SpyCEP interferes with the neutrophil response against Group A Streptococcus

Richard Ashley Lawrenson

Imperial College London

Department of Infectious Diseases and Immunity

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Abstract

This study examined how SpyCEP impaired the neutrophil response to Group A Streptococcus (GAS). It was known prior to this study that SpyCEP cleaved some of the neutrophil specific chemokines (Kurupati, *et al.* 2010, Zinkernagel, *et al.* 2008). These chemokines are known to have a variety of effects on neutrophil function, and the impact of SpyCEP on two specific effects, neutrophil homing to infection foci and NETosis, has been reported (Edwards, *et al.* 2005, Zinkernagel, *et al.* 2008).

This study identified that the substrate range of SpyCEP included all known neutrophil specific chemokines. No other substrates were found. Furthermore, the relative cleavage rate of these chemokines by SpyCEP was determined, with the most potent, CXCL8, being the optimum substrate (Mukaida, *et al.* 1998). It was hypothesised that other effects of chemokines on neutrophils, not just neutrophil homing and NETosis, would also be ablated.

Neutrophil release from the bone marrow stores is potentiated by chemokines. It was found that neutrophil release from the bone marrow was lower when mice were infected with SpyCEP-positive compared to SpyCEP-negative bacteria. Furthermore, lower levels of circulating chemokines were also found in mice infected with SpyCEP-positive GAS.

Chemokines also play a role in the activation of neutrophils, and the upregulation of receptors involved in this process (Detmers, *et al.* 1990). This study identified that neutrophil phagocytosis of SpyCEP-positive bacteria was impaired compared to SpyCEP-negative bacteria. The neutrophils incubated with SpyCEP-positive bacteria were found to have less chemokine at their surface, and lower surface expression of a phagocytosis receptor, CR3. This receptor is known to be important for GAS phagocytosis (DeMaster, *et al.* 2002) providing a mechanism for this effect.

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This work has found several novel effects of SpyCEP on neutrophil function. These impairments take place throughout the neutrophil response to GAS infection.

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Abbreviations

BSA	Bovine serum albumin
CBA	Columbia blood agar
CFU	Colony forming unit
CR1	Complement receptor 1
CR3	Complement receptor 3
CXCR1/2/4	CXC chemokine receptor 1/2/4
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbant assay
F	Forward primer
FCS	Foetal calf serum
FITC	Fluorescein Isothiocyanate
FHL1	Factor H like-1
GAS	Group A streptococcus
G-CSF	Granulocyte colony stimulating factor
HBSS	Hanks buffered saline solution
HLA	Human leukocyte antigen
HRP	Horseradish peroxidise
IC50	Concentration required to inhibit 50% of receptor binding by an agonist
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IVIg	Intravenous immunoglobulin
LB	Luria bertani
LDS-PAGE	Lithium dodecyl sulphate polyacrylamide gel electrophoresis
LFP	Low-Fluorescent hydrophobic Polyvinylidene difluoride (PVDF)
LPS	Lipopolysaccharide
NET	Neutrophil extracellular trap
NF	Necrotizing fasciitis
NK	Natural killer
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PANDAS	Paediatric autoimmune neuropsychiatric disorders associated with streptococcal infections
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	R-phycoeythrin fluorescent protein
PRR	Pattern recognition receptor

R	Reverse primer
RPMI	Roswell Park Memorial Institute
SD	Standard deviation
Sda1	Streptococcal DNAse 1
SIC	Streptococcal inhibitor of complement
SLO	Streptolysin O
SLS	Streptolysin S
SpyCEP	*Streptococcus pyogenes* cell envelope proteinase
TH	Todd Hewitt
THY	Todd Hewitt + 1% yeast extract
TNFα	Tumour necrosis factor α
TSS	Toxic shock syndrome
UV	Ultraviolet

Chapter 1 - Introduction

1.1 Group A Streptococcus biology

Group A Streptococcus (GAS) is a Gram positive human pathogen that is capable of causing a broad spectrum of disease, ranging from mild, self-limiting non-invasive conditions to severe and potentially life-threatening invasive disease. Non-invasive diseases caused by GAS are very common, yet rarely quantified as they are typically unreported. Invasive disease, whilst uncommon, can often have a high mortality rate and be difficult to treat. Furthermore GAS is also able to cause auto-immune sequelae, linked to a range of conditions (Cunningham 2000).

1.1.1 Non-invasive GAS disease and burden

The most common disease associated with GAS is tonsillo-pharyngitis. This is especially frequent amongst the young, in whom GAS colonises the throat and tonsils of the affected individual, resulting in a productive supprative infection. Disease rate follows seasonal fluctuations, being most prevalent in autumn and winter months (Cunningham 2000). Tonsillo-pharyngitis, whilst mild, causes considerable disruption, especially to schooling. Such disease receives little epidemiological attention, however one such study examining an economically developed population revealed a disease rate of 0.14 cases per child per year, with 71% of such cases receiving antibiotic treatment, and 46% missing some time from

school, with an average of 14 hours per child (Danchin*, et al.* 2004). Whilst being more common in children, GAS still causes considerable numbers of tonsillo-pharyngitis cases amongst adults, and is thought to be responsible for between 4 and 10% of adult sore throats (Bisno 1996, Komaroff*, et al.* 1986). Cases of tonsillo-pharyngitis may be associated with scarlet fever, a complication of GAS infection most typically linked to tonsillo-pharyngitis and most typically seen in children. This is characterised by a rash, strawberry tongue and desquamation of the skin. Scarlet fever is caused by superantigen expression by these non-invasive strains (Bohach*, et al.* 1990).

GAS is also responsible for pyoderma and impetigo infections, especially in less economically developed nations. These diseases involve an infection limited in depth to the epidermis, which, once established, is highly contagious (Bisno and Stevens 1996). Prevalence of such disease is highly variable, but seems to typically range between 1 and 20% in less economically developed countries, and is most common in summer months (Carapetis*, et al.* 2005).

Although these non-invasive diseases cause a disease burden directly, the major burden of non-invasive disease results from post-infection autoimmune sequelae, such as rheumatic fever. As such, the prevalence of rheumatic fever is higher in regions where non-invasive disease is more prevalent, such as in the Pacific Islands (Carapetis*, et al.* 2005).

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1.1.2 Autoimmune sequelae

1.1.2.1 Rheumatic Fever

Rheumatic fever is characterised by fever, inflammation in the joints, heart, central nervous system and skin (Cunningham 2000). Rheumatic fever is the leading cause of juvenile heart disease world-wide, and is especially prevalent in developing countries where it has an incidence of over 0.8 cases per 100 children (Guilherme and Kalil 2010). Indeed, this type of GAS disease is the leading cause of mortality of all GAS-associated conditions worldwide, and is responsible for approximately 250,000 deaths annually (Carapetis*, et al.* 2005).

Rheumatic fever is classically associated with GAS pharyngitis, and as such its prevalence is seasonal, following the trends of pharyngitis (Cunningham 2000). It is thought that such infections occasionally trigger an autoimmune attack due to cross-reacting antibodies, raised against GAS antigens such as surface M protein. Although the mechanisms which trigger autoimmunity are still unclear, it is hypothesised that these antibodies then damage endothelial and epithelial surfaces, exposing immunogenic material below, allowing a heightened immune response to proceed (Cunningham 2003). It is also thought that a variety of host factors are involved in determining susceptibility to disease, such as HLA type, but this too is poorly understood (Guilherme and Kalil 2010).

The prevalence of rheumatic fever has declined in wealthier countries due to a reduction in prevalence of pharyngitis among children and the introduction of antibiotic treatment regimes in cases of GAS pharyngitis (Bisno*, et al.* 2002). Such treatments are not used worldwide, which contributes to the continuing high incidence in developing countries.

Furthermore, with a recurrence rate of over 40%, and the cumulative nature of the damage caused by such attacks leading to valvular heart disease, a more effective method of tackling rheumatic fever worldwide is clearly required (Seckeler*, et al.* 2010). Indeed a broad spectrum vaccine against the numerous serotypes of GAS, using either conserved epitopes or a pan-serotype approach is a focus of GAS research.

1.1.2.2 Acute Post-streptococcal Glomerulonephritis

Acute post-streptococcal glomerulonephritis is primarily a disease of children and young adults, and is at its most prevalent in summer months, mirroring the epidemiology of pyoderma-type GAS infections (Bisno*, et al.* 1970). Not all serotypes of GAS are equally capable of causing acute post-streptococcal glomerulonephritis, and the prevalence of this disease has been in steady decline (Carapetis*, et al.* 2005).

As with rheumatic heart disease, the aetiology of this post-infection sequela of GAS disease is unclear. Hypotheses include the deposition of immune complexes of antibody and GAS antigen in the kidney and the generation of cross-reactive antibodies following the GAS infection (Cunningham 2000). Regardless of the cause, the disease causes extensive morbidity and mortality. Characteristic symptoms of such disease include oedema, hypertension, haematuria, and other complications associated with reduced kidney function.

1.1.2.3 Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections

An increasing body of research is accumulating linking GAS infections to compulsive tic-like disorders, categorised together as Paediatric Autoimmune Neuropsychiatric Disorders Associated with Streptococcal Infections (PANDAS). The evidence linking these disorders to GAS is variable, and the most certain correlation is found in Sydenham's Chorea (Shulman 2009), characterised by severe involuntary movements and emotional disturbances (Swedo*, et al.* 1993). More controversially, it has been suggested that obsessive compulsive disorder and Tourette's syndrome in children are also related to GAS infection, by virtue of cross-reactive antibodies against streptococcal antigens and components of the central nervous system (Swedo*, et al.* 1997). Whilst work has confirmed that some patients suffering acute attacks of such disease do indeed have circulating antibodies against surface components of GAS (Kirvan*, et al.* 2003, Kirvan*, et al.* 2006), any causal link is hard to prove, due to the very high rate of GAS infection amongst children (Kurlan*, et al.* 2008).

1.1.3 Invasive disease

Typical GAS invasive disease results in skin and soft tissue infections as described below. However, GAS may also cause pneumonia, or post-partum sepsis, a serious disease afflicting mothers immediately after childbirth. Bacteraemia, without an obvious focus of infection is frequently observed.

1.1.3.1 Cellulitis and erysipelas

By far the most common form of invasive streptococcal disease, cellulitis is a soft tissue disease, with a diffuse inflammatory response which extends into subcutaneous fat layers, without a clear demarcating line (Lopez and Lartchenko 2006). This disease is not considered as serious as the other listed invasive manifestations of GAS disease, however it is still a significant medical event. An epidemiological study of GAS infection in the UK found that such infections had a mortality rate of 16% within seven days of admission to a hospital setting, and could also act as a precursor for more severe complications of invasive GAS infection such as toxic shock syndrome or kidney damage (Lamagni*, et al.* 2008b). Erysipelas is similar to cellulitis, however features a distinct demarcation defining the infected area, and does not infiltrate so deeply into the tissues. Hospitalisation may not be required in all cases, and indeed many patients may be treated in the community with oral antibiotics (Lopez and Lartchenko 2006).

1.1.3.2 Necrotizing fasciitis

Necrotizing fasciitis (NF) is perhaps the most severe condition caused by GAS, featuring alarming clinical symptoms which are difficult to treat and a high mortality rate of approximately 32%, but is however an uncommon form of streptococcal disease. The disease causes severe pain, fever, and shock (Carapetis*, et al.* 2005) (Lamagni*, et al.* 2008b), primarily affecting adults between 16 and 60 (Lamagni*, et al.* 2008a).

The disease features GAS multiplying to very high bacterial titres and spreading rapidly along fascial planes, causing extensive necrotic destruction of subcutaneous tissue,

and often deeper connective and muscle tissue, as it progresses due to the release of toxins and disruption of normal tissue architecture.

NF typically arises following a trauma to an extremity of the body, however such trauma does not need to break the skin, and the infection may at first be unapparent, hindering initial diagnosis (Stevens 1995). Occasionally, NF may develop as a consequence of localised skin lesions, for example following varicella (chicken pox) or surgery. One must assume that the skin lesion acts as a portal of entry in such cases (Vijaykumar*, et al.* 2003).

1.1.3.3 Streptococcal Toxic Shock Syndrome

Streptococcal toxic shock syndrome (STSS) is a complication which may arise following an invasive infection by GAS. Whilst only being present in 8% of patients suffering from invasive GAS, it is a more common feature of NF, occurring in 40% of patients with this particular condition, and is also positively associated with the prior use of non-steroidal anti-inflammatory drugs (Lamagni*, et al.* 2008b). Different GAS strains produce components which are immunogenic and also express various superantigen toxins. The combined effect of such components is that the immune system becomes highly activated, releasing an arsenal of immune mediators, in turn leading to septic shock. Development of this complication is strongly linked to a fatal outcome, with 45% of patients dying when this complication is present (Lamagni*, et al.* 2008b).

1.1.4 Classification of GAS isolates

GAS isolates are normally typed by examination of a variable surface antigen, M protein, either serologically (M typing) or using DNA sequence analysis of the M protein gene, *emm* (*emm* typing). The most common invasive M-types currently found in Europe comprising just under 75% of all cases are, in descending order, M1, M28, M3, M89, M87, M12, M4, M83, M81 and M5 (Figure 1-1). The distribution of M-types throughout the world not only varies by region, but also area to area, indeed a list of most common M-types from the developing world would differ from this profoundly (Luca-Harari*, et al.* 2009). Different M-types of GAS are loosely associated with different types of disease, with M1 and M3 associated with invasive type disease, and M3 especially associated with TSS (Lamagni*, et al.* 2008b), M28 and M89 being associated with post-partum sepsis, M81 strains being associated with those suffering with pre-existing diabetes, and M12 being associated with pharyngitis (Luca-Harari*, et al.* 2009). As well as disease manifestation, there is also correlation between the expression of certain virulence factors and M-type, such as superantigens, and with M18 strains producing strikingly more protective capsule (Alberti*, et al.* 1998). As well as these correlations, the different M protein types may also represent differing M protein structures (Miller*, et al.* 1988), with differing effects on virulence (Dale*, et al.* 1996).

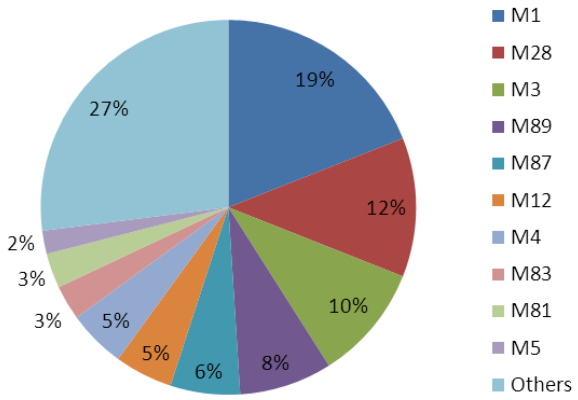


Figure 1-1 – M-type distribution in Europe

Distribution of invasive M-types in Europe, between 2003 and 2004, data taken from Luca-Harari *et al*. 2009.

Another serotypic method of typing GAS is T-typing, based on variable sequences within the bacterial pilus (Falugi*, et al.* 2008). T types correlate well with M-types, but with reduced specificity, as there are fewer T serotypes than M serotypes, and furthermore a particular strain may express several T antigens simultaneously, leading to T patterns rather than distinct types (Johnson and Kaplan 1993). Currently there is a resurgence in invasive GAS infections worldwide, associated with a hypervirulent M1T1 strain (Luca-Harari*, et al.* 2009).

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1.1.5 Treatment of GAS

1.1.5.1 Intervention

The first line treatment used in streptococcal infections is penicillin and its derivatives, to which GAS has remained sensitive. Alarmingly, reports from Canada seem to show the emergence of penicillin resistant strains via an unknown mechanism in patients who have received treatment for repeated episodes of pharyngitis (Ogawa*, et al.* 2011). The use of penicillin-type drugs to treat GAS infections while common is not universal due to allergies against β-lactam-type antibiotics. In such cases, macrolides are used, such as erythromycin. Clindamycin is also used in addition to the β-lactams when treating deep tissue infections caused by GAS (Mulla 2004). Unfortunately, the sensitivity seen using β-lactams is not seen with macrolides, with resistance being noted around the world, and being especially prevalent in some countries (Lynskey*, et al.* 2011). Of particular note is a 95% resistance rate against macrolides reported in China (Chang*, et al.* 2010).

Macrolide resistance is mediated by two distinct mechanisms. The M-phenotype refers to one such method that elicits low level protection against a narrow range of drugs, including erythromycin, but not drugs such as clindamycin, other lincosamides or streptogramin B. Such resistance is mediated by expression of a drug efflux pump (Clancy*, et al.* 1996). GAS may also use a broader specificity, high level protection method, known as the MLS_B. This method depends upon methylase modification of the ribosome target site of these drugs.

The efficacy of pooled human intravenous immunoglobulin (IVIg) has been a matter of debate with initial work in a mouse model of invasive GAS infection showing little

protective effect (Patel, *et al.* 2000). However, more recent work examining human trials and information from the clinic suggest IVIg is a useful treatment for severe GAS infections (Darenberg, *et al.* 2003, Norrby-Teglund, *et al.* 2003). The mode of action for IVIg is thought to include neutralising toxins including superantigens and opsonising bacteria with specific anti M-protein antibodies (Norrby-Teglund, *et al.* 2003).

In cases of NF however, such interventions are often insufficient to clear the infection, and invasive surgical debridement, and some cases, amputation is required to halt the spread of the disease (McHenry, *et al.* 1995). Such interventions add considerably to the morbidity of this particular type of invasive GAS disease.

1.1.5.2 Prevention of GAS disease using a vaccine

Much effort has been expended searching for a vaccine against GAS. The main focus of development has been on the variable surface M-protein, the basis for M typing. However, its variability has hampered such attempts, making the production of a broad spectrum vaccine challenging. Various reports have suggested the protective coverage of a 26-valent vaccine which reached human trials would be as low as 20% in some parts of the world (Richardson, *et al.* 2010). Furthermore, due to fears that antibodies against the M protein are the aetiological agent responsible for rheumatic fever, work examining such a vaccine type is making only slow progress (Lynskey, *et al.* 2011).

The other main tool in serotyping GAS, the pilus or T antigen is also being investigated as a vaccine candidate, as it is known there are fewer T serotypes than M serotypes (Johnson and Kaplan 1993). A 12-valent vaccine has been suggested to have the potential to provide protection against 90% of strains worldwide (Falugi, *et al.* 2008, Mora, *et al.* 2005). Other

vaccine candidates have been suggested, including SpyCEP, which are conserved virulence proteins found on the surface of GAS, and will be discussed in more detail elsewhere (Steer, *et al.* 2009).

1.2 The Immune Response

1.2.1 Barrier defences

The initial defence the host has against infection by GAS are the physical barriers provided by the skin, with its multiple layers of epidermis, and epithelial surfaces which express tight junctions. The surface of skin not only prevents GAS invasion into deeper tissues, its low pH of between 3 and 5 acts to inhibit bacterial growth on its surface. The epithelial layers are protected by mucus which acts to capture microorganisms, which are then swept out of the body by cilia (Chaplin 2010). Such defences are not breached when GAS establishes a non-invasive infection, with GAS instead colonising these surfaces. As these represent the majority of GAS infections, the efficacy of such barriers at preventing GAS invasion is evident (Cunningham 2000).

GAS enters sterile sites of the body through adhering to and then traversing epithelial cells. The initial adhesion and colonisation of these cells involves a wide variety of GAS proteins binding to host proteins fibronectin and collagen (Kreikemeyer, *et al.* 2004). GAS is then able to internalise and persist within these epithelial cells (LaPenta, *et al.* 1994). One hypothesis is that GAS will periodically invade across the epithelial layer, and establish a transient bacteraemia within the patient (Johansson, *et al.* 2010). Such events may be responsible for the less serious GAS invasive diseases cellulitis and erysipelas. Of note, necrotizing fasciitis is epidemiologically associated with blunt trauma to the afflicted area of the body (Kaul, *et al.* 1997, Stevens, *et al.* 1989). A possible explanation for this is that such muscular injuries cause increased cellular expression of vimentin, and this localised increase

may encourage the binding of GAS to these muscular sites, assuming a transient bacteraemia happens to coincide with such a trauma (Bryant, *et al.* 2006).

1.2.2 Antimicrobial peptides

Antimicrobial peptides are small, highly charged peptides, which exert microbicidal activity against bacteria, but also against fungi. Due to their small size, they can penetrate the cell wall of GAS, and interact with its membrane. They have amphiphilic structures, allowing them to interact with membranes and then cause disruption using their charge. They can be subdivided into several groups on the basis of conserved structural features (Schneider, *et al.* 2005). Two of these subgroups, the cathelicidins and the β-defensins have been demonstrated to be important in the containment of GAS infections and the prevention of invasive disease (Morrison, *et al.* 2002, Nizet, *et al.* 2001). GAS evades the activity of antimicrobial peptides in a variety of ways, including preventing their binding, and proteolytic degradation.

GAS also acts to increase the charge of its cell membrane, which so acts to repel these cationic peptides. The mechanism of this activity is to modify the teichoic acid component of the cell wall (Kristian, *et al.* 2005). Furthermore, a broad spectrum cysteine protease produced by GAS, SpeB, is capable of cleaving the potent cathelicidin, LL-37 (Nyberg, *et al.* 2004). A further virulence factor produced by GAS, streptococcal inhibitor of complement (SIC) produced exclusively by M1 strains, can inactivate LL-37 as well as other antimicrobial peptides by binding rather than by cleavage (Fernie-King, *et al.* 2004, Frick, *et al.* 2003). Interestingly, LL-37 has been shown to influence a two-component regulator of GAS, CovR/S, enhancing the expression of virulence factors regulated by this system (Gryllos, *et al.* 2008).

1.2.3 Complement

The complement system consists of a cascade of inactive serine proteases and other proteins. Upon interaction with various danger signals, protease members of this family activate, and subsequently activate further proteases and other proteins further down the cascade. A summary of the pathways is shown below (Figure 1-2)

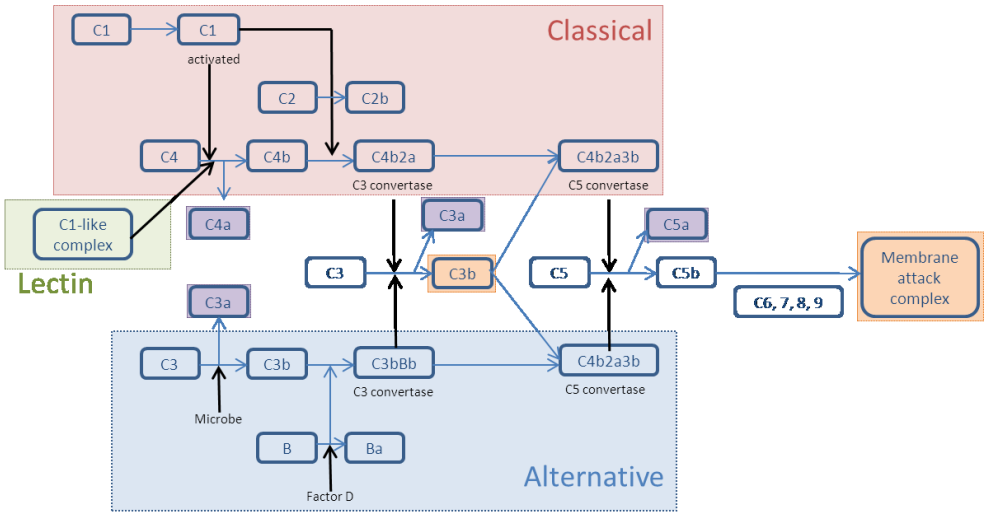


Figure 1-2– Complement pathway

Figure depicting the three complement pathways, classical, alternative and lectin. The main effector components of the complement pathway are highlighted orange. Other important immune regulating proteins are highlighted purple.

Whilst the classical pathway must be specifically activated by antibody, the alternative pathway features C3b being continuously deposited on the surface of both human and foreign cells (Zipfel and Skerka 2009). This potentially unwanted complement activation

is prevented by soluble factors such as Factor H, which detect self and deactivate complement, whilst leaving that deposited on bacteria intact (Atkinson, *et al.* 1991). Regardless of how it is activated and the intermediary steps followed, the complement cascade converges in a common set of end products which have powerful and broad spectrum antibacterial activity (Zipfel and Skerka 2009). C3b binds to the bacterial cell surface, and is a powerful opsonin, being bound by a variety of complement receptors, such as complement receptor 1 (CR1) and complement receptor 3 (CR3), which are expressed on the surface of phagocytes. CR1 is formed from a molecule of CD35, whereas CR3 is a heterodimer of CD11b and CD18; both are expressed strongly by neutrophils (Hynes 1992), and are both upregulated upon infection by the binding of the neutrophil activating chemokine CXCL8 to its receptor on the neutrophil surface (Detmers, *et al.* 1990, Paccaud, *et al.* 1990). The importance of C3b is highlighted the arsenal of virulence factors that GAS brings to bear upon it (1.3.2).

The steps passed through before activating C3 into C3b also have immune system functionality. C3a, C4a and C5a are soluble anaphlatoxins. These are pro-inflammatory molecules, which activate and recruit immune cells to sites of infection (Gasque 2004). C5a is an especially potent chemoattractant for neutrophils, cells which are known to be of vital importance in the clearance of GAS (Peveri, *et al.* 1988, Wexler, *et al.* 1983). It is hence unsurprising that GAS has virulence factors directed against such anaphlatoxins (Laarman, *et al.* 2010, Wexler, *et al.* 1985).

Additional to its function as an opsonin, C3b is also recruited to form C5 convertase, which liberates C5a and C5b. C5b interacts with other proteins of the complement cascade to form the membrane attack complex. The role of the membrane attack complex in controlling GAS is unclear. It is thought not to play a role as the thick cell wall of GAS and other Gram

positive bacteria protect them from its effects. However, GAS does express proteins which disrupt its formation (Fernie-King, *et al.* 2002).

1.2.4 Cellular recognition of pathogens

Phagocytes express pathogen recognition receptors (PRRs) which are capable of binding a broad range of ligands which are expressed by pathogens but not by the host, such as components of the bacterial cell wall, or double stranded viral RNA (Gordon 2002). Upon binding such pathogen associated molecular patterns (PAMPs), signalling cascades trigger a wide range of immune activity. One such class of PRRs are the Toll-like receptors (TLRs), which detect a variety of different PAMPs. Of importance for GAS infections are TLR1, 2, and 9, which recognise lipopeptides, lipoteichoic acid and unmethylated CpG DNA respectively, all hallmarks of bacterial infection. Interestingly, TLR4 also recognises short fragments of hyaluronic acid (Taylor, *et al.* 2004), which forms the anti-phagocytic capsule of GAS (Wessels, *et al.* 1991).

Upon binding their ligands, the TLRs recruit a barrage of adapter proteins including MyD88, activating an array of signalling cascades through the signalling kinases MAPK, PI3K and IKK onto the transcription regulator NFκB. Amongst the effects of such signalling pathways is the upregulation of CR3 (Lynam, *et al.* 1994).

1.2.5 Chemical mediators of inflammation

Chemokines are small molecule inducers of cell motility. Chemokines can be subdivided into a variety of families, and act on a variety of different cell types. All

chemokines with neutrophil activity belong to the CXC family, so named due to the characteristic arrangement of cysteine residues proximal to their amino termini (cysteine-X-cysteine) (Murdoch and Finn 2000). The 17 mammalian chemokines which belong to the CXC family can be further subdivided into two groups by virtue of the presence or absence of a glutamic acid-leucine-arginine (ELR) tripeptide motif, immediately before the first cysteine of the CXC motif. The presence of this motif is a canonical feature of neutrophil specific chemokines; all chemokines with such a motif act on neutrophils, whereas those without this motif act on other cells of the immune system (Clark-Lewis*, et al.* 1991, Hebert*, et al.* 1991) (

Systematic Name	Functional Name	ELR Motif	Responding Cell Type	Known Receptors
CXCL1	GROα	+	Neutrophil	CXCR2>CXCR1
CXCL2	GROβ	+	Neutrophil	CXCR2
CXCL3	GROγ	+	Neutrophil	CXCR2
CXCL4	PF-4	-	Fibroblast	Unknown
CXCL5	ENA-78	+	Neutrophil	CXCR2
CXCL6	GCP-2	+	Neutrophil	CXCR1, CXCR2
CXCL7	NAP-2	+	Neutrophil	CXCR1, CXCR2
CXCL8	IL-8	+	Neutrophil	CXCR1, CXCR2
CXCL9	MIG	-	T_H1/NK cell	CXCR3
CXCL10	IP-10	-	T_H1/NK cell	CXCR3
CXCL11	I-TAC	-	T_H1/NK cell	CXCR3, CXCR7
CXCL12	SDF-1α	-	All leukocytes	CXCR4, CXCR7
CXCL13	BCA-1	-	B cell	CXCR5
CXCL14	BRAK	-	Monocyte	Unknown
CXCL15	Lungkine	-	Neutrophil	Unknown
CXCL16	SCYB16	-	T cell	CXCR6
CXCL17	VCC-1	-	Dendritic cell	Unknown

Figure 1-3). These chemokines have common structural characteristics; their N termini are globular and bind the chemokine receptors, whereas their C-termini are alpha helical, and bind glycosaminoglycans. The binding of the N-termini of such chemokines to their cognate receptors, CXCR1 and CXCR2, promotes extravasation of neutrophils as the chemokine C-termini bind endothelial surfaces. After extravasation, the chemokines enable

chemotactic trafficking to infection sites and the activation of neutrophils, including the upregulation of receptors such as CR3 (Detmers, *et al.* 1990).

Systematic Name	Functional Name	ELR Motif	Responding Cell Type	Known Receptors
CXCL1	GROα	+	Neutrophil	CXCR2>CXCR1
CXCL2	GROβ	+	Neutrophil	CXCR2
CXCL3	GROγ	+	Neutrophil	CXCR2
CXCL4	PF-4	-	Fibroblast	Unknown
CXCL5	ENA-78	+	Neutrophil	CXCR2
CXCL6	GCP-2	+	Neutrophil	CXCR1, CXCR2
CXCL7	NAP-2	+	Neutrophil	CXCR1, CXCR2
CXCL8	IL-8	+	Neutrophil	CXCR1, CXCR2
CXCL9	MIG	-	T_H1/NK cell	CXCR3
CXCL10	IP-10	-	T_H1/NK cell	CXCR3
CXCL11	I-TAC	-	T_H1/NK cell	CXCR3, CXCR7
CXCL12	SDF-1α	-	All leukocytes	CXCR4, CXCR7
CXCL13	BCA-1	-	B cell	CXCR5
CXCL14	BRAK	-	Monocyte	Unknown
CXCL15	Lungkine	-	Neutrophil	Unknown
CXCL16	SCYB16	-	T cell	CXCR6
CXCL17	VCC-1	-	Dendritic cell	Unknown

Figure 1-3 – Table of CXC chemokines and their properties

Adapted from (Pease 2011)

CXCR1 and 2, which, although expressed at low levels on other immune cell lineages such as monocytes and natural killer (NK) cells, are predominantly found on the surface of neutrophils (Morohashi, *et al.* 1995). Circulating neutrophils express approximately 75000 of these receptors per cell, compared to 7500 by HL60 cells and 5000 by primary human monocytes (Grob, *et al.* 1990, Zahn, *et al.* 1997). HL60 are a leukocytic cell line which can be differentiated to resemble neutrophils. HL60 cells express phagocyctic receptors, but only low levels of chemokine receptors (Collins, *et al.* 1979). Monocytes are also phagocytic cells, but respond to a distinct range of chemokines and express accordingly different chemokine receptors (Shi and Pamer 2011).

Chemokine receptors are G-protein coupled receptors, which are themselves upregulated in number as the neutrophil progresses towards an infection site by both *de novo* expression (Manna, *et al.* 1995, Manna and Samanta 1995) and from intracellular stores (Manna and Samanta 1995). As the neutrophil response continues, CXCRs are later downregulated primarily by desensitisation and subsequent internalisation (Samanta, *et al.* 1990).

In addition to these chemotactic mediators, other soluble mediators of inflammation, cytokines, play a vital role in orchestrating the immune response. These mediators are typically thought to derive primarily from cells of the monocyte lineage. However, it must be remembered that neutrophils are a potent cytokine source and express pro-inflammatory tumour necrosis factor alpha (TNFα), interferon-1-beta (IL-1β), interferon-12 (IFN-12) and vascular endothelial growth factor (VEGF) (Scapini, *et al.* 2000).

1.2.6 Neutrophils and other cellular responders

1.2.6.1 Neutrophils

Neutrophils form part of the earliest immune response to bacterial infections, acting as rapid response bactericidal cells. These are potent antimicrobial cells, equipped with an arsenal of toxic chemicals to destroy pathogens.

Comment [RL1]:

Comment [RL2]:

1.2.6.1.1 Neutrophils and the bone marrow

The potent anti-microbial arsenal possessed by neutrophils means however that they are tightly regulated, and typically 1-2% of total neutrophil numbers circulate in the blood under normal circumstances, with the remainder being held in the bone marrow until required (Delano, *et al.* 2011, Semerad, *et al.* 2002) (Figure 1-4). Furthermore, neutrophils have an extremely short half-life of six hours (Furze and Rankin 2008a), which some have suggested is to ensure that proper membrane integrity is maintained, preventing unwanted leakage of toxic compounds (Amulic, *et al.* 2012).

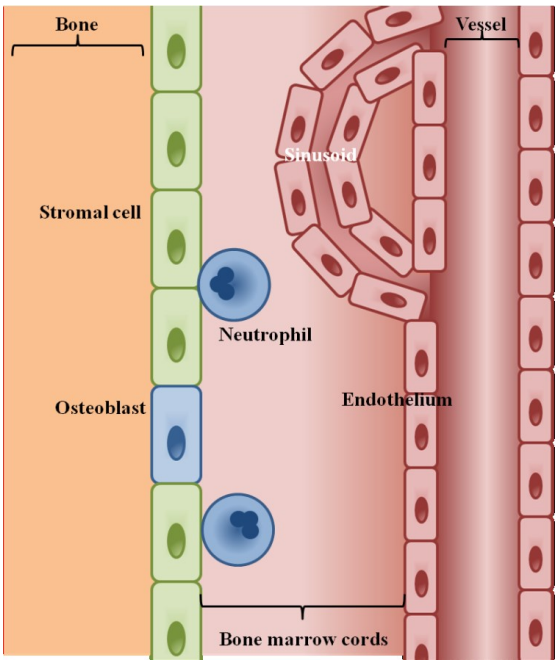


Figure 1-4 – Architecture of bone marrow

Neutrophils (blue round) and held in the bone marrow bound to stromal (green cells). Upon activation, they migrate across the sinusoidal endothelium into the circulation.

Neutrophils are held in the bone marrow bound to the stromal cells. Here they are isolated from the circulation by the sinusoidal endothelial cells, the integrity of which is critical to prevent unregulated leukocyte leakage (Burdon, *et al.* 2008). Neutrophils exit the bone marrow by crossing the sinusoidal endothelium, migrating directly through the cells, rather than the junctions between them, maintaining the integrity of the endothelium (Burdon, *et al.* 2008). Neutrophil release from the bone marrow is critically dependent on three signalling pathways. Neutrophils in the bone marrow express low levels of CXCR4, the receptor for CXCL12. It is known that ablation of CXCR4 signalling leads to neutrophils being released from the bone marrow (Eash, *et al.* 2009, Ma, *et al.* 1999). CXCL12 is

constitutively expressed at high concentrations by the bone marrow stromal cells, providing the default retention signalling (Shirozu, *et al.* 1995).

Counteracting the retention signals provided by constitutive CXCL12 expression is granulocyte colony-stimulating factor (G-CSF). G-CSF acts to reduce expression of CXCL12 by stromal cells, thus reducing the retention signals (Noursadeghi, *et al.* 2002). It is known that direct perfusion of canulated bone marrow with G-CSF leads to release of neutrophils from the bone marrow (Martin, *et al.* 2003). However, this effect is not seen using CXCR2 knock-out mice, indicating that CXCR2 signalling is also necessary for bone marrow release of neutrophils (Eash, *et al.* 2010, Kohler, *et al.* 2011). Interestingly, CXCR4 CXCR2 double knock-out mice demonstrate high levels of neutrophil circulation (Eash, *et al.* 2010), although whether this is due to enhanced release from the bone marrow, or a failure to clear old neutrophils from the blood stream, the mechanism for which also uses CXCR4 signalling, is unclear (Weisel, *et al.* 2009).

KC and MIP2, the murine chemokines which bind CXCR2, and are functional orthologues of human CXCL8, are expressed constitutively at low levels by the bone marrow endothelial cells and the osteoblasts (Eash, *et al.* 2010) balanced against the retention signals of CXCL12. These signals mutually antagonise each other such that an increase in either chemokine can exert large effects on neutrophil release (Furze and Rankin 2008b). The expression of KC and MIP-2 by the endothelial cells is enhanced in bone marrow by rising concentrations of G-CSF (Eash, *et al.* 2010). Furthermore, perfusion of either KC or MIP-2 into canulated bone marrow (Martin, *et al.* 2003); injected intravenously (Martin, *et al.* 2003) or injected intraperitoneally (Wengner, *et al.* 2008) leads to the release of neutrophils from the bone marrow. The precise mechanism of action of KC and MIP-2 is unclear, but is likely to involve provision of a concentration gradient that allows the trafficking of unbound

neutrophils out of the bone marrow (Furze and Rankin 2008b). This is summarised in Figure 1-5.

A

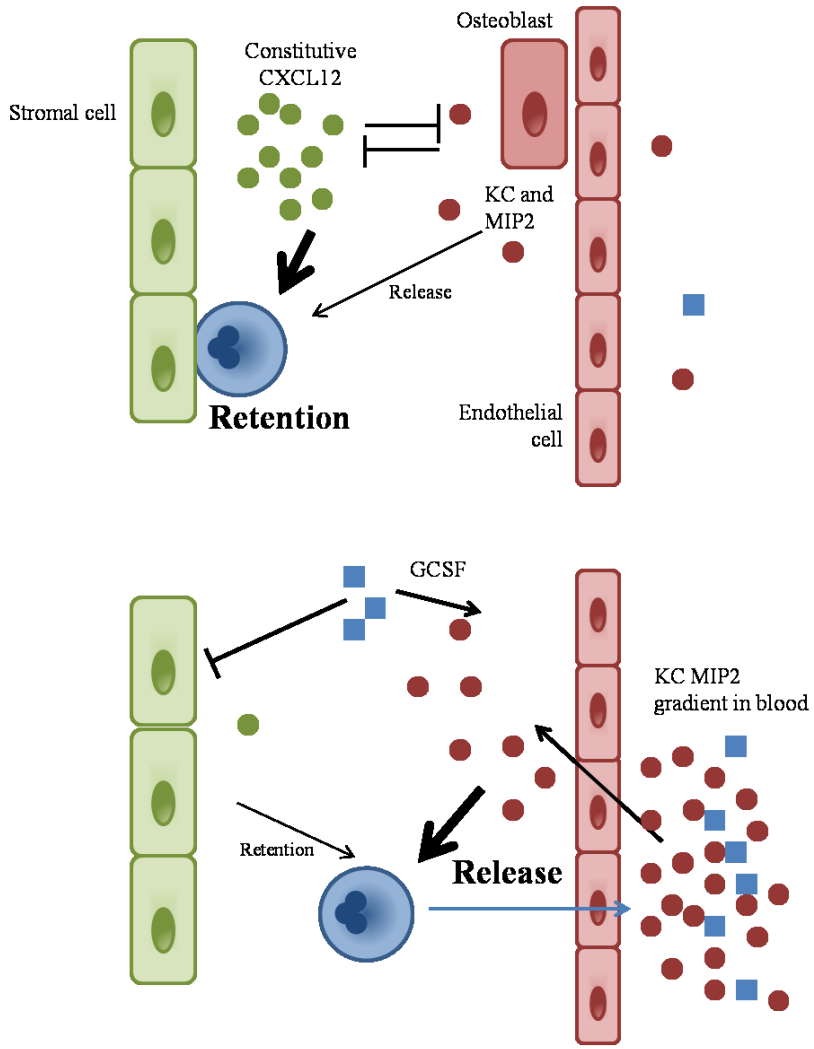


Figure 1-5 – Signalling interplay controlling neutrophil release from bone marrow

(A) – resting state. Stromal cells (green) express high levels of CXCL12 (green circles), which antagonise the KC and MIP2 (red circles) released from the endothelial cells and osteoblasts. The dominant signal is retention. (B) – infection state. KC, MIP2 (red circles) and G-CSF (blue squares) enter the blood stream. These diffuse across the sinusoidal endothelium. G-CSF reduces expression of CXCL12 and upregulates production of further KC and MIP2, leading to detachment of neutrophils from the stromal cells. KC and MIP2 form a chemokine gradient into the blood promoting neutrophil egress across the sinusoidal endothelium.

1.2.6.1.2 Neutrophil activation

After leaving the bone marrow, neutrophils begin to activate. Neutrophil activation involves the progressive release of granules from within the neutrophil and other cellular changes including increased actin mobilisation. Granules have been grouped as primary, secondary, tertiary and secretory vesicles, classically based on their staining with dyes, but are actually highly heterogeneous, in both their contents, and the timing of their release (Amulic*, et al.* 2012, Borregaard and Cowland 1997). Neutrophil activation is a progressive process, which is triggered by an array of stimuli, rather than being dependent on a single signal being received. It is driven by a variety of inflammatory mediators produced by cells local to the infection site, such as TNFα, chemokines, and directly by bacterial products such as LPS.

Granules are released from the neutrophil at varying threshold concentrations of intracellular calcium, the concentration of which is dependent on the various signalling sources neutrophils receive. As such, different granules are released throughout activation. As these granules contain a variety of surface proteins, the progressive degranulation process alters the surface expression of a variety of receptors, allowing neutrophils to egress from the bone marrow, through the peripheral blood, across the endothelial barrier and into the infection site. At the site of infection, chemokines are at much higher concentration than in the serum, and signalling from chemokine receptors via MAPK/ERK signalling pathways leads to the commencement of the oxidative burst, part of the final stages of neutrophil activation (Selvatici*, et al.* 2006).

Neutrophil granules also contain a variety of other molecules, including CXCL8. This is primarily stored in secretory vesicles, a class of vesicle released easily from the neutrophil. Accordingly, CXCL8 can be rapidly released at high concentrations upon stimulation, providing a feed forward mechanism for further neutrophil recruitment and activation (Cassatella*, et al.* 1992).

Neutrophils were once considered to be of limited productive capacity, however work has found they are able to secrete a variety of cytokines and chemokines. Many important cytokines, such as TNF-α, IL-1β and IL-12 along with chemokines CXCL10, CXCL11, MIP-1α and MIP-1β are now known to be produced by neutrophils. Whilst a detailed description of these cytokines is beyond the range of this study, the cytokines produced act to enhance various stages of the immune response. TNF-α has a broad range of functions including activation of various intracellular signalling cascades associated with an anti-pathogen state, IL-1β upregulates various adhesins on endothelial cells, assisting in the infiltration of other leukocytes, and IL-12 drives differentiation of the adaptive immune response to a T_H1 reaction, appropriate for antiviral and anti-bacterial responses. CXCL10 and 11 are chemoattractants of monocytes and MIP-1α and MIP-1β chemoattract monocytes, natural killer cells and T cells (Scapini*, et al.* 2000).

Upon infection, following their liberation from the bone marrow, neutrophils traffic to infected or damaged tissues by migrating down CXCR1 and/or 2 specific chemokine gradients (Baggiolini 1995). The importance of these two receptors in neutrophil trafficking and activation is unclear. There is some evidence to suggest that CXCR2 is involved primarily in early stages of activation, which is superseded by CXCR1 signalling as the neutrophils near the focus of the infection (Amulic *et al.* 2012). These chemokines are produced by cells at the site of infection in response to a variety of danger signals, including

pathogen associated molecular patterns (PAMPs), and are detected by neutrophil CXCR1 and 2 arranged on the cell surface in a polarized distribution (Amulic, *et al.* 2012).

After migrating to the infection site, neutrophils phagocytose opsonised particles by binding opsonins using phagocytosis receptors expressed on their surface. Degranulation upregulates the numbers of receptors involved in bacterial phagocytosis on the neutrophil surface – the neutrophils have become primed to kill bacteria or other pathogens (Thelen, *et al.* 1993). These receptors include complement receptors 1 and 3 (CR1 and 3) which bind C3b deposited on the surface of the pathogen, and receptors such as CD16 which bind opsonising antibodies. These are all upregulated during neutrophil activation by degranulation (Borregaard, *et al.* 1994, Detmers, *et al.* 1990, Elghetany 2002, Paccaud, *et al.* 1990). Receptor components are found pre-formed in a wide variety of neutrophil granules, and thus CR3 is upregulated continuously throughout neutrophil activation as neutrophils progressively degranulate (Amulic, *et al.* 2012). CR1 and FcγRIII are primarily found in early released secretory granules (Amulic, *et al.* 2012, Sengelov, *et al.* 1994). CR3 has been shown to be of critical importance in the phagocytosis of GAS. Antibody blockade of Fc receptors or CR1 have been shown to have limited impact on the phagocytosis of GAS by neutrophils, whereas blockade of CR3 caused ablation of phagocytosis (DeMaster, *et al.* 2002, Nilsson, *et al.* 2005, Weineisen, *et al.* 2004).

Neutrophils are relatively poorly studied as phagocytes, as they are difficult to study, being short lived cells and refractory to genetic manipulation (Lee, *et al.* 2003). Furthermore, the HL60 cell line which has been used as a neutrophil like immortalised cell differs from the neutrophil in several key ways, including in the expression of CXCR receptors (Collins, *et al.* 1979, Grob, *et al.* 1990, Zahn, *et al.* 1997). As described above, neutrophils have receptors able to phagocytose particles opsonised by complement or antibody. Upon binding an

opsonised particle, the specific receptors on the surface of the neutrophil cluster beneath the particle. These clustered receptors then initiate a signalling cascade leading to the recruitment and activation of actin remodelling machinery within the neutrophil. Phagocytosis by macrophages mediated by antibody opsonisation is mediated by the extension of pseudopods from the macrophage around the particle, whereas complement mediated phagocytosis appears as particles sinking into the macrophage (Allen and Aderem 1996). The subsequent phagosomes are then gradually made more antibacterial by fusion with granules filled with antibacterial agents such as myeloperoxidase and lysozyme (Nordenfelt and Tapper 2011).

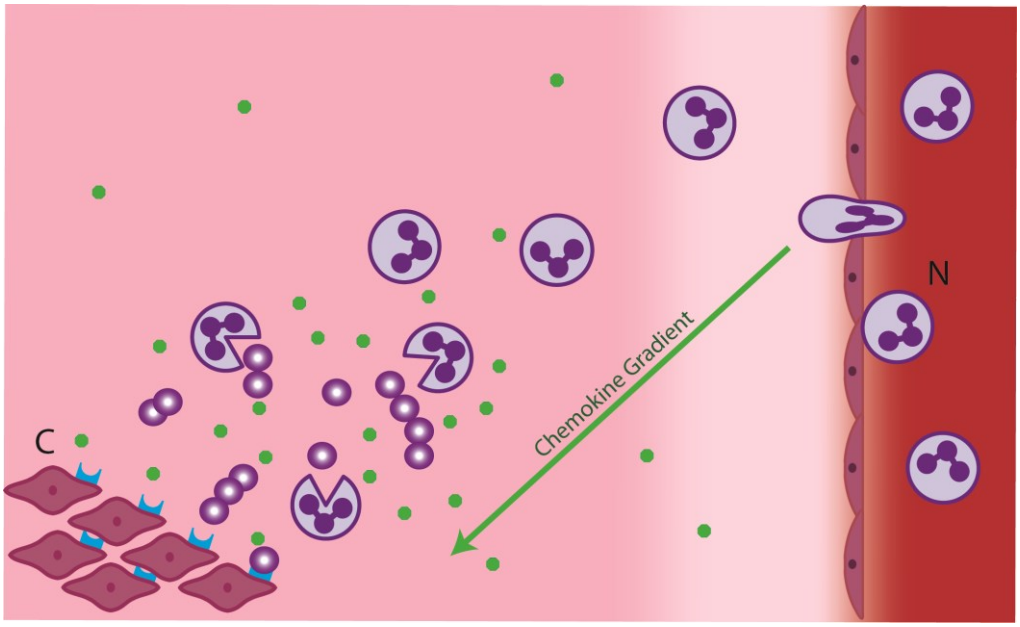


Figure 1-6 – Diagram of neutrophil trafficking

Diagram of neutrophil trafficking. Neutrophils (N) travelling in blood vessels (far right) extravasate and migrate down chemokine (green polygons) gradients towards the site of infection. At the site of infection, local cells (C) detect bacteria (purple circles) using receptors (blue) and release chemokines and other inflammatory mediators.

An activated neutrophil, as part of a unique type of cell death (NETosis), may expel neutrophil extracellular traps (NETs), consisting of chromatin studded with antimicrobial proteins (Brinkmann*, et al.* 2004). These structures have antimicrobial activity, thought to be mediated by bacteria sticking in the extended DNA, then being exposed to high concentrations of antimicrobial peptides (Papayannopoulos and Zychlinsky 2009). The importance of neutrophils in controlling GAS virulence is great, and is demonstrated by the range of virulence factors expressed by GAS to try to evade their killing (Cunningham 2000).

1.2.6.2 Macrophages and others

The other principle phagocytes of the innate immune response are macrophages. These cells derive from circulating monocytes, differentiating into cells primed to kill pathogens upon interaction with cytokines or directly with microbial products. These cells express a similar range of receptors involved in phagocytosis, and indeed much of what is known about neutrophil phagocytosis has been extrapolated from research on macrophages (Lee*, et al.* 2003, Verschoor*, et al.* 2012). Macrophages however phagocytose more slowly than neutrophils and have lower capacity to produce antimicrobial molecules than neutrophils (Nordenfelt and Tapper 2011). The phagosomes produced are made antibacterial using a different mechanism to that used by neutrophils. Rather than fusion with granules, the membrane of the phagosome modifies and eventually acidifies by fusion with the lysozome,

delivering toxic products onto the bacteria (Janeway Jr, *et al.* 2001). Macrophages are capable of a wider range of cytokine expression and furthermore can present antigens to the cells of the acquired immune response driving their expansion and activation (Verschoor, *et al.* 2012). They hence have a critical role bridging the early innate and later acquired immune response (Janeway Jr, *et al.* 2001).

Monocytes and macrophages express a different set of chemokine receptors to neutrophils, and are thus responsive to different chemokines. Whereas neutrophils express CXCR1 and 2 at very high levels, these are found at only low concentrations on the surface of monocytes (Zahn, *et al.* 1997). Instead, they express chemokine receptors CCR2 or CX_3CR1 (Geissmann, *et al.* 2003). This causes these two different phagocytes to have different chemotactic responses.

Monocytes are known to play an important role in GAS virulence. Monocyte depleted mice had much more severe infections than wild type infected mice. A non-lethal dose of GAS in wild type mice was sufficient to cause a mortality rate of 100% in mice which were monocyte depleted (Goldmann, *et al.* 2004). M1 GAS strains have also been reported to survive and replicate within macrophages by impairing fusion of the lysozome to the phagosome. These cells have been reported to provide a protective niche allowing GAS persistence (Hertzen, *et al.* 2010). GAS has not been reported to survive within neutrophils.

Other cellular mediators of the innate immune response include eosinophils, mast cells and basophils. These are also granulocytes, but are otherwise very different from neutrophils. They primarily function against large extracellular pathogens by the release of toxic compounds within their granules into the extracellular milieu. They are not phagocytes, and express different chemokine receptors from neutrophils. All these cell types are relatively minor components of the white blood cell population, but are contaminants of neutrophil

preparations. Natural killer cells are of lymphoid lineage, and target cells infected with intracellular pathogens, especially viruses. They will also not be considered further in this study (Janeway Jr*, et al.* 2001).

1.2.7 The acquired immune response

While the innate immune is responding rapidly to the onset of infection, and is normally capable of clearing the infection, a specific acquired immune response is developing against the infection. A complete description of the development of such a response is beyond the scope of this work, and has been described variously elsewhere (Janeway Jr*, et al.* 2001).

Briefly, antigen is captured by professional antigen presenting cells such as dendritic cells or macrophages, which then traffic to lymph nodes where the antigen is presented to T cells. Upon their arrival, T cells equipped with receptors specific for such antigen clonally expand and mature their receptors, enhancing their specificity. These can then in turn promote the clonal expansion and maturation of antigen specific B cells into plasma cells. These release large quantities of antibody which may also opsonise the target, or neutralise the antigen they target, by blocking the active site or effecting a conformational change (Janeway Jr*, et al.* 2001). Such responses linger in the body after the resolution of infection, conferring protection against re-infection, hence eliciting such responses is the aim of vaccination.

Opsonising antibody bound to their target antigen is bound by Fc receptors on phagocytes, which recognise the conserved, antigen independent domain of the antibody.

Opsonisation by antibody is also accompanied by driving complement deposition on the particle surface via the classical pathway of complement deposition (Zipfel and Skerka 2009).

SpyCEP interferes with the neutrophil response against Group A Streptococcus

1.3 GAS virulence mechanisms

1.3.1 Regulation of virulence

GAS tightly regulates its protein expression, including virulence factors, using a wide range of regulatory mechanisms, of which CovR/S (**c**ontrol **o**f **v**irulence) is the best characterised, and seems to be the most pluripotent, influencing expression of approximately 15% of the coding sequence (Graham*, et al.* 2006). CovR/S is a two component regulator. Such regulators consist of a sensor kinase protein (CovS), which upon sensing its stimulus phosphorylates a response regulator, and in turn acts to modulate transcription by binding DNA (CovR) (Churchward 2007). The expression profile controlled by CovR/S is complex and interacts extensively with the other regulatory mechanisms of GAS. However it can be generalised that when CovR is phosphorylated and active, it represses the expression of virulence factors involved in invasion and upregulates the expression of factors involved in persistence and colonisation. Indeed, it has been shown that invasive isolates often have a variety of mutations in *covRS* resulting in the deactivation of its regulatory system and thus allowing increased expression of invasive virulence factors. Furthermore, it has been shown to be acquired in the course of an experimental infection, in *covRS*, deactivating the regulatory system, (Sumby*, et al.* 2006, Walker*, et al.* 2007), (Cole*, et al.* 2010). Virulence factors known to be down-regulated by CovR/S include SpyCEP (Turner*, et al.* 2009a), proteins involved in capsule synthesis (Sumby*, et al.* 2006), and Sda1 (Walker*, et al.* 2007), whereas SpeB is known to be upregulated by CovR/S (Sumby*, et al.* 2006).

Mga was the first described regulatory system of GAS. It is highly conserved across GAS strains and activates expression of a wide range of virulence genes, including those encoding M protein, C5a peptidase and SIC (Kreikemeyer, *et al.* 2003), which all lie downstream of *mga*. Mga is autoregulatory, such that signalling by Mga leads to increased expression of further Mga, and it is opposed by transcription suppressors such as Rgg (Chaussee, *et al.* 2002, McIver, *et al.* 1999). The stimuli of Mga are relatively well characterised, and include elevated CO_2, iron limitation or increased temperature, common factors faced by GAS upon infecting a human host (Caparon, *et al.* 1992, McIver, *et al.* 1995, Podbielski, *et al.* 1992).

1.3.2 Defence against opsonisation

The complement system is known to be important in the phagocytosis of GAS (DeMaster, *et al.* 2002). It is hence unsurprising that GAS has mechanisms by which complement deposition is impeded. The broad specificity cysteine protease SpeB is able to directly cleave C3 and IgG, and has been shown to have a direct negative impact on phagocytosis (Lukomski, *et al.* 1998). Its specificity is sufficiently broad such that its known substrates includes some chemokines (Egesten, *et al.* 2009) and important GAS virulence factors including Sda1 (Walker, *et al.* 2007). Its importance in virulence is undermined by observations that many invasive strains express only low levels of SpeB due to mutation in CovR/S (Sumby, *et al.* 2006).

M protein has multifarious impacts on complement deposition, with variation in mechanism between M-types. The different M protein types have been shown to bind various complement-interacting factors: factor H, factor H like-1 (FHL1), C4b-binding protein and

CD46 complement regulatory protein (Horstmann, *et al.* 1988, Johnsson, *et al.* 1998, Kotarsky, *et al.* 1998). Factor H and FHL1 inhibit the alternative complement cascade, being present on host cells. They prevent the spontaneous breakdown of C3 into C3a and C3b and act to enhance the breakdown of the alternative C3 convertase, preventing further C3b deposition (Pangburn, *et al.* 1977, Weiler, *et al.* 1976, Whaley and Ruddy 1976). C4b-binding protein primarily inhibits the classical pathway of complement activation, by binding C4, preventing its inclusion into the classical C3 convertase, but does also have some activity in increasing the decay of the alternative C3 convertase (Carlsson, *et al.* 2003). CD46 directly interacts with C3b and C4b, allowing for their cleavage from the cell surface (Liszewski, *et al.* 1996). Furthermore, specific types of M protein bind to fibrinogen, which further prevents complement deposition via the classical pathway (Carlsson, *et al.* 2005, Whitnack and Beachey 1982). Considering these effects, it is not surprising that many studies have demonstrated its anti-phagocytic properties, which have been known for many years (Morris and Seastone 1955).

The polysaccharide capsule of GAS is a further anti-phagocytic factor that has been studied since the early days of GAS research (Ward and Lyons 1935). However, more modern genetic manipulation has allowed more exacting study of this virulence mechanism. The capsule comprises repeating units of *N*-acetylglucosamine and glucuronic acid (Stoolmiller and Dorfman 1969) forming hyaluronic acid. Hyaluronic acid is only weakly immunogenic, due to its similarity to hyaluronic acid in the connective tissue of the host, which is thought to explain some of its anti-phagocytosis properties (Cunningham 2000). Interestingly however, the mechanism by which capsule reduces phagocytosis does not seem to involve inhibiting complement deposition, and may instead act as a physical barrier, keeping the deposited complement on the bacterial surface from being bound by complement

receptors (Dale, *et al.* 1996). Its role in virulence has been demonstrated using acapsular mutants, which show increased phagocytosis and drastically reduced virulence in the mouse model (Wessels, *et al.* 1991).

Streptococcal inhibitor of complement (SIC) is a highly variable protein, produced by M1 type GAS strains (Akesson, *et al.* 1996). This protein binds to the C5b-C7 complex of the nascent membrane attack complex, preventing its formation (Fernie-King, *et al.* 2001). The known function of the membrane attack complex is to disrupt the outer membranes of Gram negative bacteria, hence GAS is naturally resistant. The role of SIC in GAS infection is hence unclear, however, it has also been found to bind and inactivate anti-microbial defensins, which may be the primary virulence function of this protein (Frick, *et al.* 2003).

Whilst the role of opsonisation by antibody is thought to be less important than that of complement, GAS expresses a variety of proteases which are able to cleave immunoglobulin directly. EndoS, IdeS and SpeB are all able to cleave IgG, with SpeB having cleavage activity against various subtypes of immunoglobulin (Collin and Olsen 2001a, Collin and Olsen 2001b, von Pawel-Rammingen, *et al.* 2002).

1.3.3 Defence against cellular immunity

As well as preventing opsonisation, GAS has a considerable arsenal of virulence factors to disrupt cellular immune effectors. GAS encodes a highly conserved C5a peptidase, whose expression is regulated by *mga* (La Penta, *et al.* 1994), which cleaves and inactivates the chemotactic C5a fragment produced by the complement cascade (Wexler, *et al.* 1985). This is chemotactic for neutrophils, and has been shown to impair neutrophil recruitment to the site of infection (Ji, *et al.* 1996). Its role in virulence in the mouse model has also been

demonstrated, with C5a peptidase mutants being less able to colonise the nasopharynx, and being cleared more efficiently from a sub-dermal model of infection (Ji, *et al.* 1996, Ji, *et al.* 1997). This enzyme is conserved between GAS and *Streptococcus agalactiae*, and it has been found that vaccination using C5a peptidase is protective in the mouse model against both organisms (Cleary, *et al.* 2004). A further enzyme, SpyCEP, that specifically cleaves and inactivates neutrophil chemokines plays a major role in prevention of chemotaxis and is discussed in detail in the next section.

Macrophages and neutrophils which successfully migrate to the infection site to phagocytose bacteria are subject to assault by streptolysins. Streptolysin S (SLS) is a broad spectrum cytolysin, of unknown mechanism. It is believed to accumulate in cell membranes, and form pores, causing irreversible loss of osmotic integrity. SLS has been shown to have activity against leukocytes, erythrocytes, platelets, but importantly not GAS membranes (Bernheimer and Schwartz 1965, Bernheimer 1966, Hryniewicz and Pryjma 1977). Such effects mediate the β haemolytic nature of GAS. SLS mutants are known to be less virulent in the mouse model of infection, and indeed SLS is known to lyse neutrophils (Miyoshi-Akiyama, *et al.* 2005).

Streptolysin O (SLO) is a further cytolytic toxin of GAS and has been shown to kill neutrophils and macrophages by inducing apoptosis. Its mechanism is somewhat more specific than SLS however, requiring the bacteria to be inside the cell to mediate its effect intracellularly (Timmer, *et al.* 2009). SLO forms a pore in the phagosome and allows the translocation of NAD+ glycohydrolase into the cell cytosol (Madden, *et al.* 2001), where it cleaves NAD and creates cADP ribose (Karasawa, *et al.* 1995). SLO mutants have also been found to show reduced virulence *in vivo* (Timmer, *et al.* 2009).

SpyCEP interferes with the neutrophil response against Group A Streptococcus

The final assault of neutrophils on bacteria, NETosis, is also disrupted by GAS. GAS expresses a potent DNAse, Sda1 (Aziz*, et al.* 2004), which degrades the NET, allowing the bacteria to escape (Buchanan*, et al.* 2006). This protein is repressed by an active CovR/S system, and hence is upregulated in many invasive isolates where this regulator is lost (Sumby*, et al.* 2006, Walker*, et al.* 2007). The importance of this virulence factor has been demonstrated by experiments showing it provides the driving force for the CovR/S mutation *in vivo*. It was observed that Sda1 mutants seldom acquire CovR/S mutations during *in vivo* passage compared to wild type strains (Walker*, et al.* 2007). This is unlikely to be an observation which could be applied to all GAS strains – GAS expresses many virulence factors, with large inter-strain variation in the expression of each (Turner*, et al.* 2009a).

Further work has found an additional role for Sda1 in preventing activation of neutrophils, by interfering with TLR9 signalling. This receptor recognises bacterial DNA by its characteristic high proportion of C and G residues compared to mammalian DNA, and its ligation leads to the release of proinflammatory cytokines. Sda1 has been shown to cleave extracellular bacterial DNA. Furthermore, *in vivo* studies have found that infection foci have reduced TNFα and IFNγ following infection with Sda1 positive strains compared to Sda1 mutants. Indeed, macrophages, which do not kill bacteria using DNA traps, are impaired in bacterial killing due to expression of Sda1 (Uchiyama*, et al.* 2012).

It has also been shown that M1 strains of GAS are able to survive within macrophages. This is reported to be mediated by the M1 protein itself interfering with fusion of the lysosome with the GAS containing phagosome. Survival within the macrophage by GAS is reported to potentially provide a reservoir for reactivation of infection (Hertzen*, et al.* 2010).

Of note, as a Gram positive bacterium, the classical secretion mechanisms whereby proteins are translocated across a double membrane are not applicable to GAS. For GAS and

other Gram positive bacteria, a different mechanism is required. Many GAS extracellular virulence factors are secreted out of the cell via the ExPortal (Rosch and Caparon 2005). This microdomain is found in the cell membrane of GAS, and is rich in proteins involved in translocation. Furthermore, the ExPortal plays a role in the correct folding and maturation of secreted proteins. A particular accessory factor HtrA, has been found to be vital in the SpeB proper maturation of SpeB (Rosch and Caparon 2005).

1.4 Background on SpyCEP

1.4.1 SpyCEP history and specificity of activity

The focus of this thesis are the actions of the GAS chemokine protease SpyCEP. The discovery of ***S****treptococcus* ***py****ogenes* **c**ell **e**nvelope **p**roteinase (SpyCEP) arose from observations made on clinical features of GAS disease. Patients with invasive GAS diseases were noted to have low white cell counts (Stevens*, et al.* 1989, Taylor*, et al.* 1999). Histological sections taken from patients suffering from NF showed a striking paucity of neutrophils, despite the presence of Gram positive cocci and extensive necrosis (Edwards*, et al.* 2005). The chemokine CXCL8 was initially implicated due to the vital role it plays in neutrophil chemotaxis. Whilst certain purified GAS products such as superantigens induced high CXCL8 expression from human leukocytes *in vitro*, streptococcal supernatants elicited very low levels of CXCL8. It was later found that GAS supernatants cleaved and inactivated CXCL8. The enzyme responsible was identified as SpyCEP by mass spectroscopy (Edwards*, et al.* 2005).

The SpyCEP gene was originally annotated as *prtS* due to homology to PrtS, a caseinase expressed by *Streptococcus thermophilus.* Since discovery of its activity, the gene has been variously annotated *cepA*, *scpC* and *SpyCEP*. Use of *scpC* was based on nomenclature used for the streptococcal C5a peptidases (Scp, A and B) with which SpyCEP shares homology, although not substrate specificity (Hidalgo-Grass*, et al.* 2006). As the protease has no activity against C5a peptidase, the term SpyCEP has become the preferred term.

SpyCEP interferes with the neutrophil response against Group A Streptococcus

SpyCEP is a subtilisin-like extracellular serine proteinase that cleaves and inactivates certain neutrophil chemokines in a highly specific manner. The diseases caused by GAS stem from its ability to produce an astonishing array of virulence factors, targeting a broad swathe of the immune response which the bacteria might face. Particular focus is given to neutrophils, being part of the earliest immune response to bacterial infections (Edwards*, et al.* 2005). Interestingly, several reports have indicated that NF lesions are profoundly neutrophil poor, highlighting the impact of such mechanisms on disease pathogenesis (Edwards*, et al.* 2005, Hidalgo-Grass*, et al.* 2006).

Prior to the start of this work, the substrate list of SpyCEP had not been fully resolved, However, consensus has now been reached that SpyCEP can cleave CXCL1, 2, 3, 5, 6, 7 and 8 (Zingaretti*, et al.* 2010). This set of substrates comprises the entire human neutrophil specific chemokine family. No other substrates, including other chemokines, cytokines, immunoglobulin or blood proteins have been found. Such observations indicate that SpyCEP is highly specific, targeting solely but completely the neutrophil specific chemokines (Edwards*, et al.* 2005, Hidalgo-Grass*, et al.* 2006). This activity was abrogated upon addition of Pefabloc, a stable and specific serine protease inhibitor, but not by other protease inhibitors (Edwards*, et al.* 2005).

The SpyCEP cleavage site of CXCL1, 2 and 8 has been determined by mass spectroscopy. The cleavage site of CXCL8 is between Q_{59} and R_{60}, resulting in a 59 amino acid and 13 amino acid fragment. The smaller cleavage product of CXCL8 corresponded to its C-terminal α-helix. (Figure 1-7). CXCL1 and 2 are cleaved at sites with different amino acid sequences, but analogous structural sites, with the C-terminal alpha helix being liberated in both cases (Edwards*, et al.* 2005).

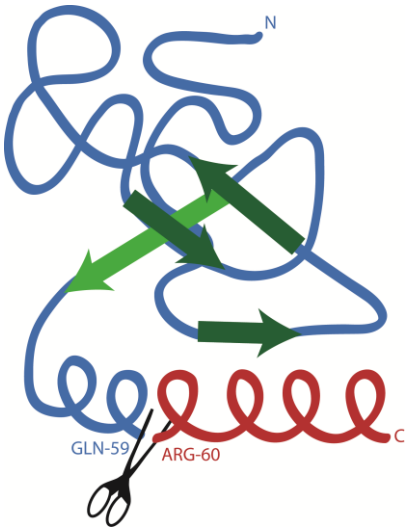


Figure 1-7 – SpyCEP cleavage site of CXCL8

Cleavage activity of SpyCEP on its substrate CXCL8. Mature SpyCEP (scissors) cleaves CXCL8 close to its C-terminus in its α-helix, liberating a 13aa helical peptide (red). Such cleavage abrogates the biological activity of the chemokine. (Goldblatt, personal communication) (Edwards *et al.* 2005)

1.4.2 Enzyme Kinetics

Enzymes such as SpyCEP classically follow Michaelis-Menten type kinetics. This model predicts that the rate of enzyme activity on a substrate is dependent on the concentration of a substrate, but at very high concentrations of substrate, the enzyme becomes saturated, breaking this link to substrate concentration. The dependence of rate of reaction on substrate concentration is illustrated in Figure 1-8.

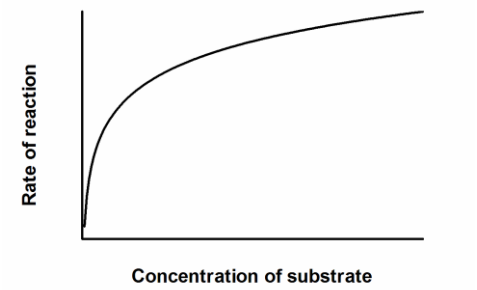


Figure 1-8 – Relationship of rate of reaction and substrate concentration.

Such kinetics are described by the Michaelis-Menten equation, where *v* is rate of reaction, v_{max} the maximum rate of reaction at excess substrate, S is the substrate and K_m is the Michaelis-Menten constant of the enzyme – the concentration of substrate required to achieve a rate of reaction which is 50% of v_{max}:

$$v = \frac{V_{\max}[S]}{K_m + [S]}$$

These constants may be calculated by performing a series of experiments using different concentrations of substrate and measuring rate of reaction over a very brief period. Under such conditions, it can be assumed that the concentration of substrate has remained constant. By plotting these results on a graph (A Scatchard Plot) with y and x axes of $1/v$ and $1/[S]$ respectively, the y intercept provides a value for $1/v_{max}$ and the x intercept a value for $-1/K_m$.

1.4.3 Structure of SpyCEP

The immature form of SpyCEP is approximately 180kDa, one of the largest proteins expressed by GAS (Edwards*, et al.* 2005). The protein can be subdivided into functional domains (Figure 1-9), based on the annotation of PrtP, a protease produced by *L. lactis* and

sharing considerable homology with SpyCEP. It is the archetype for cell envelope proteases expressed by lactic acid bacteria (Hidalgo-Grass, *et al.* 2006, Siezen 1999). These domains, despite their definition, remain poorly characterised. Being 180kDa, SpyCEP has proved difficult to express recombinantly or clone in *Escherichia coli*, hampering its structural characterisation, but also preventing complementation of mutant experiments from being performed. (Edwards, *et al.* 2005, Fritzer, *et al.* 2009, Kurupati, *et al.* 2010, Zinkernagel, *et al.* 2008). The N-terminal-most section of SpyCEP is a signal sequence, which is removed from the mature protein, and is responsible for trafficking the protein to the cytoplasmic membrane, potentiating its export (Hidalgo-Grass, *et al.* 2006).



Figure 1-9 – Schematic illustration of the structure of SpyCEP

From left (N terminus) to right (C terminus), SS – signal sequence, required for protein export, cleaved from mature enzyme, P – protease domain including catalytic triad residues (stars), PA – protease associated domain, A – A domain, B/H – B/H domain, LPXTG – sortase enzyme pentapeptide motif, required for attachment of the protein to the bacterial cell wall. The functions of the A, B and or H domain are not known (Siezen 1999).

The protease domain of the enzyme is found towards the N-terminus of SpyCEP, and is split into two separate sections by a protease-associated domain whose function is unclear (Figure 1-9). Each section of the protease domain contains some of the residues of the catalytic triad, such that either section of the protease domain alone has no activity.

The C terminal region of SpyCEP is poorly characterised. It is split into two characteristic domains, an A domain and a putative B/H domain (Hidalgo-Grass, *et al.* 2006).

This nomenclature is again based upon that which has been used for PrtP, as indeed are the putative functions assigned to these domains (Siezen 1999). The A domain is next to the protease domain, and is thought to be involved in substrate binding or specificity. Interestingly, this section of SpyCEP has been observed to be required for chemokine cleavage (Kaur, *et al.* 2010). The region between the A domain and the C terminus shares no significant homology to PrtP, hence its identity is only putatively annotated. It has no role in chemokine cleavage by recombinant SpyCEP, but may be involved in acting as a stalk, lifting the catalytic domains away from the bacterial surface above the capsule (Kaur, *et al.* 2010).

The C terminus of the protein features a further component involved in the final localisation of SpyCEP; a sortase A enzyme pentapeptide recognition motif: leucine-proline-X-threonine-glycine (LPXTG) (Navarre and Schneewind 1994). This pentapeptide is bound and cleaved by sortase A between the T and G residues whilst the enzyme also binds to, and brings into proximity, specific cell wall precursor molecules, pentaglycine bridges. Sortase A catalyses the formation of covalent bonds between the cleaved sortase motif and these pentaglycine bridges (Navarre and Schneewind 1999). Upon the integration of these SpyCEP-bound precursors into the mature GAS cell wall, SpyCEP is presented extracellularly. SpyCEP has been detected in GAS cell wall extracts using specific antibody (Turner, *et al.* 2009a). However, antibody can also be used to detect SpyCEP in the supernatant of GAS cultures implying this surface anchoring is imperfect or that SpyCEP is being liberated from the cell wall following anchoring (Edwards, *et al.* 2005). No evidence for active release of SpyCEP from the cell surface either through autocatalysis or a process involving any other GAS proteases has been found.

Antibodies raised against SpyCEP have provided evidence about its post-translational modifications. Antibodies against a C-terminal fragment of SpyCEP detected bands of

180kDa and 150kDa in GAS supernatants using immunoblotting in denaturing conditions. Similar antibodies raised against the N terminus of SpyCEP however detected bands of 180kDa and 30kDa (Edwards, *et al.* 2005). This has subsequently been proven to be a result of autocatalytic processing whereby SpyCEP cleaves itself between Q_{244} and S_{245}, liberating a 30kDa N terminus and a 150kDa C-terminus (Zingaretti, *et al.* 2010) (Figure 1-10). These bands appeared separate in the denaturing conditions of the immunoblot, proving that the two fragments were not covalently attached (Zingaretti, *et al.* 2010).

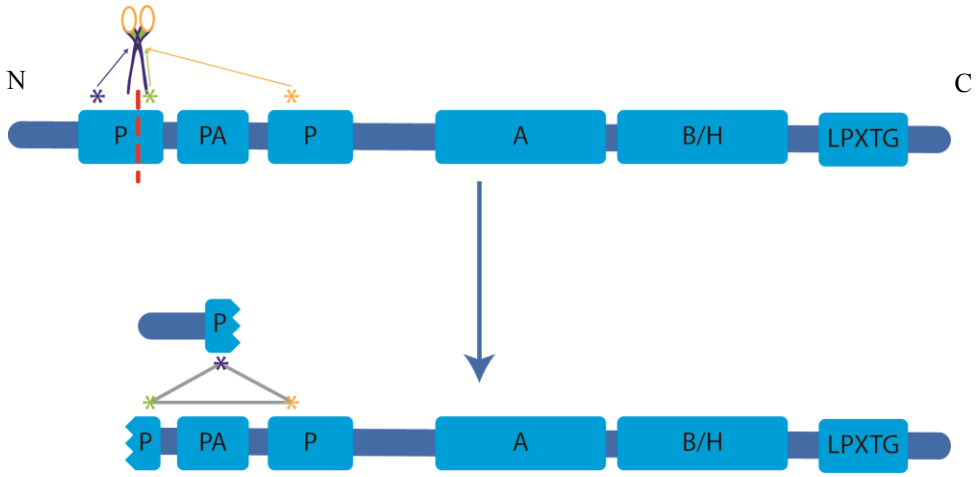


Figure 1-10 – Autocatalytic processing of SpyCEP

Autocatalytic processing of SpyCEP. Domains labelled as per Figure 1-9. Autocatalytic cleavage takes place in the protease domain splitting the catalytic triad residues between the two fragments. These domains are thought to then loosely associate. It is known that both are essential for catalytic activity

The autocatalytic nature of this modification is proven by the failure of active site mutants to cleave themselves. Furthermore, recombinant wild type SpyCEP is unable to catalytically process neighbouring active site mutants of SpyCEP, indicating that the reaction occurs internally only. The cleavage of SpyCEP into two fragments is unusual in that the

catalytic residues themselves are separated in the process. Despite this, the enzymatic mixture retains activity. It may be that SpyCEP which has not undergone autocatalytic cleavage has substrate specificity only for cleaving itself, whereas processed SpyCEP has substrate specificity for chemokines only (Zingaretti, *et al.* 2010).

1.4.4 Activity of SpyCEP *in vivo*

SpyCEP has been the subject of extensive study to determine its impact on virulence. A majority of studies have demonstrated a clear role for SpyCEP in virulence, although some have shown SpyCEP has a protective effect. Using a dermonecrotic model of GAS infection where lesion size is measured, it was found that SpyCEP-positive strains produced smaller lesions than isogenic SpyCEP-negative strains (Sumby, *et al.* 2008). Furthermore, SpyCEP was found to be protective to the mouse when GAS was injected directly into the blood stream (Sjolinder, *et al.* 2008). These studies contradict the majority of SpyCEP research, where it was found to be important in virulence, neutrophil recruitment and dissemination of infection. Indeed, reduced immunopathology caused by reduced neutrophil recruitment explains both of these contradictory results. Other work using bacterial strains which express higher levels of SpyCEP have found that SpyCEP expression enhances infection, especially when experimental challenge was given by an intramuscular route to mimic NF type invasive infections (Edwards, *et al.* 2005, Hidalgo-Grass, *et al.* 2006, Kurupati, *et al.* 2010, Turner, *et al.* 2009a). Such effects on virulence are manifested as differences in bacterial loads both locally at the infection site, and systemically throughout the mouse.

A difference in virulence and neutrophil recruitment between SpyCEP-positive and negative strains could be observed as early as three hours post infection in a thigh muscle

infection model (Kurupati, *et al.* 2010). This demonstrates the importance of neutrophils in the early response to GAS infections. These differences in virulence were seen throughout early infection to 24 hours, with higher bacterial loads and increased mortality being observed when SpyCEP was produced (Kurupati, *et al.* 2010). Other researchers have not found such clear phenotypes for SpyCEP knock-out strains. However, these groups have used different measures of infection burden, and have used strains which are less prodigious expressers of SpyCEP, resulting in a less apparent phenotype (Sjolinder, *et al.* 2008, Sumby, *et al.* 2008).

SpyCEP, like many virulence factors of GAS is regulated by CovR/S. It was found that ablation of either component of CovR/S led to a 100-fold increase in SpyCEP expression in particular strains (Turner, *et al.* 2009a). CovR/S mutations are associated with invasive disease (Cole, *et al.* 2010, Sumby, *et al.* 2006, Walker, *et al.* 2007), however this cannot be directly ascribed to increased SpyCEP expression, due to the multitude of virulence factors similarly regulated to SpyCEP by CovR/S.

Research into SpyCEP is hindered by the array of GAS virulence factors which impact on neutrophil function, including SpeB, M protein, EndoS, IdeS, SIC, capsule, C5a peptidase, SLS, SLO and Sda1, many of which are regulated also by CovR/S, meaning that GAS has considerable redundancy in evading these cells. This redundancy masks results made using knock-out mutations of any one of these virulence factors. To avoid these problems, gain of function *Lactococcus lactis* strains have been constructed and used as model organisms, mimicking GAS, but lacking their own virulence factors. Indeed *L. lactis* expresses very few extracellular proteins of its own, preventing any confounding observations (Bolotin, *et al.* 2001, Poquet, *et al.* 1998, van Asseldonk, *et al.* 1990). Such experiments have demonstrated the important role of SpyCEP (Kurupati, *et al.* 2010,

Zinkernagel, *et al.* 2008), as well as M protein (Rezcallah, *et al.* 2005), SLS (Nizet, *et al.* 2000) and SLO (Timmer, *et al.* 2009). The *L. lactis* strain expressing SpyCEP was found to be highly virulent, compared to the completely avirulent wild type *L. lactis* (Kurupati, *et al.* 2010).

Like C5a peptidase, a structurally similar, large extracellular protease, SpyCEP has been investigated as a potential vaccine candidate. It is well conserved between strains, and antibodies specific for SpyCEP have been found in the sera of convalescent GAS patients (Fritzer, *et al.* 2010). Using a recombinant fragment of the N terminus of SpyCEP, vaccination was found to be protective against systemic spread of GAS following intramuscular infection in mice. Furthermore, protection was also raised against *Streptococcus equi* which express a highly homologous protease to SpyCEP (Rodriguez-Ortega, *et al.* 2006, Turner, *et al.* 2009b).

NETosis is the final means by which neutrophils kill bacteria, expelling their chromosomal DNA studded with antibacterial proteins (Brinkmann, *et al.* 2004). NETs are known to be induced upon incubation of neutrophils with CXCL8. It was found by visualising NETs and inhibiting the activity of Sda1, that SpyCEP-negative strains induced significantly more NETs than the SpyCEP-positive wild type strains. This was confirmed to be of potential significance for virulence, reducing killing of bacteria by purified neutrophils (Zinkernagel, *et al.* 2008).

These effects on virulence stem from the ability of SpyCEP to cleave neutrophil specific chemokines. Migration is impaired, as by cleaving chemokines the means by which neutrophils extravasate and migrate to infection sites are ablated (Amulic, *et al.* 2012, Edwards, *et al.* 2005). Indeed, it is known that cleaved chemokines have no functional activity (Edwards, *et al.* 2005, Sumby *et al.* 2008 Zinkernagel *et al.* 2008), and have been

shown to be unable to bind their receptors phamacokinetically (Goldblatt, personal communication). Effects on NETosis are due to reduced activation of neutrophils caused by lack of chemokine signalling. Interestingly, no other neutrophil behaviours which are known to involve chemokines, such as release from the bone marrow, or other functions, such as neutrophil phagocytosis, have been reported to be impacted by SpyCEP

1.4.5 Related peptidases

SpyCEP orthologues can be found in variety of pathogenic Streptococci, but have generally been studied in less detail than SpyCEP itself (Table 1-1). Their conservation between species indicates that they play important roles in bacterial growth and survival, and furthermore may represent attractive vaccine targets with cross-species coverage (Cleary*, et al.* 2004, Turner*, et al.* 2009b). These putative proteases are well conserved around the protease domain itself, but with more variation in the C terminal half of the protein, where substrate specificity is speculated to be determined.

Streptococcus pyogenes

The C5a peptidase of GAS is also a large surface bound protease, with 35% identity with SpyCEP. Its role in virulence, and its conservation in *S. agalactiae* have been discussed previously.

Streptococcus pneumoniae

S. pneumoniae is a major cause of bacterial pneumonia (van der Poll and Opal 2009). This important pathogen expresses an orthologue of SpyCEP with 29% identity to SpyCEP,

PrtA. PrtA mutants have been shown to be less virulent in the mouse model of infection, but further to this, little is known about the protein, and indeed its substrate specificity is unknown. (Bethe, *et al.* 2001).

Streptococcus agalactiae

S. agalactiae is an opportunistic pathogen, of particular concern to neonates, being a cause of neonatal meningitis (Johri, *et al.* 2006). It produces a C5a peptidase, known as ScpB, which although sharing homology with SpyCEP, is a closer orthologue to the C5a peptidase of GAS. However, this protein has been reported to have degrading activity against some chemokines and fibrinogen (Bryan and Shelver 2009). A 1233aa protein of *S. agalactiae* has not been the subject of any study. This "SagCEP" has 34% identity to SpyCEP. A further protein, CspA, is described to be of importance to virulence through degradation of fibrinogen (Harris, *et al.* 2003)

Streptococcus gordonii

S. gordonii is an oral streptococcus, which is typically an avirulent commensal, although has been associated with cardiac disease (Petersen, *et al.* 2010). It produces two proteases, SGO0316 and SGO0317, with identity to SpyCEP, but which have not been annotated or studied. Proteases expressed by *S. gordonii* have been reported to play a role in competition with other oral bacteria (Wang, *et al.* 2010).

Streptococcus uberis

S. uberis expresses two proteins with homology to SpyCEP. Its C5a peptidase shares 31% identity with SpyCEP, and a hypothetical protease with 28% homology with SpyCEP.

The majority of the homology is concentrated in the protease-like domains, however there is considerable difference in the C termini of the proteins, where functions have been less clearly defined. Both these putative peptidases express sortase motifs and are hence predicted to be cell envelope proteases as SpyCEP. They however have been studied to determine their role in virulence (Ward, *et al.* 2009).

Streptococcus equi

SeCEP, expressed by *S. equi* has been studied due to its extensive homology to SpyCEP, and indeed has been shown to cleave human CXCL8, along with its equine orthologue. Work also found that vaccination against SpyCEP conferred protection against *S. equi* infection in the mouse model due to cross-reactivity of antibody (Turner, *et al.* 2009b). However, its role in the virulence of *S. equi* is unknown.

Protease	Species	Activity	Identity to SpyCEP
SeCEP	*S. equi*	Cleaves equine and human IL-8 (Turner, *et al.* 2009b)	63%
SGO0316	*S. gordonii*	Unknown	35%
SGO0317	*S. gordonii*	Unknown	36%
PrtA	*S. pneumoniae*	Important for virulence, unknown (Bethe, *et al.* 2001)	29%
CspA	*S. agalactiae*	Cleaves fibrinogen, CXC chemokines (Bryan and Shelver 2009)	39%
1233aa	*S. agalactiae*	Unknown	34%

Table 1-1 – Table showing SpyCEP orthologues

SpyCEP orthologues have been found in related pathogenic species of Streptococcus using *in silico* analysis. Their known activity and identity to SpyCEP are shown

1.5 Hypothesis and Project Aims

Chemokines are known to have an important role in neutrophil function. We hypothesise that SpyCEP, by cleaving all neutrophil specific chemokines, ablates neutrophil function in response to bacterial infection throughout their lifespan. Specifically, we will:

- Confirm that SpyCEP cleaves all neutrophil specific chemokines
- Examine if SpyCEP orthologues have similar substrate ranges
- Determine the sections of the neutrophil life cycle that SpyCEP disrupts specifically:
    - Release from the bone marrow
    - Activation and phagocytosis
- Confirm the mechanism responsible for any effects of SpyCEP
- Determine structural requirements for SpyCEP to mediate these effects

Chapter 2 - Methods

2.1 Bacterial strains

Strain	Species	Characteristics	Source
H292	GAS	Clinical strain, from patient with necrotizing fasciitis. Described in Turner *et al.* 2009 as a high SpyCEP producer	Clinical isolate
H575	GAS	Isogenic mutant of H292. *cepA* is disrupted by single cross-over recombination using a plasmid homologous to an N-terminal region of the gene.	Kurupati *et al.* 2010
H887	GAS	Isogenic mutant of H292. The sortase motif at the C terminus of SpyCEP has been disrupted by single cross-over recombination using a plasmid homologous to the sortase motif.	This study
H486	*L. lactis*	Wild type strain, containing an empty pDEST vector.	Zinkernagel *et al.* 2008
H487	*L. lactis*	Isogenic strain of H486, containing the pDEST plasmid allowing the constitutive expression of SpyCEP, and conferring erythromycin resistance.	Zinkernagel *et al.* 2008

Table 2-1 – List of strains used in this study.

2.2 Growth of bacterial strains

GAS and *L. lactis* were grown at 37^{o}C in an atmosphere of 5% CO_2. GAS was grown on Todd-Hewitt (TH) media (Oxoid, UK), solidified with 1.5% agar (Oxoid, UK). Columbia blood agar plates (CBA) (Oxoid, UK) were also used where haemolysis was required as a means to identify GAS, especially when using the murine model. In all cases, the strains (Table 2-1) were grown o/n. GAS was also grown in liquid TH media as required o/n. *L. lactis* strains were grown on TH media identically to GAS. However, the media was supplemented with 5µg/ml of erythromycin lactobionate to maintain plasmids. The strains were also grown in liquid TH media with 5µg/ml erythromycin supplementation as required. Where *E. coli* strains were used for cloning purposes, the strains were grown on Luria-

Bertani (LB) media, solidified where required with 1.5% agar. The media was supplemented with antibiotics as required, and described in more detail in the relevant chapters.

2.3 Genomic DNA Preparation (Pospiech and Neumann 1995)

50ml of the required strain was grown o/n in TH media and centrifuged for 10 minutes at 2500xg. The supernatant was removed, and the pellet was resuspended in 1ml of phosphate buffered saline (PBS) (NaCl 137mM, KCl 2.7mM, $Na_2HPO_4.2H_2O$ 10mM, KH_2PO_4 1.76mM). Cell walls were degraded with 20µl of 10000U/ml mutanolysin (Sigma Aldrich, UK) and 20µl of 100mg/ml lysozyme (Sigma Aldrich, UK) incubated at 37^{o}C for 30 minutes. The subsequent protoplasts were then centrifuged at 200xg for 10 minutes. The resultant pellet was resuspended in 500µl SET buffer, 50µl 10% SDS and 5µl of proteinase K (at 50mg/ml) (Sigma Aldrich, UK), and incubated at 55^{o}C for 2 hours, to disrupt the membranes and degrade proteins. To this, 500µl of chloroform and 175µl of 5M NaCl were added and incubated at 25^{o}C for 30 minutes. The resultant mixture was centrifuged at 13000xg for 15 minutes, and the aqueous phase collected; the organic phase containing the degraded lipid membrane, and a precipitate resolving at the interface of the layers containing proteins made insoluble at the high salt concentration. To the aqueous phase (approximately 500µl), 500µl of isopropanol was added to precipitate the DNA, and centrifuged at 16000xg for 30 minutes to pellet the DNA. This pellet was washed with 70% ethanol (another apolar solvent) at 16000xg for 10 minutes, and then allowed to air dry. The DNA was resuspended in 200µl of dH_2O, and diluted 1 in 10 prior to polymerase chain reaction (PCR). Undiluted sample typically had a DNA concentration of 50-100ng/µl when assessed by Nanodrop spectroscopy (Thermo Scientific, USA).

2.4 Plasmid preparation

Plasmid DNA was extracted from glycerol stocks (500µl stationary phase bacterial culture mixed with 500µl 50% glycerol) of Gram negative bacteria using a QIAPrep Spin Miniprep Kit (Qiagen, Germany) as per manufacturer's instructions. Briefly, 10ml of bacterial culture grown o/n was centrifuged and the pellet retained. This was resuspended in resuspension buffer then mixed with a lysis buffer. The mix was neutralised with a neutralisation buffer, causing the precipitation of the chromosomal DNA, proteins and lipids. This was centrifuged to pellet this insoluble material, and the supernatant containing the plasmid was retained. This was then passed over a charged silica membrane to bind the plasmid DNA, and was subsequently washed to remove any contaminants. The plasmid DNA was eluted into dH_2O, which due to its polarity, is able to disrupt the interactions between the DNA and the membrane.

2.5 Polymerase chain reaction (PCR) and subsequent purification

PCR for cloning reactions was done using Phusion DNA polymerase (New England Biolabs, USA). The components, including 1µM of specific primers were diluted and mixed according to manufacturer's instructions, with 1µl of appropriate genomic DNA diluted 1 in 10 in ddH_2O. Cycling conditions were: 98^{o}C 60 seconds; 30 cycles of 98^{o}C 15 seconds, (3^{o}C + the lower melting temperature of the two primers) 30 seconds, 72^{o}C 30 seconds per kilobase of target amplicon; 72^{o}C 10 minutes. Products were assessed by agarose gel electrophoresis, using gels made with TAE buffer (40mM Tris, 1mM EDTA pH8), solidified with 1% agarose and containing 1x SYBR Safe DNA gel stain (Invitrogen, USA). Prior to loading, samples were mixed with 6x loading dye, made with 30% glycerol, and dyes bromophenol blue and xylene cyanol FF (Sigma Aldrich, USA). Where required, DNA bands were purified from

agarose gels using QIAquick PCR Purification Kit (Qiagen, Germany) according to manufacturer's instructions. Briefly, bands were visualised under low power UV illumination, then excised using a clean scalpel blade. The resultant gel chips were dissolved in three volumes of a colour indicator buffer (Buffer QG) at 50^{o}C. The buffer is optimised to allow binding of the linear DNA product to supplied silica membrane columns. After binding the DNA, these columns were washed repeatedly to remove any contaminating nucleotides, enzymes, salts and agarose. The DNA was eluted using water which disrupts the polar interactions between the membrane and the DNA. Products were also purified as required using a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. This protocol is similar in principle to the previously described gel extraction protocol, using silica membrane columns and various wash steps to remove any contaminants before elution into dH_2O. The identity of the PCR products were confirmed by sequencing. Sequencing was carried out by the MRC CSC Genomics Core Laboratory using a 3730xl DNA Analyser (Applied Biosystems, USA).

2.6 Cloning of PCR products

Purified plasmids or products with restriction enzyme sites added following the PCR reaction were digested according to the manufacturer's protocol using appropriate, high fidelity, restriction enzymes (New England Biolabs, USA). Briefly, the reaction mixtures consisted of 2µl enzyme appropriate buffer, 1µl enzyme and 7µl PCR product (in ddH_2O) and 10µl ddH_2O. These were incubated at 37^{o}C for 2 hours. Following digestion, the samples were purified using a QIAquick PCR purification kit (described earlier), and DNA concentration measured using Nanodrop spectroscopy (Thermo Scientific, USA). If the subsequent ligation reaction was non-directional, the vector was dephoshorylated using Antarctic phosphatase

(New England Biolabs, USA) to reduce self-ligation. Ligation reactions were performed using Quick Ligase (New England Biolabs, USA). 1µl enzyme buffer, 1µl enzyme and 8µl DNA at a molar ratio of 3 inserts to 1 vector were mixed gently and incubated at room temperature for 1 hour. 1µl was taken and used to directly transform One Shot TOP10 Chemically Competent Cells (Invitrogen, USA). The DNA was incubated with the cells for 30 minutes at 0^{o}C, before being transferred to a 42^{o}C water bath for 30 seconds. The cells were then immediately cooled on ice and incubated with 200µl SOC media (Invitrogen, USA) for 1 hour at 37^{o}C. 100µl of the bacteria growing in SOC media was then spread onto LB agar plates supplemented with kanamycin 50µg/ml.

2.7 Construction of pSortase

pUCMut, a pUC19-based plasmid which lacks a Gram positive origin of replication and confers kanamycin resistance instead of ampicillin resistance was used as a vector to disrupt the LPXTG sortase motif of SpyCEP by single cross-over recombination. Sequence representing the C terminal 549bp of the SpyCEP gene with a premature stop codon upstream of the LPXTG motif was amplified using the primers RL0124 with a sequence GG GAATTC TGTTGT CAGGTAACAGTCTTATCTTGCC and RL0125 CC GAATTC ACAACA **CTA** GGCTTTTGCTGAGGTCGTTG. Underlined sequence indicates *EcoRI* sites introduced via PCR to allow insertion into pUCMut, and bold text indicates the introduced stop codon which is not found in the chromosomal template of *cepA*. The resultant amplicon was cloned into pUCMut, and the assembled plasmid was then transformed into GAS strain H292 and transformants were selected by kanamycin resistance. Mutants were further characterised by sequencing using primers which flank the mutagenised region to ensure replacement of the sortase coding region with a stop codon.

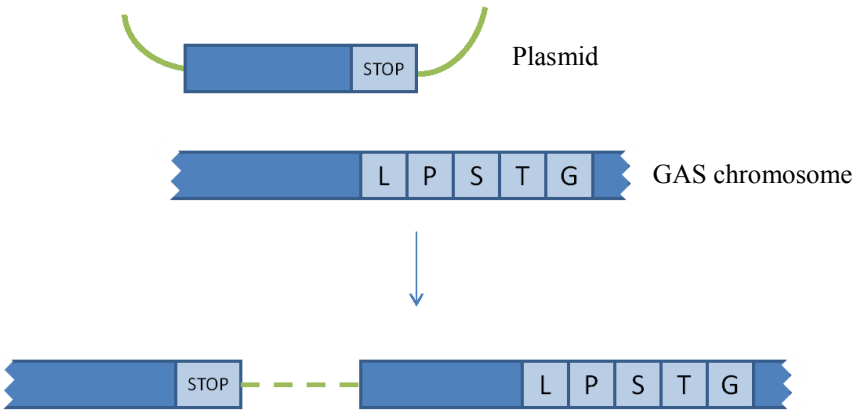


Figure 2-1 – Schematic of pSortase recombination

Diagram showing construction of strain H887 by disrupting SpyCEP sortase motif of H292. Green line indicates plasmid backbone.

2.8 Electroporation of GAS

A liquid TH media culture of the required GAS strain was grown o/n to stationary phase, with an optical density measured at a wavelength of 600nm (OD_{600}) of approximately 1.0, before being subcultured at a one in ten dilution into fresh TH media. This was grown to early logarithmic growth, with an OD_{600} of between 0.15 and 0.175. The bacteria were then harvested by centrifugation at 3500xg for 10 minutes at 4°C. The supernatant was carefully and completely removed, before the cells were washed twice in ice cold electroporation media (1mM $MgCl_2$ pH 6.5; 272mM glucose) at 3500xg for 10 minutes at 4°C. The supernatant was then removed, and the cells were resuspended in 750µl of fresh ice cold electroporation media. Electrocompetent GAS were prepared freshly prior to any electroporation. 75µl of the electrocompetent GAS were incubated with 5µl of plasmid DNA (at a concentration of 200-300ng/ml in ddH_2O) on ice; the concentration of plasmid DNA was sufficient such that at least 1µg of plasmid DNA was included in the electroporation

reaction. The cell DNA mix was transferred to a pre-chilled 1mm electrocuvette, before being electroporated at 1kV, 129Ω, 50μF. Time constants of between 3 and 4.5 milliseconds were expected. Immediately after electroporation, 1ml of freshly made TH media, enriched with 1% yeast extract (Oxoid, UK) (THY) was added, before later addition of THY to a final volume of 10ml. The bacteria were incubated at 37^{o}C for 3 hours to allow recovery. The cells were then centrifuged and resuspended in 500μl of fresh THY media. 100μl was spread onto THY plates containing erythromycin at a concentration of 2μg/ml. Included in all electroporation reactions was a control reaction using the plasmid pDL278. This plasmid confers resistance to spectinomycin, and is known to be efficiently uptaken by electrocompetent GAS. This was included to allow determination of electroporation efficiency. For such reactions, the same protocol was followed, however the strain was finally plated on media containing 50μg/ml spectinomycin.

2.9 Bacterial growth curves

Bacterial cultures were grown o/n in TH media with antibiotic where required to retain plasmids. The bacterial cultures were diluted in triplicate 1 in 20 into fresh TH media to give a final culture volume of 50ml, without antibiotic, and grown at 37^{o}C. Every 60 minutes, a 1ml sample was removed, and the OD_{600} was measured.

2.10 Lancefield antigen test

Bacterial cultures of either GAS or *L. lactis* were grown o/n in TH media supplemented with appropriate antibiotic as required. A control culture was grown where required without antibiotic to ensure that remaining antibiotic had no negative impact on the ability or not of blood to kill bacteria. The cultures were sub-cultured at a 1 in 10 dilution into fresh TH media

lacking antibiotic and allowed to reach early logarithmic phase growth (OD_{600} 0.15-0.175). These were diluted in tissue culture grade sterile PBS (Invitrogen, USA) such that GAS strains were at approximately 10^3CFU/ml, and *L. lactis* at 10^4CFU/ml. The precise starting inoculum of each strain was determined by plating the inoculum on CBA plates. 100µl of the inocula were mixed separately with freshly extracted heparinised whole blood, using approximately 30 CFU per 270µl of blood per reaction. Samples were incubated at 37^{o}C with rotation for up to 3 hours, with a sample of 50µl taken every 1 hour and plated onto CBA plates. Colonies from all plates were counted, allowing calculation of multiplication indices:

(Colonies obtained at time point x 20) / No Inocula colonies

Data points were obtained from experimental triplicates, and are representative of results obtained using 3 donors.

2.11 Enzyme Linked Immunosorbant Assays (ELISAs)

All chemokine ELISA reactions performed were carried using kits bought from manufacturers, and manufacturer's instructions were followed. Reactions performed were primarily used to measure rates of chemokine cleavage, but also to measure cytokine concentrations in murine infection models. Sample preparation and specific kits are discussed in further details in specific methods sections of results chapters. Briefly, high binding 96-well plates pre-incubated with specific antibody to the desired substrates were blocked with a bovine serum albumin (BSA) blocking buffer. Following washing each well three times using PBS containing 0.05% Tween 20, 100µl of samples together with controls and a standard of known concentrations provided by the manufacturer and diluted accordingly was added and

incubated for up to two hours to allow binding to the primary antibody. The samples were initially diluted 1 in 10 using the same dilution buffer as the standard for a pilot experiment. Adjustments were made to the dilution of the samples such that their concentrations fell within the standard curve following completion of the pilot experiment. After incubating with the primary antibody, samples were removed and after a further three washes using PBS + 0.05% Tween 20, a secondary antibody was added. An enzymatic peroxidase component was introduced, either covalently bound to the secondary antibody, or by adding a further reagent with specific binding against the secondary antibody. 50µl of colourimetric substrate of the enzyme was added and allowed to react before the reaction was stopped using 50µl 3M H_2SO_4. Protein concentration relative to a series of standards was measured by determining light absorbance at 450nm.

ELISA was also carried out to measure SpyCEP levels in bacterial supernatants. Here, bacteria cultures were grown overnight and their supernatants were collected by centrifugation at 2500xg for 10 minutes. The supernatant was filter-sterilised using a 0.2µm filter. The reagents used for this ELISA were developed and are described in Turner *et al.* 2009. Briefly, an ELISA plate was coated with 100µl of 0.4µg/ml of unlabelled anti-SpyCEP antiserum, diluted in PBS, for two hours, before washing the plate three times using PBS + 0.05% Tween 20. To the washed plate, 100µl of the prepared supernatants, diluted one in ten with PBS, were added, and allowed to bind to the antibody for 2 hours. These were again washed, before adding 100µl of biotin labelled anti-SpyCEP antibody at 20µg/ml in PBS to detect SpyCEP. 50µl of streptavidin conjugated to peroxidase was used as the enzymatic component. 50µl of colourimetric substrate of the enzyme was added and allowed to react before the reaction was stopped using 50µl 3M H_2SO_4. Protein concentration relative to a series of standards was measured by determining light absorbance at 450nm. The standards

used for this ELISA was recombinant N-terminal fragment of SpyCEP in PBS, with a maximum concentration of 2000pg/ml and a minimum concentration of 31.25pg/ml.

2.12 Chemokine cleavage rate calculation ELISA

An ELISA based method was used to measure chemokine cleavage rate. The kits used are shown in Table 2-2.

Kit	Chemokine	Manufacturer
Human GRO/MGSA Mini ELISA Development Kit	CXCL1	Peprotech (UK)
Human Groβ Mini ELISA Development Kit	CXCL2	Peprotech
DuoSet ELISA Development System CXCL5/ENA-78	CXCL5	R&D Systems (UK)
DuoSet ELISA Development System CXCL6/GCP2	CXCL6	R&D Systems
DuoSet ELISA Development System CXCL7/NAP-2	CXCL7	R&D Systems
Human IL-8 Mini ELISA Development Kit	CXCL8	Peprotech
Human CXCL16 Mini ELISA Development Kit	CXCL16	Peprotech

Table 2-2 – ELISA Kits used

No ELISA kit was available for CXCL3, hence this was not measured. CXCL16 was included as a control known not to be cleaved by SpyCEP.

The 1ml reaction mix for ELISA consisted of: 20µl of neat filtered bacterial culture supernatant (Chapter 2) or 20µl washed bacterial cells in PBS at OD_{600} 0.5 for GAS (measured at a final concentration of $3.66\text{-}5.32 \times 10^6$ CFU/ml), 1µl of specified recombinant chemokine (starting concentration 1ng/µl) (Peprotech, UK), 100µl BSA (22mg/ml), 10µl of specific cysteine protease inhibitor E64 (100µg/µl) (Invitrogen, USA) and 869µl of TH media. At 1, 2 and 24 hour time points, 75µl of sample was collected, 3µl of a specific serine protease inhibitor, Pefabloc (1µg/µl) (Invitrogen, USA) was added and the sample was placed on ice to stop the enzymatic reaction. When the samples were prepared for ELISA, 225µl of

fresh TH media was added, and the samples were plated on ELISA plates (Chapter 2) in three 100µl replicates.

In all rate reactions, controls were included which used H575, the Δ*cepA* isogenic GAS strain to allow determination of the specific contribution of SpyCEP to chemokine cleavage. The concentration of remaining chemokine was calculated at the time points measured by comparison with the included standard. The difference in chemokine concentration between the SpyCEP-positive and negative samples was calculated and this value used as the SpyCEP specific contribution to chemokine degradation. The rate was calculated from this number by, where applicable, standardising CFU to 10^6 CFU/ml, and standardising the measured concentrations of chemokine to a starting value 1ng/ml using control wells containing a known 1ng/ml of chemokine alone. Controls were also included which included the individual components of the reaction mix alone and mixed with chemokine.

2.13 Cleavage assay of substrates by SpyCEP

Cleavage assays were carried out using supernatants from 16 hour cultures or whole live cells. Supernatants were prepared by centrifugation of 5ml TH bacterial cultures at 2500xg for 10 minutes at 4°C. These supernatants were then passed through a 0.2µm filter (VWR, UK) and the filtrate collected. The supernatant was split into aliquots, and immediately stored at -20°C. The cell pellets obtained were washed twice in PBS at 16000xg for 30 seconds and resuspended in 1ml of PBS.

Recombinant chemokines used for this assay were purchased from Peprotech, UK, and reconstituted at 100ng/µl in dH_2O. 5µl of chemokine was mixed with 20µl of supernatant, or 2µl of cells (comprising approximately 10^5 CFU per sample) with 18µl of fresh TH media,

or 20µl of fresh TH media as negative control. The TH was added to the cell reactions to block the surface of the reaction tubes to which the chemokines were found to stick very readily. The assembled reactions were incubated at 37^{o}C o/n. Before being examined by LDS-PAGE analysis (see below), the samples were centrifuged at 16000xg for 30 seconds to collect any condensed sample in the tube lid, and to pellet the cells, and 20µl of supernatant was collected.

Other cleavage reactions involved using 5µl of protein extracted from activated neutrophils, 500ng murine G-CSF (Peprotech, UK), 500ng of recombinant human fibrinogen (Peprotech, UK) or 500ng recombinant human fibronectin

2.14 LDS-PAGE of cleavage reaction samples

20µl of the cleavage reaction sample was mixed with 3.5µl of LDS sample loading buffer (Invitrogen, USA) and 1.5µl of 1M DTT (Sigma Aldrich, USA). The samples were heated to 80^{o}C to help disrupt secondary structures within the protein sample before being loaded alongside 5µl of SeeBlue Plus 2 Prestained Standard (Invitrogen, UK) onto NuPAGE Novex 12% Bis-Tris gels (Invitrogen, USA). The gels was run in 1x 2- (*N*-morpholino) ethanesulfonic acid running buffer (Invitrogen, USA) supplemented with 0.5% v/v antioxidant (Invitrogen, USA) for 35 minutes at 200V constant voltage. The gels were removed and stained using NOVEX Colloidal Blue Staining Kit (Invitrogen) overnight, before being destained with water. Fibronectin was too large to be used in method. The protocol used was similar, but instead used 3-8% Tris Acetate Gels (Invitrogen, USA), and band weight was judged compared to HiMark Pre-Stained Protein Standard (Invitrogen, USA)

2.15 Immunoblotting

Protein gels were run according to the above protocol. However, rather than staining, the proteins were transferred using a wet transfer method to Hybond LFP membrane (GE Healthcare Life Sciences, UK) for 60 minutes using 100V at 4^{o}C. The membranes were blocked with PBS supplemented with 5% w/v Marvel skim milk powder and 0.05% v/v Tween 20 (Sigma Aldrich, UK) if horseradish peroxidise (HRP) conjugated secondary antibodies were to be used, or with PBS supplemented with 1% BSA (Sigma Aldrich, UK) and 0.05% Tween 20 for 1 hour if streptavidin HRP was to be used in lieu of secondary antibody when probing with biotinylated primary antibody. Following blocking, primary antibodies were added at concentrations optimised from an initial dilution of 1 in 5000 and incubated overnight at 4^{o}C with rocking. The membrane was washed three times with washing buffer (0.05% Tween 20 in PBS). HRP-conjugated secondary antibody (Invitrogen, USA) in Marvel skim milk powder blocking buffer was added at a dilution of 1 in 80000 or HRP conjugated streptavidin (R&D Systems, UK) was added at a dilution of 1 in 200 in BSA blocking solution. In both methods, secondary antibody or streptavidin was incubated for 1 hour at room temperature with rocking. After repeated washing, the membrane was developed using Amersham ECL Western Blotting Detection Kit (GE Healthcare Life Sciences, UK), and bands visualised using Hyperfilm ECL (VWR, USA). Where cell wall extracts were required for immunoblotting, the method described in Cole *et al.* 2008 was followed, where bacterial culture was washed 3 times at 16000xg in PBS, before being treated with mutanolysin and N-acetylmuraminadase as described.

2.16 Ethics Statement

All animals were handled in strict accordance with good animal practice as defined by the UK Home Office, and all animal work was approved by the local ethics committee.

Blood samples from healthy consenting volunteers were obtained through a sub collection of the Imperial College Healthcare NHS Trust Tissue Bank. The Tissue Bank is funded by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Imperial College Healthcare NHS Trust and Imperial College London and is compliant with the Human Tissues Act.

2.17 Extraction of human serum

Blood was taken into additive free polyethylene terephthalate tubes (BD Vacutainer), and blood was allowed to clot at room temperature for 30 minutes. The blood was then centrifuged at 1000xg for 10 minutes. The serum layer was collected ensuring the cell pellet was not disturbed. The serum was immediately transferred to ice, and was used on the day of extraction. Immediately prior to use, the serum was passed through a 0.2µm filter to remove any clumped proteins.

2.18 Neutrophil extraction from whole blood

Blood was collected into polyethylene terephthalate tubes containing sodium heparin (BD Vacutainer). The extracted blood was mixed with an equal volume of 3% w/v dextran Mw>100000Da (Fluka, Canada) in 0.9% NaCl (VWR, USA) solution to encourage erythrocyte rouleaux formation and thus their sedimentation. The blood and dextran solution was allowed to sediment for at least 30 minutes, until a clear interface between layers

developed. The yellow top layer consisting of serum and leukocytes was centrifuged at 250xg for 5 minutes, and the subsequent cell pellet was resuspended in an equal volume of 0.9% NaCl, before being layered onto an equal volume of Ficoll Hypaque. This was centrifuged at 700xg for 30 minutes at 20^{o}C with no brake. The pellet formed contained granulocytes and erythrocytes was resuspended in an equal volume of ice cold hypotonic 0.2% NaCl, to lyse the erythrocytes, for 30 seconds. The solution made isotonic with addition of ice cold hypertonic 1.6% NaCl, and the resultant was centrifuged to collect intact granulocytes. The process was repeated, and the cells were quantified using an Improved Neubauer Haemocytometer, and kept on ice until required for a maximum of 1 hour. The cells were judged to be at least 90% neutrophils by examination of morphology following Giemsa stain.

2.19 Monocyte extraction from whole blood

Blood was collected into polyethylene terephthalate tubes containing sodium heparin. The extracted blood was mixed with an equal volume of Hanks buffered saline solution (HBSS) (Sigma Aldrich, UK), before 30ml was layered onto 20ml Ficoll Hypaque Plus (VWR, USA). This was centrifuged at 700xg for 30 minutes at 20^{o}C with no brake, and the buffy coat layer containing predominantly B and T lymphocytes and monocytes was collected with a sterile Pasteur pipette. The cells were diluted at least two fold with HBSS, and pelleted by centrifugation at 250xg for five minutes. The cells were resuspended in 1ml of pre-heated Roswell Park Memorial Institute (RPMI) media, supplemented with 200μM L-glutamine (Invitrogen, USA). The cells were then adjusted to a concentration of 2x10^{6} cells/ml with further pre-heated glutamine supplemented RPMI, and transferred to sufficient vented tissue culture flasks (i.e. 25ml per 75cm^{2} flask) to allow the monocytes to adhere for 1 hour at 37^{o}C, 5% CO_2, ~95% humidity. Following incubation, the non-adherent cells were discarded, and

the cells were rinsed three times with further fresh HBSS to dislodge any loosely adhered cells. 5ml of fresh HBSS was added to each flask, and the adherent cells were detached from the flask surface using a cell scraper. The subsequent cell suspension was centrifuged at 250xg for 5 minutes, and resuspended in fresh HBSS. The cells were quantified using an Improved Neubauer Haemocytometer. The cells were judged to be at least 50% monocytes by analysis of forward and side scatter using a FACSCalibur (BD, USA).

2.20 HL60 propagation and differentiation (Collins, et al. 1979)

HL60 cells are a granulocyte cell line which have been used in the investigation of neutrophils (Collins *et al.* 1979). The HL60 cell line used in this study was a kind gift from Dr P. Elkington. Cells were grown in RPMI media supplemented with 10% foetal calf serum (FCS), glutamine and 1% tissue culture penicillin/streptomycin (all Invitrogen, USA) at 37^{o}C, 5% CO_2, ~95% humidity. Cell stocks were maintained by regular dilution of existing cultures into pre-warmed fresh media. When cells were required for experimentation, cells were diluted 1 in 25 into fresh media, supplemented with 1.3% dimethyl sulphoxide (DMSO) Hybri-Max (Sigma Aldrich, UK) and were grown for 72 hours to allow their differentiation into neutrophil-like cells. Following this differentiation, the cells were collected by centrifugation at 250xg for 5 minutes. The cells were then washed repeatedly at 250xg for 5 minutes in fresh tissue culture PBS to ensure all antibiotic was removed from the cells. The cells were quantified using an Improved Neubauer Haemocytometer and were stored on ice until required for a maximum of 1 hour.

2.21 Staining bacteria with fluorescein isothiocyanate (FITC)

A 5ml culture of a strain listed in Table 2-1 was grown o/n and the bacteria collected by centrifugation at 2500xg for 10 minutes at 4^{o}C. The pellet was resuspended in 500µl of pH9.0 carbonate/bicarbonate buffer containing 20µg/ml FITC (Sigma Aldrich, USA), and was incubated with shaking protected from light for 30 minutes to allow the FITC to make non-specific interactions to primary amines. Following incubation, the cells were washed twice at 16000xg for 30 seconds in tissue culture PBS (Sigma Aldrich) to remove any unbound FITC. The cells were then diluted to an OD_{600} of 0.35 with further PBS, and stored on ice, protected from light until required, for a maximum of one hour.

2.22 Flow cytometric phagocytosis assay (Bjerknes and Bassoe 1983)

To assess the phagocytosis of bacteria by purified cells, 500µl FITC-stained cells at OD_{600} of 0.35 were mixed with 1000µl of PBS and 1000µl of opsonin source (fresh human serum, specifically depleted human serum or IVIg at manufacturer's suggested dilution) and incubated at 37^{o}C with shaking protected from light for 15 minutes. Following incubation, 2500µl of freshly extracted cells at a concentration of $2x10^{6}$ cells/ml were added. Immediately after addition of cells, a sample was removed and run through a FACS Calibur flow cytometer, pre-calibrated using Calibrite 3 Beads (both BD, USA). The specific cell type being investigated was gated based on their morphology assessed by forward and side scatter. The gated cells were assessed for green fluorescence by excitation at 488nm and absorbance at 515-545nm. 20000 gated events were counted. Immediately following completion of the run, an equal volume of 0.4% v/v trypan blue (Invitrogen, USA) was added to quench extracellular fluorescence to exclude cells with adherent rather than internalised bacteria (Bjerknes and Bassoe 1984). Analysis was later performed using FloJo analysis

software. Gating to define cells positive for green fluorescence was done independently during each experiment, due to varying autofluorescence of different donor cells. Fortunately, positive and negative cells were clearly distinguishable due to the brightness of the bacteria. Later were experiments were performed where exogenous reagents were added. These were added by reducing volume of PBS included in the reaction mixture, including 10% v/v supernatant from bacterial growth, or exogenous chemokine diluted in PBS.

The addition of cells to the opsonised bacterial strains was staggered to ensure that multiple bacterial strains could be compared at the same time points using the same preparation of donor cells. Following addition of the cells to the bacteria, samples were taken every 15 minutes for 60 minutes. At all time points, immediately following completion of the sample, trypan blue was added to quench extracellular fluorescence. A neutrophil or monocyte only control was included at all time points to correct for autofluorescence. All experiments were repeated in three independent donors.

2.23 Mouse model of neutrophil release from bone marrow

5 or 6 female CD1 8 to 10 week mice were used per infection group. Bacterial strains were grown o/n in TH media containing appropriate antibiotic to maintain plasmids. Strains were washed three times in PBS at 16000xg for 30 seconds to remove any residual antibiotic and were then resuspended in sterile PBS (Invitrogen, USA) at an OD_{600} of 20 for GAS strains and 2 for *L. lactis* strains. 50µl, containing 10^8 CFU, was injected intramuscularly into the thigh without anaesthesia. Alternative experiments were carried out where 50µl of recombinant murine chemokine KC (10ng/µl) was administered intramuscularly into the thigh without anaesthesia. In all cases, a control group of six mice were used which were sham infected with 50µl of sterile PBS. At three and six hours post-infection, the mice of

each group were separately and briefly warmed before blood was taken using a tail bleed technique using a clean scalpel blade. The blood was collected and immediately mixed with sodium heparin. At 24 hours, the mice were euthanized using a rising concentration of CO_2 and blood was collected via immediate cardiac puncture. This was again transferred to sodium heparin. When using bacterial strains, the infected thigh was dissected, weighed and the tissue homogenised using sterile scissors in 5µl/mg of sterile PBS, to allow quantification of bacterial load by plating onto CBA plates.

2.24 Isolation and quantification of murine neutrophils

20µl of the freshly collected murine blood was mixed with 50µl of 3% w/v dextran Mw>100000Da (Fluka, Canada) in 0.9% NaCl (VWR, UK) solution to encourage erythrocyte rouleaux formation and thus their sedimentation. The mix was allowed to sediment for at least 30 minutes, until a clear interface between layers developed. 2µl of the upper layer was collected and used to quantify total cells using an Improved Neubauer Haemocytometer. Very small cells and cells which had taken up trypan blue (Invitrogen, USA) were excluded. A further 18µl was mixed with 30µl of PBS and 1µl of Purified Rat Anti-Mouse CD16/CD32 (Mouse Fc Block) Clone 2.42G2 (BD Pharmingen, USA) to prevent non-specific antibody binding by Fc receptors for 10 minutes on ice. Following this, 1µl of PE conjugated Rat Anti-Mouse Ly6G (BD Pharmingen, USA)(Wengner*, et al.* 2008) to stain neutrophils was added or an appropriate isotype control (BD Pharmingen, USA) and then incubated on ice for 30 minutes protected from light. Following incubation, the cells were washed three times in tissue culture grade PBS at 1000xg for 3 minutes, before being resuspended in a further 500µl of PBS.

Following staining, the cells were run through a FACSCalibur, pre-calibrated using Calibrite 3 Beads (BD, USA). Cells were initially sorted by forward and side scatter to exclude remaining erythrocytes and debris. The remaining cells were analysed using the FL2 channel (excitation 488nm, emission 564-606nm) to measure the surface levels of CD11b (Ly6G). Neutrophils are CD11b bright, monocytes CD11b dim and lymphocytes CD11b negative, hence the proportion of bright cells was calculated using FloJo software. This was combined with data from total cell counts to allow calculation of total neutrophil circulation.

2.25 Luminex assay to cytokine concentration

6 female CD1 mice aged 8 to 10 weeks were used per infection group. Strains were grown o/n in TH media containing appropriate antibiotic to preserve plasmids. Strains were washed three times in PBS to remove any residual antibiotic and were then prepared in tissue culture sterile PBS (Invitrogen, USA) at an OD_{600} of 20 for GAS strains and 2 for *L. lactis* strains. In all cases 50µl, containing approximately 10^8 CFU was injected intramuscularly into the thigh without anaesthesia. A control group of six mice were used which were sham infected with 50µl of sterile tissue culture PBS. At 6 hours, the mice were euthanized using a rising concentration of CO_2 and blood was collected via immediate cardiac puncture and allowed to clot for 30 minutes. The infected thigh was dissected and the tissue homogenised in 5µl/mg of tissue culture grade PBS, to allow quantification of bacterial load by plating onto CBA plates. Furthermore, the draining superficial inguinal lymph node and spleen were also dissected; the lymph node was homogenised in 200µl of PBS, and the spleen in 10µl/mg of PBS before being plated.

The clotted blood was centrifuged at 16000xg for 30 minutes. The serum layer was collected, transferred into aliquots and stored at -20^{o}C. 50µl of each of these samples was

analysed using a Mouse Cytokine 20plex Panel (Invitrogen, USA) following the manufacturer's protocol. Briefly, a mix of beads conjugated to antibodies against the 20 cytokines of the panel was added to a membrane base 96 well plate. To these, the samples - or a serially diluted standard mix of each of the 20 cytokines - were added and incubated with the beads for 2 hours to allow binding. The residual sample was removed under a vacuum, leaving the substrates attached to the beads. A mix of secondary antibodies against the 20 cytokines, each with unique fluorescent conjugates, was then added, and allowed to bind the substrates. The binding levels of each secondary antibody were measured using a Luminex platform Bio-Plex 200 system (Bio-Rad, UK). Bio-Plex manager software (version 5.0) was used to construct standard curves and calculate unknowns by comparison with the standard curves.

2.26 Confocal microscopy of phagocytosis of GAS by neutrophils

GAS strains were grown o/n in 5ml of TH media, and were then washed twice at 16000xg for 30 seconds in tissue culture PBS (Invitrogen, USA). The bacteria were resuspended in 500µl of PBS supplemented with 1% w/v BSA (Sigma Aldrich, USA) and 0.2% w/v FITC conjugated rabbit polyclonal antibody against Streptococcus group A antigen (Abcam, UK) or an isotype control. Following incubation for 30 minutes at 4^{o}C protected from light, the bacteria were washed three times in PBS followed by centrifugation at 16000xg for 30 seconds, before being finally resuspended at an OD_{600} of 7.0 in PBS supplemented with 1% w/v BSA. The bacteria were opsonised in an equal volume of fresh whole human serum as previously described. Following opsonisation, 50µl of the fluorescent bacteria was mixed with 50µl of freshly extracted human neutrophils at a final concentration of $2x10^{7}$ neutrophils/ml.

Immediately after mixing, 10µl of bacteria and cells were spotted onto a glass microscope slide and were visualised using a confocal microscope under bright field and a 488nm laser to visualise green fluorescence.

2.27 Depletion of serum of opsonin

Complement was depleted from serum by heating to 56^{o}C for 30 minutes, before being immediately cooled on ice (Morgan 2000). To the serum, two volumes of tissue culture grade PBS were added to mimic the dilution during depletion of antibody. To deplete antibody from serum, 1ml of serum was diluted with 1ml of tissue culture grade PBS. The serum was passed over a Nab Protein A/G Spin Column (Thermo Scientific, USA) three times to bind the IgG. A further 1ml of PBS was added to the column to wash any unbound serum off the column. Where serum was to be depleted of both complement and serum, serum was first depleted of antibody, before being heat inactivated. In all cases the serum was diluted in two volumes of tissue culture grade PBS either through the protocol, or prior to use. For experiments using complement or antibody depleted serum, the opsonisation protocol was modified slightly from the general protocol described earlier. Rather than using 500µl of bacteria mixed with 1000µl of PBS and 1000µl of serum, 500µl of bacteria were mixed with 2000µl of depleted serum.

2.28 Measuring opsonisation of bacteria

Bacteria grown o/n in TH media were washed in PBS and collected by centrifugation at 16000xg for 30 seconds and resuspended in PBS at an OD_{600} of 0.35. 50µl of bacteria were mixed with 100µl of PBS and 100µl of freshly extracted whole serum, and incubated with shaking for 15 minutes at 37^{o}C (to mimic the opsonisation protocol used for the flow

cytometric phagocytosis assay). Following incubation, the bacterial cells were washed three times in PBS and collected by centrifugation at 16000xg for 30 seconds, before being resuspended in 100µl of PBS supplemented with 1% BSA and placed on ice. To these, 1µl of Purified Rat Anti-Mouse CD16/CD32 (Mouse Fc Block) (BD, USA) was added as a source of non-specific non-staining antibody to reduce effects of antibody degradation by GAS. 0.5µl of each conjugated antibody or appropriate isotype controls were also added to measure deposition of opsonins: Goat anti human IgG FITC (Invitrogen, USA) conjugate was used to measure antibody deposition, and PE conjugated Anti-human complement C3b mouse monoclonal antibody (Thermo Scientific, Canada) to measure C3b deposition. This was incubated on ice, protected from light for 30 minutes. The bacteria were then washed three times in PBS and collected by centrifugation at 16000xg for 30 seconds, before being resuspended in 500µl of PBS. The samples were stored on ice before being analysed using a FACSCalibur flow cytometer (BD, USA), pre-calibrated using Calibrite 3 Beads (BD, USA). Compensation was performed using singly stained samples. 20000 bacterial events were measured, and the median fluorescence intensity of both green and red fluorescence was measured using FL1 and FL2 channels, using FloJo software for subsequent analysis. Experiments were repeated in triplicate using three independent serum donors.

2.29 Antibody staining of neutrophils

Neutrophils and bacteria were prepared as previously although with unlabelled bacteria. Following thirty minutes of incubation of bacteria with cells at 37^{o}C, 100µl was removed and immediately placed on ice. To this 1µl of relevant conjugated antibody was added, or isotype control, and incubated on ice protected from light for 30 minutes with occasional mixing. The antibodies used in the study were anti-Human CD11b (Mac-1) FITC conjugate (Invitrogen,

USA) and anti-Human IL-8/FITC (eBiosciences, UK). After incubation, the cells were washed three times at 1000xg for 3 minutes to remove unbound antibody. Cells were resuspended in 500µl of tissue culture grade PBS and stored on ice until analysed using a FACSCalibur flow cytometer (BD, USA), pre-calibrated using Calibrite 3 Beads (BD, USA). Cells were initially positively gated on the basis of their morphology, as judged by forward and side scatter. 20000 of these cells were counted and the median fluorescence intensity of green fluorescence was measured in comparison to isotype controls. Data were later analysed using FloJo software. Experiments were repeated in triplicate using independent neutrophil donors. Further experiments were performed using recombinant human chemokines CXCL5, 8 and 10 (Peprotech, UK) before measuring the surface levels of CD11b. For this, 50µl of freshly extracted neutrophils at a concentration of $2x10^6$ were mixed with chemokine serially diluted in 50µl of tissue culture grade PBS (Invitrogen, USA) and incubated at 37^oC for 15 minutes, before being placed on ice. Staining and subsequent flow cytometric analysis was identical to that described above.

2.30 Isolation of neutrophil proteins

Freshly extracted neutrophils at a concentration of $3x10^7$ cells/ml were lysed in 1ml of lysis buffer (150mM NaCl (VWR, USA), 1% Triton X-100 (Sigma Aldrich, USA), and 50mM Tris (VWR, USA) pH8). It was found that without the addition of a broad range of small molecule protease inhibitors, no CD11b could be isolated, thus Calbiochem protease inhibitor cocktail III (Novagen, USA) was included at 10% v/v of final volume (Edwards *et al.* 2005). However, protease inhibitor cocktail III includes serine protease inhibitors, at a sufficient concentration to inhibit SpyCEP activity, hence the lysate was passed through a Amicon Ultra 10kDa protein column (Millipore, USA) to remove the protease inhibitors.

2.31 Preparation of a chemokine receptor antagonist

Chemokine receptor CXCR2 antagonist PD0220245 99% (Axon Medchem, Netherlands) was reconstituted at 107µg/ml in dH_2O, twenty times the IC_{50} of CXCL8 binding to CXCR1 (Li, *et al.* 2003) as a ten times concentrated stock. The antagonist was used for experiments measuring phagocytosis of FITC labelled bacteria and in bacterial growth curves. For phagocytosis assays the antagonist was added at 1 times working concentration (10.7µg/ml) to the neutrophils for 15 minutes at 37^{o}C prior to the addition of the opsonised bacteria. For growth curve work, the antagonist was added at 0.5, 1 and 2 times working concentration 90 minutes subsequent to the initial sub-culturing step. Growth curve experiments with the antagonist were repeated in triplicate.

2.32 Expression of recombinant CEP5 protein

Turner *et al*. 2009 expressed an N terminal fragment of the *cepA* gene, CEP5, which proved able to effectively vaccinate mice against GAS infection. The pET100 plasmid used in this work, which expresses CEP5 with a 6xHis tag under the control of an IPTG inducible promoter, was extracted and transformed into BL21 expression *E. coli* cells (Invitrogen, USA) using standard transformation protocol. These cells were transferred into fresh liquid LB media immediately after recovery in SOC media (Invitrogen, USA) and grown overnight with ampicillin supplementation to maintain the plasmid. After 16 hours of growth at 37^{o}C with shaking, the bacteria were subcultured at a 1 in 20 dilution into fresh LB media containing antibiotic. After the bacteria had reached an OD_{600} of 0.5-0.8, further antibiotic was added with 1mM of IPTG to induce expression. After three hours, the bacteria were harvested by centrifugation at 2500xg for 10 minutes. The bacterial pellet was lysed using

3ml of BugBuster Protein Extraction Reagent (Novagen, USA) per 50ml of original bacterial culture, with vigorous shaking for 20 minutes. The resultant lysate was centrifuged at 16000xg for 20 minutes to pellet any insoluble debris and the supernatant was applied to a pre-charged nickel resin column. The column was prepared using a Novagen Histag Kit (Novagen, USA) following manufacturer's instructions. Briefly, per gravity flow column, 2ml of a slurry of nickel ion resin was washed with 3ml of dH_2O, 5ml of a charge buffer, then 3ml of binding buffer to optimise the binding of the His tag to the Ni resin. 3ml of bacterial lysate was applied to each column twice, before a further 10ml of binding buffer was added to enhance the histidine-nickel interactions. The column was then washed with 12ml of wash buffer before the CEP5 was eluted in elution buffer. The elutate was immediately mixed with an excess of PBS to prevent degradation of the protein in the elution buffer. The protein was dialysed and concentrated by repeated centrifugation and PBS buffer replacement using Amicon Ultra 10kDa protein columns (Millipore, USA). The protein was concentrated to 2mg/ml in tissue culture grade PBS.

2.33 Vaccination model

4-6 week old Balb/C mice were used (Turner*, et al.* 2009b). One group of 15 received the full vaccination protocol, and 15 mice received a sham vaccination protocol, with the aim of challenging mice in each group with either H292 or H887. The full vaccination protocol used 25µl of 2mg/ml (50µg) CEP5 protein in sterile PBS that was thoroughly mixed with 25µl of Freund's Complete (first prime vaccination) or Incomplete (second and third boost vaccination) adjuvant (Turner*, et al.* 2009b). The first prime vaccination was given at day 0 of the experiment. At day 14 and 21, boost vaccinations were given. The sham vaccinees underwent the same procedures, but were injected with PBS + adjuvant rather than

recombinant protein. In all cases, vaccination was intramuscular into the thigh tissue, alternating between thighs throughout the protocol. At day 24, tail bleeds were obtained from 3 mice in each group (Chapter 4), allowed to clot for 30 minutes, and then centrifuged at 16000xg for 20 minutes. Collected serum was used to confirm immunity using a dot blot method. On day 28, mice were challenged with GAS strains (washed three times in tissue culture grade PBS) at an OD_{600} of 20. 50µl, containing approximately 10^8 CFU was injected intramuscularly into the thigh without anaesthesia. Six mice received H292, six received H887 and three a sham PBS infection. At twenty four hours post-infection, the mice were euthanized using a rising concentration of CO_2 and blood was collected via immediate cardiac puncture. This was transferred to sodium heparin. The infected thigh was dissected and the tissue homogenised in 5µl/mg of tissue culture grade PBS, to allow quantification of bacterial load by plating on CBA plates. Furthermore, the draining inguinal lymph node, spleen and liver were also dissected; the lymph node was homogenised in 200µl of PBS, and the spleen and liver in 10µl/mg of PBS before being plated. The blood was also plated.

2.34 Statistical analysis

Any statistical analysis was performed using GraphPad Prism 5. All data sets were non-parametric; where two groups were compared, Mann-Whitney tests were performed. For comparison between more than 2 groups, a Kruskal Wallis test (non-parametric one way ANOVA) was performed. P values of less than 0.05 were considered statistically significant, and is labelled throughout this work with *, P values of less than 0.01 are indicated by **, and less than 0.001 indicated ***

Chapter 3 - Results: Substrate specificity and kinetics of SpyCEP and its orthologues

3.1 Introduction

Previous work on SpyCEP has investigated its substrate range and role in GAS virulence. The first substrates discovered were human CXCL8, and its murine orthologues MIP-2 and KC (Edwards, *et al.* 2005). These were implicated due to clinical observations that neutrophils were scarce at infection foci during necrotizing fasciitis. Chemokines play a vital role in the early immune response, activating and attracting anti-microbial neutrophils to the site of infection. Other neutrophil chemoattractants were subsequently found to also be substrates of SpyCEP. CXCL1, CXCL6 (Sumby, *et al.* 2008) and CXCL2 (Kurupati, *et al.* 2010) were next discovered. Whilst these chemokines include many of the neutrophil specific chemokines, they do not include many other chemokine ligands that bind either chemokine receptor expressed by the neutrophil (Table 3-1). A major aim of this chapter was to explore the substrate range of SpyCEP with particular regard for the chemokines cleaved and rate of cleavage.

Systematic name	Functional name	ELR motif	Responsive cell type	Known receptor specificity
CXCL1	GROα	+	Neutrophil	CXCR1>CXCR2
CXCL2	GROβ	+	Neutrophil	CXCR2
CXCL3	GROγ	+	Neutrophil	CXCR2
CXCL4	PF-4	-	Fibroblast	unknown
CXCL5	ENA-78	+	Neutrophil	CXCR2
CXCL6	GCP-2	+	Neutrophil	CXCR1, CXCR2
CXCL7	NAP-2	+	Neutrophil	CXCR1, CXCR2
CXCL8	IL-8	+	Neutrophil	CXCR1, CXCR2
CXCL9	MIG	-	natural killer cell	CXCR3
CXCL10	IP-10	-	natural killer cell; macrophage	CXCR3

Table 3-1- List showing CXC1-10 chemokines

Adapted from (Pease 2011)

Subsequent to the commencement of this study, recombinant SpyCEP was expressed, and used to determine that, CXCL3, 5, 7 and also to a limited extent CXCL10 could be cleaved by SpyCEP (Zingaretti, *et al.* 2010) in addition to the previously determined substrates. With the exception of CXCL10, this forms a more rational list of substrates, including all chemokines able to bind CXCR1 and/or 2, offering an explanation of specificity. CXCL10 has been shown to be directly antibacterial towards GAS, hence this apparent cross-reactivity may be beneficial for GAS (Egesten, *et al.* 2007). Importantly, SpyCEP-cleaved substrates were identified in those studies using the recombinant protein only, and human substrates cleaved by native SpyCEP were unknown except for CXCL8, CXCL1, CXCL2 and CXCL6 at the start of this project (Edwards, *et al.* 2005, Hidalgo-Grass, *et al.* 2006, Sumby, *et al.* 2008).

3.2 Results

3.2.1 Substrate range of SpyCEP

To investigate the substrate range of SpyCEP, two strains of GAS were used, H292, a high SpyCEP producing clinical isolate, and H575, an isogenic *cepA* mutant of H292. The expression of SpyCEP by H292, and its ablation in H575 were confirmed using immunoblotting with an antibody raised against the N-terminus of SpyCEP (Results not shown but have been repeated previously (Kurupati*, et al.* 2010)). To ensure that the role of SpyCEP was not to allow the maturation of an alternative protease exported via the ExPortal, SpeB levels of an equal volume of H292 and H575 supernatants were measured using immunoblotting. SpeB has previously been used as a marker for external protein maturation (Cole*, et al.* 2007). Supernatant was taken from both strains of GAS grown in liquid media at various growth phases: mid-logarithmic, late-logarithmic and stationary (overnight growth). In each case, the amount of SpeB was identical between H292 and H575, showing that SpyCEP ablation did not affect the function of the ExPortal (Figure 3-1).

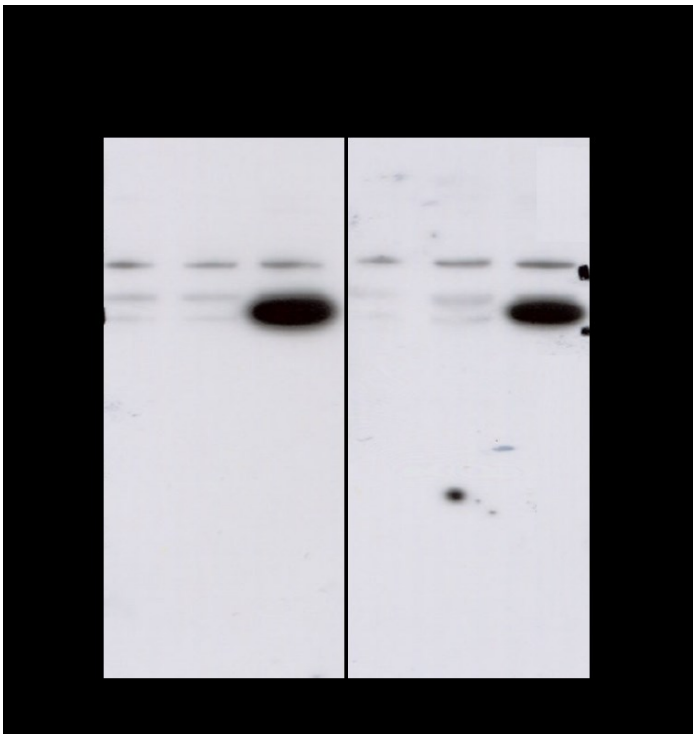


Figure 3-1– Immunoblotting of isogenic bacterial supernatants against SpeB

Supernatant of bacterial cultures were collected at specified stages of growth, subject to LDS-PAGE and transferred to hydrophobic membrane, before being probed using anti-SpeB mAb. SpeB is known to be expressed in stationary phase. o/n overnight culture

The known substrate range of SpyCEP has expanded from the originally described activity against CXCL8 to include some of the other CXC chemokines (Edwards*, et al.* 2005, Kurupati*, et al.* 2010). This list does not include all the ELR+ chemokines, nor does it include all those chemokines which bind CXCR1 and 2. A list of such chemokines is shown in Table 3-1. Linear polypeptides representing sequences flanking the CXCL8 cleavage site are not cleaved by SpyCEP (Kurupati, unpublished data). As no common primary or secondary structural features have been found in common between the known substrates of SpyCEP, the structural basis for substrate specificity remains unknown. To determine the wider substrate

range of SpyCEP, all known CXCL chemokines were incubated with the both supernatant and washed whole cells from H292 and H575, and the samples analysed by gel electrophoresis.

When incubated with supernatant from wild type GAS H292 o/n, CXCL1, 2, 3 and 8 were cleaved, as evidenced by a clearly defined reduction in the apparent molecular weight compared to untreated chemokine (Figure 3-2). Similar work examining SpeB cleavage of chemokines showed chemokines incubated with SpeB completely disappeared from protein gels, indicative of the non-specific mode of action of SpeB (Egesten*, et al.* 2009). Such activity was not observed using these strains. The band of reduced size appears to demonstrate that one discrete cleavage reaction had taken place. CXCL5 and CXCL6 underwent partial cleavage by SpyCEP, evidenced by two bands, one at the same molecular weight as the native chemokine, one of a reduced molecular weight. These reductions in weight were not seen when incubated with the SpyCEP-negative GAS H575, showing that SpyCEP alone is responsible for this apparent cleavage (Figure 3-2). CXCL 4, 7, 9 and 10 were not cleaved by either strain (Figure 3-2), nor were CXCL 11-16 (results not shown).

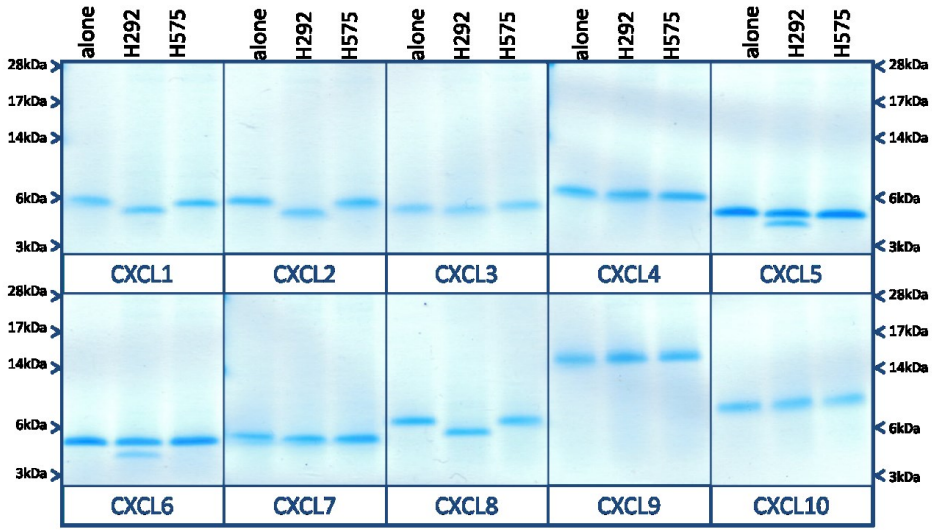


Figure 3-2 – Chemokine cleavage by GAS supernatant

500ng of the chemokines CXCL1-10 (Table 3-1) were incubated with supernatant of either wild type *GAS* (H292) or an isogenic *cepA* mutant (H575). These were examined by protein electrophoresis and stained with colloidal blue.

The same range of chemokines, when incubated with washed live cells of H292 or H575 o/n, revealed an extended substrate range compared with using supernatant alone. Once again, H575 was not able to cleave any chemokines, demonstrating all the cleavage effects seen were due to SpyCEP. In addition to those cleaved by supernatant, CXCL5, 6 and 7, and to a lesser extent CXCL10 were cleaved by H292 cells (Figure 3-3). Interestingly, CXCL5 was cleaved yielding two bands, perhaps indicating two SpyCEP cleavage sites within this chemokine. The range found comprises the entire repertoire of CXC chemokines which bind to CXCR1 and/or 2 and contain the ELR motif, essential for neutrophil chemotaxis (Table 3-1), along with CXCL10 at a low level. CXCL4 and 9 were not cleaved by SpyCEP. These chemokines neither bind CXCR1 or 2, nor have an ELR motif. These findings indicate that the probable substrate recognition sequence of SpyCEP is very similar to that of CXCR1/2.

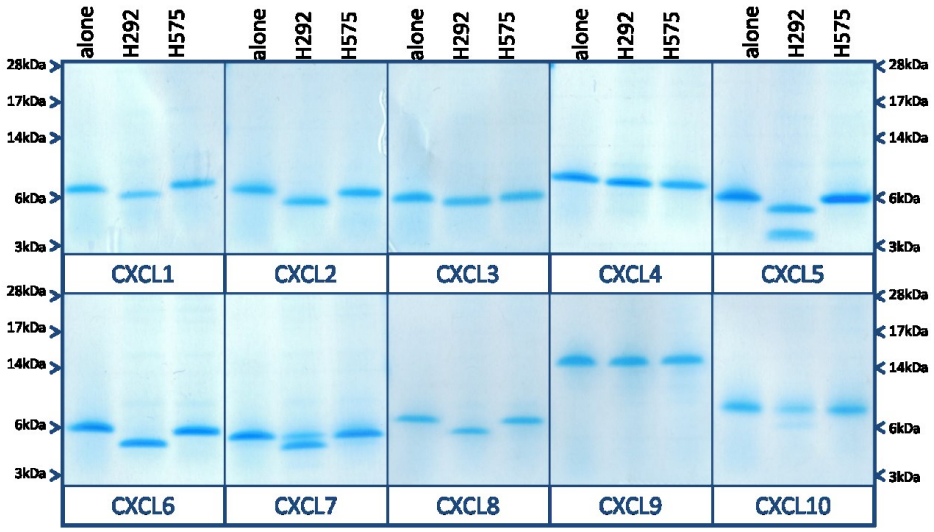


Figure 3-3 - Chemokine cleavage by GAS cells

500ng of the chemokines CXCL1-10 (Table 3-1) were incubated with 2µl of either washed wild type *GAS* cells (H292) or an isogenic *cepA* mutant (H575). These were examined by protein electrophoresis and stained with colloidal blue.

In addition to these substrates, other proteins were tested to examine any further cleaving activity of SpyCEP. These included IL-1β, IL-6, complement protein C3, collagen, fibrinogen, fibronectin and RANTES (Results not shown). No evidence of any cleavage was found using this method. This, along with previous work showing that the substrate range of SpyCEP is limited to the CXC chemokines indicates that the substrate range of SpyCEP includes all, but is restricted to, the ELR+ CXC neutrophil tropic chemokines (Edwards, *et al.* 2005, Hidalgo-Grass, *et al.* 2006, Kurupati, *et al.* 2010, Sumby, *et al.* 2008). Subsequent to this work, full length SpyCEP was expressed recombinantly in *E. coli* and its substrate range was investigated (Zingaretti, *et al.* 2010). The substrate range in that study was identical to that found in this work, including the slight cleavage of CXCL10. The authors used the recombinant protein to investigate structural features of SpyCEP. As the recombinant protein

showed substrate range identical to SpyCEP expressed by GAS, we can be confident that the recombinant protein is highly similar to SpyCEP expressed by GAS. Thus we can have confidence that the structural conclusions drawn in this work are applicable to SpyCEP expressed by GAS.

L. lactis strains H486 and H487 have previously been used as a model organism for *in vivo* work to examine the effects of SpyCEP on virulence (Kurupati*, et al.* 2010). These strains contain a plasmid carrying the *cepA* gene (H487) with its native promoter and LPXTG sortase motif (pDESTcepA) (Zinkernagel*, et al.* 2008), or the empty plasmid (H486). Previous work using this strain has shown that SpyCEP is essential and sufficient for virulence in soft tissue infection (Kurupati*, et al.* 2010).

The expression of SpyCEP by the SpyCEP-positive *L. lactis* strain H487, and its absence from the SpyCEP-negative *L. lactis* strain H486 was confirmed using immunoblotting (Results not shown) (Zinkernagel*, et al.* 2008). These strains were investigated for their chemokine cleavage to further confirm that the effects observed were solely due to direct SpyCEP activity, rather than a combination of streptococcal proteases that are processed or activated by SpyCEP. They were further investigated to confirm that they were an appropriate model organism to use in subsequent studies. Supernatant of H486 and H487 was incubated with the entire repertoire of CXC chemokines o/n, and it was observed that the supernatant of H487 could cleave CXCL 1, 2, 3 and 8, whereas the H486 showed no such activity (Figure 3-4).

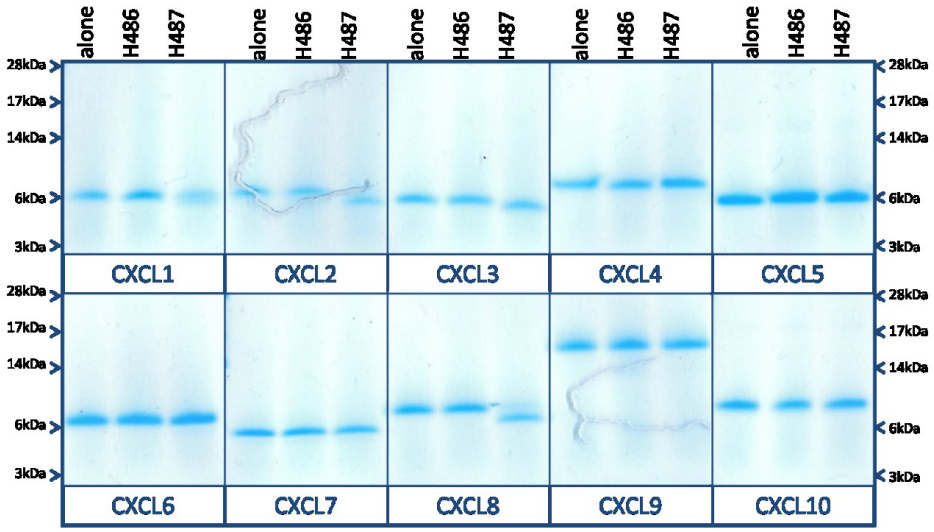


Figure 3-4 – Chemokine cleavage by L. lactis supernatant

500ng of the chemokines CXCL1-10 (Table 3-1) were incubated with supernatant of either SpyCEP-negative *L. lactis* (H486) or SpyCEP-positive *L. lactis* (H487). These were examined by protein electrophoresis and stained with colloidal blue.

The experiment was repeated using washed live cells. Here all the neutrophil tropic chemokines underwent some cleavage (Figure 3-5). CXCL10 was not cleaved by *L. lactis*, a difference compared to GAS, and once again, CXCL4 and 9 are not cleaved.

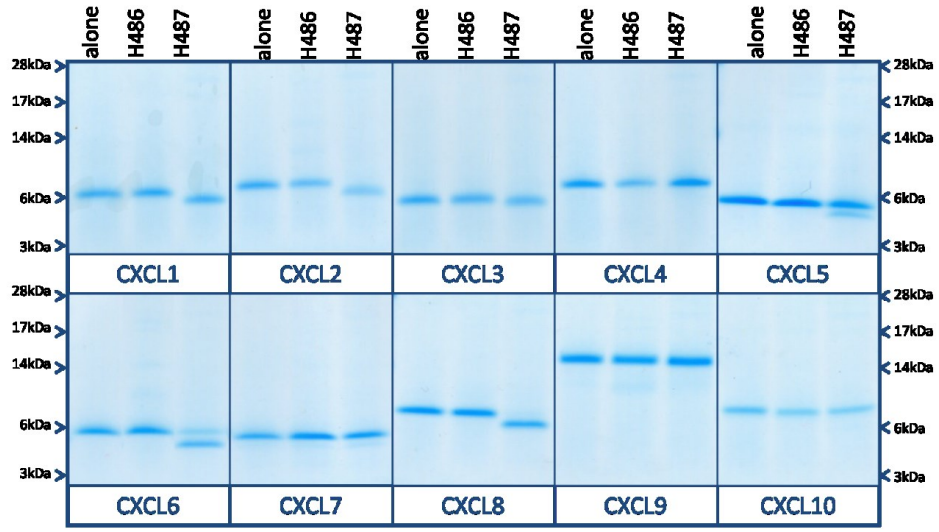


Figure 3-5 – Chemokine cleavage by L. lactis cells

500ng of the chemokines CXCL1-10 (Table 3-1) were incubated with 2µl of either washed wild type *L. lactis* cells (H486) or containing pDESTcepA (H487). These were examined by SDS-PAGE and stained with colloidal blue.

These data, when coupled to the results seen previously with GAS, confirmed that SpyCEP is responsible for the chemokine cleavage observed, and further demonstrate that the specificity of SpyCEP mirrors that of CXCR1/2. However, chemokine cleavage by *L. lactis* seemed less efficient than that by GAS. For instance, *L. lactis* cells were only able to partially cleave CXCL6, and intact chemokine seems to be the dominant form with CXCL5 and CXCL7, whereas these were all fully cleaved by GAS H292 cells. The expression level of SpyCEP by these strains was examined to ensure that these differences were simply due to differences in expression. Supernatants of H292, H575, H486 and H487 were examined by ELISA using antibody specific against the N terminus of SpyCEP (Figure 3-6).

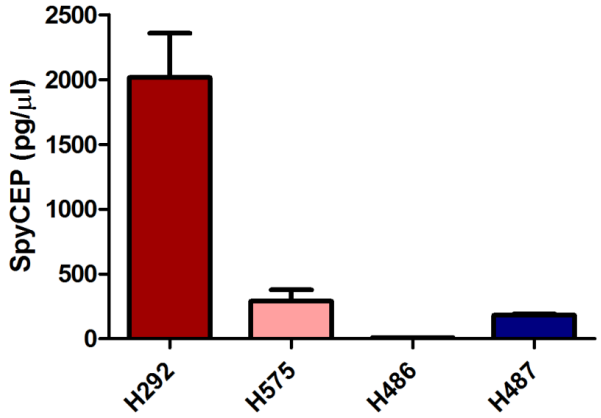


Figure 3-6 – ELISA measuring SpyCEP in bacterial supernatants

Supernatants collected after 16 hours of growth of each of the four strains were tested by ELISA to measure concentration of SpyCEP. Bars indicate the average of three separate wells. Data represents the mean of three separate experiments (+standard deviation).

As expected, GAS strain H292 expresses much more SpyCEP than H487, offering an explanation of the differences observed in substrate cleavage. SpyCEP is also detectable in the supernatant of H575. This is because H575 is able to make an N terminal fragment of SpyCEP which is detected by the antibody used in this ELISA.

3.2.2 Rate of chemokine cleavage by SpyCEP

The work of this chapter, coupled with work by other groups (Zingaretti*, et al.* 2010), demonstrated conclusively that SpyCEP cleaved all neutrophil specific CXCL chemokines, CXCL1, 2, 3, 5, 6, 7 and 8. However, the results of the protein gel electrophoresis suggested that SpyCEP was not able to cleave all chemokines equally efficiently. Several chemokines were only partially cleaved by bacterial supernatant, whereas others seemed to be fully

cleaved. Work was hence undertaken to determine the cleavage rates by SpyCEP of these chemokines. The cleavage rate of CXCL8 has been calculated in previous work using bacterial supernatant incubated with chemokine for 24 hours. The rate of reaction of most chemical reactions is dependent on the concentration of substrate, unless the substrate is at very high, saturating, concentrations, thus as a reaction continues, its rate will vary. Measuring rate of reaction over a long duration will produce an average of a constantly changing reaction rate (Figure 3-7). However, the studies typically used to measure Michaelis-Menten constants using exceptionally short reaction times are not applicable for this work, as we cannot measure the emergence of the cleaved chemokine products.

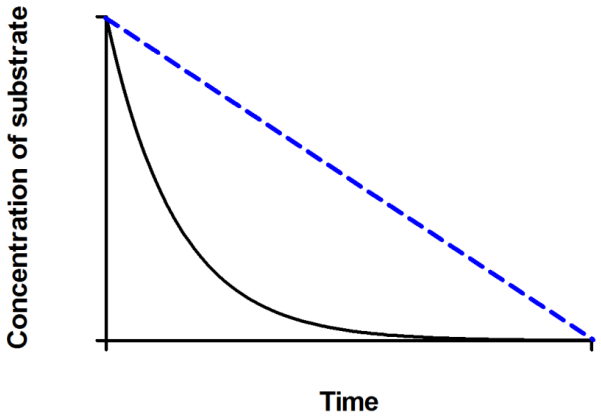


Figure 3-7 – Idealised rate of reaction curve

As the concentration of substrate declines over time, the gradient (rate of reaction) decreases. Blue dotted line indicates average rate of reaction which is measured

Edwards *et al*. used an ELISA based method to determine the cleavage rate of CXCL8. This method is limited in that we can only measure the loss of substrate rather than the emergence of product. Unfortunately, as we have no straightforward method to measure the emergence of the product, the ELISA based method was also used in this study using kits

which were readily available, for CXCL1, 2, 5, 6 and 8. ELISA kits for CXCL3 and 7 were not readily available.

This method was dependent upon the specific antibodies in the ELISA kits being able to specifically bind only uncleaved recombinant chemokine, but not SpyCEP cleaved recombinant chemokine. Immunoblotting was hence carried out to confirm this using recombinant chemokine alone and chemokine treated with either H292 or H575 cells o/n. Chemokine alone and chemokine treated with SpyCEP-negative cells were detected by the specific antibodies as expected at the predicted full length molecular weight. Chemokine incubated with SpyCEP-positive cells either disappeared completely from the immunoblot, or appeared as a fainter band of identical molecular weight to uncleaved product, indicative that the cleavage reaction had not gone to completion. In all cases, no chemokine was detected at a lower molecular weight (Results not shown). These results show that the all the antibodies to be used in the ELISA kits are specific for uncleaved chemokine, and can thus be used to measure rate of reaction.

The method used briefly comprised incubating 1ng/ml of recombinant chemokine with bacterial supernatant or washed cells for 1, 2 and 24 hours. The reaction was stopped using specific protease inhibitors and ice, based on previous studies (Edwards *et al.* 2005). The remaining chemokine concentration was measured using ELISA. The difference in the remaining chemokine between the SpyCEP-positive and negative strains was measured to allow a measure of SpyCEP specific activity. As the rate of enzymatic reaction is dependent on chemokine concentration, the shortest time point where chemokine concentration differences could be accurately measured for all substrates were used. Conversely, the ELISA-based method measures the loss of a substrate rather than the emergence of a product,

and as such, we had to wait for a sufficiently long time point so as to achieve a significant loss of the substrate to be detectable.

3.2.2.1 Chemokine cleavage rates by GAS supernatants

At 1 and 2 hour timepoints, no SpyCEP specific cleavage was detectable against any chemokines except CXCL8. However, by 24 hours, SpyCEP specific activity could be detected. Edwards, *et al.* also used a 24 hour timepoint to measure chemokine cleavage. The fastest rate of reaction was observed for SpyCEP cleaving CXCL8, with a rate of 1.5pg/hr/µl (Figure 3-8). This compared with 10pg/hr/µl over the first 8 hours of incubation reported in the previous work (Edwards, *et al.* 2005), indicating the method used in this study produced comparable results to those previously reported. CXCL6 was cleaved at a rate of 1.0pg/hr/µl, CXCL2 at 0.4pg/hr/µl and CXCL1 at 0.3ph/hr/µl. This result was somewhat unexpected, Figure 3-2 shows CXCL1 and CXCL2 being cleaved completely, whereas CXCL6 appeared only to be partially cleaved. More accordingly with Figure 3-2, CXCL5 had the slowest cleavage rate at 0.1pg/hr/µl. The rate of rate reaction measured closely mirrors the order of discovery of substrates. CXCL8, 1 and 2 were among the first discovered, whereas CXCL5 was only found to be a substrate in this work and using recombinant SpyCEP (Zingaretti*, et al.* 2010).

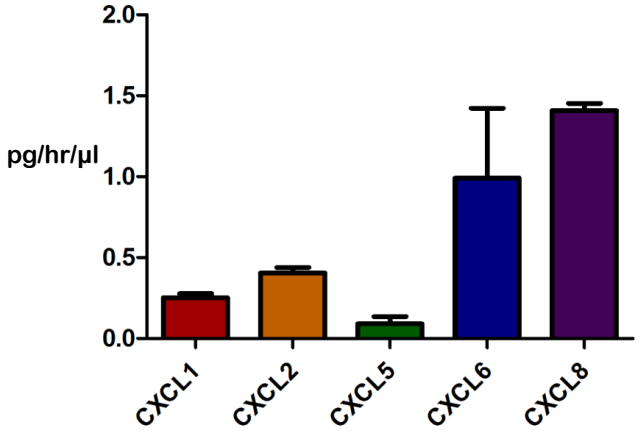


Figure 3-8 – Chemokine cleavage rates by GAS supernatant

GAS supernatants were incubated with 1ng/ml of chemokine for 24 hours, and the average rate of reaction was calculated. Error bars indicate standard deviation of three independent experiments.

3.2.2.2 Chemokine cleavage rates by whole GAS cells

Whole washed cells were next incubated with the recombinant chemokine. As the cells used in this method were alive, they were actively producing SpyCEP, so along with SpyCEP bound to the cell surface, soluble SpyCEP was also present at a rising concentration. Thus, this is a more accurate representation of bacteria in an infection site than measuring rate using supernatant. All chemokines were observed to have undergone measurable cleavage after just one hour, thus this was used to calculate rate. Whilst this means that the values obtained were a more accurate measure of rate of reaction when substrate concentration is 1ng/ml, the values cannot be directly compared to those for the bacterial supernatants. Trends however remain comparable.

The fastest cleavage rate using bacterial cells was again measured for CXCL8, at 709pg/hr/10^6CFU. CXCL2 was cleaved at a rate of 115pg/hr/10^6CFU and CXCL1 at 110pg/hr/10^6CFU. CXCL6 was cleaved more slowly at rate of 50pg/hr/10^6CFU, and CXCL5 at 27pg/hr/10^6CFU (Figure 3-9). This data matches better the chemokine cleavage data obtained using protein gel electrophoresis, whereby CXCL1, 2 and 8 were cleaved by SpyCEP containing supernatants and washed cell preparations, and CXCL5 and 6 were only cleaved partially. The CXCL16 negative control, known not to be a substrate of SpyCEP, showed no cleavage.

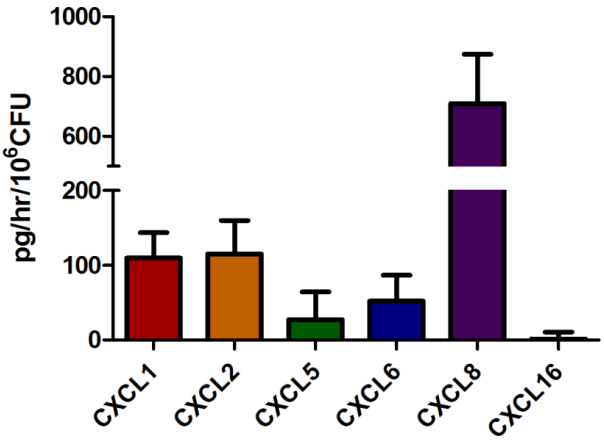


Figure 3-9 – Chemokine cleavage rate by live GAS cells

Live GAS cells were incubated with 1ng/ml of chemokine for 1 hour, and the average rate of reaction was calculated. Error bars indicate standard deviation of three independent experiments.

3.2.3 SpyCEP orthologues in species related to GAS

Previous work has identified various SpyCEP orthologues in related pathogenic streptococci which share secondary sequence homology (Turner, unpublished data; Table 1-1). All the proteases examined have a very similar structure to SpyCEP, consisting of an N-terminal signal sequence, a protease domain bisected by a PA (protease associated) domain, with a C-terminal sortase motif. Their homology is concentrated around the protease domain, with the greatest variation being in the C-terminal region of the protein.

Supernatants of the related streptococcus *S. pneumoniae* were incubated with a variety of substrates including IL-1β, IL-6, C3, collagen and RANTES, as well as the CXCL chemokines, to determine if any cleavage was observed. However, the resultant gel electrophoresis analysis did not demonstrate cleavage of any CXCL chemokines, or the other substrates tested. Supernatant of *S. pneumoniae* was however able to cleave the α and β chains of fibrinogen, an observation that has not previously been observed, which we speculate may be attributable to the SpyCEP orthologue PrtA (Figure 3-10) (Hermans and McDonagh 1982). *S. pneumoniae* undergoes autolysis, leading to the release of cytosolic proteins into the supernatant, hence many putative enzymes may mediate the observed cleavage (Martner*, et al.* 2009). Although biochemical purification might allow identification of the responsible protease, the development of an *L .lactis* strain expressing PrtA would allow definitive identification of its substrates.

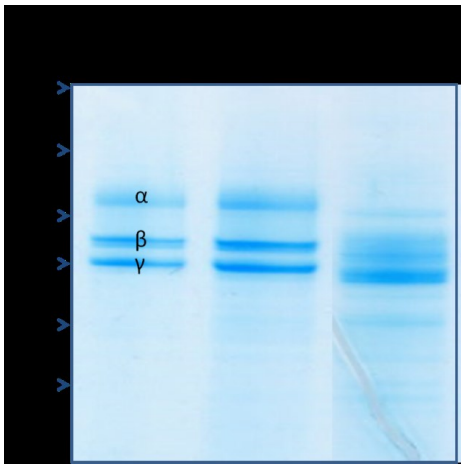


Figure 3-10 – Fibrinogen degradation by S. pneumoniae supernatant

500ng of the fibrinogen was incubated with 7µl of either supernatant from overnight growth of either *GAS* (H292) or *S. pneumoniae* (H400). These were examined by SDS-PAGE and stained with colloidal blue.

For many of the target Gram positive pathogens, genetic manipulation is very difficult and potential virulence implications of these proteases may be missed due to redundancy between virulence factors. The data above, coupled with previous work (Kurupati*, et al.* 2010, Zinkernagel*, et al.* 2008) demonstrate the advantages of *L. lactis* as a heterologous expression system for Gram positive proteases.

3.3 Discussion

3.3.1 Substrate range of SpyCEP

This section of the study examined the substrate range of SpyCEP and the rate at which SpyCEP was able to cleave these substrates. Previous work using GAS products reported an incomplete list of SpyCEP substrates and failed to provide a coherent description of the specificity of SpyCEP. Interestingly, work examining CspA of *S. agalactiae* identified a similar substrate range of the CXCL chemokines (Bryan and Shelver 2009). Here however, the protease was not limited to the neutrophil specific chemokines, cut the chemokines at many sites indicating poor site specificity, and failed to cleave CXCL8 at all. The authors could not offer explanations for these findings (Bryan and Shelver 2009).

Work by Zingaretti *et al.* 2010 provided a more complete and logical substrate list, using a recombinant SpyCEP preparation, however these findings had not been repeated using a GAS strain. This work found an identical substrate range to that found by Zingaretti *et al.*, including the low level cleavage of CXCL10 by GAS cells. This work hence validates the findings made using the recombinant enzyme. Furthermore, it provides the logical substrate list for SpyCEP; it cleaves all proteins that bind to chemokine receptors CXCR1 and CXCR2, and presumably its active site shares some structural homology with the binding domains of these receptors. Chemokines bind to their cognate receptors in a two-step process, firstly, binding GAGs with their C termini, before the N terminus binds to the receptor itself. This two-step binding process provides an explanation as to why cleaving a small fragment from chemokine can prevent their ability to bind and trigger receptor signalling (Edwards *et al.*

2005, Zingaretti *et al.* 2008) – SpyCEP may interfere with the initial GAG interaction. By cleaving all chemokines which act on neutrophils (Table 3-1), we hypothesise that SpyCEP will have wide ranging activity on the function of neutrophils in an infection setting.

It seemed likely that the variable substrate patterns found using the GAS and *L. lactis* supernatants and cells was due to varying concentrations of SpyCEP. This especially impacted on the results observed using poorly cleaved substrates, such as CXCL5. It was indeed found that GAS supernatants contained much more SpyCEP than those of the SpyCEP+ve *L. lactis* strain (Figure 3-6). Indeed, H292, the SpyCEP-positive GAS strain used in this work was originally highlighted due to its exceptionally high SpyCEP expression (Edwards*, et al.* 2005). If other groups examining substrate specificity have utilised a strain which expresses less SpyCEP, it would be expected that the apparently poorly cleaved substrates such as CXCL5 would be overlooked. Cell preparations did not have SpyCEP expression levels measured using ELISA, as ELISAs cannot be performed against cell bound proteins.

It is unclear why SpyCEP-positive *L. lactis* strain H487 produces so much less soluble SpyCEP than wild type GAS H292. The mechanism by which SpyCEP is released from the surface of GAS is unclear. It is hypothesised to occur due to the sortase mechanism being too slow to catalyse reactions using all the SpyCEP produced. However, it may be that GAS actively releases SpyCEP from its surface using an enzyme not expressed by *L. lactis*. It is known that SpyCEP does not catalyse its own release from the GAS cell wall (Zingaretti*, et al.* 2010).

3.3.2 Rate of chemokine cleavage by SpyCEP

The rate calculations made in this section further support the idea that the newly confirmed substrates of SpyCEP are those which are least efficiently cleaved by SpyCEP. The reason for this reduced rate of substrate cleavage may be caused by the ability of SpyCEP to bind the substrate, or its ability to release cleaved products. The rates of reaction found show that CXCL8 is the optimal substrate of SpyCEP with the fastest cleavage rate. A second group of substrates including CXCL1, 2 and 6 appear as a group with similar cleavage rates, cleaved more slowly than CXCL8, yet better substrates than CXCL5. This pattern matches the order of discovery of proteins as substrates of SpyCEP, CXCL8 was the first discovered, followed by CXCL1, 2 and 6, with CXCL5 only being found to be a substrate using recombinant SpyCEP or in this study using the live cells of a high SpyCEP producing strain.

It is unfortunate that rate calculations could not be carried out using CXCL3 and 7. These have also not been previously observed as SpyCEP substrates using GAS products, hence it would be expected that their rate of reaction would be slow. CXCL7 remains only partially cleaved when incubated with GAS cells, so it would be expected that its cleavage rate would be even slower than that of CXCL5 (Figure 3-3). Such results would have further validated this method.

The ELISA based method used to determine rates of reaction has been previously used by other groups (Edwards*, et al.* 2005), however does have limitations. The classical way to determine enzyme kinetics and rate constants has been to perform short time point experiments measuring the rate of emergence of product at various substrate concentrations. By using such short time points, the assumption that the concentration of substrate does not

change throughout the period measured can be safely made. Unfortunately, we did not have a method to measure the emergence of all the chemokine products, and thus had to measure the loss of substrate. Antibodies have been made against the C terminus of cleaved CXCL8, which only detect cleaved CXCL8. While this would be useful in future work, without similar such antibodies against the other cleaved products, fair comparisons cannot be made. This method, by definition, cannot assume constant concentration of substrate, and rate constants cannot be determined.

The values obtained using this method are not constants, rather they are the rates of reaction observed when using a particular set of experimental conditions and a particular concentration of substrate. Although this limits their utility in predicting the cleavage rate when using different substrate concentrations, they do allow us to compare the individual substrates, as the experimental conditions did not change. The concentrations of each substrate were set at a concentration of 1ng/ml, rather than a fixed number of moles, in order to better fit the outputs made by the ELISA kits. However, due to the structural similarity between these substrates, including in their molecular weight, the effect on the rates calculated would be very small.

The rates calculated demonstrated a very large discrepancy between the rates per µl of supernatant and per 10^6CFU. This was caused by two separate effects. The values calculated for the supernatant were taken over 24 hours, and are hence an average of relative fast rates early in the time course and much slower rates later in the time course (Figure 3-7), whereas the values calculated using cells were over a one hour time course. The other factor influencing rate calculations is the difference in amount of SpyCEP in the reaction mixtures. An immunoblot was carried out loading the same amount of washed cells and supernatant as used in the rate reactions. The SpyCEP specific bands seen using the cell preparation were

much brighter than those seen using the supernatants, indicating there is indeed much more SpyCEP in the cell preparation (Results not shown). Thus, the two separate experiments cannot be compared. As the washed live cells were producing SpyCEP throughout the experiment, they more accurately mimic an infection setting of SpyCEP activity.

Chapter 4 - Results: Impact of SpyCEP in the very early phase of the neutrophil response to GAS infection

4.1 Introduction

Neutrophils are cells capable of delivering highly toxic compounds to infection sites with accuracy against the invading organism. Their potency against pathogens is such that they must be tightly regulated to prevent damage against tissue. Neutrophils are produced at high levels in the bone marrow, where they are stored until required to clear infection. It is estimated that in a healthy human, 98% of neutrophils are stored in the bone marrow (Amulic *et al.* 2012). They are retained in the bone marrow through a balance between CXCR4 and CXCR2 signalling (Figure 1-5). Furthermore, they have an extremely short half-life of six hours (Furze and Rankin 2008b), which some have suggested is to ensure that they maintain proper membrane integrity, preventing unwanted leakage of these antimicrobial toxic compounds from neutrophil granules (Amulic*, et al.* 2012).

Work has shown that CXCL chemokines have a role in potentiating the release of neutrophils from the bone marrow as summarised in Figure 1-5. The CXCR2 signalling by CXCL chemokines acts to both inhibit CXCR4 signalling (which constitutively acts to hold neutrophils in the bone marrow), and to provide a gradient to leave the bone marrow. This balance between CXCR4 and CXCR2 signalling means that a small increase in CXCR2 agonists gives rise to a large increase in circulating neutrophils (Furze and Rankin 2008b).

Neutrophils are known to play a vital role in clearing GAS infections, Furthermore, patients with severe GAS infections are frequently neutrophil poor (Edwards*, et al.* 2005, Stevens*, et al.* 1989, Taylor*, et al.* 1999), further suggesting the importance of neutrophils in

preventing the establishment of severe infection. By cleaving these chemokines, SpyCEP provides a potential mechanism that explains the clinical observations frequently made by clinicians treating severe GAS infections. In this chapter, I sought to determine whether GAS infection could result in mobilisation of neutrophils from the bone marrow, and to investigate whether SpyCEP, a CXCR2 chemokine inactivator, might impede this process.

4.2 Results

4.2.1 SpyCEP expression impacts on circulating neutrophil levels

To test this hypothesis, mice were injected intramuscularly with SpyCEP-positive and negative strains of GAS and *L. lactis*. At three, six and twenty four hours post infection, peripheral blood was taken from the mice, depleted of erythrocytes, and a total white cell count made. The cells were then stained using a fluorescently conjugated antibody against Ly6G, a highly expressed surface protein of murine neutrophils (Wengner, *et al.* 2008). The results following infection with *L. lactis* are shown in Figure 4-1.

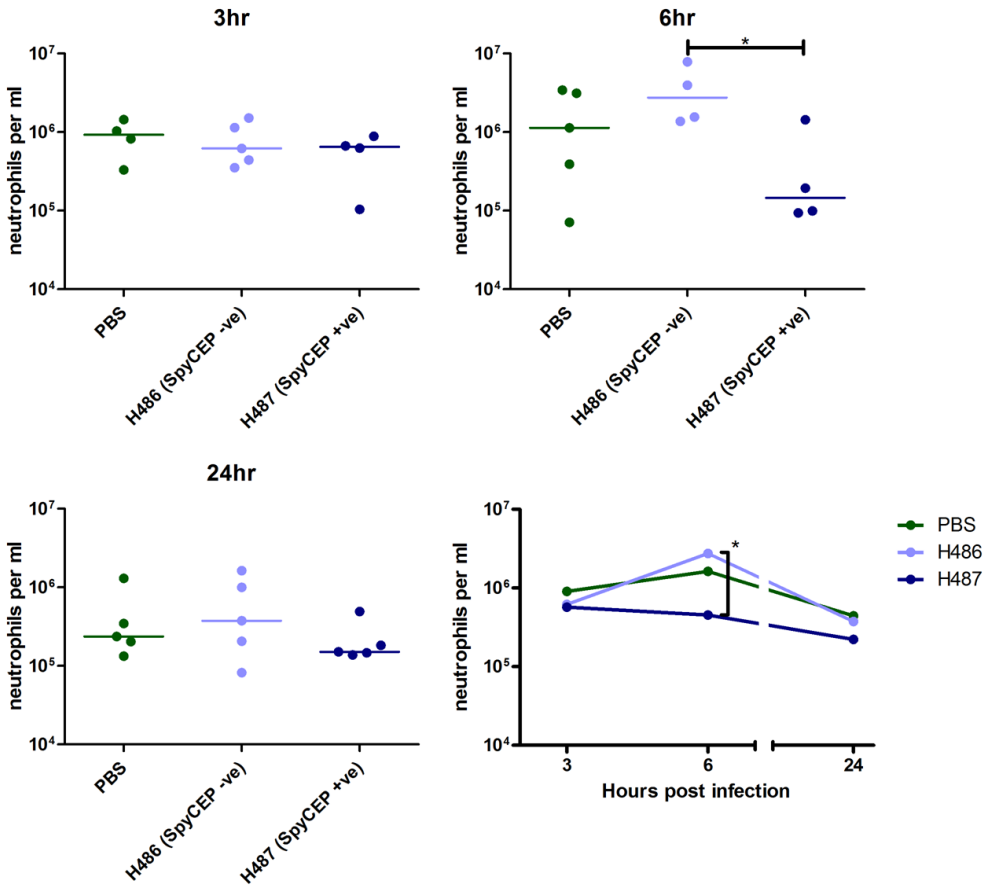


Figure 4-1 – Peripheral neutrophil levels following L. lactis infection

Five female CD1 mice were injected with 10^8 CFU of H486 (wild type *L. lactis* – SpyCEP-negative – pale blue), H487 (SpyCEP +ve *L. lactis* – dark blue) or sham infected with PBS (green). Mouse blood was collected by tail bleed at 3 and 6 hours and cardiac puncture at 24 hours, and peripheral circulating neutrophils counted by flow cytometry. * = $P<0.05$ by Mann Whitney analysis. Time course plot shows medians alone.

At 24 hours post infection, the mice were euthanized and the infected thigh tissue was homogenised to allow determination of bacterial load. The results are shown in Figure 4-2.

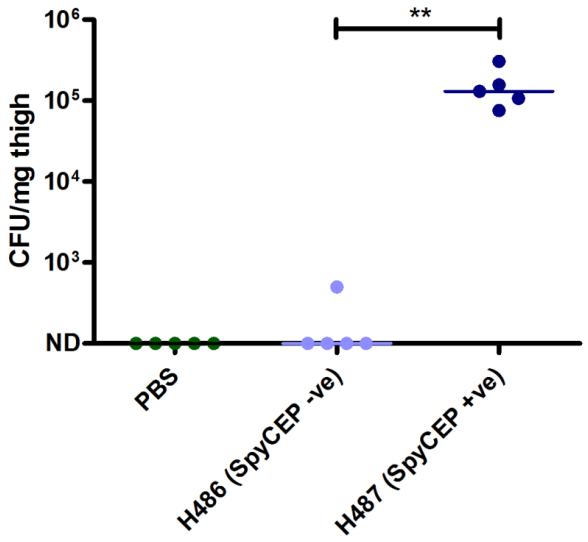


Figure 4-2 – L. lactis CFU recovered 24 hours post infection of thigh

24 hours post infection, the mice were euthanized and thigh tissue was collected and homogenised. Colony forming units observed are shown. ** = P<0.01 by Mann Whitney. Lines indicate median ND: not detected (<200CFU/mg).

At three hours, peripheral circulating neutrophil levels in both challenged groups and the PBS group were similar. However at 6 hours, neutrophils counts increased in mice challenged with H486 SpyCEP-ve *L. lactis*, whereas H487 SpyCEP+ve *L. lactis* challenged mice had significantly fewer neutrophils. The results are consistent with SpyCEP having an impact on neutrophil levels in the peripheral blood. The PBS sham infected mice also had increased circulating neutrophils at 6 hours. This was probably due to the method of blood extraction, using a tail bleed method. This process performed at 3 hours would lead to an acute phase response itself, which was probably being detected at 6 hours. Later work was

performed measuring cytokine levels in mice at six hours which had not been subjected to tail bleeding. In the latter case, the sham infected mice had low levels of cytokines such as IL-6, a typical acute phase response indicator, compared to mice infected with bacteria (4.2.3. Cytokine levels in peripheral blood following infection).

At 24 hours, mice infected with SpyCEP-ve H486 had completely cleared their infection, whereas those infected with SpyCEP+ve H487 retained a substantial bacterial load at the infection site. This would suggest that the difference in neutrophil count seen at 6 hours is not due to a lower level of H487 infection compared to H486, and instead further indicates the importance of the neutrophil response in the clearance of bacterial infections.

At 24 hours post infection, all groups showed low peripheral neutrophil counts. This may indicate the typical resting state of neutrophil counts in mice. However, the blood for this experiment was obtained using a cardiac puncture technique rather than tail bleed. As a result, care should be taken when comparing the results at 24 hours to those obtained at 3 and 6 hours. *L. lactis* strains express no virulence factors of their own. We hypothesised that there would hence be a more striking phenotype difference between the strains, that any differences in peripheral neutrophil counts caused by SpyCEP expression would be more clearly displayed using *L. lactis* than the corresponding SpyCEP+/- GAS strains. However, the experiment was repeated using GAS, with results shown in Figure 4-3.

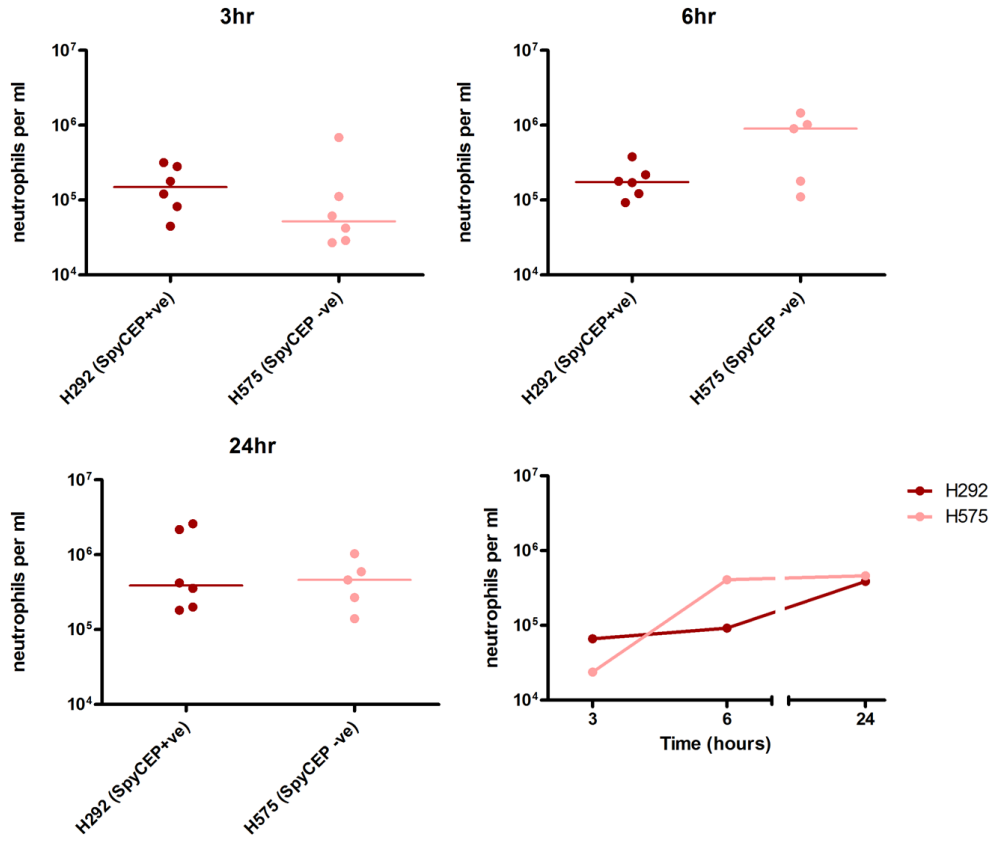


Figure 4-3 - Peripheral neutrophil levels following GAS infection

Six female CD1 mice were injected with 10^8 CFU of H292 (wild type GAS – SpyCEP-positive – dark red) or H575 (SpyCEP -ve GAS – pink). Mouse blood was collected at 3,6 and 24 hours, and peripheral circulating neutrophil counted. Time course plot shows medians alone.

The results obtained at 3 and 6 hours post infection closely resembled those obtained using the *L. lactis* strains. At three hours post infection, both groups of mice demonstrated low levels of neutrophils, with little difference between the strains. At 6 hours post infection, mice infected with the SpyCEP-ve GAS strain appeared to mount a more substantial neutrophil response in the circulation compared with mice infected with wild type GAS,

however this difference was not statistically significant. This result was not unexpected; GAS produce an array of virulence factors, including factors such as streptolysin O which acts to lyse neutrophils (Timmer, *et al.* 2009), potentially masking any differences between the strains. The trend here however does seem to be maintained compared to *L. lactis.*

At 24 hours, all infected mice infected with both strains showed an increased number of circulating neutrophils. As previously mentioned, the mode of blood extraction was changed between 6 and 24 hours, so comparisons between these time points must be made cautiously. It may be that the GAS, being much more virulent than *L. lactis*, elicits a more sustained innate immune response, which continues throughout the duration of the infection. Indeed, when thighs were homogenised at 24 hours, both strains exhibited an active infection, in contrast to *L. lactis* infected mice which cleared the infection.

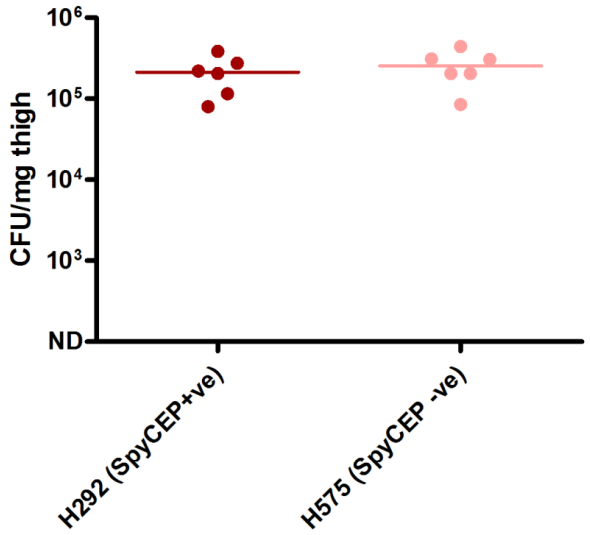


Figure 4-4 - GAS CFU recovered 24 hours post infection of thigh

24 hours post infection, the mice in Figure 4-3 were euthanized and thigh tissue was collected and homogenised. Colony forming units observed are shown. Lines indicate median.

This work measured peripheral blood neutrophil levels following infection, rather than directly measuring release from the bone marrow. It was assumed that these neutrophils have entered the circulation from the bone marrow, the sole known source of neutrophil reserve; however alternative sources of neutrophils were not excluded.

4.2.2 Intramuscularly injected CXC chemokines increase circulating neutrophils

Previous work where CXC chemokines have been definitively shown to be important in driving the emergence of neutrophils from the bone marrow used an infusion based method, whereby chemokines were administered into bone marrow and the output from the bone marrow could be measured (Martin*, et al.* 2003). A further method used intraperitoneal injection of chemokine into the mouse model, allowing subsequent collection of infiltrated cells, although this study therefore measured the additive effects of both neutrophil mobilisation and recruitment into the peritoneum (Wengner*, et al.* 2008). Whilst these methods are informative to our study of the role of SpyCEP in neutrophil release from the bone marrow, I wanted to determine whether chemokine released at a site of infection distant from the bone marrow was also able to induce increased neutrophil mobilisation into the circulation. This was important to confirm, as bacterial products have not been reported to affect neutrophil release in this way previously. I hypothesised that SpyCEP produced by GAS at the site of infection would cleave and inactivate chemokines at the site of infection and systemically. This would prevent chemokine translocation across the endothelial barriers both at the site of infection and in the bone marrow. To emulate this, chemokine was directly administered into the muscle without bacterial infection.

500ng of the murine CXCL8 functional orthologue KC was injected into the thigh muscle while a sham injection of PBS was administered to a control group. This chemokine and amount was chosen based on previous work examining neutrophil release by chemokines (Wengner*, et al.* 2008). In this experiment, significantly more neutrophils were detected in the peripheral blood of the mouse 3 hours post inoculation with KC compared to PBS. At 6 and 24 hours, neutrophil counts between these groups were identical. The mice were weighed at the end of the experiment to ensure that the chemokine injection had not impacted on the general health of the mice and so confound these findings. Both groups were observed to have gained a small amount of weight over the course of the experiment (Figure 4-6).

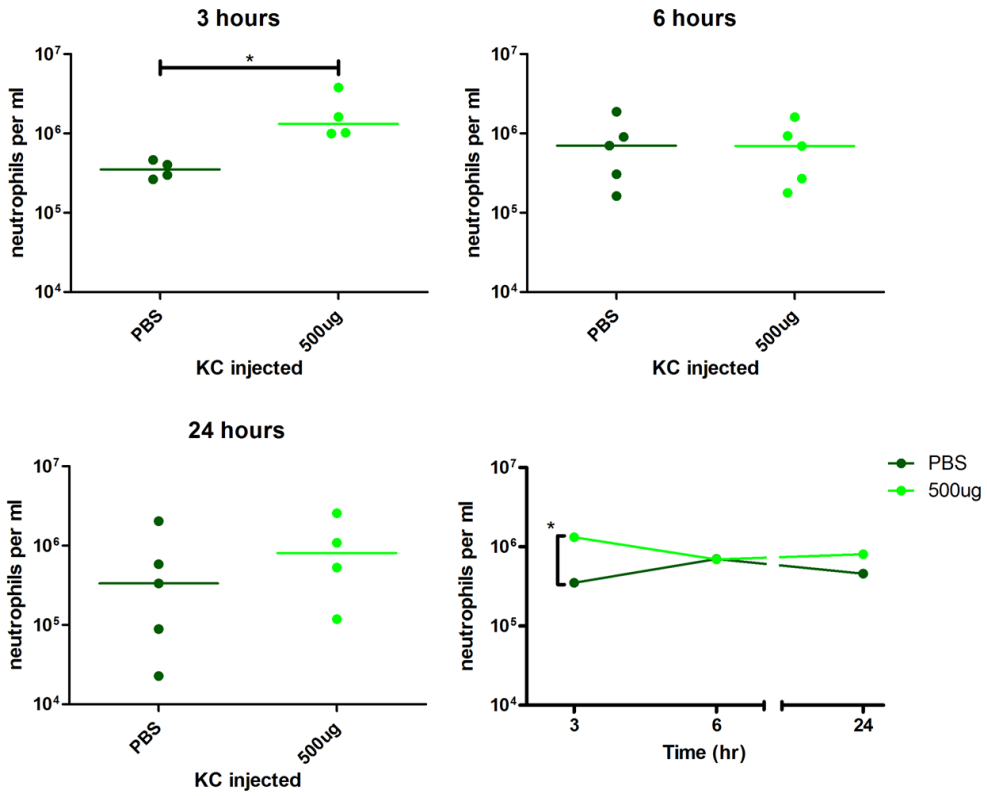


Figure 4-5 - Peripheral neutrophil levels following chemokine KC injection

Five female CD1 mice were injected with 500ng of chemokine KC (bright green) or sham injected with PBS (dark green). Mouse blood was collected at 3,6 and 24 hours, and peripheral circulating neutrophil counted. * = P<0.05 by Mann Whitney analysis. Time course plot shows medians alone.

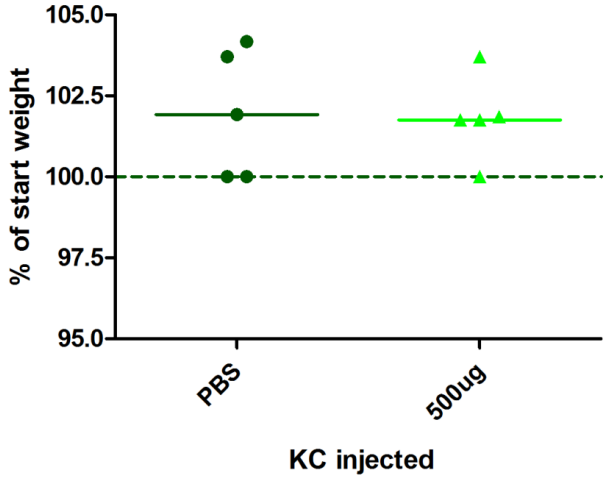


Figure 4-6 – Mouse weight 24 hours post injection with PBS or chemokine KC

24 hours following the injection of Figure 4-5, the mice were weighed and this was compared to their starting weight. Horizontal lines indicate median.

The effect on circulating neutrophil levels using chemokine was observed more quickly post injection than using bacterial infection. This was not unexpected; time would be required for a chemokine response to develop in response to the infection in the mouse tissue, whereas pre-formed chemokine was injected in this instance. Again, at 6 hours, the PBS infected mice show an increase in neutrophil levels, most likely due to a response against the tail bleed performed at three hours. At 6 hours, both groups of mice demonstrated similar peripheral neutrophil counts. As there was no active infection and chemokines were not being actively expressed, it is likely that the chemokine injected was either broken down or bound to receptors and internalised, although KC levels were not measured following the injection. The results supported the hypothesis that chemokines produced at a site distant to the marrow can elicit systemic mobilisation of neutrophils into the circulation, and provide a mechanism for the action of SpyCEP on neutrophil mobilisation.

4.2.3 Cytokine levels in peripheral blood following infection

Neutrophils are often considered to be cells, which whilst carrying highly toxic anti-microbial chemicals, have limited productive capability. However, they are known to release important cytokines required for the establishment of later elements of the immune response (Scapini*, et al.* 2000). It was hence hypothesised that those strains which reduce peripheral neutrophil levels through SpyCEP expression would also reduce other early cytokines of the immune response.

Furthermore, the explanations offered in section 4.2.1 assume that there is a difference in the circulating levels of murine neutrophil specific chemokines, such as KC, in the mouse blood following infection with SpyCEP-positive and negative bacterial strains. The concentrations of many important cytokines and chemokines, including KC, were thus measured 6 hours following infection with SpyCEP-positive and negative bacterial strains. Six female CD1 mice per group were infected with 10^8CFU (Figure 4-7) of the SpyCEP-positive and negative GAS or *L. lactis* strains. 6 hours post infection, mice were euthanized, blood extracted by cardiac puncture, and the infected thigh and organs were removed and homogenised to allow determination of bacterial load (Figure 4-7). The mice were not tail bled at 3 hours as had been done in the previous experiments.

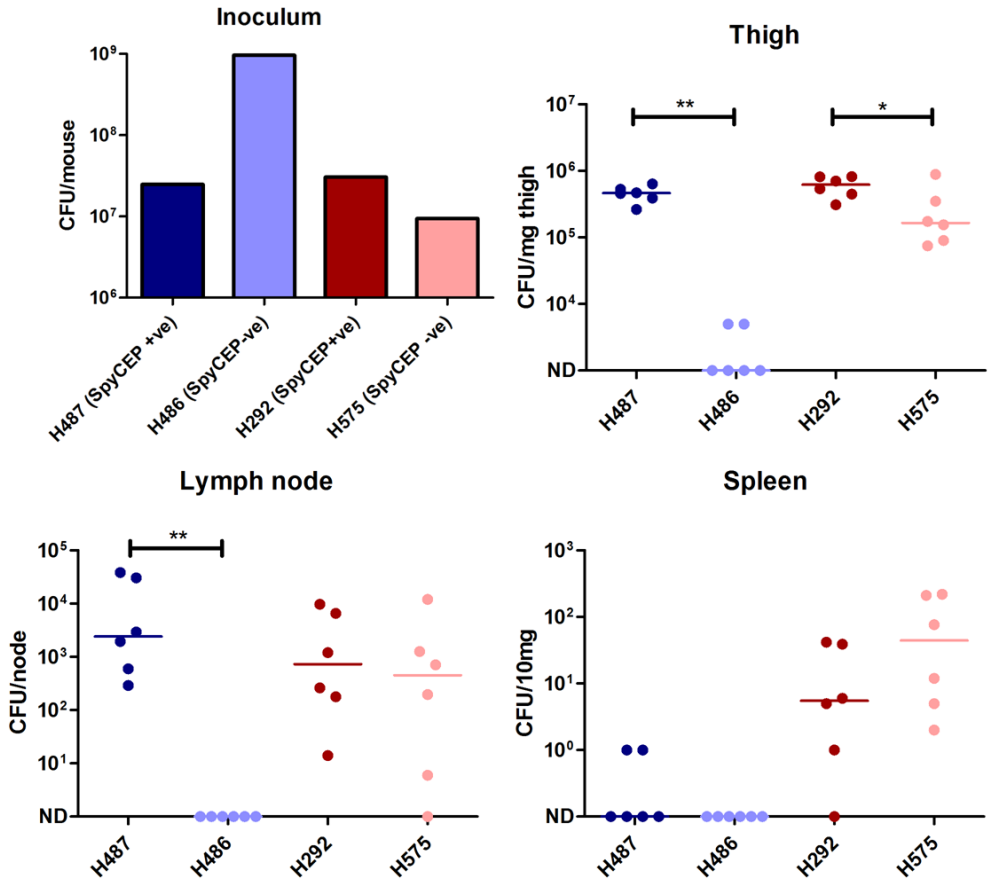


Figure 4-7 – Bacterial load 6 hours post infection with SpyCEP +/- L. lactis or GAS

Six CD1 female mice were infected intramuscularly with either H487 (SpyCEP+ve *L. lactis* – dark blue), H486 (wild type *L. lactis* SpyCEP-ve – pale blue), H292 (wild type GAS, SpyCEP+ve – dark red) or H575 (SpyCEP-ve GAS – pale red). The inoculum of each strain is shown in the top left graph. 6 hours post infection, the mice were euthanized and thigh tissue, spleen and inguinal lymph nodes harvested and homogenised. Bacterial loads were quantified. Horizontal lines indicate median. P values determined by Mann Whitney analysis, * = P<0.05, **=P<0.01. PBS groups not shown, no bacteria detectable ND: not detectable.

Mice were infected with H487, H292 and H575 (SpyCEP+ve *L. lactis,* SpyCEP+ve GAS and SpyCEP-ve GAS respectively). All were injected with a similar CFU of bacteria,

between 2 and $4x10^7$ CFU. Mice infected with wild type SpyCEP-ve *L. lactis* H486 however received over 1 log fold more bacteria than mice infected with the SpyCEP producing strain H487. Despite this, at 6 hours, H486 was almost entirely cleared by the mice, with no bacteria detectable anywhere, except a very low level in the infection sites of two mice. The SpyCEP+ve *L. lactis* strain caused a productive infection in the thigh tissue that had spread to the locally draining lymph node, further highlighting the importance of SpyCEP in virulence.

GAS isogenic strains H292 and H575 also caused infection in the thigh and the local draining lymph node similar to SpyCEP+ve *L. lactis*, but also caused more sustained systemic infection, with spread to the mouse spleen. Differences between SpyCEP+ve and –ve GAS strains were less obvious than those observed between isogenic *L. lactis* strains, although there were significantly more bacteria in the thighs of mice infected with the SpyCEP-positive strain in this experiment. This is indicative that SpyCEP is important in GAS virulence, although clearly additional GAS virulence mechanisms allow H575 to cause infection. Sera from infected mice were then analysed to measure circulating levels of KC and G-CSF, another cytokine known to have a central role in releasing neutrophils from the bone marrow (Figure 4-8).

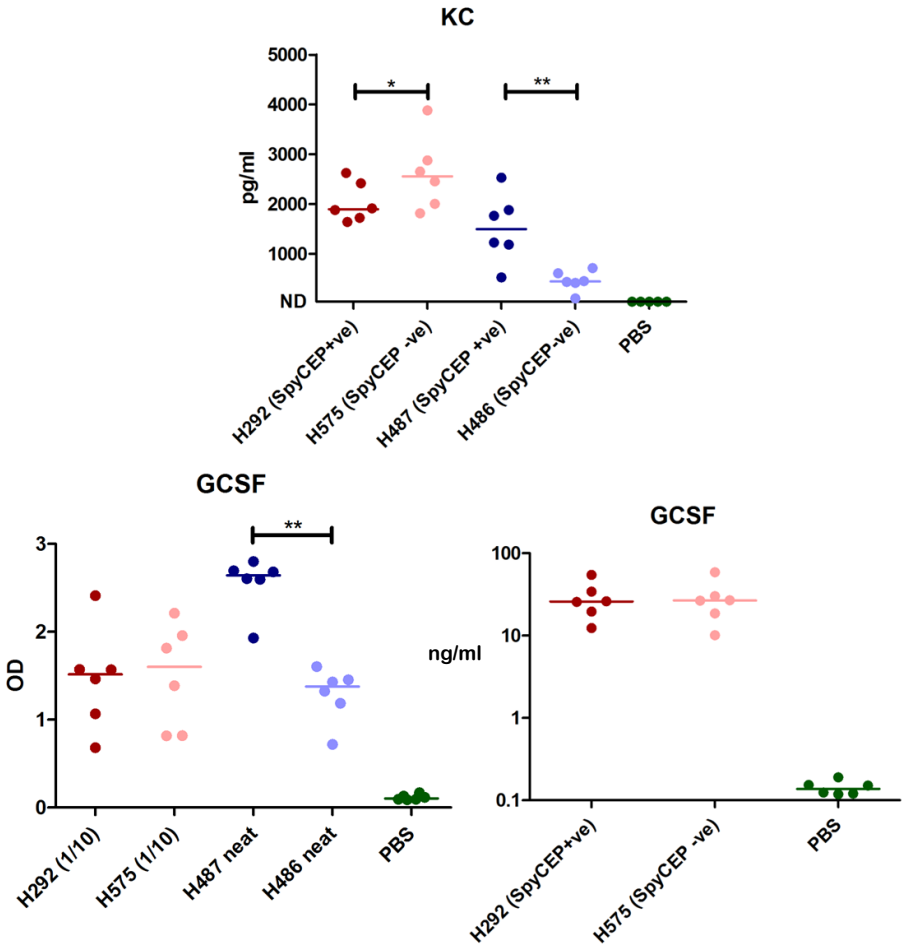


Figure 4-8 – Serum concentrations of KC and G-CSF 6 hours post infection

Six CD1 female mice were injected intramuscularly with either H487 (SpyCEP+ve *L. lactis* – dark blue), H486 (wild type *L. lactis* SpyCEP-ve – pale blue), H292 (wild type GAS, SpyCEP+ve – dark red), H575 (SpyCEP-ve GAS – pale red) or PBS (dark green). 6 hours post infection, the mice were euthanized and blood collected and processed to serum. KC and G-CSF were quantified using Luminex and ELISA respectively. ND represents below 500pg/ml. Horizontal lines indicate median. P values determined by Mann Whitney analysis between paired strains, * = $P<0.05$, **=$P<0.01$.

As previously reported, mice infected with SpyCEP-positive GAS had less circulating serum KC than mice infected with SpyCEP-negative GAS (Kurupati*, et al.* 2010). Again, as previously reported, the reverse observation was seen using the *L. lactis* strains, with significantly less KC circulating in the SpyCEP-negative strains (Kurupati*, et al.* 2010). However, as mice infected with SpyCEP-negative *L. lactis* had cleared the infection (Kurupati*, et al.* 2010), lower KC levels were expected. The G-CSF measurement using *L. lactis* was unsuccessful, as serum was used at too high a concentration, and hence G-CSF readings were above the standard curve. This experiment used all the serum collected, hence the assay could not be repeated. The optical densities of the ELISA observed are shown in Figure 4-8, as a surrogate for quantification. The results were similar to those seen with KC, with more G-CSF detectable in the SpyCEP-positive infected mice. Again, as the SpyCEP-negative *L. lactis* infection had been cleared, the lower observed levels of G-CSF were expected. G-CSF could be quantified in the mice infected with GAS, and no difference was observed between SpyCEP-positive and negative strains. All infected mice showed increased levels of both cytokines compared to sham PBS infected mice.

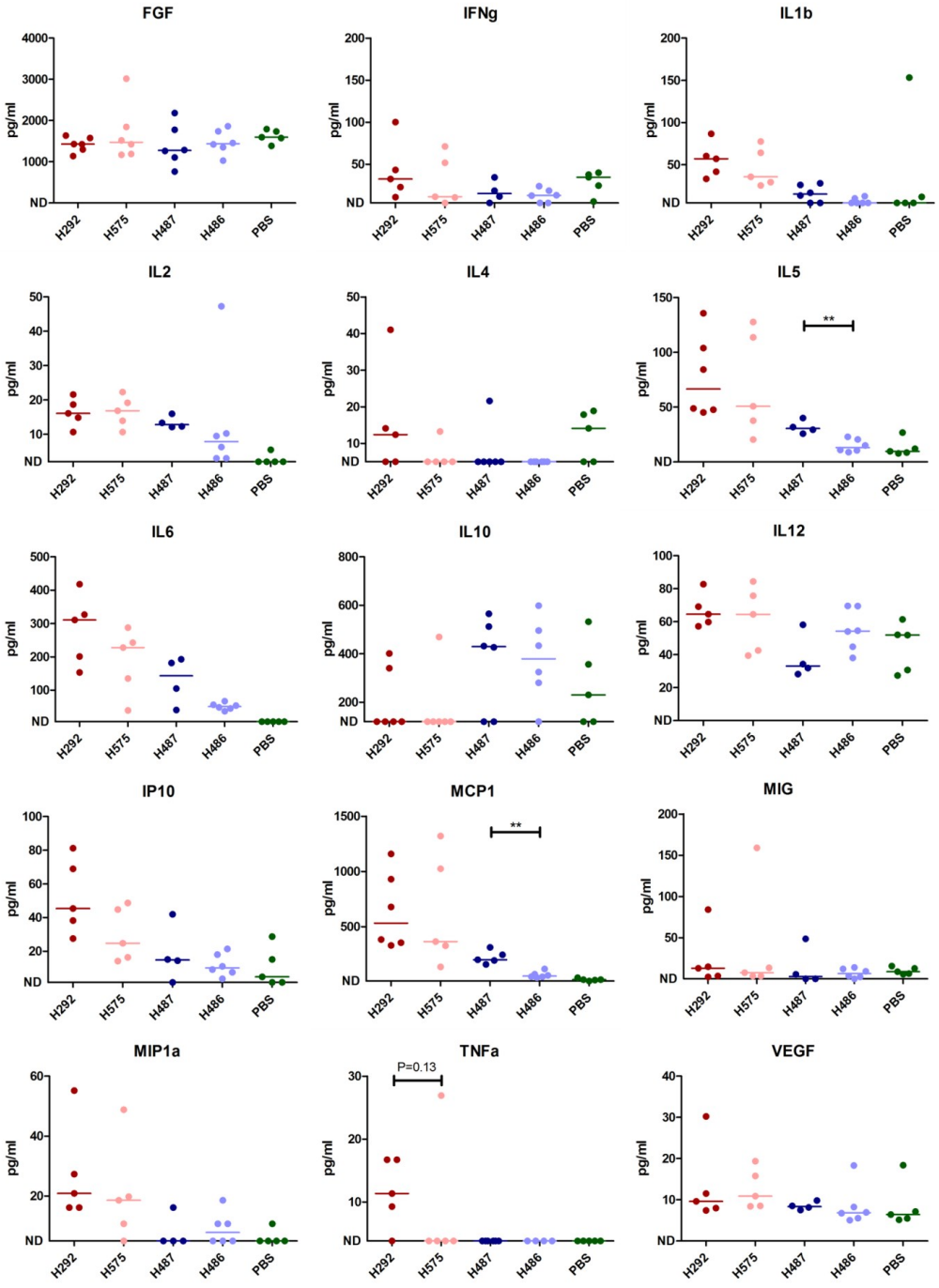


Figure 4-9 – Serum concentrations of cytokines 6 hours post infection

Six CD1 female mice were infected intramuscularly with either H487 (SpyCEP+ve *L. lactis* – dark blue), H486 (wild type *L. lactis* SpyCEP-ve – pale blue), H292 (wild type GAS, SpyCEP+ve – dark red), H575 (SpyCEP-ve GAS – pale red) or PBS (dark green). 6 hours post infection, the mice were euthanized and blood collected and processed to serum. Cytokines were quantified using Luminex. Horizontal lines indicate median. P values determined by Mann Whitney analysis between paired strains, * = $P<0.05$, **=$P<0.01$.

A Luminex assay was carried out to measure other murine chemokines. No significant differences between mice infected with SpyCEP-positive and negative strains were observed. This was expected for cytokines such as VEGF, a growth factor released in hypoxia to encourage growth of the vasculature. We expected to see more inflammatory chemokines, such as IL-1β, IL-12 and TNFα, in strains infected with SpyCEP-negative mice, due to their increased peripheral neutrophils (Scapini*, et al.* 2000). However the relative contribution of circulating neutrophils balanced against infection severity was of uncertain importance. There was a trend for higher cytokine levels in mice infected with SpyCEP-positive strains, perhaps due to these mice having a more severe infection, however this was most marked, but not significant, with regard to TNFα and GAS infection.

A similar trend was observed using the *L. lactis* strains, with a trend towards higher cytokine concentrations where SpyCEP was present. Mice infected with SpyCEP-negative *L. lactis* had cleared the infection by six hours (Figure 4-7), hence explaining the lower concentrations of inflammatory cytokines. Indeed, mice infected with SpyCEP-positive *L. lactis* H487 had significantly higher serum concentrations of IL-5 and MCP1, GMCSF, IL-1α. IL-13 and IL-17 were also measured, but the concentrations were too low to be detected in all mice.

4.3 Discussion

4.3.1 Circulating neutrophil levels

The results in Figure 4-1 show that *L. lactis* strains expressing SpyCEP are able to significantly reduce the number of neutrophils circulating in peripheral mouse blood compared to the response seen to wild type *L. lactis*. This is despite the fact that there are significantly more SpyCEP expressing *L. lactis* in the mouse thigh (Figure 4-2). Similar results were seen using the GAS strains, although the difference in circulating neutrophils was not sufficient to reach statistical significance (Figure 4-3). The *L. lactis* strains examined express no virulence factors of their own, thus any difference observed between the two strains must be being directly mediated by SpyCEP. Such a system allows us to clearly and cleanly study the effects of SpyCEP. GAS express a range of virulence factors which are unaffected between the SpyCEP-negative and positive strains, including factors which are known to act on neutrophils, such as Streptolysin O (SLO) (Timmer*, et al.* 2009). Such other virulence factors make observations about virulence factors more challenging, explaining why the differences observed using GAS were not considered statistically significant.

The observations made are interesting when considered together with earlier research examining the effects of SpyCEP on infiltration of infection foci by neutrophils (Kurupati*, et al.* 2010). *In vivo* work has demonstrated that the migration of neutrophils through endothelium into the infection site is disrupted by SpyCEP, a finding that has been confirmed *in vitro* using transwell assays with known numbers of cells (Edwards*, et al.* 2005, Zinkernagel*, et al.* 2008). This study adds to previous findings; not only are neutrophils

deficient at leaving the circulation to reach the infection focus, but there are also fewer neutrophils entering the blood at the time of infection, before any migration takes place. It would be interesting to look at the later stages of neutrophil function such as bacterial killing to see if these are also disrupted by SpyCEP. The very final stage in the bactericidal lifespan of the neutrophil, release of neutrophil extracellular traps (Brinkmann*, et al.* 2004), has indeed been shown to be impaired in SpyCEP-positive strains (Zinkernagel*, et al.* 2008).

Chemokines have been previously reported to have a role in neutrophil release from the bone marrow (Martin*, et al.* 2003). The mechanism described has separate chemokine axes either retaining or encouraging neutrophil release from the bone marrow, mediated by CXCR4 and CXCR2 signalling respectively. These signals are mutually antagonistic, such that a small change in one has a large overall effect on neutrophil release (Furze and Rankin 2008a). Prior to this study, this chemokine interplay has not been studied in the context of a bacterial infection. The results obtained in this study fit with these previous reports. GAS expressing SpyCEP does indeed reduce the circulating level of CXCR2 binding chemokine KC in the mouse serum (Figure 4-8).

Considering that wild type *L. lactis* not expressing SpyCEP was cleared by 6 hours, it is interesting that neutrophils were not detected at higher numbers in the peripheral blood at three hours (Figure 4-1). Furthermore, by 6 hours post infection, systemic KC was much lower in mice infected with wild type *L. lactis* than SpyCEP expressing *L. lactis* (Figure 4-8). This is most likely explained by the choice of time points in this study. It may have been too early to see a neutrophil response at three hours, but too late to see a KC response at six hours. The chemokine response may well be peaking at 2-3 hours following infection with wild type *L. lactis* strain. The neutrophil peak will lag behind this at 4-5 hours with concomitant bacterial killing (and subsequent loss of KC production), and may well have returned to

normal levels by 6 hours. This seems likely, as the timing of the neutrophil response seen using GAS matches that of *L. lactis* (Figure 4-3). Furthermore the administration of recombinant KC would lead to an immediate spike in circulating levels, circumventing the time required to express endogenous KC, allowing the difference in neutrophil release being detected at just three hours (Figure 4-5).

We considered the possibility that the main neutrophil response seen in this experiment was in reaction to the tail bleed technique itself at three hours, caused by an acute phase response to the injury. Soluble SpyCEP produced by bacterial strains would have been equally able to neutralise chemokine release triggered by tail bleeding as chemokine release from thigh muscle infection. Importantly however, there were global differences in neutrophil numbers between three and six hours, suggesting that the bleed technique could not alone account for such differences (Figure 4-1). Nonetheless, there was a rise in neutrophil numbers at six hours even in response to PBS.

It is also interesting that in mice infected with GAS, G-CSF is greatly upregulated compared to mice injected with PBS (Figure 4-8) at 6 hours post infection. G-CSF alone is known to induce neutrophil release. It would thus be expected that the mice infected with either strain of GAS would have many more neutrophils (Figure 4-3) circulating than those in the PBS control (Figure 4-1). G-CSF measurements were taken without performing a tail bleed at three hours, and thus the circulating G-CSF in the PBS control group may well be much higher in the neutrophil count experiment than the cytokine measurement experiment.

The results obtained using SpyCEP-positive and negative strains were supported by the results obtained using intramuscular injection of murine chemokine KC (Figure 4-5). As expected, chemokine injected into the muscle was able to elicit an increase in circulating neutrophils at three hours. This effect had completely disappeared at six hours post injection,

with very similar numbers of circulating neutrophils between the two groups. This was presumably due to clearance of the chemokine by six hours, and loss of subsequent effect. The idea that KC remains active in the mouse for a short period only is strengthened by the data observed measuring levels of KC during *L. lactis* infection (Figure 4-8).

4.3.2 Cytokine levels in peripheral blood following infection

As expected, the serum levels of KC, a substrate of SpyCEP, were lower in mice infected with a GAS strain expressing SpyCEP than those infected with a SpyCEP mutant strain (Kurupati*, et al.* 2010). The same result was not seen using *L. lactis*, as the wild type strain had been cleared from the infected mice before the six hour time point (Figure 4-7), and thus KC levels were returning to resting state. This was also observed in a previously published study (Kurupati*, et al.* 2010). It is hypothesised that had levels of KC been measured at three hours, the same pattern as was observed with GAS would have been measured, with higher chemokine levels in the absence of SpyCEP.

It was expected that as neutrophil mobilisation and recruitment was affected by SpyCEP, the levels of cytokines and other chemokines associated with neutrophils might also be affected (Scapini*, et al.* 2000). This was however not observed. Global differences were not observed between mice infected with different strains of GAS, although there was a trend towards higher levels of pro-inflammatory cytokines in the mice infected with SpyCEP-positive GAS, indicated by higher TNFα in the SpyCEP-positive group (Figure 4-9). The same trend was observed using the *L. lactis* strains, and indeed two pro-inflammatory cytokines IL-5 and MCP1 were significantly higher in the serum of mice infected with SpyCEP-positive *L. lactis (*Figure 4-9). We hypothesise that the burden of infection, which

was greater in mice infected with SpyCEP producing strains, is the major contributory factor of proinflammatory cytokine levels, rather than circulating neutrophil counts.

Differences in immunology between mice and humans limit the conclusions which can be drawn from such studies, especially when studying neutrophils. Neutrophils play a much less important role in the mouse immune response than in human. Indeed neutrophils make up just 10% of the resting mouse leukocyte count, compared to up to 70% in humans. Furthermore, mice produce fewer chemokines for neutrophils, and only express the CXCR2 receptor (Amulic*, et al.* 2012). Nevertheless, the results obtained in this study, especially those measuring peripheral neutrophil counts, are consistent with clinical data from human infection, strengthening their validity (Edwards*, et al.* 2005).

Chapter 5 - Results: Phagocytosis of GAS by neutrophils

5.1 Introduction

Work in Chapter 4 of this study has demonstrated that SpyCEP is able to disrupt neutrophil release from the bone marrow, an early stage in the nascent neutrophil response against a GAS infection. Previous work using the intramuscular model of invasive infection using SpyCEP-positive and negative strains of *L. lactis* and GAS suggested that SpyCEP may prevent killing of bacteria (Kurupati*, et al.* 2010). This was in part confirmed by work demonstrating reduced killing of SpyCEP-positive GAS by NETs (Zinkernagel*, et al.* 2008).

Phagocytosis by neutrophils is a poorly studied field, due to the fact they are not amenable to genetic manipulation, and have only a short lifespan. Proposed mechanisms of neutrophil phagocytosis are hence closely based on those derived from studying macrophages, despite the differences in these cells (Lee*, et al.* 2003). Neutrophils predominantly phagocytose opsonised particles, using receptors able to bind deposited antibody or complement. The receptors then cluster beneath the particle, forming a signalling platform beneath which cell changes can occur. The internalisation mechanism of an antibody opsonised particle is different from that of a complement opsonised particle. Apart from different receptors being used, the actin mediated cell changes required are also different, with complement opsonised particle apparently sinking inside the cell, whereas antibody opsonised particles are uptaken by extending pseudopods (Greenberg and Grinstein 2002).

GAS is known to bear many defences against both antibody and complement mediated phagocytosis. Antibodies against GAS have been explored in a variety of ways including a shaving proteolysis technique to find surface exposed GAS proteins that are recognised by antibody (Rodriguez-Ortega, *et al.* 2006), and using pooled convalescent sera to probe a panel of target GAS antigens (Fritzer, *et al.* 2010). Serotype defining antibody against the M protein is thought to be especially important, and despite its variability, is the subject of many vaccine efforts (Lynskey, *et al.* 2011). It is the major surface protein of GAS, and is itself anti-phagocytic. *L. lactis* unlike GAS, does not express factors to actively prevent opsonisation, but it is coated in a polysaccharide pellicle, which is known to be anti-phagocytic by an unknown mechanism (Chapot-Chartier, *et al.* 2010). *L. lactis* does not infect humans or mice, and hence antibodies are not expected to have been raised against this bacterium. Complement deposition does not require prior exposure to the pathogen however. As *L. lactis* was efficiently cleared from a mouse model of infection (Figure 4-2), we assume that it must be successfully opsonised by complement, although an alternative mechanism of killing, such as by anti-microbial peptides may also explain these observations.

5.2 Results

5.2.1 Lancefield Assay using SpyCEP-positive and negative strains

To examine the reasons underlying failed killing of bacteria in mice exposed to SpyCEP-expressing bacteria, we re-examined thin sections from thighs of GAS or *L. lactis* infected mice in collaboration with Professor Ken Smith (Royal Veterinary College).

From the microscopy images (Figure 5-1), it was apparent that neutrophils that had entered the areas infected with SpyCEP-negative strains were able to engulf many Gram positive cocci – often up to 10 per cell, while other neutrophils had not engulfed any visible bacteria. This was interesting and suggests that neutrophils undergoing phagocytosis enhance their phagocytic capacity. Neutrophils undergoing phagocytosis have been reported to release high levels of chemokine, much of which binds to the source neutrophil, potentially explaining these observations (Bazzoni*, et al.* 1991, Marie*, et al.* 1997), and implying that endogenous chemokine is important for phagocytosis. In contrast, where bacteria were expressing SpyCEP, there were, as predicted, fewer apparent neutrophils. For instance, there are no visible neutrophils in the sections infected with SpyCEP positive strain H292, unlike sections infected with SpyCEP negative H575. Even where neutrophils had reached the infected field, there was no evidence of interaction or phagocytosis of bacteria (Figure 5-1). This strongly suggested a potential role for SpyCEP in the prevention of phagocytosis.

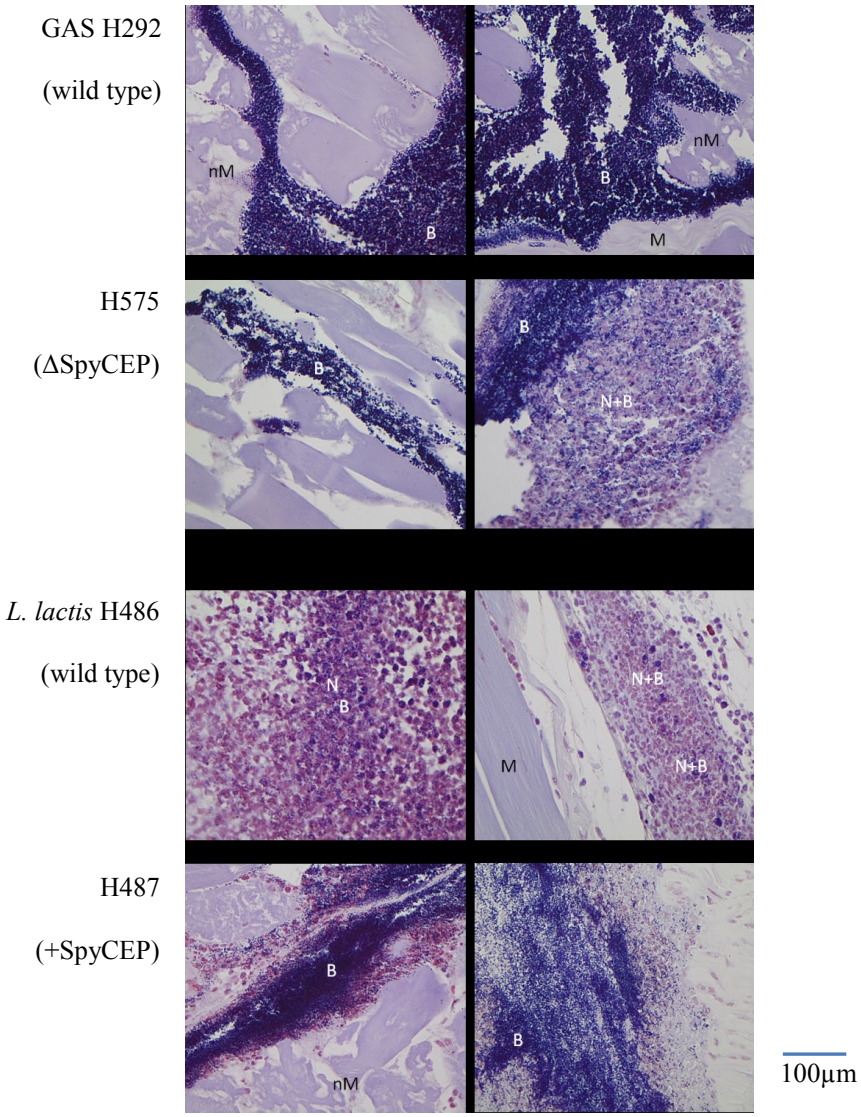


Figure 5-1 – Histology taken from infected mouse thighs

Mice were infected for 3 hours with H486 (SpyCEP-ve *L. lactis*), H487 (SpyCEP+ve *L. lactis*), H292, (SpyCEP+ve GAS) or H575 (SpyCEP-ve GAS). Mice were then euthanized and thigh tissue collected for histology. Sections were Gram stained. B indicates bacteria, M muscle and N neutrophils. Lower case n indicates necrosis. Representative sections shown. Magnification 400x.

SpyCEP interferes with the neutrophil response against Group A Streptococcus

Phagocytosis of GAS is classically measured using a Lancefield assay, where a small number of GAS CFU are inoculated into a volume of blood and incubated at 37^{o}C with rolling for 3 hours. The blood provides complement and antibody to opsonise the bacteria, and the phagocytes in the blood then phagocytose and kill the GAS. If the bacteria are able to grow in the blood, the donor is considered non-immune to that particular serotype. A limitation of this assay is that the number of colonies obtained at the end of the experiment is determined both by the rate of killing of the bacteria and the simultaneous rate of growth of the bacteria (ie the net growth).

Approximately 30 CFU of H292, a wild type, high SpyCEP producing M81 strain, and its isogenic SpyCEP mutant H575 were inoculated separately into freshly extracted blood from three donors, in experimental triplicates. In all donors, no bacteria were detectable at any time point in the experiment (Figure 5-2), consistent with all donors being immune to this serotype.

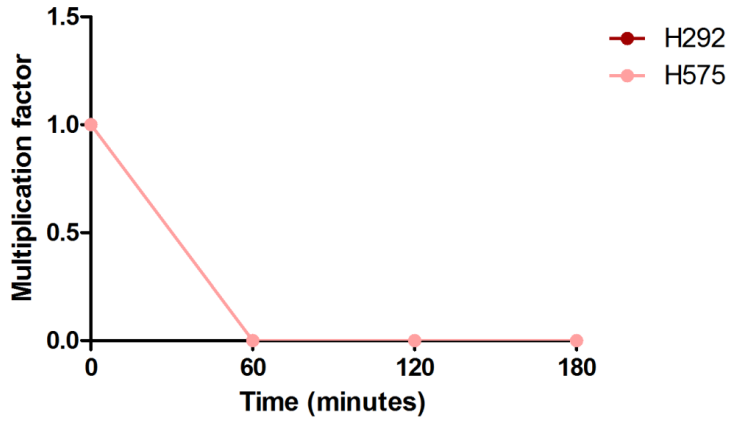


Figure 5-2 – Lancefield assay using GAS strains

Approximately 30 CFU (1 CFU/µl) of H292 (SpyCEP+ve GAS) and H575 (SpyCEP-ve GAS) were inoculated into 270µl of freshly extracted blood from three different donors in experimental triplicate. At 60, 120, and 180 minutes, a 100µl sample was taken and plated to allow quantification of bacteria.

As *L. lactis* is not a pathogen of humans, it was hoped that these strains could be used in a whole blood assay as antibody against *L. lactis* was not expected to be present (Bolotin, *et al.* 2001, Poquet, *et al.* 1998, van Asseldonk, *et al.* 1990). A comparison was made between SpyCEP expressing *L. lactis* and the isogenic wild type strain; results were expressed as a multiplication factor (ratio of bacteria after : input culture bacteria).

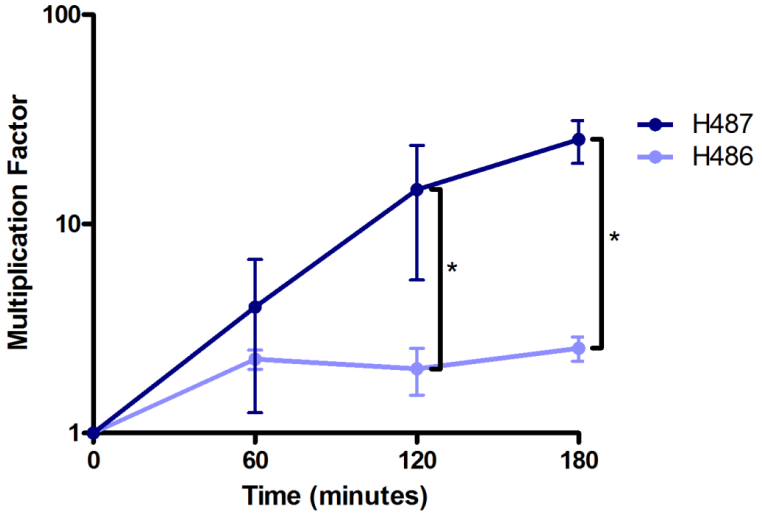


Figure 5-3 – Lancefield assay using L. lactis strains

Approximately 300 CFU of H487 (SpyCEP+ve *L. lactis*) and H486 (SpyCEP-ve *L. lactis*) were inoculated into 270µl of freshly extracted blood from three different donors in experimental triplicate. At 60, 120, and 180 minutes, a sample was taken and plated to allow quantification of bacteria. $P<0.05$ between the two strains at specific time points as assessed by Mann Whitney. Data shows the mean of three experimental replicates and the mean of three different donors.

Unlike GAS, the *L. lactis* strains survived and grew in whole blood. The *L lactis* strain expressing SpyCEP grew significantly better than the wild type organism in blood. As the strains are not known to have any differences in their growth (Zinkernagel, *et al.* 2008), we hypothesise that the difference was due to an enhanced ability of the SpyCEP expressing strain to evade killing. 300 rather than 30 CFU were used in this experiment which may explain why these strains grew compared to GAS. A drawback of this assay was that growth of GAS was a requirement. Whilst it appears that SpyCEP was protective against killing in

blood, presumably by phagocytes, a different assay was needed which specifically measured phagocytosis, and could be used to examine the GAS strains.

5.2.2 SpyCEP impacts on phagocytosis as assessed by flow cytometry

A technique has been previously described where bacterial phagocytosis was measured using flow cytometry (Bjerknes and Bassoe 1983). Briefly, bacteria are fluorescently labelled, opsonised and then mixed with purified leukocytes of interest. Thus cells that have phagocytosed bacteria become fluorescent themselves and can be distinguished by flow cytometry. Subsequent addition of trypan blue acts to quench fluorescent signalling due to extracellular bacteria, allowing the differentiation of cells which have actually undergone phagocytosis from those that have bacteria attached to their surface. To determine whether this protocol would be useful using GAS, a pilot experiment was performed using confocal microscopy, to confirm that cells did indeed become fluorescent in the protocol (Figure 5-4).

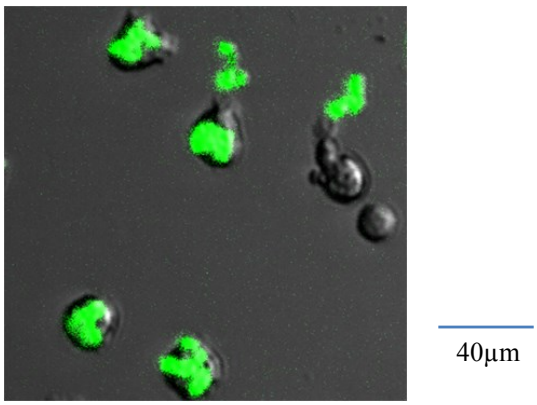
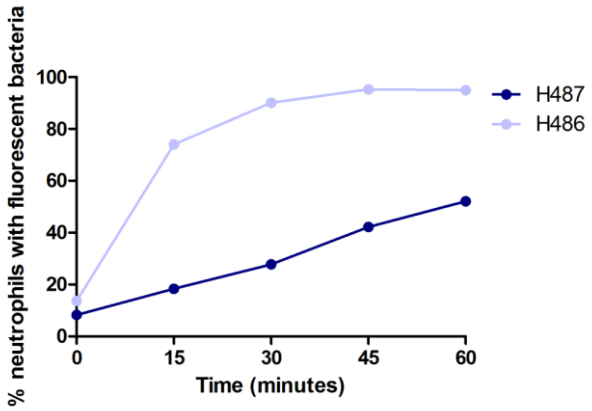


Figure 5-4 – Confocal microscopy of fluorescent GAS and neutrophils

GAS cells were stained with FITC, opsonised using whole serum and incubated with freshly extracted neutrophils.

It was observed that neutrophils were themselves not fluorescent, that bacteria could be made fluorescent using FITC, and upon phagocytosis of these bacteria, neutrophils became fluorescent (Figure 5-4). Thus flow cytometry analysis was performed to specifically measure phagocytosis of bacteria using purified human neutrophils opsonised with human serum. The *L. lactis* strains H487 and H486 (SpyCEP+ve and –ve respectively) were initially used, as these strains had previously demonstrated differences using the Lancefield assay. The bacteria were first labelled non-specifically using FITC, prior to opsonisation in serum and neutrophil co-incubation. To ensure that any signals observed were not due to labelled bacteria binding to the neutrophil outer membrane, trypan blue was included in the sample to quench extracellular fluorescence (Bjerknes and Bassoe 1984). The results are shown in Figure 5-5.

A



B

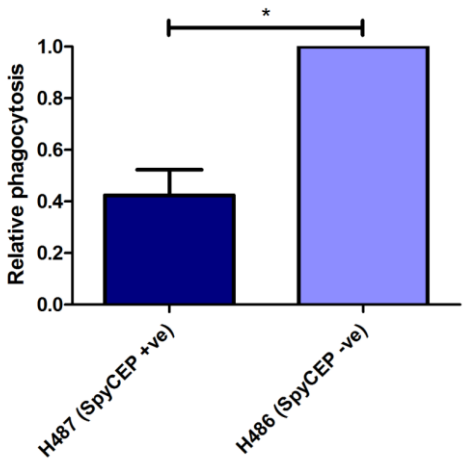


Figure 5-5 – Phagocytosis of L. lactis by purified neutrophils

(A): Percentage of neutrophils positive for fluorescence following incubation with FITC-stained H486 (wild type) and H487 (SpyCEP+ve) *L. lactis* for 15, 30, 45 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology). Data representative of three separate experiments using different donors. (B): Phagocytosis levels of the three donors 30 minutes post addition of bacteria to neutrophils. Values shown are phagocytosis levels for SpyCEP-positive strain observed, relative the

values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD, * = P<0.05 (Mann Whitney).

The wild type *L. lactis* SpyCEP-negative H486 was rapidly phagocytosed, with approximately 75% of neutrophils emitting a fluorescent signal 15 minutes post infection, reaching a plateau of 95% by 45 minutes. Conversely, the SpyCEP-positive *L. lactis* strain H487 was much more slowly phagocytosed, with only 20% of neutrophils having phagocytosed labelled bacteria at 15 minutes and only 45% by 60 minutes (Figure 5-5). This difference was preserved between the different donors.

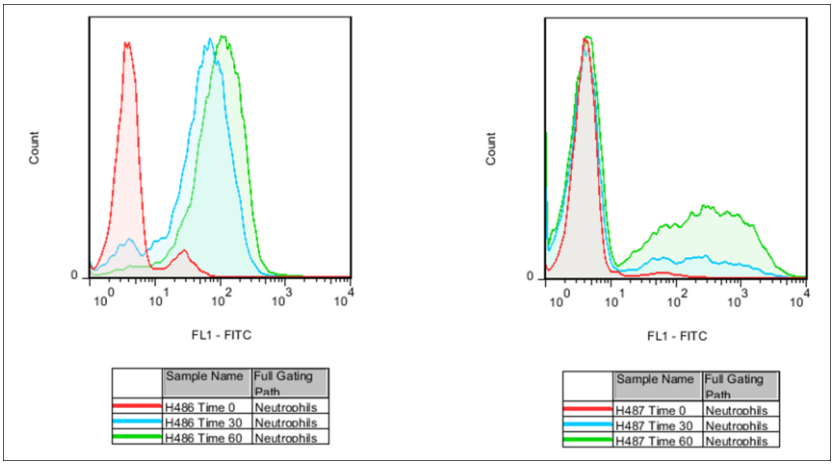


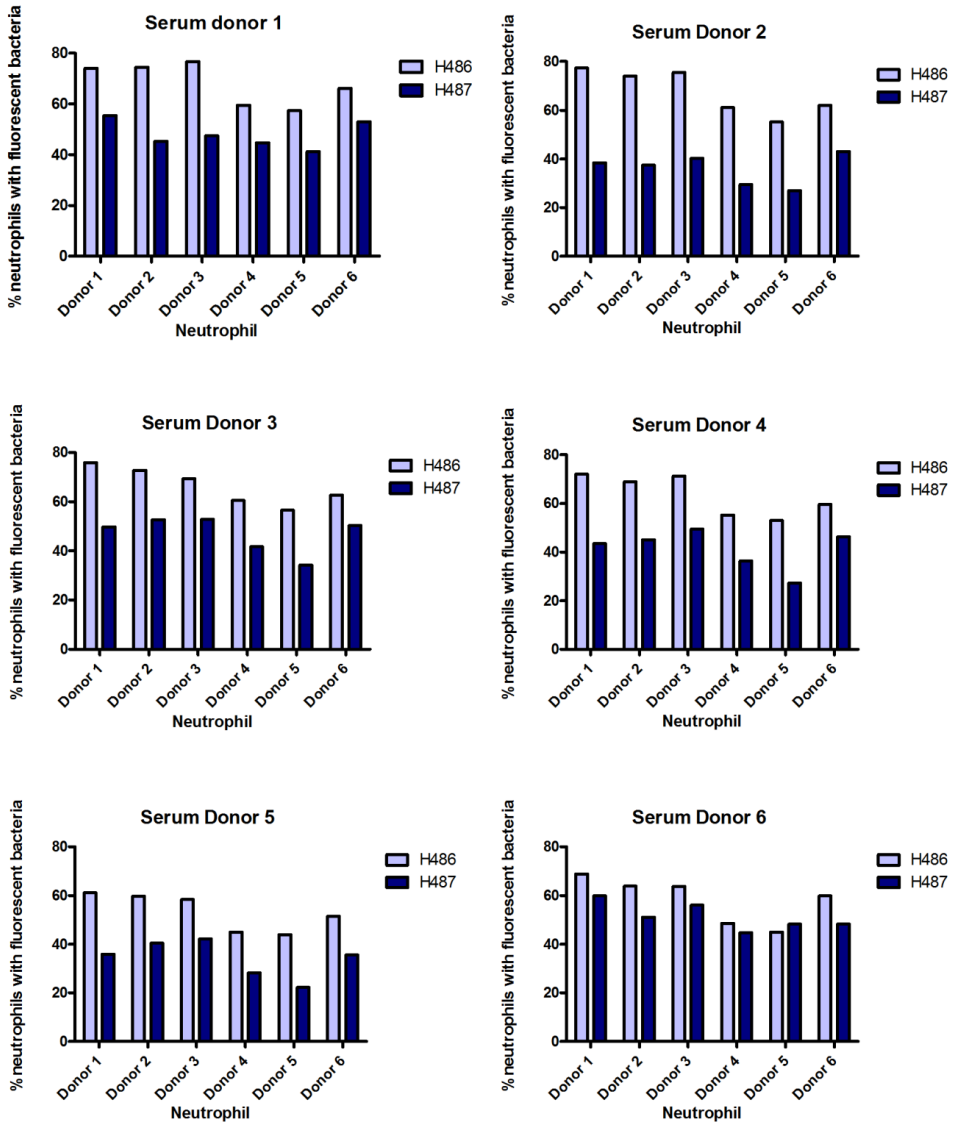
Figure 5-6 – Histograms of green fluorescent signal after phagocytosis of L. lactis

Histogram of events and FL1 channel FITC detection at 0, 30 and 60 minutes. Left panel indicates results using H486 (SpyCEP-ve), right panel H487 (SpyCEP+ve). Line colours indicate time points: red 0 minutes, blue 30 minutes, green 60 minutes incubation of bacteria with neutrophils.

There was a slightly increased median fluorescence intensity (MFI) among neutrophils that had internalised H487, and this may be the result of uneven staining between H486 and H487 (Figure 5-6). Despite this, it was clear that far fewer neutrophils had internalised bacteria when exposed to H487. The results shown in Figure 5-5 were

representative of three independent donors, but a further experiment was performed using six different donors where neutrophils of the six donors were mixed with the six different sera collected, to ensure this result was robust and repeatable.

A


Serum donor 1
Serum Donor 2
Serum Donor 3
Serum Donor 4
Serum Donor 5
Serum Donor 6
% neutrophils with fluorescent bacteria
Neutrophil
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor 6
H486
H487
0
20
40
60
80

B

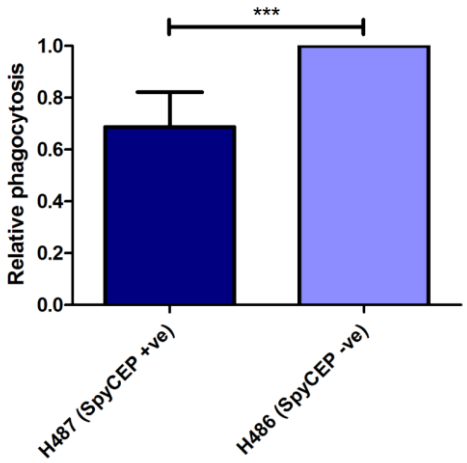


Figure 5-7 - Phagocytosis of L. lactis by purified neutrophils using a panel of donors

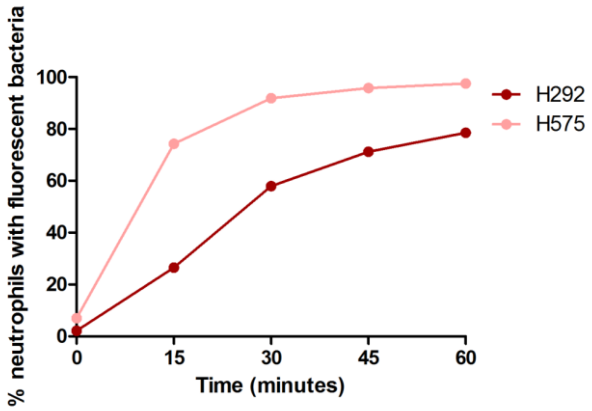
(A): Percentage of neutrophils positive for fluorescence following incubation with FITC-stained H486 (wild type) and H487 (SpyCEP +ve) *L. lactis* for 30 minutes. Each bar 20000 gated events (gating made around neutrophils based on morphology). (B): Phagocytosis levels of the six donors with six sera (36 samples in total) 30 minutes post addition of bacteria to neutrophils. Values shown are phagocytosis levels for SpyCEP-positive strain observed, relative the values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD, * = P<0.0001 (Mann Whitney).

Among 5 out of 6 donors, SpyCEP-negative *L. lactis* was phagocytosed more than the isogenic SpyCEP-positive *L. lactis*. Interestingly, not all samples demonstrated this difference, and indeed when opsonised using the serum of donor 6, the differences between the two strains were smaller or non-existent (Figure 5-7). The interperson variability in the results appeared to be primarily due to serum. Although antibodies to *L. lactis* were not expected among human donors, it is feasible that strong anti SpyCEP activity could explain this anomaly. This data however shows that the findings are robust and observed in the majority of donors.

SpyCEP interferes with the neutrophil response against Group A Streptococcus

Using both flow cytometry and the Lancefield assay, SpyCEP has been proven to interfere with the phagocytosis of *L. lactis*. It was next decided to test whether these observations could also be made using SpyCEP+ve and –ve GAS strains (Figure 5-8), given that the Lancefield assay could not be used for the M81 serotype.

A



B

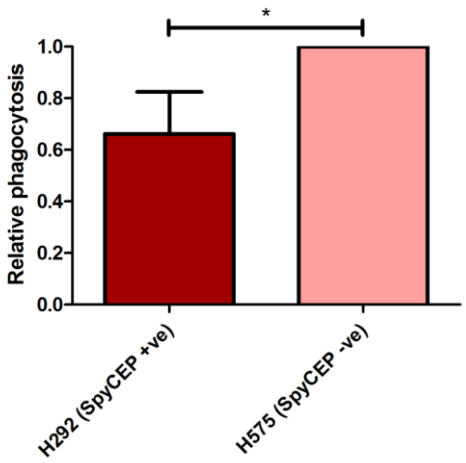


Figure 5-8 – Phagocytosis of GAS by purified neutrophils

(A): Percentage of neutrophils positive for fluorescence at following incubation with FITC-stained H292 (wild type) and H575 (SpyCEP-ve) GAS for 15, 30, 45 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology). Data representative of three separate experiments using different donors. (B): Phagocytosis levels of the three donors 30 minutes post addition of bacteria to neutrophils. Values shown are phagocytosis levels for SpyCEP-positive strain observed, relative the

values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD, * = P<0.05 (Mann Whitney).

SpyCEP-negative H575 was more quickly phagocytosed than the wild type SpyCEP expressing counterpart H292 (Figure 5-8). Approximately 75% of neutrophils incubated with H575 were positive for a fluorescent signal 15 minutes post infection, reaching a plateau of 95% by 45 minutes. Neutrophils co-incubated with SpyCEP-positive GAS strain H292 demonstrated green fluorescence among 25% of the population 15 minutes post infection, rising to 75% positive by 60 minutes. As expected and seen throughout this study, the difference between the SpyCEP-positive and negative GAS strains was less obvious than seen using the *L. lactis* strains, but was still clearly measurable and significant.

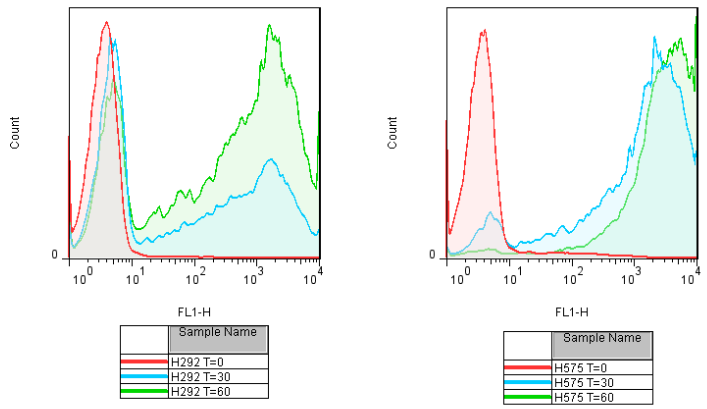


Figure 5-9 - Histograms of green fluorescent signal after phagocytosis of GAS

Histogram of events and FL1 channel FITC detection at 0, 30 and 60 minutes. Left panel indicates results using H292 (SpyCEP+ve), right panel H575 (SpyCEP-ve). Line colours indicate time points: red 0 minutes, blue 30 minutes, green 60 minutes incubation of bacteria with neutrophils.

The MFI of neutrophils that had phagocytosed bacteria was similar between the two GAS strains, again indicating that the reduced percentage of cells positive for green fluorescence was not due to a technical difficulty of staining (Figure 5-9).

5.2.3 Impact of serum depletion of antibody and complement on phagocytosis of SpyCEP +/- strains

Bacteria in the experiments of the previous section were opsonised using whole human serum. Although neutrophils are able to phagocytose some unopsonised particles (Lee*, et al.* 2003), this opsonisation source was found to be vitally important for phagocytosis of the strains tested here. An experiment was performed using foetal calf serum (FCS) rather than human serum with *L. lactis* strains. This serum contains only very low levels of bovine antibody, and has been heat inactivated, which disrupts the complement system (Morgan 2000). No phagocytosis was observed (Figure 5-10).

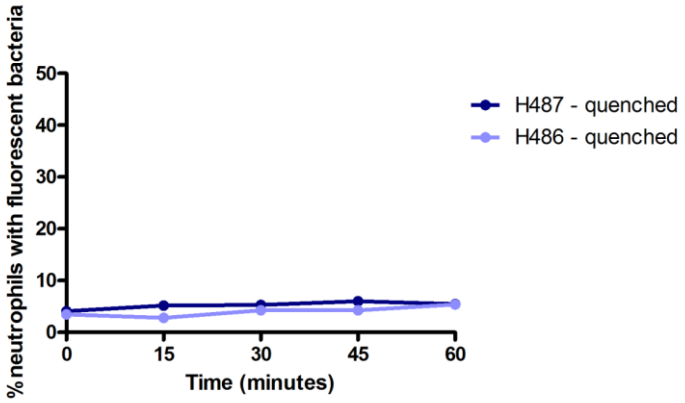


Figure 5-10 – Phagocytosis of L. lactis without opsonisation source

Percentage of neutrophils positive for fluorescence following incubation with FCS incubated FITC-stained H487 (SpyCEP+ve) and H486 (SpyCEP-ve) *L. lactis* for 15, 30, 45 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology), and was performed once.

As previous work (Figure 5-7) showed that differences in phagocytosis between donors were primarily due to sera, it was decided to determine what particular opsonin in the sera was important for phagocytosis of the strains. Initially, each of the four bacterial strains were incubated in freshly extracted whole human serum, as per the phagocytosis experiments, before being probed using fluorescently conjugated antibodies against human antibody or C3b, an important opsonin of the complement cascade (Figure 5-11).

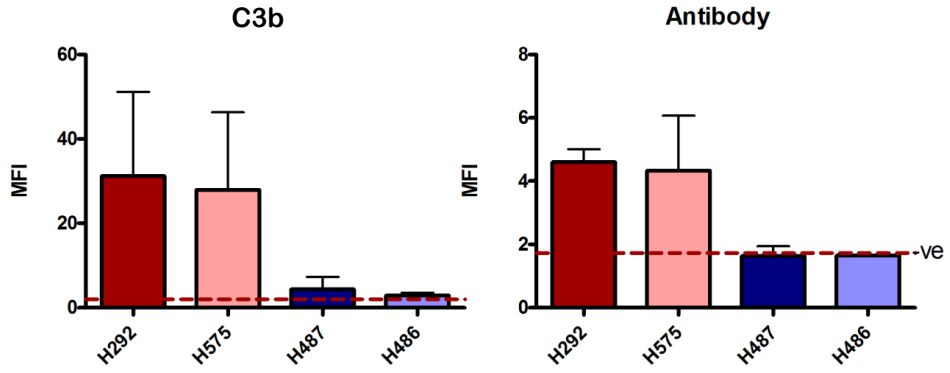


Figure 5-11 – Opsonisation of GAS and L. lactis

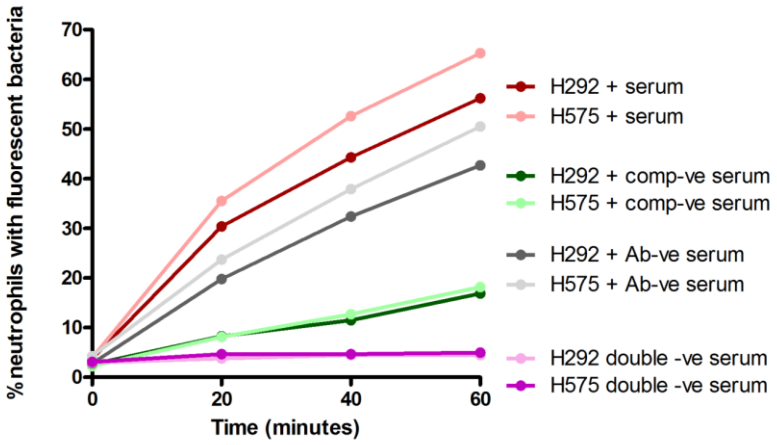
GAS strains H292 and H575 (SpyCEP+ve and SpyCEP-ve respectively) and *L. lactis* strains H487 and H486 (SpyCEP+ve and SpyCEP-ve respectively) were incubated with whole human serum for 15 minutes. This was removed and cells were probed using fluorescently conjugated antibody against bound C3b (left panel) and IgG (right panel). Bars indicate mean and error bars SD of three independent experiments.

SpyCEP did not affect the binding of either complement or antibody to either the GAS or *L. lactis* strains, with no significant differences observed within the pairs of strains (Figure 5-11). GAS was observed to bind more complement than *L. lactis*. *L. lactis* is an avirulent bacterium that produces no virulence factors of its own (Bolotin, *et al.* 2001, Poquet, *et al.* 1998, van Asseldonk, *et al.* 1990). Furthermore it is coated in a polysaccharide pellicle, which is known to be anti-phagocytic (Chapot-Chartier, *et al.* 2010). This is interesting when considering the results of Figure 4-7, where wild type *L. lactis* was cleared within six hours. This bacterium is avirulent, and hence seems to require very little response from the immune system to clear the infection. This might explain why *L. lactis* able to express SpyCEP causes severe disease in the mouse model, being almost refractory to the immune system, but now expressing a virulence factor. *L. lactis* also was not bound by antibodies using the sera tested

in this experiment, although human antibodies were not expected as this organism does not cause disease in humans. GAS was opsonised by antibody, being a common pathogen of humans. However, as both these values had only low median fluorescence intensities, it is possible that this method of measuring antibody deposition is not very sensitive and limited binding of antibodies against *L. lactis* may be missed. Furthermore, Figure 5-7 shows that phagocytosis of *L. lactis* is varied among donors, and thus a minority of donors may express anti-*L. lactis* antibodies.

Fresh human serum was then depleted of complement by heat inactivation or antibodies by passage over columns bound with antibody binding proteins (Chapter 2). Double depleted sera were also produced by combining these methods. GAS phagocytosis by neutrophils was found to be optimal when both complement and antibody were present in whole serum (Figure 5-12). A slight reduction in phagocytosis was observed when antibody was depleted from the serum. Complement depletion had a more profound impact on phagocytosis, and only 15% of neutrophils demonstrated fluorescence at 60 minutes post-coinoculation. There was little difference between the SpyCEP-positive and negative strains using this serum. However, as phagocytosis rates were so low, it may be that this method was not sensitive enough to determine any differences here. Double depleted sera demonstrated no phagocytosis (Figure 5-12). When this experiment was repeated, high levels of phagocytosis were observed despite depleting the serum using the same method. Double depleted serum still phagocytosed 60% as well as whole serum. The trends observed were the same, but the fold changes were different due to this high residual phagocytosis (Figure 5-12).

A



B

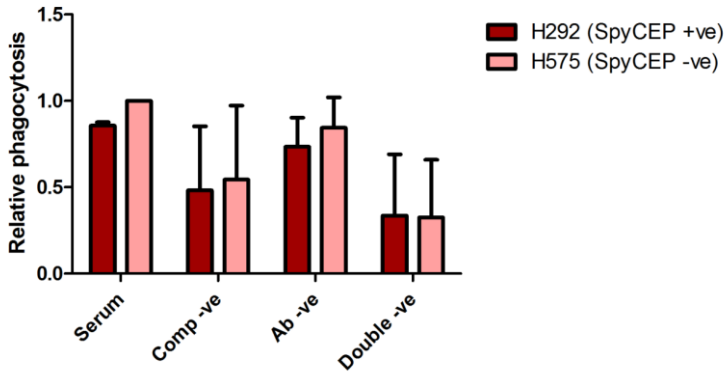


Figure 5-12 – Phagocytosis of GAS opsonised with depleted sera

(A): Percentage of neutrophils positive for fluorescence at following incubation with FITC-stained H292 (wild type) and H575 (Δ*cepA*) GAS opsonised in variously depleted sera for 20, 40 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology). Data from one of two separate experiments using different donors. (B): Phagocytosis levels of the two donors 40 minutes post addition of bacteria to neutrophils. Values shown are phagocytosis levels, relative to the values obtained for SpyCEP-negative strain in each experiment opsonised with whole serum. Error bars indicate SD, * = $P<0.05$ (Mann Whitney).

Similarly prepared sera were also used with the *L. lactis* strains. Here the results using antibody depleted sera were less repeatable, with some antibody only sera able to opsonise successfully whereas others could not. This was probably due to these donors containing an antibody which could opsonise *L. lactis* whereas others did not have these antibodies. Two results are shown in Figure 5-13, showing both types of donor.

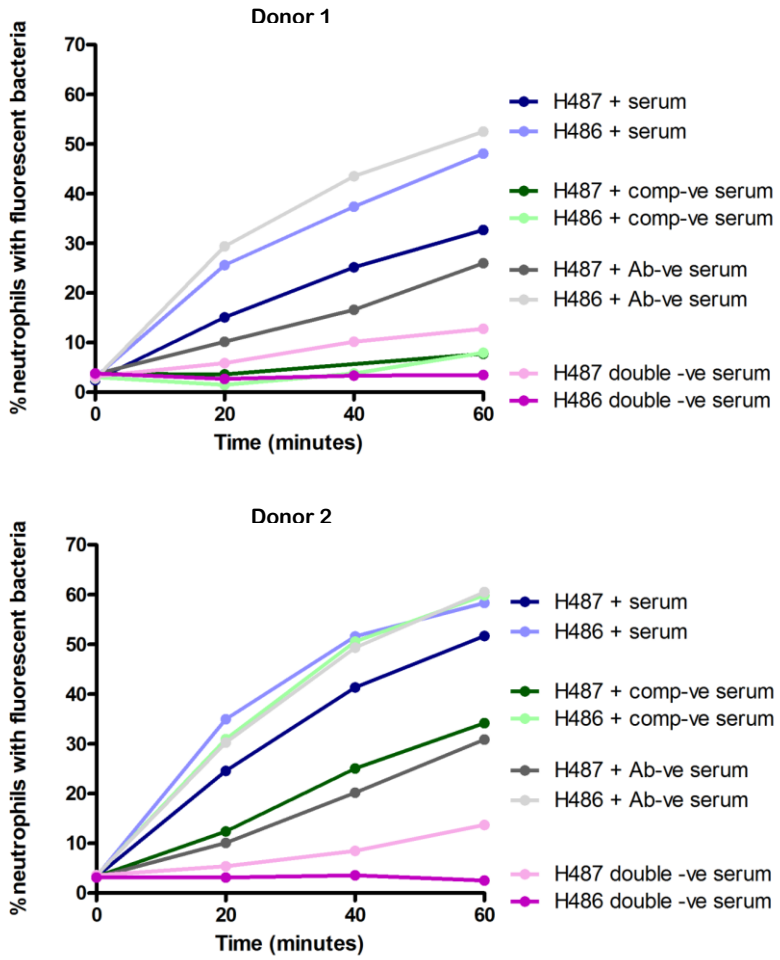


Figure 5-13 – Phagocytosis of L. lactis opsonised with depleted sera

Percentage of neutrophils positive for fluorescence at following incubation with FITC-stained H486 (wild type) and H487 (SpyCEP+ve) *L. lactis* opsonised in variously depleted sera for 20, 40 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology). Data shows two of three donors.

Figure 5-13 shows the phagocytosis of *L. lactis* by two different donors. In both donors, bacteria were effectively opsonised where whole sera were used, and this was inhibited by SpyCEP. Neither strain was effectively phagocytosed when opsonised with

double depleted sera. When antibody alone was depleted, in both sera, the remaining complement was able to opsonise the bacteria, and subsequent phagocytosis was inhibited by the presence of SpyCEP.

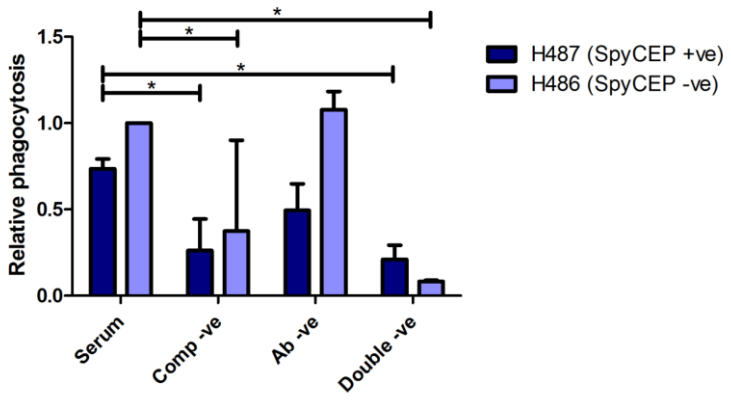


Figure 5-14 – Relative phagocytosis of L. lactis opsonised with depleted serum

Phagocytosis levels of the three donors (Figure 5-13) 40 minutes post addition of bacteria to neutrophils. Values shown are phagocytosis levels, relative to the values obtained for SpyCEP-negative strain in each experiment opsonised with whole serum. Error bars indicate SD, * = $P<0.05$ (Mann Whitney).

The data showed that complement is very important in the opsonisation of GAS and *L. lactis* strains. Antibody appears to have a limited opsonic role against GAS compared to complement with antibody depleted serum allowing considerable residual phagocytosis, unlike complement depleted or double depleted serum (Figure 5-14).

5.3 Discussion

5.3.1 SpyCEP impacts on neutrophil phagocytosis

The Lancefield assay was originally used to determine whether or not a particular blood sample demonstrated immunity to a particular serotype of GAS. In this study, we attempted to use it as measure of phagocytosis. This assay however is not a measure of phagocytosis, rather it is a measure of two competing factors, bacterial growth and bacterial killing. Using GAS strains, the method proved to be uninformative, with all donors used able to kill both SpyCEP+ve and SpyCEP-ve GAS strains within one hour. The SpyCEP+ve and –ve *L. lactis* strains yielded an interesting result however, in that significantly more bacteria survived if SpyCEP was present than where absent. The difference between GAS and *L. lactis* was presumably due to the specific donors used having immunity against GAS infection due to exposure, but not against *L. lactis* which does not cause disease.

Although this assay proved to be unhelpful when trying to measure differences in phagocytosis using GAS strains, the results with *L. lactis* did appear more instructive. However, this assay was not designed to allow quantification of phagocytosis, rather a yes/no answer to immunity. Figure 5-15 shows the problems with using this method. This Figure has been drawn to simulate results from a Lancefield assay, where bacteria are killed at a constant rate, and are growing logarithmically with a doubling time of 30 minutes after a 30 minute lag phase. Such a model suggests that the starting CFU is very important in the results obtained, and differences in killing capacity between two strains are only revealed clearly

when an optimal CFU is used. The results of Figure 5-2 and Figure 5-15 indicated that a more quantitative and specific measure of phagocytosis was required.

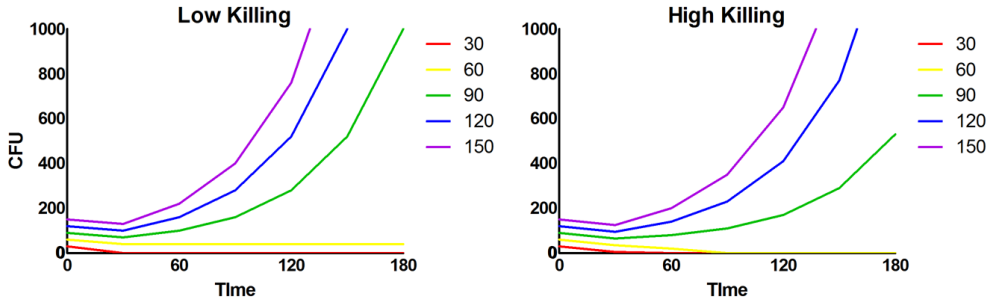


Figure 5-15 – Hypothetical Lancefield assay results

CFU recovered using a model Lancefield assay, whereby bacteria are killed at constant rate (60 CFU/hr), while bacteria were modelled to grow logarithmically following a 30 minute lag phase. Each line represents a different starting titre

To this end, the flow cytometric method of determining phagocytosis (Bjerknes and Bassoe 1983) was used. Such a method is not dependent on bacterial growth or killing for its results, only upon the internalisation of bacteria. Indeed this method provides no information about the killing of bacteria, and any effect that SpyCEP may have on this process. A large difference in neutrophil phagocytosis between SpyCEP+ve and –ve *L. lactis* strains could be observed, with the SpyCEP-positive strain being phagocytosed considerably less well than the SpyCEP-positive wild type strain (Figure 5-5). As the only known substrates of SpyCEP are chemokines, this result suggests that by cleaving chemokines, SpyCEP inhibits neutrophil phagocytosis of *L. lactis*. Confocal microscopy confirmed that fluorescence of neutrophils was caused by internalised bacteria

The FITC labelling procedure is non-specific, and is mediated by covalent binding to primary amines. These are only found at the amino terminus of proteins. As FITC is a small molecule, with a molecular weight of 389Da, and is binding only to the very terminal-most amino acid of proteins, it is unlikely that it disrupts the protein function of the strains. *L. lactis* expresses very few extracellular proteins of its own, and has a polysaccharide pellicle repeating unit outer coat (Chapot-Chartier*, et al.* 2010). The SpyCEP-negative strain is hence predictably dimmer than the SpyCEP-positive strain due to number of FITC binding targets (Figure 5-6), and both strains are dimmer than the GAS strains (Figure 5-9). However, this has not prevented the method from being useful, as the dimmest bacteria were still sufficiently bright to allow positive and negative groups of neutrophils to be clearly distinguished by flow cytometry (Figure 5-6).

The results obtained using this method were broadly repeatable using different donors, with wild type *L. lactis* being significantly better phagocytosed by neutrophils than SpyCEP expressing *L. lactis* (Figure 5-7). However, some interperson variation was observed, mediated by differences in sera rather than cells. This implied that opsonisation of bacteria represented the source of any interperson differences. It is possible that donor 5 had antibodies against SpyCEP, as these have been reported following GAS infection (Fritzer*, et al.* 2010). By neutralising this virulence factor with antibody, any difference attributable to that antibody would disappear. These interperson differences meant that throughout this chapter, only representative rather than pooled data could be shown, and statistics were performed on relative differences between the SpyCEP positive and negative strains within experiments.

The differences in neutrophil phagocytosis between SpyCEP+ve and –ve strains of GAS were smaller than those seen using *L. lactis*, presumably due to other virulence factors

expressed by GAS which also target neutrophils. Nevertheless, a clear difference in the phagocytosis of these two strains was clearly observed by flow cytometry (Figure 5-8).

Previous work has reported that SpyCEP expression protects GAS from killing by purified neutrophils. This was assessed by measuring viable CFU following incubation with purified neutrophils. Such observations were attributed to SpyCEP preventing NETosis by neutrophils, reducing bacterial killing (Zinkernagel*, et al.* 2008). In addition to preventing NETosis, this study revealed that SpyCEP is also able to prevent killing by neutrophils by preventing phagocytosis. NET based killing of bacteria is extracellular, thus signal from FITC labelled bacteria within NETs is quenched by the trypan blue. Hence this difference in NETosis is not responsible for the observed differences in bacterial phagocytosis.

5.3.2 Impact of serum depletion of complement and antibody on phagocytosis by neutrophils

Work in this section has demonstrated that SpyCEP-positive and negative strains of the same species of bacteria were opsonised with antibody and complement to a similar level. GAS is known to make virulence factors which impair opsonisation and thus phagocytosis (1.3.2 - Defence against opsonisation), but the differences observed in this study of phagocytosis by neutrophils are not due to differences in opsonisation. GAS demonstrated greater opsonisation by C3b, an opsonin of the complement system, than *L. lactis*. Complement is deposited on the surface by both a reversible random process, and using a microbe specific process. By expressing its repetitive polysaccharide pellicle rather than a multi protein surface of several virulence factors, *L. lactis* seemed to be protected from at least one of these modes of complement deposition as evidenced by the low complement

deposition seen on the *L .lactis* strains (Figure 5-11). The pellicle has been reported to be anti-phagocytic previously (Chapot-Chartier*, et al.* 2010), and these results suggest a mechanism by which this may occur, acting to prevent complement deposition.

The three serum donors used displayed antibody binding against only GAS (Figure 5-11). The result here was expected, as GAS causes regular human infection, whereas *L. lactis* does not. Although some specific donors may have antibody against *L. lactis* due to occupational exposure, the three donors used in this particular study did not (Figure 5-11).

In contrast to the donors used in Figure 5-11, it was apparent that one donor in further experiments could successfully opsonise *L. lactis* with antibody alone resulting in phagocytosis by neutrophils (Figure 5-13). All donors used for these studies were laboratory workers, thus it is conceivable that occupational exposure has resulted in antibodies being raised against *L. lactis.* It is hence likely that the results where antibody was not able to opsonise *L. lactis* are more representative of the general population. Complement was cases able to successfully opsonise *L. lactis* in all cases. Phagocytosis by neutrophils, whether opsonised by whole serum, complement, or, where applicable, antibody, was inhibited by SpyCEP, indicating a general inhibition rather than pathway specific mechanism.

GAS was optimally opsonised for neutrophil phagocytosis when both complement and antibody were present. When antibody was depleted, complement was still able to opsonise GAS well, and phagocytosis by neutrophils in both these experiments was inhibited by SpyCEP expression (Figure 5-12). Antibody was able to opsonise GAS slightly and subsequent phagocytosis by neutrophils was not inhibited by SpyCEP expression. Such results raise interesting questions about the role and target of antibody in opsonisation of GAS. The serotype specific antibody raised against GAS is against the M protein. The M protein is itself anti-opsonic by interfering with complement deposition and its subsequent

interaction with the complement receptors on neutrophils (Peterson*, et al.* 1979, Weis*, et al.* 1985). Thus, antibody against this protein could enhance phagocytosis by both direct opsonisation, or by neutralising this protein, enhancing complement deposition. Confusingly, a further role of opsonising antibody is to provide a focal point for complement deposition, enhancing the process. Thus, determining the precise function of the antibody is very difficult. Interestingly, other researchers have found that blockade of antibody receptors on the surface of neutrophils had no impact on phagocytosis, whereas blockade of complement receptors caused a complete ablation of phagocytosis (Nilsson*, et al.* 2005).

As antibody depleted serum containing only complement had reduced neutrophil phagocytosis compared to whole serum, the antibody clearly aided opsonisation when complement was present. However, phagocytosis of bacteria opsonised with antibody alone was very poor. These results suggest that the antibody raised against GAS acts primarily to enhance complement deposition or its interaction with complement receptors. When using antibody and complement depleted serum, both strains were scarcely phagocytosed by neutrophils, indicating that the depletion methods used in this study were effective.

The only known substrates of SpyCEP are neutrophil specific chemokines. Hence, the mechanism by which SpyCEP inhibits neutrophil phagocytosis presumably involves a chemokine dependent process. Variation in antibody specificity and opsonisation between donors makes its study is challenging. However, the complement system seems very important in the phagocytosis of both *L. lactis* and GAS, and results between donors are much more repeatable. Furthermore, complement mediated neutrophil phagocytosis was always disrupted by SpyCEP expression.

Chapter 6 - Results: SpyCEP impedes phagocytosis by neutrophils by interfering with complement receptor 3

6.1 Introduction

In the previous chapter, I made the novel finding that SpyCEP was able to impede phagocytosis of bacteria. In this chapter, I aim to elucidate the mechanism responsible for this. Recent work has shown that chemokines which have been cleaved by SpyCEP may demonstrate reduced binding to CXCR1 and CXCR2 (Goldblatt personal communication). Signalling after the binding of these receptors by their ligands is known to be important in neutrophil activation (Amulic*, et al.* 2012). This includes the upregulation of complement receptor 3 (CR3), known to be vitally important in phagocytosis of GAS (DeMaster*, et al.* 2002, Detmers*, et al.* 1990, Nilsson*, et al.* 2005, Weineisen*, et al.* 2004).

As such, I hypothesise that cleavage of chemokines by SpyCEP reduces the activation of neutrophils by preventing chemokine signalling, reducing surface expression of CR3. As CR3 is known to be important in GAS phagocytosis, such a hypothesis would explain the results observed in the previous chapter. However, importantly, no effects on phagocytosis have been reported when using chemokine receptor antagonist, such as the drug reparixin and neutralising antibodies (Stillie*, et al.* 2009). This suggests that the link between ligand binding of CXCR1/2 and phagocytosis is unclear.

Effects on neutrophil activation by SpyCEP have been previously reported, though only in situations where activation was induced by exogenous CXCL8. For example, SpyCEP-expressing strains were able to prevent neutrophil NETosis (Zinkernagel 2008), and SpyCEP

inhibited shedding of CD62L, a feature characteristic of neutrophil activation mediating binding to activated endothelium (Edwards, *et al.* 2005).

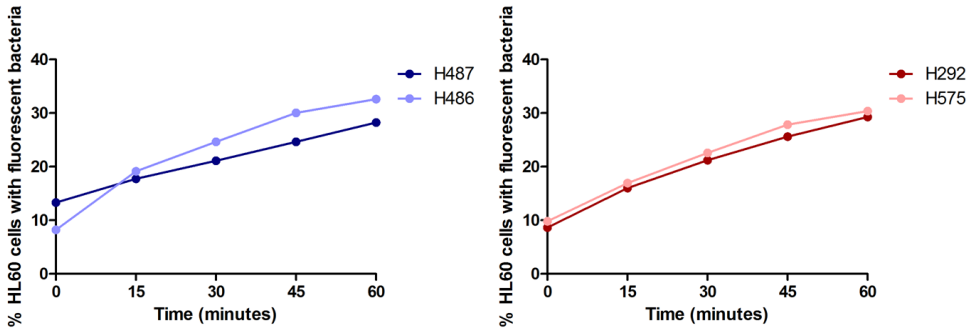
To fully elucidate the mechanism behind SpyCEP-impaired phagocytosis, my hypothesis was tested using flow cytometric assays using phagocytes which are relatively unresponsive to CXCR1 and 2 chemokine ligands, by measuring chemokine levels in reaction mixtures, and by measuring complement receptor levels on the surface of neutrophils.

6.2 Results

6.2.1 Internalisation of bacteria by other cell types

To determine whether the observed impact of SpyCEP on neutrophil phagocytosis could be explained by a generalised impact on phagocytosis or internalisation, the effect of SpyCEP on other cell types was determined. HL60 cells are an immortalised leukocyte cell line, which can be induced to differentiate into neutrophil-like phagocytic cells, and which express receptors for C3b and the Fc region of antibodies, allowing phagocytosis of opsonised particles (Collins*, et al.* 1979). HL60 cells however express approximately one tenth of the number of CXCR receptors that are expressed by primary human neutrophils (Grob*, et al.* 1990, Zahn*, et al.* 1997). HL60 cells are hence less responsive to CXCL chemokines, the substrates of SpyCEP, than human neutrophils.

A



B

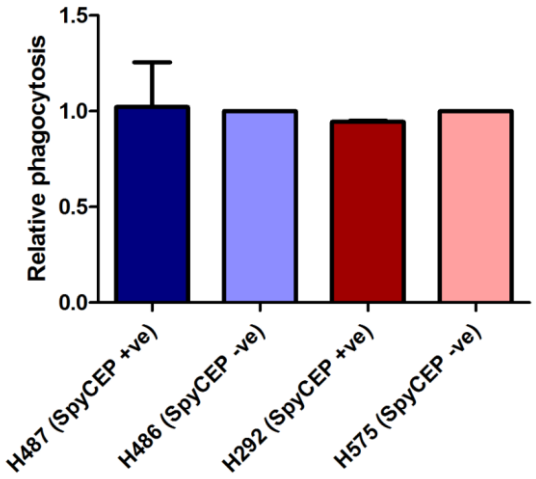


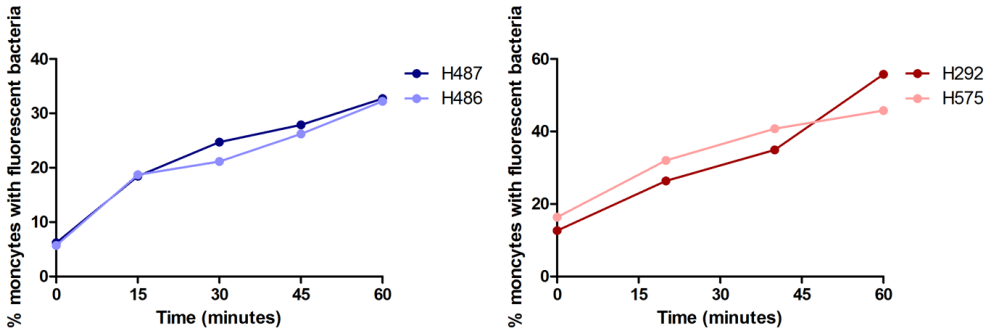
Figure 6-1 - Impact of SpyCEP on HL60 cell phagocytosis of bacteria

(A): Percentage of HL60 cells positive for fluorescence following incubation with FITC-stained *L. lactis* strains H486 (wild type) and H487 (SpyCEP+ve) or GAS strains H292 (SpyCEP+ve) and H575 (SpyCEP-ve) for 15, 30, 45 and 60 minutes. Each time point represents 20000 gated events (gating made around HL60 cells based on morphology). Data representative of two separate experiments using different cell preparations. (B): Relative phagocytosis of SpyCEP+ bacteria compared to their isogenic SpyCEP negative strain by HL60 30 minutes after the addition of bacteria. Data combined from two experiments. Values

shown are phagocytosis levels for SpyCEP positive strain observed, relative to the values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD.

Unlike the situation using fresh human neutrophils in Chapter 5, SpyCEP-positive and negative *L. lactis* and GAS strains were equally phagocytosed by HL60 cells (Figure 6-1). The *L. lactis* strains were both phagocytosed by 20% of the HL60 cells in 15 minutes, rising to approximately 30% by 60 minutes. Phagocytosis occurred more slowly than had been observed using freshly extracted neutrophils. The GAS strains were phagocytosed at a similar rate to the *L. lactis* strains, again reaching 30% cells positive for phagocytosis by 60 minutes, regardless of SpyCEP expression. These results indicate that the mechanism by which SpyCEP impairs phagocytosis in neutrophils cannot be replicated in HL60 cells. Despite the similarity in range of phagocytosis receptors expressed by HL60 and neutrophils, the much lower expression of CXCR1 and 2 receptors by HL60 provides a possible explanation for these results, through an effect of SpyCEP on CXCR1/2 signalling. HL60 are also however not genuine phagocytes, but a cell line, and may have alterations at several points in the phagocytosis signalling in addition to near absent CXCR1/2 signalling. To determine if SpyCEP-mediated effects on phagocytosis could be observed in all *bona fide* phagocytes, regardless of CXCR1/2 signalling, freshly extracted primary monocytes were also evaluated in phagocytosis assays (Figure 6-2).

A



B

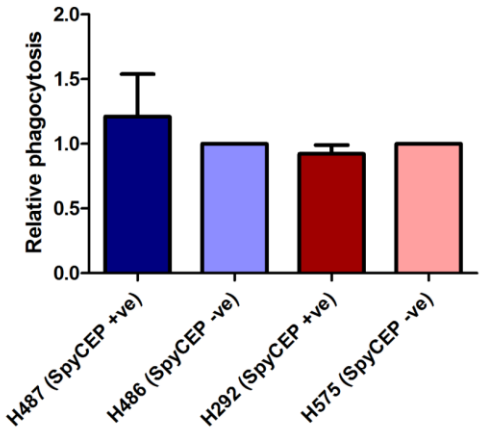


Figure 6-2 – Monocyte phagocytosis

(A): Percentage of monocytes positive for fluorescence following incubation with FITC-stained *L. lactis* strains H486 (wild type) and H487 (SpyCEP+ve) or GAS strains H292 (SpyCEP+ve) and H575 (SpyCEP-ve) for 15, 30, 45 and 60 minutes. Each time point represents 20000 gated events (gating made around monocytes based on morphology). Data representative of three separate experiments using different donors. (B): Monocyte phagocytosis levels 30 minutes post addition of bacteria to monocytes (n=3 donors). Values shown are phagocytosis levels for SpyCEP-positive strain observed, relative the values obtained for isogenic SpyCEP-negative strain in each experiment. Error bars indicate SD.

SpyCEP-positive and negative *L. lactis* and GAS strains were equally phagocytosed by monocytes (Figure 6-2). The *L. lactis* strains were phagocytosed by 20% of the monocytes in 15 minutes, rising to approximately 35% by 60 minutes. Approximately 25% of monocytes incubated with the GAS strains demonstrated fluorescence at 20 minutes post inoculation. 50% of the monocytes were positive for phagocytosis by 60 minutes. These results indicate that the mechanism by which SpyCEP impairs phagocytosis is also not seen using monocytes. Like HL60, monocytes are also low expressers of CXCR1 and 2 compared to neutrophils (Shi and Palmer 2011). Hence the similarity of trends between the monocytes and the HL60 cells, and their differences from neutrophils, were expected. Together these results suggest that SpyCEP mediates an effect on neutrophil phagocytosis through the CXCR1 and 2 receptor pathways, as only phagocytes which express high levels of these receptors are affected by SpyCEP. As the ligands for these receptors form the substrate range of SpyCEP, such an observation is not unexpected. Furthermore, it is known that CXCR1 and 2 signalling leads to the upregulation of receptors involved in GAS phagocytosis (DeMaster*, et al.* 2002, Detmers*, et al.* 1990, Nilsson*, et al.* 2005, Weineisen*, et al.* 2004).

The phagocytosis of GAS by monocytes was further investigated using depleted sera. This was to determine which opsonisation pathways are important in the phagocytosis of GAS by monocytes. Macrophages are known to be important phagocytes of GAS in infection (Goldmann*, et al.* 2004).

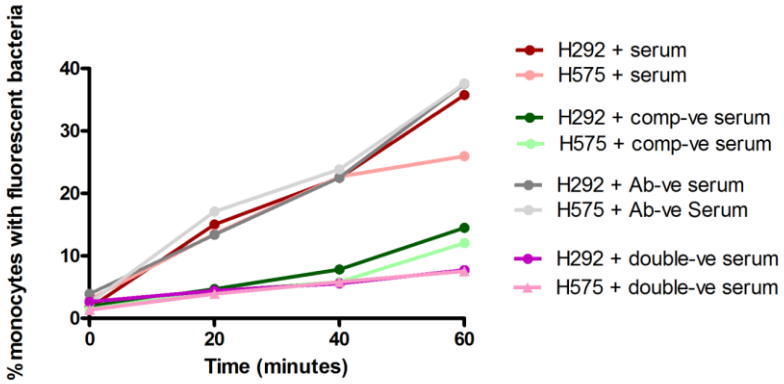


Figure 6-3 – Monocyte phagocytosis of GAS opsonised with depleted sera

Percentage of primary human monocytes positive for fluorescence following incubation with FITC-stained GAS strains. H292 (wild type) and H575 (Δ*cepA*) GAS were opsonised in variously depleted sera. Samples for flow cytometry were obtained 20, 40 and 60 minutes after co-incubation. Each time point represents 20000 gated events (gating made around monocytes based on morphology) and the experiment was performed once.

The results obtained using depleted sera to opsonise GAS for phagocytosis by monocytes were similar to those obtained for neutrophil phagocytosis using similarly depleted sera (Figure 6-3). Antibody depletion from the serum in this experiment had little impact on phagocytosis compared to complement depletion. Both SpyCEP-positive and SpyCEP-negative GAS strains were phagocytosed by approximately 40% of monocytes by 60 minutes using whole and antibody depleted serum. Complement depletion however had a profound impact on phagocytosis, with only 15% of monocytes demonstrating fluorescence at 60 minutes post inoculation. Double depleted sera demonstrated no phagocytosis (Figure 6-3). In all cases, the expression of SpyCEP had no impact on phagocytosis by fresh primary monocytes. Taken together, there was no evidence that SpyCEP expression could influence uptake of bacteria by HL60 cells or monocytes.

Work has been performed by other investigators which suggested that SpyCEP prevents internalisation of GAS into epithelial cells, which do not express CXCR1 or CXCR2, highlighting a potential role for SpyCEP entirely distinct from any effect on chemokines (Andreoni and Zinkernagel, Abstract at XVIII Lancefield Symposium). These investigators found their observation to be dependent on SpyCEP retaining its active protease site, but not on chemokine cleavage, indicating, an alternative substrate and mechanism of SpyCEP activity. This raised the possibility that the phenomenon observed in neutrophils was not a function of phagocytosis, but might be replicated in other cell types which express low levels of CXCR1 and 2. Although we did not specifically evaluate uptake of bacteria by epithelial cells, as had been undertaken by Andreoni and colleagues, we did not find that SpyCEP had a generalised effect on internalisation based on data from HL60 and monocytes.

6.2.2 Investigating the impact of CXCR receptors on neutrophil phagocytosis

The experiments using other cell types indicated that SpyCEP may impair phagocytosis via disruption of CXCR1 and 2 dependent processes. An approach was followed whereby an antagonist against the chemokine receptors CXCR1 and 2 was included prior to the addition of bacteria to neutrophils to further investigate this hypothesis. By blocking signalling through these receptors, it was hypothesised that the effects of SpyCEP could be mimicked, and the wild type *L. lactis* strain protected from phagocytosis to the same extent as SpyCEP-positive *L. lactis*.

The CXCR antagonist was however a coloured chemical, which absorbed the laser light of the flow cytometer, and emitted light over a wide spectrum. Although this prevented

the antagonist having utility in flow cytometric assays, it was hoped that the reagent could be used for Lancefield assays which are not colourimetric using *L. lactis*. However, the antagonist was found to have antibacterial activity against *L. lactis* at working concentrations (Figure 6-4).

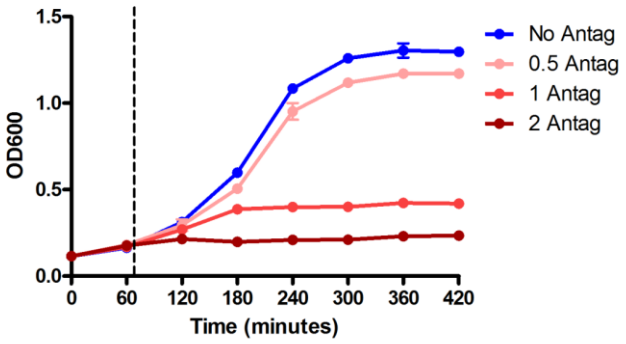


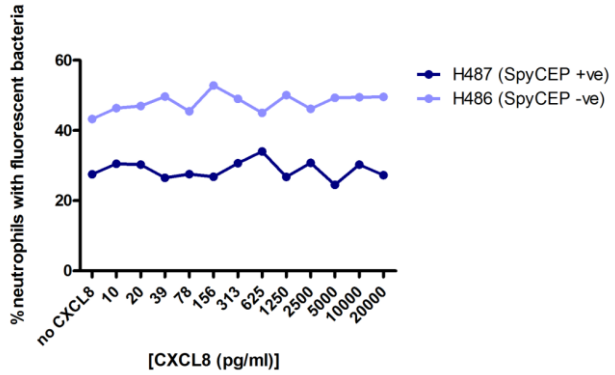
Figure 6-4 – Growth curve of L. lactis with CXCR antagonist

Growth curve of SpyCEP-negative *L lactis* (H486) over 420 minutes. After 60 minutes, antagonist (PD0220245 99% (Axon Medchem, Netherlands)) was added (dashed line) at 0.5x, 1x or 2x the working concentration (1x working concentration was 10.7µg/ml), and bacterial growth measured over 420 minutes. Results show mean of 3 repeats, error bars indicate standard deviation

As experiments where antagonising the binding of chemokine to their receptors failed to produce meaningful results, an alternative strategy was used. Here, soluble, exogenous chemokine was added to the cells, to overwhelm the cleavage effects of SpyCEP and thus overcome the SpyCEP-positive phenotype. CXCL8, as the most abundant inflammatory CXCL chemokine, with the highest specific activity on neutrophils (Mortier, *et al.* 2011), was used for initial experiments. Furthermore, the recombinant CXCL8 used for this experiment has been reported to be cleaved relatively slowly by SpyCEP, but still has biological activity

as indicated by chemotaxis assays (Goldblatt, personal communication). It is also known that cleaved chemokines are have low affinity for their chemokine receptors (Goldblatt personal communication). This experiment was performed to prove that differences in the amount of chemokine in the neutrophil phagocytosis reactions were the cause of the observed phenotype.

A



B

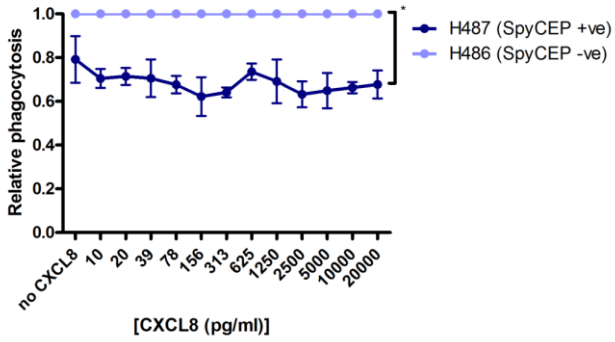


Figure 6-5 – Neutrophil phagocytosis of L. lactis with exogenous CXCL8 titration

(A): Percentage of human neutrophils positive for fluorescence following incubation with FITC-stained H486 (wild type) and H487 (SpyCEP+ve) *L. lactis* incubated with serial dilutions of CXCL8 for 30 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology). Data representative of three separate experiments using different donors. (B): Phagocytosis by neutrophils from the three donors 30 minutes post addition of bacteria to neutrophils with different concentrations of CXCL8. Values shown are phagocytosis of SpyCEP-positive strains, relative to the values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD, * = $P<0.05$ (Mann Whitney). All values were significant.

SpyCEP interferes with the neutrophil response against Group A Streptococcus

Despite the clear impact of SpyCEP expression by *L. lactis* on phagocytosis, the addition of exogenous of soluble CXCL8 had no effect on phagocytosis of *L. lactis* (Figure 6-5). SpyCEP also cleaves the other CXCL chemokines which are specific for neutrophils. Hence, the experiment of Figure 6-5 was repeated using a chemokine cocktail of all the known substrates of SpyCEP, to ensure that other chemokines were not responsible for the phenotype previously observed.

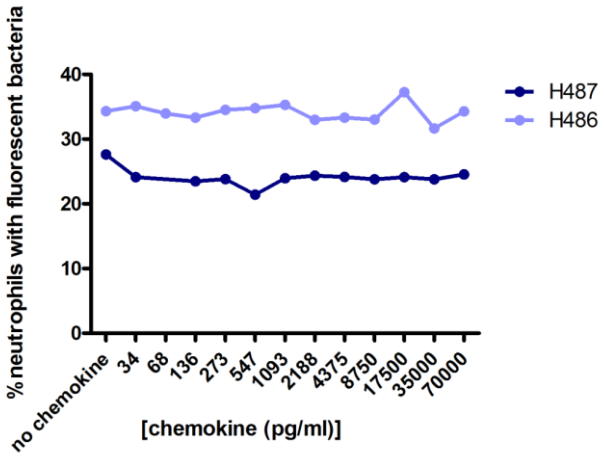


Figure 6-6 - Neutrophil phagocytosis of L. lactis with exogenous chemokine cocktail titration

Percentage of neutrophils positive for fluorescence following incubation with FITC-stained H486 (wild type) and H487 (SpyCEP+ve) *L. lactis* incubated with serial dilutions of a chemokine cocktail including an equal concentration of all known SpyCEP substrates (CXCL1, 2, 3, 5, 6, 7 and 8) for 30 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology) and performed once.

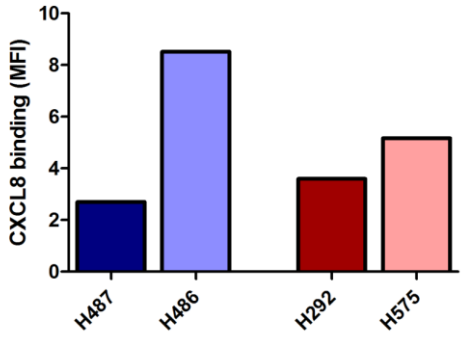
Again, addition of exogenous chemokine did not impact on the phenotype actually conferred by SpyCEP (Figure 6-6). The chemokines used for these experiments were recombinant, and hence may not have the same biological activity as neutrophil released chemokines, due to potential differences in folding or post-translation modification. It is

known for instance that the 77aa form of CXCL8 (Peprotech) used in this experiment is a less potent neutrophil chemoattractant than the 72aa form (Mortier*, et al.* 2011).

The results observed in Chapter 5 were achieved independently of any exogenous chemokine, hence any chemokine dependent effects were due to differences in endogenous chemokine levels. CXCL8 ELISAs were performed separately on the opsonising serum and the supernatant of the neutrophil preparation alone, as these represented the only two potential sources of exogenous or pre-formed soluble chemokine. Furthermore, a typical phagocytosis reaction was prepared, using the *L. lactis* strains H487 (SpyCEP-positive) and H486 (SpyCEP-negative). An aliquot of reaction supernatant was removed every 15 minutes and a specific serine protease inhibitor (Pefabloc) was then added to the aliquot to prevent any further SpyCEP cleavage activity. The samples were all analysed by ELISA to measure concentrations of soluble CXCL8. In all cases, no CXCL8 could be detected (Results not shown). The sensitivity of this ELISA was 16pg/ml.

As neutrophils are known to release large quantities of preformed CXCL8 by degranulation rapidly upon encountering bacterial products (Cassatella*, et al.* 1992), the failure to find soluble CXCL8 in any of these situations, including neutrophils incubated with opsonised SpyCEP-negative *L. lactis*, was surprising. A flow cytometric approach was next used to measure CXCL8 expressed on the surface or binding to the surface of the neutrophils, as such CXCL8 would not be detected using the previous ELISA method. A fluorescently conjugated antibody against CXCL8 was used, and median fluorescence intensity thus provided a measure of chemokine at the cell surface (Figure 6-7).

A



B

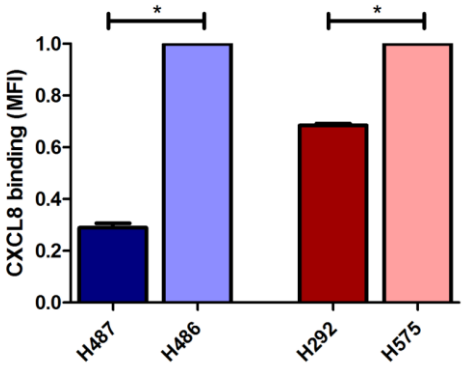


Figure 6-7 – CXCL8 at the neutrophil surface

(A): Human neutrophils were incubated with *L. lactis* H487 (SpyCEP+ve), H486 (SpyCEP-ve) or GAS strains H292 (SpyCEP+ve) or H575 (SpyCEP-ve) for 30 minutes and CXCL8 levels on the neutrophil surface were measured using FITC conjugated anti CXCL8. Each point shows median fluorescence intensity relative to isotype control and represents 20000 gated events (gating made around neutrophils based on morphology). Results representative of three independent experiments. (B): CXCL8 binding levels of all tested serum/neutrophils 30 minutes post addition of bacteria to neutrophils. Values shown are CXCL8 levels, relative to the values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD, * = $P<0.05$ (Mann Whitney).

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Neutrophils that had been incubated with SpyCEP-negative *L. lactis* were observed to have higher levels of CXCL8 at the cell surface, compared to SpyCEP-positive *L. lactis*. This experiment did not determine whether different amounts of CXCL8 were bound to the chemokine receptors expressed on the surface of neutrophils. The same results were seen using GAS strains, with more CXCL8 attached to the cell surface of neutrophils incubated with SpyCEP-negative GAS compared to SpyCEP-positive GAS. As no soluble CXCL8 could be detected using the ELISA method, it seems likely that the CXCL8 detected is endogenously produced by neutrophils upon interacting with bacterial components, which then binds rapidly with high avidity to the surface of the neutrophil, leading to neutrophil activation

A further experiment was performed to strengthen the hypothesis that by cleaving chemokines, SpyCEP was able to prevent neutrophil activation and thus phagocytosis. Supernatants from SpyCEP-expressing bacteria are also able to cleave chemokines (Figure 3-2). Thus, SpyCEP-negative strains should be protected from phagocytosis by the addition of soluble SpyCEP expressed by an alternative strain. This experiment was performed to confirm that the protease activity of SpyCEP is sufficient to prevent phagocytosis. Initially, SpyCEP-positive and negative *L. lactis* strains were incubated with supernatants from GAS strains. These were used as they were known to contain higher levels of SpyCEP than the supernatants of the *L. lactis* strains (Figure 3-6). Both *L. lactis* strains were however protected by both of the GAS supernatants, regardless of the presence or absence of SpyCEP. This was likely due to the large number of other soluble virulence factors expressed by GAS, which are able to prevent the relatively weakly opsonised *L. lactis (*Figure 5-11) from being phagocytosed (Results not shown).

The experiment was hence repeated using the supernatants from *L. lactis*. This provided a source of SpyCEP free from other soluble virulence factors which might interfere with phagocytosis (Figure 6-8), but with a very much lower concentration of SpyCEP.

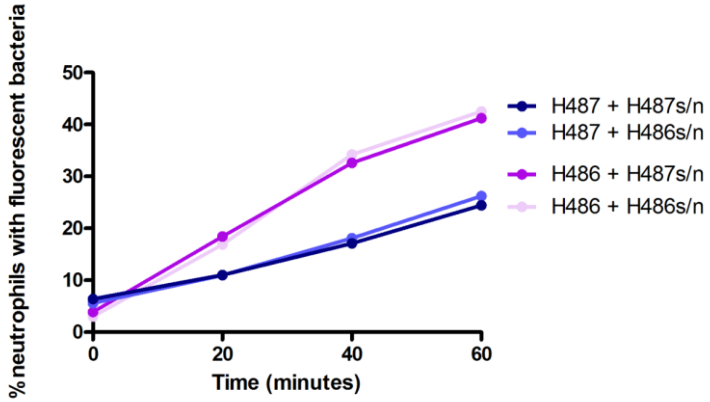


Figure 6-8 – Neutrophil phagocytosis of L. lactis incubated with soluble SpyCEP

Percentage of neutrophils positive for fluorescence following incubation with FITC-stained H486 (wild type) and H487 (SpyCEP+ve) *L. lactis* incubated with supernatant from SpyCEP+ or SpyCEP- *L. lactis* strains for 20, 40 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology) and the study was performed once.

The data suggested that soluble SpyCEP could not protect *L. lactis* against neutrophil phagocytosis. We had hypothesised that chemokine cleavage was mediating the observed difference in phagocytosis, hence this result was unexpected. As soluble SpyCEP had no effect on phagocytosis, it seemed likely that bacterial cell surface expressed SpyCEP was critical for this effect, potentially interacting and inactivating chemokines exposed on the surface of neutrophils in order to evade phagocytosis. Work was carried out in the next chapter using a GAS strain which expresses only soluble SpyCEP.

6.2.3 Assessing the levels of CD11b on the neutrophil surface

The results obtained in Chapter 5 demonstrate that SpyCEP is able to disrupt neutrophil phagocytosis, and work in this chapter shows SpyCEP is able to reduce the binding of CXCL8 to the neutrophil surface. Thus, the hypothesis that the mechanism by which SpyCEP impacted on phagocytosis was by cleaving chemokines has been supported. Chemokines play a role in neutrophil activation by binding the chemokine receptors CXCR1 and CXCR2 expressed at high levels on the surface of neutrophils (Grob*, et al.* 1990). Following CXCL binding of these receptors, neutrophils are known to release granules that contain further chemokines including CXCL8 (Scapini*, et al.* 2000), components of the complement receptor heterodimers, including CR3, (Detmers*, et al.* 1990, Paccaud*, et al.* 1990), and Fc receptors (Elghetany 2002). By cleaving chemokines, it was hypothesised that SpyCEP may prevent the upregulation of surface expression of receptors involved in phagocytosis, and thus prevent phagocytosis. As complement has been shown to be vitally important for GAS phagocytosis, both in this work and by other investigators (DeMaster *et al*. 2002) neutrophil cell surface expression of complement receptors was measured.

Initial experiments were carried out to confirm that the chemokine substrates of SpyCEP, but not other CXCL chemokines were able to activate neutrophils, and result in expression of complement receptors. Neutrophil activation is classically measured by the surface expression of CD11b (Nicholson*, et al.* 2007), which acts as a surrogate for all activation-induced surface markers (Elghetany 2002). Further, CD11b is itself part of CR3 (Hynes 1992), the blockade of which has been reported to prevent GAS phagocytosis (DeMaster*, et al.* 2002). Blockade of other complement receptors including CR1 has been reported to have little effect on GAS phagocytosis (Nilsson*, et al.* 2005, Weineisen*, et al.*

2004). Hence, by measuring CD11b surface levels, a receptor known to be critically involved in GAS phagocytosis is being measured, furthering the utility of this approach. CXCL8 and CXCL5, both agonists of CXCR1 and 2, and substrates of SpyCEP were incubated with neutrophils along with CXCL10, known to not bind neutrophils and not be a substrate of SpyCEP. Surface CD11b was labelled using a specific fluorescently conjugated antibody, and fluorescence intensity was measured by flow cytometry (Figure 6-9).

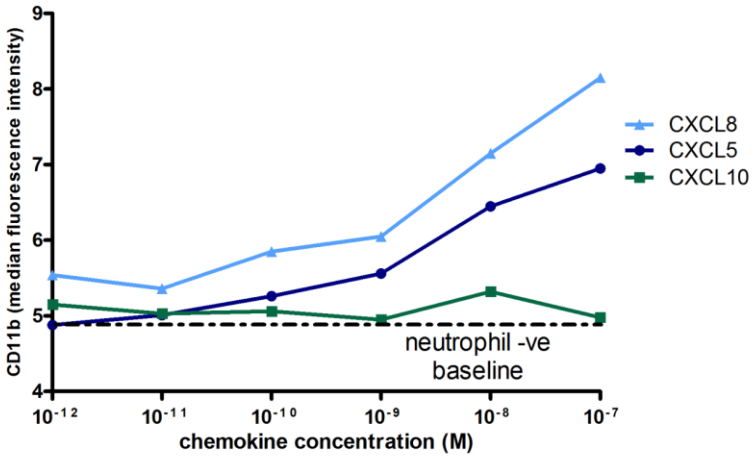


Figure 6-9 – Neutrophil CD11b levels following incubation with CXCL chemokines

Human neutrophils were incubated with varying concentrations of CXCL chemokines for 30 minutes, and CD11b levels measured using FITC-conjugated anti-CD11b. Each point shows median fluorescence intensity relative to the isotype control and represents 20000 gated events (gating made around neutrophils based on morphology), and was performed once.

Neutrophil specific CXCL chemokines (CXCL5 and CXCL8) increased surface levels of CD11b compared with a non-neutrophil specific chemokine CXCL10 (Figure 6-9). Further work was performed to examine whether SpyCEP-cleaved chemokines were also capable of increasing CD11b levels on the cell surface. It was found that chemokine incubated with the supernatant of SpyCEP-negative GAS strain H575 raised CD11b significantly more than

chemokine incubated with supernatant from SpyCEP-positive GAS strain H292. In both cases however, CD11b was raised more than chemokine alone, presumably due to neutrophils being activated by bacterial products in the supernatants and generic inflammatory mediators within the TH media itself (Figure 6-10).

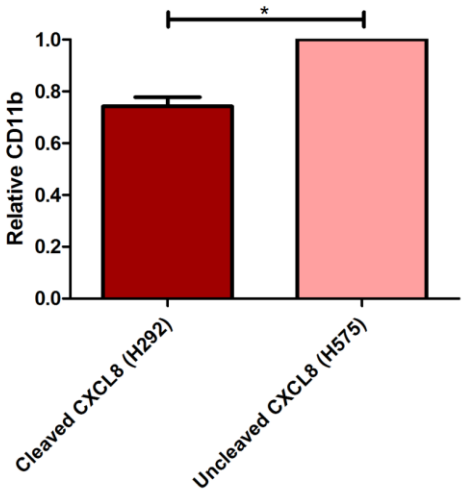


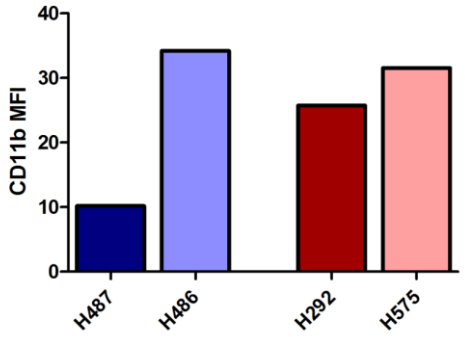
Figure 6-10 – CD11b on neutrophil surface in response to cleaved and uncleaved CXCL8

10nM of CXCL8 incubated o/n with TH supernatant from GAS strains H292 (SpyCEP +ve) or H575 (SpyCEP –ve) was incubated with human neutrophils for 30 minutes. CD11b levels measured using FITC-conjugated anti-CD11b. Each point shows median fluorescence intensity relative to a control using H292 and H575 supernatants combined alone, and represents 20000 gated events (gating made around neutrophils based on morphology). Values shown are CD11b levels, relative the values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD, * = P<0.05 (Mann Whitney).

Comment [RL3]: SS thinks I should have used RPMI

Next, measurements were taken to determine the levels of CD11b on the neutrophil surface following co-incubation with SpyCEP-positive and negative strains of bacteria (Figure 6-11).

A



B

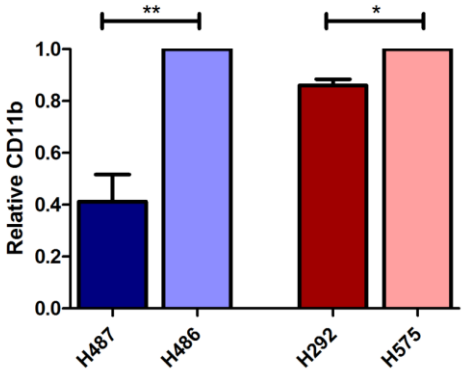


Figure 6-11 – Neutrophil CD11b surface expression following incubation with bacteria

(A): Neutrophils were incubated with *L. lactis* strains H487 (SpyCEP+ve) and H486 (SpyCEP-ve), or GAS strains H292 (SpyCEP+ve) and H575 (SpyCEP-ve) for 30 minutes. CD11b levels were measured using FITC conjugated anti CD11b, compared to a FITC labelled isotype control. Each point shows median fluorescence intensity relative to a PBS control and represents 20000 gated events (gating made around neutrophils based on morphology). Results representative of 5 independent experiments using *L. lactis* strains and 3 using GAS strains. (B): CD11b levels of all tested donors 30 minutes post addition of bacteria to neutrophils. Values shown are CD11b levels, relative to values obtained for SpyCEP-negative strain in each experiment. Error bars indicate SD, * = $P<0.05$, **=$P<0.01$ (Mann Whitney).

SpyCEP interferes with the neutrophil response against Group A Streptococcus

SpyCEP expressing bacteria caused neutrophils to express less CD11b on their surface than SpyCEP-negative strains (Figure 6-11). This difference was more clearly seen using *L. lactis* strains than the GAS strains, but both were still significant. Taking CD11b as a surrogate of activation, the data suggest that SpyCEP expressing strains were able to prevent neutrophil activation. As CD11b is a phagocytosis receptor itself important for phagocytosis of GAS (DeMaster*, et al.* 2002, Nilsson*, et al.* 2005, Weineisen*, et al.* 2004), reduced levels provide a direct mechanism by which SpyCEP expressing bacteria impede phagocytosis compared to their SpyCEP-negative counterparts.

Whilst chemokine cleavage is the most likely mechanism that underlies reduced CD11b and neutrophil activation, further investigations were undertaken to determine whether this was indeed the case. Firstly, the alternative explanation that SpyCEP directly degrades CD11b was explored. CD11b was extracted from activated neutrophils and incubated with high SpyCEP containing GAS supernatants (Figure 3-6) or a negative control to determine whether SpyCEP could directly cleave this protein. Immunoblotting was performed in triplicate using a CD11b antibody, and the band density at approximately 100kDa (full length CD11b) was measured (Figure 6-12).

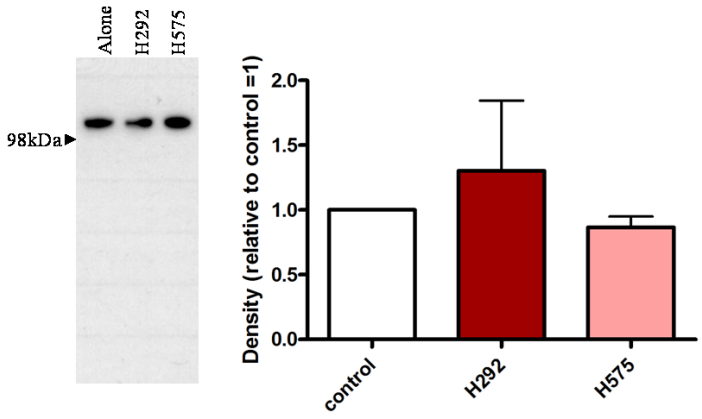


Figure 6-12 – Densitometry of CD11b incubated with SpyCEP

CD11b extracted from neutrophils was incubated overnight with GAS supernatants of various concentrations or alone. Above shows neat results obtained with neat supernatant. This was then immunoblotted using an antibody against CD11b in independent triplicate. The three immunoblots were analysed using ImageJ, and mean band density plotted relative to a CD11b alone control included in each experiment. Bars indicate standard deviation.

CD11b was present at the same concentration whether incubated with SpyCEP-positive or negative supernatants (Figure 6-12), confirming that SpyCEP does not cleave CD11b. Taken together, the data showed that SpyCEP expression caused reduced neutrophil surface CR3, but that there was no evidence that this was related to SpyCEP cleavage of CD11b. Rather the reduced CD11b, could be attributed to cleavage of neutrophil surface-expressed CXCL chemokines.

Comment [RAL4]: POSTSS – She wonders if something else that upregulates CD11b could be used, and show that SpyCEP has no effect.

6.3 Discussion

6.3.1 Phagocytosis by other cell types

GAS is an extracellular pathogen, although several studies have reported that GAS is able to invade a diverse range of cells, including epithelial cells (LaPenta*, et al.* 1994) and cultured endothelial cells (Greco*, et al.* 1995). Importantly, internalisation into such cells is undergone by a tiny minority of bacteria, and the contribution to pathogenicity is unclear (Cunningham 2000). Cell invasion has been reported to be of importance in GAS virulence, acting as a reservoir for persistent infections, although this has primarily been associated with GAS survival within macrophages (Hertzen*, et al.* 2010). Indeed GAS has been reported to be killed very efficiently upon phagocytosis by neutrophils (Sullivan and Mandell 1980).

There are two conflicting reports regarding the impact that SpyCEP makes on the invasion of eukaryotic cells by GAS. One report demonstrated that SpyCEP can act as an invasin, its expression allowing enhanced entry of SpyCEP-expressing killed Group B Streptococcus and SpyCEP-coated latex beads into endothelial cells (Kaur*, et al.* 2010). In contrast, another group (Andreoni and Zinkernagel, Abstract at XVIII Lancefield Symposium) have reported that SpyCEP acts as an anti-invasin, preventing the invasion of epithelial cells by GAS. Zinkernagel *et al.* 2008 also describe SpyCEP as an anti-adhesin. One of the starting points for work in this chapter was that the reduced entry of SpyCEP expressing strains into neutrophils may represent an example of SpyCEP preventing GAS "invading" neutrophils, rather than reducing neutrophil phagocytosis of GAS.

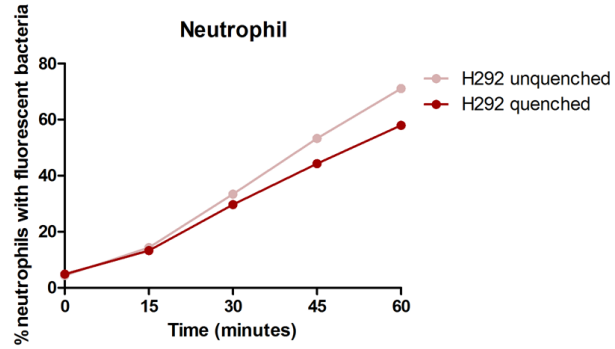
To examine whether this might explain the observations of this study, and to provide support for the hypothesis that SpyCEP prevents phagocytosis by interfering with CXCR mediated upregulation of phagocytosis receptors, other phagocytic cells were used for

phagocytosis experiments. The HL60 leukocyte cell line can be differentiated to resemble a cell with neutrophil like characteristics. HL60 phagocytose opsonised particles and express complement receptors and antibody receptors akin to neutrophils (Collins*, et al.* 1979). However, they express low levels of CXCR1 and 2, and are thus poorly responsive to neutrophil specific chemokines (Grob*, et al.* 1990, Zahn*, et al.* 1997).

SpyCEP-positive and negative *L. lactis* strains were phagocytosed equally well by HL60 cells, although much less well than was observed using freshly extracted human neutrophils (Figure 6-1). Phagocytosis by these cells was not affected by the expression of SpyCEP. These phagocytic cells express only low levels of CXCR1 and 2, and thus enzymatic activity which disrupts this pathway would be much less noticeable using these cells than using neutrophils (Grob*, et al.* 1990, Zahn*, et al.* 1997). Such a result hence strengthens the hypothesis that by cleaving chemokines, SpyCEP inhibits full activation of neutrophils mediated by chemokines, preventing the full upregulation of receptors involved in phagocytosis. If the mechanism did not depend on chemokine cleavage, rather the presence of SpyCEP on the GAS surface, an observed difference in phagocytosis by HL60 cells would have been expected.

The ability of HL60 cells to phagocytose particles was less than that of freshly extracted neutrophils. Neutrophils are known to be particularly efficient phagocytes, able to phagocytose particles very quickly (Nordenfelt and Tapper 2011). Indeed, a noteworthy difference between neutrophils and HL60 cells was the level of extracellular signal that was quenched by addition of trypan blue (Figure 6-13). These results show that bacteria were bound to the surface of HL60, but were not phagocytosed efficiently. Neutrophils, upon binding bacteria to their surface were much more efficient at phagocytosis (Figure 6-13).

A



B

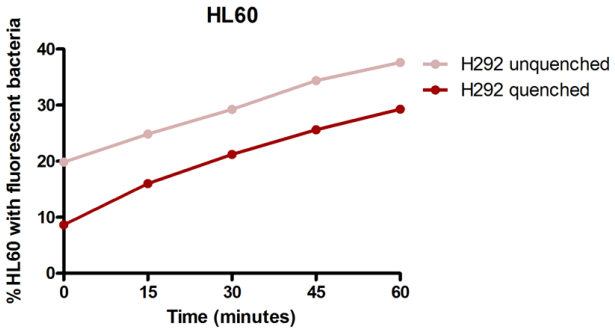


Figure 6-13 – Comparison of fluorescence quenching between neutrophils and HL60

Percentage of neutrophils (A) and HL60 cells (B) positive for fluorescence following incubation with FITC-stained H292 (wild type GAS) for 15, 30, 45 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology). Measurements were taken at each time point without quenching, then trypan blue was added to quench extracellular signal. Data representative of three separate experiments

HL60 cells are a cell line, and thus the experiments were repeated using freshly extracted monocytes. These are genuine human phagocytes, which express complement and antibody binding receptors, but, like HL60, few CXCR1 and 2 receptors (Shi and Pamer 2011). It was hypothesised that these cells would give similar results to those obtained using

HL60 cells, such that if SpyCEP-mediated its effects on phagocytosis by cleaving chemokines, phagocytosis by these cells should be unaffected.

SpyCEP-positive and negative *L. lactis* and GAS strains were both equally phagocytosed by monocytes (Figure 6-2). Monocyte phagocytosis was slow compared to neutrophils, which has been previously reported (Nordenfelt and Tapper 2011). Together, these results show that SpyCEP does not act as a broad spectrum anti-invasin on the surface of GAS, as has been suggested. Instead, SpyCEP acts to specifically prevent neutrophil specific phagocytosis. As SpyCEP is known to have cleavage activity against neutrophil specific chemokines, this provides a likely source for the specificity of cell type of which SpyCEP can impair phagocytosis.

Phagocytosis of GAS by macrophages is reportedly a vital feature in successful clearance of experimental GAS infections. Indeed macrophage deficient mice had a significantly more severe infection and a much higher mortality rate than wild type mice (Goldmann*, et al.* 2004). In this chapter, the contribution of complement and antibody to this vital process was examined. It was determined that, similar to neutrophils, depletion of antibody had only a small impact on phagocytosis. However, ablation of complement had a major impact on phagocytosis. The double depleted serum allowed no phagocytosis, demonstrating the effectiveness of the serum depletion methods. The importance of complement to phagocytosis of GAS identified complement receptors as a likely basis for SpyCEP-mediated inhibition of phagocytosis.

The main role of antibody in opsonisation of GAS may be to enhance complement deposition, thus without complement, antibody can only poorly opsonise GAS. Indeed the direct blockade of Fc receptors is known to have no impact on GAS phagocytosis (Nilsson*, et al.* 2005). The GAS strain used throughout this study was an *emm*/M81 strain. Whilst this M

protein type has not been studied extensively, variation between biological activities of M-types is known. There are broadly two groups of M protein, both of which inhibit complement deposition, but using different mechanisms (Carlsson*, et al.* 2005), thus impact of antibody in the sera may be different between these M protein types.

6.3.2 Assessing the impact of chemokines on neutrophil phagocytosis

The results of this chapter suggested that SpyCEP disrupts chemokine-mediated neutrophil activation. This was hypothesised to prevent full upregulation of receptors involved in phagocytosis. Initially an antagonist of CXCR1 and 2 was used to try to mimic the prevention of chemokine signalling that was hypothesised to be mediating the SpyCEP effect on phagocytosis. However, this coloured compound made the neutrophils fluorescent in all channels available on the FACS Calibur machine used in this study. Furthermore, the antagonist was antibacterial, hence was of no use for assays measuring bacterial growth in blood. Later, serine protease inhibitors were added to block SpyCEP activity in flow cytometry phagocytosis experiments using IVIg-opsonised bacteria. However, these inhibitors proved toxic to neutrophils (Results not shown). Other antagonists of CXCR1 and 2 exist, but were not used in this study.

As chemokine receptor antagonists had failed to provide results to validate or disprove our hypothesis, we sought to use an alternative approach by determining whether any effect of SpyCEP could be overcome using an excess of chemokine. The addition of excess exogenous chemokine to the phagocytosis reactions upon the addition of the bacteria was hypothesised to increase the phagocytosis of the SpyCEP-positive strains. The recombinant CXCL8 used for this experiment has been reported to be cleaved relatively

slowly by SpyCEP, but still has biological activity as indicated by chemotaxis assays (Goldblatt, personal communication). Hence, despite the presence of proteolytic SpyCEP activity, some CXCL8 would remain intact to bind neutrophils, especially when using higher concentrations. It is also known that this particular cleaved chemokine does not antagonise the binding of intact chemokines to the CXCR1 and 2 receptors (Goldblatt, personal communication).

Surprisingly, exogenous chemokine had no effect on phagocytosis (Figure 6-5). The chemokine used in this experiment however has been reported to be a relatively poor substrate for SpyCEP compared to other sources of recombinant CXCL8. Whilst it was used for this reason, this may also mean this chemokine has a poor affinity for CXCR1 and 2. CXCL8 undergoes extensive processing, and is found in a variety of lengths, with varying biological activity (Mortier*, et al.* 2011, Van Damme*, et al.* 1990). Indeed, the 77aa acid form of CXCL8, used in this experiment, is known to be less chemotactic than the 72aa version (Mortier*, et al.* 2011). It may also be that simply not enough chemokine was added in these experiments to overwhelm the cleavage by SpyCEP.

The inclusion of all the neutrophil specific chemokines also had no effect on phagocytosis. Again, processing is known to play an important role in their activity; however, these results were still highly unexpected. Indeed, comparable concentrations of a different recombinant chemokine of 77aa to those added in these experiments caused a measurable increase in CD11b concentration within 30 minutes (Figure 6-9). A unifying explanation for these observations is that phagocytosis by neutrophils requires endogenous chemokine.

If the hypothesis explaining SpyCEP-mediated inhibition of phagocytosis was correct, a higher chemokine concentration ought to have been measurable in the phagocytosis reaction mixtures between the strains which lack SpyCEP compared to the isogenic SpyCEP-

positive strains. Initially, CXCL8, the most abundant CXCL chemokine (Mortier*, et al.* 2011), was measured in the freshly extracted serum and supernatant around freshly extracted neutrophils alone. No CXCL8 was measurable in either fluid. As the sera used were from healthy donors, and the neutrophils had been extracted using sterile technique, this inflammatory chemokine would be at only very low concentrations.

However, soluble CXCL8 was also not detectable in the supernatant taken from phagocytosis reactions, including experiments where opsonised SpyCEP-negative bacteria were mixed with neutrophils. Addition of bacteria to neutrophils leads to the exocytosis of secretory vesicles that are quick to degranulate, and contain CXCL8 (Amulic*, et al.* 2012), hence a measurable level of CXCL8 was expected in the supernatants (Cassatella*, et al.* 1992).

A greater amount of CXCL8 could be measured following neutrophil incubation with SpyCEP-negative bacteria compared to SpyCEP-positive strains but this difference was only detected at the surface of neutrophils. This result suggests that following the release of CXCL8 from neutrophil granules, the chemokine is very rapidly or perhaps immediately bound by the neutrophils. This was likely an artefact of the reaction conditions used; where neutrophils are the only human cell present, the reaction mixture is being agitated, and they are at high concentrations. What could not be determined was whether the CXCL8 was bound to its receptors or was simply associated with the surface of the neutrophil.

To confirm that cleavage of chemokines by any form of SpyCEP was responsible for the observed differences in phagocytosis between SpyCEP-positive and negative strains, bacterial supernatants were included in the phagocytosis reaction mixes (Figure 6-8). GAS supernatants were initially added to *L. lactis* strains, as wild type GAS strain H292 is known to produce high concentrations of soluble SpyCEP (Figure 3-6). However, it was found that both supernatants from SpyCEP-positive and negative GAS strains were able provide

considerable protection for both *L. lactis* strains. This was presumably due to other soluble virulence factors expressed by GAS (Results not shown).

Experiments using the supernatants from *L. lactis* strains demonstrated that soluble SpyCEP produced by SpyCEP-positive *L. lactis* strain H487 offered no protection against phagocytosis. These results suggested that surface expression of SpyCEP was essential for its observed effect on phagocytosis (Figure 6-8). However, SpyCEP-positive *L. lactis* strain H487 is known to produce only very low levels of soluble SpyCEP (Figure 3-6), and thus the SpyCEP concentration may have been too low to mediate any biologically relevant effects.

6.3.3 A mechanism by which SpyCEP inhibits phagocytosis

The only known function of SpyCEP is the cleavage of ELR+ CXCL chemokines which are specific for neutrophils. Specificity for neutrophils is due to their binding affinity for CXCR1 and CXCR2 which are expressed in high levels on the surface of neutrophils (Grob*, et al.* 1990). It is known that CXCL8, a substrate of SpyCEP which binds with high affinity to CXCR1 and CXCR2, upregulates CD11b in a dose responsive manner (Detmers*, et al.* 1990). CD11b is part of the complement receptor 3 (CR3) heterodimer (Hynes 1992). This receptor has previously been shown to be vitally important for GAS phagocytosis (DeMaster *et al.* 2002) which coupled with the observations made here that complement is required for GAS phagocytosis, provides a mechanism by which SpyCEP was hypothesised to act. However, previous work showing CXCR receptor blockade have not reported reduced phagocytosis, but have instead focussed on reduced neutrophil recruitment (Stillie*, et al.* 2009).

CD11b is found preformed in a variety of neutrophil granules, which are released upon progressive activation of neutrophils. Neutrophil activation is a gradual continuum, whereby different stimuli, including CXCL8, act in concert to gradually increase the state of activation (Borregaard, *et al.* 1994). CD11b is classically used as a measure of neutrophil activation. Due to its presence in many classes of granules which are released at various stages of activation, its release onto the cell surface continues throughout activation (Amulic, *et al.* 2012). Its upregulation is also accompanied by the upregulation of other phagocytic receptors, such as complement receptor 1 and antibody receptors for phagocytosis, specifically FcγRIII.

Figure 6-9 confirmed the earlier work (Detmers, *et al.* 1990) which showed that CXCL8 was able to upregulate CD11b in a dose dependent manner. A CD11b response was seen when CXCL8 reached 1nM (approximately 8000pg/ml), a concentration typically exceeded in infection (Kurupati, *et al.* 2010). A similar dose dependent response was observed using increasing concentrations of another neutrophil CXCR ligand, CXCL5. Both these ligands are substrates of SpyCEP, and their ability to upregulate CD11b was ablated following SpyCEP cleavage (Figure 6-10). This result was expected, as work currently underway at Imperial College demonstrates that SpyCEP cleavage ablates the binding of the chemokines to their receptors (Goldblatt personal communication). CXCL10, known not to be a ligand for these receptors, was unable to increase CD11b expression, demonstrating this was a specific effect that required CXCR1/2 activation.

The increase in CD11b was observed thirty minutes subsequent to the addition of chemokine. This was also reported by Detmers 1990, and can occur this rapidly as the CD11b is pre-formed in granules within the neutrophil. These granules are known to be able to exocytose very quickly following detection of a stimulus (Detmers, *et al.* 1990).

The surface levels of CD11b were also measured following incubation with the different SpyCEP-positive and negative strains (Figure 6-11). SpyCEP-negative *L. lactis* was found to induce neutrophils to upregulate surface CD11b more strongly than the SpyCEP-positive strain. The same pattern of results was observed using the GAS strains, although with reduced difference between the isogenic GAS strains. The SpyCEP-positive strains activated the neutrophils less than the SpyCEP-negative strains. It was confirmed that SpyCEP could not directly cleave CD11b using densitometry analysis of Western blots (Figure 6-12).

Neutrophils are activated by a variety of stimuli including exposure to both bacteria and chemokine (Amulic*, et al.* 2012). The bacterial strains used in this experiment were isogenic, apart from their expression of SpyCEP. It hence seems unlikely that bacterial stimuli being received by the neutrophil are different between the SpyCEP-positive and negative strains, and instead differences in chemokine signalling are the more likely source of activation differences. As complement is known to be important for opsonisation of *L. lactis* and GAS (Figure 5-12 and Figure 5-13), and CD11b is known to be a component of a complement receptor, the reduced CD11b levels seen with SpyCEP positive bacteria also provides a mechanism by which SpyCEP inhibits phagocytosis. Furthermore, it is known that neutralisation of CD11b using antibody can prevent phagocytosis of GAS by neutrophils, indicating its importance in GAS phagocytosis (Nilsson*, et al.* 2005). Thus, taken together, these results provide a mechanism by which SpyCEP could interfere with phagocytosis, illustrated in Figure 6-14.

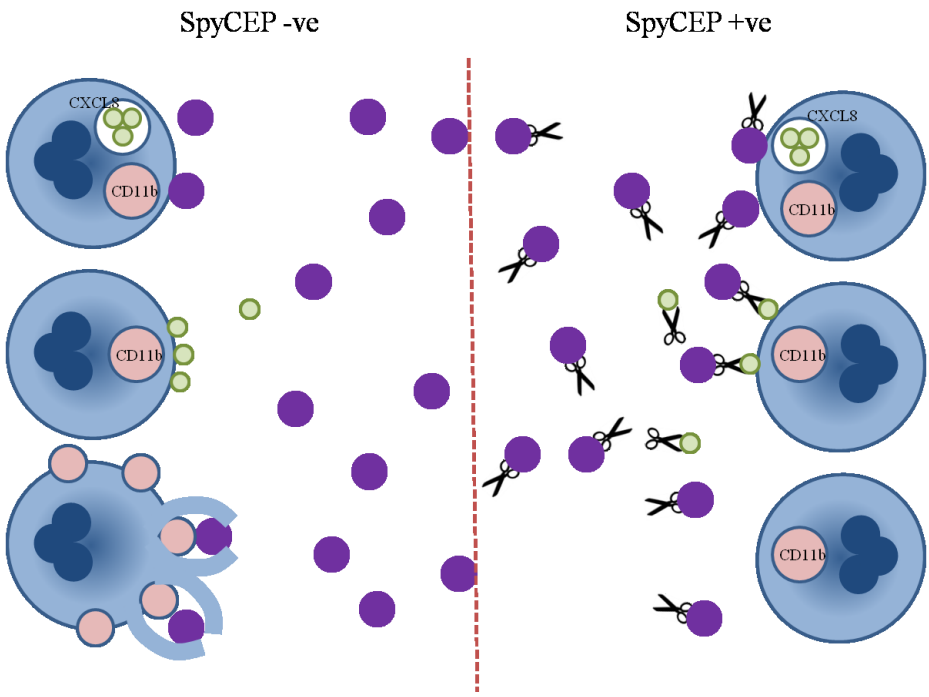


Figure 6-14 – Proposed mechanism by which SpyCEP disrupts phagocytosis

Left of dashed line indicates SpyCEP-negative strain, right of line indicates SpyCEP-positive strain. LEFT (moving down diagram): Bacteria (purple) interact with neutrophils causing some activation. Neutrophils release CXCL8 (green circles) from their granules. This causes further activation, and the upregulation of CD11b. CR3 bind complement opsonised bacteria leading to phagocytosis. RIGHT (moving down diagram). Bacteria (purple) interact with neutrophils causing some activation. Neutrophils release CXCL8 (green circles) from their granules, which is cleaved by SpyCEP (scissors). CD11b is hence not upregulated.

Chapter 7 - Results: The effects of localisation of SpyCEP on function and immune response to GAS

7.1 Introduction

The C terminus of SpyCEP features an LP(X)TG motif which acts as a substrate for the bacterial sortase enzymes. These covalently attach SpyCEP to the cell wall. H292 is noteworthy in that it transcribes very high levels of the SpyCEP gene, and releases large amounts of this protein into the supernatant, in addition to binding to the cell wall (Turner*, et al.* 2009a). All strains known to express high amounts of SpyCEP release the protein into the supernatant. Whether the release of SpyCEP requires active catalysis or simply reflects the overwhelming of the sortase system is unclear, although it is known that release is not autocatalytic (Zingaretti*, et al.* 2010). The effect of SpyCEP release on virulence is also unknown. The amino acid sequence SpyCEP of H292 differs from other sequenced strains in that it has a two amino acid deletion proximal to the LP(X)TG motif.

```
     N                                    1600        1610          C
H292 VKVGDASDSTGDHKVMSKNNSQALTASATPTKTTTS--AKALPSTG
H305 VKVGDASDSTGDHKVMSKNNSQALTASATPTKSTTSATAKALPSTG
```

Figure 7-1 – Amino acid sequence of H292 compared to typical SpyCEP sequence

Amino acid alignment of SpyCEP from GAS strain H292 used throughout this study, and from strain H305, which expresses a SpyCEP with a typical C-terminus. Grey indicates sortase motif, blue indicates a 2 amino acid difference. Sequences from NCBI.

SpyCEP interferes with the neutrophil response against Group A Streptococcus

Previous work in this study and others (Kurupati*, et al.* 2010) has demonstrated that SpyCEP has both systemic and local effects. SpyCEP released locally can affect neutrophil release from the bone marrow, in association with reduced concentrations of systemic neutrophil chemokines implying that soluble SpyCEP has a role in virulence distant from the focus of infection (Figure 4-1, Figure 4-8). Negative effects on phagocytosis (Figure 5-5) however depended upon bacterial surface bound SpyCEP, and could not be reproduced by addition of soluble SpyCEP (Figure 6-8). It was thus hypothesised that both surface bound and soluble SpyCEP contributed separately to virulence in GAS infection. I postulated that a strain constructed without a SpyCEP sortase motif would survive less well at the site of infection, but result in otherwise similar effects systemically when compared with an isogenic control strain of GAS.

I further postulated that loss of surface-expressed SpyCEP might abrogate any protective effect of SpyCEP vaccination against invasive GAS infection. However, if the mechanism of vaccine efficacy required neutralising anti-SpyCEP activity, rather than opsonisation of bacteria, then vaccination would impact similarly on infection caused by bacteria expressing SpyCEP on the surface or bacteria that released SpyCEP.

7.2 Results

7.2.1 Construction and *in vitro* characterisation of SpyCEP sortase mutant (H887)

Cell wall extracts and supernatants from H292 and H887 were prepared and analysed by immunoblotting using an anti SpyCEP antibody. It was observed that strain H887 had more SpyCEP in its supernatant than H292. H887 however had much reduced SpyCEP in the cell wall fraction compared to H292, although a small amount was still detectable, likely related to the export of SpyCEP through the cell membrane or due to contamination of cell wall preparations with large amounts of SpyCEP in the culture supernatant (Figure 7-2). Furthermore, non-specific Van der Waals type interactions between SpyCEP and other large GAS cell wall proteins may also lead to some carry over during the preparation of the cell wall extracts. This was as expected, and confirmed that SpyCEP expression was not quantitatively affected following insertion of the plasmid, but the location of expression was as predicted. Hence SpyCEP was present wholly in the culture supernatant rather than being cell surface anchored.

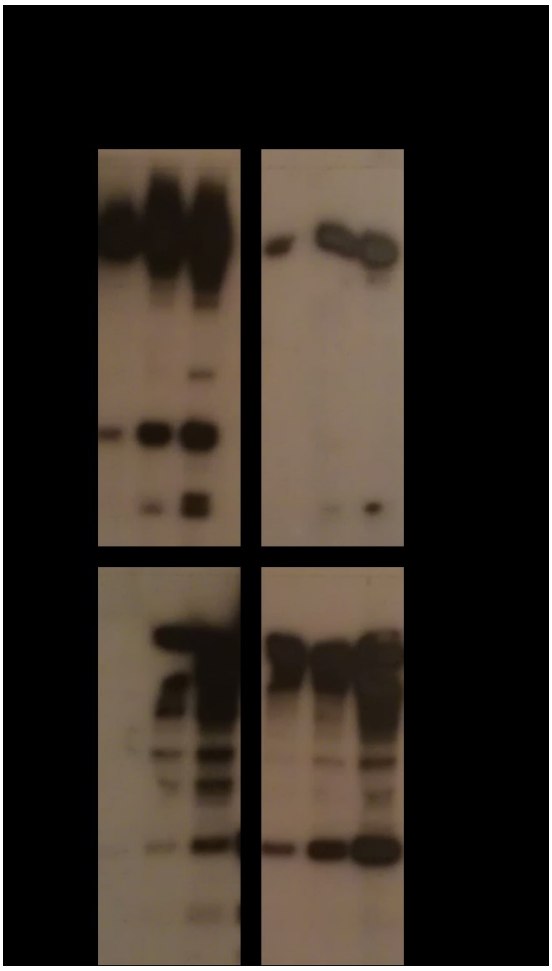


Figure 7-2 – Immunoblots showing SpyCEP localisation is GAS strains H292 and H887

Supernatants and cell wall extracts were collected from GAS cultures of wild type strain H292 and isogenic sortase motif mutant strain H887 at early, mid and late log. SpyCEP levels were measured using an antibody raised against an N terminal segment of SpyCEP. Equivalent protein amounts were loaded for each strain, as assessed by spectroscopy.

Supernatants were also tested to ensure that SpyCEP produced by strain H887 retained its enzymatic activity. Cleavage assays were performed using all the known CXCL chemokine substrates of SpyCEP No differences in cleavage activity between H292 and H887 were observed, although slightly more cleavage was observed using the supernatants of

sortase negative strains, indicative of the greater amount of SpyCEP present in the supernatant (Figure 7-3).

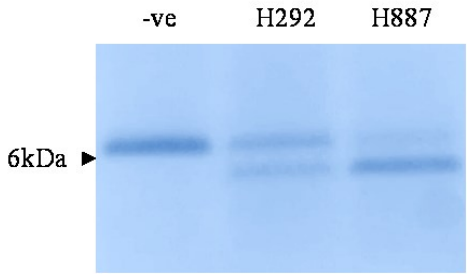


Figure 7-3 - Chemokine cleavage by supernatants of GAS strains H292 and H887

200ng of the chemokine CXCL6 was incubated with 8µl of wild type GAS supernatant (H292) or an isogenic SpyCEP sortase mutant (H887), each grown to an OD on 1.0. These were examined by protein electrophoresis and stained with colloidal blue. CXCL6 shown to illustrate the slightly increased cleavage by the sortase mutant strain.

H887 was characterised prior to its use in virulence assays. Initial studies examined its growth in TH media to ensure that the disruption of the sortase motif of SpyCEP had no indirect effects on GAS growth (Figure 7-4).

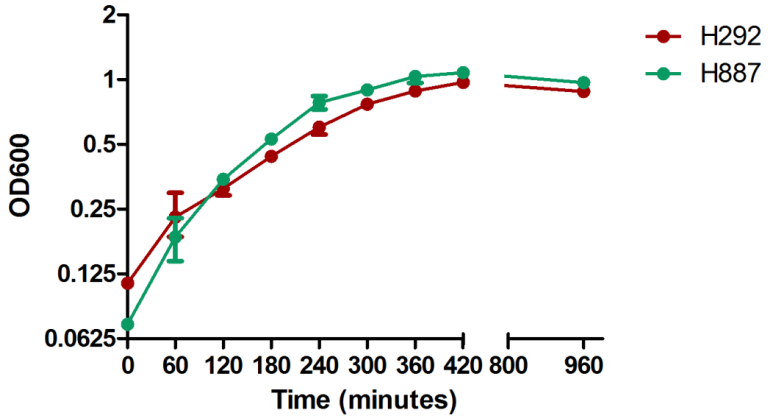
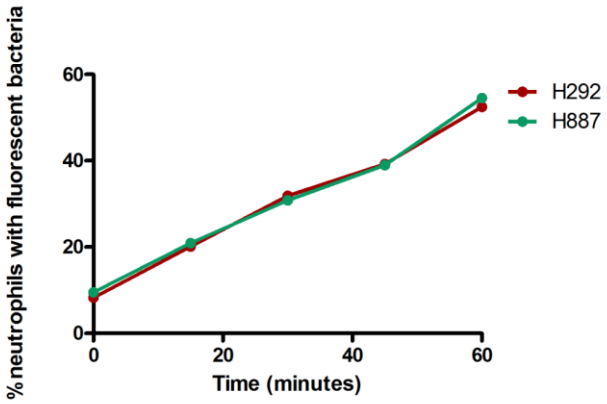


Figure 7-4 – Growth curve of H292 and H887

Cultures grown o/n were subcultured 1 in 10 into fresh TH media in triplicate. At 60 minute intervals, a sample was taken and the OD_{600} measured. Results indicate mean and standard deviation of three experiments.

The two isogenic strains were labelled using FITC and ability to affect phagocytosis was compared using purified human neutrophils. No difference was seen between the two strains, with 50% of neutrophils containing fluorescent bacteria after sixty minutes (Figure 7-5).

A



B

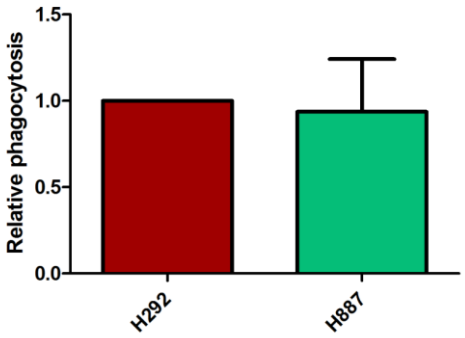


Figure 7-5 – Neutrophil phagocytosis of GAS strains H292 and H887

(A): Percentage of neutrophils positive for fluorescence following incubation with FITC-stained H292 (wild type) and H887 (Δsortase) at 15, 30, 45 and 60 minutes. Each time point represents 20000 gated events (gating made around neutrophils based on morphology). Data representative of three separate experiments using different donors. (B): Phagocytosis from the two experiments 30 minutes post addition of bacteria to neutrophils. Values shown are phagocytosis levels for sortase-positive strain (H292), relative to values obtained for sortase-negative strain (H887) in each experiment. Error bars indicate SD.

This finding surprisingly disproved the hypothesis generated in Chapter 6 that surface bound SpyCEP was required to prevent phagocytosis, although Figure 7-3 suggests there is some SpyCEP on the surface of H887. The tenet that inhibition of phagocytosis required surface-bound SpyCEP stemmed from the observation that supernatant from SpyCEP-positive *L. lactis* was unable to prevent phagocytosis (Figure 6-8). However, *L. lactis* produces only a small amount of soluble SpyCEP (Figure 3-6), providing a potential explanation as to why the *L. lactis* supernatant could not prevent phagocytosis. Clearly in a setting where soluble secreted SpyCEP is abundant, phagocytosis inhibition does occur readily.

7.2.2 *In vivo* studies to determine if soluble SpyCEP has a specific role

In this study, the systemic effects of SpyCEP have been noted to specifically prevent the release neutrophils from the bone marrow, while the local effects include impairment of neutrophil recruitment (Edwards*, et al.* 2005) and impairment of phagocytosis (this study). To determine if GAS secreting only soluble SpyCEP might differentially affect distant and local events following infection compared with wild type GAS, pilot experiments were conducted where mice were infected intramuscularly with wild type H292 or sortase motif negative H887 GAS strains. At six hours, neutrophils were counted in the peripheral blood.

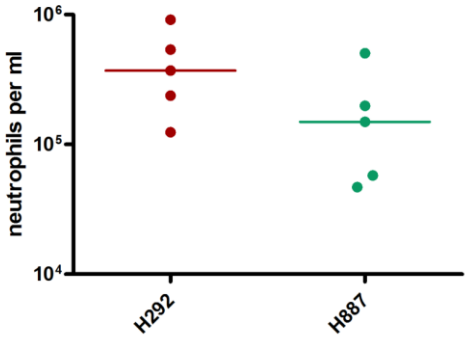


Figure 7-6 - Peripheral neutrophil levels following H292 and H887 infection

Five female CD1 mice were injected with approximately 10^8 CFU of GAS strains H292 (wild type) or H887 (SpyCEP sortase mutant). Mouse blood was collected at 6 hours and peripheral circulating neutrophil counted.

We had hypothesised that H887 would release more soluble SpyCEP, which seemed to be supported by immunoblotting (Figure 7-2). This would have a greater systemic effect, and thus have a greater impact on neutrophil release. It was found that there were more circulating neutrophils in mice infected with strain H292 than strain H887. However, this difference was not statistically significant. This experiment was necessarily a pilot study, designed to power a more substantive experiment. The data shows that 16 mice per group would be required to show a 60% reduction in peripheral neutrophil numbers. H292 releases large amounts of soluble SpyCEP itself. It would be interesting to compare H887 with a strain unable to release SpyCEP from its surface, and with strain H575.

GAS strains H292 and H887 were also compared with regard to local and systemic bacterial survival: in this instance a third experimental group was included to allow comparison with the SpyCEP-negative isogenic strain H575 (Figure 7-7). It was expected that by releasing all its SpyCEP from the cell surface, H887 would survive less well at the site of infection, with an unpredictable effect on systemic spread.

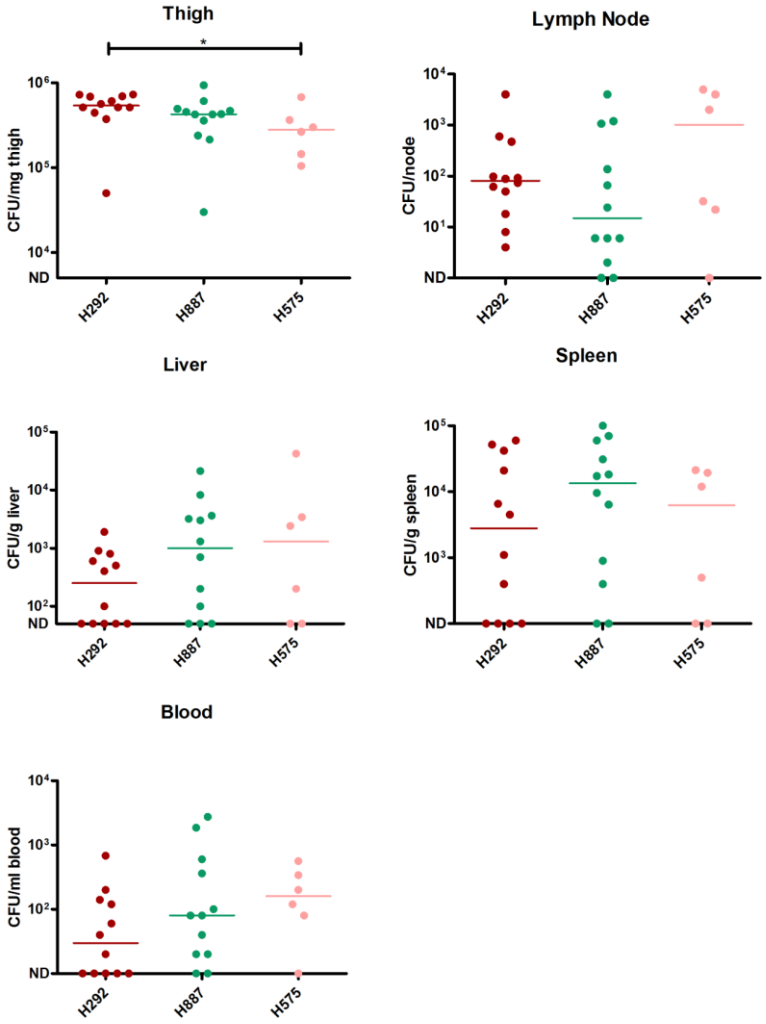


Figure 7-7 - Bacterial load 24 hours post infection with GAS strains H292 and H887

Groups of 12 CD1 female mice were infected intramuscularly with either H292 (wild type GAS, SpyCEP+ve), H887 (sortase negative SpyCEP) or six mice with H575 (SpyCEP-ve GAS). 24 hours post infection, the mice were euthanized and organs harvested and homogenised. Bacterial loads were quantified. Horizontal lines indicate median. P values determined by One way ANOVA analysis, * = $P<0.05$.

Strains H887 and H292 caused very similar levels of infection in the mouse model (Figure 7-7). Hence, surface binding of SpyCEP on GAS had little impact on the virulence of GAS. H292 is known to readily release large concentrations of SpyCEP into the supernatant, thus the difference in SpyCEP release between these two strains may not be sufficient to see any differences in bacterial systemic spread. Specifically however, there was no evidence that cell surface expression of SpyCEP was essential for local bacterial survival. Mice infected with SpyCEP-negative strain H575 were included as an indicator for the role of SpyCEP, and did demonstrate a difference in bacterial survival in the thigh, consistent with previous reports (Kurupati*, et al.* 2010). Interestingly the SpyCEP-negative mutant spread as well as the SpyCEP positive strains in this experiment, although numbers in this group were only 6, in contrast to the earlier report, underlining the subtlety of effect of single virulence factors on GAS pathogenesis. None of the other differences in bacterial load measured between H292 and H887 infected mice were significant.

It is known that the N terminus of SpyCEP can be used as a vaccine to protect against GAS and *S. equi* infection (Turner*, et al.* 2009b); however the mode of action of this protection is unclear. SpyCEP is cell surface-expressed, and antibodies can recognise cell-surface expressed proteins (Turner*, et al.* 2009b). However, antibody-mediated protection could stem from opsonising antibodies against this surface bound protein, or from neutralisation of the action of SpyCEP. H887 was used as a tool to examine this protection. As the sortase negative SpyCEP strain H887 has no SpyCEP bound to its surface, only neutralising antibodies against SpyCEP would offer protection against this strain.

An N terminal fragment of SpyCEP, CEP5, which was previously used for vaccination studies (Turner*, et al.* 2009b) was expressed recombinantly, purified, and injected with adjuvant into the thighs of mice. A further group of mice received PBS with adjuvant as

a sham vaccine. The mice received an initial prime, followed by two boost vaccinations. Immunity was confirmed prior to infection by tail bleeding three mice from each group. Serum was collected and used as a primary antibody for immunoblotting against recombinant CEP5 spotted onto membrane (Figure 7-8).

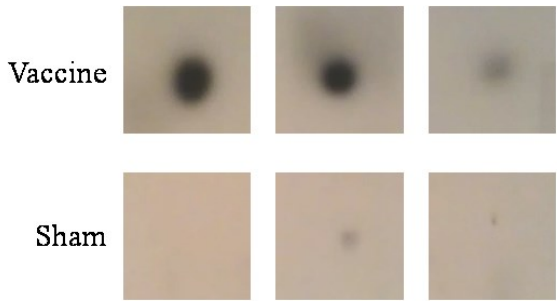


Figure 7-8 – Dot immunoblot using sera of vaccinated mice

Balb/C female mice were injected intramuscularly with either recombinant CEP5 with Freund's adjuvant (15 mice), or a sham vaccine of PBS and adjuvant (15 mice). Three days following second boost vaccination, three vaccinated and three sham vaccinated mice were randomly selected and tail bleeds were performed. The sera was collected and used as primary antibody against recombinant CEP5. Upper three panels using vaccinated mice, lower three panels, sham vaccinated mice.

Having vaccinated the mice successfully, the sham and vaccine group were then challenged with either wild type GAS H292 (7 mice) or sortase negative SpyCEP GAS H887 (8 mice). 24 hours post infection, the mice were euthanized, and the organs harvested to allow determination of bacterial load.

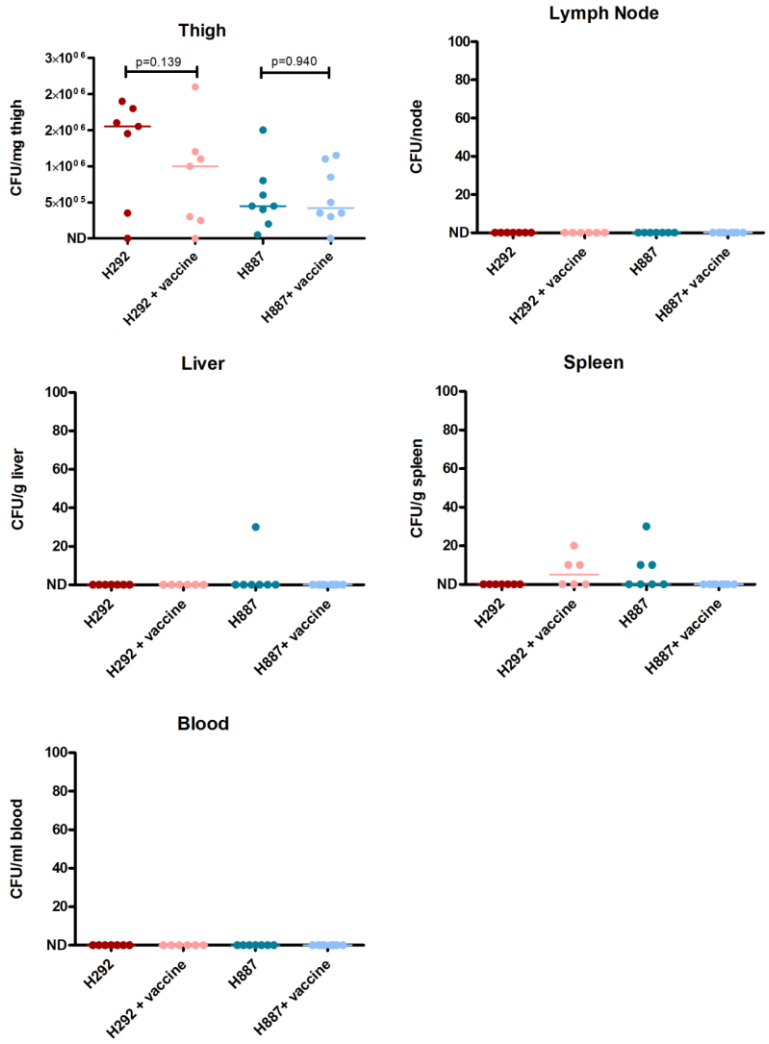


Figure 7-9 – GAS CFU recovered following challenge of CEP5 or sham vaccinated mice with H292 and H887

Balb/C female mice were injected intramuscularly with either recombinant CEP5 with Freund's adjuvant (15 mice), or a sham vaccine of PBS and adjuvant (15 mice). Seven days following second boost vaccination, the mice were challenged with $5x10^7$CFU of either H292 (wild type) (7 mice per group) or H887 (SpyCEP sortase negative) (8 mice per group) GAS strains. 24 hours following infection, mice were euthanized, organs collected and then

homogenised to determine bacterial load. Bars indicate median. P values determined using Mann Whitney within groups.

Previous vaccination studies found little effect of vaccination at the site of infection, but a much greater protective effect against distant sites (Turner*, et al.* 2009b). In this experiment, none of the mice developed a measurable invasive infection, hence protective effects against dissemination could not be measured – in all cases no bacteria were detected. The results are however consistent with previous work showing a reduction in bacterial burden in the thigh muscle following vaccination, compared to sham vaccination, which was not seen in mice infected with H887. This result suggests that the antibody response to the vaccine has a mainly opsonising role in deep tissue infection. Group sizes were small however and such an observation would need to be confirmed in a larger study where dissemination to distant tissues was seen consistently.

Interestingly, in this experiment, a difference in local infection was observed between mice infected with H292 and mice infected with H887. The GAS strain that did not express surface SpyCEP survived less well in the muscle tissue, as was hypothesised previously. The difference observed may be related to the different experimental setting, whereby mice had been primed for an immune response with sham vaccination, or may simply be the fact that in-bred mice were used in vaccination studies, rendering it easier to determine biological differences in response to infection between groups using smaller group sizes.

7.3 Discussion

SpyCEP is released in large concentrations from the surface of GAS strain H292. It is known that SpyCEP is antigenic, and antibodies are raised against SpyCEP following natural infection (Fritzer, *et al.* 2010). Furthermore, it known that vaccination against SpyCEP is protective against invasive GAS soft tissue and respiratory infection (Rodriguez-Ortega, *et al.* 2006, Turner, *et al.* 2009b). As GAS largely retains SpyCEP on the cell wall (Edwards, *et al.* 2005), it was hypothesised that surface bound SpyCEP must enhance virulence. We earlier demonstrated that the addition of soluble SpyCEP from *L. lactis* was not protective against phagocytosis (Figure 6-8). It was thus hypothesised that a strain unable to retain SpyCEP on its surface would survive less well at the site of infection, but might exert systemic effects at distant sites to the same or greater extent than a wild type GAS strain.

To test this hypothesis, a strain was constructed where the sortase motif, which acts as a substrate for sortase enzyme-mediated attachment to the cell wall, was replaced with a stop codon. The method used single cross-over recombination to alter the chromosomal sequence. Such a method generates stable integrants in GAS strains, and thus antibiotic is not needed to maintain this mutation (Kurupati, *et al.* 2010). It is also much easier than allelic replacement which requires two recombination events to occur. As such, single cross-over recombination is useful for *in* vivo studies, and does not require antibiotic dosing to maintain mutations.

The amino acids closer to the C terminus of the protein than the sortase motif were also lost following insertion of this plasmid. However, these residues are lost following processing by the sortase enzyme of wild type SpyCEP, hence protein function was not expected to be impaired. Indeed the C-terminal mutant strain was able to cleave the same substrates as the wild type SpyCEP (Figure 7-3). The approach appears to have been effective if removing

SpyCEP from the cell wall of GAS, although a small amount of SpyCEP remains associated with the bacterial wall (Figure 7-3). The sortase-negative SpyCEP strain was also observed to have very similar growth in TH media compared to the wild type strain (Figure 7-4). As such, the strains could be used for *in vitro* and *in vivo* work, without the results being influenced by growth defects, alongside the isogenic mutant H575 that lacks functional SpyCEP. Ultimately, a strain that could not release SpyCEP from the cell surface would be a desirable complimentary tool to develop, however construction of such a strain would require insight into the mechanisms permitting release and secretion of SpyCEP.

Phagocytosis experiments were carried out comparing the sortase motif-negative SpyCEP strain with wild type GAS. No difference in neutrophil phagocytosis was observed between the two strains (Figure 7-5). This refuted the hypothesis that surface bound SpyCEP was required to prevent neutrophil phagocytosis. This hypothesis was based on the results of an earlier experiment (Figure 6-8), where soluble SpyCEP from SpyCEP-positive *L. lactis* H487 was unable to inhibit phagocytosis of SpyCEP-negative *L. lactis* strain H486. However, H487 is known to produce only low levels of soluble SpyCEP (Figure 3-6), thus SpyCEP may not reach high enough levels in H487 supernatant to provide protection against phagocytosis. We were unable to test GAS supernatants in a similar way because GAS supernatants contain a range of other reagents capable of interfering with phagocytosis. The observations of this chapter also strengthen the hypothesis that SpyCEP prevents phagocytosis by cleaving chemokines (Figure 6-14). Soluble SpyCEP is able to cleave chemokines, hence soluble SpyCEP ought to be able to prevent neutrophil phagocytosis.

In vivo studies were also carried out using the sortase negative SpyCEP strain, H887. Initially release of neutrophils into the peripheral blood was measured. It was hypothesised that by releasing more soluble SpyCEP, H887 would have greater systemic effects, and hence

have a more profound effect on neutrophil release from the bone marrow than wild type GAS. Although fewer neutrophils were measured in the peripheral blood following intramuscular infection with H887 than with wild type H292, this difference was not significant (Figure 7-6), and the data require confirmation with a larger experiment. Levels of systemic and soft tissue chemokines should also be measured to determine if H887 results in greater systemic cleavage of KC and MIP-2.

Wild type GAS strain H292 is known to release large amounts of soluble SpyCEP (Figure 3-6). Thus differences in soluble SpyCEP levels between H292 and sortase negative SpyCEP strain H887 are likely to be small. Furthermore, KC, which is cleaved by SpyCEP and is involved in neutrophil release from the bone marrow, is initially made locally at the infection focus. Thus, a locally high level of SpyCEP in the infection site may be more important than high levels of circulating SpyCEP and may indeed impact on systemic levels of chemokine (Figure 7-10).

Having not detected any significant differences in neutrophil release, the virulence of the wild type GAS strain H292 and the sortase negative SpyCEP stain H887 were compared using the intramuscular invasive disease model of mouse infection. Although we hypothesised that the sortase negative SpyCEP strain would survive less well at sites of infection due to enhanced phagocytosis, the earlier results showing H887 and H292 were phagocytosed equally *in vitro* suggested this hypothesis was incorrect (Figure 7-5), or that enough SpyCEP remained associated with the surface of H887 (Figure 7-3).

The sortase negative SpyCEP strain resulted in a slightly lower bacterial load at the site of infection, however the difference was not statistically significant. Furthermore, the difference between the median infection loads of these two infections was less than one log, hence any biological significance is also doubtful. A small difference in bacterial load was

observed when comparing the sham vaccinated mice infected with the two GAS strains in Figure 7-9. Any differences between the two strains may be due to the chemokine gradient established in infection, as highlighted in Figure 7-10, rather than any direct impairment of phagocytosis. The binding of SpyCEP to the surface of GAS serves as a focus for SpyCEP concentration, thus reducing chemokine concentration most profoundly around the bacteria, disrupting normal chemokine gradients. As phagocytosis between the two strains was identical when testing human neutrophils *in vitro* (Figure 7-5), it would be inconsistent to attribute differences in local growth observed *in vivo* to impairment of phagocytosis. It is however important to note that the *in vitro* and *in vivo* experiments differed in terms of species (human vs. mouse).

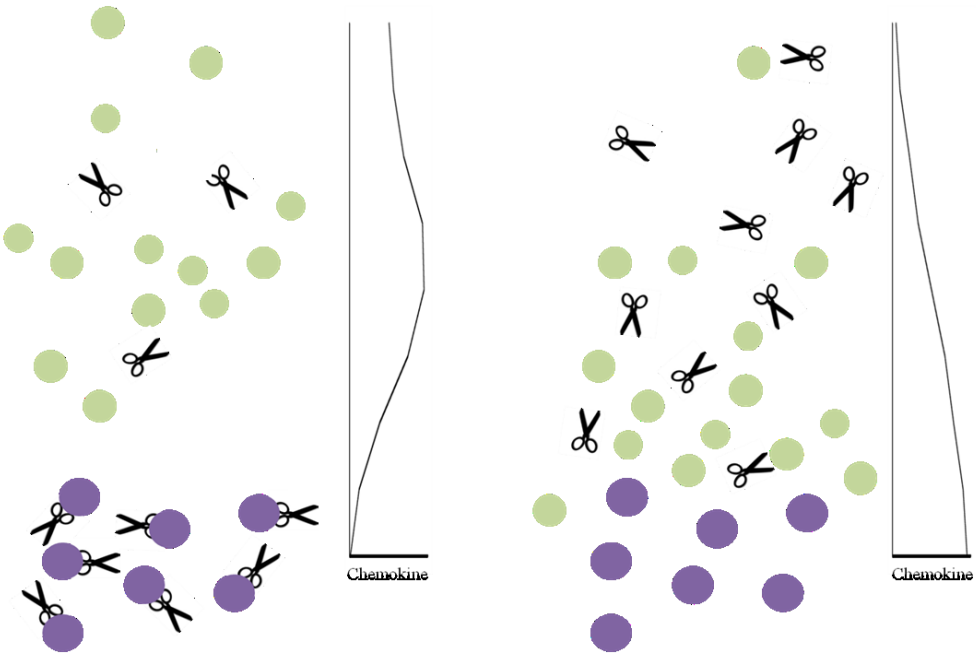


Figure 7-10 – Putative chemokine gradients around H292 and H887

GAS strains (purple) express SpyCEP (scissors). Chemokine (green) is cleaved at a faster rate close to the bacteria. Graph shows chemokine levels at distances away from the infection focus. Left panel, wild type GAS strain H292, right sortase negative SpyCEP strain H887.

While the SpyCEP-negative strain, H575, was recovered at a significantly lower level from the infected thigh than the wild type GAS strain H292, H575 spread to distant sites was surprisingly not impaired (Figure 7-7). This contrast with data obtained previously in our laboratory and may reflect simple differences in the timing of samples in relation to bacterial growth (Kurupati*, et al.* 2010), the use of outbred mice, and the subtlety of single virulence factor contributions to virulence when considering pathogens such as GAS which produce a multitude of factors that can influence virulence. Specifically, the greatest difference seen

with regard to SpyCEP impact on neutrophils and bacterial clearance was observed very early in infection at 3-6h (Kurupati*, et al.* 2010).

The antibody response raised against SpyCEP following vaccination was also studied using the sortase negative SpyCEP strain, H887. If the antibody raised was neutralising SpyCEP, it was expected that the burden of infection caused by strain H887, and wild type GAS strain H292 would be equally impaired by vaccination. However, if the antibody was opsonising, the vaccination would preferentially be protective against H292. Previous work using SpyCEP as a vaccine in an invasive model of infection found that the vaccine was primarily protective against systemic spread from the focus of infection at the 24 hour time point (Turner*, et al.* 2009b).

This study found that the vaccinated mice infected with H292 appeared to have a slight, but not significant, protection against GAS infection at the infection focus. This protection was not evident using strain H887. In this study, the mice infected with H887 had a significantly lower bacterial burden in the infected thigh than those infected with wild type GAS strain H292, likely due to the mechanism described in Figure 7-10. The slight protection seen using strain H292 but not seen using strain H887 implies that the antibody protection raised in the mice was opsonising GAS rather than neutralising SpyCEP. Unfortunately, the mice in this experiment did not suffer systemic infection, thus the protective effect of the vaccine could not be fully assessed. This is likely due to the inocula used in this experiment being lower than used in other experiments, due to unpredictable variations in GAS growth. Based on the results obtained, upon repeating this experiment, it would be expected that the vaccine was able to protect mice against systemic spread of wild type GAS strain H292, but not against the spread of sortase negative SpyCEP GAS strain H887.

We considered the possibility that, by shedding SpyCEP in its soluble form, GAS may actively inhibit any opsonising action of pre-existing anti-SpyCEP antibody. Although this study cannot address this directly, vaccination experiment comparing H292 with a strain that did not release SpyCEP at all would be illuminating. The development of tools to systematically address the structure function of SpyCEP activity has been hampered by difficulties in cloning the full length protein in both *E. coli* and *L. lactis*. Indeed, those who report success have excluded the LPXTG motif in constructs (Kaur*, et al.* 2010, Zingaretti*, et al.* 2010, Zinkernagel*, et al.* 2008). The development of a non-LPXTG SpyCEP construct in GAS is an advance in this respect but the *in vivo* studies reported herein are small and require confirmation in future work.

Chapter 8 - Discussion and Future Work

8.1 Conclusions

This study has enhanced our understanding of SpyCEP and its effects on neutrophils. Prior to this study, the substrate range of SpyCEP was incompletely described, including, some but not all, neutrophil specific chemokines (Edwards*, et al.* 2005, Kurupati*, et al.* 2010, Sumby*, et al.* 2008). However, a structural basis for specificity could not be described based upon this list; some chemokines which could bind CXCR2 were not included, but the range was not limited to those chemokine which could bind CXCR1. This work, and subsequent work (Zingaretti*, et al.* 2010), has found that all chemokines which bind CXCR1 and 2 with high affinity are cleaved by SpyCEP (Figure 3-3). As these substrates all share regions of considerable structural homology, including a pair of acidic residues at the SpyCEP cleavage site, and are able to bind the same receptors, the named substrates represent a more rational list.

Chemokine	ELR	Cleaved by SpyCEP	Sequence
CXCL4	-	-	LQAPLYKKIIKKLL
CXCL9	-	-	PDSADVKELIKKVE
CXCL11	-	-	PKSKQARLIIKKVE
CXCL12	-	-	PKLKWIQEYLEKAL
CXCL1	+	+	PASPIV**KK**IIEKML
CXCL3	+	+	PASPMV**QK**IIEKIL
CXCL5	+	+	PEAPFL**KK**VIQKIL
CXCL6	+	+	PEAPFL**KK**VIQKIL
CXCL8	+	+	PKENWV**QR**VVEKFL

Figure 8-1 – Chemokine sequence alignment

List of chemokines used in this study detailing their aligned C termini. Chemokines highlighted blue are cleaved by SpyCEP. The bold residues indicate the acidic residues which form the cleavage site of SpyCEP.

This study also provided rates of reaction for SpyCEP cleavage of each if its substrates. CXCL8 was found to be the substrate which is cleaved most rapidly (Figure 3-9). This is the most potent, and most abundant neutrophil specific chemokine, hence optimal cleavage of this substrate is consistent with optimal disruption of neutrophil function (Mortier*, et al.* 2011). The other substrates were cleaved more slowly. Interestingly, the ranking of their cleavage rate from fastest to slowest matches the order of discovery of the substrates; the faster cleaved substrates were the earlier discovered. The strain used in this study is noted for its exceptional SpyCEP expression (Turner*, et al.* 2009a). The slower cleaved substrates may have been overlooked in previous studies which used less prodigious SpyCEP producers.

The discovery of the substrate range of SpyCEP, and the failure to detect any other substrates, demonstrates that SpyCEP is a highly specific protease with consequent predictably specific effects on GAS virulence. SpyCEP-chemokines have specificity for neutrophils alone, and subsequently this study has found that neutrophils are the only cells affected by SpyCEP with regard to effects on mobilisation from bone marrow and

phagocytosis. Other studies have shown that GAS expression of SpyCEP is upregulated following ablation of the GAS CovR/S virulence regulation axis, a hallmark of invasive GAS strains (Turner, *et al.* 2009a).

These observations are very different from SpeB, the other protease produced by GAS which also reportedly shows chemokine degrading activity (Egesten, *et al.* 2009). SpeB is highly unspecific, cleaving a very broad range of substrates, including the C5a peptidase, another large GAS cell surface protease (Berge and Bjorck 1995). It also tends to be expressed by non-invasive strains and is markedly down-regulated or switched off in strains demonstrating *covRS* mutations in contrast to SpyCEP (Turner, *et al.* 2009a).

Neutrophils are known to play a key early role in the clearance of bacterial infections, and indeed SpyCEP is known to be a critical virulence factor for GAS in the mouse model of infection (Kurupati, *et al.* 2010, Sumby, *et al.* 2008, Zinkernagel, *et al.* 2008). Chemokines are vital in the release of neutrophils from bone marrow reserves (Martin, *et al.* 2003), their trafficking across the endothelial barrier (Baggiolini 1995) and their activation including upregulation of phagocytic receptors (Detmers, *et al.* 1990) (Figure 8-2). Whilst the effects of SpyCEP on neutrophil trafficking and also on NETosis, a final attempt at bacterial killing as neutrophils apoptose (Brinkmann, *et al.* 2004, Zinkernagel, *et al.* 2008), are known, the other important functions of chemokines on neutrophils had not been explored prior to this study (Edwards, *et al.* 2005).

The original description of SpyCEP-mediated cleavage of CXCL8 highlighted the C-terminal cleavage of the molecule, yielding a roughly 6kD residual protein with intact N terminal and di-sulphide bonds. Chemokines such as CXCL8 bind their cognate 7 transmembrane domain spanning receptors via the N terminal ELR motif, thus an effect of cleavage on binding and activation of receptors was not predicted, Indeed, the impact of

SpyCEP cleavage on neutrophil recruitment can be solely attributed to loss of the C terminal of CXCL8, which is essential for transmigration of chemokine across endothelial cells, and presentation at the endothelial or luminal surfaces of blood vessels (Middleton, *et al.* 1997). Notwithstanding uncertainty regarding the impact of C-terminal cleavage of chemokines on neutrophil binding and activation, we hypothesised that additional chemokine-dependent events could also be disrupted, with unknown functional consequences *in vivo*. A more profound disruption of neutrophil function would also explain the profound impact of SpyCEP on bacterial virulence (Kurupati, *et al.* 2010).

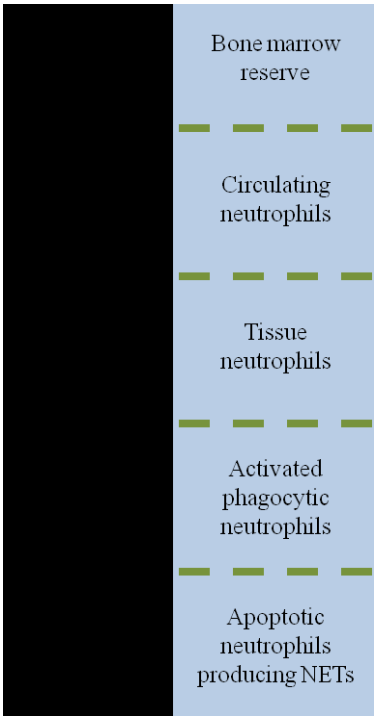


Figure 8-2 – Development of a neutrophil response to infection

Neutrophils are held quiescent in the bone marrow by constitutive production of CXCL12 (Shirozu, *et al.* 1995). The first stage of a developing neutrophil response to

bacterial infection is the release of these cells from these stores. This study confirmed that a local increase in neutrophil specific murine chemokines was able to increase circulating neutrophil levels (Wengner*, et al.* 2008) (Figure 4-5). It was accordingly also found that expression of SpyCEP by GAS or *L. lactis* was able to reduce the numbers of circulating neutrophils early after infection (Figure 4-1; Figure 4-3). This difference was seen despite the SpyCEP expressing *L. lactis* strain causing a significantly more severe infection (Figure 4-7). It was concluded that by cleaving the chemokines expressed locally at the site of infection, SpyCEP reduced systemic chemokine concentrations, interfering with neutrophil release from the bone marrow. The reduced neutrophil release means that fewer neutrophils reach the blood and are able to cross the endothelial barrier to reach the infection focus. This, combined with the known reduced trafficking of neutrophils across the endothelial barrier, also attributable to SpyCEP, provides a double level of protection to the bacteria (Figure 8-3). Such effects lead to potentially large impacts on disease severity.

Throughout the study, the isogenic *L. lactis* strains provided clearer phenotypes than the GAS strains. *L. lactis* is a non-pathogenic species of bacteria, which expresses few extracellular proteins of its own (van de Guchte*, et al.* 1992). This contrasts with GAS which produces a wide range of virulence factors, including many that act on neutrophils. As such GAS strains which lack SpyCEP still have a considerable arsenal to bring to bear on the neutrophil response. Whilst a phenotype difference between SpyCEP-positive and negative GAS strains can still be clearly observed, the difference was inevitably smaller than that observed between the *L. lactis* strains. The *L. lactis* strains have been previously reported to display a clearer phenotype than the GAS strains *in vivo* (Kurupati*, et al.* 2010, Zinkernagel*, et al.* 2008).

The hypothesis that SpyCEP may interfere with neutrophil release from the bone marrow stemmed from clinical observations in patients with very severe GAS disease who have frequently demonstrated paradoxically low neutrophils counts at presentation to hospital. (Shiranee Sriskandan, personal communication). This has been borne out in non-human primate models of invasive GAS disease and strengthened by the data from this study, shown in Figure 4-8. These data showed that the mice infected with a SpyCEP-expressing GAS strain had reduced systemic levels of the murine neutrophil chemokine KC compared to mice infected with an isogenic SpyCEP-negative strain 6 hours post infection. This effect could not be reproduced using the *L. lactis* strains, due to the huge difference in *L. lactis* infection severity; the SpyCEP-negative strain had been completely cleared by six hours (Figure 4-7), and thus chemokine levels were returning to normal precluding any comparison with mice infected with the SpyCEP-positive strain. All the other chemokines and cytokines measured were found to be at similar concentrations between the strains, although TNFα and IL-6 appeared to be higher in the SpyCEP-positive GAS strains. These are markers of acute disease, and high concentrations tend to be associated with poor outcome of infection.

Importantly, the reduced concentrations of systemic chemokine were specifically associated with a reduction in circulating neutrophils. Previously it was reported that CXCL chemokines were central to mobilisation of neutrophils from the bone marrow (Wengner*, et al.* 2008), and experiments conducted in this study have confirmed that KC can mobilise neutrophils into the peripheral blood in the absence of infection (Figure 4-5). The observed reduction in circulating neutrophils in mice infected with SpyCEP-producing bacteria was not associated with a change in systemic G-CSF levels (Figure 4-8), suggesting that chemokine cleavage by SpyCEP provided the sole reason for the difference.

This study has demonstrated that the first stage of the developing neutrophil response - release from the bone marrow - is disrupted by SpyCEP. Previous studies have indicated that the second stage, extravasation is disrupted (Edwards*, et al.* 2005, Kurupati*, et al.* 2010), leading to a reduction in tissue neutrophils through reduced recruitment across endothelium (Figure 5-1). We next set about to determine if the third stage, activation leading to phagocytosis, was also disrupted by SpyCEP. The classical Lancefield assay was initially used to measure phagocytosis. However this method was only useful when examining the *L. lactis* strains (Figure 5-2; Figure 5-3). The donors used had immunity against the GAS strains, and thus the bacteria were all killed, illustrating a common limitation of the Lancefield assay. As *L. lactis* is not a human pathogen (van de Guchte*, et al.* 1992), it was predictable that the human donors would not have immunity against this pathogen.

Although antibody-mediated immunity against *L. lactis* was not present in early experiments, it did appear to be present in some of the donors used for later experiments. As *L. lactis* is not a human pathogen, presumably an acquired antibody response against it is unusual. The donors used in these experiments were laboratory workers, and thus potentially occupationally exposed to *L. lactis*. It was hypothesised that using donors from an external source would lead to more consistent results, and a complete lack of antibody mediated opsonisation.

As the Lancefield assay proved unsuccessful at measuring differences in phagocytosis between SpyCEP-negative and positive strains, a more quantitative method of measuring bacterial phagocytosis by flow cytometry (Bjerknes and Bassoe 1983) which has previously been used successfully with GAS (Ji*, et al.* 1998, Schnitzler*, et al.* 1997) was employed. The method measured the gain of fluorescence by phagocytes incubated with fluorescent bacteria. This method clearly and reproducibly showed that SpyCEP expression by both *L. lactis* and

GAS reduced phagocytosis by neutrophils (Figure 5-5; Figure 5-7; Figure 5-8). As such, these results confirmed that SpyCEP inhibits all phases of the neutrophil response to infection. Indeed, the effects of SpyCEP are additive. SpyCEP reduces circulating neutrophils, so there are fewer neutrophils to extravasate; SpyCEP inhibits extravasation, so there are fewer neutrophils to phagocytose bacteria; SpyCEP inhibits phagocytosis by those few neutrophils which do enter tissue (Figure 8-3).

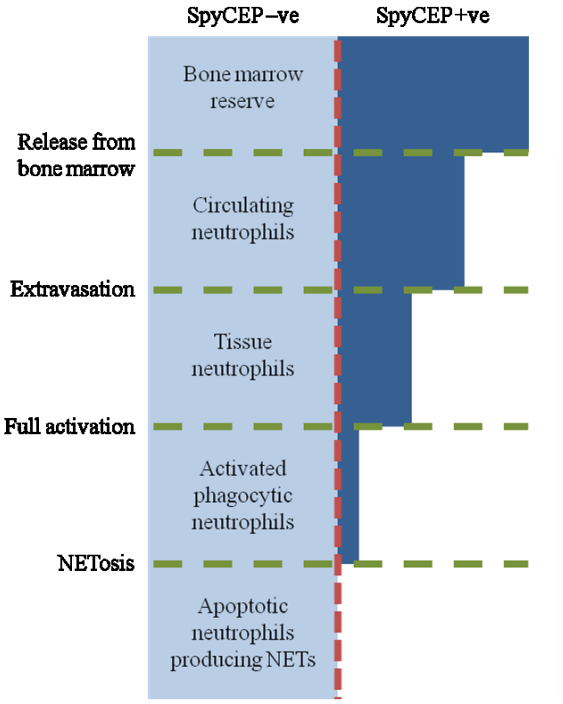


Figure 8-3 – Progressive impact of SpyCEP on neutrophil function

SpyCEP-positive bacteria (RIGHT of panel) inhibit progressive stages of neutrophil function, leading to profound ablation of neutrophil function, compared to SpyCEP-negative bacteria (LEFT of panel).

Having determined that SpyCEP acts to inhibit phagocytosis of opsonised bacteria, the methodologies were used to further examine phagocytosis of GAS and describe the

mechanism by which SpyCEP inhibits phagocytosis by neutrophils. Neutrophil phagocytosis of both *L. lactis* and GAS was found to be dependent on opsonisation (Figure 5-10). *L. lactis* was found to be opsonised equally by whole serum and antibody depleted serum (complement positive). Complement depleted serum (antibody positive) was found to only occasionally allow phagocytosis (Figure 5-13). This data indicated that complement is the required opsonin for *L. lactis* phagocytosis, and further shows that antibodies against *L. lactis* are uncommon.

Neutrophil phagocytosis of GAS was found to be optimal in whole serum containing both complement and antibody. Complement depleted serum (antibody positive), was found to allow low levels of phagocytosis, whereas antibody depleted serum (complement positive) was able to opsonise the bacteria such as to allow good, but sub-optimal phagocytosis. These data indicates that complement is very important for opsonisation, but this is enhanced by antibody. Antibody alone was only slightly opsonising (Figure 5-12).

These results raise interesting questions about the role of antibody in GAS immunity. The Lancefield assay was used to determine whether a blood sample was immune to GAS, defined as having serotype specific antibodies against the variable M protein, a major GAS antigen. The donors used in this study were largely immune to the serotype of the GAS strain used in this study (M81), as demonstrated by the Lancefield assays carried out (Figure 5-2). Indeed, this immune blood was able to rapidly kill the GAS. Despite this, antibody alone from the same group of broadly immune donors was not able to effectively opsonise the bacteria and complement was needed, a feature of the innate immune response, present in both immune and non-immune blood. The M protein is itself anti-phagocytic, preventing proper complement deposition and its interaction with complement receptors (Peterson*, et al.* 1979, Weis*, et al.* 1985). Based on the results obtained in this study, the serotype specific

antibody against M protein seems to primarily function not by opsonising the bacteria directly, but by overcoming this inhibition, enhancing complement deposition. This conclusion is further supported by earlier work. It has been determined that direct blockade of Fc receptors which mediate antibody opsonised phagocytosis had no effect on phagocytosis of GAS, unlike blockade of complement receptors (Nilsson*, et al.* 2005).

The mechanism by which SpyCEP inhibits neutrophil phagocytosis was hypothesised to involve chemokines, as these form the only known substrates of SpyCEP. Chemokines are known to have a role in the activation of neutrophils; a process which includes the upregulation of CR3 receptors that are involved in the phagocytosis of GAS (DeMaster*, et al.* 2002, Detmers*, et al.* 1990). It was confirmed that neutrophil-specific chemokines increased the surface levels of CD11b (Figure 6-9), and it was found that SpyCEP-expressing strains induced lower levels of CD11b on the neutrophil surface than their SpyCEP-negative counterparts (Figure 6-11). It was confirmed that SpyCEP had no direct cleavage activity against CD11b (Figure 6-12), or on opsonisation of bacteria (Figure 5-11).

These observations provided a mechanism by which SpyCEP could retard neutrophil phagocytosis (Figure 6-14). By cleaving neutrophil specific chemokines, neutrophils fail to completely activate and upregulate phagocytosis receptors of known importance in GAS phagocytosis. The effects on phagocytosis are seen over very short time periods, suggesting that SpyCEP is rapidly cleaving chemokine (Figure 5-5). As such, differences in chemokine levels in the phagocytosis reaction mixtures would be detectable. It was indeed found that neutrophils incubated with SpyCEP-negative strains had more CXCL8 bound to their surface than those incubated with SpyCEP-positive strains (Figure 6-7).

Paradoxically however, the addition of excess CXCL8 failed to have any effect on phagocytosis. The addition of CXCL8 with the addition of bacteria to the neutrophils was

hypothesised to upregulate CD11b, as had been previously demonstrated (Figure 6-9), thus increasing phagocytosis. Despite SpyCEP beginning to cleave this chemokine immediately, the cleavage rates found in Figure 3-9 suggested that SpyCEP could not cleave all this chemokine within the time period of this experiment, especially where high concentrations of chemokine were used. The results could also not be explained by cleaved chemokine antagonising receptor binding (Goldblatt, personal communication), nor would it seem likely that the recombinant chemokine had no biological activity (Figure 6-9). Other experiments to examine the hypothesis of Figure 6-14 that chemokine cleavage prevents phagocytosis, including the addition of soluble SpyCEP and a chemokine receptor antagonist, also failed to provide support for the hypothesis. However, these could be explained by technical problems.

Despite being contradicted by the results of where soluble exogenous chemokines were added, the hypothesis proposed is supported by work using other cells. It was found that other phagocytes, which unlike neutrophils, express low numbers of CXCR1 and 2 receptors (Grob*, et al.* 1990, Zahn*, et al.* 1997) were unaffected in their ability to phagocytose bacteria by the presence of SpyCEP (Figure 6-1; Figure 6-2). These cells express very similar phagocytosis receptors to neutrophils (Collins*, et al.* 1979, Shi and Pamer 2011), and accordingly have similar dependence on complement to opsonise both GAS and *L. lactis* (Figure 6-3). Hence any direct effect that SpyCEP might have had on phagocytosis receptors or opsonisation would have been seen using all types of phagocytic cell. The effects of SpyCEP on phagocytosis are specific for neutrophils; the chemokine substrates of SpyCEP are specific for neutrophils, and it is known that the binding of these substrates to neutrophils upregulates a receptor known to be important for GAS phagocytosis (DeMaster*, et al.* 2002).

An alternative hypothesis explaining the impairment of phagocytosis has been suggested based on observations that SpyCEP inhibits invasion of epithelial cells by GAS, in

a chemokine independent manner (Andreoni, personal communication). This observation was dependent upon SpyCEP being present on the bacterial surface, and enzyme being catalytically active. It could be hypothesised that SpyCEP is acting to prevent "invasion" of neutrophils by GAS. However, if this were the case, the impairment would be seen using the other phagocytes. Furthermore, a strain of GAS mutated such that it can express only soluble SpyCEP would be phagocytosed more efficiently than a wild type strain with surface bound SpyCEP. This was not observed to be the case (Figure 7-5). This strain was however found to survive slightly less well in the invasive mouse model at the infection focus (Figure 7-9), which we hypothesise was due to differences in chemokine gradients in the infection site (Figure 7-10).

In conclusion, this study has found that SpyCEP specifically cleaves all neutrophil specific chemokines. The effects of this on neutrophil function are profound, impacting cumulatively on neutrophil release from the bone marrow, and phagocytosis, as well as neutrophil trafficking and NETosis which had previously been described (Edwards *et al.* 2005, Zinkernagel *et al.* 2008). These effects specifically target neutrophils due to their high expression of chemokine receptors CXCR1 and 2, and as such the effects of SpyCEP on neutrophil function are mediated by ablated chemokine signalling.

8.2 Future work

Future investigation into the impacts of SpyCEP would be facilitated by being able to genetically manipulate and express SpyCEP recombinantly in *E. coli*. Attempts to achieve this aim were pursued in this study, but ultimately proved unsuccessful. Indeed, attempts to obtain a shuttle vector containing the SpyCEP gene, *cepA*, from collaborators in Switzerland were abortive due to cryogenic stocks being non-viable. Many investigators have reported difficulties in manipulating *cepA* through *E. coli* (Edwards*, et al.* 2005, Fritzer*, et al.* 2009, Kurupati*, et al.* 2010, Rodriguez-Ortega*, et al.* 2006, Sjolinder*, et al.* 2008, Sumby*, et al.* 2008, Zinkernagel*, et al.* 2008). This has impacted on cloning and expression of SpyCEP in *L. lactis* since all manipulations are generally undertaken in *E. coli.* However, some recent work has included successful manipulation of SpyCEP by expressing sub-domains that appear to self-associate and form functional enzyme (Kaur*, et al.* 2010, Zingaretti*, et al.* 2010), hence there is precedence for such manipulation being performed. Future work would thus include trying to genetically manipulate SpyCEP, or establish collaborations to use these successfully created strains.

Recombinant SpyCEP would be used to allow more precise quantification of the rate of chemokine cleavage. Current work has allowed quantification of SpyCEP cleavage rates in specific reaction mixtures. Using recombinant SpyCEP would allow excess concentrations of SpyCEP to be used, finding maximal cleavage rates for each substrate, at a specific substrate concentration, providing a constant value for reference. We would also like to determine why the cleavage rates of these chemokines are different. Using the recombinant SpyCEP, we could determine binding affinities between the enzyme and the ligand. We would also like to

crystallise the protein, allowing full structural determination; with improved techniques, this may be possible using lower quantities of protein than were previously required.

Recombinant SpyCEP would also be used to further examine its effects on neutrophil release. Enzyme alone, chemokine alone and enzyme mixed with chemokine could be injected intramuscularly into the mouse to confirm that chemokine cleaved by SpyCEP could not mobilize chemokines, as this could not be confirmed in the current study using the reagents currently available.

We would also use the recombinant SpyCEP to further confirm the hypothesis regarding the mechanism of SpyCEP disruption of phagocytosis. Earlier work using bacterial supernatants as sources of recombinant chemokine failed to demonstrate that the introduction of soluble SpyCEP could protect SpyCEP-negative strains. The GAS strains contained too many other virulence factors, and the *L. lactis* supernatants contained only very low concentrations of SpyCEP. Thus recombinant SpyCEP would be ideal to use to repeat this experiment.

Genetic manipulation of SpyCEP would also allow us to perform structure function analysis on SpyCEP. Previous work where SpyCEP has been genetically manipulated has revealed information about its structure (Zingaretti*, et al.* 2010), and the minimal domains required for proteolytic activity (Kaur*, et al.* 2010). We would like to mutate the active site to ensure that SpyCEP requires its enzymatic activity for all the phenotypes observed in this study, as a means of further proving that chemokine cleavage is mediating the effects observed. We would also investigate the C terminus of this protein, which has been indicated not to be important for enzymatic activity (Kaur*, et al.* 2010).

Being able to genetically manipulate *cepA* in *E. coli* would also allow us to better investigate the impact of the localisation of SpyCEP. In this study, we could mutate the

sortase motif of chromosomally encoded GAS *cepA* using single cross-over recombination. However, as GAS strains typically release large amounts of SpyCEP, such mutagenesis could only be expected to produce a limited phenotype. The *L. lactis* strains do not release much soluble SpyCEP, thus a sortase motif negative *cepA* in this species would be more instructive. We would use site directed mutagenesis to alter the *cepA* encoding plasmid in *E. coli* before re-transforming *L. lactis*. Using these strains, we would repeat vaccine studies, trying to achieve a more systemic infection than achieved in this study. Previous work using this vaccine identified that the main protection offered was against systemic infection (Turner*, et al.* 2009b).

Cloning difficulties were also encountered when trying to manipulate the orthologues of SpyCEP found in other organisms. The orthologue found in *S. pneumoniae* appears particularly interesting, as a novel fibrinogen cleavage effect was reported in this study (Figure 3-10). As a known function of the SpyCEP orthologue in *S. agalactiae* is fibrinogen cleavage, investigating the *S. pneumoniae* orthologue PrtA would be interesting. However, without genetic manipulation of *S. pneumoniae* or expressing PrtA in *L. lactis* such studies could not occur.

Further work which would not require genetic manipulation of SpyCEP could be carried out which would also be useful to support the hypotheses from this study. We would investigate cytokine and chemokine levels following infection with SpyCEP-positive and negative *L. lactis* strains at shorter time points than those used in this study. By six hours, the SpyCEP-negative *L. lactis* infection had been completely cleared, and thus cytokine and chemokines were returning to resting levels. As the *L. lactis* strains showed greater phenotypic differences than GAS strains, these earlier time points may reveal differences in

the immune response against these two strains which have been missed, and which could thus be further investigated using the GAS strains.

The impact of neutrophil release from the bone marrow has been described based on the increase in circulating neutrophils. Whilst it seems highly unlikely that these neutrophils came from anywhere except the bone marrow, future work to confirm their origin would be important. Previous work has used canulated murine femurs, adding stimuli in the supportive media, and measuring the output (Wengner*, et al.* 2008). This method provides certainty that emerging cells have originated from the bone marrow. Recombinant SpyCEP, chemokine and SpyCEP plus chemokine could be used in such a study and the output from the marrow measured.

The contradictory data obtained where soluble chemokine failed to have any impact on phagocytosis needs to be investigated further. Further work here would be to use alternate forms of recombinant chemokine, including the more bioactive 72aa form (Mortier*, et al.* 2011). Other further work would be to activate the neutrophils with chemokines prior to adding bacteria, allowing chemokines to bind neutrophils before any SpyCEP activity could occur.

Observations have been made in this study regarding the role of antibody in GAS immunity. The results in this study have led us to hypothesise that immunising antibody primarily functions to enhance complement deposition. This would be investigated using the methods used in this study, using the depleted sera.

Chemokine ligands for CXCR1 and 2 have been implicated in a variety of autoimmune diseases linked to aberrant neutrophil function, such as chronic obstructive pulmonary disorder, asthma (Pease and Sabroe 2002) and Crohn's disease (Morain*, et al.* 1981). Accordingly, small molecule antagonists against these receptors have been the subject

of drug investigation. Indeed many of these drugs, such as Repertaxin, are the subject of current study determining efficacy (Pease and Horuk 2012). With its exquisite substrate specificity, limited to agonists of CXCR1 and 2, SpyCEP may be an alternative to the antagonists being studied.

A major barrier to SpyCEP being used therapeutically is finding an efficient and safe means to deliver the protein to the site where required. *L. lactis*, which is being studied as a potential vector for vaccination due to its avirulence, was transformed into a lethal pathogen upon expression of SpyCEP (Kurupati*, et al.* 2010, van de Guchte*, et al.* 1992, Zinkernagel*, et al.* 2008). As such, using a live vector would be a dangerous approach.

As a recombinant protein, SpyCEP would have poor bioavailability, due to action of proteases (Shaji and Patole 2008), and would need to be injected directly into the site required. Interestingly, SpyCEP expression by bacteria injected directly into the blood stream or using a dermonecrotic model of infection was found to reduce inflammation and thus be protective to the mouse (Sjolinder*, et al.* 2008, Sumby*, et al.* 2008). SpyCEP has repeatedly been shown to be difficult to make recombinantly, and needs nearly all of its domains to retain chemokine cleaving function (Kaur*, et al.* 2010), making production of SpyCEP for therapeutic use challenging. If recombinant SpyCEP could be made in large amounts in *E. coli*, structural studies may allow functional analogues to be designed, overcoming the issues of SpyCEP for therapy, whilst maintaining its specificity and efficacy.

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