory cytokine response which is consistent with reports that bacterial load is correlated with severity of disease (Hackett et al., 2002).

CRP increases inflammatory cytokine production by PBMCs in response to the gram positive pathogen *S. pneumoniae* by dramatically increasing the production of IL-1β and IL-6 (Mold and Du Clos, 2006). This increase in pro-inflammatory cytokine production limits bacteraemia and increases survival in mouse models of streptococcal infection, with the cytokines IL-6, TNFα and IL-1β identified as important components of this protection (Mold et al., 1981; van der et al., 1997). The pathogenesis of meningococcal infection is very different, however, primarily due to the presence of LPS in the cell wall of this Gram negative species. LPS, along with other cell surface components, potently activates the inflammatory cascade of sepsis that can result in organ failure (the major cause of fatality amongst meningococcal septicaemia patients). Thus, it would not be advantageous for the host if CRP increased the secretion of IL-6 in response to meningococci, and this hypothesis was supported by data from this chapter in which CRP did not cause a statistically significant increase in TNFα secretion by macrophages in response to meningococci.

Surprisingly, IL-6 secretion by U937-derived macrophages incubated with IFNγ and LPS was approximately double the amount secreted by THP-1-derived macrophages in these conditions, indicating an important difference in inflammatory activation of these two cell types. This disparity in inflammatory responses between THP-1 and U937 cells has been demonstrated experimentally by profiling the expression of NFκB target genes by the two cell lines in response to LPS (Sharif et al., 2007) and care must, therefore, be taken in interpreting results from cell lines such as these.
6.3.2.3 IL-1β secretion

The pro-inflammatory cytokine IL-1β is also pivotal to the acute phase response, enhancing the production of acute phase proteins by the liver and stimulating fever generation by the hypothalamus. Excessive or sustained production of IL-1β during sepsis can, however, result in hypotension, multiorgan failure, hypoalbuminuria, and neutrophilia which contribute to the mortality of sepsis (Pruitt et al., 1995). Because of the importance of this cytokine in the pathology of inflammatory disease, antagonists for the IL-1 receptors have been successfully used to treat inflammatory diseases such as arthritis (Hallegua and Weisman, 2002). Here, incubation of macrophages with meningococci caused a greater increase in IL-1β compared to the positive control treatments in the cell line-derived macrophages at the low MOI, but levels were more comparable in the high MOI experiments. This indicates that the lower bacterial loads were better at inducing IL-1β at the 24 hour time point (although this does not rule out an earlier peak in IL-1β levels at the higher MOI).

In these experiments, secretion of IL-1β in response to meningococci was over ten times lower in PBMC-derived macrophages than in either cell line at both low and high MOI infections. This observation is in agreement with the findings of a recent transcriptomic study by Kohro et al., comparing gene expression in macrophages differentiated from the THP-1 cell line and PBMC-derived macrophages in which it was shown that IL-1β was reciprocally regulated by differentiation in the two types of macrophage. In that study, PBMC-derived macrophages had reduced expression of IL-1β after differentiation from monocytes whereas THP-1 monocytes showed an increased expression of IL-1β upon PMA-induced differentiation (Kohro et al., 2004). Additionally, the secretion of IL-1β by THP-1-derived macrophages after incubation with IFNγ and LPS was over twice the amount secreted by U937-derived macrophages. Caution must, therefore, be taken in extrapolating data from individual cell-lines as extreme reactions can be peculiarities associated with a single cell line or PMA treatment.

At the low MOI, in both THP-1- and PBMC-derived macrophages, the IL-1β response to CRP-opsonised meningococci was moderately higher than the response to non-opsonised meningococci but this difference was not statistically significant. Individual analysis of each donor, however, revealed that 5 out of 5 donors produced more IL-1β in response to CRP-opsonised meningococci as compared to non-opsonised meningococci, indicating that CRP could augment
the IL-1β response to meningococci. Increasing the sample size to a larger population of donors may reveal a statistically significant increase in IL-1β response to meningococci in the presence of CRP, which would indicate an increased pro-inflammatory response seen for this cytokine which was not seen for IL-6 or TNFα. In these experiments, free CRP did not itself initiate a statistically significant increase in IL-1β, although differently designed experiments have recently shown that certain concentrations of CRP can cause increased IL-1β which will be discussed later (Singh et al., 2006; Nabata et al., 2007).

6.3.2.4 IL-10 secretion

IL-10 is largely considered to be an anti-inflammatory cytokine, originally known as the "cytokine synthesis inhibitory factor" because of its effects on inhibiting proinflammatory cytokine production and surface co-stimulatory molecule expression by macrophages (Moore et al., 2001). IL-10 secretion by macrophages in response to pathogens is thought to act as a negative feedback mechanism to limit tissue damage by downregulating the production of proinflammatory cytokines (De Waal Malefyt et al., 1991). However, several mouse studies have indicated that IL-10 may have pro-inflammatory functions under certain conditions, as although systemic administration of IL-10 prevents type-1 diabetes in non-obese diabetic (NOD) mice, when the gene is transgenically expressed in the mouse pancreas, it can accelerate the onset of diabetes (Pennline et al., 1994; Hill and Sarvetnick, 2002). In the studies detailed here, non-opsonised and CRP-opsonised meningococci caused the largest increase in IL-10 secretion in all cell types, but secretion by U937- and THP1-derived macrophages was over tenfold lower than PBMC-derived macrophages at both low and high MOI. Interestingly, PBMC-derived macrophages incubated with meningococci at the low MOI secreted approximately twice as much IL-10 as PBMC-derived macrophages incubated with the high MOI of meningococci. This data indicates that the anti-inflammatory signal may be reduced by higher numbers of bacteria, permitting extreme inflammation during conditions of high bacterial load.

In PBMC-derived macrophages, a greater amount of IL-10 was induced by CRP-opsonised meningococci in 4 out of 5 donors as compared to levels induced by non-opsonised meningococci, but these differences were small and
not statistically significant. By extending this study to a larger number of donors, the immunomodulatory effect of CRP on IL-10 in response to meningococci would be further elucidated. CRP may, therefore, have a protective role in reducing the damage caused by the extreme amplification of pro-inflammatory cytokines induced by meningococci.

Other soluble pattern recognition receptors are known to have modulatory effects on the inflammatory response to meningococci. MBL, for example, has been shown to enhance IL-10 production in response to *N. meningitidis*, limiting damage to the host, although it also increases production of the pro-inflammatory cytokine IL-1β (Sprong *et al.*, 2004). Inflammation is a complex balance between pathogen destruction and host damage, especially in the case of meningococcal disease. Even small changes in levels of individual cytokines may have a significant impact on the overall cytokine profile and subsequent inflammatory response. The results from these studies imply that CRP, by virtue of increasing the IL-10 response to meningococci, could have a role in limiting inflammatory damage to the host.

### 6.3.2.5 The effect of free CRP on macrophage cytokine secretion

The studies detailed in this chapter demonstrated that free CRP at a final concentration of 5μg/ml or 10μg/ml did not significantly increase the secretion of any of the cytokines tested. Reports by other groups using azide-free, LPS-free, human CRP have found that free CRP can cause the secretion of relatively low amounts of TNFα, IL-6 and IL-1β in macrophages. These studies, however, used different experimental designs (eg. 12.5 and 25 μg/ml CRP for 24 hours or 5μg/ml for 12 hours) and although observed increases were statistically significant, they were very small (hundreds of picograms per 10 million cells) compared to the equivalent of tens of nanograms per 10 million PBMC-derived macrophages treated with positive control in the experiments detailed in this chapter (Singh *et al.*, 2006; Nabata *et al.*, 2007). This disparity between the data published on CRP-stimulated cytokine production could be caused by differences in experimental procedures and further exploration is needed to shed light on this fascinating area.
6.3.3 Further experimentation

During this investigation, the surface activation markers measured consisted of CD11b, CD11c, CD14, ICAM-1, HLA-DR and the FcγRI receptor CD64. CRP is known to bind to both FcγRI and FcγRII on monocytes and macrophages and FcγRII has been identified on the surface of U937- and THP-1- and PBMC-derived macrophages (Fleit and Kobasiuk, 1991; Huang et al., 2000). FcγRII expression in response to CRP-bound meningococci was not investigated in this study because preliminary FACS staining experiments by a final year student supervised by myself and Dr Kikki Bodman-Smith found that less than 1% of THP-1-derived macrophages were positive for FcγRII staining either by IV.3 or FL18.26 antibodies, and this level of expression was not increased by IFNγ activation (data not shown).

It would be very interesting to investigate the effect of CRP-opsonised meningococci on PBMC-derived macrophage FcγRII expression because FcγRI and FcγRII have both been identified as important for macrophage cytokine responses to CRP-bound S. pneumoniae (Mold et al., 2006). In the study by Mold et al., CRP opsonisation increased the levels of TNFα and IL-1β produced by human macrophages in response to S. pneumoniae, and this increase was no longer observed when a mutant protein of CRP that binds PC but cannot bind to Fcy receptors was used instead of native CRP. Blockade by antibodies specific for either FcγRI or FcγRII both reduced the cytokine responses in those assays. Human macrophages express 2 types of FcγRlla allele with different affinities for CRP which may affect the expression response of different donors with different haplotypes. The study by Mold et al. also reported that individuals homozygous for the high affinity CRP receptor FcγRlla R-131 showed a greater cytokine response to CRP-opsonised streptococci than individuals homozygous for the lower affinity H-131 allele. It would, therefore, be appropriate to haplotype donors by PCR and compare the cytokine and FcγRII expression responses to CRP-bound meningococci in donors of different haplotype.

As CRP-opsonisation increased the association of N. meningitidis with macrophages, it follows that macrophages incubated with CRP-opsonised meningococci may contain a greater number of meningococci than macrophages incubated with unopsonised meningococci. The effect on macrophage activation marker or cytokine secretion could, at least in part, be due to the increased bacterial load of the macrophage rather than the properties of the bound CRP.
itself. This could be verified experimentally by normalising the bacterial load. As it was demonstrated that increased association of macrophages with CRP-bound meningococci could be reduced by blocking Fcγ receptors with human IgG, it would also be interesting to block Fcγ receptors on the surface of the macrophage before infection and to investigate the effect of reduced bacterial load on activation marker expression and cytokine production.

Although it was demonstrated here that CRP-opsonisation did not affect cytokine responses to meningococcal challenge of macrophages, pre-treatment of both macrophages and meningococci with CRP may provide a different result. Other research has shown that pre-treatment of PBMC-derived macrophages with 25μg/ml CRP reduces the macrophage IL-10 response to LPS as well as increasing the production of inflammatory cytokines IL-6 and TNFα (Singh et al., 2006). This is the reverse to the experiments with CRP-opsonised meningococci presented here. Further work is required to determine if pre-treatment of macrophages with CRP as well as opsonisation with meningococci affects macrophage inflammatory cytokine secretion in response to meningococci.

Surface markers and inflammatory cytokines were quantified by flow-cytometry based methods in order to measure the amounts of protein produced by the cells. Although transcription of cytokine and receptor messenger RNA can be measured sensitively by quantitative-reverse-transcriptase-PCR, this does not necessarily correlate with protein expression. Transcriptional studies of surface markers and cytokines would, however, be of interest and would complement the data presented in this study.

Although the focus of this chapter was on surface marker expression and extracellular cytokine production, this is only a small component of the possible effects on macrophage activation. Cross-linking of Fcγ receptors in response to bacterial uptake is known to activate intracellular signalling cascades that can result in the production of anti-bacterial reactive oxygen and nitrogen species. Investigations into the effect of CRP-opsonised meningococci on macrophage respiratory burst activity would, therefore, be of great interest. Other important activation signals such as macrophage chemokines (which potently attract leucocytes to the site of inflammation) including macrophage inflammatory proteins (MIP) and macrophage chemoattractant proteins (MCP) and other adhesion molecules, such as macrophage adhesion molecule (MAM) should
also be measured in further studies. Although beyond the scope of this project, it would also be of interest to investigate whether CRP-opsonisation of meningococci affects antigen presentation and T cell activation. Co-culture experiments of naïve T cells with macrophages pre-exposed to non-opsonised and CRP-opsonised meningococci could reveal if CRP is capable of potentiating meningococcal antigen presentation by macrophages.

6.3.4 Conclusions

In conclusion, the data presented in this chapter support the theory that *N. meningitidis* can activate macrophages and initiate a proinflammatory response by virtue of its pathogen-associated molecular patterns (as macrophages were activated in the absence of immunoglobulin or complement). Meningococci induced the expression of macrophage adhesion molecules and decreased the expression of FcγRI and CD11b (at a high MOI) as well as increasing production of the proinflammatory cytokines TNFα, IL-6 and IL-β and moderately increasing secretion of the anti-inflammatory IL-10. These findings were consistent with observations in the literature surrounding the inflammatory response to the meningococcus.

Although CRP-opsonisation had little effect on macrophage surface marker and cytokine production in response to meningococci at the high MOI of 200:1, at a lower MOI of 10:1, a specific phenotype for macrophages incubated with CRP-opsonised meningococci was described. This evidence suggests that CRP may be capable of increasing macrophage HLA-DR expression, which would increase the presentation of antigens and accelerate the specific immune response. The evidence also suggests that CRP-opsonisation may increase IL-10 and IL-1β production in response to meningococci, although the limited numbers of human donors used in these experiments have not yielded statistically significant results for these parameters. Further investigation will confirm whether or not CRP-opsonisation can increase antigen presentation and limit the "cytokine storm" of the inflammatory response to meningococcal infection by dampening down some proinflammatory cytokines and promoting a mild increase in anti-inflammatory cytokines, making CRP an immunomodulator of meningococcal disease.
CHAPTER SEVEN

FINAL DISCUSSION AND CONCLUSIONS
7.1 General discussion

Meningococcal disease remains a serious threat to the world’s population, with a safe, effective and broad-acting serogroup B vaccine still awaiting production. Despite ample research to identify meningococcal antigens as vaccine candidates, many mysteries still remain about the pathogenesis of meningococcal disease which could hold important information on how to better direct vaccines and treatments. Both specific and innate immunity are important in the response to meningococcal infection, with many innate defences provided by pattern recognition molecules such as CRP. The aims of this thesis were to define and characterise the binding of CRP to \( N. meningitidis \) and to uncover some of the immunological consequences of this binding in terms of cellular and non-cellular innate immune defences. Armed with this information, the ultimate aim was to determine whether CRP binding to \( N. meningitidis \) is of net benefit to the pathogen or to the host. This chapter summarises the progress made in characterising the binding between CRP and \( N. meningitidis \) and in uncovering the effects of this binding on serum killing, phagocyte uptake and macrophage activation by \( N. meningitidis \). In light of these novel findings, future lines of investigation are discussed, followed by a new hypothesis on the role of CRP in innate immune responses to \( N. meningitidis \).

7.1.1 Characterisation and consequences of CRP binding to \( N. meningitidis \)

These investigations have revealed that CRP binds to piliated \( N. meningitidis \) and that this binding is of low-affinity, calcium-dependent and specific for PC. The binding was characterised using paraformaldehyde-fixed meningococci and was also shown to occur with live meningococci. As predicted, CRP binding to the fixed organism resulted in a significantly increased association of the meningococcus with macrophages and neutrophils. In preliminary investigations, CRP-opsonisation also increased the association of meningococci with dendritic cells. Fc\( \gamma \) receptors were implicated in the increased association of bacteria with macrophages, because blocking of Fc\( \gamma \) receptors abrogated the CRP-mediated association with the meningococci. In contrast to this, CRP opsonisation of live meningococci did not increase association of meningococci with macrophages and preliminary studies indicated that there was no effect on killing of the bacteria within the macrophage. This suggests that
CRP binding of *N. meningitidis* may not directly facilitate an increased clearance of live meningococci by phagocytes during an infection, but that does not rule out other methods of increasing immune protection such as activation of the macrophages' inflammatory and antigen presentation responses.

CRP-opsonisation had no effect on complement-mediated killing of *N. meningitidis* either in the presence or absence of IgG. These results were a little surprising given that commensal species of *Neisseria*, which express PC on their LPS are known to exhibit increased serum killing in the presence of CRP, with a direct correlation between increased PC expression and increased CRP-mediated serum killing (Serino and Virji, 2002). The fact that PC on the pilus of the meningococcus does not facilitate CRP-mediated serum killing may be a useful survival strategy for the pathogen. PC is obviously an important molecule for bacteria inhabiting the human respiratory tract (as it is present on the surface of many species that colonise the area) but PC is a target for both bactericidal antibodies and CRP. In fact, the immunogenicity of this moiety has led to the suggestion of incorporating PC in potential broad-spectrum vaccines against PC-expressing respiratory pathogens such as *N. meningitidis*, *H. influenzae* and *S. pneumoniae* (Bay et al., 2004). The fact that *N. meningitidis* is not subject to the increased serum-killing by CRP that commensals experience is yet another important difference between closely related species of different pathogenicity. It is tempting to speculate that the location of PC on the pilus, remote from the bacterial cell wall and the low avidity of the binding interaction are the causal factors which prevent CRP-mediated complement fixation resulting in MAC formation and bacterial lysis of *N. meningitidis*. It could, however, transpire that CRP bound to PC on the pilus can recruit the first components of the complement cascade, but that activation is not sufficient for full MAC formation (as binding of individual complement components was not examined in the serum killing studies).

The investigation into the activation of macrophages incubated with CRP-opsonised *N. meningitidis* at a MOI of 10 bacteria per macrophage indicated that CRP-opsonisation may modulate the response of macrophages to the meningococcus. In particular, HLA-DR expression could increase with CRP-opsonisation of *N. meningitidis*, which if confirmed, would mean that CRP can increase meningococcal antigen presentation by macrophages. The implications of this theory are that CRP could potentiate the specific immune response to
meningococcal antigens by increasing presentation of antigen to T cells which could, in turn, stimulate the production of bactericidal antibodies from specific B cells, leading to a more rapid adaptive response.

Macrophage studies also indicated a possible CRP-specific phenotype for cytokine production in response to meningococci. IL-10 secretion in response to meningococci by macrophages from the majority of donors was increased by CRP-opsonisation of the bacteria (not statistically significant). Meningococcal sepsis is characterised by high levels of inflammation which can lead to vascular coagulation and organ damage. If the pattern seen for PBMC-derived macrophages in these assays were to continue in a larger population study, it would indicate that CRP can modulate the inflammatory response to meningococci by increasing macrophage production of this predominantly anti-inflammatory cytokine. This suggests that CRP is working to limit damage to the host, caused by the "cytokine storm" as IL-10 works as a negative feedback stimulus, targeting local cells to reduce their production of pro-inflammatory cytokines such as TNFα, IL-1, IL-6 and IL-8.

In contrast to this possible anti-inflammatory effect, CRP-opsonisation also caused a trend towards increase in IL-1β production by macrophages in response to meningococci (not statistically significant). A larger population study may overcome the issues of inter-donor variation to confirm the correlation between CRP-opsonisation and increased IL-1β production. Such a pattern would mean that CRP binding to the meningococcus may also direct inflammation by increasing IL-1β, a cytokine known to augment the acute phase response by recruiting neutrophils and monocytes to the site of infection and to increase production of CRP by the liver (Zhang et al., 1995). A CRP-mediated increase in IL-1β production by macrophages in response to meningococci would be concurrent with findings for the gram positive bacteria S. pneumoniae, which causes greater macrophage IL-1β production when opsonised by CRP (Mold and Du Clos, 2006). Further investigation is needed to confirm the net effect of CRP-opsonisation on the inflammatory cytokine response of macrophages to meningococci.

Preliminary studies indicated that CRP-opsonisation can increase association of meningococci with both neutrophils and dendritic cells. Neutrophils play pivotal roles in fighting blood infections such as meningococcal septicaemia because they are the most numerous leucocytes of the blood and
are unparalleled in their killing ability for phagocytosed pathogens. The study did not include an investigation into neutrophil activation or intracellular killing, but the CRP-mediated increased association of meningococci with these cells suggests that increased bactericidal killing may occur, dependent on the ability of the meningococcus to survive within the neutrophil. Despite recent work highlighting the importance of the meningococcal capsule in promoting intracellular survival of *N. meningitidis* within macrophages and non-phagocytic cells (Spinosa *et al.*, 2007), there is little literature reporting survival of *N. meningitidis* within neutrophils. It has been observed, however, that some strains are better at evading neutrophil killing than others (Estabrook *et al.*, 1992). There is also much evidence that the closely related species *N. gonorrhoeae* can survive, and even replicate within neutrophils (Casey *et al.*, 1979; Parsons *et al.*, 1981; Casey *et al.*, 1986; Simons *et al.*, 2005), thus the effect of CRP-opsonisation on intracellular killing of *N. meningitidis* deserves further study.

Dendritic cells exhibited a moderate increase in association with paraformaldehyde-fixed meningococci when opsonised by CRP in preliminary experiments. Dendritic cells have received much attention in meningococcal research because of their important role in orchestrating specific immune responses to meningococcal antigens, much needed in the development of efficacious vaccines against serogroup B disease. Human CRP has recently been shown to increase uptake of opsonised *S. pneumoniae* by murine dendritic cells. Furthermore it increased antigen presentation and subsequent memory IgG responses to streptococcal antigens in recipient mice, which were then protected from subsequent challenge with virulent streptococci (Thomas-Rudolph *et al.*, 2007). It is possible that CRP-mediated uptake of *N. meningitidis* by dendritic cells could afford similar increases in meningococcal antigen presentation. This would, in turn, mean that CRP could increase antibody responses to meningococcal antigens and would, therefore, be considered highly useful in protecting the host from meningococcal infection.
7.1.2 Further avenues of investigation

These studies into the effects of CRP binding to *N. meningitidis* on immune responses to meningococcal infection have led to many potential avenues of further investigation. Several ideas have been discussed at the end of chapters 3 to 6 but additional lines of investigation will now be explored.

Firstly, the majority of assays in these investigations utilised paraformaldehyde-fixed meningococci to overcome the variation of working with live cultures and to allow techniques to be employed outside of containment level 2* facilities. Although it was confirmed that CRP was able to bind to live meningococci as well as fixed cells, the affinity of the interaction with live organisms has not been characterised and preliminary experiments indicated that the CRP-mediated increase in association of fixed meningococci with macrophages was not repeated by live meningococci. It would be appropriate to determine the effects of CRP-opsonisation on macrophage activation by live bacteria, uncovering useful information on how macrophages respond differentially to live and dead meningococcal cells. Studies into the effects of CRP-opsonised fixed meningococci are still relevant to infection because despite being inert, dead meningococci can activate inflammation by LPS and other surface structures. Perhaps even more importantly, these surface structures may be useful targets for antibody responses to live meningococci.

Marked differences were observed in macrophage responses to CRP-opsonised *N. meningitidis* at an MOI of 200 bacteria per macrophage when compared to a lower MOI of 10 bacteria per macrophage, both in terms of the activation markers expressed and cytokines secreted. It was not possible to determine from these experiments whether these differences were due to the higher CRP concentration or the lower number of bacteria used in the lower MOI experiments. If more time and resources were available, the assays would have been repeated changing only one variable at a time. Experiments with the closely related *N. gonorrhoeae* have shown that changing the dose of bacteria from 10 bacteria per macrophage to 0.1 bacteria per macrophage diminishes TNFα production whilst maintaining IL-8 production by monocytes, thus MOI can have a considerable impact on leucocyte responses to pathogenic *Neisseria* (Patrone *et al.*, 2006). It would be interesting to examine activation marker and cytokine secretion by macrophages incubated with non-opsonised and CRP-opsonised meningococci at a range of lower multiplicities of infection to
investigate whether an even lower MOI could stimulate a greater contrast in the macrophage response to meningococci opsonised with a range of concentrations of CRP.

Fcγ receptors were implicated in the CRP-mediated increased association of meningococci with phagocytes, but the relative roles of each receptor type have not yet been investigated. FcγRI and FcγRII are both known to mediate uptake of bacteria into phagocytes and could be separately inactivated by antibody blockade or specific small interfering RNA (siRNA) inactivation of receptor genes to study the relative importance in each receptor type. An alternative strategy would be to transfect Fcγ receptors into cell lines that do not normally express these receptors, such as COS cells which were used in previous studies to investigate the phagocytic uptake of CRP-opsonised erythrocytes (Bodman-Smith et al., 2004). These methods could be employed in order to discover which receptor or receptors are crucial for the CRP-mediated uptake of meningococci.

A better understanding of the involvement of Fcγ receptors in the uptake of CRP-bound meningococci may also lead to related studies examining the effect of CRP-opsonisation of meningococci on the invasion of Fcγ-expressing non-phagocytic cells, such as airway epithelial cells (Salik et al., 1999). Epithelial cells of the mucosa are sites of pathogen invasion and are also considered to have a function in antigen sampling to stimulate local T cells (Oei et al., 2004). CRP is known to be present in the respiratory epithelium and secreted by epithelial cells (Gould and Weiser, 2001). It would, therefore, be highly relevant to discover if CRP-opsonisation increased uptake of meningococci into epithelial cells in vitro. Conversely, CRP-opsonisation may result in decreased uptake of meningococci by epithelial cells due to masking of other important ligands for initial attachment, such as the pilus tip adhesin PilC which is thought to bind to CD46 on host cells (Kallstrom et al., 1997). It is also possible that N. meningitidis may utilise the PC present on its pilus for attachment, as has been reported for other PC-expressing pathogens of the respiratory tract. PC on the surface of S. pneumoniae and H. influenzae, for example is a known ligand for the receptor for platelet activating factor rPAF on the surface of epithelial cells (Cundell et al., 1995; Swords et al., 2000). Attachment of these two species to respiratory epithelial cells is inhibited by CRP by masking the PC epitopes on the surface of the organisms (Gould and Weiser, 2002) and this could be relevant to
N. meningitidis attachment. Interestingly, the study by Gould and Weiser reported that CRP was unable to diminish attachment of either species to epithelial cells in the presence of lung surfactant, which is common in the terminal airways. This suggests that CRP may not protect against pathogen attachment in this area of the respiratory tract, affording an advantage for invasion at this site.

CRP opsonisation increased association of meningococci with neutrophils, and various functions of neutrophils that could be examined with respect to activation by CRP-opsonised meningococci were discussed in chapter 5. In addition to increasing bactericidal activity, neutrophil activation contributes to the vascular damage and multi-organ failure associated with severe meningococcal sepsis. CRP has been indicated as an immunomodulator of inflammatory activation of neutrophils, with high acute phase levels of CRP tending to down regulate neutrophil activation and limit damage caused by neutrophils during infection (Mortensen and Zhong, 2000). It would be appropriate to examine the role of CRP in modulating granulocyte activation in response to meningococci. This would involve measuring neutrophil respiratory burst, inflammatory cytokine secretion and surface marker expression in response to CRP-opsonised meningococci.

When considering the role of CRP in immune responses in the blood, it would also be relevant to examine the effects of CRP-opsonised meningococci on phagocytosis by blood monocytes (which may also phagocytose CRP-opsonised bacteria via FcγR). With further time and resources available, studies of the interaction of CRP-opsonised N. meningitidis with monocytes would have been undertaken to measure effects on phagocytic uptake, intracellular killing, respiratory burst activity, surface marker changes and inflammatory cytokine secretion which may all affect pathogen survival and host damage.

The effect of CRP-opsonised meningococci on dendritic cell activation is of great interest because of the role of dendritic cells in initiating and directing specific immune responses to bacteria. CRP has recently been shown to increase dendritic cell activation and antigen presentation when bound to S. pneumoniae, helping to protect mice from streptococcal infections (Thomas-Rudolph et al., 2007). Discovering the role of CRP in dendritic cell responses to N. meningitidis will be conducive to a better understanding of specific immune responses initiated by meningococci. Both live and dead bacteria are present.
during an infection. Consequently, the effect of the viability of the meningococci should be considered throughout investigations into the effect of CRP-opsonisation on dendritic cell surface marker expression, cytokine secretion and antigen presentation in response to meningococci. It has recently been shown that dendritic cells respond differently to live and killed meningococci (Jones et al., 2007). In the study by Jones et al., live meningococci failed to initiate the maturation response stimulated by fixed meningococci (namely the up-regulation of CD40, CD86, HLA-DR and MHC class 1 molecules). Live meningococci stimulated IL-12 and IL-10 production by dendritic cells but showed much lower association with macrophages than fixed bacteria. Thus it will be important to examine the response of dendritic cells to CRP-opsonised meningococci that are alive as well as fixed to look for differences in dendritic cell response.

When considering the central role of dendritic cells in directing specific immune responses against pathogens, it is important to consider the co-evolution of host and pathogen to discover if host defences are being hijacked for pathogen virulence. When examining the effect of CRP-opsonisation of meningococci on dendritic cell function, it will be particularly important to look at intracellular killing since any meningococci surviving inside dendritic cells could be trafficked through the blood and lymph systems and cause further damage to the host. Thus in future experiments, intracellular killing and dendritic cell apoptosis should also be examined.

7.2 Proposed model for the role of CRP in the innate immune response to *N. meningitidis*

By combining the results of the assays performed in this investigation with published data on the effects of CRP binding on immune responses to other bacteria, a model for the potential roles of CRP in the innate immune response to *N. meningitidis* is now proposed. The model is applied to two important areas of meningococcal infection; invasion at the respiratory epithelium and survival in blood.

Figure 7.1 depicts the proposed role of CRP in immune defence from meningococci at the respiratory epithelium. PC is clearly a crucial moiety for respiratory tract colonisation as it is abundant in commensal and pathogenic bacteria resident in the nasopharynx, but the marker is highly immunogenic and is targeted by anti-PC antibodies of both IgG and salivary IgA. Additionally, PC
displayed on these organisms can be bound by CRP present at the respiratory epithelial surface either secreted endogenously by epithelial cells, or transported from the blood stream through a more permeable epithelial layer during inflammation.

Figure 7.1 Proposed model of CRP interactions with *N. meningitidis* at the mucosal surface

CRP bound to PC on the pilus of *N. meningitidis* at the mucosal surface may not amplify complement-mediated lysis of pathogenic *Neisseria* (a) because of the remoteness of PC from the bacterial membrane. CRP can, however, increase phagocytic uptake by macrophages (b) and dendritic cells (c) possibly allowing greater phagocytic killing and/or presentation of antigens to T cells (f). Conversely, CRP may be utilised by the meningococcus to accelerate invasion of epithelial cells expressing FcyR (d). It is postulated, however, that this could be counteracted by inhibition of pilus mediated attachment to epithelial cells (e). CRP-mediated uptake of *N. meningitidis* may result in modulation of proinflammatory cytokine production (f) and increased antigen presentation (g).
Complement proteins are known to be exuded from plasma into the respiratory tract and are a valuable defence against invading pathogens which can activate the classical, alternative and lectin complement pathways (Persson et al., 1998). CRP amplifies complement-mediated killing of other bacterial species, but our experiments revealed no effect of CRP on complement-mediated killing, possibly due to the remoteness of the CRP binding site from the cell wall of the meningococcus. CRP is, therefore, unlikely to increase complement-mediated killing at the mucosal surface (figure 7.1, a). In contrast, CRP opsonisation of *N. meningitidis* could increase FcγR-mediated uptake of bacteria into alveolar macrophages (b) and dendritic cells (c) which are resident sentinels in the respiratory epithelium and lumen. This CRP-mediated uptake of meningococci by phagocytes is clearly of benefit to the host. CRP opsonisation could, however, be detrimental to the host if it increased FcγR-mediated uptake of meningococci by respiratory epithelial cells (d). The effect of CRP-opsonisation on uptake by non-phagocytic cells remains to be studied but may be influenced by a reduction in CD46-mediated attachment of meningococcal pilus to the cells (e). CRP-mediated uptake of meningococci by phagocytes protecting the respiratory epithelium could influence downstream responses of these cells. These investigations indicate that this could include increased antigen presentation by HLA-DR up-regulation (g) which would accelerate the specific immune response against meningococcal antigens. Further to this, the CRP-mediated uptake could also modulate the inflammatory response by influencing the inflammatory cytokines secreted by resident macrophages (f). CRP-opsonisation may increase the production of the pro-inflammatory IL-1β but also the anti-inflammatory IL-10 in response to the meningococcus, potentially resulting in a net reduction in inflammatory signals which would reduce damage at the site of infection. In conclusion; this model predicts that CRP is protective against invasion of meningococci at the respiratory epithelium where it can result in increased phagocytosis of *N. meningitidis* by professional phagocytes. Despite the lack of evidence to suggest that this increases intracellular killing, we propose that the increased antigen presentation could be protective by accelerating specific immune responses to clear the meningococcus with IgA, and IgG–dependent complement-mediated killing and cytotoxic T cell mediated killing as well as limit local tissue damage by increasing IL-10 production.
Figure 7.2 applies the model to the bloodstream where very high levels of CRP are achieved during the acute phase response and CRP-opsonised meningococci will encounter blood monocytes and neutrophils. The data from this thesis suggests that CRP bound to PC on the pilus may not be sufficient to increase cell lysis by activation of the classical complement cascade (a), but uptake could be increased into neutrophils via FcγR recruitment (b). As monocytes also express FcγR, it is possible that CRP may increase uptake into these leucocytes and may additionally affect intracellular killing mechanisms (c). Depending on the cytokines and cell surface markers induced, this may increase or decrease inflammation and the consequent sepsis of meningococcæmia (d). Clearly, this model is more speculative than the mucosal model, as limited data was collected on the effect of CRP-opsonisation on neutrophil responses to meningococci and monocytes were not included in the analysis. The model is useful, however, in highlighting the importance of further investigation which will uncover crucial information about the modulatory capacity of CRP in the innate immune response to *N. meningitidis* in the blood.

Figure 7.2 Proposed model of CRP interactions with *N. meningitidis* in the bloodstream. Serum CRP bound to PC on the pilus may not amplify complement-mediated lysis of *N. meningitidis* (a) because of the remoteness of PC from the bacterial membrane. CRP may, however, increase phagocytosis of opsonised meningococci by neutrophils (b) and possibly monocytes (c) and may influence the activation markers and production of inflammatory cytokines (d) to direct the inflammatory response.
In conclusion, these investigations have revealed a novel role for CRP in the protective immune response to *N. meningitidis*. CRP can bind to piliated, PC-expressing meningococci in a calcium-dependent manner and this binding facilitates increased association of (fixed) organisms with several professional phagocytes. This increased association results in specific changes to the activation of macrophages, suggesting that CRP may be an important innate protection against meningococcal infection alongside the more well known soluble pattern recognition receptor mannose-binding Lectin. It is possible that CRP may also cooperate with other plasma lectins *in vivo*, as has been seen in the horseshoe crab (Ng *et al.*, 2007), making its role even more important in defence against bacterial invasion. The *in vitro* studies undertaken here have uncovered many interesting actions of CRP in the immune response to the meningococcus, several of which may help the host fight meningococcal infection by increasing clearance of bacteria, increasing antigen presentation and limiting inflammatory damage to host cells. It is clear from the studies undertaken that CRP has many protective effects for the host and further experiments to explore the effect of CRP-opsonisation on leucocyte recognition and killing of meningococci may confirm that CRP binding to *N. meningitidis* is of net benefit to the host during a meningococcal infection.
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