

**Targeting the streptococcal virulence
factor SpyCEP as a
novel therapeutic approach**

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Statement of originality

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Abstract

The *Streptococcus pyogenes* cell envelope protease (SpyCEP) is a crucial virulence factor involved in the immunomodulation of the innate immune response to *S. pyogenes* infection. SpyCEP rapidly and efficiently cleaves the entire family of ELR⁺ CXC chemokines, resulting in a chronic paucity of infiltrating neutrophils to the site of *S. pyogenes* infection. As such, expression of SpyCEP is highly associated with driving invasive disease and poor clinical outcome in streptococcal associated diseases, such as necrotizing fasciitis.

The aim of this thesis was to determine the need for inhibition of SpyCEP activity, and to then identify potential inhibitors and characterise their activity, both *in vitro* and *in vivo*, as an adjunctive treatment in invasive streptococcal infection. This required further interrogation of the role of SpyCEP in streptococcal disease and exploration of novel aspects of SpyCEP function.

The work has provided the first quantification of SpyCEP production *in vivo*, in murine models of *S. pyogenes* infection and in human samples from clinical *S. pyogenes* infection. Bioactive SpyCEP was detected and quantified in patient samples, and it was demonstrated that enzyme activity is independent of bacterial viability.

Biochemical and structural tools were leveraged to analyse multiple recombinant SpyCEP constructs. The work provided the first evidence that the C-terminal domain of SpyCEP is necessary for CXCL8 substrate binding and attributed novel proteolytic activity to the C-terminus alone, independent of the N-terminus. A novel kinetic assay was employed to quantify and compare activity of different SpyCEP constructs and demonstrated the C-terminal activity to be 1500-fold less efficient than full length SpyCEP.

High throughput screening in collaboration with an industrial partner facilitated discovery of two compounds with novel SpyCEP inhibitory activity. It was shown that the compounds ablate SpyCEP cleavage of CXCL8. Inhibitory activity against SpyCEP was superior to any previously known inhibitor but was not unique; the inhibitors demonstrated activity against a panel of archetypal serine proteases, including in one case the similar streptococcal protease ScpA (C5a peptidase). *In vitro* characterisation of one inhibitor confirmed functional recovery of CXCL8 mediated neutrophil chemotaxis following SpyCEP treatment and demonstrated a modest protective effect *in vivo*.

This work has provided a greater understanding of the catalytic activity of SpyCEP, its expression in human disease, and has elucidated new mechanisms of SpyCEP proteolytic function. Underlining a central role for SpyCEP in preventing neutrophil recruitment even in the setting of antibiotic treatment. Novel inhibitors of SpyCEP have been identified, providing a platform for future research and the development of improved compounds that can provide adjuvant treatment for severe invasive disease.

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List of abbreviations

ARF	Acute rheumatic fever
BSA	Bovine serum albumin
CBA	Columbia blood agar
CC	Cysteine-cysteine
CFU	Colony forming unit
CTAB	C-terminal antibody
CWE	Cell wall extract
CXC	Cysteine-X-cysteine
CXCR	CXC chemokine receptor
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELR	Glutamic acid-leucine-arginine
EndoS	Endo- β -N-acetylglucosaminidase
FDA	US Food and Drug Administration
FITC	Fluorescein isothiocyanate
FL-1	Fibroblast-stimulating lipopeptide - 1
GAGs	Glycosaminoglycans
GAS	Group A streptococcus
HA	Hyaluronic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IC ₅₀	Substrate concentration at which 50% of enzyme activity is inhibited

IgG	Immunoglobulin G
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IVIG	Intravenous immunoglobulin
kDa	kiloDalton
K_{cat}	Substrate turnover number
K_M	Michaelis–Menten constant
LB	Luria-Bertani
MES	2-(N-morpholino) ethanesulfonic acid
mg, ml, mM	Milligram, millilitre, millimolar
MOPS	3-(N-morpholino) propanesulfonic acid
MT	Mutant
MWCO	Molecular weight cut-off
NAD	Nicotinamide adenine dinucleotide
NET	Neutrophil extracellular traps
ng, nl, nM	Nanogram, nanolitre, nanomolar
NMR	Nuclear magnetic resonance
PA	Protease-associated
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pg, pl, pM	Picogram, picolitre, picomolar
pIC_{50}	$-\log (IC_{50})$
P/S	Penicillin / Streptomycin
PSGN	Post-streptococcal glomerulonephritis
qPCR	Quantitative polymerase chain reaction
RHD	Rheumatic heart disease
RPM	Revolutions per minute
RPMI	Roswell park memorial institute media
SD	Standard deviation
Sda1	Streptococcal DNase 1

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEC-MALLS	Size exclusion chromatography – multi-angle laser light scattering
SEM	Standard error of the mean
Ska	Streptokinase
SLO	Streptolysin O
SLS	Streptolysin S
SpeB	Streptococcal pyrogenic exotoxin B
SpyCEP	<i>Streptococcus pyogenes</i> cell envelope protease
STSS	Streptococcal toxic shock syndrome
TAE	Tris-acetate buffer
TB	Terrific broth
TCA	Trichloroacetic acid
TCS	Two-component regulatory system
THB	Todd-Hewitt broth
TLR	Toll-like receptor
TMB	3,3',5,5'-Tetramethylbenzidine
TNF α	Tumour necrosis factor alpha
μg , μl , μM	Microgram, microlitre, micromolar
UV	Ultraviolet
WT	Wildtype
yFMI	Forward migration index

1 Introduction

1.1 *Streptococcus pyogenes*

Streptococcus pyogenes or group A streptococcus (GAS) is a Gram-positive, β -haemolytic, extracellular coccus and the etiological agent responsible for a wide range of human disease. The clinical manifestations of *S. pyogenes* have been described historically for over 2 millennia with Hippocrates describing erysipelas and subsequent tissue necrosis in the 5th century BC (1). However, it was not until the late 19th and early 20th century following the isolation of bacteria from clinical samples that *S. pyogenes* was characterised, named, and associated with human disease (2-7). Ffol

1.1.1 Streptococcal disease burden

S. pyogenes is an obligate human pathogen responsible for a wide range of self-limiting, mild, and severe diseases, including pharyngitis, pyoderma, bacterial sepsis, streptococcal toxic shock syndrome, necrotizing fasciitis, and rheumatic fever. This spectrum of disease results in a large global morbidity and mortality across both high and low-income countries, with at least 517,000 deaths annually (8). Streptococcal disease manifestation can be broadly differentiated into 3 sub-categories, non-invasive disease, autoimmune sequelae and invasive disease.

Non-invasive disease

The majority of streptococcal infections present as non-invasive disease. These superficial *S. pyogenes* cases are usually self-limiting or highly treatable with antibiotic intervention. However, these infections are prevalent globally and so consequently have a high economic and health care impact. *S. pyogenes* largely colonises the human nasopharynx and skin, as such, the largest burden in terms of case numbers is attributable to streptococcal throat infections, with an estimated 616 million cases per year (9). Indeed *S. pyogenes* is the leading global

etiological agent of acute bacterial pharyngitis (10). Infection can occur in both children and adults, with *S. pyogenes* the causative agent of 15-30% of child and 5-20% of adult pharyngitis cases. However, asymptomatic throat carriage of *S. pyogenes* is skewed towards children, with a prevalence of 5-21% compared to ~1% in adults (11-15). The streptococcal pharyngitis burden is dependent on season, with cases peaking late in the winter and at the beginning of spring (16, 17), and typically results in a self-limiting infection characterised by inflammation of the tonsils and the pharynx. However, on occasion, pharyngitis can also be associated with additional downstream streptococcal disease manifestations, such as the development of scarlet fever in children. This disease, characterised by fever, skin rash and ‘strawberry tongue’ results from the expression of superantigens by the *S. pyogenes* strain causing pharyngitis (18, 19). Whilst historical infections were associated with mortality, intervention with antibiotics now results in very few fatalities (18). Repeated streptococcal pharyngitis can also be associated with autoimmune sequelae described below.

In addition to pharyngitis, *S. pyogenes* causes superficial skin infections, with at least 111 million cases of pyoderma reported per year (8). Spread through skin contact, the incidence of pyoderma is affected by both personal hygiene and climate; as such pyoderma disproportionately affects patients in low socio-economic settings and equatorial or tropical climates (18). Indeed, the highest global prevalence of *S. pyogenes* related impetigo is found across the Pacific islands and in aboriginal Australians (8).

Autoimmune sequelae

One of the greatest burdens of streptococcal disease is attributable to autoimmune sequelae of streptococcal infection, in particular acute rheumatic fever (ARF) and associated rheumatic heart disease (RHD). Indeed, ARF is the leading cause of child heart disease globally, with 300,000-350,000 cases in children aged 5-14 annually (18). ARF is highly associated with *S.*

pyogenes pharyngitis in temperate regions and, although pathogenesis is not understood, has been thought to be caused by antibodies raised against the *S. pyogenes* surface M protein, cross reacting with host proteins found in the heart and skeletal system. This is hypothesised to result in inflammation of joints and inflammation of the heart which can subsequently lead to damage of cardiac structures and ultimately mortality, with 233,000 deaths attributed to RHD annually (8). Other autoimmune sequelae such as acute post-streptococcal glomerulonephritis (PSGN) also contribute to global burden. While the pathophysiology of PSGN is not entirely elucidated, it is likely linked to auto-reactive *S. pyogenes* antibody deposition in the kidneys, and can lead to kidney impairment and failure with a mortality rate of 0.02-0.4/100,000 cases (18).

Invasive disease

A less common manifestation of *S. pyogenes* infection is invasive disease, the dissemination of bacteria to tissue sites that are usually sterile. These infections occur after *S. pyogenes* breaches the epithelial barrier and are usually associated with penetrating soft tissue and muscle infections such as cellulitis and necrotizing fasciitis / myositis, as well as streptococcal toxic shock syndrome and bacterial sepsis, although infections with no portal of entry are recognised. Whilst the incidence of invasive streptococcal disease is low, approximately 3.5/100,000 people per year (20), the disease prognosis is poor. Indeed, despite antibiotic intervention invasive streptococcal disease carries a 20% mortality within the first 7 days of infection (9). The incidence of invasive disease has however been increasing since the 1980s, with invasive disease now resulting in at least 163,000 annual deaths and 663,000 new cases per year globally (8, 21).

1.1.2 Necrotizing fasciitis

One manifestation of invasive disease is necrotizing fasciitis, a serious and life-threatening condition characterised by the destruction of subcutaneous, muscle and connective tissues by the rapid and uncontrolled spread of a pathogen through fascial planes. The condition is classified into 4 categories based on the etiological agent causing disease, a polymicrobial infection, a Gram-positive, Gram-negative, or fungal infection. Incidence of necrotizing fasciitis caused by *S. pyogenes* varies by country, however can range from 0.08 - 0.6 per 100,000 population (22), with 500 cases per year estimated in the United Kingdom (23). Despite treatment, in high income countries the mortality rate of necrotizing fasciitis remains high, approximately 32% (24).

Disease presentation may begin with pain and swelling at the site of a skin breach; however, an open wound is not a prerequisite for disease manifestation, with 20% of patients presenting with no visible wound and an additional 20% presenting with a history of blunt trauma (25). Blistering and discolouration of the skin may follow with purple and black colouration seen in 32% of patients (26); importantly however, these skin presentations are a sign of late disease. Necrotizing fasciitis may present earlier with systemic manifestations analogous to sepsis indicators, with tachycardia, pyrexia, and hypotension usually apparent. The cardinal symptom, however, is pain.

Early intervention is vital, with delayed treatment highly associated with increased mortality (27). Surgical intervention, including substantial tissue debridement and amputation is often required due to the aggressive nature of the infection, alongside empirical antibiotic treatment. Intravenous immune globulin (IVIG), a therapeutic treatment consisting of pooled human immunoglobulins from over 1000 donors, is also administered in some settings. Although primarily administered as a neutralising agent for *S. pyogenes* pyrogenic exotoxins in

streptococcal toxic shock syndrome (STSS), IVIG has been shown in a systematic review and metaanalysis to reduce patient mortality to 15.7% (28). Hyperbaric oxygen therapy has been used in some settings as an adjunctive treatment for necrotizing fasciitis, although a consensus on efficacy has not been reached (29). The pathogenesis of streptococcal necrotizing fasciitis is varied with several streptococcal virulence factors contributing to the proteolysis of, and dissemination through host tissues. Of relevance to the current work, it has been demonstrated that a single virulence factor alone, the *Streptococcus pyogenes* cell envelope protease (SpyCEP), can drive lethal invasive disease, and a necrotizing phenotype through heterologous expression in an otherwise avirulent bacterium (30).

1.1.3 Clinical treatment

There are currently no licenced *S. pyogenes* vaccines, as such, infections are largely controlled by antibiotic treatment. *S. pyogenes* is extremely sensitive to penicillin, with only very rare and sporadic cases of reduced penicillin susceptibility reported in China, India, Japan, and the US (31-34). As such, penicillin, and β -lactam derivatives are the first line treatment for *S. pyogenes* infection. Patients with penicillin allergy are alternatively prescribed macrolides, such as erythromycin, or, in cases of erythromycin resistance, clindamycin. Clindamycin is also prescribed as an adjuvant to penicillin in cases of invasive skin and soft tissue infection due to its suppressive effects on known streptococcal virulence factors (35-38). Macrolides, however, do not exhibit the same continuity of sensitivity as seen in β -lactams. Resistance to macrolides is apparent in 5-20% of *S. pyogenes* isolates, with China reporting resistance in up to 95% of strains (39). Treatment in severe invasive disease, such as necrotizing fasciitis, is not limited to antibiotic administration and includes direct surgical intervention and adjuvant therapies such as IVIG and, frequently, intensive care support in ~20% of cases.

1.1.4 *S. pyogenes emm* type classification

The largest and most universally adopted method for *S. pyogenes* classification is by sequencing of the *emm* gene, which encodes the variable surface antigen and virulence factor, M protein - specifically its hypervariable N-terminus. There are currently over 200 unique *emm* types globally, however their distribution is not homogeneous and *emm* type varies by country and region (40). Across Europe the five most common *emm* types M1, M28, M3, M89 and M87 account for over 50% of sequenced *S. pyogenes* strains, albeit that most strains that are genotyped are from invasive disease cases (41). Globally the most prevalent serotypes are M1, M12, M28, M3, M4 (40).

There is an association between *emm* genotypes, disease manifestation, and tissue tropism. In fact, *emm* typing can loosely group *S. pyogenes* into skin or throat ‘specialists’ (42). However, within these two broad categories there are a number of *emm* types that are more associated with specific disease; M1, M3, M11, M12, M28 are responsible for 50% of invasive *S. pyogenes* infections for example, with M1 and M3 the most associated with increased mortality (20, 43) and M1 highly associated with necrotizing fasciitis (44). Equally, M12 is more associated with pharyngitis and M3 with streptococcal toxic shock syndrome (41, 45). Importantly, *S. pyogenes emm* types do not only differ by the hypervariable region of the M protein; there are also a number of further genetic and proteomic differences between strains which help contribute to their disease profiles. M18 isolates, for example, make a significantly increased amount of hyaluronic capsule, that leads to resistance to neutrophil-mediated phagocytosis and an increased virulence profile relative to unencapsulated mutants (46, 47).

Since the 1980s, a dominant *S. pyogenes* lineage has emerged, designated M1T1. This clone has spread globally and is associated with the increased invasive disease seen worldwide. Genetic analysis of the clone has identified a 5% divergence from the closest related M1 isolate

(48). This divergence introduced a number of novel features which contribute to enhanced pathogenesis. These include the acquisition of bacteriophage-associated virulence factors Sda1 and the superantigen SpeA2, as well as a recombination event which enhanced production of two co-expressed streptococcal toxins, SLO and NADase. Multiple works have established that the emergence of the M1T1 clone is associated with an increase in streptococcal pathogenesis and invasive disease. The M1T1 clone is now identified as the leading cause of invasive *S. pyogenes* infections globally, and as such it is used as a paragon for invasive streptococcal research (21, 48-51).

1.2 The human innate immune response to *S. pyogenes*

Neutrophils

Neutrophils are key leukocytes in the innate immune response to early infection and are often regarded as the first line of defence against invading pathogens. With 10^{11} neutrophils produced daily, they are the most abundant white blood cell and exhibit multiple vital cellular functions (52, 53). Neutrophils can act directly to protect against pathogens e.g., via phagocytosis, cytotoxic degranulation, release of extracellular traps, but can also act indirectly, by modulation of the inflammatory response. These multi-faceted anti-pathogenic functions are crucial to controlling infection and as such, it is not a surprise that many pathogens, including *S. pyogenes*, have evolved mechanisms to subvert this cell type for increased *in vivo* survival.

CXCL8 and neutrophil chemotaxis

Neutrophils are actively recruited to infected or damaged tissue via the process of chemotaxis, a mechanism of directed cell migration in response to a stimulus. One major family of signalling proteins which induce such neutrophil migration are chemotactic cytokines, or chemokines. These signalling molecules are characterised into 2 major subsets based on two conserved N-terminal cysteine residues; CC, (where the cysteine residues are adjacent) and

CXC (where X represents an amino acid interspersed between the two cysteine residues). Chemokines are directly responsible for the migration of a variety of immune cells and stimulation of the inflammatory response.

One subset of the CXC group, further characterised by a glutamate, leucine, arginine (ELR) motif upstream of the CXC motif, specifically acts upon neutrophils and is comprised of the chemokines CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7 and exemplified by the most well-characterised molecule, CXCL8. Host cells at the site of an infection, such as epithelial cells or tissue resident macrophages, release chemokines such as CXCL8 into the extracellular milieu. CXCL8 is actively taken up at the abluminal (outer) surface of blood vessels, transported across endothelial cells, and thereafter presented on the luminal surface via binding of the C-terminal chemokine domain to the endothelial cell surface glycosaminoglycans (GAGs) (54). From this position, bound to the endothelial lumen, CXCL8 is able to interact directly with neutrophils in circulation, recruiting them from the blood into the infected tissue.

Initially, ligands on circulating neutrophils transiently bind selectins on the endothelial surface, this permits a slow neutrophil 'rolling' movement along the luminal surface. Upon neutrophil interaction with endothelial-bound CXCL8, neutrophil surface integrins LFA-1 and Mac-1, bind to endothelial surface molecules ICAM-1 and VCAM-1 and halt neutrophil rolling. Once neutrophils are no longer rolling and are bound to the endothelial surface, they are able to transmigrate the endothelium through intracellular junctions into the interstitial space (55, 56). Once there, neutrophil pseudopodia, extended into the surrounding environment, can bind extracellular chemoattractants, such as CXCL8, causing downstream cellular actin nucleation and polymerisation, driving neutrophil movement in the direction of increased CXCL8 concentration (57, 58).

The neutrophil interaction with external ELR⁺ CXC chemokines is mediated by the surface G protein-coupled receptors CXCR1 and CXCR2. These receptors, whilst not solely expressed on neutrophils, are highly abundant on the neutrophil cell surface with approximately 75,000 receptors per cell (59). In contrast to chemokine GAG binding described above, which requires the chemokine C-terminal, it has been shown that the globular chemokine N-terminal domain is vital for mediating receptor binding, with the ELR motif additionally conferring CXCR1 and CXCR2-specific binding (60-63). Not all members of the ELR⁺ chemokine family however bind both the CXCR1 and CXCR2 receptor. CXCL1, CXCL5, CXCL6, CXCL7 and CXCL8 do demonstrate cross receptor binding, while CXCL2 and CXCL3 only bind CXCR2 (64-66).

CXCR1 and CXCR2 receptors share 77% homology, and both elicit neutrophil chemotaxis, however downstream signalling cascades from receptor activation vary between the receptors. Neutrophil interaction with CXCL8 is not limited to directing chemotaxis, CXCL8 signalling can induce additional anti-pathogenic mechanisms. Indeed, CXCR1 activation is highly associated with induction of the neutrophil oxidative burst and release of reactive oxygen species, and CXCR2 activation with the release of proteases, namely elastase and matrix metalloproteinase (67).

1.2.1 Complement based chemotaxis

CXC chemokines are not the sole molecules to elicit neutrophil chemotaxis; both complement factors and anaphylatoxins C3a and C5a have been associated with neutrophil migration (56, 68, 69). These proteins are both by-products of the complement cascade, an important function of the innate immune system, comprising an array of small proteins and proteases which help promote clearance of bacterial pathogens (56). C3a and C5a are both products of enzymatic

cleavage of parent molecules C3 and C5 and demonstrate differential activity. C5a is recognised as the more stable and biologically potent chemoattractant and acts an 'end target' stimulus (56, 58). In contrast, the ability of C3a to function as a neutrophil chemoattractant is more contentious in the literature, with publications in disagreement (68-71). Both molecules however do exhibit functions additional to chemotaxis; inducing mast cells to release histamines and TNF α , increasing the permeability of blood vessels and increasing smooth muscle contraction (56). These functions help the local inflammatory response and help phagocytes reach the site of infection.

1.2.2 LL-37 mediated chemotaxis

An additional molecule which has been shown to elicit neutrophil chemotaxis is LL-37. LL-37 is a small antimicrobial peptide utilised by the innate immune system to defend against pathogen invasion. LL-37 is produced by multiple cell types including epithelial cells lining the respiratory tract, skin, and multiple immune cells; macrophages, neutrophils, and natural killer cells (72). It has a multifaceted role in immunomodulation and has been shown to promote chemotaxis of neutrophils and eosinophils via the formyl-peptide receptor FPR2 (73, 74). However, LL-37 is traditionally recognised as the only member of the human cathelicidin family and for its direct antimicrobial activity. LL-37 is produced via cleavage of a precursor molecule (hCAP18) by kallikreins in keratinocytes or the serine protease, proteinase 3, in neutrophils to release LL-37 as a small 4.5 kDa α -helix (73). The subsequent antimicrobial action is attributed to LL-37 membrane binding, modulation and toroidal forces exerted on the lipid bilayer (72). LL-37 is increasingly described as an extremely pleiotropic molecule across a wide range of immune functions, acting as an antibacterial, antiviral, antifungal, chemoattractant, stimulator and modulator of cytokine releases. This breadth of action is likely

mediated through interaction with multiple cell receptors and in different biological niches (73).

1.3 Virulence of *Streptococcus pyogenes*

As with all human pathogens *S. pyogenes* utilises a vast number of proteins which contribute to both its *in vivo* survival and pathogenesis. These effector proteins encompass a broad range of important cellular functions, from epithelial cell adhesion and invasion, nutrient acquisition, and environmental survival. Importantly, however, *S. pyogenes* also uses many of these proteins for interaction with the host human immune system, in order to facilitate bacterial persistence and dissemination.

1.3.1 Subversion of the innate immune system

S. pyogenes is an obligate human pathogen and as such has evolved numerous mechanisms of evading the host immune response to infection. Mechanisms are multi-faceted, however typically involve direct subversion of immune factors or modulation of the immune response. Neutrophils, being the primary immune cell at the site of infection, are strongly targeted by *S. pyogenes*, which has developed multiple mechanisms of disrupting neutrophil action and opsonophagocytosis.

A primary virulence factor for immune evasion is the streptococcal M protein. The serotype dependent protein is foremost a ubiquitously expressed cell surface protein and vital streptococcal virulence factor. It has multiple roles in neutrophil evasion; different M proteins can bind host factors such as C4b, factor H, fibrinogen, and plasminogen to limit the deposition of complement and, theoretically, subsequent complement mediated phagocytosis (75-80). However, M protein can also inhibit the correct maturation of the neutrophil phagosome (81).

Another phagocytic defence is the streptococcal capsule. Most, but not all, genotypes of *S. pyogenes* are encompassed by a hyaluronic acid capsule, structurally identical to hyaluronic acid found in humans, enabling the camouflage of bacteria by molecular mimicry, and promoting resistance to phagocytosis (50, 82-84). The physical layer of capsule additionally blocks several surface epitopes from antibody-based recognition, as well as blocking the deposition of complement and increasing the ability of *S. pyogenes* to survive in neutrophil NETs (83, 85, 86).

S. pyogenes further encodes two distinct cell surface peptidases which cleave chemotactic factors responsible for the migration of neutrophils to the site of infection. The first identified protease was C5a peptidase which actively cleaves both C5a and C3a (87, 88), thus reducing the recruitment of neutrophils and limiting the response to local infection. The second protease identified was SpyCEP, which cleaves the entire family of ELR⁺ CXC chemokines, causing a subsequent reduction in neutrophil migration. The activity of SpyCEP is the main focus of this thesis and will be described in greater detail in section 1.5.

S. pyogenes also releases factors which display direct action against the neutrophil. Streptolysin O (SLO) is an extracellular cytolysin released by *S. pyogenes* which helps facilitate tissue invasion and neutrophil death. The oligomerisation of SLO enables the formation of large pores in host membranes, both in epithelial cells and leukocytes; as such, cells lose membrane integrity and cell death is induced (89, 90). This pore-forming action leads to direct killing of host cells but can additionally enable *S. pyogenes* to escape the endosome during cell invasion (91). *S. pyogenes* expression of NAD-glycohydrolase synergises with SLO to further increase cell damage. NAD-glycohydrolase is co-expressed with SLO and enters host cell through SLO dependent pores, subsequently depleting cellular energy stores through the depletion of NAD and enhancing cell death (92). SLO however, is not the only secreted toxin which lyses

neutrophils and host cells. Streptolysin S (SLS) also enables *S. pyogenes* to evade the innate immune system by lysing neutrophils, among other cell types, and contributing to the pathogenicity of *S. pyogenes* (93, 94). Indeed, the broad lytic profile of SLS towards host cells has been associated with driving tissue necrosis during invasive disease (95).

These virulence factors, along with a plethora of others including immunoglobulin degrading proteins, extracellular proteases, secreted toxins, and host binding proteins, combine to form an impressive collection of mechanisms to aid *S. pyogenes* with its survival, pathogenesis, and immune evasion. However, it is no surprise that such an extensive and redundant repertoire is apparent in *S. pyogenes*; it is an obligate human pathogen and must stay one step ahead of the immune system for successful dissemination.

1.3.2 Regulation of virulence

S. pyogenes employs a vast arsenal of potent virulence factors and, as such, utilizes a wide range of regulatory systems to maintain homeostasis. There are at least thirty independent transcriptional regulators and thirteen two-component regulatory systems (TCS) responsible for controlling *S. pyogenes* gene expression (96). These regulators respond to a number of external stimuli; chemical, pH, host factors, to regulate and adapt to the microenvironments *S. pyogenes* encounters during its infection course.

One of the best characterised regulatory systems is the two-component control of virulence (CovR/S) system. This regulator consists of a membrane bound sensor kinase (CovS) responsible for sensing external stimuli and subsequently phosphorylating a downstream cytoplasmic response molecule (CovR). Post-phosphorylation, CovR can dimerise and bind to DNA promotor regions, regulating gene expression. The system additionally comprises an

accessory transmembrane pseudokinase, RocA, which interacts with CovS greatly increasing the phosphorylation of CovR (97). The CovR/S system is colloquially known as the streptococcal master regulator, due to its direct or indirect transcriptional control of approximately 15% of the *S. pyogenes* genome (98). This includes a wide range of virulence factors including; SpyCEP, Sda1, SpeB, SLO, SLS, Ska, EndoS, HA capsule (98-102), as well as a number of regulons involved environmental adaptation (18). Furthermore, the CovR/S system regulates an additional 18 stand-alone transcriptional regulators (96) - increasing the scope of its downstream control.

Under ‘normal’ cellular conditions the CovR/S system represses the expression of many of the streptococcal virulence factors under its control. However, in response to external stimuli, such as the human anti-microbial peptide LL-37, or a reduced extracellular Mg^{2+} concentration (103, 104), the repression is released allowing for the upregulation of vital proteins for bacterial survival. The mechanism is yet to be fully elucidated however, it is currently hypothesised that these external stimuli inhibit the interaction between the membrane bound CovS and RocA proteins, resulting in a drastic reduction in cytoplasmic phosphorylated CovR and so transcription of virulence factors is de-repressed (97).

Importantly, it has been shown through animal passage and *in vitro* observations of invasive strains, that *S. pyogenes* isolates with mutations in the CovR/S system are highly associated with more invasive disease and hypervirulence. Indeed, over 15% of invasive *S. pyogenes* isolates from clinical infection display mutations in either CovR or CovS (105-107). Previous work has hypothesised that these CovR/S mutants are likely positively selected for during invasive infection, due to upregulation of critical virulence factors involved with bacterial dissemination and evasion of the innate immune system (105, 108-111).

1.4 Vaccines

1.4.1 M protein-based vaccines

There is a worldwide demand for a safe and effective vaccine against *S. pyogenes*. Globally, severe *S. pyogenes* infections kill 517,000 people per year with the greatest burden attributed to acute rheumatic fever and rheumatic heart disease (8). Despite decades of vaccine research dating back to the early 1920's (112) there are currently no licenced vaccines for *S. pyogenes*. Historically, antigen discovery work has largely focused on the M protein; a coiled α -helical protein incorporated into the bacterial cell wall and required for serotyping of *S. pyogenes*. Unfortunately, due to safety concerns arising from human trials in the 1960s, where 3 patients progressed to develop acute rheumatic fever within 2 years of vaccination (113, 114), the US Food and Drug Administration (FDA) banned the administration of *S. pyogenes* products in human trials. This ultimately stymied clinical vaccine development until the ban was lifted in 2006. Since then, there has been a resurgence in *S. pyogenes* vaccine research and several immunogenic cell wall-exposed antigens have been assessed in non- M protein-based vaccine studies. Two of the leading candidates are the cell envelope proteinases C5a peptidase and SpyCEP, both ubiquitous and highly conserved antigens which facilitate *S. pyogenes* pathogenesis and disease progression.

1.4.2 Non-M protein vaccines

Since its identification as a key streptococcal virulence factor and immuno-modulatory protein responsible for the cleavage of neutrophil specific chemotactic cytokines by Edwards *et al* in 2005 (115), SpyCEP has been included in a number of GAS vaccine designs. It was initially identified as a potentially protective antigen during a cell surface proteomic mass spectrometry screen for potential vaccine candidates (116). The authors subsequently demonstrated that

intraperitoneal immunisation with recombinant SpyCEP adjuvanted with Freund's conferred a 70% survival rate to mice challenged with a 90 % lethal intranasal dose of an M23 *S. pyogenes*.

Subsequent work demonstrated that immunisation with SpyCEP in Freund's adjuvant induced SpyCEP specific serum IgG with neutralizing activity (117). This immunisation did not reduce bacterial burden from the site of infection however it conferred enhanced protection against *S. pyogenes* dissemination in both an intranasal and intramuscular infection model. Indeed, immunised mice showed reduced serum IL-6 levels and reduced bacterial recovery from the spleen and liver. It was additionally demonstrated that SpyCEP had the potential to induce cross-species protection; with immunisation also reducing bacterial systemic spread following a *S. equi* intramuscular infection in mice.

SpyCEP was again highlighted as an important vaccine candidate in a systematic search for 'conserved, highly expressed, well exposed, and *in vivo* immunogenic surface proteins' (118). The proteins identified in this screen resulted in a multi-antigen preparation combining SpyCEP with 2 additional proteins, SpyAD and Streptolysin O. This 'combo' vaccine demonstrated cross serotype (M1, M6, M12, M23) protection *in vivo*, conferring 50-80% survival following intranasal and intramuscular challenge and significantly reduced bacterial growth in a subcutaneous air pouch infection model. Immunisation with the combo vaccine was shown to induce SpyCEP specific antibodies capable of neutralizing CXCL8 cleavage.

SpyCEP immunisation has additionally been demonstrated to reduce the signal of a bioluminescent reporter, M75, *S. pyogenes* strain after an intranasal challenge and elicit a SpyCEP specific serum IgG response (119). However, the authors note at the end of the challenge there was no significant difference in nasopharyngeal CFU between the SpyCEP immunised and control mice.

Although SpyCEP was initially identified as a potential vaccine candidate through so-called ‘reverse vaccinology’ (116), SpyCEP was also identified by an immunoproteomic screen using a highly protective preparation of affinity-purified pooled human IVIG that enabled bacterial clearance in both mice and human blood (120). SpyCEP alone was as effective as a 7-component vaccine (Spy7) when evaluated in murine invasive M1 and M3 infections (121).

SpyCEP has also been used to augment the efficacy of M protein-based vaccines, in relation to highly invasive strains. Pandey *et al* describe an M protein based, minimal B cell epitope vaccine conjugated to the diphtheria toxoid named J8-DT (122). This vaccine proved effective against pyoderma and following bacteraemia, however, was ineffective against *S. pyogenes* strains that had undergone mutation in the two-component virulence regulation system, CovR/S. The subsequent addition of a SpyCEP polypeptide into their J8-DT vaccine displayed effective protection against these hypervirulent strains (123). The vaccine was further refined to contain only a minimal epitope of SpyCEP that was required to elicit protection against virulent CovR/S mutants (124).

Whilst there is currently no vaccine in human trials containing either C5a peptidase or SpyCEP, one vaccine has been assessed in a non-human primate pharyngeal model of infection (125). Intramuscular immunisation of the combo 5 vaccine composed of C5a peptidase, SpyCEP, Arginine deiminase, streptolysin O and trigger factor, elicited a robust antigen specific antibody response in Rhesus macaques and reduced both pharyngitis and tonsillitis following an M1 intranasal challenge. Importantly, the authors showed that antibodies raised against SpyCEP during immunisation do not confer protection by a traditional opsonophagocytic mechanism. Instead, they provide protection by inhibition of SpyCEP activity. An attempt was made to increase the potency and immunogenicity of the combo 5 vaccine by conjugating 4 of the recombinant proteins to a Toll-like receptor (TLR) 2 antagonist, fibroblast-stimulating

lipopeptide -1 (FL-1). This resulted in up to 10-fold increases in IgG specific antibody responses compared to non-conjugated antigens, however these values were similar to serum antibody levels when originally adjuvanted with alum (126).

These vaccine data strongly highlight the potential of targeting SpyCEP in *S. pyogenes* vaccines and demonstrate that targeting SpyCEP activity is an effective method of conferring protection against invasive disease.

1.5 The *Streptococcus pyogenes* cell envelope protease SpyCEP

1.5.1 SpyCEP identification

Clinical observations have highlighted that patients presenting with lethal invasive *S. pyogenes* infection demonstrate reduced levels of neutrophils at infection sites compared with other Gram-positive bacterial infections (115, 127). Indeed, post debridement analysis of tissues from both *S. pyogenes*-associated necrotizing fasciitis and myonecrotic infections show a striking paucity of neutrophils infiltrating the infected tissue, particularly in patients who do not survive (115, 127). As such, an interaction between *S. pyogenes* and CXCL8, a central chemokine involved in early neutrophil recruitment during infection was initially hypothesised. This hypothesis was supported by the paradoxical observation that human PBMCs, when stimulated by *S. pyogenes* supernatants, produced significantly lower levels of CXCL8 than expected, despite containing multiple products shown to potently induce CXCL8 when expressed recombinantly (128, 129). In 2005, Edwards *et al* subsequently identified the *S. pyogenes* cell envelope protease (SpyCEP), an enzyme which specifically cleaves CXCL8 resulting in both reduced neutrophil activation and migration (115).

1.5.2 SpyCEP structure

SpyCEP is expressed as a 1647 amino acid, 180 kDa, cell envelope protease which is found both anchored in the streptococcal cell wall and secreted into the cellular environment – a release which is not currently understood. SpyCEP is classified as a subtilisin-like serine protease and categorised into the SB sub clan of the S8 protease family as defined by the MEROPS data base of proteolytic enzymes (130). Structurally, it shares a high degree of sequence identity with several other homologous cell envelope associated proteases from both pathogenic Gram-positive bacteria and lactic acid bacteria - most notably the streptococcal C5a peptidase. Initial structure function studies to identify and characterise domains within SpyCEP

were dependent upon direct comparison to annotations of these homologous proteins. However, recent crystallization studies have solved the SpyCEP structure to 2.8 and 2.2 Å resolutions respectively (131, 132), allowing for a greater resolution and characterisation of the 9 specific domains of SpyCEP.

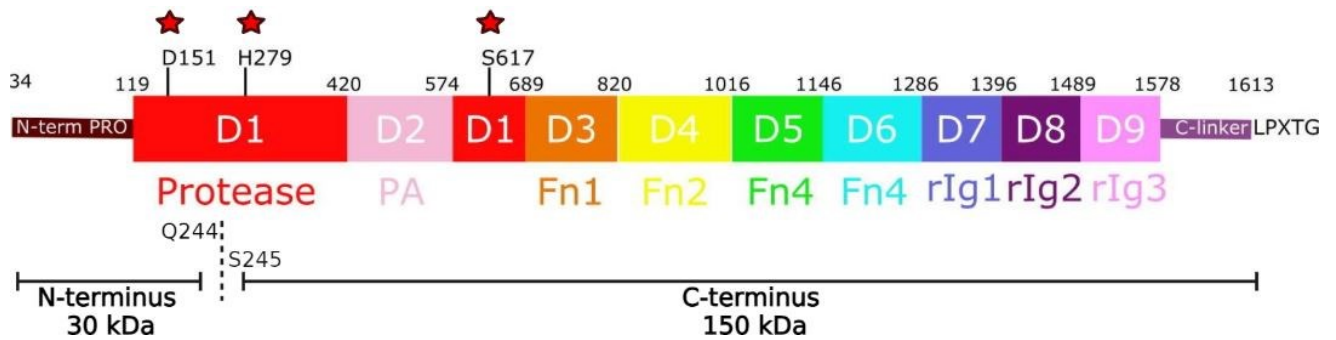


Figure 1-1. Structural schematic of SpyCEP.

Schematic of the domain architecture of SpyCEP. From left to right – N-terminal pro domain. D1, the protease domain containing the catalytic triad required for enzyme activity, D151, H279, S617 (red stars). D2, the protease associated domain, inserted within the protease domain. D3-6, four fibronectin-like domains. D7-9, three reverse immunoglobulin like domains followed by a C-linker and LPXTG motif for cell wall anchoring. Autocatalytic cleavage between Q244 and S245 (dotted line) generates two separate polypeptides, a 30 kDa N-terminus and a 150 kDa C-terminus. Figure adapted from McKenna *et al* (132).

As typical for subtilisin-like serine proteases, SpyCEP is targeted to the cellular periphery and translocated across the cell membrane by an N-terminal signalling peptide (aa 1-33), a peptide which is subsequently cleaved during the maturation of SpyCEP (133, 134). A disordered N-terminal pro domain (aa 34-119) follows prior to the main protease domain (aa 119-618) and the inserted protease-associated (PA) domain (aa 420-574). The protease domain harbours the catalytic machinery required for enzyme activity, containing three vital residues comprising the catalytic triad, Aspartic acid 151, Histidine 279 and Serine 617. These residues are

characteristic of the S8 serine protease family and, following enzyme maturation, are situated in the SpyCEP active site. The function of the inserted PA domain is currently speculative. Structural studies highlight slight mobility in the domain and situate it directly above the enzyme active site. Coupled with evidence from other subtilisin PA domain studies, this suggests a possible role in additional substrate recognition and specificity (131, 132).

The protease domain additionally contains an autocatalytic cleavage site between position Q244 and S245. SpyCEP is unique in that it processes itself into two distinct polypeptides: a 30 kDa N-terminal polypeptide containing the D151 catalytic residue and a 150 kDa C-terminal polypeptide containing the H279 and S167 residues. These proteins subsequently non-covalently reassociate to form the full-length wildtype enzyme (135). Whilst this conformational change appears to be the dominant architecture *in vitro*; denaturing conditions liberate the two separate polypeptides. Mutational studies of the autocatalytic region have shown that self-processing is not necessary for enzyme activity. SpyCEP ‘locked’ as a single polypeptide effectively cleaves CXCL8 with efficacy comparable to wildtype enzyme (136).

Subsequent to the protease domain are four (fn1-4) fibronectin like-domains, the functions of which have not been fully elucidated. Structural analysis has determined that the 4 domains contain secondary structures which may help enable interdomain contacts and maintain correct domain orientation. It is also hypothesised that these may act as a ‘stalk’ to extend the protease domain away from the cell wall to increase proteolysis (132).

Following the 4 fibronectin like-domains are 3 reverse immunoglobulin like domains (rIg1-3). Whilst their SpyCEP-specific role has not been explicated, they are a common domain found in other Gram-positive pilin structures assembled in a sortase-dependent manner where they can play a role in host cell adhesion (132, 137). These domains are followed by a LPXTG motif which permits SpyCEP to anchor into the cell wall in a sortase-dependent manner (138).

1.5.3 SpyCEP function *in vitro*

Whilst CXCL8 was the first described SpyCEP substrate, subsequent studies have expanded the SpyCEP substrate repertoire to include the entire family of ELR⁺ CXC chemokines, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7 and CXCL8 (30, 115, 139, 140). The precise cleavage sites for CXCL1, CXCL2 and CXCL8 have been identified by mass spectrometry, between Q65-K66, K60-K61 and Q59-R60 respectively (30, 115). Although the primary sequence of these cleavage sites differs between substrates, they maintain a high degree of homology in their secondary structure. In all cases, SpyCEP cleaves immediately upstream of the chemokine C-terminal α -helix, which is subsequently released post-cleavage, in the case of CXCL8 as a 13 amino acid product. Whilst this cleavage does leave the chemokine N-terminal largely intact, liberation of the C-terminus reduces the CXCR1/CXCR2 receptor binding affinity and activation in addition to ablating CXCL8 translocation to the endothelial lumen and GAG binding (54, 139). As such, the SpyCEP mediated C-terminal cleavage results in a reduction of CXCR1 and CXCR2 mediated neutrophil chemotaxis and subsequently a chronic dearth of infiltrating neutrophils to the site of *S. pyogenes* infection (115, 140).

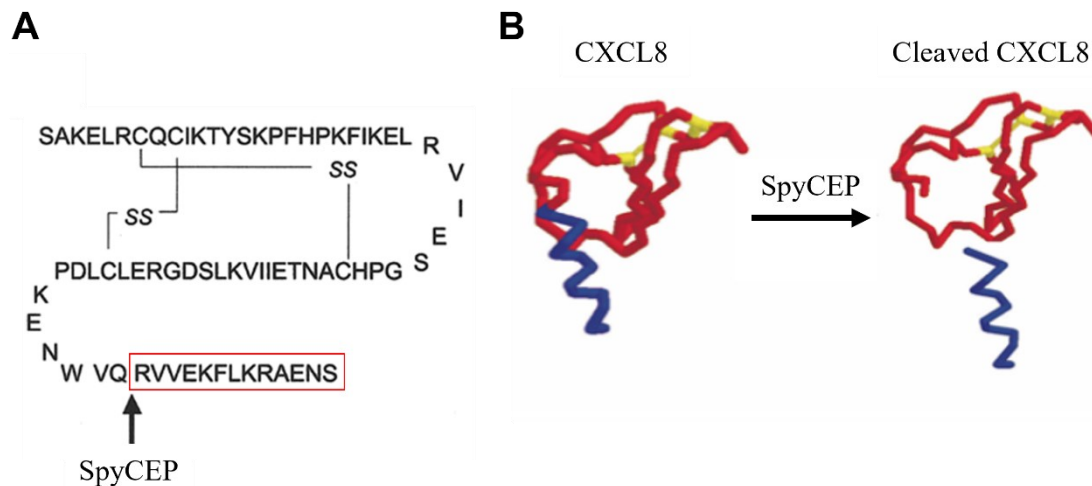


Figure 1-2. The SpyCEP cleavage site of human CXCL8.

The structural sequence (A) and structural diagram (B) highlighting the SpyCEP cleavage site of human CXCL8. The 13 amino acid C-terminal α -helix RVVEKFLKRAENS is released post cleavage.

Figure adapted from Edwards *et al* (115).

Interestingly, the first non-CXC chemokine substrate for SpyCEP has recently been described. Work has demonstrated that SpyCEP can cleave the human cathelicidin LL-37 (141). This cleavage ablates LL-37 induced neutrophil chemotaxis however retains LL-37 antimicrobial action (141). There is a degree of redundancy in LL-37 inactivation, as two additional streptococcal virulence factors, SpeB and SIC, have also been shown to inactivate the antimicrobial peptide (142-144). SpyCEP activity against LL-37 may be explained by the high degree of structural similarity between the chemokine C-terminal α -helix released upon SpyCEP cleavage and the C-terminal alpha helix structure of LL-37.

Due to the pleiotropic nature of CXC chemokines, downstream effects of chemokine inactivation are not limited to neutrophil chemotaxis. SpyCEP has been demonstrated to reduce CXCL1, CXCL6 and CXCL8-based neutrophil activation, production of neutrophil reactive oxygen species and bacterial killing by neutrophil extracellular trap formation (115, 140, 145).

1.5.4 Association of SpyCEP with streptococcal disease

SpyCEP directly inactivates chemokines, signalling molecules that are integral to the correct function of leukocytes of the innate immune system. As such, SpyCEP expression can alter the pathogenesis of *S. pyogenes*. Indeed, studies using clinical isolates and patient sera show that *S. pyogenes* SpyCEP transcription and CXCL8 cleavage activity are associated with invasive disease severity and disease outcome (146).

SpyCEP expression is under direct negative repression by the two component CovR/S regulatory system (117, 145). Previous work has demonstrated that *S. pyogenes* isolates with mutations in this regulatory system have markedly upregulated SpyCEP expression, in excess of a 100-fold increase in transcription (111, 146). Increased expression of SpyCEP translates to a measurable *in vivo* phenotype, with SpyCEP expression significantly associated with both increased bacterial dissemination from the site of streptococcal infection, increased resistance to bacterial clearance, and invasive disease (30, 111, 140, 145, 147). Importantly, the heterologous expression of SpyCEP in an otherwise avirulent *Lactococcus lactis* strain has successfully driven lethal necrotizing infection *in vivo* (30). While SpyCEP expression helps to drive invasive infection, bacterial dissemination, and confers resistance to neutrophil influx, SpyCEP expression may be detrimental to early non-invasive infection. SpyCEP expression is associated with decreased binding and invasion of epithelial and endothelial cells, in addition to reducing biofilm formation (148).

As described in section 1.3.2, the CovR/S system regulates up to 15% of the streptococcal genome. Transient modulation of this system in response to external stimuli is vital for bacterial proliferation in different niches during streptococcal infection. Data indicates that over expression of SpyCEP during nasopharyngeal carriage or early pharyngeal infection is

detrimental and as such, tight repression by the CovR/S system is necessary. If the streptococcal infection breaks the epithelial barrier and initiates an invasive infection it is likely the CovR repression of SpyCEP is modulated to allow for an increased chance of immune system evasion.

1.5.5 SpyCEP enzymatic kinetic activity

Prior to this thesis almost no work has been done to quantify the catalytic properties of SpyCEP, largely due to issues arising with recombinant protein expression, and the cumbersome techniques required to quantify natural substrate cleavage. Indeed, most work has focused on disease association and the impact of SpyCEP on *S. pyogenes* pathogenesis. However, previous work has demonstrated by SDS-PAGE analysis that differential cleavage rates are apparent across the entire ELR⁺ CXC substrate range (139). Using a fixed concentration of whole bacteria to cleave chemokine, work demonstrated that that all chemokines are not cleaved equally. Indeed, CXCL1, CXCL2, CXCL3, CXCL7 and CXCL8 were cleaved to completion, whereas CXCL5 and CXCL6 were only partially cleaved over the same time course (139). While these data did not specifically quantify SpyCEP cleavage rates, they do highlight the likely differential binding and cleavage affinities SpyCEP has for each substrate. Preliminary data from this thesis has further built upon these initial findings (132); a greater knowledge of enzyme kinetics is important for understanding the proteolysis of a substrate by an enzyme. Increased biochemical understanding of the interaction between SpyCEP and CXCL8 would facilitate improved protease inhibitor design.

Thesis rationale

Due to its highly conserved nature and its multifaceted role in *S. pyogenes* infection, SpyCEP has been included in several recent *S. pyogenes* vaccine designs. Immunisation with SpyCEP has been shown to successfully elicit a SpyCEP-specific neutralising antibody response, providing protection against systemic bacterial dissemination and reducing disease severity in intramuscular and skin infection models (117, 118, 123, 124, 149). Recent results from a non-human primate infection model (125), where SpyCEP was utilised as a component of a combination vaccine, demonstrated anti-SpyCEP antibodies do not provide protection by opsonisation. Rather, they inhibit SpyCEP cleavage activity against human chemokines. These published data underline the potential of targeting SpyCEP therapeutically, especially in invasive infections where SpyCEP contributes strongly to driving disease pathogenesis and progression.

Project hypothesis

Where a bacterial virulence factor, such as SpyCEP is the driving force behind disease pathogenesis, that virulence factor is a promising candidate for therapeutic targeting.

2 Materials and methods

2.1 Materials

2.1.1 Bacterial isolates

Isolate no.	Species	Description and genetic nomenclature	Source
H292	<i>S. pyogenes</i>	Clinical isolate containing a CovR mutation - obtained from a lethal necrotizing fasciitis infection. (M81 CovR _{MT})	Turner <i>et al</i> (146)
H575	<i>S. pyogenes</i>	Isogenic <i>cepA</i> deletion mutant of H292 (M81 CovR _{MT} Δ <i>cepA</i>)	Kurupati <i>et al</i> (30)
H486	<i>L. lactis</i>	Wild type isolate, harbouring an empty pDEST control plasmid. Confers erythromycin resistance. (Lactis _{vector})	Zinkernagel <i>et al</i> (140)
H487	<i>L. lactis</i>	Isogenic complementation mutant of H486, harbouring a pDEST plasmid for the constitutive expression of <i>cepA</i> . Confers erythromycin resistance. (Lactis _{pcepA})	Zinkernagel <i>et al</i> (140)
H584	<i>S. pyogenes</i>	Clinical isolate, CovR/S wild type, from lethal puerperal infection (M1 CovR/S _{WT})	Turner <i>et al</i> (150)
H1317	<i>S. pyogenes</i>	Isogenic derivative of H292 with the C-terminal LPTXG anchor domain replaced with 6x – Histidine tag to permit soluble SpyCEP release and purification. (M81 CovR _{MT} released <i>cepA</i>)	Richard Lawrenson and Mark Reglinski (This thesis)
H1464	<i>S. pyogenes</i>	Isogenic <i>cepA</i> deletion mutant of H584 (M1 CovR/S _{WT} Δ <i>cepA</i>)	This thesis

H1565	<i>S. pyogenes</i>	Isogenic derivative of H584, resultant from a single <i>in vivo</i> passage, containing a CovR mutation (A-C at codon 65) (M1 CovR _{MT})	Lamb <i>et al</i> (151)
H1567	<i>S. pyogenes</i>	Isogenic <i>cepA</i> deletion mutant of H1565 (M1 CovR _{MT} Δ <i>cepA</i>)	This thesis

Table 2-1. List and origin of bacterial isolates utilised in this study.

2.1.2 Primers

Primer name	Primer sequence (5'- 3')	Primer use
EcoR1	ATAGCGAATTCAACAGACAAAAACCTCCGCT	Cloning of upstream region of homology for <i>cepA</i> deletion in <i>S. pyogenes</i> H584 and H1565
Kpn1	ATAGGGTACCTTGTGATTGCTCTTTTTCACGA	
Sal1	AGACGTCGACCAAAGCGCAAAGAGACAACA AA	Cloning of downstream region of homology for <i>cepA</i> deletion in <i>S. pyogenes</i> H584 and H1565
Pst1	ATAGCTGCAGAACTGGGGGTGTCTCCGT	
LCT0	CTTCGCCACAATCCGTATGA	Confirmation of successful gene deletion in <i>S. pyogenes</i> H584 and H1565
HYPO	TCGTATTTGTGCGAGACGCCT	

Table 2-2. Table of primers utilised in this study.

2.1.3 Ethical approval for human and animal samples

Use of fluid samples from patients with suspected infection, that had been routinely collected as part of clinical care, were surplus to diagnostic requirement, and would be otherwise discarded was approved by the West London Research Ethics Committee as a non-consented study ("Microbial Products in Infection", REC reference 06/Q0406/20). Samples were linked to routinely collected pathology and patient administration data then anonymised prior to any research being undertaken.

Human neutrophils from healthy donors were obtained after informed donor consent from an approved subcollection of the Imperial tissue bank (Wales REC3 17/WA/0161; local approval R12023).

All animal experiments were carried out in strict adherence to the Animal (Scientific Procedures) Act 1986 by a Home Office personal licence holder (I6E0E3928). All experiments were conducted on 6 – 10-week-old, female, C57BL/6 J mice (Charles River) in accordance with an approved United Kingdom Home Office Project Licence, P4C824899.

2.2 Methods - Chapter 3

2.2.1 Bacterial culture

S. pyogenes was cultured for 16 hours at 37°C in 5% CO₂ on either solid media; Columbia blood agar plates (CBA) (E&O Laboratories) or in liquid media, Todd-Hewitt broth (THB) (Oxoid).

L. lactis was cultured for 16 hours at 30°C under normal atmospheric conditions. For solid media *L. lactis* was cultured on 1.5% agar M17 (Oxoid) supplemented with 0.2 µm filtered glucose and lactose (ThermoFisher) to a final concentration of 0.5%, with 5 µg/ml erythromycin (ThermoFisher) added to maintain the pDEST plasmid when required. For liquid culture, the conditions remained consistent as above, albeit without 1.5% agar.

Commercially sourced XL10 - Gold *E. coli* strains (Agilent) were used for plasmid cloning and cultured in Luria-Bertani broth (LB) supplement with either 400 µg/ml Kanamycin (ThermoFisher) and 100 ug/ml ampicillin (ThermoFisher) as required at 37°C, 225 rpm under normal atmospheric conditions.

For growth curve analysis, bacteria were cultured for 16 hours and sub-cultured 1:10 into fresh media. *S. pyogenes* sub-cultures were grown under standard conditions above, with 1ml of culture removed every hour to measure optical density at 600_{nm}.

2.2.2 *S. pyogenes* genomic DNA extraction

(adapted from Pospiech *et al* (152))

S. pyogenes was cultured for 16 hours in 10 ml THB and centrifuged for 10 minutes at 2,500 xg. The bacterial pellet was re-suspended in 1 ml of sterile PBS (Gibco) and transferred to an eppendorf tube. 1 ml of bacterial suspension was incubated for 10 minutes at 37°C with 20 µl mutanolysin (10,000 U/ml) (Sigma Aldrich) and 20µl lysozyme (100 mg/ml) (Sigma Aldrich). Suspension was pelleted at 200 xg for 10 minutes and re-suspend in SET buffer (75 mM NaCl, 35 mM EDTA, 20 mM Tris-HCL, pH 7.5) supplemented with proteinase K (Sigma Aldrich) to a final concentration of 0.5 mg/ml and SDS (Sigma Aldrich) to 1% (w/v). Bacterial suspensions were incubated at 55°C for 2 hours with gentle agitation prior to the addition of 175 µl 5 M NaCl and 500 µl Chloroform (Sigma Aldrich) and further incubated at room temperature for 30 minutes. The mixture was centrifuged at 16,000 xg for 15 minutes and the aqueous layer transferred to new Eppendorf. An equal volume of isopropanol (Sigma Aldrich) was added to precipitate the genomic DNA before centrifugation at 16,000 xg for 5 minutes. Pelleted DNA was washed in 70 % ethanol (Sigma Aldrich) and re-pelleted at 16,000 xg for 5 minutes. Ethanol was removed and the pellet allowed to air dry for 20 minutes at room temperature. DNA was resuspended in 100 µl molecular grade water and quantified on a Nanodrop (Thermo Fischer).

2.2.3 *S. pyogenes* supernatant preparation and cell wall extraction

50 ml of 16-hour bacterial cultures were pelleted at 2,500 xg for 10 minutes. The supernatants were aspirated and passed through a 0.22 µm syringe filter (Sartorius) for sterilisation. Dependent on the experimental design, supernatants were concentrated through centrifugal filter units with a 10 kDa cut off (Amicon) to a maximum of 25X concentrate as per manufacturer's instructions. For preparation of the bacterial cell wall components, the bacterial

pellet was re-suspended in 1 ml of 30% (w/v) raffinose (Sigma Aldrich) and centrifuged for 5 minutes at 10,000 rpm. The resultant pellet was re-suspended in 1 ml of cell wall extraction buffer (10 mM Tris-HCL, 30% raffinose, 100 U/ml mutanolysin (Sigma Aldrich), 1 mg/ml lysozyme (Sigma Aldrich) and incubated for 3 hours at 37°C with gentle inversion. The bacterial suspension was centrifuged at 13,000 rpm for 10 minutes, the supernatant removed, filter sterilised through 0.22 µm filters (Sartorius) and dialysed into PBS at 4°C for 16 hours using 2 kDa MWCO cassettes (Thermo Fisher). Total protein concentrations were subsequently quantified by Nanodrop (Thermo Fisher).

2.2.4 Polymerase chain reaction (PCR)

PCR reactions were carried out using GoTaq Green Master Mix (Promega). 2 µl DNA template (≤ 250 ng) was added to 12.5 µl master mix, with 1 µl of upstream and 1 µl downstream primers (1 µM) and 13.5 µl molecular grade water (VWR). Reactions were added to a T100 Thermal Cycler (Bio-Rad) and DNA amplified using the following settings:

Initial denaturation: 95°C, 3 minutes	
Denaturation: 94°C, 30 seconds	
Annealing: 50-60°C, 1 minute	} 30 X
Extension: 72°C, 1 minutes 15 seconds – 10 minutes	
Final extension: 72°C, 5 – 11 minutes	
Hold: 4°C, indefinite	

For PCR products which were involved in downstream cloning, reactions were cleaned and purified using a PCR purification kit (Qiagen) Briefly, 5 volumes of buffer PB were added to 1 volume of PCR sample, loaded onto QIAquick column, and centrifuged at 17,900 xg for 1 minute. The column was washed with 750 µl of PE buffer and centrifuged twice at 17,900 xg for 1 minute. To elute DNA, 60 µl of molecular grade water was added to the column, allowed

to stand for 1 minute and then centrifuged at 17,900 xg for 1 minute. Eluted DNA was quantified using a Nanodrop (Thermo Fisher)

2.2.5 DNA electrophoresis

DNA was visualised and separated by size using standard agarose electrophoresis. 2 µl of DNA (~100 ng) was mixed with 6X DNA loading dye (NEB) and loaded onto 1% agarose (Sigma Aldrich) TAE gels stained with SYBR safe (Thermo Fisher) diluted 1:10,000. DNA was separated at 135 V for 40 minutes alongside a 1 Kb Plus DNA ladder (Invitrogen). Bands were visualised on a UV transilluminator or LiCOR odyssey FC.

2.2.6 DNA sequencing

DNA bands which required subsequent sequencing or downstream purification were excised from the agarose gel using a scalpel and cleaned using a QIAquick gel extraction kit (Qiagen). Briefly, the extracted agarose band was dissolved at 50°C for 10 minutes in 3 times its weight of QG buffer. After the gel slice was fully dissolved, an equal volume of isopropanol was added, and the mixture transferred to a QIAquick spin column (Qiagen). The column was centrifuged at 17,900 xg for 1 minute, washed with 500 µl QG buffer and centrifuged again. The column was equilibrated for 5 minutes in PE buffer and centrifuged twice at 17,900 xg for 1 minute. DNA was eluted off the column by the addition of 60 µl molecular water, 5-minute room temperature incubation and centrifugation at 17,900 xg for 1 minute. Purified DNA was subsequently quantified on a Nanodrop (Thermo Fisher).

2.2.7 Plasmid purification

Plasmids were extracted and purified from *E. coli* using Qiagen plasmid miniprep kits according to manufacturer's instructions. Briefly, *E. coli* was cultured for 16 hours and pelleted

at 6,800 xg for 3 minutes. Bacterial pellets were resuspended in 250 µl P1 buffer and mixed with an equal volume P2 buffer. The mixture was incubated for 5 minutes at room temperature with gentle inversion prior to the addition of 350 µl N3 buffer and mixed by thorough inversion. The suspension was centrifuged at 17,900 xg for 10 minutes, the supernatant removed, loaded onto QIAprep spin columns, and centrifuged at 17,900 xg for 1 minute. The columns were washed with 500 µl PB buffer, centrifuged at 17,900 xg for 1 minute, washed with 750 µl PE buffer and centrifuged for a further 2 minutes. Plasmids were eluted off the column by the addition of 60 µl molecular water, 5-minute room temperature incubation and centrifugation at 17,900 xg for 1 minute. Purified plasmids were subsequently quantified on a Nanodrop (Thermofischer).

2.2.8 Electroporation of *S. pyogenes*

S. pyogenes for electroporation was cultured overnight for 16 hours before sub-cultured (1:10) in 100 ml fresh pre-warmed THB supplemented with hyaluronidase (Sigma Aldrich) to 30 ug/ml final concentration. *S. pyogenes* was grown until an OD 600_{nm} of between 0.15-0.175 and centrifuged at 2500 xg for 10 minutes. Supernatant was removed and bacterial pellet was resuspended in 10 ml ice cold electroporation media (1 mM MgCl₂, 272 mM glucose, pH 6.5). Suspension was pelleted again at 4°C, 2500 xg for 10 minutes and washed again in 10ml ice cold electroporation media. Bacteria were re-pelleted and resuspended in 750 µl ice cold electroporation media (bacteria were now competent). 5 µl plasmid DNA (minimum 1.5 µg) were added to 75 µl competent cells and transferred to pre-chilled 1 mm electroporation cuvette (Thermofischer) and electroporated at 1 KV, 150 Ω, 50 µF with a time constant of 3.0-4.5 seconds. 1 ml of pre warmed THB was added to the cuvette and the entire volume then transferred to 9 ml pre-warmed THB. Bacteria were cultured for 3 hours at 37°C, 5% CO₂ before pelleted at 2500 xg for 10 minutes and resuspended in 200 µl THB. Bacteria resuspensions

were plated out on THB agar supplemented with 400 µg/ml kanamycin and incubated for 16 hours 37°C, 5% CO₂ for selection of transformed bacteria.

2.2.9 Allelic exchange by homologous recombination

To construct SpyCEP allelic replacement mutants H1464 and H1567, from CovR/S wild type and CovR parent strains H584 and H1565, a 520bp fragment of the *cepA* gene and a 485bp fragment of the intergenic region between *cepA* and hypothetical protein M5005_Spy0343 was amplified and cloned into the suicide vector pUCMUT to produce pUCMUTcepA. The forward primer: 5'- 20 ATAGCGAATTCAACAGACAAAAACCTCCGCT-3', and reverse primer: 5'- 21 ATAGGGTACCTTGTGATTGCTCTTTTTCACGA-3', incorporated *EcoRI* and *KpnI* restriction sites into 5' and 3' ends respectively. And the forward primer: 5'- AGACGTCGACCAAAGCGCAAAGCGACAACAAA-3', and reverse primer: 5'- ATAGCTGCAGAACTGGGGGTGTCTCCGT-3', incorporated *Sall* and *PstI* restriction sites into the 5' and 3' ends. Plasmids were propagated in *E. coli* XL-10 gold (Stratagene) and the construct electroporated into *S. pyogenes* H584 or H1565 and inserted into the chromosome by homologous recombination. PCR analysis demonstrated a double recombination event between chromosomal *cepA* and the 520bp and 485bp fragment. The insertion fully negated CXCL8 cleavage activity and production of SpyCEP, measured by CXCL8 cleavage assays and western blots respectively.

2.2.10 SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis)

Samples for analysis were added to 10X 1 M dithiothreitol (DTT) (Thermofischer) and 4X Bolt SDS sample buffer (Invitrogen) before heating to 70°C for 10 minutes. Samples were loaded into precast Bolt Bis-Tris, 8, 10, 12, 4-12% polyacrylamide gels (Invitrogen) alongside a SeeBlue Plus” pre-stained ladder (Thermofischer) buffered with MES or MOPS depending on size separation required. Samples were separated at 165 V for 35 minutes (MES buffer) or 45 minutes (MOPS buffer) and stained for 16 hours in either InstantBlue (Sigma Aldrich) or PageBlue (Thermofischer) prior to detaining in deionized water for 16 hours.

2.2.11 Immunoblotting

Protein samples that were not used for SDS-PAGE analysis were processed for downstream immunoblotting. Proteins were transferred onto 0.22 µm nitrocellulose membranes (Thermofischer) using a using an iBlot2 (Thermofischer) and the following conditions: 1-minute 20 V, 3 minutes 23 V and 1 minute at 25 V. Membranes were subsequently blocked at room temperature with gentle agitation for 1 hour in blocking buffer (PBS with 5% skimmed milk powder (Sigma) and 0.1% Tween). Blocked membranes were incubated for 16 hours at 4°C with primary antibodies diluted in blocking buffer with gentle agitation. Primary antibodies used: Rabbit anti-CEP-5 (117) (1:1000), rabbit anti-ENWVQ (1:1000), mouse anti-human CXCL8 (1 µg/ml) (R&D systems) and sheep IgG anti-LL-37 (2 µg/ml) (R&D systems). Membranes were washed in PBS Tween 0.05% and incubated with secondary antibodies diluted in blocking buffer for 1 hour at room temperature with gentle agitation. Secondary antibodies used: goat anti-rabbit IgG A680nm (1:7,500) (LI-COR), goat anti-mouse IgG A790nm (1:7,500) (LI-COR), rabbit anti-sheep IgG (Abcam) (1:40,000) and goat anti-rabbit - HRP (1:80,000) (Sigma-Aldrich). Membranes were re-washed and visualised on the LiCor

Odyssey Fc (Invitrogen) with ECL Prime substrate (Amersham) for chemiluminescence detection, as per manufacturers' instructions.

For fluorescent immunoblotting, the SDS-PAGE and immunoblot protocols were followed as above, with the following adaptations. 4X fluorescent loading dye (Invitrogen) was used for SDS-page and antibodies from two different species were used simultaneously for primary antibodies and antibodies linked to two fluorophores were used for secondary detection. Membranes were visualised on the LiCor Odyssey Fc by excitation in the 600, 700 and 800nm channels.

2.2.12 Semi-quantitative immunoblotting

To quantify SpyCEP in both clinical samples and from *S. pyogenes* isolates, SpyCEP activity against CXCL8 was compared to the activity of a known recombinant SpyCEP standard. SpyCEP standard (4-0.5 ng), whole bacteria, bacterial cell wall extracts, bacterial supernatants or clinical samples were incubated with 150 ng CXCL8 (Almac) at 37°C for 20 minutes. Reactions were stopped by the addition of DTT, LDS and heating to 70°C for 10 minutes as described in the SDS-page protocol above. Reactions involving antibiotic treated bacteria were carried out as above with a pre-treatment of 1 mg/ml Penicillin G (Thermo Fisher) for 2 hours at 37°C.

All quantification was carried out post chemiluminescence immunoblotting by direct comparison to a SpyCEP standard curve, calculated on Image Studio Lite software (LiCor) according to manufacturer's protocols.

2.2.13 Enzyme-linked immunosorbent assay (ELISA)

R&D systems DuoSet ELISA kits were used for detection of human CXCL1, CXCL6 and CXCL8 and murine CXCL1. 96 well plates (Costar) were coated with capture antibody and

incubated overnight at room temperature. Plates were washed three times in wash buffer (PBS-T 0.05%) by a Thermo Fisher Wellwash microplate washer. Wells were blocked in 300 µl PBS 1% BSA for a minimum of 2 hours at room temperature before being re-washed and loaded with 100 µl of prepared samples or standards and incubated at room temperature for 2 hours. Standards were loaded in a 1:2 serial dilution from concentration 2000 pg/ml – 15.625 pg/ml or 1000 pg/ml -7.8125 pg/ml ELISA dependent. The wells were re-washed and incubated with detection antibody for 2 hours at room temperature. After incubation, the wells were washed and incubated with 100 µl Streptavidin – HRP for 20 minutes at room temperature before being washed and incubated with 100 µl TMB. The reactions were stopped with 100 µl 1M H₂SO₄ and the optical density at 570 nm subtracted from 450 nm as measured on a BMG FLUOstar Omega plate reader.

2.2.14 *In vivo* intramuscular infection

The bacterial inoculum was prepared on solid media culture as described above. Bacteria were scrapped off media with a sterile loop and re-suspended in sterile PBS. Bacteria were washed three times by re-suspension in sterile PBS and centrifugation at 2500 xg for 5 minutes. Bacterial pellets were resuspended in sterile PBS to create the inoculum at a final concentration of 2×10^9 CFU/ml. Mice were sedated via inhalation of isoflurane anaesthetic and infected with 50 µl of inoculum ($\sim 1 \times 10^8$ CFU) into the rear right hindlimb. Mice were monitored to ensure clinical endpoints were not reached; ruffled fur, abnormal respiration, reduced mobility to a few steps and $\geq 20\%$ weight loss. At 24 hours post infection, mice were culled by cardiac puncture under terminal anaesthesia and cardiac blood collected in heparinised tubes for analysis. Mouse tissues: thigh, liver, spleen, and both inguinal and axillary lymph nodes from the ipsilateral and contralateral sides were harvested by necropsy. Organs were homogenised

in sterile PBS by a FastPrep-24 5G (MP Biomedical) using the following settings: lymph nodes 4.0m/sec for 20 seconds, liver and spleen 4.0 m/sec for 30 seconds and thigh 4.5m/second for 40 seconds. Homogenised tissues and cardiac blood were plated on CBA agar to enumerate *S. pyogenes* CFU.

2.3 Methods - Chapter 4

2.3.1 Cloning and purification of recombinant SpyCEP constructs

Codon-optimized SpyCEP gene constructs were expressed in *Escherichia coli* using synthetic gene sequences (GenScript) from Spy_0416 in the SF370 *S. pyogenes* M1 genome (135, 153).

SpyCEP constructs were cloned into the vector pET-24B and expressed in BL21 (DE3) competent *E. coli*, cultured in Terrific Broth (TB) medium supplemented with 50 µg/mL kanamycin at 37°C, 200 rpm, for 3 hrs. The cultures were induced with 0.5mM IPTG, cooled to 15 °C and incubated at 200 rpm for 16 hours before lysis by sonication on ice. The expressed constructs were purified by Ni-IDA affinity chromatography as per the manufacturer's instructions. SpyCEP protein was concentrated and further purified by size exclusion chromatography on a HiLoad Superdex 200 prep grade column (GE Healthcare).

The resultant panel of recombinantly expressed SpyCEP constructs is shown in Table 4-1. The R.E (re-associated enzyme) construct required the re-association of the SpyCEP N and C-termini carried out by equimolar co-incubation of both constructs at 37°C for 30 minutes in 40 mM Tris-HCL pH 7.5, 0.1 mM CHAPS, 1 mM DTT, 0.1% BSA, 75 mM NaCl.

2.3.2 Construction of recombinant *S. pyogenes* expressing histidine tagged SpyCEP

To permit release of soluble His-tagged SpyCEP by *S. pyogenes*, the C-terminal anchor domain of SpyCEP was replaced by a stop codon in strain H292, a strain that makes abundant SpyCEP (146). A 549 base pair region immediately upstream of the SpyCEP cell wall anchor motif was amplified from *S. pyogenes* H292 genomic DNA using the primers :5'GGGAATTCTGTTGTCAGGTAACAGTCTTATCTTGCC -3' and 5'CCGAATTCACAACACTAGGCTTTTGCTGAGGTCGTTG -3'. *EcoRI* restriction sites were incorporated at the terminal ends of the primer sequences. The amplified DNA was cloned into the homologous recombination plasmid pUCMUT to produce vector pUCMUT_{CEP} which was transformed into One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher) according to the manufacturer's instructions. The nucleotide sequence of the SpyCEP C-terminus was subsequently further modified to encode a hexa-histidine tag by inverse PCR using pUCMUT_{CEP} as the template and the primers 5'-TATCCTAGGTAGTGTGTTGTGAATTCGTAATCATGGTCATAG-3' and 5'-TATCCTAGGATGATGATGATGATGATGATGGGCTTTTGCTGAGGTCGTTG-3'. The amplification was performed using GoTaq Long PCR Master Mix (Promega) according to the manufacturer's instructions. *AvrII* restriction sites, incorporated at the terminal ends of the primer sequences, facilitated re-circulation of the amplified plasmid. The presence of a hexa-his sequence in the modified pUCMUT_{CEP} construct (denoted pUCMUT_{CEP-HIS}) was confirmed by Sanger sequencing using the pUCMUT sequencing primers 5'-GACAGCAACATCTTTGTGAAAGATGG-3' and 5'-CATTAATGCAGCTGGCACGAC-3'. The pUCMUT_{CEP-HIS} construct was introduced into H292 by electroporation and crossed into the chromosome by homologous recombination as previously described (47) to generate strain H1317. Secretion of histidine tagged SpyCEP into the culture supernatant of H1317 was confirmed by western blotting (data not shown). To purify SpyCEP, *S. pyogenes* H1317 was grown in Todd-Hewitt broth (Oxoid) overnight at 37°C with 5% CO₂. The culture was pelleted

at 2500 xg for 10 minutes and the supernatant sterilised using Amicon 0.22 μ M filters, then concentrated using 15 ml Amicon 100 kDa centrifuge filter columns and purified by nickel column affinity chromatography (Novagen His-Bind Resin) as per the manufacturer's instructions.

2.3.3 Chemokine cleavage by SpyCEP

Recombinant SpyCEP constructs were initially screened for CXCL8 cleavage activity by fluorescent immunoblotting. 150 ng of human CXCL8 (R&D Systems) was incubated with SpyCEP constructs a 1:50 molar ratio in favour of chemokine, for 2 hours at 37°C. Fluorescent immunoblotting was subsequently carried out as described in section 2.2.11.

For SDS-PAGE analysis of SpyCEP cleavage of human chemokines CXCL1, CXCL6 and CXCL8 (R&D Systems), 5 μ M of chemokine were incubated with an equal volume of SpyCEP constructs at a final molar ratio of 1:5- 1:5000, in favour of chemokine. for 2 hours at 37°C. SDS-PAGE analysis was subsequently carried out as described in section 2.2.10

SpyCEP activity against LL-37 was assessed by an 18 hour, 37 °C incubation of 500ng human LL-37 (R&D Systems) with SpyCEP constructs at a 1:10 molar ratio in favour of LL-37. Reactions were, stopped, separated, and blotted onto 0.22 μ m nitrocellulose as above and incubated overnight at 4°C in blocking buffer supplemented with 2 μ g/ml polyclonal sheep IgG anti-LL-37 (R&D Systems). Membranes were washed in wash buffer (PBS with 0.05% Tween) and incubated in blocking solution with rabbit anti-sheep IgG (Abcam) at 1:40,000 dilution for 1 hour at room temperature and visualised on LiCor Odyssey Fc (Invitrogen).

2.3.4 Enzyme linked immunosorbent assay (ELISA)

Catalytic activities of SpyCEP constructs were measured through detection of remaining intact CXCL8 substrate following incubation with SpyCEP by ELISA (R&D Systems human

CXCL8 DuoSet) as per manufacturer's instructions and described in 2.2.13. For cleavage time courses of full length SpyCEP, 50 pM or 100 pM SpyCEP were incubated with an equal volume of 20 nM human CXCL8 and CXCL1 respectively (R&D Systems) and incubated at room temperature for 30 minutes. Reactions were halted at defined timepoints with the addition of concentration of Pefabloc (Sigma-Aldrich) to a final concentration of 2 mg/ml.

The activity of C-terminal SpyCEP against CXCL8 was additionally assessed by ELISA. 100 nM of human CXCL8 (R&D Systems) was incubated with an equal volume of full length and C-terminal SpyCEP constructs at a final molar ratio of 1:5 – 1:1000, in favour of chemokine. Cleavage reactions were halted at desired time points, 0, 5, 10, 15, 30 and 60 minutes by the addition of Pefabloc (Sigma-Aldrich) to a final concentration of 2 mg/ml. The remaining uncleaved CXCL8 was measured by ELISA (R&D Systems human CXCL8 DuoSet), as described in section 2.3.4.

2.3.5 Mass spectrometry

Analysis of CXCL8 cleavage was assayed on a SCIEX API6500 triple quadrupole electrospray mass spectrometer coupled to a high-throughput robotic sample preparation and injection system, RapidFire200 (Agilent Technologies). CXCL8 substrate, the 13 amino acid CXCL8 SpyCEP cleavage product RVVEKFLKRAENS, and a heavy atom substituted internal standard of CXCL8 SpyCEP cleavage product RV[U-¹³C₅ ¹⁵N-VAL]-EKF-[U-¹³C₆ ¹⁵N-Leu]-KRAENS) were monitored by mass spectrometry. The mass spectrometer was operated in positive electrospray MRM mode, and transitions (Q1/Q3) for each species were optimised to give m/z as follows: CXCL8, 1048.7/615.3, CXCL8 cleavage product 526.2/211.1, internal standard 530.5/211.1. A dwell time of 50 ms was used for the MRM transitions. The mass spectrometer was operated with a spray voltage of 5500 V and at a source temperature of 650 °C.

To assay CXCL8 cleavage over time 6.25-2000 nM chemokine were loaded into a 384 well plate. Fixed concentrations of SpyCEP constructs to a final concentration of, 250pM SpyCEP or 40nM C-terminal fragment were added to the plate and reactions stopped at desired time points, 0-240 minutes, by addition of stop solution (1% formic acid) supplemented with 1 μ M heavy atom substituted CXCL8 internal standard and centrifuged 3000 rpm for 10 minutes. Assay plates were transferred onto RapidFire200 integrated to the API6500 mass spectrometer. Samples were aspirated under vacuum directly from 384-well assay plates for 0.6 s. The samples were then loaded onto a C18 solid-phase extraction cartridge to remove non-volatile buffer salts, using HPLC -grade water 0.1% (v/v) formic acid at a flow rate of 1.5 mL/min for 4 s. The retained analytes were eluted to the mass spectrometer by washing the cartridge with acetonitrile HPLC-grade water (8:2, v/v) with 0.1% (v/v) formic acid at 1.25 mL/min for 4 s. The cartridge was re-equilibrated with HPLC-grade water 0.1% (v/v) formic acid for 0.6 s at 1.5 mL/min

2.3.6 Kinetic analysis

A linear regression analysis of the initial five time points during a cleavage reaction of 6.25-2000 nM CXCL8 by an equal volume of 250 pM full length SpyCEP (0, 1, 2, 3, 4 minutes) or 40 nM C-terminal SpyCEP (0, 15, 30, 45, 60 minutes) were calculated. Chemokine cleavage rates were plotted against substrate concentration and kinetics were derived from Michaelis-Menten graphs $Y = V_{\max} * X / (K_M + X)$ and K_{cat} equation $Y = E_T * k_{\text{cat}} * X / (K_M + X)$ where E_t = enzyme concentration as fitted by GraphPad Prism 8.0.2

2.4 Methods - Chapter 5

2.4.1 Inhibitor IC₅₀ values

Inhibitor 1, inhibitor 2 and Pefabloc were diluted from stock concentrations to 200 μ M, 1687.4 μ M and 208.76 mM respectively, in molecular grade water (VWR) supplemented with 2% DMSO (Thermo Fisher) to standardise the DMSO levels in all samples. 50 μ l of each inhibitor were added in triplicate to 50 μ l 200 pM SpyCEP, diluted in H₂O 2% DMSO, centrifuged at 1,000 xg for 1 minute and incubated at room temperature for 30 minutes. 50 μ l was removed from each sample and added to 50 μ l 40 nM CXCL8 (R&D systems), centrifuged for 1 minute at 1,000 xg and incubated at room temperature for 30 minutes. CXCL8 alone incubated with H₂O 2% DMSO was included as a positive control. Cleavage reactions were stopped by the addition of Pefabloc (Sigma Aldrich) to each well to a final concentration of 2 mg/ml. Samples were diluted 200 – fold in reaction buffer (40 mM Tris-HCL pH 7.5, 0.1% BSA, 75 mM NaCl) and loaded on CXCL8 ELISA plates (R&D systems) for chemokine quantification as described above.

2.4.2 Inhibition of FITC- labelled casein cleavage

4 μ M thrombin (Sigma-Aldrich), chymotrypsin (Sigma-Aldrich), elastase (Sigma-Aldrich) and subtilisin (Sigma-Aldrich) were incubated with an equal volume of either 0.4, 4, 40, 400 or 4000 μ M inhibitor 1 or inhibitor 2 for 30 minutes at room temperature. A vehicle control consisting of the highest DMSO concentration present for inhibitor 1 and inhibitor 2, 2.98 % and 4.74% respectively and 4 μ M Pefabloc (Sigma Aldrich) were included for controls. Remaining protease activity against FITC-labelled casein was assessed with the protease fluorescent detection kit (Sigma Aldrich) as per manufacturers instruction. Briefly, in experimental triplicate, 10 μ l of inhibited samples or 10 μ l molecular grade water was incubated with 20 μ l (0.5% w/v) FITC-casein substrate and 20 μ l incubation buffer (20 mM

sodium phosphate with 150 mM sodium chloride, pH 7.6) for 60-minutes at 37 °C with gentle agitation (200rpm). After incubation 150 µl of 0.6N Trichloroacetic Acid (TCA) solution (Sigma Aldrich) was added to each sample and incubated, without light, for 30-minutes at 37 °C with gentle agitation (200 rpm). Samples were centrifuged at 10,000 xg for 10 minutes and 2 µl removed and added to 200 µl assay buffer (500 mM Tris-buffer, pH 8.5) in a black 96 well plate (Greiner). Fluorescence of each sample was read on FLUOstar Omega (BMG Labtech) at excitation 485 nm and emission 535 nm

2.4.3 Size exclusion chromatography – multi-angle laser light scattering (SEC-MALLS)

All experiments were conducted at room temperature on a system comprising a Wyatt miniDAWN TREOS multi-angle light scattering detector and a Wyatt Optilab T-rEX refractive index detector coupled with a Superose 6 10/300 GL (G.E. Healthcare). SpyCEP was analysed at 2 mg/ml (11 µM) in 40 mM Tris pH 7.5, 3% DMSO without and with a 10-fold molar excess of inhibitor 1 (100 µM). Enzyme and inhibitor were incubated at room temperature for 20 minutes prior to analysis.

2.4.4 WaterLOGSY nuclear magnetic resonance (NMR)

NMR samples for the WaterLOGSY experiment consisted of 100 µM inhibitor 1 with 2 µM SpyCEP, ScpA, CXCL8 in 40 mM Tris pH 7.5, 3% D6-DMSO and 10% D2O. Enzyme and inhibitor were incubated at room temperature for 20 minutes prior to analysis. The WaterLOGSY mixing time was 1.5 s and all experiments were recorded at 298 K. A spin lock of 20 ms was applied to the C5a and CXCL8 samples to suppress the protein signal. All

spectra were recorded on a Bruker Avance III HD 800 MHz spectrometer equipped with a triple-resonance cryoprobe.

2.4.5 Neutrophil isolation and purification

Freshly drawn venous blood was collected from healthy donors in lithium heparin anticoagulant tubes (BD Bioscience). Human neutrophils were selectively isolated using a MacsXpress human neutrophil isolation kit (Miltenyl Biotec) according to manufacturer's instruction. Briefly, the supplied lyophilised pellet was resuspended in 2 ml of buffer A and added to 2 ml of buffer B. 4 ml of resuspended pellet was added to 8 ml of whole human blood and incubated for 5 minutes with head over tail rotation at 12 rpm. 14 ml suspension was added to the MacsXpress magnet and allowed to separate over 15 minutes at room temperature. The supernatant was removed and centrifuged at 400 xg for 10 minutes to pellet neutrophils and erythrocytes. The remaining erythrocytes were removed by osmotic lysis - 30 second wash in a hypotonic solution (0.2% NaCl) followed by neutralization in an equal volume of hypertonic solution (1.6% NaCl). The neutrophils were then pelleted at 400 xg for 10 minutes and resuspended in chemotaxis buffer (RPMI, 25 mM HEPES, 0.1% BSA). An aliquot of neutrophils was stained with an equal volume of 0.4% Trypan blue (Gibco), enumerated by light microscopy and adjusted to desired concentration in chemotaxis buffer.

2.4.6 SpyCEP inhibition reactions

To create a stock of inhibited SpyCEP for subsequent use in neutrophil chemotaxis experiments, 1 nM SpyCEP was incubated with an equal volume of 1 mM inhibitor 1, DMSO vehicle control or molecular water (VWR) for 30 minutes at 37°C. An equal volume of 5 µM human CXCL8 (R&D Systems) was added to the resultant mixtures and incubated for 20 hours at 37°C. Aliquots of each reaction were analysed by SDS-PAGE, as described in 2.2.10, to ensure expected chemokine cleavage and inhibition.

2.4.7 Modified Boyden chamber

Corning HTS Transwell 96 well (pore size 5µm) plates were blocked with blocking buffer (RPMI 25 mM HEPES, 1% P/S, 1% BSA) for a minimum of 1 hour at room temperature, 80 µl for the upper chambers and 235 µl for the lower chambers. Human neutrophils were selectively purified from drawn human venous blood with MacsXpress human neutrophil isolation kit (Miltenyl Biotec) as described in 2.4.5 and adjusted to 1.25×10^6 cells/ ml (100,000 cells / 80 µl) in chemotaxis buffer (RPMI 25mM HEPES, 1% P/S, 0.1% BSA).

80 µl of neutrophils (100,000 cells) were added to the upper chambers of the Transwell plate and incubated for 4.5 hours at 37°C, 5% CO₂ and a further 30 minutes at room temperature to equilibrate the plate to ambient temperature. Samples and standards were removed from the lower chamber and transferred to a white luminescence 96 well plate (Greiner). An equal volume of CellTiter-Glo (Promega) was added to the samples and incubated for 10 minutes at room temperature with constant agitation, 400 rpm. The luminescence of each sample was read on a FLUOstar Omega (BMG Labtech) and readings compared to the standard curve for quantification.

2.4.8 Neutrophil migration by TAXIScan

Human neutrophils were freshly harvested and purified as per section 2.4.5 and resuspended in chemotaxis buffer (RPMI 25mM HEPES, 1% P/S, 0.1% BSA) to 5×10^5 neutrophils / ml. The chemotaxis assay was carried out according to manufacturer's instructions (154). Briefly, 1 µl neutrophil suspension was loaded into the proximal well of a 12 channel TAXIScan chip and 1 µl of 100 nM of CXCL8 (R&D systems) was loaded into the distal well. Neutrophils were aligned adjacent to a 5 µm high, 253 µm long channel, separating the proximal and distal wells. Individual neutrophil migration was manually tracked by the ImageJ (National Institutes of Health) manual tracker plugin (Fabrice Cordelieres, Institut Curie). Tracking was started after

the centre of mass of a neutrophil passed the tracking start line, Figure 2-1. The neutrophil migration was monitored in real time over a 1- hour, room temperature time course and halted after 1 hour had elapsed or the neutrophil centre of mass passing the tracking end line. Neutrophils that were already passed the tracking start line at the commencement of the assay were not analysed.

Images were captured every 1 minute and compiled for processing by the Chemotaxis and Migration tool (Ibidi). Quantitative chemotaxis parameters used for experimental comparisons are defined in Figure 2-2 below.

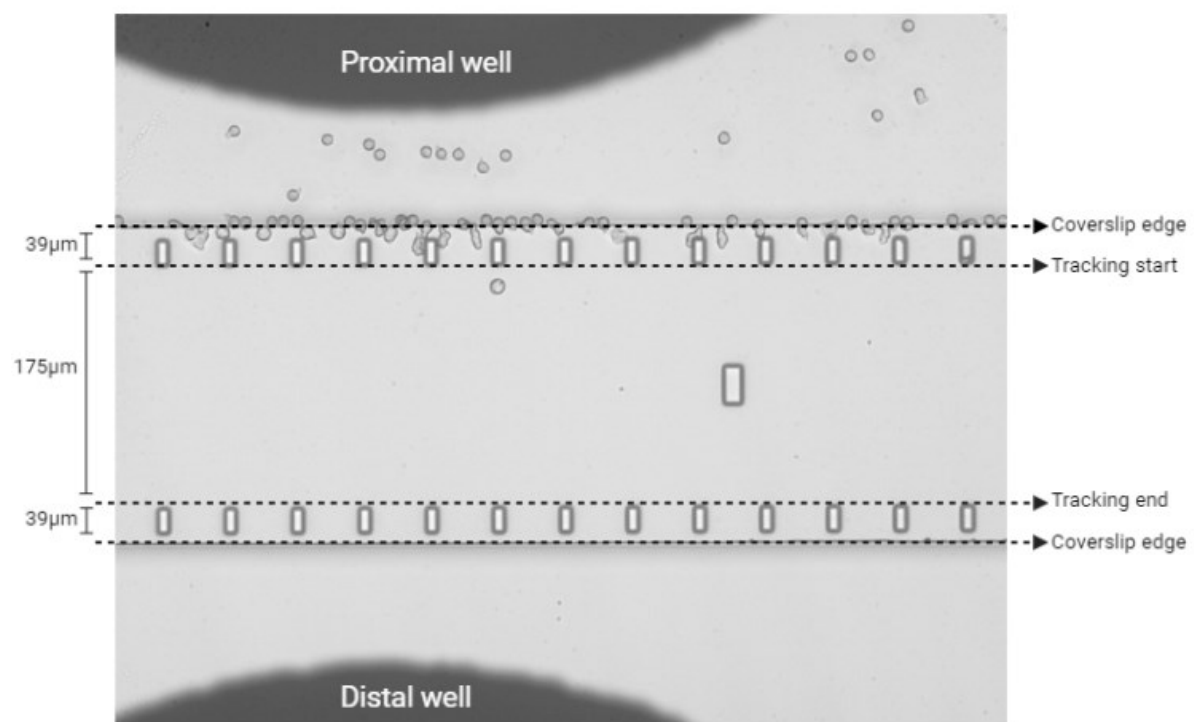
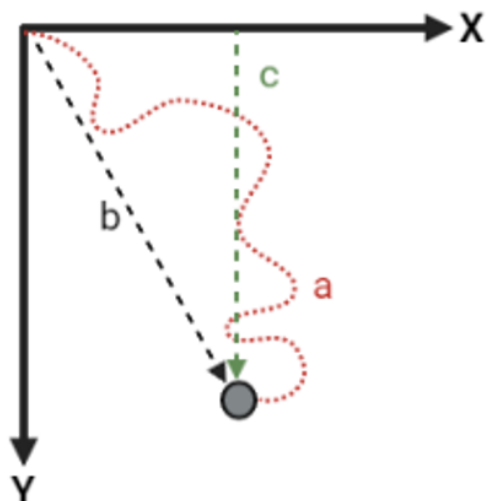


Figure 2-1. Visual representation of TAXIScan assay

Neutrophils were loaded into the proximal well and were aligned at the coverslip edge. 1 µl of CXCL8 (100 nM) was loaded into the distal well and the time lapse camera started. Neutrophils were manually tracked for 1 hour at room temperature when the centre of mass of the neutrophil passed the tracking start line and the neutrophil migrated into the 175µm migration space. Tracking was stopped when either the neutrophil centre of mass passed the tracking end line or 1-hour had passed. Neutrophils present within the 175µm space at the commencement of the assay were omitted from analysis.



Migration parameter	Description	Value
Track length	Total distance migrated	a
Euclidean distance	Straight line distance from start to end point	b
Velocity	Track length / time taken (t)	a / t
Directionality	Euclidean distance / track length	b / a
Forward migration index (yFMI)	Y axis displacement / Euclidean distance	c / b

Figure 2-2. Schematic for Ibidi neutrophil migration parameters

Description and calculation of quantitative neutrophil chemotaxis parameters, track length (a) (μm), Euclidean distance (b) (μm), velocity (a / t) ($\mu\text{m} / \text{minute}$), Directionality (b / a) and forward migration index (yFMI) (c / b). All parameters are calculated in respect to a neutrophil start position at X = 0, Y = 0.

2.5 Methods - Chapter 6

2.5.1 Intramuscular infection

Intramuscular infection models were carried out in strict accordance with protocol outline in 2.2.14

2.5.2 Intranasal infection

Intranasal infections were carried out as per the protocol 2.2.14, however with the following adaptations. The bacterial inoculum was re-suspended to a concentration of $\sim 1 \times 10^{10}$ CFU/ml and administered to mice by inhalation whilst under isoflurane sedation. 5 μ l were administered per nostril comprising an inoculum dose of $\sim 1 \times 10^8$ CFU. The entire nasopharynx, lungs, cervical lymph nodes and spleen were harvested at necropsy and homogenised as per 2.2.14, with lungs processed at 4.0 m/ second for 3 seconds and nasopharynx 4.5m/second for 40 seconds.

2.5.3 Drug preparation and administration

Inhibitor 1 was reconstituted to 100 mg/ml (134.25 mM) in 100% DMSO (Thermofischer) and stored at -20 °C. On the day of administration an aliquot was thawed, and concentration adjusted to 1.5 mg/ml with water for injection (B. Braun Medical). A 100% DMSO sample was additionally diluted as above for vehicle control dose. 100 μ l inhibitor 1 (150 μ g / dose), 100 μ l inhibitor control, or 100 μ l water for injection were administered by intraperitoneal route 2-hour pre-bacterial infection. For infection models with multiple doses, addition doses were administered every 6 hours.

Erythromycin lactobionate for infusion (ADMIPHARM) was reconstituted to 50 mg/ml in water for injection (B. Braun Medical) and stored at 4 °C. On the day of infection erythromycin

was diluted to 50 µg/ml in 0.9% saline for injection (B. Braun Medical) and administered by 100 µl intraperitoneal injection 2 - hours before injection.

3 Results - Quantification of SpyCEP in clinical samples

3.1 Introduction

It has been repeatedly shown that SpyCEP plays a prominent role in the pathogenesis of *S. pyogenes* and driving invasive infection (30, 115, 134, 140, 146, 155). Indeed, heterologous expression of SpyCEP in an otherwise avirulent *L. lactis* infection model leads to systemic disease, a necrotizing phenotype and death (30). In these severe necrotizing infections, in both mouse and human histopathology, a marked reduction in neutrophil infiltrate is seen at the sites of *S. pyogenes* infection, in comparison with other types of bacterial infection (30, 156, 157). The prevailing hypothesis is that SpyCEP significantly impacts the innate immune response during early infection, by reducing neutrophil chemotaxis and activation and therefore the subsequent phagocytosis and clearance of *S. pyogenes*. SpyCEP's proteolytic activity against neutrophil specific chemokines and the dissection of its role in the neutrophil response to *S. pyogenes* infection has been a major target for investigation since its discovery (115).

Conversely, the *in vivo* expression of SpyCEP is a topic that has been less well researched. The large disparity between isolates ability to cleave CXCL8 is well recognised, and previous work has utilised qPCR to highlight the differential expression of SpyCEP between invasive and non-invasive strains (146). However, to date no work has determined SpyCEP levels in clinical invasive disease and only preliminary results are available for *in vitro* SpyCEP expression levels (30). An understanding of SpyCEP abundance and expression during clinical disease will underpin development of therapeutic inhibitors and may identify clinical infection phenotypes that might respond best to such inhibitors. Furthermore, although SpyCEP had shown promise as a vaccine antigen in mice, demonstration of expression during human

infection will provide useful rationale for its inclusion in a future multicomponent vaccine for use in humans.

In the first chapter of this work, a panel of isogenic SpyCEP positive and negative *S. pyogenes* strains was derived which permitted interrogation and confirmation of SpyCEP function *in vivo*. The isogenic strains were used to develop and validate assays to detect SpyCEP and SpyCEP bioactivity *in vitro* and *in vivo* using experimental models. This paved the way to detection of SpyCEP in samples from patients with invasive *S. pyogenes* infection and quantification of SpyCEP in those infections.

3.2 Results

3.2.1 Deletion of *cepA* in *S. pyogenes* isolates H584 and H1565

To experimentally assess the impact of SpyCEP throughout this project, both *in vitro* and *in vivo*, it was necessary to derive a panel of isogenic *S. pyogenes* strains that differ only in the production of SpyCEP. Isolate H584, a clinical M1 strain obtained from a lethal puerperal *S. pyogenes* infection and H1565, a virulent derivative, recovered post single animal passage of H584 were initially chosen. Both isolates were of the M1T1 background and selected due to the lineages' global dissemination and high association with invasive disease. Whole genome sequencing confirmed that although H584 had a wildtype two component CovR/S regulator, the derivative H1565 had a non-synonymous mutation (A-C) at codon 65 in *CovR*, as such H1565 expressed high levels of SpyCEP compared with H584. The two isogenic strains enabled us to generate a panel of further isogenic mutants to interrogate the role of SpyCEP in different phenotypic backgrounds. The *cepA* gene encoding SpyCEP was replaced via homologous recombination with the *Aph A3* gene encoding a kanamycin resistance cassette using the suicide pUCMUT plasmid (158).

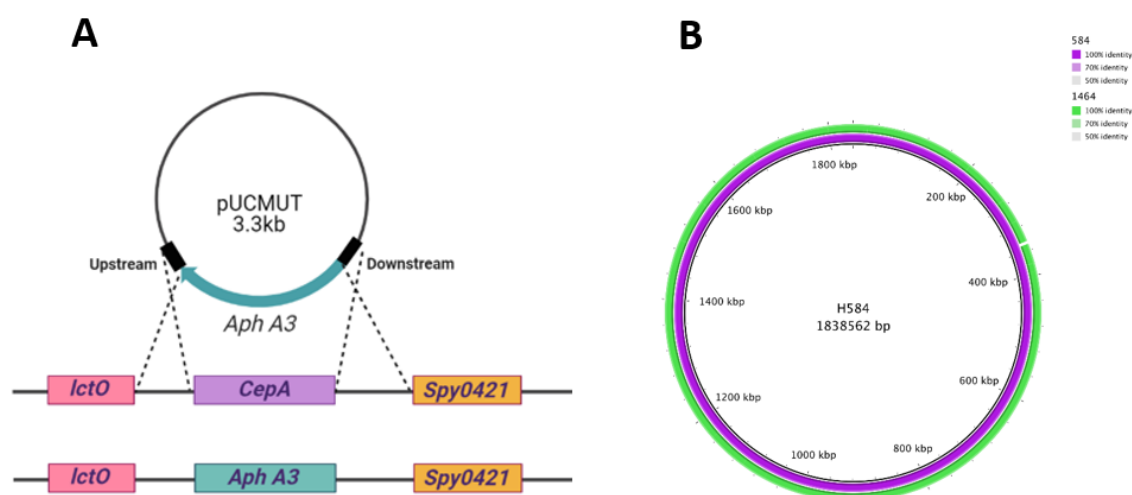


Figure 3-1. Allelic replacement strategy for *cepA*

A. Intergenic regions directly upstream and downstream of the *cepA* gene were cloned into the suicide pUCMUT vector to enable homologous gene replacement with *Aph A3*. **B.** Representative Brigg diagram displaying percentage whole genome sequence identity between *S. pyogenes* strains H584 (M1 CovR/S_{WT}) and H1464 (M1 CovR/S_{WT} Δ *cepA*). Successful knock out of *cepA* gene in H1464 is shown by genome gap above 400kbp marker.

CepA in both H584 and H1565 was successfully deleted resulting in isogenic knockout strains H1464 and H1567 respectively. Gene deletion was confirmed by PCR amplification of the region spanning *lctO-cepA-Spy0421* (appendix). Whole genome sequencing of the derivative strain, H1464, was undertaken and sequences compared with the parent strain H584 to ensure that no additional mutations were introduced during the cloning process (Figure 3-1 B).

The new M1T1 isogenic *cepA* knockouts supplemented our existing panel of isogenic strains altered in SpyCEP production. Firstly, a high SpyCEP producing M81 CovR mutant, H292, and its corresponding *cepA* mutant, H575 - which expresses an inactive N-terminal SpyCEP product (30). Secondly, *L. lactis* isolate, H487, constitutively expressing plasmid encoded SpyCEP was derived from H486, a *L. lactis* isolate containing an empty vector control.

Combined, the panel of isolates provided an ideal platform to assess the role of SpyCEP in *S. pyogenes* infection. Full details of the isolate panel can be found in Table 2-1.

To confirm that the mutants were not actively producing SpyCEP, we isolated and purified the components of both the bacterial cell wall and the bacterial supernatants from all isolates after overnight growth in rich culture medium and immunoblotted to detect SpyCEP (Figure 3-2).

Denatured immunoblotting with an anti-SpyCEP (anti-CEP-5) antibody detected multiple SpyCEP specific fragments in both the cell wall isolations and the supernatants of SpyCEP + isolates, as has been previously published (30, 117) . The parent M1 strain H584 expressed surprisingly little detectable SpyCEP despite possessing manifest CXCL8 cleaving activity; concentration of the supernatant 25-fold was necessary to detect SpyCEP. However, the SpyCEP-negative mutant H1464 made no detectable SpyCEP (Figure 3-2 A). In contrast, the CovR derivative, H1565 produced a larger amount of SpyCEP detected as a major band in the cell wall at ~150kDa that represents the main C-terminal fragment of SpyCEP (Figure 3-2 B). This band disappeared in the mutant H1567 (Figure 3-2 B). The CovR mutant H292 produced similar amounts of SpyCEP while as expected the mutant H575 produced a truncated variant (Figure 3-2 C). Finally, SpyCEP production by *L. lactis* strain H487 was confirmed, mainly in the supernatant (Figure 3-2 D). The 25-fold concentration highlighted the multiple breakdown products of SpyCEP in supernatant under these conditions, shown by the smeared banding.

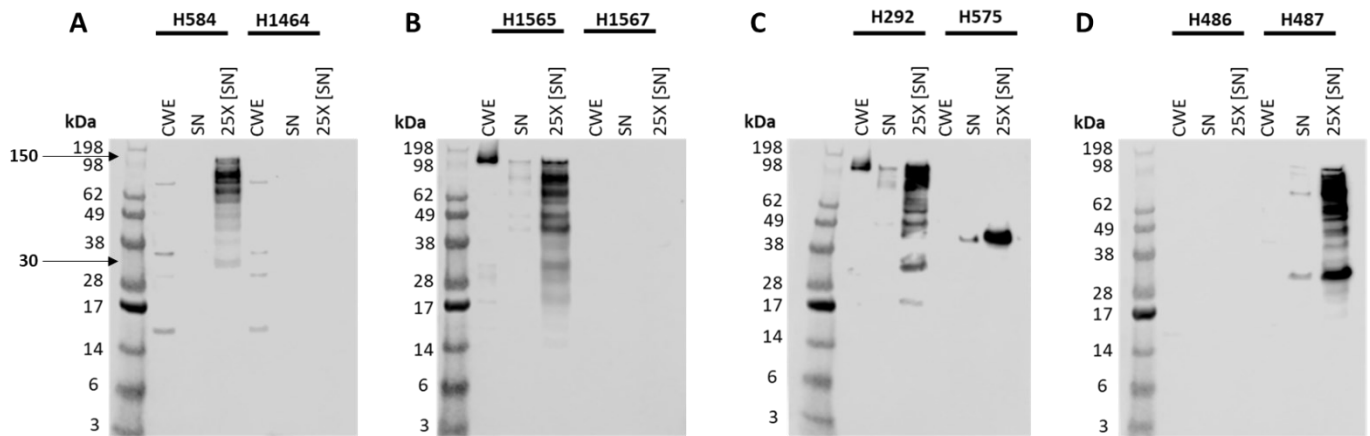


Figure 3-2. Comparative immunoblots of isogenic SpyCEP deletion mutants

Anti-SpyCEP immunoblots of 100 µg/ml cell wall extract (CWE), 10 µl supernatant (SN) and 10 µl 25X concentrated supernatant (25X[SN]) of **A.** H584 (M1 CovR/S_{WT}) and H1464 (M1 CovR/S_{WT} Δ *cepA*). **B.** H1565 (M1 CovR_{MT}) and H1567 (M1 CovR_{MT} Δ *cepA*). **C.** H292 (M81 CovR_{MT}) and H575 (M81 CovR_{MT} Δ *cepA*). **D.** H486 (Lactis_{vector}) and H487 (Lactis_{*pcepA*}). SpyCEP banding detected by CEP-5 immunised rabbit serum (1:1000) and goat anti-rabbit IgG HRP (1:80,000). Expected 150 kDa and 30 kDa banding indicated by arrows.

We further analysed the newly created M1 mutants to assess whether allelic replacement of the *cepA* gene in H584 or H1565 caused any fundamental differences in bacterial growth. Isolates were cultured in THB broth and growth was monitored by optical density (600_{nm}) over time. There was no significant change in the growth profiles of either SpyCEP deletion mutant compared to their parent strain, in the CovR/S WT or CovR mutant background (Figure 3-3).

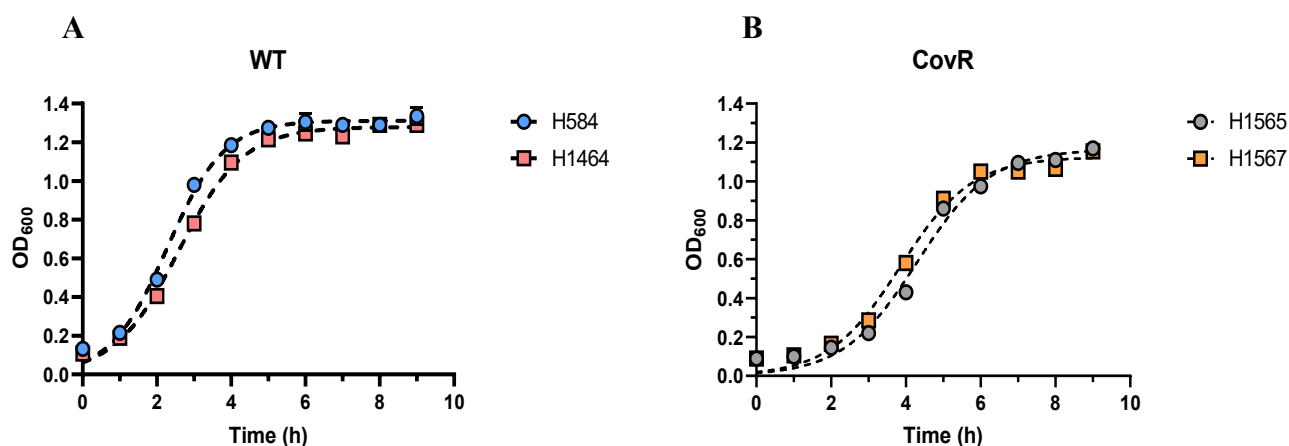


Figure 3-3. Growth curves of isogenic SpyCEP deletion mutants

S. pyogenes 9-hour growth in THB at 37°C, 5% CO₂, measured at OD_{600nm} of **A.** H584 (M1 CovR/S_{WT}) and isogenic SpyCEP deletion mutant H1464 (M1 CovR/S_{WT} $\Delta cepA$) and **B.** H1565 (M1 CovR_{MT}) and isogenic SpyCEP deletion mutant H1567 (M1 CovR_{MT} $\Delta cepA$). Data points represent mean of n=2 biological replicates and data were fitted with a logistic growth regression.

3.2.2 Bioassay for SpyCEP activity and quantification

Having validated the new M1 SpyCEP deletion panel, it became clear that direct detection of SpyCEP using antibodies might be challenging as the protease was subject to degradation and western analysis was reliable only using purified bacterial cell wall extract, making *in vivo* detection in complex tissue samples difficult. Furthermore, SpyCEP expression levels are unequal across isolates and not static during infection, with differential regulation and mutations likely to strongly influence the amount of SpyCEP being produced by *S. pyogenes*; increased expression levels being strongly associated with invasive disease (146) .

Previous work has attempted to quantify SpyCEP using a single antibody sandwich ELISA utilising the anti-SpyCEP antibody used for immunoblotting in Figure 3-2, with H292 and H487 reported to release 69 and 12 ng/ml SpyCEP into the growth supernatant respectively (30). However, a single antibody self-sandwich ELISA has sensitivity and specificity

drawbacks, especially in assaying clinical samples. Additionally, the multiple fragmented banding of SpyCEP seen in direct SpyCEP immunoblotting would not be conducive to quantification. It was also clear from published work that the activity of SpyCEP was easy to detect via cleavage of CXCL8 in contrast to the amount of detectable protein present, i.e., even when little is visible on western analysis (140). A method to detect SpyCEP bio-activity was therefore developed, exploiting a rabbit C-terminal antibody (CTAB) (159), raised against the CXCL8 C-terminal neo-epitope peptide, ENWVQ. Based on the hypothesis that the C-terminal peptide of any protein is linear and therefore exposed to antibody recognition, it was hypothesised that the epitope would be exposed to antibody binding only after SpyCEP-specific cleavage of CXCL8. Initial evaluation of the ENWVQ antibody was conducted by Dr Jenny Goldblatt (PhD thesis, 2018) and it was confirmed that the antibody specifically recognised SpyCEP-cleaved CXCL8 but not intact CXCL8.

Using immunoblotting and densitometry to detect generation of cleaved substrate (cleaved CXCL8, identified using the ENWVQ antibody), SpyCEP quantification from test samples containing unknown amounts of SpyCEP was possible by comparison to CXCL8 samples co-incubated with a standard of known concentrations of recombinant SpyCEP.

3.2.3 Quantification of SpyCEP from *S. pyogenes* isolates

We used the SpyCEP bioassay to quantify cell surface SpyCEP as well as SpyCEP released into the supernatant by the isolate panel. Whole bacteria were isolated from the growth supernatant by centrifugation and washed to ensure measurement of only cell wall bound enzyme. Whole live bacteria (using a range of suspension densities) were incubated with 150 ng of exogenous CXCL8 at 37°C for 20 minutes before reactions were stopped and blotted with the CTAB anti-ENWVQ to detect the appearance of cleaved CXCL8. A range of

recombinant SpyCEP standard concentrations was also co-incubated with CXCL8 to quantify SpyCEP on the bacterial cells tested.

The bioassay successfully detected the production of cleaved CXCL8 (6 kDa band) from exogenous full length CXCL8 (8 kDa) following co-incubation with SpyCEP-producing isolates (Figure 3-4 A-D). Cleaved CXCL8 was absent when CXCL8 was incubated with Δ SpyCEP isolates (Figure 3-4 E). The quantity of cleaved CXCL8 varied between isolates; the two CovR mutant strains (H1565 and H292) produced significantly more cleaved CXCL8 at lower densities of bacteria. A 500-fold dilution of H1565 bacterial suspension successfully cleaved CXCL8 (Figure 3-4 B) and a 1000-fold dilution H292 bacterial suspension cleaved CXCL8 (Figure 3-4 C). In contrast, the CovR/S WT H584, and the *L. lactis* expressing SpyCEP H487, only produced cleaved CXCL8 when incubated with the highest suspension of bacteria, a 10-fold dilution (Figure 3-4 A, D). These results validated our bioassay and confirmed that bacterial surface associated SpyCEP was bioactive and expressed by all our SpyCEP + isolates; the findings also confirmed *L. lactis* can successfully anchor SpyCEP to the bacterial wall.

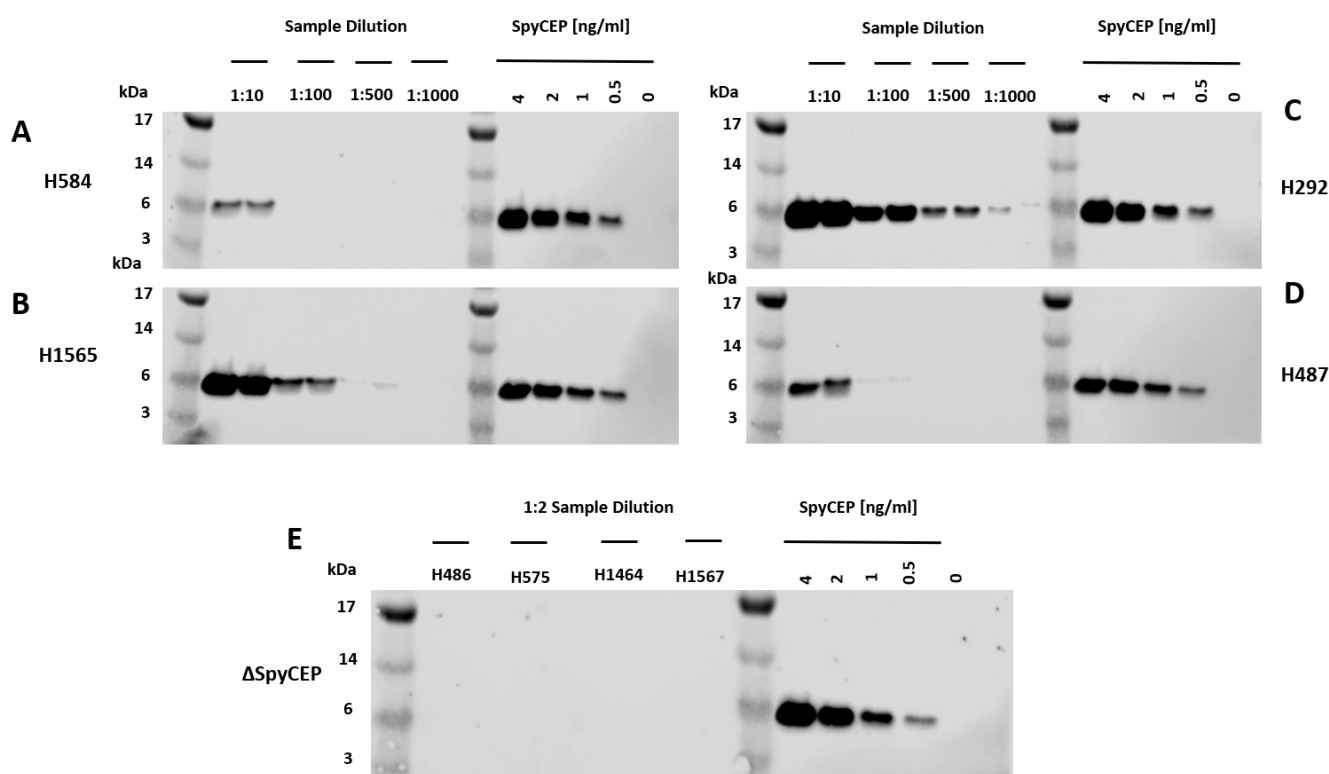


Figure 3-4. Cleaved CXCL8 production by bacterial cell surface SpyCEP

Immunoblots showing the appearance of cleaved CXCL8 (6 kDa) after a 20-minute, 37°C incubation of 150 ng CXCL8 with SpyCEP expressing isolates **A.** H584 (M1 CovR/S_{WT}), **B.** H1565 (M1 CovR_{MT}), **C.** H292 (M81 CovR_{MT}), **D.** H487 (*Lactis_{pcpA}*) or **E.** isogenic SpyCEP mutants H486 (*Lactis_{vector}*), H575 (M81 CovR_{MT} Δ *cepA*), H1464 (M1 CovR/S_{WT} Δ *cepA*), H1567 (M1 CovR_{MT} Δ *cepA*). N = 2 biological duplicates are shown for each bacterial suspension shown (A-E).

Standard concentrations of SpyCEP (0-4 ng/mL) were also co-incubated with 150 ng CXCL8

Having established that the bacterial surface attached SpyCEP was biologically active, bacterial supernatants were then co-incubated with CXCL8 to quantify the soluble SpyCEP released during growth (Figure 3-5). Interestingly, we could not detect SpyCEP activity in even the most concentrated supernatant of H584 (Figure 3-5 A), even after experimental replication (data not shown), and despite demonstrating that H584 does express SpyCEP. The data suggest soluble SpyCEP shed by H584 is below the level of detection of the bioassay; it may be that SpyCEP is confined to the cell surface in low expressing strains. Supernatants from H1565 and

H487 successfully cleaved CXCL8 at the lowest concentration tested, 1.56X (Figure 3-5 B, C). Due to the high levels of accumulated SpyCEP we required further dilution of the H292 supernatant to aid with more accurate quantification. At a 5-fold dilution (0.2X concentration), the lowest assayed, cleaved CXCL8 was still detected (Figure 3-5 D) As expected, the supernatants from Δ SpyCEP isolates did not cleave CXCL8, even at the highest fold concentration, 12.5X (Figure 3-5E).

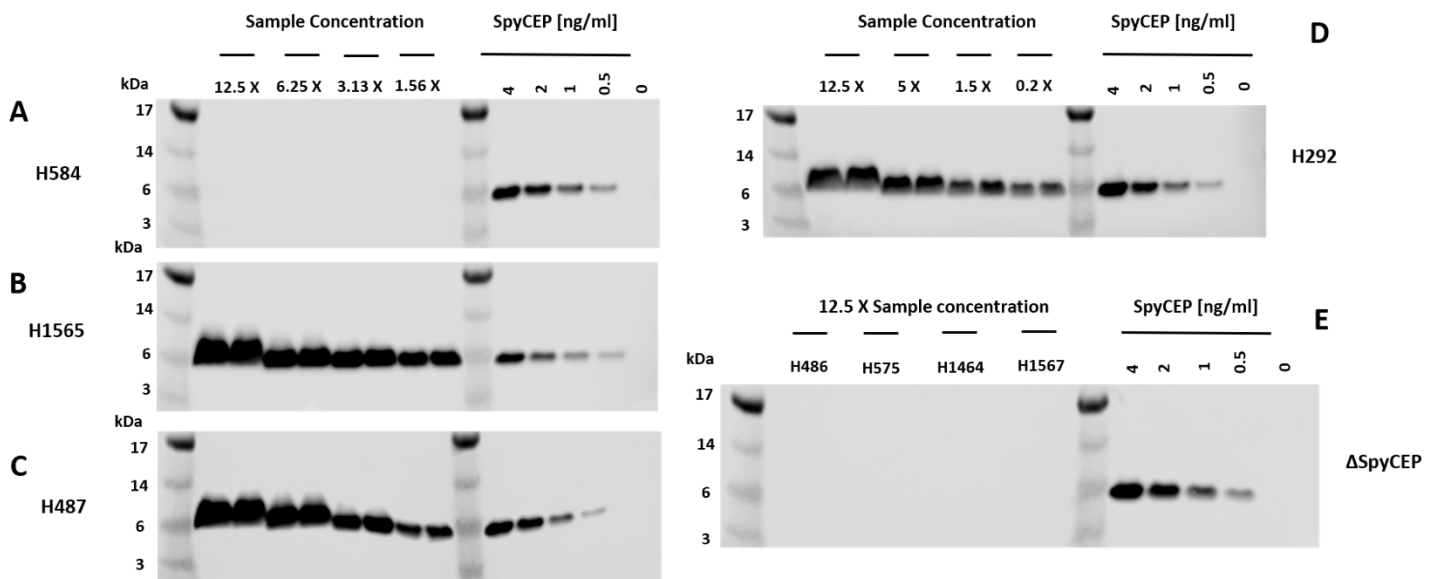


Figure 3-5. Cleaved CXCL8 production by secreted SpyCEP

Immunoblots showing the appearance of cleaved CXCL8 (6 kDa) after a 20-minute, 37°C incubation of 150ng CXCL8 with concentrated bacterial supernatants. Supernatants collected from SpyCEP expressing isolates **A.** H584 (M1 CovR/S_{WT}), **B.** H1565 (M1 CovR_{MT}), **C.** H487 (*Lactis_{spcepA}*), **D.** H292 (M81 CovR_{MT}) or **E.** isogenic SpyCEP mutants H486 (*Lactis_{vector}*), H575 (M81 CovR_{MT} Δ *cepA*), H1464 (M1 CovR/S_{WT} Δ *cepA*), H1567 (M1 CovR_{MT} Δ *cepA*). N = 2 biological duplicates are shown for each concentration of bacterial supernatant, 12.5-fold – 0.2-fold (A-E).

Standard concentrations of SpyCEP (0-4 ng/mL) were also co-incubated with 150 ng CXCL8 and the resultant generation of cleaved CXCL8 is shown on the right side of each panel. Densitometry was used to calculate the amount of SpyCEP in each setting.

Combined with the information obtained from the standard concentration of SpyCEP, the western blots above can be used directly for quantification. However, for inter-strain comparison standardisation is required to account for differences in late phase growth density; this is especially apparent between *S. pyogenes* and *L. lactis* which can grow to 2 log folds greater density than *S. pyogenes*. To quantify SpyCEP, we utilised the standard curve of cleaved CXCL8 produced by a known concentration of recombinant SpyCEP and normalised the results to the bacterial CFU input.

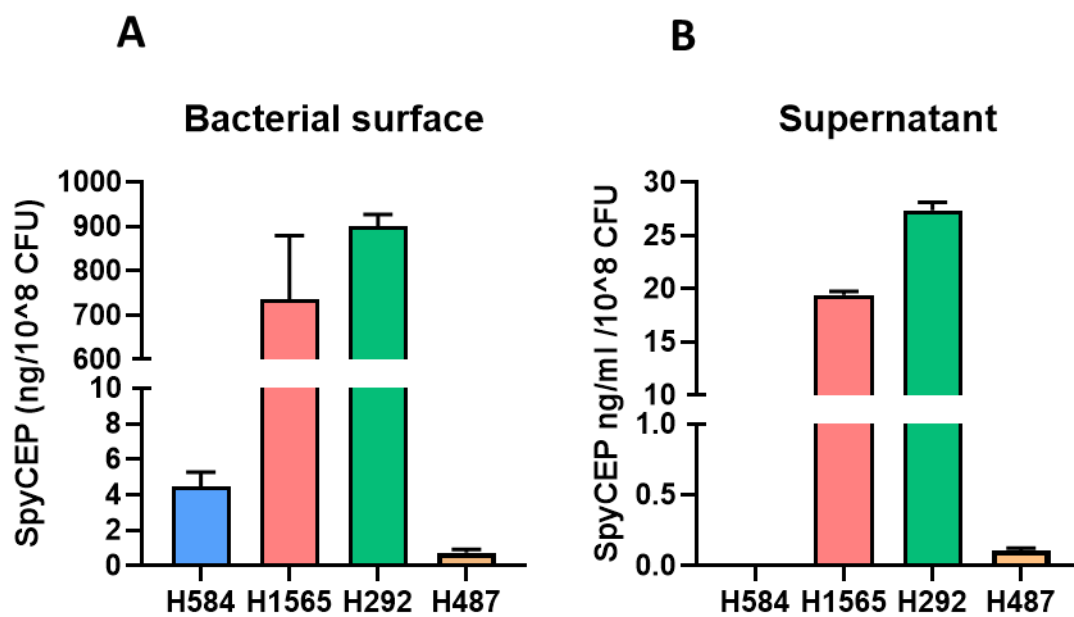


Figure 3-6. Absolute SpyCEP quantification from SpyCEP expressing bacterial isolates.

A. Quantification of cell wall associated SpyCEP in H584 (M1 CovR/S_{WT}), H1565 (M1 CovR_{MT}), H292 (M1 CovR_{MT}), and H487 (*Lactis_{pepA}*). **B.** Quantification of extracellular released SpyCEP from H584 (M1 CovR/S_{WT}), H1565 (M1 CovR_{MT}), H292 (M1 CovR_{MT}), and H487 (*Lactis_{pepA}*). N = 2 experimental replicates per strain, error bars show SD.

Our results show that across all isolates, more SpyCEP is retained on the bacterial surface than released into the growth medium. H584 expressed 4.49 ng/10⁸ CFU bioactive SpyCEP on the bacterial surface but supernatant had values below the limit of detection of our bioassay. As expected, both CovR mutants produced significantly more SpyCEP: H1565 expressed an average of 735.35 ng/10⁸ CFU bioactive SpyCEP at the cell surface and 19.4 ng/ml/10⁸ CFU in the supernatant. H292 surface expression of SpyCEP was 900.81 ng/10⁸ CFU and 27.39 ng/ml/10⁸ CFU SpyCEP in supernatant. When normalised to bacterial density results for H292 supernatant were in line with the previous published results, 69 ng/ml. *L. lactis* produced the lowest quantities of SpyCEP despite a constitutively active promoter; surface expression was 0.76 ng/10⁸ CFU, while soluble expression was 0.11 ng/ml/10⁸ CFU. The latter value is in accordance with the previous reported value of 12 ng/ml in neat supernatant measured semi-quantitatively by western blotting (30) - H487 reached 3.3x10⁹ CFU/ml in overnight cultures which equates to 3.63 ng/ml SpyCEP.

3.2.4 Quantification of SpyCEP from experimental infection *in vivo*

Quantification of native SpyCEP expression *in vitro* by isogenic *S. pyogenes* isolates provided validation of the antibody-based approach to a SpyCEP bioassay. However, although the anti-ENWVQ antibody performed well, yielding specific bands when tested with pure, cleaved CXCL8, there was a high likelihood of cross-reaction if combined with complex tissues containing multiple mammalian proteins. As a major intention was to develop an assay to accurately quantify the SpyCEP production in human tissues during infection, we first examined whether we could detect SpyCEP in samples from *S. pyogenes* infected mice, as this allowed the use of isogenic mutant *S. pyogenes* strains and the evaluation of antibody specificity in complex tissues.

Female C57BL/6J mice were infected via rear hindlimb intramuscular injection with either H1565 or the isogenic Δ SpyCEP mutant, H1567, $n = 5$ per group. The mice were culled 24-hour post infection and both circulating cardiac blood and infected thigh tissue were harvested for processing of serum and bacterial enumeration. Mice with the highest systemic bacterial burden; 3×10^6 and 8×10^5 CFU/ml for H1565 and H1567 respectively were chosen for immunoblotting analysis to detect SpyCEP activity.

Homogenised thigh tissue from mice infected with H1565 successfully cleaved exogenous CXCL8 at all tested dilutions; this cleavage was not seen in thigh samples infected with SpyCEP-negative H1567 (Figure 3-7A). The bioassay was additionally able to detect SpyCEP activity in serum, with detectable cleaved CXCL8 present at the highest concentration tested, 1:10 (Figure 3-7 B). SpyCEP production by H1565 *in vivo* was measured as 176.5 ng/ml of thigh homogenate and 1.24 ng/ml of serum.

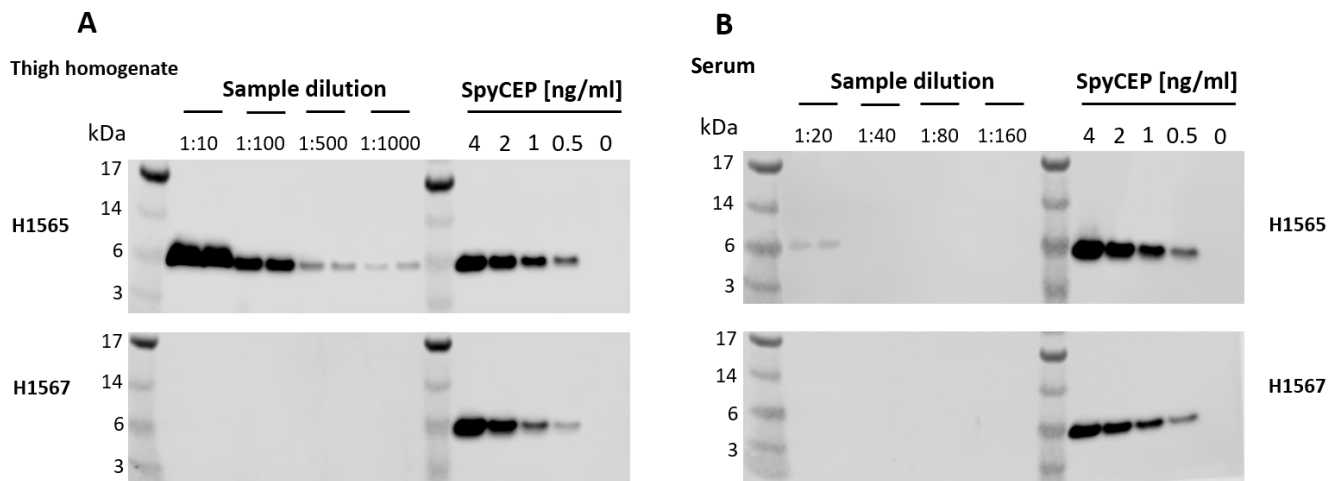


Figure 3-7 Quantitative immunoblots for SpyCEP activity in samples from infected murine tissues

Production of cleaved CXCL8 (6 kDa) from exogenous CXCL8 (8 kDa) incubated with either **A.** homogenised murine thigh or **B.** Serum isolated from murine blood following intramuscular infection with *S. pyogenes* H1565 (M1 CovR_{MT}) or H1567 (M1 CovR_{MT} Δ cepA) for 24 hours. N= 2 experimental replicates per sample dilution Standard concentrations of SpyCEP (0-4 ng/mL) were also co-incubated with 150 ng CXCL8 and the resultant generation of cleaved CXCL8 is shown on the right side of each panel. Densitometry was used to calculate the amount

Previous work has posited that SpyCEP may have systemic effects, distal to the infection site (30). To assess SpyCEP's effect on chemokine at both the local soft tissue infection site and in the systemic circulation we measured chemokine levels directly by ELISA. In our mouse model it was necessary to measure the functional murine CXCL1 SpyCEP substrate homologue KC, a potent murine neutrophil chemoattractant, rather than CXCL8, due to mice lacking this chemokine. Thigh homogenates from mice infected with H1565 contained significantly less KC when compared to tissue from mice infected with H1567; median values were 1.50 and 13.90 ng/ml respectively (Figure 3-8 A). KC measurements from sera isolated from cardiac blood, showed a trend in KC reduction after infection with the SpyCEP producing strain H1565

compared to H1567; median values were 95.75 and 165.0 ng/ml respectively. However, these results were not significant $p = 0.15$ (Figure 3-8 B).

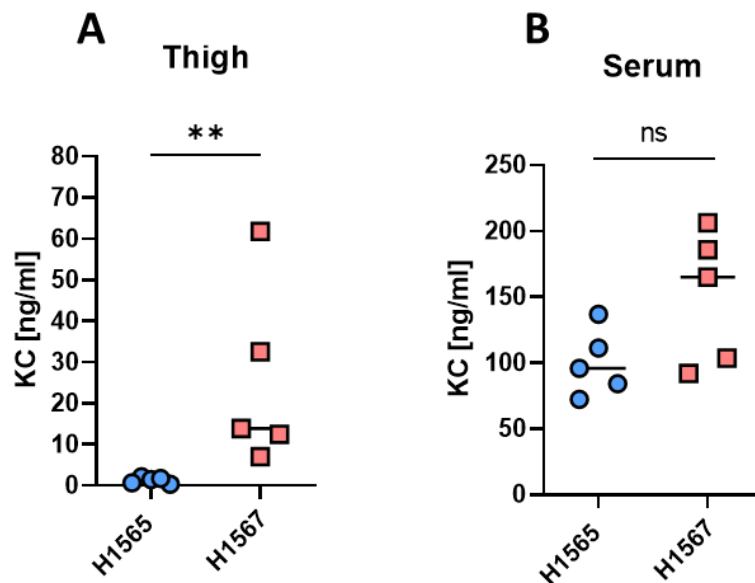


Figure 3-8. KC levels in *S. pyogenes* infected mouse samples

KC levels in **A.** mouse thigh homogenates or **B.** mouse serum, post 24-hour IM infection with *S. pyogenes* strain H1565 (M1 CovR_{MT}) and H1567 (M1 CovR_{MT} $\Delta cepA$). Symbols represent individual mice, $n = 5$ per group, bar represents mean value, ns $p < 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, determined by Mann-Whitney test.

3.2.5 Quantification of SpyCEP from human clinical samples

Having successfully established and validated the bioassay we wanted to now extend our focus to quantifying SpyCEP in human clinical samples. Biofluid samples from a recently published study examining host and streptococcal proteome from the sites of confirmed *S. pyogenes* invasive infections were used (160). The sample details are outlined in Table 3-1; and comprised of tissue exudates (soft tissue infection), joint aspirates (septic arthritis), and pleural fluid (empyema) from infections associated with multiple *S. pyogenes emm* types. The bacterial load originally present in each sample was quantified by qPCR, since some samples had been acquired after antibiotics had been administered preventing bacterial culture. In the published

proteomic study (160), a total of 19 distinct streptococcal proteins were detected by mass spectrometry, however SpyCEP was not among them.

Ethical approval had been previously obtained to measure microbial products such as SpyCEP in anonymised clinical samples that were linked to limited relevant clinical data, as described 2.1.3.

Fluid no.	Type of fluid	Infection	Isolate source	Emm type	<i>S. pyogenes</i> DNA (copies/ μ l)
F1	Tissue exudate	Necrotising fasciitis	Tissue	emm 4	1.96×10^4
F2	Tissue exudate	Necrotising fasciitis	Tissue	emm 28	7.10×10^5
F3	Knee aspirate	Septic arthritis	Aspirate	emm 4	1.45×10^7
F4	Knee aspirate	Septic arthritis	Aspirate	emm 4	9.73×10^4
F5	Pleural fluid	Empyema	Aspirate	emm 1	1.45×10^7
F6	Knee aspirate	Reactive arthritis	Blood	emm 28	ND
F7	Pleural fluid	Empyema	Aspirate	emm 1	1.64×10^4
F8	Pleural fluid	Empyema	Aspirate	emm 1	5.37×10^8
F9	Hip aspirate	Septic arthritis	Blood	emm 77	3.52×10^8
F10	Knee aspirate	Septic arthritis	Aspirate	emm 89	3.23×10^6
F11	Tissue exudate	Necrotising fasciitis	Tissue	emm 89	6.11×10^4
F12	Tissue exudate	Necrotising fasciitis	Tissue	emm 1	2.05×10^5
F13	Tissue exudate	Necrotising fasciitis	Tissue	emm 1	1.66×10^6
F14	Elbow aspirate	Septic arthritis	Aspirate	emm 28	1.23×10^6
F15	Knee aspirate	Septic arthritis	Blood	emm 89	1.71×10^4
F16	Tissue exudate	Necrotising fasciitis	Tissue	emm 4	1.33×10^7
F17	Tissue exudate	Necrotising fasciitis	Tissue	emm 1	1.09×10^6

Table 3-1. Clinical fluid samples from confirmed invasive *S. pyogenes* infections

ND, genome copy number below 6.79×10^2 . Table adapted from Edwards *et al* (160)

Using the bioassay previously described, we attempted to detect SpyCEP activity in the samples by co-incubation of 10 μ l of biofluid with 150 ng exogenous CXCL8 and monitoring the cleavage of exogenous CXCL8 by immunoblot. In 11/17 samples we detected no SpyCEP activity (appendix) however in 6/17 samples we detected the appearance of cleaved CXCL8 at

6 kDa (Figure 3-9), despite SpyCEP not being identified by mass spectrometry. A knee aspirate (F3) and tissue exudate (F16) both from *emm* 4 infections displayed CXCL8 cleavage activity despite 80-fold dilution (Figure 3-9 A, F). Samples from two *emm* 1 infections, pleural fluid (F5) and tissue exudate (F12) and an *emm* 89 tissue exudate (F11) cleaved CXCL8 at a 160-fold dilution (Figure 3-9 B, D, E). One sample was of particular note; F8, pleural fluid from an *emm* 1 infection, showed substantial cleavage of CXCL8 even at 800-fold dilution (Figure 3-9 C). The SpyCEP concentration in these samples ranged from 4.45 – 873.5 ng/ml and is summarised in Table 3-2.

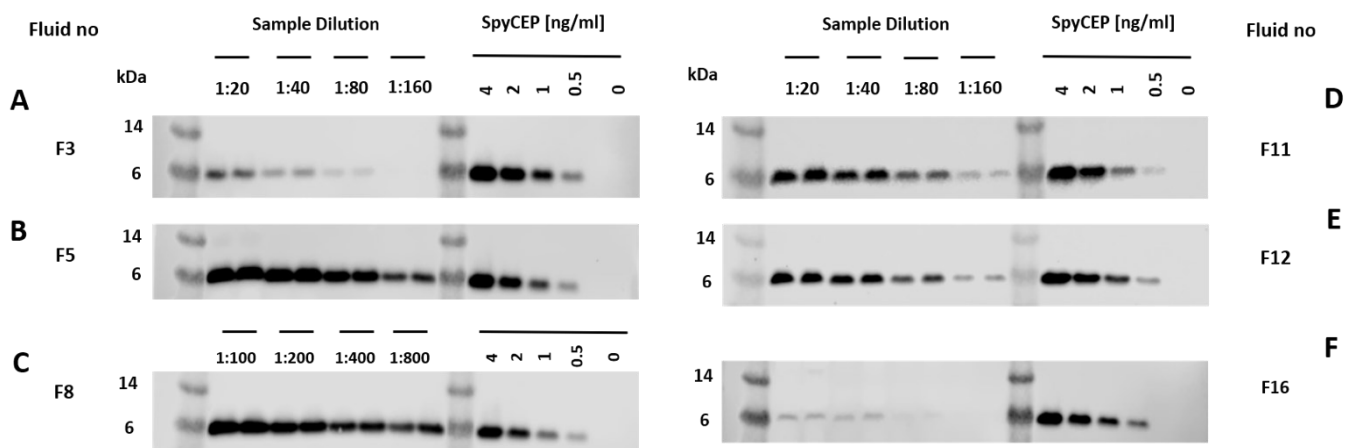


Figure 3-9. Cleaved CXCL8 production from human clinical samples

Immunoblots showing the appearance of cleaved CXCL8 (6 kDa) after a 20-minute, 37°C incubation of 150 ng exogenous CXCL8 with human clinical samples from confirmed *S. pyogenes* infections **A.** F3 (knee aspirate), **B.** F5 (pleural fluid), **C.** F8 (pleural fluid), **D.** F11 (tissue exudate), **E.** F12 (tissue exudate), **F.** F16 (tissue exudate). **A, B, D, E, F** n=2 experimental duplicates for each of the dilutions 1:20 – 1:160. **C** n=2 experimental duplicates for each of the dilutions 1:100 – 1:800. Standard concentrations of SpyCEP (0-4 ng/mL) were also co-incubated with 150ng CXCL8 and the resultant generation of cleaved CXCL8 is shown on the right side of each panel. Densitometry was used to calculate the amount of SpyCEP in each setting.

Fluid no.	Type of fluid	Infection	Emm type	<i>S. pyogenes</i> DNA (copies/ μ l)	SpyCEP (ng/ml)
F3	Knee aspirate	Septic arthritis	<i>emm</i> 4	1.45×10^7	12.76
F5	Pleural fluid	Empyema	<i>emm</i> 1	1.45×10^7	214.6
F8	Pleural fluid	Empyema	<i>emm</i> 1	5.37×10^8	873.5
F11	Tissue exudate	Necrotising fasciitis	<i>emm</i> 89	6.11×10^4	84.4
F12	Tissue exudate	Necrotising fasciitis	<i>emm</i> 1	2.05×10^5	54.8
F16	Tissue exudate	Necrotising fasciitis	<i>emm</i> 4	1.33×10^7	4.45

Table 3-2. Absolute quantification of SpyCEP in human clinical samples

Concentration of SpyCEP (ng/ml) measured in human fluid samples F3, F5, F8, F11, F12, and F16 listed in Table 3-1.

Previous experiments using isogenic SpyCEP negative strains in broth culture and murine infection had confirmed that the cleavage of CXCL8 and the appearance of the 6 kDa band was specific to SpyCEP alone; SpyCEP is the only enzyme known to cleave CXCL8 specifically at the Q₅₉R₆₀ site to reveal the neo-epitope ENWVQ. To ensure that the appearance of the 6 kDa band in human tissues was specific to *S. pyogenes* infection, and not an assay artefact or nonspecific cleavage by a human protease, 17 additional clinical samples; 7 abdominal fluids, 5 pleural fluids and 5 joint fluids collected from patients with confirmed non-streptococcal infections were subjected to the same bioassay as above. There was no detectable 6 kDa band corresponding to cleaved CXCL8 present in any fluid from non-streptococcal infections (appendix). As the samples were from human infections, it was additionally important to ensure that the detected cleaved CXCL8 (Figure 3-9) was the result of *de novo* cleavage of exogenously added chemokine rather than any remaining background cleaved CXCL8 in the sample. To ensure this was not the case, the all-fluid samples F1-17, were re-assayed at neat and 1:20 dilutions with no added exogenous CXCL8. As before, there was no detected 6 kDa CXCL8 cleavage product (appendix). These data provide the first systematic measurement of SpyCEP concentrations in human invasive *S. pyogenes* samples.

3.2.6 SpyCEP quantification in a patient case study

Despite detection and quantification of SpyCEP in clinical *S. pyogenes* infections samples, the fluid samples above, obtained by interception of routine diagnostic samples, only allowed measurement of SpyCEP levels at a single time point during infection. To monitor SpyCEP levels during the course of an invasive infection samples spanning a patient's infection course were required. Just prior to the commencement of this project a clinical sample series became available that allowed longitudinal measuring of SpyCEP levels.

A middle-aged patient, with a 6-day history of fever and intense leg pain associated with bilateral compartment syndrome was admitted to Hammersmith hospital. Anonymised routine clinical samples linked to routine clinical data were obtained at days 1 -6 of admission. The patient collapsed on day 2 hospitalisation, was found to have systemic acidosis, and required intensive care, artificial ventilation, and hemofiltration. Fasciotomies were performed. On day three of admission the patient was diagnosed with necrotizing fasciitis following a blood culture taken from day 1 which yielded streptococci. The patient underwent extensive surgical debridement on days 3, 4 and 5 of admission. This included the bilateral amputations. The patient was treated with intravenous antibiotics and pooled intravenous immunoglobulin (IVIG) from day 2 of his admission however he died on day 6 of his admission. Exudate fluid from muscle samples and aspirates from blisters surplus to diagnostic requirement were available for analysis.

Utilising the SpyCEP bioassay previously described bioactive SpyCEP present in patient samples was measured. In 5/7 tissue samples assayed from admission day 3, 5, 6 measurable SpyCEP activity was detected (Figure 3-10). SpyCEP activity was not found in any patient serum samples from admission day 1-6 (data not shown).

All 4 tested samples from the initial debridement on day 3 of admission - upper thigh (Figure 3-10 A), skin and subcutaneous tissue from the fasciotomy site (Figure 3-10 B), deeper muscle tissue from the fasciotomy site (Figure 3-10 C) and foot blister aspirate (Figure 3-10 D), were positive for SpyCEP activity. SpyCEP was quantified at 9.09, 196.1, 21.15 ng/g tissue and 26.17 ng/ml blister aspirate respectively. Distal blisters from the right flank and upper left chest were drained on day 5 of admission and found to have no measurable SpyCEP activity (Figure 3-10 E-F). However, subcutaneous tissue debrided from the right flank on day 6 of admission was positive for SpyCEP activity (Figure 3-10 G), with 61 ng/g tissue detected.

Subsequent whole genome sequencing of the *S. pyogenes* strain isolated from the patient's infection revealed a mutation in the CovS regulator. We utilised the bioassay to quantify the bacterial surface and released SpyCEP levels as previously, using bacteria isolated from both muscle and blister fluid. There was little variation in SpyCEP production independent of the bacterial source. Bacteria isolated from the blister yielded a mean surface active SpyCEP concentration 681 ng/ 10^8 CFU, mean released active SpyCEP concentration 0.039 ng/ 10^8 CFU. Bacteria harvested from muscle sample: mean surface SpyCEP concentration 540.8 ng/ 10^8 CFU, mean released SpyCEP concentration 0.038 ng/ 10^8 CFU (graphs not shown). These results aligned closely with our values previously quantified from CovR mutants H292 and H1565.

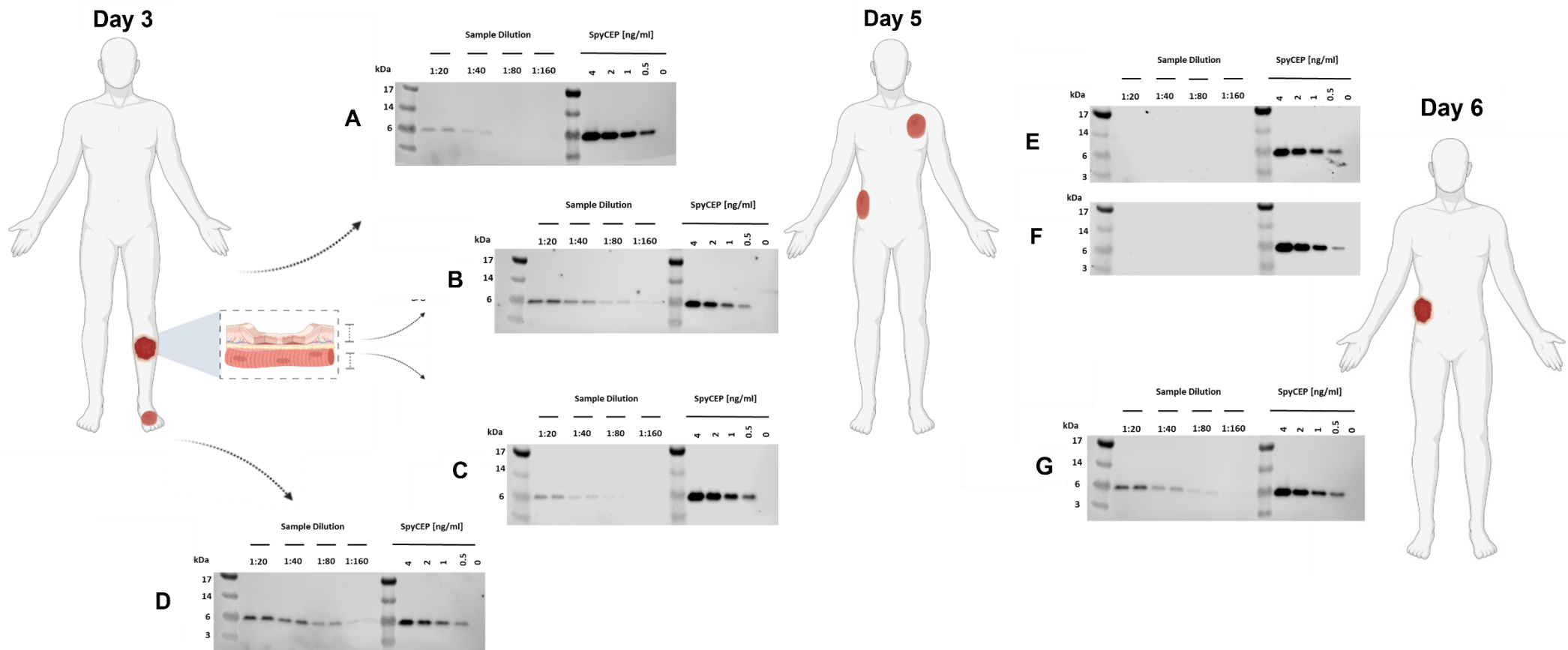


Figure 3-10. Longitudinal quantification of SpyCEP during a *S. pyogenes* necrotizing fasciitis infection

Immunoblots showing the production of cleaved CXCL8 (6 kDa band) after a 20-minute, 37°C incubation of 150 ng exogenous CXCL8 with 10 µl necrotizing fasciitis clinical samples collected from day 3, 5 and 6 of a patient's hospital admission. Clinical samples were collected from multiple infection sites: **A**. Muscle tissue above fasciotomy site, **B**. Dermis and subcutaneous tissue from fasciotomy site, **C**. Deep muscle tissue from fasciotomy site, **D**. Aspirate from foot blister, **E**. Aspirate from right flank blister, **F**. Aspirate from left chest blister, **G**. Subcutaneous tissue from right flank. N = 2 experimental replicates for each dilution 1:20 – 1:160 (A-G). Standard concentrations of SpyCEP (0-4ng/mL) were also co-incubated with 150 ng CXCL8 and the resultant generation of cleaved CXCL8 is shown on the right side of each panel. Densitometry was used to calculate the amount of SpyCEP in each setting.

The patient received IVIG, a treatment consisting of pooled immunoglobulins from at least 1000 separate donors. IVIG is primarily administered to prevent toxin mediated streptococcal toxic shock and to increase the opsonophagocytosis of *S. pyogenes* (161, 162). However, it has also been shown to bind SpyCEP during a screening of IgG reactive *S. pyogenes* cell wall components (120) and to neutralise SpyCEP activity (163). The sample series that demonstrated active SpyCEP despite treatment with IVIG was unexpected and provided an opportunity to examine the impact of IVIG treatment on SpyCEP in *S. pyogenes* necrotizing infection.

We utilised a human IgG ELISA to measure the systemic IgG levels in the patient's serum samples from day 1-6 of admission; in addition, we quantified IgG levels in samples debrided and aspirated from infection sites (Figure 3-11). On day 1 of admission, serum IgG was 7.09 mg/ml which fell to 3.96 mg/ml on day 2. Post IVIG administration on day 2, serum IgG rose steadily to a peak of 19.31 mg/ml on day 4, before falling rapidly on day 5 to 8.4 mg/ml and 8.08 mg/ml on day 6 (Figure 3-11 A). Serum IgG levels remained consistently above measurable IgG from blister aspirates; the highest value was 4.56 mg/ml from right flank blister on day 5 (Figure 3-11 B) and muscle homogenate IgG; highest value 0.26 mg/ml in muscle tissue debrided on day 3 (Figure 3-11 C).

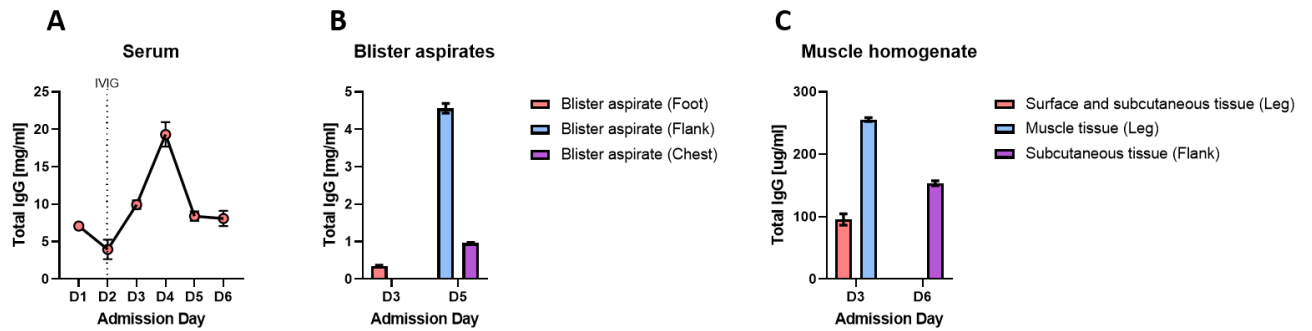


Figure 3-11. Effect of IVIG on systemic IgG concentration

Total human IgG levels as measured by ELISA in **A.** Patient serum from day 1 – 6 of admission, **B.** Blister aspirates from a foot, flank and chest blister aspirated on admission day 3 and 5. **C.** Muscle homogenate from dermis and subcutaneous tissue from fasciotomy site, deep muscle tissue from fasciotomy site and flank subcutaneous tissue. N= 3 technical replicates per data point, error bars represent SD. Dotted line representative of IVIG administration on admission day 2.

3.2.7 SpyCEP activity persists post bacterial death

We were surprised to find quantifiable SpyCEP activity in 5/17 fluid samples and 5/7 patient samples, despite the previous failure to identify SpyCEP using mass spectrometry (160), and the antibiotic treatments and interventions patients are usually subjected to. In the patient case study for example, antimicrobial therapy with beta lactam antibiotics had successfully cleared bacteraemia, however, the disease continued to progress and SpyCEP activity was still detected in the final day 6 sample. This raised the hypothesis that SpyCEP activity and chemokine cleavage might persist even after bacterial killing.

To test this hypothesis, we assessed the ability of SpyCEP on the surface of both live and penicillin treated *S. pyogenes* to cleave CXCL8. We additionally modified the bioassay by multiplexing the secondary antibodies for the immunoblot and conjugating both antibodies to a different fluorophore. This created a two-colour multiplex immunoblot which could

simultaneously detect intact CXCL8 and cleaved CXCL8. Prior to the bioassay, half the bacterial population was treated with penicillin to simulate antibiotic treatment *in vivo* and viability was assessed through plating to confirm killing.

When incubated with exogenous CXCL8 both live bacteria H584 and H1565 produced cleaved CXCL8 (green 6 kDa band), this activity could be titrated out through serial dilution of the bacteria (Figure 3-12 B). As expected, the CovR mutant, H1565, produced more cleaved CXCL8 than the CovR/S wild type, H584. However, unexpectedly, the penicillin-treated (dead) bacteria produced cleaved CXCL8 to an equal extent as untreated (live) bacteria. The appearance of the green 6 kDa cleaved CXCL8 band was no different across all bacterial suspension dilutions regardless of whether exogenous CXCL8 was incubated with live or non-viable bacteria. These data suggest that SpyCEP present on dead bacteria cleaved CXCL8 as effectively as SpyCEP on live bacteria. These data raise important questions pertaining to bacterial clearance during *S. pyogenes* infection and antibiotic failure in serious infection, pointing to a potential role for SpyCEP inhibition.

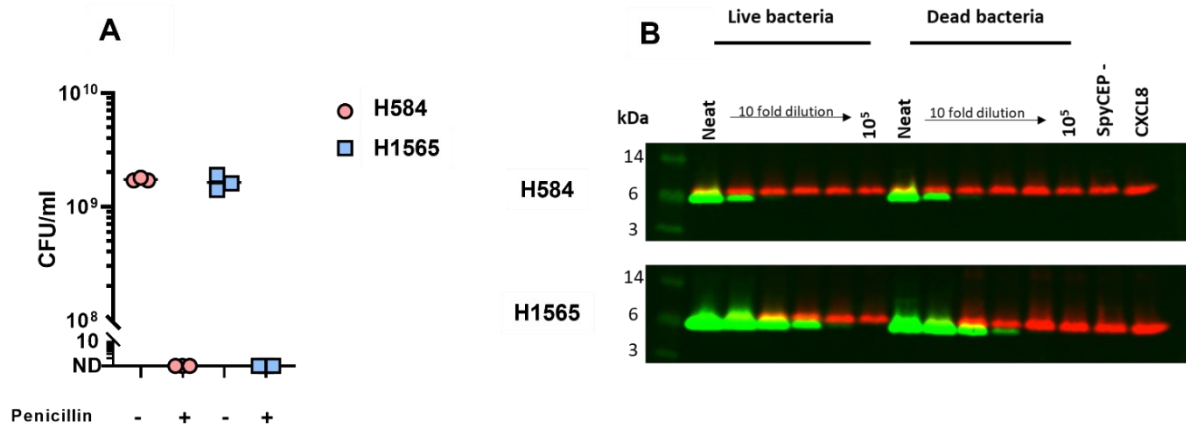


Figure 3-12. SpyCEP activity persists beyond *S. pyogenes* viability

A. 2-hour, 37 °C incubation of H584 (M1 CovR/S_{WT}) and H1565 (M1 CovR_{MT}) treated with 2mg/ml penicillin or PBS control. *S. pyogenes* CFU and viability was subsequently assessed by rich solid phase culture. **B.** Multiplex fluorescent immunoblot showing the appearance of cleaved CXCL8 (green 6 kDa band) and disappearance of full length CXCL8 (red 8 kDa band) after a 20-minute 37 °C incubation of 150 ng CXCL8 with a 10-fold serial dilution of live or dead (penicillin treated) H584 and H1565. Live isogenic SpyCEP negative controls, H1464 (M1 CovR/S_{WT} Δ *cepA*) and H1567 (M1 CovR_{MT} Δ *cepA*) were used for each isolate respectively. Immunoblot is representative of one independent experiment.

3.3 Discussion

Isogenic SpyCEP deletion mutants

In this chapter we successfully deleted the gene encoding SpyCEP by allelic exchange in clinically relevant M1 isolates of both CovR/S WT and CovR mutant background. We confirmed the mutations using whole genome sequencing then used immunoblotting to determine SpyCEP protein expression by this new panel of M1 isolates - in comparison with isogenic M81 *S. pyogenes* and *L. lactis* strains derived in earlier work. We identified bioactive functional SpyCEP on the bacterial cell surface and secreted SpyCEP in the extra cellular growth medium. We have further shown that the heterologous expression of SpyCEP in *L. lactis* isolate H487 is permissible for SpyCEP incorporation into the cell wall. The panel of isogenic M1 and other isolates represent extremely useful tools in analysing SpyCEP function *in vitro* and *in vivo* during this thesis and for subsequent work on *S. pyogenes* pathogenesis.

Quantification of SpyCEP

The primary aim of this chapter was to robustly quantify the surface expression and extracellular release of SpyCEP from *S. pyogenes* isolates and to assess the *in vivo* levels of SpyCEP during invasive infection. Although a polyclonal antibody to a large recombinant fragment of SpyCEP (anti-CEP5) permitted identification of SpyCEP in western immunoblots, the sensitivity was low, despite knowing that CXCL8 cleaving activity was present. To enable quantification of bioactive SpyCEP, we developed a specific bioassay for SpyCEP activity that was suitable for analysing human clinical samples. The sensitivity of the assay is underlined by successful quantification of SpyCEP in samples where mass spectrometry analysis could not detect the protein.

The data showed that the greatest abundance of SpyCEP is attached to the cell wall, on the bacterial (Figure 3-6), and that mutations in CovR can increase the cell wall-associated

SpyCEP 160 – 200-fold. While the repression of SpyCEP expression by CovR has been highlighted previously, this is the first time that SpyCEP protein expression change has been quantified. Comparative data for absolute protein concentration of cell wall anchored streptococcal virulence factors is limited. However previous work in the host laboratory has estimated the highly abundant cell wall-associated M protein to be 69.4 ng/ 10⁸ CFU in strain H584 [Leung, MSc thesis, 2019], a ~15-fold increase over SpyCEP by weight.

Unsurprisingly, the data showed an increase in the SpyCEP concentration released into the growth medium between M1 CovR/S WT and CovR mutants; from undetectable levels to 19.4 ng/ml/10⁸ CFU. The mechanism of SpyCEP release from the cell wall is currently not well understood. Both streptococcal and human proteases have been hypothesized to play a role in ectodomain shedding of SpyCEP, however due to lack of published data no consensus has been reached. Whilst this work does not address the mechanism of SpyCEP cell wall release, quantification of shed SpyCEP is important in helping to understanding streptococcal pathogenesis. Prior work has demonstrated that SpyCEP may influence chemokines distal to the infection site, possibly due to circulating soluble SpyCEP (30). This may have dramatic implications on the early innate immune response. Both KC and MIP-2, mouse homologues of SpyCEP substrates CXCL1 and CXCL2, have been shown in animal models to strongly mobilise neutrophils from the bone marrow and into circulation (164). Soluble SpyCEP may interfere with this process, affecting neutrophil bone marrow release in addition to local neutrophil recruitment, though this was not addressed in the current work. Whilst we did detect SpyCEP activity in the circulating serum of H1565 infected mice, (inferred from ability of serum to cleave exogenous CXCL8), these mice were bacteraemic and so activity may be attributable to bacterial cell wall associated SpyCEP shed into the sample, rather than SpyCEP circulating from the main site of infection. It was notable however that circulating CXCL1

(KC) levels were reduced in mice infected with SpyCEP-producing strains, consistent with a systemic SpyCEP effect. We did not detect SpyCEP activity in serum from the patient case study, even in serum obtained prior to IVIG. This may be because the density of bacteria at the site of clinical infection is less than in an experimental model, and subject to the effects of antibiotic treatment; notably however the patient was not bacteraemic.

In this chapter we have quantified SpyCEP levels in clinical samples for the first time. Infections in which SpyCEP was detected were caused by M1, M4 and M89 *S. pyogenes*, the 1st, 5th and 6th most common genotypes in high income countries (17). There was a general trend for SpyCEP concentration to correlate with *S. pyogenes* genome copies in the clinical samples (Spearman correlation, $P=0.0003$); sample F8 had the highest SpyCEP quantification and the highest bacterial burden. However, there were outliers: samples F11 and F12 produced measurable SpyCEP despite low *S. pyogenes* genome content, yet samples apparently containing a higher content of *S. pyogenes* did not. Variation in SpyCEP production may result from transient modulation or mutation of the CovR/S system, greatly influencing SpyCEP levels, a possibility that has not been evaluated. Sampling practises may also skew the relation between bacterial burden and SpyCEP. Empyema samples for example may be obtained relatively promptly upon hospitalisation, shortly before or after initiation of antibiotic treatment, whereas recognition of necrotizing fasciitis, and subsequent debridement may occur after >24h of antibiotic treatment. Finally, although pleural fluid is provided as a neat sample, joint and tissue aspirates are commonly diluted by saline at the time of sample acquisition. Nevertheless, our work shows that SpyCEP can be abundantly expressed at infection sites and, albeit based on a single example, levels of bioactive SpyCEP can remain high throughout the course of a necrotizing infection despite antimicrobial treatment (Figure 3-10).

In addition to measuring SpyCEP, we also measured total IgG levels in infected samples to determine if IVIG administration had led to an increment in levels. The literature on the efficacy of IVIG administration during severe invasive streptococcal disease is somewhat polarised. Three studies retrospectively examining the effect of IVIG use in invasive infections found no significant reduction in patient mortality, though one examined all necrotising soft tissue infections rather than *S. pyogenes* alone (165-167). Conversely, many clinicians promote IVIG intervention for *S. pyogenes* infection, largely based on the potential to inhibit soluble superantigenic exotoxins. Other studies have shown that IVIG is associated with increased patient survival during invasive infection, although randomised controlled clinical trials have proven challenging (168-170). A recent systematic review and metanalysis has shown IVIG use is associated with a reduction in patient mortality from 33.7% to 15.7% (28).

What is consistent however, is the limited research into the interaction of IVIG and streptococcal proteins. Reglinski *et al* (120) described the *S. pyogenes* surface proteome that binds to IVIG and highlighted that SpyCEP is recognised and bound by pooled human immunoglobulins. The authors, among others, have proceeded to show that vaccination with SpyCEP is protective against experimental *S. pyogenes* infection and that immunisation can successfully produce neutralising antibodies against SpyCEP. (116-118, 121, 123). IVIG is also known to contain inhibiting antibodies to SpyCEP, that prevent CXCL8 cleavage (163).

A primary reason for measuring IVIG in patient samples was to assess whether exogenous IVIG, with SpyCEP neutralisation ability, could reach thrombotic necrotizing tissue at the site of *S. pyogenes* infection when administered intravenously. Previous work suggests that an IVIG concentration of at least 1 mg/mL is required to promote opsonophagocytosis of *S. pyogenes* by human neutrophils (120). However, IgG measured in muscle homogenate was 260 µg/ml, one day post IVIG administration, compared with 9.93mg/ml in the serum. This may

explain a lack of meaningful neutralisation of SpyCEP activity at the site of infection; it is speculated that the large size of IgG molecules may preclude penetration of soft tissues that are subject to high compartment pressures and vasoconstrictor drugs. Blister aspirates distant from the site of infection and serum contained higher IgG concentrations, thus the potential to neutralise SpyCEP may be greater, however, this cannot be confirmed without neutralisation studies.

Interestingly, data from a non-human primate model of infection has now shown that vaccine induced anti-SpyCEP antibodies do not protect by traditional opsonophagocytic means, instead they work by inhibiting the activity of SpyCEP against chemokines (125). This is likely to be the case for antibodies from pooled immunoglobulins that recognise SpyCEP. These data highlight the potential of therapeutically targeting SpyCEP in invasive diseases where SpyCEP is known to strongly contribute to pathogenesis, such as necrotizing fasciitis (30, 140).

The requirement for a SpyCEP-specific therapeutic intervention, above and beyond antibiotic treatment, is further highlighted by the results from the patient case study and the discovery that SpyCEP activity persists beyond *S. pyogenes* cell death. The patient had received broad spectrum antibiotics that successfully cleared the bacteraemia, however the soft tissue infection still progressed and upon death the soft tissues had high levels of measurable SpyCEP activity, despite few if any viable streptococci remaining. Separate *in vitro* experiments clearly demonstrated that killing of streptococci with penicillin had no impact on the abundance of bioactive SpyCEP at the bacterial surface. The persisting ability of SpyCEP to cleave human chemokines effectively, despite bacterial killing, may present a chronic obstacle to phagocyte-mediated bacterial clearance and may explain the large numbers of persisting *S. pyogenes* seen in histopathological sections from cases of lethal necrotising infection (30, 115). Often any neutrophil infiltrate is observed to be ‘held back’. Large numbers of antibiotic-killed *S.*

pyogenes may readily protect any minority of live bacteria through bystander SpyCEP action. Hence, despite treatment and systemic clearance of bacteria, local clearance may still be impaired, providing a protected niche for continued disease progression that neutrophils cannot access. A specific, targeted therapeutic agent, capable of binding and neutralising soluble and cell wall bound SpyCEP could have measurable impact on both slowing and treating invasive streptococcal disease.

The robust quantification of SpyCEP in this chapter will provide a useful metric in helping develop novel, SpyCEP targeting, therapeutics that will be explored in the next chapter of this thesis

4 Results – Characterisation of enzymatic activity – relation to structure and inhibitor development

4.1 Introduction

SpyCEP has distinctive structural features. It autocatalytically processes itself into two discrete polypeptides, each containing a share of the three catalytic residues required for chemokine cleavage. As a result, SpyCEP non-covalently re-associates its N and C – terminal proteins to form the active enzyme - a maturation process that is unique to SpyCEP among streptococcal proteases (135). Bar these mechanistic data, there has been a historical dearth of research into the structural characterisation of SpyCEP, largely hindered by an inability to reproducibly express recombinant protein. Therefore, the biophysical characterisation of SpyCEP has been limited to description of the CXC chemokine substrate range, with very little known about the enzymatic function of SpyCEP.

During the course of this thesis the crystal structure of SpyCEP was successfully solved to a 2.8Å resolution (131) and further improved to a 2.2Å resolution (132), in part driven by increasing interest in SpyCEP as a vaccine candidate. These breakthrough publications have shed light on the structural domains and features present in SpyCEP, allowing for deeper insights into how the structure of SpyCEP might affect protease activity. However, experimental data are required to characterise and quantify enzyme activity, in order to advance the development of anti-SpyCEP therapeutics. Increased understanding of the SpyCEP-CXCL8 interaction, would facilitate downstream inhibitor or vaccine design.

In the second chapter of this work, the first aim was to stably express multiple recombinant SpyCEP constructs in order to interrogate the domains necessary for enzymatic activity. The

second aim was to develop and validate one or more assays to characterise the enzymatic activity of the SpyCEP constructs. A final aim was to use a high-throughput assay of SpyCEP activity to run a large, industrial compound screen to identify potential SpyCEP inhibitors.

The majority of the data described in this chapter was collected while on secondment at GSK, Stevenage.

4.2 Results

4.2.1 Expression and activity of recombinant SpyCEP

Due to the longstanding and problematic history with recombinant SpyCEP expression, both in local and global academic laboratories; protein expression was outsourced commercially. The SpyCEP sequence from the *emm 1 S. pyogenes* genome SF370 was codon optimised and synthesised by GenScript prior to recombinant expression in *E. coli* as part of a Discovery Partnership with Academia Collaboration (DPAC) at GSK Stevenage, as described in 2.3.1. SpyCEP constructs were designed and expressed representing full-length enzyme (SpyCEP³⁴⁻¹⁶¹³); the N-terminal polypeptide (SpyCEP³⁴⁻²⁴⁴); and the C-terminal polypeptide (SpyCEP²⁴⁵⁻¹⁶¹³). Constructs were also generated with alanine substitutions to replace catalytic residues in the N-terminal fragment (SpyCEP³⁴⁻²⁴⁴ D151A); the C-terminal fragment (SpyCEP²⁴⁵⁻¹⁶¹³ S617A); and the full-length enzyme (SpyCEP³⁴⁻¹⁶¹³ D151A, S617A). Constructs and nomenclature are summarised in Table 4-1.

SpyCEP construct	SpyCEP amino acid sequence
SpyCEP	Full length SpyCEP ³⁴⁻¹⁶¹³
SpyCEP ^{DASA}	Full length double mutant SpyCEP ³⁴⁻¹⁶¹³ D151A, S617A
N-terminal (N)	N-terminal fragment SpyCEP ³⁴⁻²⁴⁴
C-terminal (C)	C-terminal fragment SpyCEP ²⁴⁵⁻¹⁶¹³
Re-associated SpyCEP (R.E)	N-terminal (N) + C-terminal (C) fragments
N-terminal ^{DA} (N ^{DA})	N-terminal mutant fragment SpyCEP ³⁴⁻²⁴⁴ D151A
C-terminal ^{SA} (C ^{SA})	C-terminal mutant fragment SpyCEP ²⁴⁵⁻¹⁶¹³ S617A
s.pSpyCEP	SpyCEP from <i>S. pyogenes</i> supernatant

Table 4-1. Recombinant and native SpyCEP constructs

Seven GenScript SpyCEP constructs were recombinantly expressed in *E. coli*. The s.pSpyCEP construct was expressed natively and purified from *S. pyogenes* supernatant, see Table 2-1 and 2.3.2.

To ascertain biological activity of the recombinant SpyCEP proteins all constructs were screened for CXCL8 cleavage activity using the 2-colour fluorescent bioassay developed in chapter 3. It was expected that full length and re-associated constructs would retain activity provided that the catalytic residues were intact. Conversely, it was expected that individual N or C-terminal fragments, and constructs with mutated catalytic residues would lack activity. SpyCEP constructs were incubated with CXCL8 for 2 hours at 37°C and subsequently reactions were immunoblotted with antibodies to detect cleaved CXCL8 (red 6 kDa band) and intact CXCL8 (green 8 kDa band) (Figure 4-1). Natively produced SpyCEP (s.pSpyCEP), isolated from the supernatant of *S. pyogenes* was included as a positive control. As expected, both full length recombinant SpyCEP and natively expressed SpyCEP successfully cleaved CXCL8, this cleavage was not apparent when CXCL8 was incubated with the double catalytic mutant, SpyCEP^{DASA}. The independently expressed N and C-termini, when re-associated (R.E SpyCEP), successfully cleaved CXCL8 to levels that were comparable to full-length enzyme and native SpyCEP. The observed cleavage activity was in concordance with the natural maturation process for SpyCEP and with previously published data (135). When assayed for independent activity, the N-terminal polypeptide could not cleave CXCL8, however, unexpectedly, the C-terminal construct of SpyCEP successfully cleaved CXCL8, although cleavage was not complete (both red and green banding present). The observed proteolytic activity was negated when the catalytic residue S617 was substituted with an alanine; no cleaved CXCL8 was present when the C-terminal^{SA} construct was incubated with CXCL8. Interestingly, the activity of this construct was enhanced, equal to wild type levels, when reassociated with the catalytically inactive N-terminal^{DA} construct. Importantly, the inactive N-terminal^{DA} construct was incapable of restoring proteolytic activity when reassociated with the inactive C-terminal^{SA} construct. These data suggest the C-terminal catalytic serine residue is vital for enzymatic activity while the N-terminal aspartate residue may be dispensable. These

data additionally show that, using the immunoblot assay, the C-terminal of SpyCEP alone is capable of CXCL8 proteolytic activity, a novel finding.

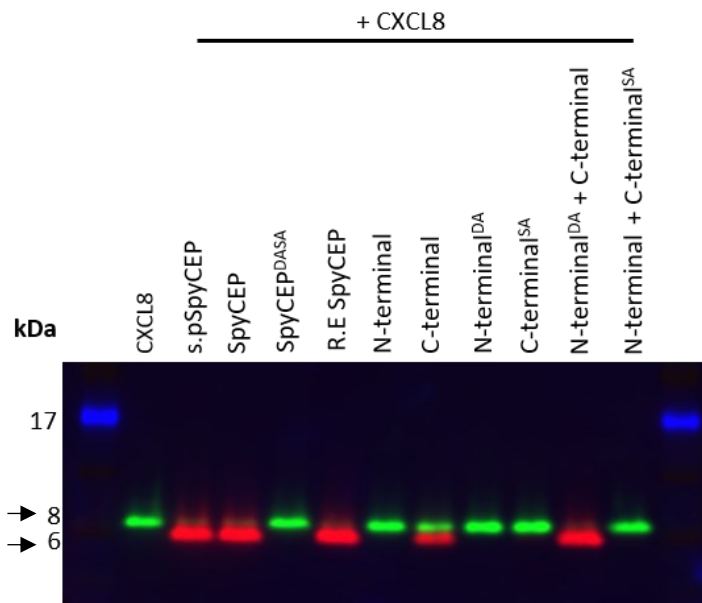


Figure 4-1. CXCL8 cleavage activity of recombinant SpyCEP constructs

Two colour immunoblot showing cleavage of 150ng CXCL8 incubated for 2 hours at 37 °C either alone (2nd lane) or with the panel of SpyCEP constructs at a 1:50 molar ratio in favour of chemokine. 8 kDa banding represents intact CXCL8 (anti-CXCL8 antibody); 6 kDa red banding represent cleaved CXCL8 (anti- ENWVQ). 17 kDa molecular weight marker in blue, 8 and 6 kDa highlighted by arrows. Figure is representative of two immunoblots.

In order to determine that the C-terminal activity detected in the bioassay was not an artefact of the immunoblot, which necessarily uses a large amount of reagent to enable visualisation and requires at least 2-hour incubation, a secondary method was used. Proteolytic activity of the C-terminal construct was assessed in a CXCL8 ELISA, an assay where SpyCEP activity is detected through a reduction in measured chemokine i.e., relies on a reduction in substrate. Full length SpyCEP and C-terminal constructs were incubated with CXCL8, and the remaining intact CXCL8 was measured at 5, 10, 15, 30 and 60-minutes post incubation. The C-terminal constructs were assayed at multiple molar ratios to CXCL8, ranging from 1:5 – 1:250, C-

terminal: CXCL8 respectively. Activity of full length SpyCEP was measured at a reduced concentration, 1:1000 and the inactive C-terminal^{SA} mutant assessed at the highest concentration, 1:5.

Full length SpyCEP rapidly and efficiently cleaved CXCL8 to near completion, over 50% of the CXCL8 input was cleaved after 5 minutes of incubation and only 6.25% remained after 1 hour (Figure 4-2). Proteolytic activity was also detected when testing the C-terminal constructs, with a dose-dependent cleavage profile evident. At 1:5 molar ratio, the C-terminal of SpyCEP showed steady cleavage of CXCL8 over a 60-minute period; 9% of the CXCL8 input was cleaved after 5 minutes, 25% after 30 minutes and by 60 minutes 42% of the CXCL8 had been cleaved. At the lowest concentration tested, 1:250, the C-terminus had cleaved 9% of the starting CXCL8 by 60 minutes. As expected, the C-terminal^{SA} catalytic mutant did not display enzyme activity in this assay, despite being assayed at the highest concentration, 1:5. After a 60-minute incubation the full length and C-terminal SpyCEP construct at all concentrations, bar 1:250, had significantly cleaved more CXCL8 compared to the C-terminal^{SA} catalytic mutant.

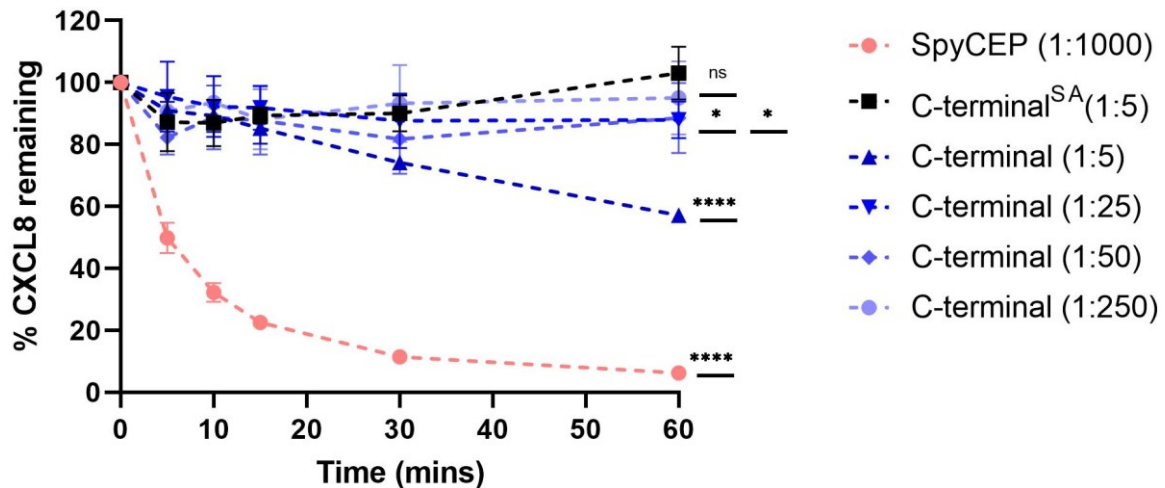


Figure 4-2. Cleavage activity of SpyCEP and C-terminal SpyCEP²⁴⁵⁻¹⁶¹³ constructs using CXCL8 ELISA

SpyCEP constructs were co-incubated with CXCL8. Graphs show residual CXCL8 over a 60-minute time course at room temperature, using SpyCEP at a 1:1000 ratio to CXCL8; C-terminal at 1:5 – 1:250 molar ratio to CXCL8; and C-terminal S617A mutant at a 1:5 molar ratio to CXCL8. Reactions were halted at specific timepoints by the addition of Pefabloc (2mg/ml), n=6 experimental replicates for each construct, data points show means, error bars represent SD. ns $p > 0.05$, * $p \leq 0.05$, **** $p \leq 0.0001$, at 60 minutes as determined by ordinary one-way ANOVA.

These data confirmed the immunoblot results and underlined that SpyCEP activity against CXCL8 can be conferred by the C-terminus of the protein. The data further highlight the importance of the catalytic 617 serine residue in enzyme activity.

Proteolysis of CXCL8 has been the paragon for SpyCEP substrate activity, however chemokine cleavage is not confined to CXCL8; the substrate range encompasses all neutrophil-specific ELR+ CXC chemokines, in addition to a newly discovered substrate, LL-37 (141). To determine if the recombinant SpyCEP constructs showed activity against a wider range of substrates and to additionally examine the C-terminal activity against these ligands, SpyCEP

constructs were incubated with CXCL1, 6 and 8 and chemokine cleavage was examined by SDS-page due to a lack of reagents to detect cleaved CXCL1 and CXCL6.

CXCL1, 6 and 8 were incubated for 2 hours at 37 °C with either full length SpyCEP or C-terminal SpyCEP at a range of molar ratios, 1:5 –1:5000, enzyme: chemokine respectively (Figure 4-3). Catalytically inactive mutants SpyCEP^{DASA}, C-terminal^{SA} and the inactive N-terminal were included as controls and assayed at the highest concentration, 1:5.

Both CXCL1 and CXCL8 were cleaved to completion by all molar ratios of full length SpyCEP, shown by the reduction in chemokine size compared to the chemokine control. In contrast, CXCL6 was only cleaved by SpyCEP up to a 1:500 molar ratio, no cleavage was present at 1:5000. As expected, the SpyCEP^{DASA} construct was inactive in this assay against all chemokines (Figure 4-3 A). Conversely, the SpyCEP C-terminal fragment was required at a much greater concentration than full length enzyme for detectable activity. Proteolytic cleavage of both CXCL1 and CXCL8 by the C-terminal fragment was only evident at the highest concentration assayed (1:5) and no C-terminal activity was detected against CXCL6 at any tested concentrations (Figure 4-3 B).

To ascertain whether the SpyCEP C-terminal was capable of cleaving CXCL6 at all, or whether its substrates were limited to CXCL1 and CXCL8, we further incubated CXCL6 with the C-terminal for an extended 20-hour incubation at 37°C (Figure 4-3 C). Under this prolonged incubation we detected partial cleavage, represented by the double chemokine bands, at the highest concentration of C-terminal, 1:5.

These data confirmed that the proteolytic activity of the SpyCEP C-terminal is not limited to the preferred substrate CXCL8. However, the results do highlight the marked difference in cleavage efficiency between full length enzyme and C-terminal construct in addition to the varied cleavage efficiency across the CXC substrate range.

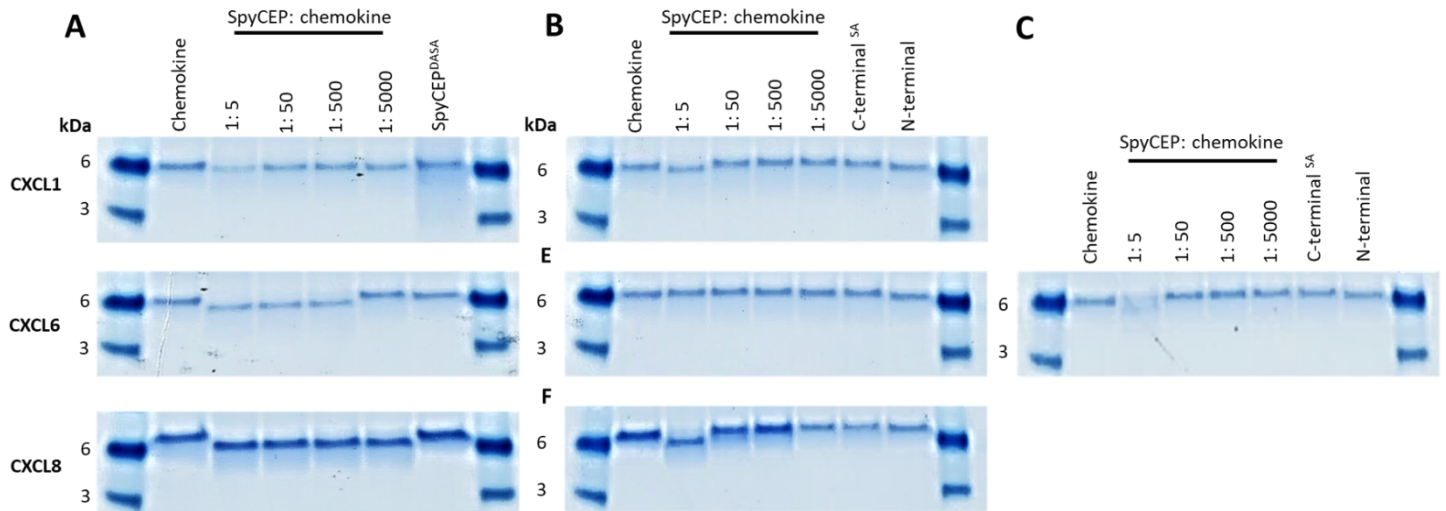


Figure 4-3. Comparative cleavage of CXCL1, CXCL6 and CXCL8 by SpyCEP and C-terminal SpyCEP

SDS-page analysis of 5 μ M human CXCL1, CXCL6 or CXCL8 cleavage at 37°C for 2-hours by an equal volume of **A.** full length SpyCEP or **B.** C-terminal SpyCEP at a 1:5 – 1:5000 molar ratio (SpyCEP: chemokine). **C.** SDS-page analysis of 5 μ M human CXCL6 cleavage at 37°C for 20-hours by an equal volume of C-terminal SpyCEP at a 1:5 – 1:5000 molar ratio (SpyCEP: chemokine). SpyCEP^{DASA}, C-terminal^{SA} and N-terminal controls were all assayed at the highest 1:5 molar ratio. Representative of 2 experiments

To determine whether C-terminal SpyCEP also had activity against the newly discovered non-chemokine substrate of SpyCEP, LL-37, we assessed its cleavage by immunoblot. Full length SpyCEP, the independently expressed N and C-terminal constructs and the catalytic mutants of each were incubated with LL-37 for a prolonged 18-hour incubation at 37°C, cleavage was measured by a reduction in LL-37 molecular weight (Figure 4-4).

Under these conditions' full length SpyCEP was capable of degrading LL-37, as shown by the decrease in LL-37 molecular weight in lane 2. This size shift is in accordance with the published 1 kDa reduction of LL-37 after SpyCEP cleavage (141). No other SpyCEP construct, including the C-terminal fragment, displayed proteolytic activity against LL-37.

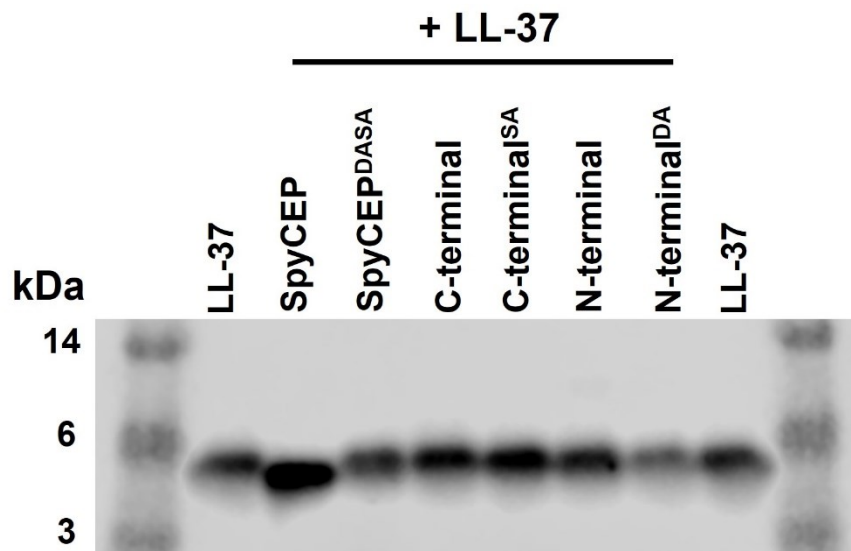


Figure 4-4. Immunoblot of LL-37 cleavage by recombinant SpyCEP constructs

Immunoblot of 5.56 μ M human LL-37 incubated for 18 hours at 37 °C either alone (lane 1 and 8) or with a panel of SpyCEP constructs at a 1:10 molar ratio in favour of LL-37. 4.5 kDa full length LL-37 and 3.5 kDa SpyCEP cleaved LL-37 bands detected with 2 μ g/ml sheep IgG polyclonal LL-37 antibody and rabbit anti-sheep IgG antibody (1:40,000). Figure is representative of 2 immunoblots.

As demonstrated, there are disparities in SpyCEP activity across its substrate range; this is an observation that has been previously recognised, but not quantified (139). Utilising the two preferred substrates identified in the data obtained, CXCL1 and CXCL8, we further investigated the kinetics of SpyCEP-mediated chemokine cleavage, assessing the cleavage of chemokine over time by ELISA. 20nM of CXCL8 and 20nM of CXCL1 were incubated at room temperature with an equal volume of 50pM or 100pM of full length SpyCEP respectively (Figure 4-5). Remaining chemokine was quantified at specified time points over a 30-minute

reaction. 50pM SpyCEP rapidly cleaved 20nM CXCL8; 85% of chemokine was cleaved after a 10-minute incubation (Figure 4-5 A). Proteolysis of CXCL1 occurred at a slower rate, despite the reaction containing twice the concentration of SpyCEP; 100pM SpyCEP only cleaved 24% of the CXCL1 input after a 10-minute incubation (Figure 4-5 B). By 30 minutes incubation, 50pM SpyCEP had cleaved 92% of the CXCL8 input compared to 100pM SpyCEP cleaving 47% of the CXCL1 input.

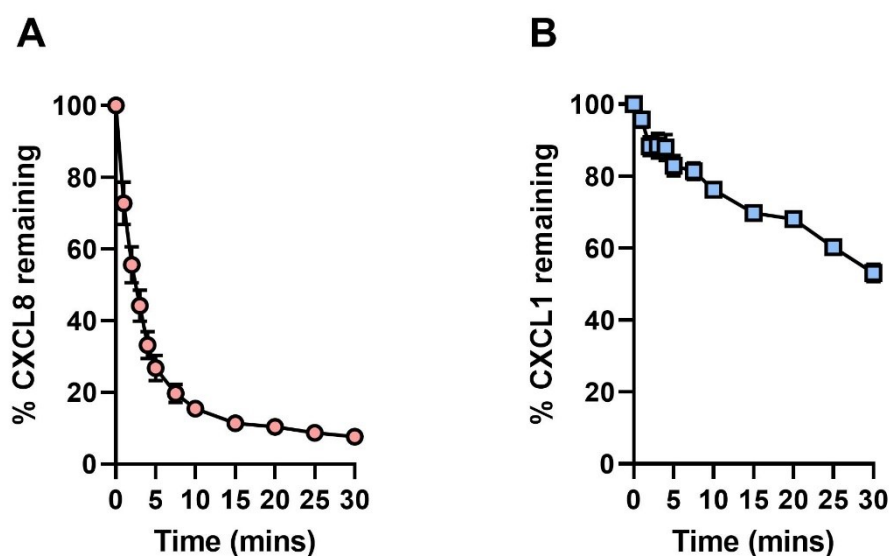


Figure 4-5. Cleavage of CXCL8 and CXCL1 by recombinant SpyCEP

30-minute, room temperature cleavage time course of an equal volume of: **A.** 20 nM CXCL8 by 50 pM SpyCEP and **B.** 20 nM CXCL1 by 100pM SpyCEP. Cleavage reactions were halted at specified timepoints by the addition 2 mg/ml Pefabloc (final concentration) prior to detection by ELISA. N=6 biological replicates for each data point, error bars represent SD of mean values.

Linear regression was applied to data obtained in the initial 5 minutes of each reaction – where SpyCEP cleavage occurs at maximum rate- to calculate the CXCL1 and CXCL8 turnover under these conditions. 50 pM of SpyCEP was capable of cleaving 2.84 nM of CXCL8 per minute, in contrast to 100 pM SpyCEP cleaving 0.62 nM CXCL1 per minute. These data, the results

from our SDS-PAGE cleavage analysis, and previously published chemokine cleavage gels (139), strongly highlight that CXCL8 is the preferred substrate for SpyCEP.

4.2.2 Kinetic parameters of full length and C-terminal SpyCEP

Whereas western blotting and SDS-PAGE provide opportunity to detect generation of a specific cleavage product, these methods cannot easily be used for kinetic analyses and require large amounts of reagent. Current CXCL8 ELISAs can only detect substrate disappearance and, since chemokines are small and may be subject to non-specific proteolytic events, they may not be sufficiently accurate for measurement of catalysis and are difficult to undertake rapidly. Modifications of the assay are possible, however, there are intrinsic specificity drawbacks to monitoring the disappearance of chemokines as they are prone to oligomerisation and non-specific surface binding. In order to more precisely compare the activity of the recombinant SpyCEP constructs and evaluate their differing enzymatic efficiencies a more robust, high throughput method of measuring SpyCEP activity was required, that examined the generation of cleavage product. i.e., via the detection of the 13 amino acid cleavage product released after SpyCEP specific CXCL8 proteolysis.

Development of a high-throughput screen for SpyCEP activity

In collaboration with GSK Stevenage, a mass-spectrometry based assay was designed and validated to monitor the appearance of the 13 amino acid portion of the C-terminal α -helix of CXCL8 that is released upon cleavage by SpyCEP. Automated processing of 384 well plates into a robotic sampler and mass spectrometry injection system, enabled massive upscaling of SpyCEP cleavage reactions. This high throughput technology facilitated precise measurements of CXCL8 cleavage which enabled experimental description of SpyCEP kinetics.

250 pM of full length SpyCEP and 40 nM C-terminal SpyCEP were incubated with a range of CXCL8 concentrations, from 6.25- 2000 nM. Cleavage by full length SpyCEP was assayed over a 30-minute, room temperature, time course, in comparison to 4-hour, room temperature, time course for C-terminal SpyCEP. As expected from our previous results, both SpyCEP constructs produced a quantifiable CXCL8 cleavage product, which accumulated as cleavage time increased (Figure 4-6). During a 30-minute incubation, 250 pM of full length SpyCEP was able to cleave the 6.25 – 250 nM CXCL8 to completion. CXCL8 concentrations above 250 nM were incompletely cleaved (Figure 4-6 A). 40 nM of C-terminal SpyCEP was also able to cleave 6.25-250 nM CXCL8, however over a 4-hour incubation. With CXCL8 inputs that exceeded 250 pM, incomplete cleavage of CXCL8 was observed (Figure 4-6 B). These cleavage data strongly highlight the differential catalytic efficiency between full length SpyCEP and C-terminal SpyCEP. Both enzymes cleaved 250 nM CXCL8 to completion however the C-terminal required an additional 3.5 hours and a 160-fold increase in protein concentration.

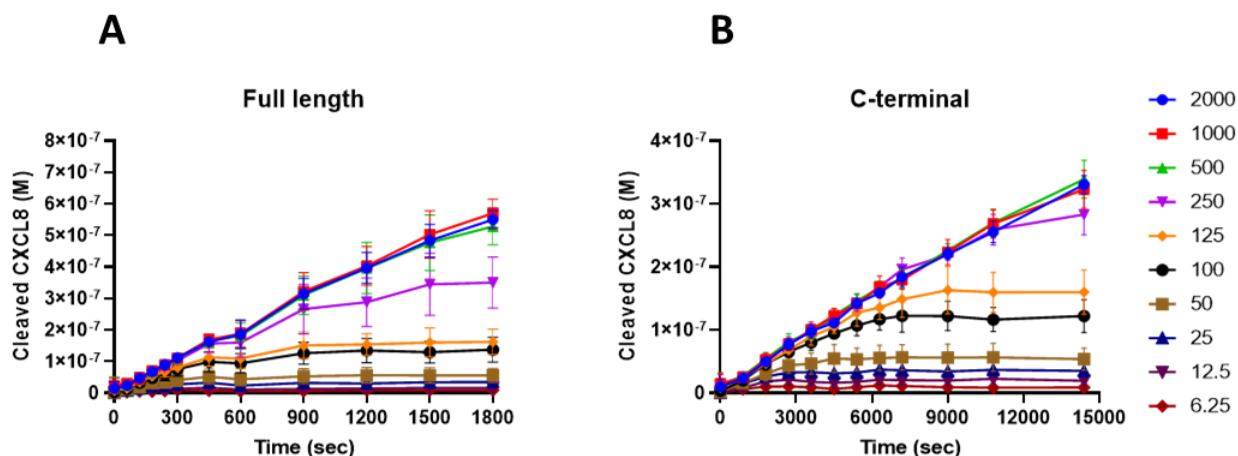


Figure 4-6. CXCL8 cleavage by full length and C-terminal SpyCEP constructs measured by mass spectrometry

A. Production of the 13 amino acid species cleaved from CXCL8 by 250 pM of full length SpyCEP over 30 minutes at room temperature, following incubation with 6.25 nM – 2000 nM CXCL8, 1: 250 – 8000 molar ratio (SpyCEP: CXCL8). **B.** Production of the 13 amino acid species by 40 nM SpyCEP C-terminal fragment over a 4 h room temperature incubation, following incubation with 6.25 nM – 2000 nM CXCL8, 1: 0.15 – 50 molar ratio (SpyCEP: CXCL8). N=5 experimental replicates per data point, error bars represent SD of mean values.

In order to directly compare the catalytic parameters of each enzyme construct, linear regression of the first 5 timepoints of each cleavage reaction above was analysed, where the rate of reaction remains at maximum. These data can be fitted against the CXCL8 substrate concentration to plot Michaelis-Menten graphs (Figure 4-7) from which we can calculate the quantifiable kinetic parameters, K_M and K_{cat} . This allow us to directly compare activity of the two enzymes.

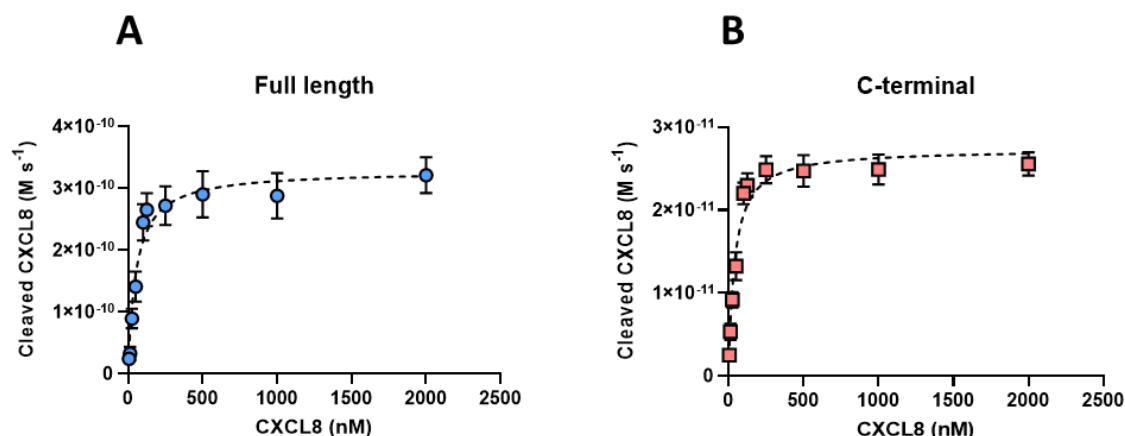


Figure 4-7. Michaelis-Menten plots of full length and C-terminal SpyCEP activity against CXCL8

Data points represent the mean change in cleaved CXCL8 production over time ($M s^{-1}$) by **A.** full length SpyCEP and **B.** C-terminal SpyCEP, as calculated from the initial velocity from CXCL8 cleavage (**Figure 4-6**). Error bars represent SD.

Construct	$K_{cat} \pm SD (s^{-1})$	$K_M \pm SD (nM)$	$K_{cat}/K_M (M^{-1} s^{-1})$
SpyCEP	1.34 ± 0.61	53.49 ± 10.36	2.46×10^7
C-terminal	0.00068 ± 0.000027	40.98 ± 7.2	1.67×10^4

Table 4-2. Full length and C-terminal SpyCEP kinetics.

Kinetic parameters K_{cat} and $K_M \pm SD$ for full length SpyCEP and C-terminal SpyCEP derived from Michaelis-Menten graphs $Y = V_{max} * X / (K_M + X)$ and K_{cat} equation $Y = ET * k_{cat} * X / (K_M + X)$ where Et = enzyme concentration, $2.5 \times 10^{-10} M$ for full length SpyCEP and $4 \times 10^{-8} M$ for C-terminal SpyCEP

The K_M results for full length SpyCEP and C-terminal SpyCEP with native substrate CXCL8 were 53.49 and 40.98 nM respectively. These nanomolar results highlight an extremely high binding affinity for the CXCL8 substrate, with similar values suggesting a similar binding affinity for substrate for the two enzyme constructs. Conversely, the K_{cat} values differed

greatly, from 1.34 molecules per second for full length enzyme to 2.45 molecules per hour for the C-terminal enzyme, a 1,970-fold reduction. In summary, the data underlined greatly reduced activity of the C terminal SpyCEP fragment, compared with full length SpyCEP, but binding of the CXCL8 substrate could be accounted for in large part by the C-terminal alone.

4.2.3 Industrial compound screen for a SpyCEP inhibitor

Having shown successful activity and characterised the kinetic parameters of recombinant SpyCEP, the constructs and high throughput assay were exploited to screen a large panel of potential SpyCEP inhibitors. Chapter 3 highlights a very real clinical need, over and above antibiotics, for therapeutics that target SpyCEP in invasive disease. The collaboration with industry provided a unique opportunity to exploit a large compound library to screen for molecules with inhibitory activity. The immunoblot assay developed in chapter 3 of this thesis could be used to screen for inhibitors of SpyCEP. However, due to the low throughput nature of immunoblotting it would be extremely reagent intensive and time consuming to screen any great number of molecules. The ELISA-based assay, monitoring the disappearance of CXCL8 could also be used, however the more robust and specific high throughput mass spectrometry assay developed in tandem with GSK during this chapter, provided an ideal system to leverage for a rapid, large-scale screening.

Having helped establish the MS assay as part of this doctoral project, the large-scale screen was wholly led and undertaken by the industrial partner based in the UK and USA, as contractually stipulated, whereby any inhibitors identified would then be available for testing. The practical aspects of screening for inhibitory molecules were carried out at GSK Stevenage and occurred in 4 separate stages as illustrated in (Figure 4-8) These experiments were

performed in parallel to this thesis entirely by GSK research scientists but as a collaboration with our laboratory. Firstly, a library of >1 million compounds were streamlined into a smaller library of 44,748 molecules, that were predicted to have a greater chance of inhibitory activity. This bespoke library consisted of compounds with properties consistent with potential serine protease inhibition, for example, molecules containing short peptides that mimicked the CXCL8 cleavage site, electrophiles, transition state analogues and known protease inhibitors. An additional set of compounds was also added that represented the structural diversity of the larger initial library to maximise the possibility of inhibitor discovery [personal communication, GSK].

The second stage of compound selection consisted of a single concentration inhibitory screen. This provided an initial highlight of any molecules showing potential inhibitory activity. 50µM of each compound was incubated with 100pM SpyCEP for 30 minutes at room temperature. The resultant preparation was then incubated with 50nM CXCL8 for 30 minutes at room temperature and the production of cleaved CXCL8 measured by mass spectrometry. 1068 / 44,748 screened molecules were identified as potential inhibitors and were carried into a third screening stage.

The tertiary screening consisted of an 11-point dose response curve for each compound, assayed using the above conditions, with 5µM of each compound used as the highest concentration. 48 / 1068 molecules from this screen showed a robust response and reproducible inhibition of SpyCEP activity. These 48 molecules were then further streamlined based on their availability, water solubility, aggregation potential, NMR and LCMS purity and CXCL8 inhibitory activity; to leave 2 compounds (Figure 4-9).

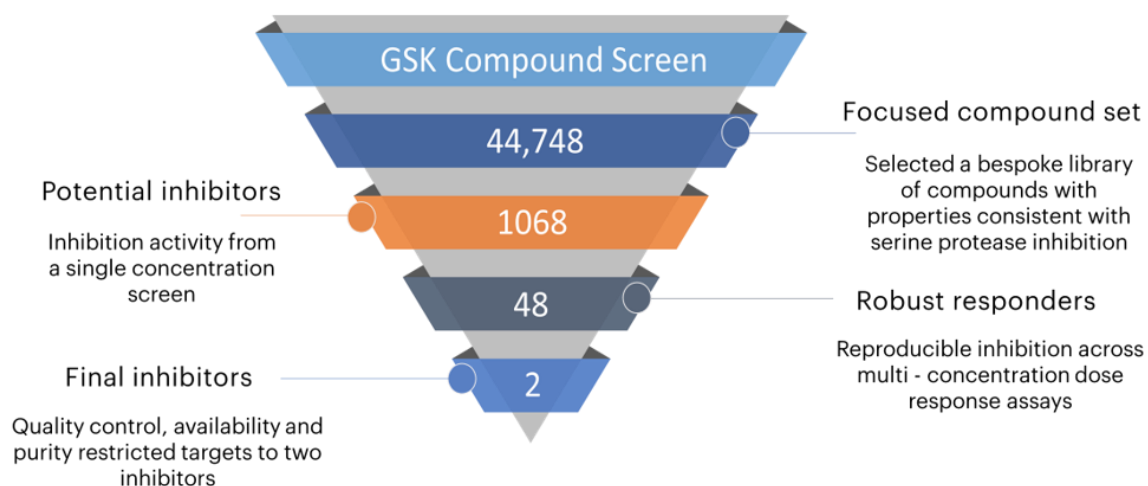


Figure 4-8. Graphical illustration of GSK compound screen

4 stage mass spectrometry screening of 44,748 compounds to identify molecules with anti-SpyCEP activity.

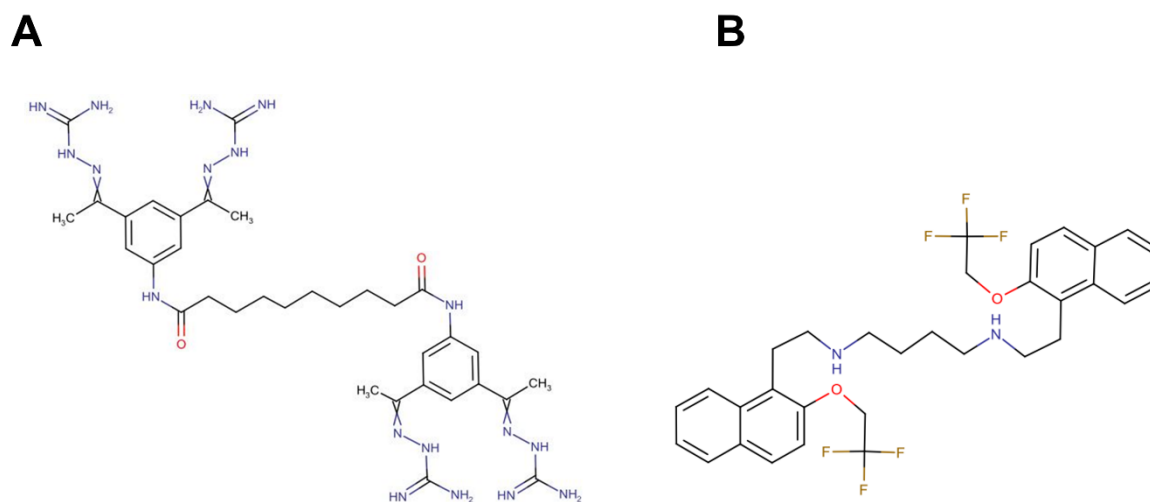


Figure 4-9. Chemical structure of SpyCEP inhibitor 1 and 2

A. Inhibitor 1 and **B.** inhibitor 2, identified from a GSK compound screen of 44,748 molecules as potential SpyCEP inhibitors

The two most promising compounds were transferred to Imperial College London for further *in vitro* and *in vivo* assessment and are referred to henceforth as inhibitor 1 and inhibitor 2.

4.2.4 Discussion

Activity of recombinant SpyCEP

During this chapter a number of recombinant SpyCEP constructs were successfully cloned and expressed commercially, then their proteolytic activity against CXCL1, CXCL6 and CXCL8 was assessed using a range of assays. It was found that SpyCEP cleaves CXCL8 more rapidly than CXCL1, and that CXCL1 is cleaved more rapidly than CXCL6. Whilst additional SpyCEP substrates, such as CXCL2, CXCL3, CXCL5, and CXCL7 were not assessed, it is assumed that enzymatic activity of the recombinant constructs will be conserved. The results fit with the hypothesis that CXCL8 is the major target for SpyCEP, and that cleavage of the additional ELR⁺ CXC chemokines is due to a high degree of structural homology between the proteins (139). This reasoning may explain, in part, why LL-37 can act as a substrate; we have confirmed during this chapter that LL-37 can be cleaved by high concentrations of SpyCEP over a prolonged incubation. However, given the cleavage conditions, it is unlikely these results are relevant *in vivo*, particularly as *S. pyogenes* expresses two other virulence factors which inactivate LL-37: SpeB and SIC (142-144). The structure of the LL-37 monomer is, however, an α -helix, and so shares secondary structural homology with the portion of the C-terminal α -helix of CXCL8 that is released upon SpyCEP cleavage. It is likely that SpyCEP activity is due to these structural similarities between the molecules.

The novel finding from analysis of the SpyCEP constructs during this chapter, is the unexpected activity of the SpyCEP C-terminal. It was found that the C-terminus of SpyCEP can cleave CXCL1, CXCL6 and CXCL8 independent of the presence of the N-terminus. These data conflict with a previously published report demonstrating no measurable proteolytic activity attributed to the C-terminus (135). The failure of the earlier report to detect activity is likely due to the increased concentration and incubation period required for C-terminal activity.

C-terminal activity was entirely unexpected; however, it is not without precedent. The MEROPS database, an archive of proteolytic enzymes, currently classifies SpyCEP as a member of the S8 serine protease family within the SB protease clan (130). There are a number of proteases with this clan that utilise different amino acids within their respective catalytic triads, serine, histidine and glutamate or serine, glutamate, and aspartate for example. Significantly, there are also proteases from other clans which rely on a catalytic dyad instead of catalytic triad for proteolysis. Among others, these proteins use serine and histidine or lysine and histidine as their catalytic residues. Our data showing proteolytic activity of the C-terminus suggest that SpyCEP is capable of chemokine degradation based on a catalytic dyad of histidine and serine, although with greatly reduced efficacy.

There is also precedent for protease activity despite mutations to the catalytic residues. Systematic mutational studies of the catalytic triad of serine proteases have shown that even enzymes with all three sites mutated, can still catalyse the breakdown of a peptide bond at 10^3 fold above the background rate (171-173). This is due to the catalytic power of the enzyme not solely being supplied by the catalytic triad. Additional machinery can contribute to catalysis, other residues surrounding the catalytic pocket can reduce the fall in activity resultant from a catalytic residue mutation, and water molecules can potentially ‘moonlight’ as a missing functional group (173). Further, the oxyanion hole, a region of the active site that is composed of backbone NH groups and amino acid side chains may provide substrate stabilisation and help drive catalysis (173). It is these supplementary features of serine proteases which may help mitigate catalytic triad mutations and maintain a level of proteolysis.

The impact of C-terminal activity on *S. pyogenes* pathogenesis *in vivo* has not been assessed, however it likely to be negligible. The rate of enzyme cleavage was extremely slow, 2.45 molecules of CXCL8 per hour, and it is unlikely that the C-terminus will be present

independent of the N-terminal *in vivo*. However, the discovery does provide functional insights relating to both enzyme activity and to facilitating rational vaccine and therapeutic design. Firstly, the findings point to the C terminus of SpyCEP providing the major substrate binding site for CXCL8. Secondly, in relation to functional inhibition, the majority of *S. pyogenes* vaccines targeting SpyCEP are based on immunisation with the CEP-5 protein (amino acid 35 – 587) (117, 118, 121, 123, 132, 149), a polypeptide which spans the N- and part of the C-terminal domain. Due to the antibody response from these vaccines providing protection by inhibiting SpyCEP activity rather than opsonisation, future vaccine and targeted therapies may have increased efficacy by solely targeting the C-terminal.

Kinetics of active SpyCEP constructs

We have shown by SDS-PAGE experiments that cleavage of chemokine by the C-terminal is far less efficient than by full length enzyme. Furthermore, we have assessed and quantified the catalytic parameters of these active SpyCEP constructs to allow for direct comparison of activity. Our mass spectrometry data showed that full length SpyCEP has a K_M of 53.49 nM and K_{cat} of 1.34 molecules per second and the C-terminus has a K_M of 40.98 nM and K_{cat} of 2.45 molecules per hour. The K_M value is a direct measure of the concentration of substrate required to reach half the maximal velocity of an enzyme; as such it is an indirect measure of enzyme: substrate binding affinity - the lower the K_M , the greater the binding affinity. K_M values between full length SpyCEP and the C-terminal SpyCEP construct were not significantly different, however both were in the low nanomolar range. These low values were somewhat surprising, as K_M values are often reported to be in the μM -mM range, however they are not unprecedented. The serine protease Factor Xa, part of the prothrombinase complex which cleaves prothrombin to thrombin has a K_M value of 150 nM (174) and the serine

protease, human Kallikrein 6 has a K_M reported to be 300 nM (175). These data suggest that SpyCEP has a high binding affinity for CXCL8, which is likely conferred by the C-terminal domain and not dependent on the presence of the N-terminal polypeptide. C-terminal domain substrate binding is consistent with mechanistic data from the streptococcal C5a peptidase and other lactococcal CEPS (133, 176).

The K_{cat} however, also referred to as the turnover number, was markedly different between full length SpyCEP and the C terminal. C-terminal SpyCEP was only able to cleave 2.45 molecules of CXCL8 per hour, a marked reduction in cleavage compared to 1.34 molecules per second for full length SpyCEP. These data show that despite the C-terminal being strongly implicated in binding of substrate, and capable of enzymatic activity alone, the N-terminal is required for optimal activity. Interestingly, data from our immunoblotting screen (Figure 4-1), show that under those assay conditions, enzymatic activity is recovered to wild type levels by the addition of the N-terminal^{DA} inactive mutant. These results indicate the aspartate residue at position 151 may be dispensable and the N-terminal structure itself required for full activity. However, this hypothesis was not evaluated with quantifiable data in this thesis.

These two kinetics parameters can additionally be used to calculate the enzyme specificity constant (K_{cat} / K_M), a value which describes the cleavage efficiency of an enzyme. Our data show full length SpyCEP has an enzyme specificity constant of $2.46 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$. This value is in the order of magnitude expected for serine proteases (173). C-terminal SpyCEP has a value of $1.67 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, demonstrating the C-terminus is ~1500 times less efficient at cleaving CXCL8 than the full-length enzyme. This reduction in efficiency is in concordance with published kinetic data from mutational studies of the aspartate residue in the serine protease subtilisin, from *Bacillus amyloliquefaciens* (177).

SpyCEP inhibitors

The second major output from this chapter was the identification of two potential SpyCEP inhibitors from an industrial screen of 44,748 compounds; utilising the mass spectrometry based SpyCEP activity assay developed in this chapter. Data received from GSK indicated that these molecules successfully inhibited SpyCEP activity, however they could not elucidate the molecules' mechanism of action. Failures in both surface plasma resonance and differential scanning fluorimetry experiments at our industrial partner laboratories meant that successful binding or interaction of inhibitors with SpyCEP could not be confirmed [personal communication]. Consequently, inhibition of SpyCEP activity may have been an artefact of CXCL8 inhibitor binding, a hypothesis examined in chapter 5 of this thesis.

A literature search on the two compounds highlighted publications and relevant that was relevant for downstream functional assessment of the inhibitors. Whilst there was no data for inhibitor 2, inhibitor 1 had a publication record from 1995, where it was first developed as a selective inhibitor of nitric oxide production by inflammatory macrophages and demonstrated protection in mice against a lethal endotoxin challenge (178). It was shown to further suppress proinflammatory cytokines TNF- α , IL-1 β , IL-6, MIP-1 α and MIP-1 β (179) and subsequently discovered to be suppressing TNF- α translation efficiency and blocking p38 map kinase activation by targeting the kinase, c-Raf (180-182), as well as protecting mice from polymicrobial sepsis (183). It was not, however, described as a serine protease inhibitor.

Due to its anti-inflammatory profile, inhibitor 1 has been evaluated as a therapeutic treatment option in a number of human diseases. It has been assessed as an adjunctive treatment in combination with high doses of IL-2 in a phase 1 clinical trial for the treatment of renal cancer and melanoma (184). This was subsequent to *in vivo* data showing co-administration of inhibitor 1 and IL-2 protected against fatal IL-2 related toxicity at doses required for anti-

tumour effect (185). It has been additionally assessed in phase 1 and phase 2 trials as a therapeutic treatment for patients with moderate to severe Crohn's disease (182, 186, 187) as well as being explored as a protective agent against post – endoscopic retrograde cholangiopancreatography (ERCP) related hyperamylasaemia and acute pancreatitis (188). It is additionally being explored in a variety of pre-clinical works, assessing its protective role in arthritis, cancer metastasis, Parkinson's and Alzheimer's disease and Haemophilus influenzae and malarial infection (189-194). Inhibitor 1 is now seen in the literature as a general inhibitor of the inflammatory response in mice and human. The current proposed mechanism for *in vivo* action is twofold, by direct inhibition of c-Raf and subsequent p38 map kinase activation (181, 182) and additionally by direct stimulation of the efferent vagus nerve, activating the cholinergic anti-inflammatory pathway (195). This pathway leads to downstream suppression of cytokines resultant from the release of acetylcholine throughout organs of the reticuloendothelial system (196). The anti-inflammatory action and sepsis protection profile of inhibitor 1 raises important questions for therapeutic use in severe *S. pyogenes* infections and will be discussed in chapter 6 of this thesis.

In this chapter, successful expression of recombinant SpyCEP was achieved and it was determined that chemokine cleavage activity can be conferred by the C-terminus of SpyCEP. The kinetic parameters of both the full length SpyCEP and C-terminal SpyCEP fragment were calculated for the first time, highlighting a likely C-terminal substrate binding domain and high binding affinity of SpyCEP and CXCL8. We have developed a robust, high-throughput assay for SpyCEP activity and utilised it to identify 2 potential SpyCEP inhibitors which we will assess in the following chapters.

5 Results – *In vitro* assessment of inhibitor 1 and 2

5.1 Introduction

Novel therapeutic inhibitors targeting SpyCEP have the potential to be an important adjunctive treatment for invasive *S. pyogenes* infection. Despite targeted antibiotic therapy, invasive streptococcal disease still carries a 20% mortality within the first 7 days of infection (9), as such, requirement for additional treatment modalities are high. As discussed in Chapter 1.4, research into streptococcal vaccines is ongoing, however there are currently no vaccines licensed for use, despite a global demand. Adjunctive therapeutics are an alternative mechanism for treating streptococcal disease, and, in some settings, are currently in clinical use - intravenous immunoglobulin therapy for example. However, there are currently no SpyCEP-specific inhibitors in either pre- or post-clinical development, indeed, there is only a single published serine protease inhibitor with activity against SpyCEP, Pefabloc (115).

Pefabloc is a broad acting, non-reversible serine protease inhibitor, which reacts with the catalytic serine residue (S617), disabling proteolytic activity. Despite being marketed as a non-toxic alternative to PMSF and other serine protease inhibitors, Pefabloc cannot be used in cell culture above 0.25mM. The requirement for SpyCEP inhibition is in excess of 4mM, as such, the therapeutic use of Pefabloc is severely limited by its toxicity. Though, it still remains useful as an *in vitro* resource and provides a base level for comparative inhibitory action.

Inhibition screening carried out in chapter 4 of this thesis has identified two molecules with properties consistent with SpyCEP inhibition. In the fifth chapter of this work, we interrogated whether the inhibition of CXCL8 cleavage was an artefact of inhibitor: CXCL8 binding and assessed whether the inhibitory molecules interacted directly with SpyCEP. We examined the efficacy of inhibitor 1 and inhibitor 2 in comparison to Pefabloc and assessed whether the

inhibitors are SpyCEP specific or elicit broader inhibitory activity. We subsequently examined the downstream effect CXCL8 cleavage on neutrophil migration and examined whether inhibitor 1 can rescue a specific SpyCEP induced reduction in neutrophil chemotaxis.

5.2 Results

5.2.1 Comparative efficacy of SpyCEP inhibitors

To determine which compound was more effective at inhibiting SpyCEP cleavage and to compare their efficacy to the only known SpyCEP inhibitor, Pefabloc, the activity of each inhibitor was measured across a range of concentrations. This provided a dose response curve of activity which further enabled the determination of half maximal inhibitory concentration values, IC_{50} , for each inhibitor (the concentration at which 50% of activity is inhibited). These values facilitated the direct comparison of inhibitor activity against SpyCEP.

Two-fold serial dilutions of inhibitor 1, inhibitor 2 and Pefabloc, starting at 100 μ M, 1.6 mM and 0.2 M respectively were incubated with an equal volume of 200 pM SpyCEP for 30 minutes at room temperature. Post incubation, CXCL8 was added to a final concentration of 20 nM and incubated for a further 30 minutes before cleavage reactions were stopped by the addition of excess Pefabloc at an 8.35 mM final concentration. Intact CXCL8 remaining in each reaction was quantified by CXCL8 ELISA and compared to the CXCL8 starting input (Figure 5-1). Inhibitor IC_{50} and pIC_{50} values for each inhibitor were calculated and reported in Table 5-1.

Inhibition of SpyCEP was evident for inhibitor 1, inhibitor 2, and Pefabloc, however compounds displayed varying efficacies (Figure 5-1). Inhibition became evident for inhibitor 1 at concentrations above 0.78 μ M, inhibitor 2 above 6.59 μ M and Pefabloc above 1630.94 μ M, with inhibitor IC_{50} values determined as 1.05, 46.12, 2616 μ M respectively. pIC_{50} values are defined as the $-\log (IC_{50})$ and transform data to a logarithmic scale for ease of direct comparison, with an increase in value of 1 representing a 10-fold increase in efficacy. pIC_{50} values for inhibitor 1, inhibitor 2 and Pefabloc were calculated at 5.98, 4.34 and 2.58 respectively. 46.12-fold less inhibitor 1 was required to inhibit half of SpyCEP activity when compared to inhibitor 2. However, both inhibitors 1 and 2, were far more effective at negating

CXCL8 cleavage activity than Pefabloc, with 2015.24 and 46.88- fold less required to inhibited 50% of SpyCEP activity. These data are in concordance with initial values collected during the GSK inhibitor screen and are promising for downstream therapeutic use.

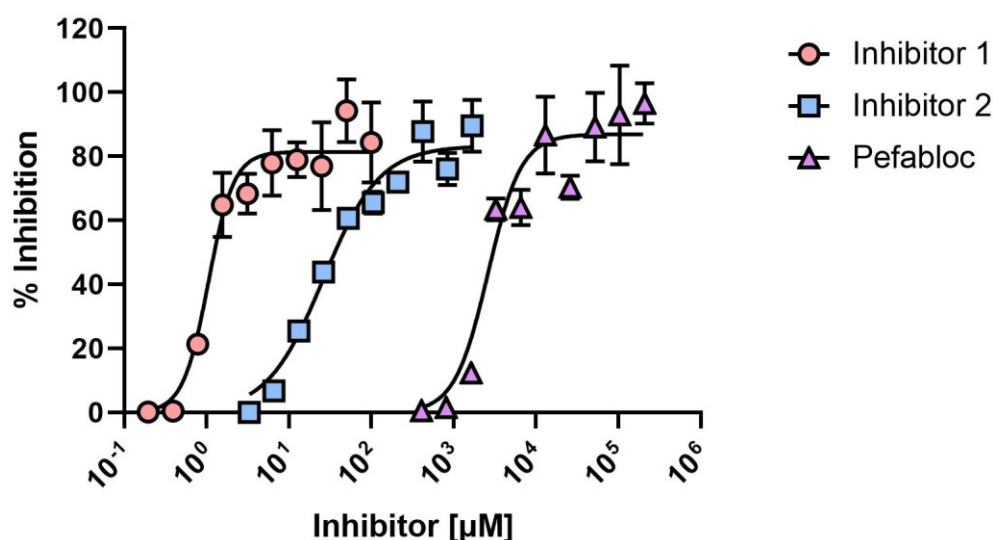


Figure 5-1. Inhibition of CXCL8 cleavage by inhibitor 1, 2 and Pefabloc

Dose response curves of a 30-minute, room temperature cleavage, of 20 nM CXCL8 by an equal volume of 100pM SpyCEP. SpyCEP was pre-treated with an equal volume of either 100 – 0.2 μM inhibitor 1 (red circles), 1687.4 μM – 3.3 μM inhibitor 2 (blue squares) or 208.7 – 0.4 mM Pefabloc (purple triangles) for 30 minutes at room temperature. Reactions were halted by the addition of 8.35 mM Pefabloc. Input and residual CXCL8 was quantified by ELISA for percentage inhibition calculation. N = 4 experimental replicates per data point, error bars represent SEM. Data is fitted by a 4-parameter variable slope regression for inhibitor IC₅₀ calculation.

Inhibitor	IC ₅₀ ± SEM (μM)	pIC ₅₀
Inhibitor 1	1.05 ± 0.19	5.98
Inhibitor 2	46.12 ± 34.68	4.34
Pefabloc	2616 ± 695.20	2.58

Table 5-1. Inhibitor IC₅₀ and pIC₅₀ values

Concentration of inhibitor 1, 2 and Pefabloc required to inhibit 50% of 100 pM SpyCEP activity against 20 nM CXCL8 (IC₅₀), as calculated from (Figure 5-1). pIC₅₀ values are calculated utilising the IC₅₀ and the equation, pIC₅₀ = -log (IC₅₀).

5.2.2 Inhibitor 1 and inhibitor 2 show broad specificity

Both inhibitor 1 and inhibitor 2 were identified by screening for inhibition of SpyCEP specific activity on CXCL8. However, this does not indicate that the molecules are specific for SpyCEP alone, as they may have a broader inhibitory profile. To examine whether inhibitors 1 and 2 could inhibit the activity of other enzymes we first assessed whether they displayed activity against the streptococcal serine protease, C5a peptidase (ScpA). Like SpyCEP, C5a peptidase is a cell envelope associated virulence factor responsible for immunomodulation, cleaving complement factors C5a and C3a (87, 88, 197, 198). Both SpyCEP and C5a peptidase are members of the S8 subtilisin-like serine protease family and as such share common structural domains and a 34% sequence identity. These considerations highlight C5a peptidase as an ideal candidate to assess cross-protease activity of inhibitor 1 and 2.

500 pM SpyCEP and ScpA were pre-incubated with an equal volume of 0.5 mM inhibitor 1, 2, respective DMSO vehicle controls or molecular grade water for 30-minutes at 37 °C. The resultant preparations were further incubated with an equal volume of 5µM native human substrates, C3a and C3a for ScpA and CXCL8 for SpyCEP, for 20 hours at 37 °C. Both ScpA and SpyCEP cleavage of respective substrates results in a reduction of chemokine molecular weight. As such, cleavage reactions were subsequently separated by SDS-page and inhibition of protease activity visualised by Coomassie staining.

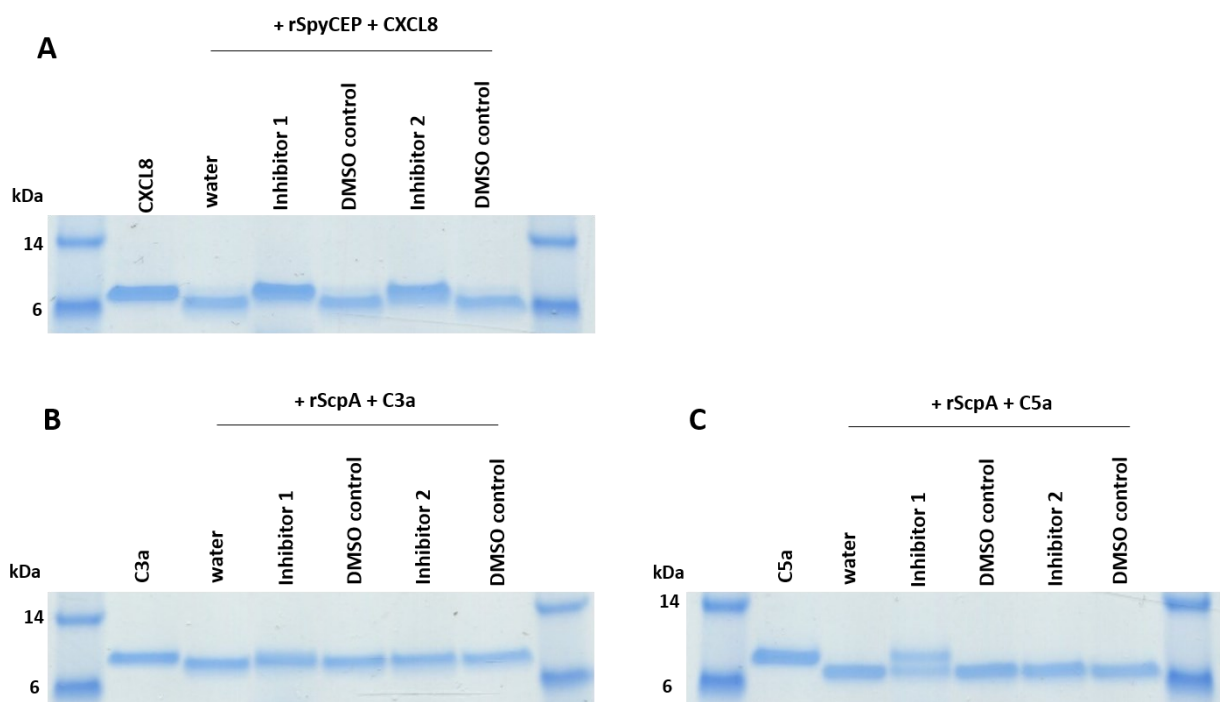


Figure 5-2. Action of inhibitor 1 and 2 on SpyCEP and C5a peptidase activity

SDS-page analysis of a 20-hour, 37°C, cleavage reaction of equal volumes of **A.** 500 pM SpyCEP and 5 μ M human CXCL8 **B.** 500 pM C5a peptidase and 5 μ M human C3a or **C.** 500 pM C5a peptidase and 5 μ M human C5a. Cleavage reactions follow an enzyme pre-treatment with either molecular water, inhibitor 1, inhibitor 2 or respective DMSO controls at 10⁶:1 molar ratio (inhibitor: enzyme) for 30 minutes at 37°C.

As expected from the ELISA data in Figure 5-1, pre-treatment of SpyCEP with inhibitor 1 or 2 successfully halted the cleavage of CXCL8, there was no reduction in the chemokine size indicative of cleavage, this activity was independent of the inhibitor vehicle controls (Figure 5-2 A). Unexpectedly, we further observed inhibition of C5a peptidase in our assay. When incubated with ScpA, inhibitor 1 fully abrogated cleavage of C3a, no reduction in chemokine molecular weight was apparent when compared to the C3 only control, a result not seen with inhibitor 1 DMSO vehicle control (Figure 5-2 B). In contrast, ScpA treatment with inhibitor 2 did not protect against C3a cleavage, a drop in band size is evident after incubation (Figure 5-2 B). Inhibitor 1 displayed further activity against ScpA cleavage of C5a, partially inhibiting

cleavage as indicated by the double C5a banding (Figure 5-2 C). This activity was specific to inhibitor 1 and not seen in the DMSO vehicle control. In accordance with its action against C3a, inhibitor 2 displayed no activity against C5a cleavage, indeed treatment of ScpA with inhibitor 2 provided comparable cleavage of both complement factors compared to the ScpA only control.

Activity of inhibitor 1 against C5a peptidase was surprising, however could be rationalised by considering the high degree of homology between the two cell envelope proteases. In order to further examine whether the inhibitors' substrate range was limited to streptococcal cell envelope proteases or whether they displayed a broader inhibitory profile, we assayed their ability to inhibit a range of archetypal serine proteases.

Inhibitory activity against, subtilisin, chymotrypsin, elastase and thrombin were assayed using FITC-labelled casein as a substrate. Proteolytic cleavage of FITC-casein produces smaller fragments which, unlike the full-length substrate, are insoluble under acidic conditions. This allowed us to utilise a high throughput assay where proteolytic cleavage resulted in a reduction in measurable fluorescent intensity. Inhibitors 1, 2, respective DMSO vehicle controls and the broad acting serine protease inhibitor, Pefabloc were pre-incubated with 4 μ M of serine proteases at a range of molar ratios (1: 1 – 1000: 1) in favour of inhibitor, for 30-minutes at 37 °C. FITC-casein substrate was subsequently incubated with the enzyme: inhibitor mixture and proteolytic cleavage carried out for 1 hour at 37 °C. Reactions were halted by the addition of TCA to precipitate fluorescent cleaved peptides and the remaining fluorescence, representative of the uncleaved substrate, quantified.

Both inhibitor 1 and inhibitor 2 displayed dose dependent inhibition of elastase, subtilisin and chymotrypsin and inconclusive results for thrombin, (Figure 5-3A-H). At a 1000: 1 molar ratio of inhibitor to enzyme, both inhibitors 1 and 2, reduced the fluorescent signal resultant after

incubation with elastase, subtilisin and chymotrypsin by half, with statistically significant results for subtilisin (Figure 5-3 E-F). Pefabloc inhibition of both elastase and chymotrypsin was more effective than inhibitor 1 and 2 in this assay (Figure 5-3 A-B,E-F), however both inhibitors 1 and 2 outperformed Pefabloc at inhibiting subtilisin (Figure 5-3 C-D), despite Pefabloc being assayed at an equal molar excess, 1000: 1. These data align with the published reports of Pefabloc IC_{50} for subtilisin being higher than elastase and chymotrypsin, 1.8 mM versus 0.53 mM and 0.04 mM respectively (199). Results for inhibitor 1, 2 and Pefabloc were indistinguishable from the molecular water negative control (dashed line) when assessing inhibitor action against thrombin. This result was expected as casein is not a substrate of thrombin, and as such inhibition of thrombin could not be determined by this assay. These data, from both the C5a peptidase and serine protease panel screen, highlight the pan-serine protease activity of both inhibitor 1 and inhibitor 2. These data strongly underline potential off target effects which may occur when inhibitors are used therapeutically.

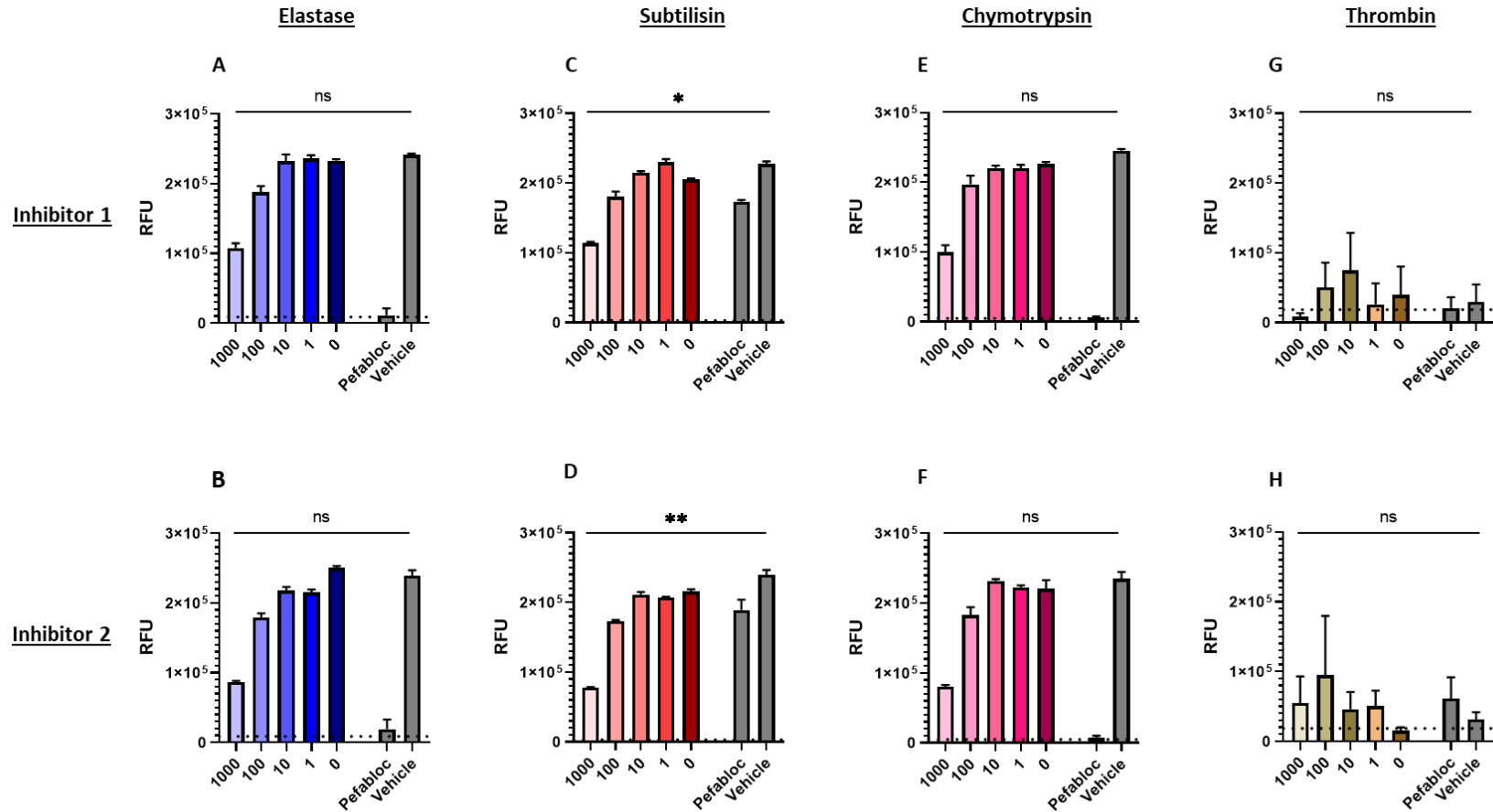


Figure 5-3. Inhibition of archetypal serine proteases by inhibitor 1 and inhibitor 2

Relative fluorescence intensity (RFU) of remaining FITC-casein substrate after cleavage by 4 μ M **A-B**. Elastase, **C-D**. Subtilisin, **E-F**. Chymotrypsin, **G-H**. Thrombin, pre-treated with inhibitor 1 or inhibitor 2 for 30 minutes at 37 $^{\circ}$ C. Inhibitor 1 and 2 were incubated with proteases at 0, 1, 10, 100, or 1000-fold molar excess in favour of inhibitor. Pefabloc was included as a positive control and assayed at the highest 1000-fold molar excess and DMSO vehicle control included at the highest DMSO % present. Background fluorescence is indicated by dash line. N=3 experimental replicates, errors bars represent SD, ns $p > 0.05$, * $p \leq 0.5$, ** $p \leq 0.1$ as determined by Kruskal-Wallis

Despite inhibitor 2 demonstrating activity against SpyCEP and our panel of serine proteases, it was decided to continue *in vitro* assessment with inhibitor 1 only. Initial supply of inhibitor 2 was extremely limited and without a chemical synthesis pipeline to create more, stock would not last to further *in vitro* or future *in vivo* therapeutic assessment, unlike the commercially available inhibitor 1.

5.2.3 Interaction of inhibitor 1 with cell envelope proteases

It is important to note that despite the inhibition of SpyCEP activity, as measured by monitoring the appearance of cleaved CXCL8 substrate by mass spectrometry, we cannot exclude the hypothesis that inhibitory activity may be an artefact of an inhibitor: CXCL8 interaction. Such an interaction would sequester CXCL8 from SpyCEP cleavage and result in the reduced appearance of cleaved substrate. Indeed, there are currently no experimental data for successful inhibitor: enzyme binding. To determine whether inhibitor 1 directly interacts with SpyCEP, C5a peptidase or CXCL8, we assessed their binding by WaterLOGSY (200), an observational NMR methodology for monitoring protein interaction with small molecules and a versatile technique for ligand screening. These data were collected by a project collaborator, Dr Sophie McKenna, in the Department of Life Sciences, Imperial College London,

1D NMR spectra were obtained for 100 μ M inhibitor 1 alone and WaterLOGSY 1D spectra for inhibitor 1 pre-incubated with 2 μ M CXCL8, SpyCEP or C5a peptidase. Each 1D spectra of inhibitor: protein mixture (blue line) was compared to inhibitor 1 alone (red line) and peaks representative of inhibitor 1 functional groups separated and highlighted by colour (Figure 5-4 A). Interaction of inhibitor 1 with protein is demonstrated by a 180-degree polarisation of peaks, inverting from a positive to negative phase; non-interaction is shown by peaks remaining in a positive phase.

Spectra for inhibitor 1 alone and inhibitor 1 incubated with CXCL8 remained consistent across all peaks. No peak inversion was measured at 7.7 ppm for interaction with inhibitor 1 aromatic protons, 2.3, 1.6, or 1.1 ppm for interaction with CH₂ protons or 2.1 and 1.3 ppm for interaction with CH₃ protons (Figure 5-4 B). These data strongly suggest there is no interaction between CXCL8 and inhibitor 1. In comparison, spectra from inhibitor 1 pre-incubated with SpyCEP demonstrate an opposite result to CXCL8: inhibitor 1. Incubation with SpyCEP causes a direct negative polarisation across all highlighted functional groups, including aromatic, CH₂ and CH₃ protons (Figure 5-4 C). These data highlight a strong interaction of inhibitor 1 and SpyCEP. Such data highlighting interaction is also evident when examining the C5a peptidase and inhibitor 1 spectra. Peak inversion can be seen at 7.7 ppm indicting interaction of C5a peptidase with inhibitor 1 aromatic protons in addition to peak inversion of the CH₂ protons proximal to the C=O functional group, the four CH₃ groups at 2.1 and 1.3 ppm and all other CH₂ groups, 1.1 and 1.6 ppm (Figure 5-4 D). These data may suggest there is a strong interaction of both C5a peptidase and SpyCEP with inhibitor 1.

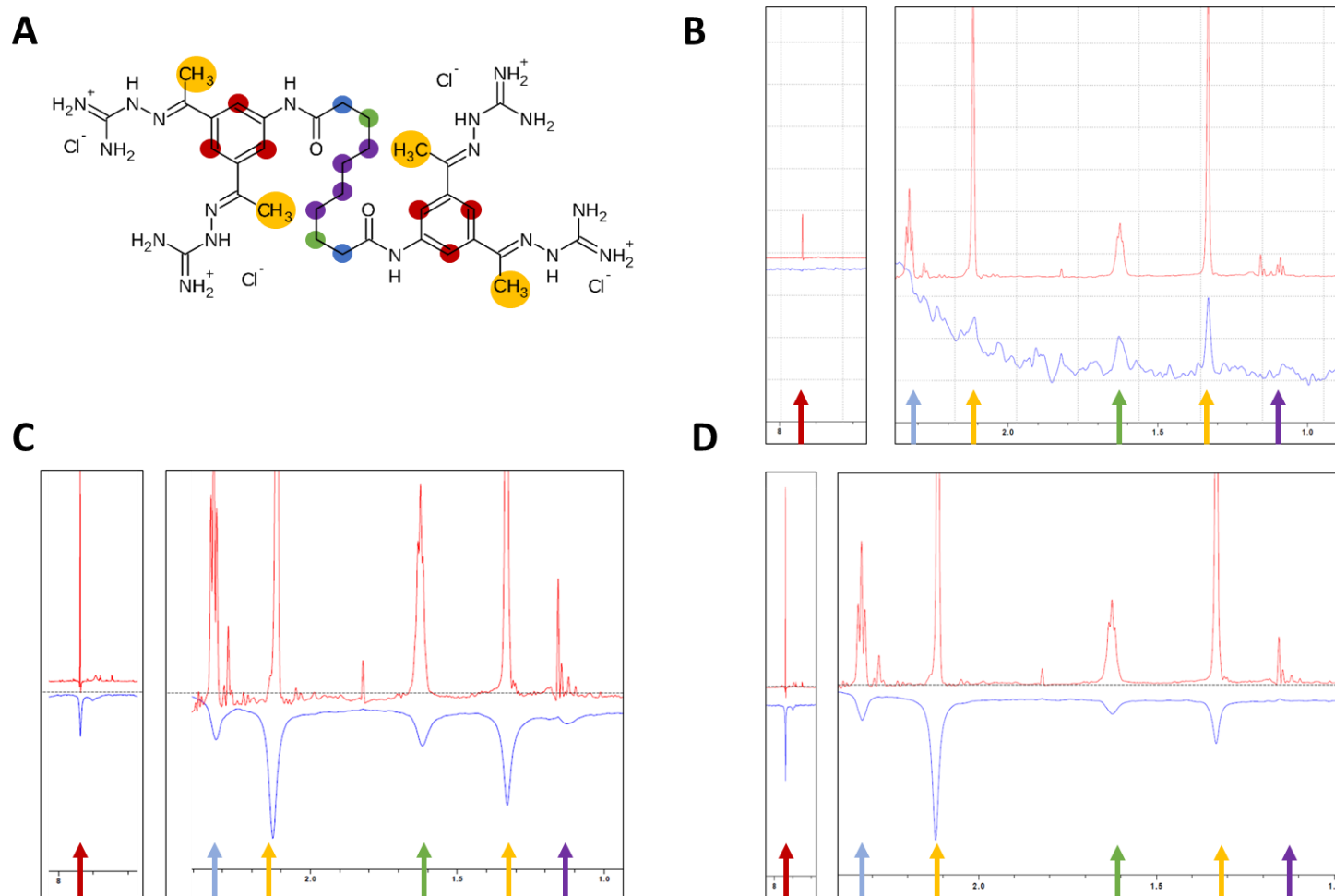


Figure 5-4. 1D NMR waterLOGSY spectra for inhibitor: protease interaction

A. Structural chemical schematic of inhibitor 1 highlighting functional groups observed by WaterLOGSY. Protons from inhibitor 1 aromatic ring coloured (red), CH₃ group (yellow), CH₂ group proximal to C=O (light blue), adjacent CH₂ group (green) and remaining CH₂ groups purple.

B-D. 1D NMR spectrum of 100 μM inhibitor 1 alone (red line) or WaterLOGSY spectra of 100 μM inhibitor 1 incubated with **B**. 2 μM CXCL8, **C**. 2 μM SpyCEP or **D**. 2 μM C5a peptidase (blue lines). Coloured arrows represent the position of protons in inhibitor 1 that relate to specific peaks.

Having successfully confirmed an interaction of inhibitor 1 with SpyCEP, we further interrogated a potential binding and inhibition mechanism. The structure of inhibitor 1 as seen in (Figure 4-9 A) is highly symmetrical and as such, raised the hypothesis that inhibitor 1 activity could be a function of enzyme dimerization. To assess this, 11 μ M SpyCEP was incubated with 100 μ M inhibitor 1, or buffer control and the resultant molar mass measured by size exclusion chromatography-multi angle laser light scattering (SEC-MALLS) (Figure 5-5), successful dimerization would result in SpyCEP elution at an increased molar mass.

The SEC-MALLS assay reported SpyCEP (pink line) molecular mass as 176.6 kDa, with an error of $\pm 0.2\%$; these data are calculated from the average molar mass across the elution peak (black line). SpyCEP incubated with a near 10-fold molar excess of inhibitor 1 (blue line), measured 174.9 kDa with an error of $\pm 0.2\%$. These molecular weight values of SpyCEP are in concordance with published data. No protein was eluted at any further volume and as such these data indicate that inhibitor 1 does not induce the dimerization of SpyCEP under these conditions.

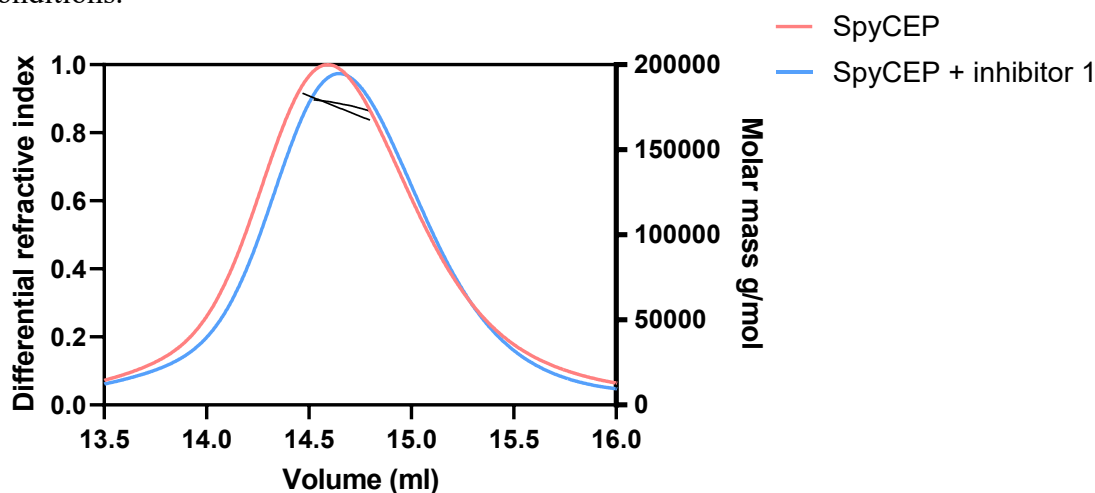


Figure 5-5. Size exclusion chromatograph of SpyCEP and inhibitor 1 interaction

Average molar mass (black line) of 11 μ M SpyCEP alone (pink line) or SpyCEP pre-incubated with 100 μ M inhibitor 1 (blue line). Data are representative of 1 experiment

5.2.4 The effect of inhibitor 1 on CXCL8 mediated neutrophil chemotaxis

To determine whether the *in vitro* inhibition of CXCL8 cleavage, seen in the SDS-PAGE and ELISA data above, translates to a phenotypic response, we assessed the ability of inhibitor 1 to block SpyCEP neutralisation of CXCL8-mediated neutrophil chemotaxis. We quantified this effect using two experimental assays, firstly by assessing neutrophil migration in a modified Boyden chamber and secondly by real time, live tracking, of migrating neutrophils using a TAXIScan assay (154).

SpyCEP was incubated with either molecular grade water, inhibitor 1, or DMSO vehicle control for 30 minutes at 37°C before an elongated incubation with CXCL8 for 20 hours at 37°C. The resultant reactions were analysed by SDS-page (Figure 5-6 A) to ensure correct chemokine cleavage and correct inhibition of cleavage, prior to use in neutrophil chemotaxis experiments.

To assess migration in a Boyden chamber, 100,000 freshly isolation neutrophils from human donors were loaded into the upper wells of the chemotaxis chamber. SpyCEP, inhibitor 1, vehicle or water control cleavage reactions, containing 10nM of CXCL8 were loaded into the bottom wells of the chemotaxis chamber. The total number of neutrophils which actively migrated through a polycarbonate membrane (5 µm pores) that actively separated the upper and lower chambers, was enumerated after a 5-hour, 37°C incubation (Figure 5-6 B).

As expected, SpyCEP actively cleaved CXCL8, with chemokine proteolysis successfully inhibited by pre-treatment of SpyCEP with a 10⁶ molar excess of inhibitor 1. This inhibitory activity was independent of any activity attributable to DMSO vehicle (Figure 5-6 A). These chemokine preparations were used in all chemotaxis assays below and allowed for dissection of the downstream effect of inhibitor 1 on SpyCEP CXCL8 cleavage.

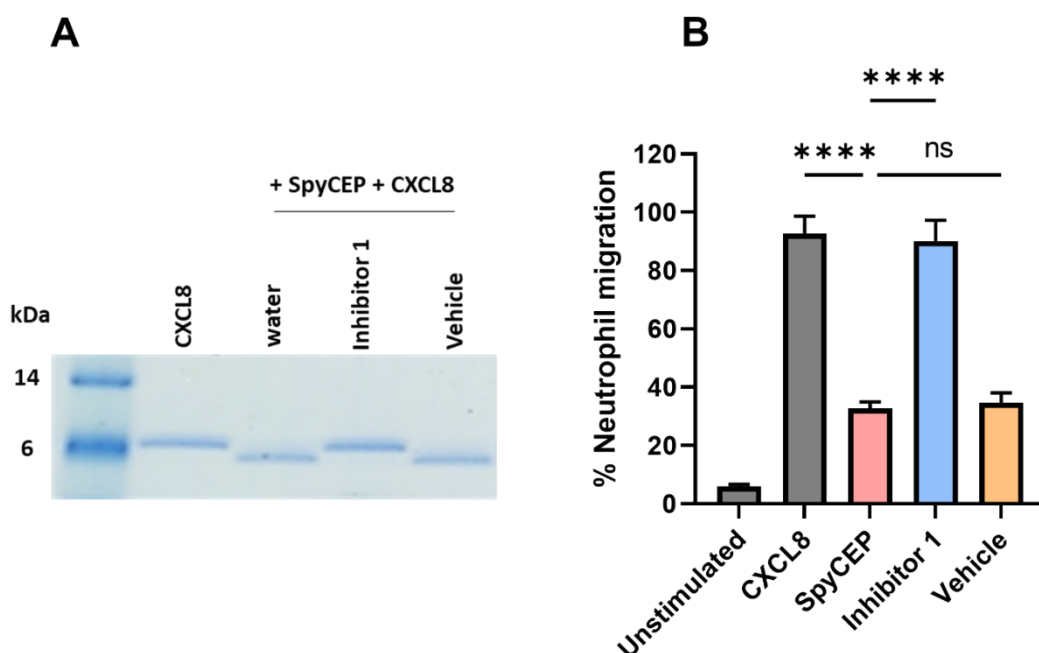


Figure 5-6. Inhibition of CXCL8 cleavage and neutrophil chemotaxis by inhibitor 1

A. SDS-page analysis of a 20-hour, 37°C, cleavage reaction of 5 μ M human CXCL8 by an equal volume of 500 pM SpyCEP, pre-treatment with either molecular water, inhibitor 1 or DMSO vehicle control at 10⁶:1 molar ratio (inhibitor: enzyme) for 30 minutes at 37°C.

B. Percentage of neutrophils actively migrating across a transwell membrane (5 μ m pores) over 5-hours, at 37°C, 5% CO₂. Migration was assessed unstimulated, in response to a 10nM CXCL8 gradient or 10nM CXCL8 after cleavage with SpyCEP pre-treated with either, molecular water, inhibitor 1 or DMSO vehicle control at 10⁶:1 molar ratio (inhibitor: enzyme) for 30 minutes at 37°C. Data are representative of mean migration values from 3 separate experiments utilising 3 independent neutrophil donors. Error bars represent SEM, ns $p > 0.05$, **** $p \leq 0.0001$, as determined by ordinary one-way ANOVA.

In the presence of no stimulus, only 5.98% of neutrophils actively migrated across the polycarbonate membrane, from the upper to lower chamber. This number was significantly increased to 92.68% in the presence of 10 nM CXCL8 in the lower chamber. As expected SpyCEP mediated cleavage of CXCL8 significantly reduced the active migration of neutrophils to 32.68% and inhibition of SpyCEP significantly restored migrating neutrophil numbers, equal

to wild type levels, 90.07%. DMSO vehicle control displayed no inhibitory activity and did not restore neutrophil migration, percentage migration 34.69%, this value is not significantly different to chemotaxis after SpyCEP CXCL8 cleavage.

The Boyden chamber is a useful tool for quantifying total percentage neutrophil migration, however there are number of aspects of chemotaxis it does not assess, for example the directional movement and speed of neutrophils. These, and other migration related parameters, can be calculated by analysing real time neutrophil migration via the TAXIScan assay. For detailed methodology see section 2.4.8. Briefly, purified neutrophils are aligned along the leading edge of a chemotaxis chamber, with a 5 μm height ceiling. Upon addition of 1 μL of chemokine to the far end of the slide, 260 μm distal from the neutrophils, a chemokine gradient is formed which the cells detect. To reach the source, the cells must actively crawl under the 5 μm slide to migrate in the direction of chemokine. Neutrophil migration can be monitored via light microscopy in real time, with a time lapse camera in operation to track each individual neutrophil. The data from each neutrophil migration track are collated using the manual tracking software on Image J and analysed using the IBIDI chemotaxis tool. This tool outputs functional neutrophil migration parameters, see section 2.4.8.

The migration of freshly isolated neutrophils from human donors was monitored over a 1- hour period in response to a control of 1 μ L of 100 nM CXCL8 and 1 μ L of a CXCL8 cleavage reaction (Figure 5-6 A). Images were captured at 1-minute intervals and the migration tracks manually collated. Neutrophil migration in unstimulated conditions were markedly and visibly different, neutrophils migrated a shorter distance and with less directionality compared to conditions with CXCL8 present (Figure 5-7 A). However, subtle differences in neutrophil migration under the remaining conditions were harder to resolve with the eye and required computational analysis to differentiate.

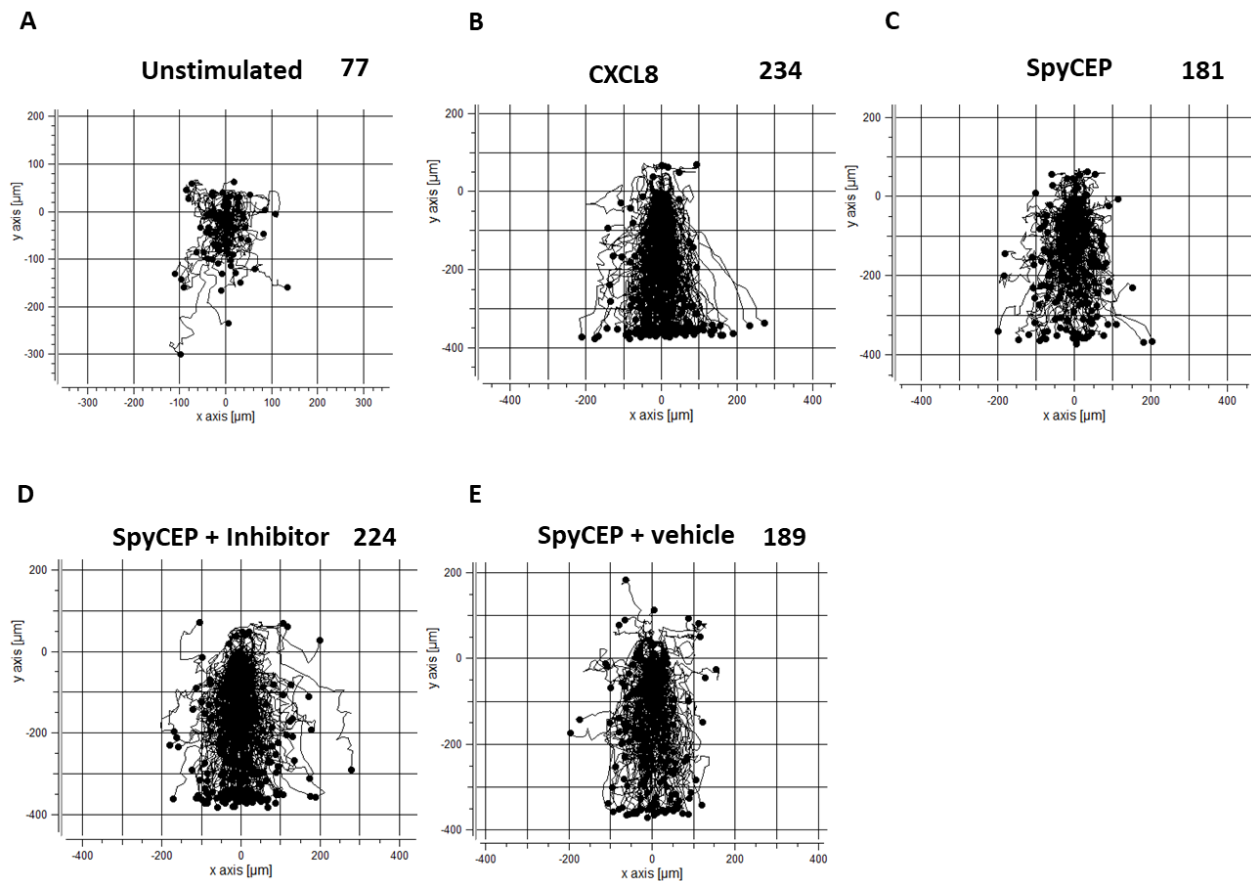


Figure 5-7 Neutrophil migration tracks

Manual tracking of individual neutrophils over a 1-hour, room temperature migration in response to no stimulus (n=77), 100 nM CXCL8 (n=234) or after 100 nM CXCL8 cleavage with SpyCEP pre-treated with either, molecular water (n=181), inhibitor 1 (n=224) or DMSO vehicle control (n=189) at 10⁶:1 molar ratio (inhibitor: enzyme) for 30 minutes at 37°C. Figure is representative of 3 individual experiments utilising 3 independent neutrophil donors, with each condition assayed in duplicate. Neutrophils have been overlayed at starting point x=0, y=0 and n = total pooled neutrophils from 3 donors.

Utilising the chemotaxis analysis tool from IBIDI we quantified the migration parameters, velocity, Euclidean distance, directionality, and the forward movement index, enabling comparison of migration conditions and statistical analysis (201).

The first parameter assessed was neutrophil velocity, a value which does not consider the directional of movement, only speed of migration. As expected, neutrophils stimulated with 100 nM CXCL8 moved significantly faster than those unstimulated, 7.4 $\mu\text{m minute}^{-1}$ compared to 12.38 $\mu\text{m minute}^{-1}$, respectively (Figure 5-8 A). Cleavage of chemokine by SpyCEP significantly reduced the neutrophil migration speed to 8.91 $\mu\text{m minute}^{-1}$, a reduction which was significantly blocked upon SpyCEP treatment with inhibitor 1 to 10.71 $\mu\text{m minute}^{-1}$. This impairment was not seen with the DMSO vehicle control.

Secondly, we analysed the Euclidean distance travelled by each neutrophil, a parameter which measures the straight-line distance from the neutrophil starting point to end point. The results followed the same trend as velocity, on average stimulated neutrophils migrated a further distance than unstimulated, 75.57 μm and 279.4 μm and the distance was significantly reduced upon chemokine cleavage by SpyCEP to 190.7 μm . The Euclidean distance travelled was significantly increased post SpyCEP incubation with inhibitor 1 to 254.8 μm , a result which

was not seen with the vehicle control, 202.9 μ m. The third parameter assessed was the neutrophil directionality, the ratio of the Euclidean distance travelled compared to the total distance travelled. It is a value of the straightness of neutrophil travel and followed the same result trend in results as the neutrophil velocity and Euclidean distance travelled.

The final and most important parameter for consideration is the forward movement index (yFMI), it is a direct measure of neutrophil migration in response to the chemokine gradient. A value of 1 indicates a straight-line migration directly parallel to the chemokine gradient, values in (Figure 5-8 D) are negative due to neutrophil migration from the top to bottom of the chamber. Neutrophils yFMI values were significantly increased upon stimulation with CXCL8, -0.25 for unstimulated and -0.68 for stimulated neutrophils. The yFMI was significantly decreased upon SpyCEP treatment of CXCL8, yFMI -0.51. Treatment of SpyCEP with inhibitor 1 significantly increased the yFMI to -0.61 representative of a significant increase in specific neutrophil chemotaxis. This increase was not apparent after SpyCEP treatment with DMSO vehicle control.

These neutrophil chemotaxis data from both Boyden chamber analysis and TAXIScan tracking highlight the significant effect that SpyCEP cleavage of CXCL8 has on recruitment of neutrophils. These data also show that pre-treatment of SpyCEP with inhibitor 1 successfully halts the cleavage of CXCL8 and leaves its ability to recruit neutrophils intact.

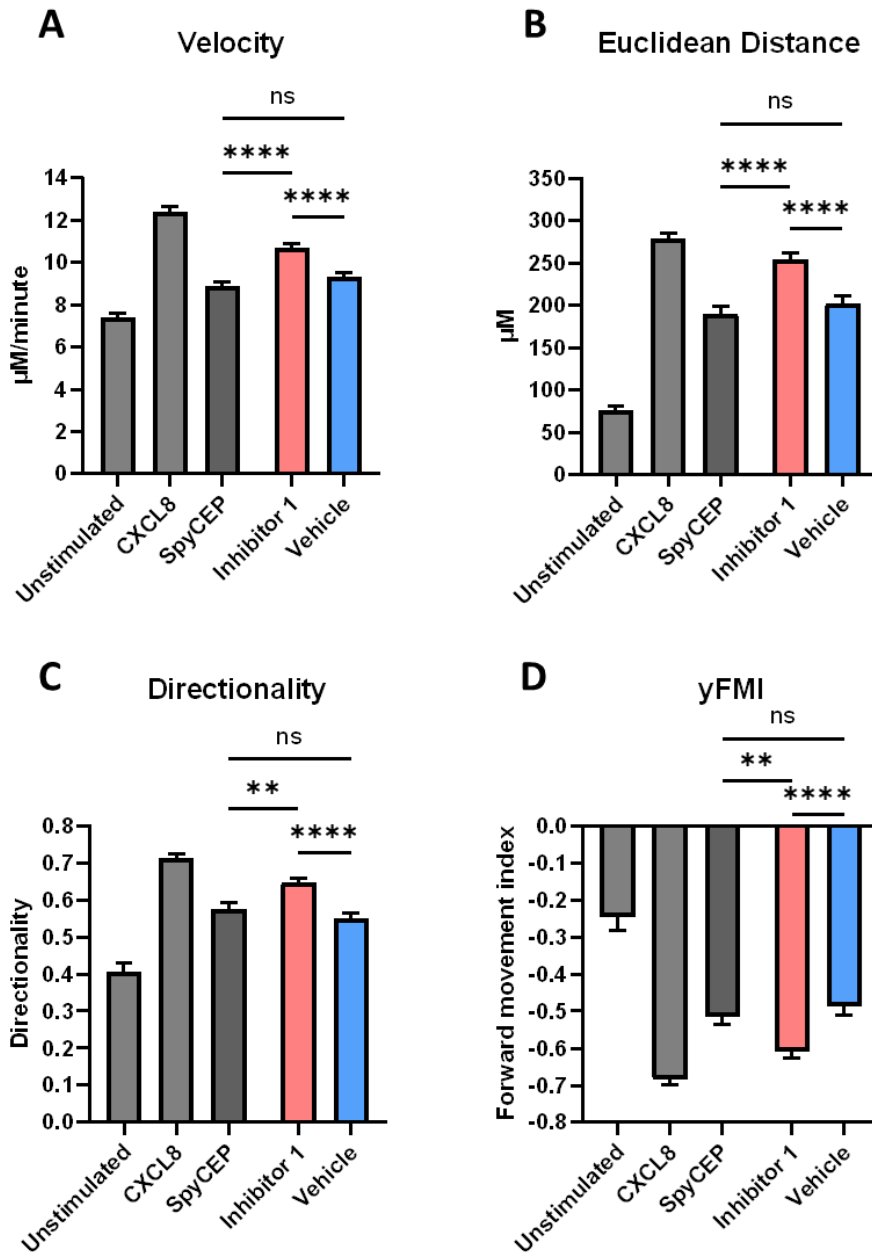


Figure 5-8. Chemotaxis parameters calculated from neutrophil migration tracks in Figure 5-7

IBIDI chemotaxis parameters from neutrophil migration tracks collated from 3 independent neutrophil donors showing neutrophil **A.** velocity, **B.** Euclidean distance travelled, **C.** directionality and **D.** forward migration index (γ FMI).

Data are representative of the mean values from 3 experiments. Error bars represent SEM, ns $p > 0.05$, ** $p \leq 0.01$, **** $p \leq 0.0001$, as determined by ordinary one-way ANOVA.

5.3 Discussion

In vitro assessment of CXCL8 cleavage activity

During the fifth chapter of this thesis, we have directly compared the *in vitro* efficacy of the newly identified SpyCEP inhibitors 1 and 2 to the only known previous SpyCEP inhibitor, Pefabloc. Both inhibitor 1 and 2 were more effective than Pefabloc, requiring 2000-fold less inhibitor 1 and 46-fold less inhibitor 2 to emulate the same 50% inhibitory action as Pefabloc. However, both molecules were still required in great excess to optimally inhibit SpyCEP, an approximate excess of 10^6 and 10^7 for inhibitor 1 and 2 respectively, compared to 10^8 for Pefabloc. These values are a useful comparator for *in vitro* efficacy and highlight a strong rationale for continued assessment of inhibitor 1; the lower an inhibitor IC_{50} , the more likely it may have a measurable effect *in vivo*. However, it must be noted that there are a number of other aspects that must be considered for therapeutic administration that will affect its *in vivo* efficacy, for example toxicity, route of administration and bioavailability. These may strongly limit the *in vivo* efficacy and as such, differences in *in vitro* IC_{50} values may not be associated with differences in therapeutic outcome.

Pan-serine protease activity of inhibitor 1 and 2

An important consideration highlighted in this chapter is the apparent non-specific action of both inhibitor 1 and inhibitor 2. Pre-treatment of ScpA by inhibitor 1 entirely abrogated ScpA mediated cleavage of C3a and partially inhibited cleavage of C5a. Inhibition of either complement factor was not seen with pre-treatment with inhibitor 2. These were promising initial results, cleavage of C3a and C5a by ScpA has significant effects of neutrophil activation, chemotaxis, and phagocytosis (88). As such, inhibition of both ScpA and SpyCEP would be fortuitous and provide a multipronged approach to help rescue neutrophil function during streptococcal infection.

Activity of inhibitors against ScpA and SpyCEP did however, raise the question of whether inhibitors elicit a broad activity against other serine proteases. Indeed, when activity was subsequently assessed against a panel of archetypal serine proteases, both inhibitor 1 and inhibitor 2 inhibited casein cleavage by chymotrypsin, subtilisin and elastase, with statistically significant effects seen against subtilisin. Both ScpA and SpyCEP are classified as subtilisin-like serine proteases, as such, increased inhibitory action against subtilisin, over other serine proteases, could be attributable to an increased homology between the enzymes. The FITC-casein cleavage protocol only assessed inhibitors up to a one-thousand-fold molar excess of inhibitor to protease and the exact IC_{50} values for each enzyme was not assessed for direct comparison. However, while the inhibitory activity across proteases cannot be compared due to the relative nature of the fluorescence measurements, comparisons can be drawn to the Pefabloc control in each experiment. Our data show inhibitor 1 and 2 both outperform Pefabloc at inhibiting subtilisin. However, both inhibitors are outperformed against chymotrypsin and elastase by Pefabloc. Nevertheless, in each case both inhibitor 1 and 2 displayed measurable signs of inhibitory activity as concentrations of inhibitor increased.

These data strongly highlight the potential of off target if either antagonist were to be administered as a therapeutic. Indeed, 184 / 588 proteases in the human degradome are serine proteases, the second largest class after metalloproteases, with > 2% of the total human genome encoding proteases (202). As such, the importance of proteolytic enzymes in human biology cannot be overstated including and importantly their role in inflammation and the innate immune system. Human serine proteases are vital for a plethora of immune functions, modulation of leukocyte chemotaxis, bacterial killing by neutrophil extracellular traps, neutrophil phagocytosis, direct neutrophil degranulation and processing of the antimicrobial peptide LL-37 for example (203-205). Disruption to any of these systems, in addition to many

other vital host processes which are reliant on host serine proteases, may cause multiple downstream effects on the pathophysiology to infection. It must also be noted that these effects may be additionally compounded by the already established action of inhibitor 1 against p38 map kinase activation and the subsequent downstream effects caused by its anti-inflammatory profile. Important future work on *in vitro* assessment of inhibitor 1, should include inhibitor action against a wide range of human serine proteases, in addition to expansion of the protease assessment panel to include other proteolytic enzyme families, in order to validate the breadth of inhibitor action.

Assessment of inhibitor 1 protein interaction

Due to unsuccessful surface plasma resonance and differential scanning fluorimetry experiments, our collaborators at GSK were unable to confirm a direct interaction of inhibitor 1 and SpyCEP. As such, it was important to rule out CXCL8: inhibitor 1 binding as a causative mechanism behind the SpyCEP inhibition seen in our mass spectrometry activity assay. WaterLOGSY, an NMR based methodology which utilises the nuclear overhauser effect to monitor the difference in transfer of magnetisation between water and target molecules in the presence and absence of the protein of interest, was an ideal experimental approach. There were no measurable interactions seen between CXCL8 and inhibitor 1, and strong interactions with both C5a peptidase and SpyCEP. These data support a direct mechanism of binding and inhibition of enzymatic function and demonstrate a specific interaction of inhibitor 1 with SpyCEP and C5a peptidase for the first time. The WaterLOGSY data coupled with the activity of inhibitor 1 against SpyCEP, C5a peptidase, subtilisin, elastase and chymotrypsin in 5.2.2, suggest there is likely to be a homologous mechanism of inhibition occurring. Whether through a direct attack on the catalytic serine, conformational manipulation of the active site or by other means, inhibitor 1 does display broad activity across serine proteases. Additional cross-family

activity was not assessed in this chapter, however future work utilising enzymes from alternative protease families may help further interrogate the inhibitor 1 mechanism of action.

One potential mechanism of action that was addressed in this chapter was enzyme inactivation by the induction of SpyCEP dimerization, an avenue explored due the highly symmetrical nature of inhibitor 1. Size exclusion chromatography permitted examination of SpyCEP molecular weight when incubated with and without inhibitor 1, after binding was confirmed by the WaterLOGSY experiment. Protein was not detected in any elutes at any molar mass other than that expected for full length SpyCEP, nor was there a significant difference in molecular weight when SpyCEP was incubated with inhibitor 1. Due to the small molecular weight of inhibitor 1, <1 kDa and intrinsic error margins in this system, it was not possible to discern inhibitor 1 binding by measuring an increase in SpyCEP molecular weight. However, this assay was successful in confirming the inhibitor did not induce the dimerization SpyCEP. Further work to assess binding mechanisms were beyond the scope of this thesis.

Effect of inhibitor 1 on neutrophil chemotaxis

Having established the inhibitor 1 was interacting with SpyCEP and that CXCL8 cleavage could be reproducibly inhibited *in vitro*, it was necessary to determine whether inhibition of chemokine cleavage had downstream effect on neutrophil function. CXCL8 is a potent neutrophil chemoattractant and histopathology of invasive streptococcal disease reproducible show a chronic lack of infiltrating neutrophils, as such a modified Boyden chamber to assess neutrophil chemotaxis was an ideal methodology to discern downstream inhibitor effects. Pre-treatment of SpyCEP with inhibitor 1 significantly recovered neutrophils chemotaxis, equal to CXCL8 only control, a phenotype that was not seen in inhibitor vehicle control. To further dissect the effect on neutrophils we additionally assessed their chemotaxis via TAXIScan, an assay which visually tracked individual neutrophils as they migrated in response to stimulus.

This methodology resulted in multiple chemotactic parameters for assessment of neutrophil migration. Pre-treatment of SpyCEP with inhibitor 1, subsequent to CXCL8 cleavage, significantly increased the neutrophil migration velocity, the Euclidean distance travelled, the directionality and most importantly the forward movement index, a direct measurement of migration in response to a stimuli. These data, from both assays of neutrophil migration, strongly indicate that if therapeutic intervention during invasive disease successfully ablated SpyCEP cleavage of CXCL8, neutrophil chemotaxis to the site of infection would be restored. There are other aspects of the interaction of CXCL8 with neutrophils which could further be assessed, for example the activation and release of endogenous CXCL8 or the induction of neutrophil phagocytosis. However, these assays were not evaluated in this chapter. It can be assumed with a degree of confidence however, that complete inhibition of CXCL8 cleavage would likely result in normal function of all downstream CXCL8 specific effects.

In conclusion, in this chapter we have successfully confirmed that inhibitor 1 binds to both SpyCEP and C5a peptidase, cementing its role as a true protease inhibitor. We have determined that both inhibitors 1 and 2 successfully negate SpyCEP activity, with a greater efficacy than the only published inhibitor, Pefabloc. Indeed, inhibitor 1 is over two thousand-fold more effective. We have found that both inhibitors exhibit a pan-serine protease inhibition profile and are capable of inhibiting a range of archetypal serine proteases. Importantly, we have further shown that inhibitor 1 can successfully negate the effect of SpyCEP on CXCL8-mediated neutrophil migration, a mechanism central to neutrophil function which lack thereof is associated with severe streptococcal infection.

6 Results – *In vivo* assessment of inhibitor 1

6.1 Introduction

SpyCEP expression is significantly associated with invasive *S. pyogenes* disease, systemic infection, and death (30, 115, 134, 140, 146). It has been strongly associated with driving a necrotizing phenotype in an otherwise avirulent *L. lactis* infection model (30) and its proteolytic activity associated with reduced neutrophil clearance of bacteria from a sterile infection site (30, 115). These clinical observations, in addition to ubiquitous cell surface expression, strongly identify SpyCEP as a target for therapeutic intervention in invasive disease. There are currently no licenced therapeutic agents that specifically target SpyCEP, and whilst antibody-based IVIG treatment has been shown to inhibit SpyCEP activity *in vitro* (163), the large size of immunoglobulins may limit their ability to penetrate highly necrotic and thrombotic tissue. However, although smaller F(ab')₂ fragments, derived from SpyCEP-neutralising IVIG, may penetrate tissues more effectively than IVIG, they appear unable to inhibit SpyCEP CXCL8 cleavage (163).

Chapters 4 and 5 of this thesis outlined the discovery, validation, and analysis of a small (0.89 kDa) SpyCEP inhibitor which successfully abrogates proteolytic enzyme activity and rescues neutrophil chemotaxis in response to a CXCL8 stimulus. Work in this chapter aimed to determine the potential impact of the inhibitor on SpyCEP activity *in vivo*. To provide a biological ‘gold standard’ comparator, bacterial isolates with or without SpyCEP expression were used. The first step was to develop a suitable infection model in mice, where disease phenotype was highly dependent on SpyCEP production, using challenge isolates. The next step was to examine the impact of therapeutic administration of inhibitor 1 in these models of streptococcal and lactococcal infection.

Infection models

There are a number of published infection models which emulate invasive streptococcal disease and display a necrotizing phenotype, although those with a focus on SpyCEP largely involve subcutaneous or intramuscular inoculation of bacteria (30, 140). Previously published data from intramuscular infection models using both *S. pyogenes* and *L. lactis* underpins the rationale for inhibitor development and therefore the intramuscular route was chosen as the primary model for the following work. A 24-hour infection time course was initially chosen based on published work, utilising the high SpyCEP expressing M81 strain, H292 and its isogenic SpyCEP mutant H575, demonstrating that SpyCEP expression was associated with an increased local tissue burden and increased spread of *S. pyogenes* to the lymphatic system (30). Routes of bacterial inoculation are outlined in (Figure 6-1), however, briefly, mice were infected either by intramuscular injection into the right hindlimb, or by inhalation of bacteria via both nostrils. Systemic tissues including the spleen, liver, lung, and blood were harvested at necropsy to quantify bacterial spread and invasion from the site of inoculation. The expression of SpyCEP has recently been associated with increased bacterial survival during *S. pyogenes* lymphatic transit (147), as such, the local and distal draining lymph nodes were additionally harvested to identify evidence of lymphatic bacterial metastasis. Where antibiotic or therapeutic intervention was necessary, agents were administered by intraperitoneal injection 2-hours prior to infection and if multiple dosing required, every 6-hours hence.

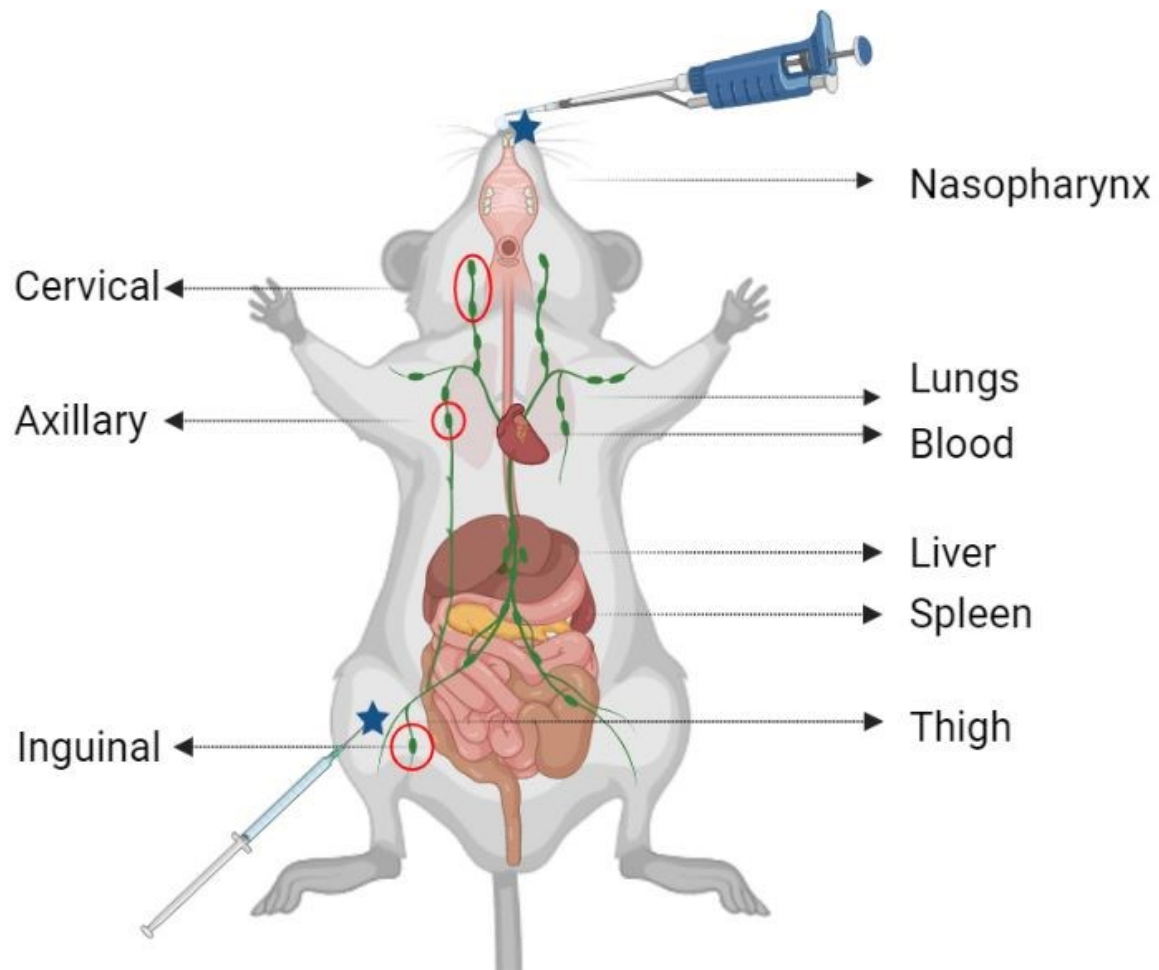


Figure 6-1. Schematic of *in vivo* intramuscular and intranasal infection routes and tissues harvested by necropsy

Intramuscular infection model. Mice were inoculated by direct injection into the right hindlimb (blue star). Upon termination, cardiac blood was drawn from the heart and a necropsy was performed for tissue harvesting. The inguinal lymph node, which drains directly from the infection site (red circle), and the axillary lymph node (red circle), a secondary draining node from the ipsilateral side of the mouse were harvested. The inguinal and axillary lymph nodes on the contralateral side were additionally harvested as controls and as a measure of systemic infection. At necropsy the liver, spleen and infected thigh tissue were collected for bacterial enumeration.

Intranasal infection model. Mice were inoculated by inhalation (blue star) into both nostrils, 5 μ l each. Cardiac blood was harvested upon termination and a necropsy was performed. Bilateral cervical lymph nodes (red circle), spleen, lungs, and the entire nasopharynx were collected for bacterial enumeration.

Dosing and pharmacokinetics of inhibitor 1

Having been evaluated in phase 1 and phase 2 human trials, the tolerability, toxicity, and pharmacodynamics of inhibitor 1 are already known (184). Reports of prior use in murine models of cancer metastasis, Parkinson's, and Alzheimer's disease (192-194, 206, 207) facilitated *in vivo* assessment of inhibitor 1 in streptococcal disease. Intravenous use of inhibitor 1 in humans is well tolerated when administered daily over 5 days, though some patients developed injection related phlebitis (184). The maximum tolerated dose for inhibitor 1 was not reached in this study and was defined as greater than the highest dose given, > 32 mg/m² (184). Increased doses were not administered due to the study pool consisting of melanoma and renal cancer patients however, *in vivo* toxicology studies in rats have noted nephrotic histological abnormalities only when inhibitor 1 was given daily for 2 weeks at high doses, 20 mg/kg (120 mg/m²) (184). The half-life of inhibitor 1 ranged from 1.02 ± 0.35 to 1.42 ± 0.67 hours, with no indication that half-life varied with dose escalation. The highest human dose of inhibitor 1, 32 mg/m², is equivalent to 865 µg/kg, which for an average 60 kg human would be 50 mg per dose. One 32 mg/m² dose resulted in a peak serum concentration (C_{max}) of 6.2 ± 0.8 µg/ml.

In vivo measurement of SpyCEP in *S. pyogenes*-infected tissue samples, 3.2.4, revealed a concentration of ~1nM in infected thigh tissues. Inhibitor IC₅₀ experiments, 5.2.1, have shown that a 10⁶ molar excess of inhibitor 1 is required to optimally inhibit SpyCEP activity – to inhibit 1 nM SpyCEP would therefore require a C_{max} of 1 mM (890.75µg/ml), which is ~ 140-fold more than the maximum dose given to humans. To achieve a maximum concentration of 1 mM inhibitor 1 in mice would require a dose of approximately 40 mg/kg. This dose is double the dose given to rats (20 mg/kg) during toxicology studies in which rats developed nephrotic histological abnormalities, albeit after daily dosing. As a result, we elected to administer 150

µg of inhibitor 1 per dose, in line with the highest dose previously used safely in mice, which is 8 mg/kg (193).

6.2 Results

6.2.1 Assessment of M1T1 *S. pyogenes* in 24-hour streptococcal infections

To determine whether M1T1 *S. pyogenes* strain H584 (M1 CovR/S_{WT}), and its isogenic *cepA* deletion mutant, H1464 (M1 CovR/S_{WT} Δ *cepA*), were suitable isolates to use in murine models for inhibitor assessment, we first examined whether H584 caused a clinical phenotype that could be quantifiably attributed to SpyCEP alone. We compared both strains in exploratory nasopharyngeal and intramuscular infection models to identify the infection route that was most dependent on SpyCEP to drive disease. Female C57BL/6 mice were infected either intramuscularly (Figure 6-2) or intranasally (Figure 6-3) with H584 or its isogenic SpyCEP deletion mutant, H1464. The infection was permitted to progress for 24-hours at which time the mice were culled, organs harvested and bacteria from tissues enumerated.

Intramuscular infection resulted in no significant difference between infected groups in bacterial burden in the local soft tissue (Figure 6-2 A), draining lymphatic nodes (Figure 6-2 B-C) or blood at the 24h time point (Figure 6-2 D-H). Mice infected with the SpyCEP deficient mutant H1464, displayed a slight reduction in *S. pyogenes* recovered from the systemic organs compared to SpyCEP-expressing parent strain. Median spleen burden was reduced 5.9-fold and liver burden reduced from 19.31 CFU/100mg to 0 CFU/100mg, however the differences were not statistically significant.

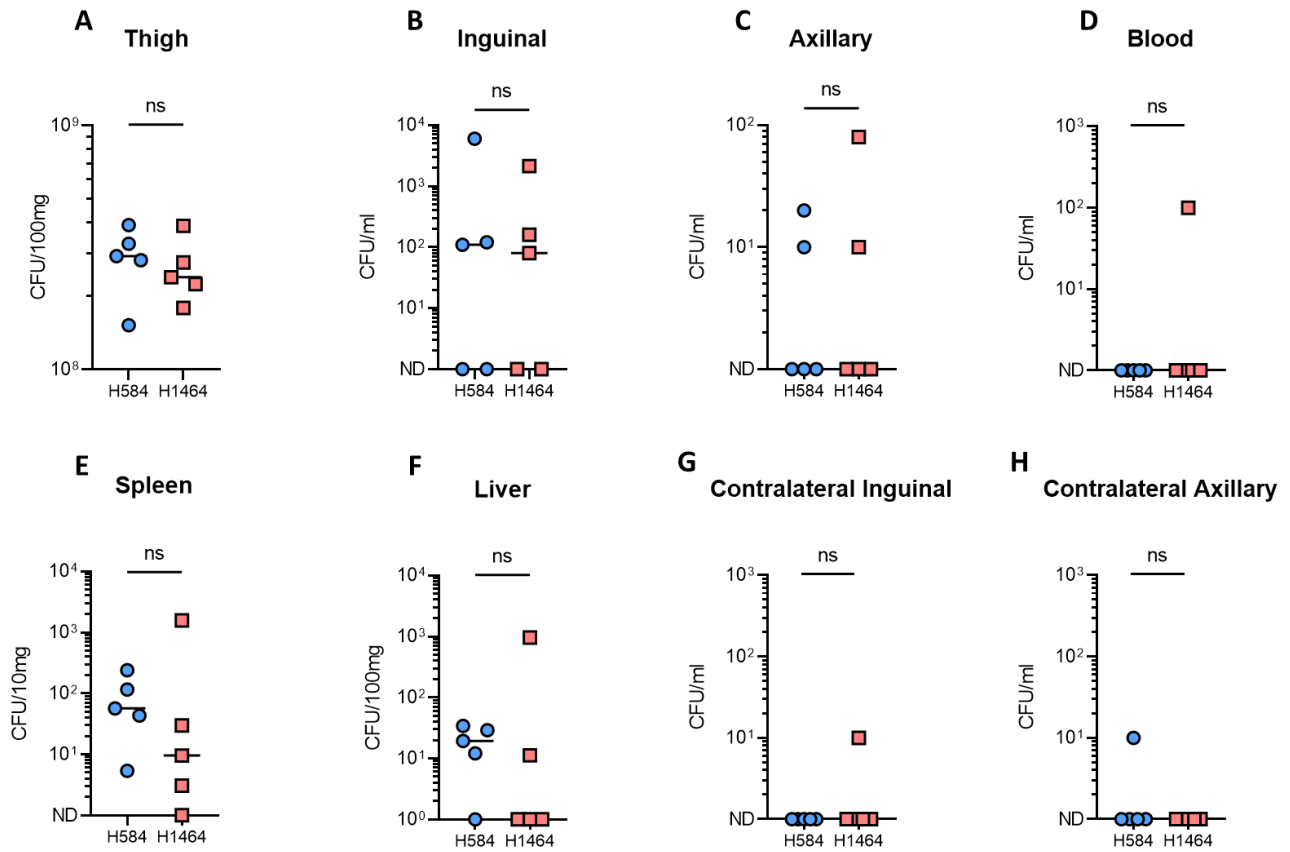


Figure 6-2. Recovery of viable *S. pyogenes* after 24-hour intramuscular infection with H584 and isogenic *cepA* mutant, H1464

Female, C57BL/6 mice were inoculated with 6.5×10^7 CFU H584 (M1 CovR/S_{WT}) (blue circles) or 9.5×10^7 CFU H1464 (M1 CovR/S_{WT} $\Delta cepA$) (red squares) into the rear hindlimb and culled 24 hours post infection. *S. pyogenes* was recovered and enumerated by bacterial culture from sites A-H. n = 5 mice per group, bar represents median value, ns p > 0.05 determined by Mann-Whitney U test.

Intranasal infection with H584 and H1464 produced similar results to the intramuscular model at 24 hours. SpyCEP expression did not affect bacterial clearance from the infected nasopharynx (Figure 6-3 A), nor bacterial burden in the draining lymphatic system (Figure 6-3 B), lungs (Figure 6-3 C) or blood (Figure 6-3 D-E), although median bacterial counts were higher across all examined tissues when infected with SpyCEP positive isolates.

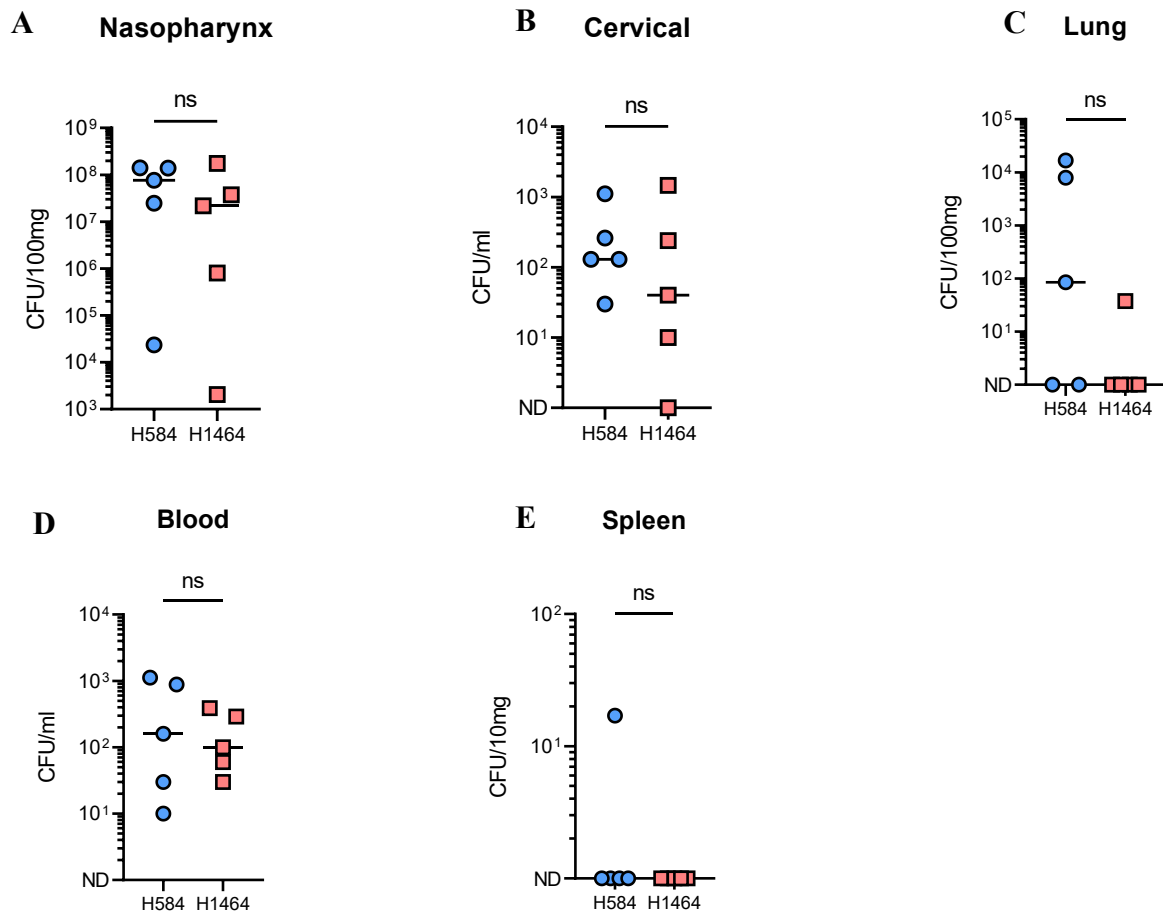


Figure 6-3. Recovery of viable *S. pyogenes* after 24-hour intranasal infection with H584 and isogenic *cepA* mutant, H1464

Female, C57BL/6 mice were inoculated with 3×10^7 CFU H584 (M1 CovR/S_{WT}) (blue circles) or 2×10^7 CFU H1464 (M1 CovR/S_{WT} Δ *cepA*) (red squares) by inhalation into both nostrils and culled 24 hours post infection. *S. pyogenes* was recovered and enumerated by bacterial culture from sites A-E. n = 5 mice per group, bar represents median value, ns p > 0.05 determined by Mann-Whitney U test.

These exploratory studies suggested that the phenotype exhibited by H584 is not sufficiently dependent on SpyCEP to be a useful model infection, when evaluating a SpyCEP-specific therapeutic intervention. The findings are likely due to the somewhat limited SpyCEP expression by H584, which has a wild type CovR/S regulator, as measured in 3.2.3, as well as

the myriad of other virulence factors produced by M1 *S. pyogenes*. The need for an inhibitor of SpyCEP is mostly related to the high SpyCEP producing isolates associated with rapidly progressive necrotising fasciitis, which often have mutations in CovR/S that result in upregulation of SpyCEP. We therefore undertook further exploratory studies using instead the isogenic (animal passaged) CovR mutant, *S. pyogenes* H1565 (M1 CovR_{MT}) and its isogenic *cepA* deletion strain, H1567 (M1 CovR_{MT} $\Delta cepA$).

Over a 24-hour intramuscular infection, significant differences in bacterial burden were not seen between groups, in any examined tissues (Figure 6-4) including the local soft tissue, draining inguinal and axillary lymph nodes (Figure 6-4 A-C). However, whilst not significant, there were large reductions in median bacterial burden in all organs examined as a surrogate of systemic bacterial dissemination (Figure 6-4 D-H). Median values for SpyCEP expressing strains compared to SpyCEP negative strains were 12.9-fold higher in the blood (Figure 6-4 D), 2.5-fold higher in the liver (Figure 6-4 E), 2.7-fold higher in the spleen (Figure 6-4 F), 6.3-fold higher in the contralateral inguinal (Figure 6-4 G) and 1.7-fold higher in the contralateral axillary lymph node (Figure 6-4 H). These data suggest that SpyCEP is playing a measurable role in driving invasive disease progression in this model; as exploratory studies, the experiments were insufficiently powered to show a significant difference. It is also likely that the global de-repression of the CovR/S regulon, resulting in mass upregulation of numerous virulence factors, conceals the SpyCEP-specific effect.

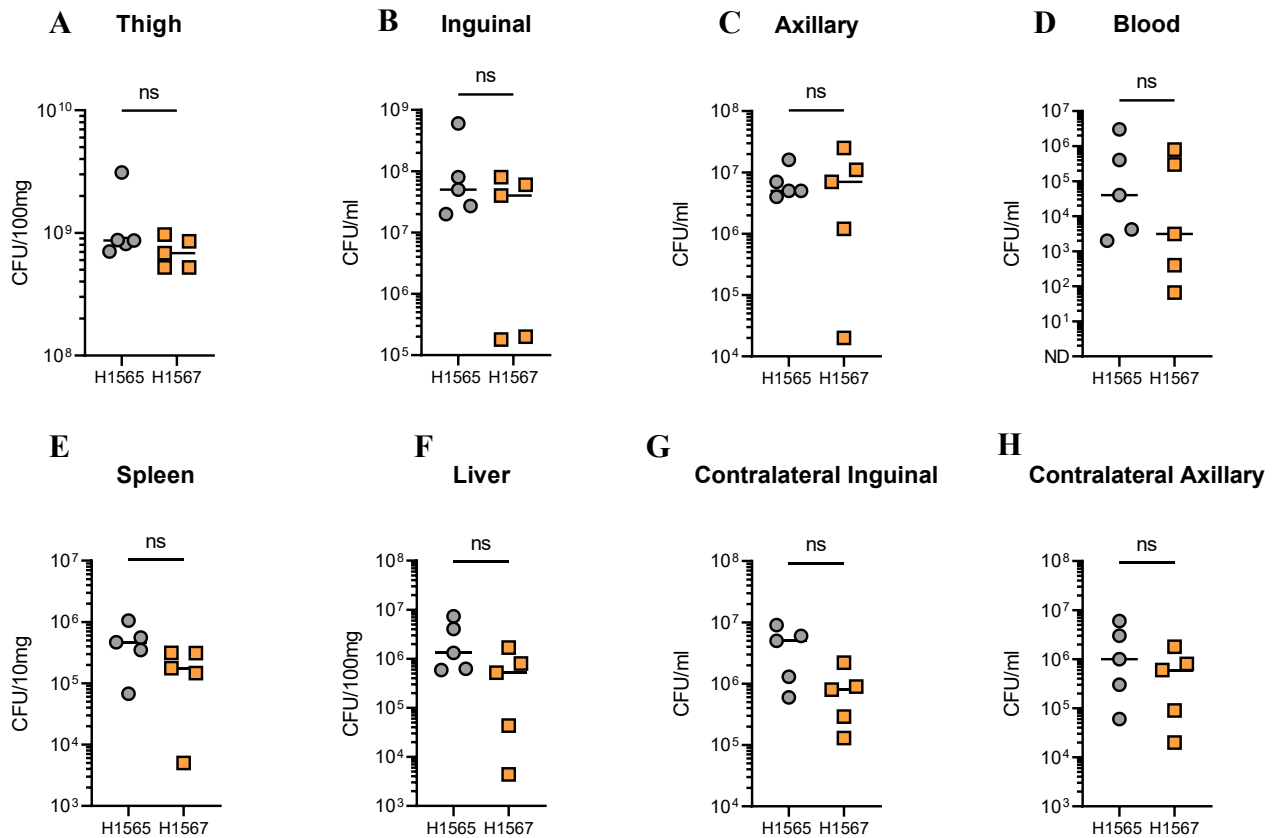


Figure 6-4. Recovery of *S. pyogenes* after 24-hour intramuscular infection with H1565 and isogenic *cepA* mutant, H1567

Female, C57BL/6 mice were inoculated with 3×10^8 CFU H1565 (M1 CovR_{MT}) (grey circles) or 1.67×10^8 CFU H1567 (M1 CovR_{MT} $\Delta cepA$) (yellow squares) into the hindlimb and culled 24 hours post infection. *S. pyogenes* was recovered and enumerated by bacterial culture from sites A-H. n = 5 mice per group, bar represents median value, ns p > 0.05 determined by Mann-Whitney U test.

H1565 and H1567 were then compared in an intranasal model of infection. In line with previous data, there was no statistically significant difference between SpyCEP positive or negative isolates (Figure 6-5). Interestingly however, the median values in the intranasal infection model showed a trend of increased burden in the absence of SpyCEP. These pilot results may align with previously published reports demonstrating SpyCEP expression is detrimental for *S.*

pyogenes adherence and endothelial and epithelial cell invasion (148), although the differences observed herein were not significant.

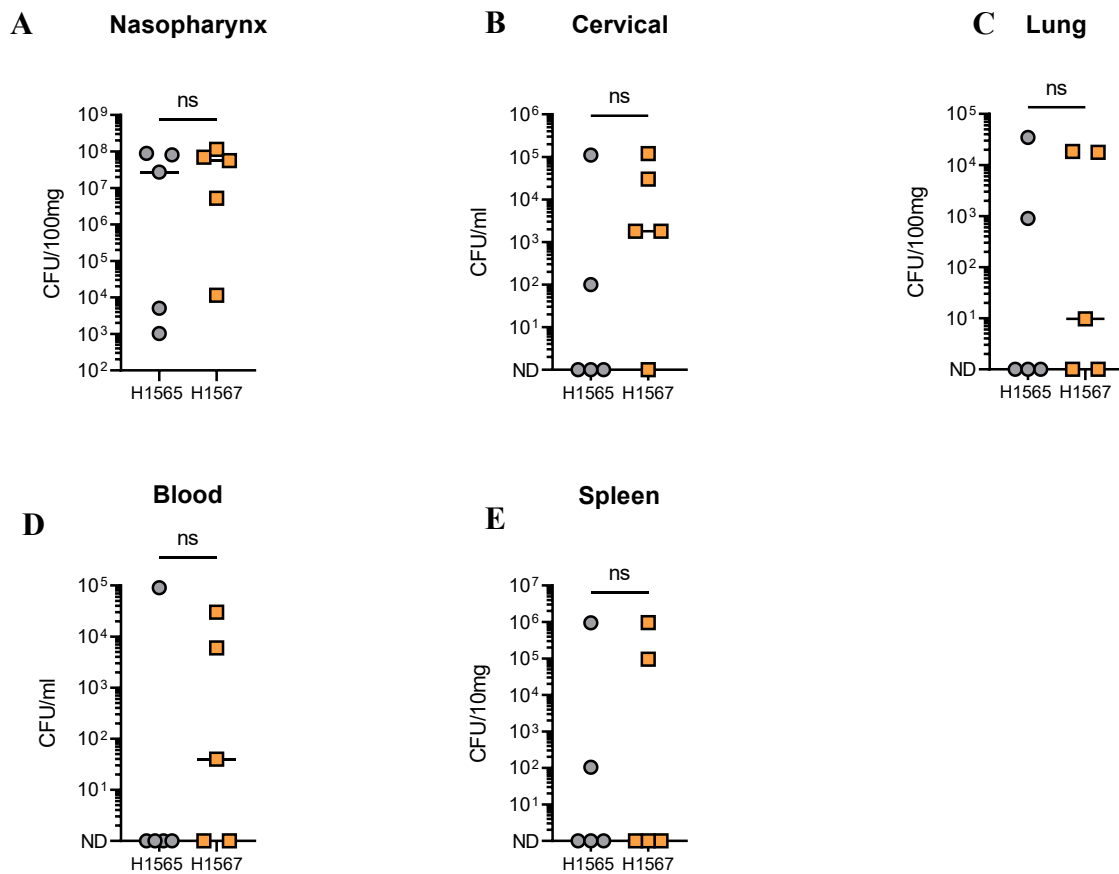


Figure 6-5. Recovery of *S. pyogenes* post 24-hour intranasal infection with H1565 and isogenic *cepA* mutant, H1567

Female, C57BL/6 mice were inoculated with 8.67×10^7 CFU H1565 (M1 CovR_{MT}) (grey circles) or 4.33×10^7 CFU H1567 (M1 CovR_{MT} Δ *cepA*) (yellow squares) by inhalation into both nostrils and culled 24 hours post infection. *S. pyogenes* was recovered and enumerated by bacterial culture from sites A-E. n = 5 mice per group, bar represents median value, ns p > 0.05 determined by Mann-Whitney U test.

Earlier experimental data identifying a role for SpyCEP highlighted that SpyCEP may play a role in the early stages of invasive infection; by 24-hours other virulence factors may become more important. Whilst exploratory data over 24-hours gave an indication that intramuscular infection with the CovR mutant H1565 might provide a suitable model, it was decided to adapt

the model to examine the role of SpyCEP at an earlier stage of infection. Recently, SpyCEP expression has been directly linked to increased bacterial survival in the lymphatic system during early infection (147) while heterologous SpyCEP expression in a *L. lactis* infection model can drive bacteraemia within 7-hours of infection (30). The SpyCEP-dependent lactococcal infection model which has been previously used to strongly implicate SpyCEP in driving invasive disease was therefore selected for initial evaluation in a small pilot study (30). SpyCEP is the only known virulence factor expressed by the recombinant *L. lactis* strain and as such was predicted to provide the clearest phenotype between SpyCEP positive and negative isolates. Intranasal infection was not assessed with this model.

Mice were infected by the intramuscular route with either H486, a wild type *L. lactis* isolate containing an empty control vector, (Lactis_{vector}), or H487, an isogenic strain constitutively expressing plasmid encoded SpyCEP, (Lactis_{pcepA}). C57/BL6 mice were given intraperitoneal erythromycin, to maintain plasmid expression, 2-hours prior to infection. The infection was halted 7-hours post infection and organs were harvested for bacterial enumeration (Figure 6-6). In line with previously published data, SpyCEP expression was clearly required for invasive infection. Mice infected with SpyCEP expressing H487 had a median of 8.66×10^7 CFU/100mg in the soft tissue infection site, a statistically significant 94-fold increase over the control strain (Figure 6-6 A). SpyCEP expression further permitted the metastasis of *L. lactis* throughout the local and distal lymphatic system, a feature that was not present in mice infected with SpyCEP negative strains, (Figure 6-6 B-C). SpyCEP expression additionally resulted in the seeding of *L. lactis* into the systemic circulation, with bacteria detected in the blood of 1/3 mice and in all harvested mice spleens (Figure 6-6 D-E).

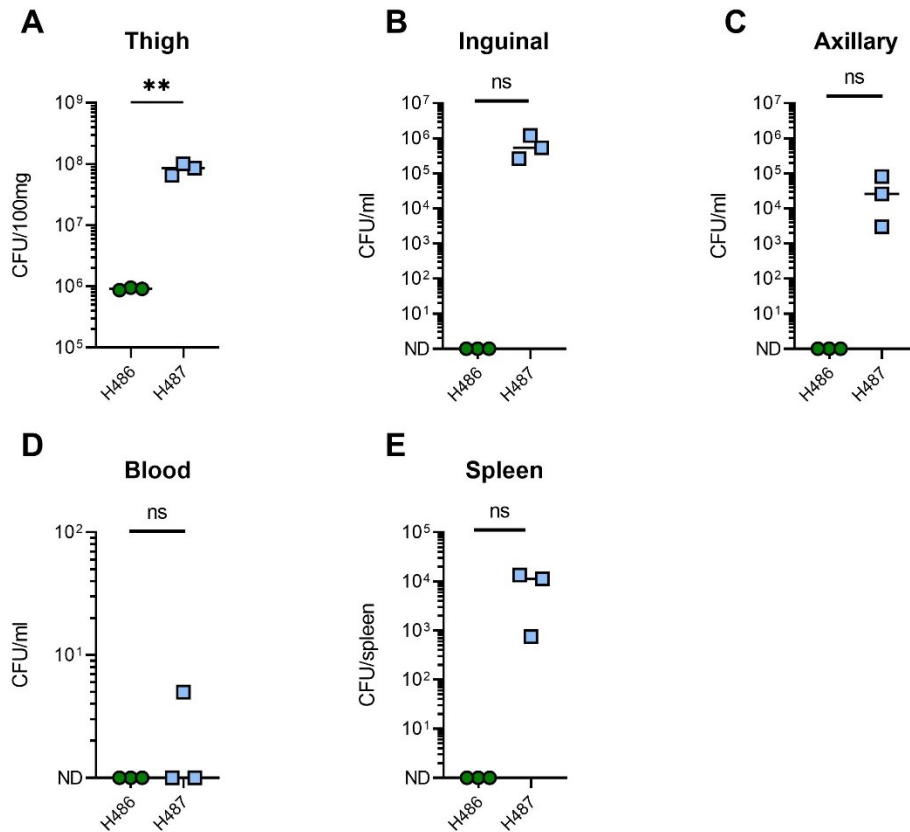


Figure 6-6. Recovery of *L. lactis* after 7-hour intramuscular infection with H486 and isogenic *cepA* expression strain, H487

Female, C57BL/6 mice were inoculated with 1.42×10^7 CFU H486 (Lactis_{vector}) (green circles) or 2.2×10^7 CFU H487 (Lactis_{cepA}) (blue squares) by intramuscular injection and culled 7 hours post infection. *L. lactis* was recovered and enumerated by bacterial culture from sites A-E. n = 3 mice per group, bar represents median value, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$ determined by Mann-Whitney U test.

Due to the considerable and significant SpyCEP-dependent phenotypic differences highlighted in this infection model, it was selected as a first candidate to begin assessing therapeutic intervention with inhibitor 1.

6.2.2 Administration of inhibitor 1 in a *L. lactis* intramuscular infection

It was decided to evaluate inhibitor 1 in an infection model with and without SpyCEP present, to check how the inhibitor behaved *in vivo* and ensure that any downstream results would be specifically attributable to SpyCEP inhibition and not to other, non-specific off target effects. This was particularly important to determine, inhibitor 1 is known to impair c-Raf, p38 map kinase signalling and TNF- α translation which could likely impact the response to infection.

Female C57BL/6 mice were pre-administered erythromycin and either inhibitor 1, or control reagent (water or the DMSO vehicle) by intraperitoneal injection. Two hours later, intramuscular infection followed with *L. lactis* isolate H486 containing the empty expression control vector. Infections were halted at 7-hours post inoculation and organs were harvested to assess bacterial dissemination. As expected, there were no significant differences in bacterial burden at the local infection site, or in bacterial dissemination through the lymphatic and systemic system between any treated or control groups (Figure 6-7). H486 was recovered from the local draining lymph nodes, a feature not seen in (Figure 6-6), however this is likely due to the increased inoculum used in this infection.

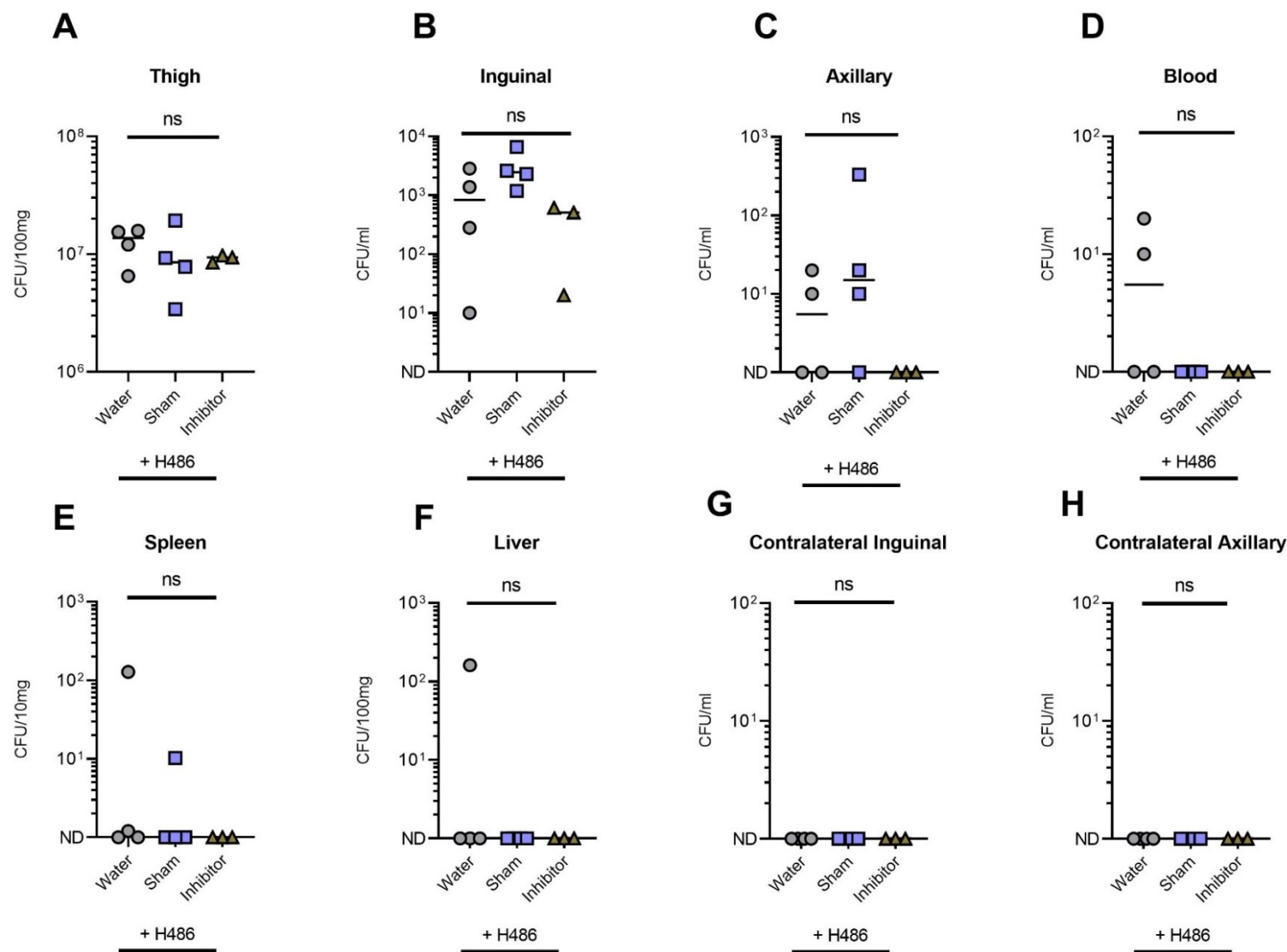


Figure 6-7. Recovery of *L. lactis* post 7-hour intramuscular infection with H486 treated with either water, sham control, or inhibitor 1

Female, C57BL/6 mice were pre-treated with 100 μ l water (light grey circles), 100 μ l 1.5% DMSO water (blue squares) or 100 μ l 1.5% DMSO 1.5mg/ml inhibitor 1 (brown triangles) by intraperitoneal injection, 2-hours prior to an intramuscular inoculation of 9.0×10^7 CFU *L. lactis* H486 (Lactis_{vector}). Mice were culled 7-hours post infection and *L. lactis* was recovered and enumerated by bacterial culture from sites A-H. n = 3-4 mice per group, bar represents median value, ns p > 0.05 determined by Kruskal-Wallis test.

Having established that administration of the inhibitor was not having measurable off target effects on bacterial dissemination, use of the compound was assessed in an infection model in the presence of SpyCEP. The sample size was increased for adequate statistical power, as calculated from the group size required for statistical significance ($p < 0.05$) in the spleen during SpyCEP positive and negative *L. lactis* infection, (Figure 6-6 E). As detailed above, mice were pre-treated with erythromycin, and either inhibitor 1, DMSO vehicle control or water were given, followed 2-hours later by inoculated via intramuscular injection with the *L. lactis* expressing SpyCEP strain, H487.

As seen in pilot infections, SpyCEP expression by *L. lactis* resulted in widespread bacterial dissemination throughout the lymphatic and systemic system (Figure 6-8). This was highlighted by the presence of bacteria in contralateral lymph nodes, a result only possible through systemic bacterial spread. Pre-treatment of mice with inhibitor 1 had a statistically significant effect on the bacterial burden at the site of infection, when compared to untreated mice or mice treated with DMSO vehicle control. Therapeutic use of inhibitor 1 resulted in a 1.7 and 2.1-fold reduction in median bacterial thigh load when compared to untreated and vehicle treated mice respectively (Figure 6-8 A). Administration of inhibitor 1 did not result in a reduction in bacterial spread to local and distal lymph nodes (Figure 6-8 B-C), nor was there a measurable difference in the bacterial load in the circulating bloodstream (Figure 6-8 D). However, there was a significant reduction in median bacterial recovery from the spleen when inhibitor 1 intervention was compared to the vehicle control: 5.63-fold reduction in median load. A 2.4-fold reduction was also seen when compared to untreated mice, however this result was not significant (Figure 6-8 E). There were no significant differences present in the remaining systemic organs or tissues tested (Figure 6-8 F-H).

These data demonstrate a SpyCEP-specific *in vivo* effect of inhibitor 1. Whilst only providing a moderate reduction in bacterial load, the results indicate that inhibitor 1 can penetrate infected tissue to neutralise SpyCEP activity at the site of infection and promote bacterial clearance.

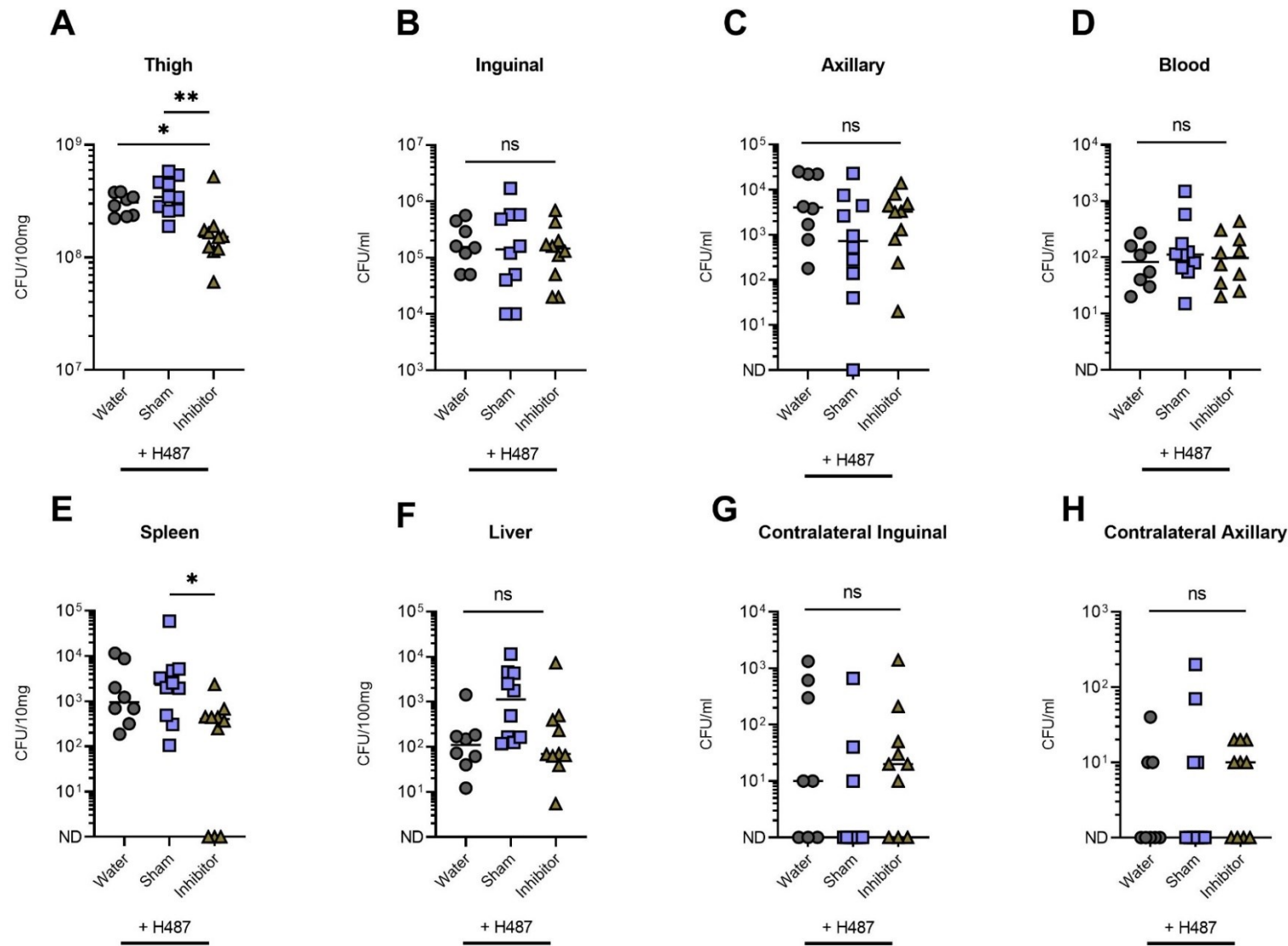


Figure 6-8. Recovery of *L. lactis* post 7-hour intramuscular infection with H487 treated with either water, sham control, or inhibitor 1

Female, C57BL/6 mice were pre-treated with 100μl water (dark grey circles), 100μl 1.5% DMSO water (blue squares) or 100μl 1.5% DMSO 1.5mg/ml inhibitor 1 (brown triangles) by intraperitoneal injection, 2-hours prior to an intramuscular inoculation of 6.28×10^7 CFU *L. lactis* H487 (*Lactis_{pcepA}*). Mice were culled 7-hours post infection and *L. lactis* was recovered and enumerated by bacterial culture from sites **A-H**. n = 8-10 mice per group, bar represents median value, ns p > 0.05, * p ≤ 0.05, ** p ≤ 0.01, determined by Kruskal-Wallis test.

A single dose of inhibitor 1, pre-administered prior to infection, showed a modest effect in the animal model and raised the question of whether a second dose may increase therapeutic efficacy. Toxicology and pharmacokinetic studies have shown that the inhibitor is metabolised rapidly *in vivo* (~1 hour half-life), is well tolerated, and has been administered at higher doses, albeit in rats. Two 150 µg doses of inhibitor 1 were therefore evaluated in a pilot experiment, administered by intraperitoneal injection, 6-hours apart. The first dose, 2-hours before infection was given alongside erythromycin as before, and a second dose was administered 4-hours post infection. A water bolus control group and DMSO vehicle control group were included and dosed according to the same schedule. As per previous experiments, infections were halted at 7-hours (Figure 6-9). An additional control group infected with control *L. lactis* strain, H486, and pre-administered with a water bolus was also added for comparison.

Surprisingly, inhibitor 1 did not measurably affect the bacterial load recovered from the infected thigh (Figure 6-9 A), nor was there a significant effect on bacterial dissemination to the local and draining lymph nodes (Figure 6-9 B-C). When assessing systemic dissemination however, there was a consistent reduction in median recovered bacterial loads in every organ and tissue assessed, though this was not significant (Figure 6-9 D-H). In the circulating blood *L. lactis* load was reduced 2.8 and 4.8-fold when inhibitor treatment was compared to the water or DMSO controls respectively. Bacterial reduction in the systemic organs was more marked, with a 12.1 and 9.1-fold reduction in the spleen and 10.4 and 4.4-fold reduction in the liver compared to the two control groups. This reduction was also evident in the contralateral lymphatics where no bacteria were recovered after inhibitor treatment.

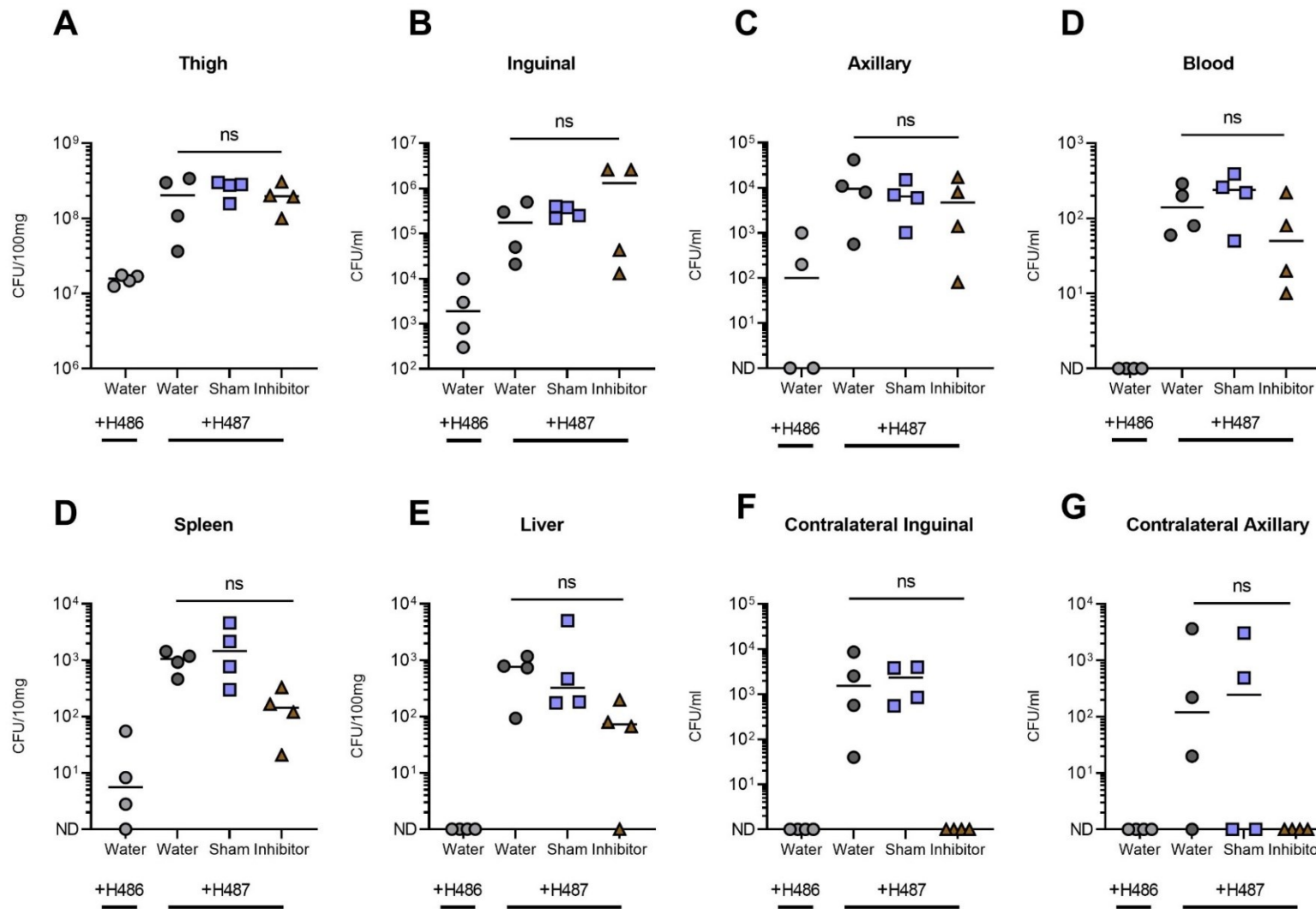


Figure 6-9. Recovery of *L. lactis* post 7-hour intramuscular infection with H486 or H487 treated with 2 doses of either water, sham control, or inhibitor 1.

Female, C57BL/6 mice were pre-treated with 100 μ l water (light and dark grey circles), 100 μ l 1.5% DMSO water (blue squares) or 100 μ l 1.5% DMSO 1.5mg/ml inhibitor 1 (brown triangles) by intraperitoneal injection, 2-hours prior and 4- hours post an intramuscular inoculation of 1.15×10^8 CFU *L. lactis* H486 (Lactis_{vector}) or 8.67×10^7 CFU *L. lactis* H487 (Lactis_{pcepA}). Mice were culled 7-hours post infection and *L. lactis* was recovered and enumerated by bacterial culture from sites A-H. n = 4 mice per group, bar represents median value, ns p > 0.05 determined by Kruskal-Wallis test.

These data suggested that an additional dose of inhibitor 1 may increase immune clearance and control of bacterial which have spread to the systemic system. Notably the experiments were pilot studies and insufficiently powered to rule in or rule out an effect.

6.2.3 Administration of inhibitor 1 in a *S. pyogenes* intramuscular infection

Establishment of inhibitor activity, albeit modest, in the *L. lactis* infection model led to the question of whether efficacy could be demonstrated during a streptococcal infection. A streptococcal model with the greatest possible SpyCEP phenotype was needed, to determine the efficacy of inhibitor 1. To achieve this, an M81 high SpyCEP producing strain, H292, was employed, as a replacement for the M1T1 isolates examined earlier in this chapter. H292 produces greater concentrations of SpyCEP than the CovR mutant, H1565, as quantified in 3.2.3. Further, whilst H292 was isolated from a lethal infection, the M81 background is not as highly associated with invasive disease as M1T1. These considerations provided us with an increased opportunity to measure inhibitor 1 effect in a streptococcal infection.

Mice were infected with *S. pyogenes* strain, H292 (M81 CovR_{MT}) and control group was infected with isogenic *cepA* mutant, H575 (M81 CovR_{MT} Δ *cepA*). One dose of inhibitor 1, water or DMSO sham was administered by intraperitoneal injection 2 hours before infection to the mice infected with H292 (Figure 6-10).

Although minimal effect was seen at the site of infection, administration of inhibitor 1 significantly reduced bacterial burden in the draining lymph node in mice infected with H292, compared to those pre-treated with sham control or water only (5.7-fold reduction and 1.74-fold reduction respectively) (Figure 6-10 B). Inhibitor 1 further significantly reduced *S. pyogenes* recovery from the secondary draining axillary lymph node, with a 9.2 and 10.8-fold

reduction compared to water and DMSO controls respectively (Figure 6-10 C). Although significant reductions were measured in the lymphatics, there were no significant effects of inhibitor 1 on bacterial load in any systemic organs or tissues examined (Figure 6-10 D-H).

Mice infected with the SpyCEP deficient H575 isolate provided insight into the contribution that SpyCEP makes to infection phenotype in this strain; mice infected with H575 had a significantly reduced bacterial burden at the local infection site, and in draining lymph nodes where SpyCEP expression was associated with 8.73-fold increase in inguinal lymph node spread. However, SpyCEP deficiency had no effect on systemic spread to other organs or blood (Figure 6-10 A). The results were therefore partially emulated by inhibitor-1 which resulted in reduced ipsilateral lymph node spread but did not affect systemic spread.

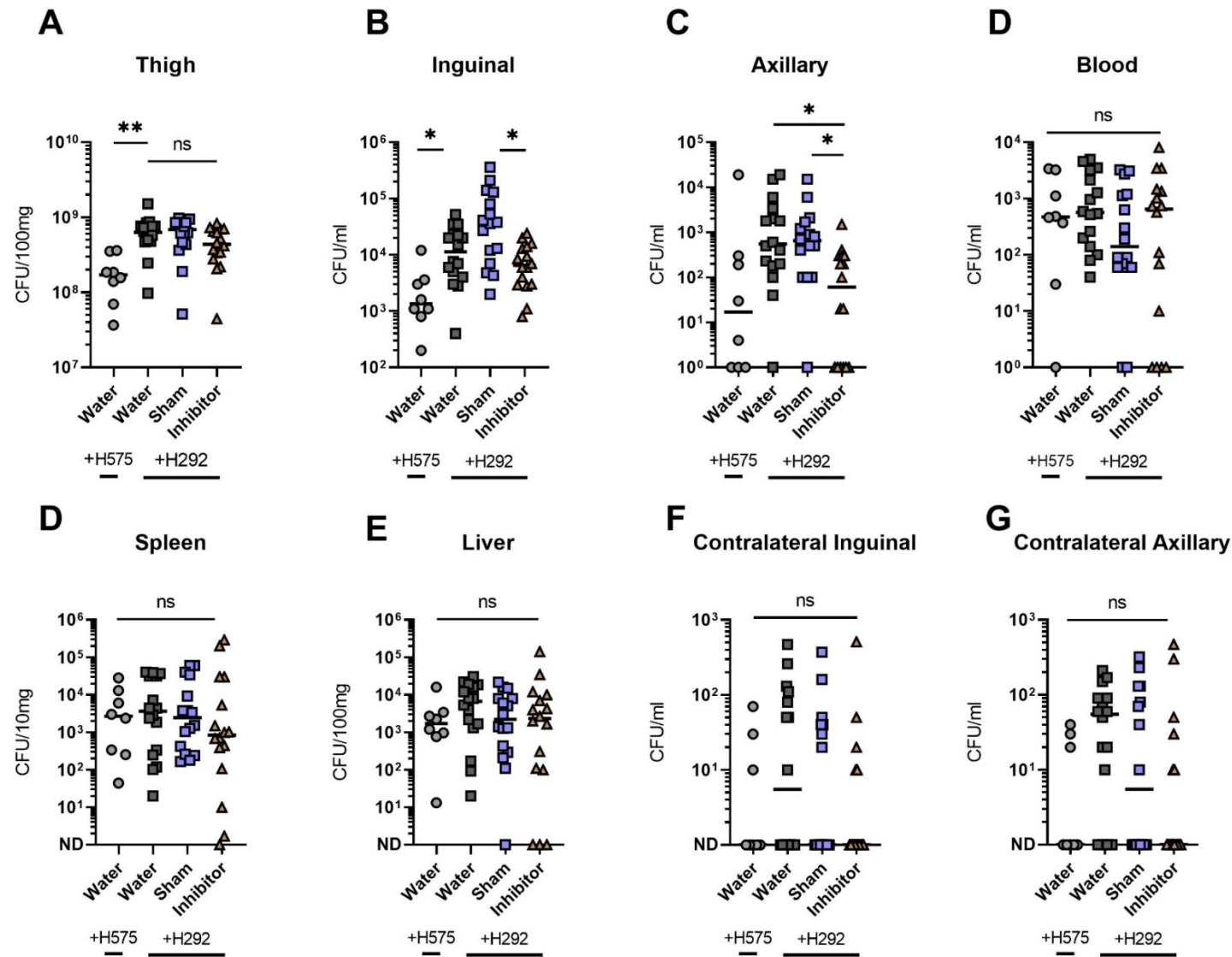


Figure 6-10. Recovery of *S. pyogenes* post 7-hour intramuscular infection with H575 or H292 treated with either water, sham control, or inhibitor 1.

Female, C57BL/6 mice were pre-treated with 100μl water (light grey circles and dark grey squares), 100μl 1.5% DMSO water (blue squares) or 100μl 1.5% DMSO 1.5mg/ml inhibitor 1 (brown triangles) by intraperitoneal injection, 2-hours prior into an intramuscular inoculation of 1.05×10^8 CFU *S. pyogenes* H292 (M81 CovR_{MT}) or 1.27×10^8 CFU *S. pyogenes* H575 (M81 CovR_{MT} ΔcepA). Mice were culled 7-hours post infection and *S. pyogenes* was recovered and enumerated by bacterial culture from sites A-H. n = 8-16 mice per group, bar represents median value, ns p > 0.05, * p ≤ 0.05, ** p ≤ 0.01, determined by Kruskal-Wallis test.

As with previous results from the lactococcus model, the moderate effects of a single pre-administered dose of inhibitor 1 led to assessment of 2 doses of inhibitor 1. As before, dosing was administered 6 hours apart, 2 hours before intramuscular infection and 4 hours after (Figure 6-11). As seen in single dose experiments, when comparing H292 with H575, SpyCEP expression increased median bacterial burden in the thigh by 3.78-fold, however, 2 doses of inhibitor-1 did not emulate this reduction in bacteria (Figure 6-11 A). Inhibitor administration did, however, significantly reduce bacterial spread to the draining inguinal lymph node when compared to the DMSO control group, (5.77-fold reduction), and when compared to the water bolus control group, (4.15-fold reduction), although the latter result was not significant (Figure 6-11 B). In contrast to results from the single dose infection, inhibitor 1 did not significantly reduce bacterial burden in the axillary lymph node and in concordance with single dose, results did not affect blood, spleen, or liver burden (Figure 6-11 D-F). Interestingly, however, inhibitor 1 did produce a significant reduction in bacterial burden in the contralateral lymph node compared with both water and DMSO control, a 25 and 180-fold reduction respectively (Figure 6-11 G). Median bacterial burden was additionally reduced 205 and 75- fold in the contralateral axillary lymph node, though this result was not significant (Figure 6-11 H)

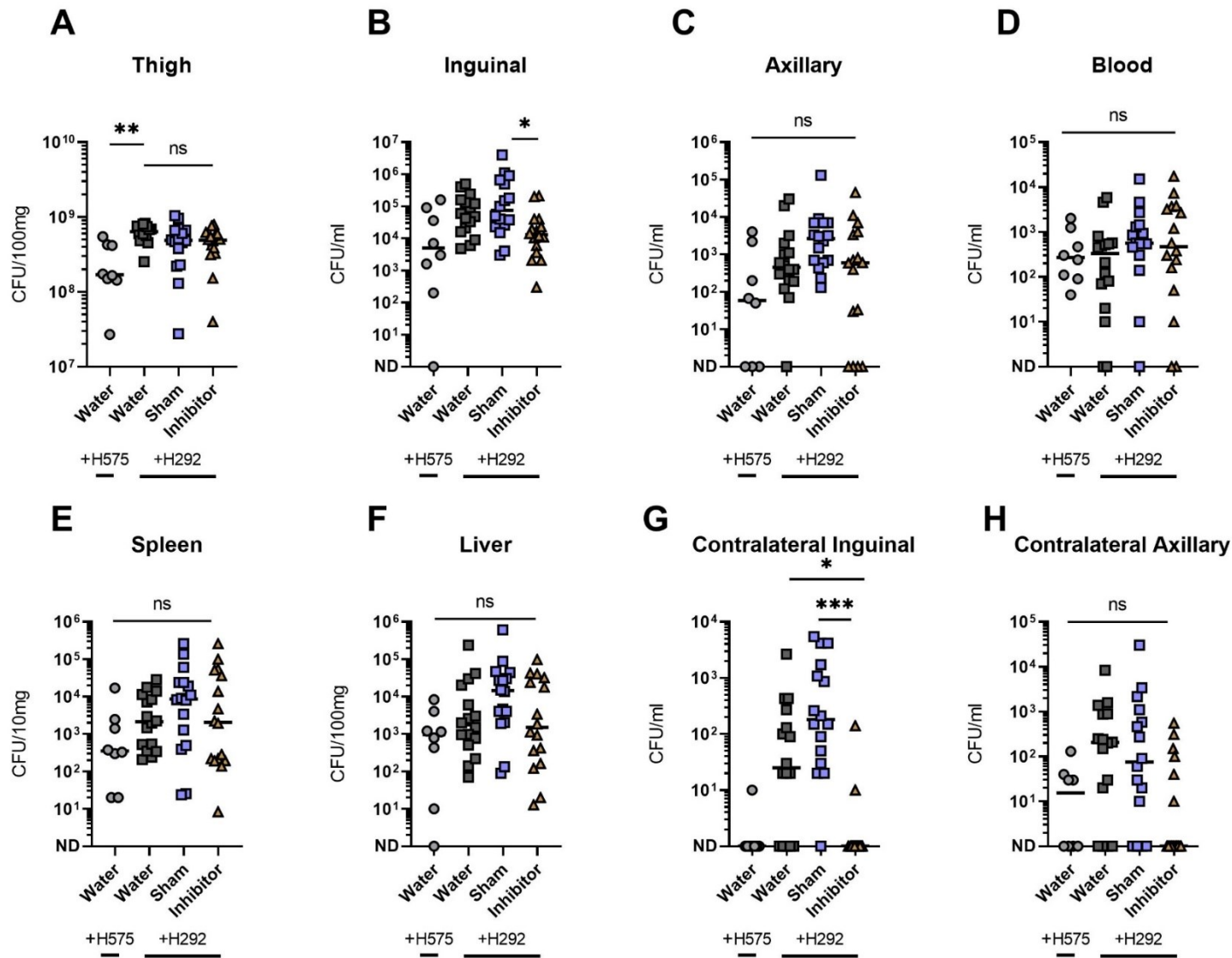


Figure 6-11. Recovery of *S. pyogenes* post 7-hour intramuscular infection with H575 or H292 treated with 2 doses of either water, sham control, or inhibitor 1.

Female, C57BL/6 mice were pre-treated with 100μl water (light grey circles and dark grey squares), 100μl 1.5% DMSO water (blue squares) or 100μl 1.5% DMSO 1.5mg/ml inhibitor 1 (brown triangles) by intraperitoneal injection, 2-hours prior or 4-hours post an intramuscular inoculation of 1.54×10^8 CFU *S. pyogenes* H292 (M81 Cov_{MT}) or 1.35×10^8 CFU *S. pyogenes* H575 (M81 Cov_{MT} Δ*cepA*). Mice were culled 7-hours post infection and *S. pyogenes* was recovered and enumerated by bacterial culture from sites A-H. n = 8-16 mice per group, bar represents median value, ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ determined by Kruskal-Wallis test.

6.3 Discussion

Assessment and identification of suitable infection models

In this chapter, the infection phenotypes of the two M1T1 *S. pyogenes* isolates and their isogenic SpyCEP deletion mutants have been characterised *in vivo*. In the infection model using CovR/S wild type isolates, H584 and H1464, SpyCEP expression had limited effect on disease outcome when evaluated solely on bacterial clearance at 24-hours. Despite a high inoculum, and despite being a clinical isolate associated with a lethal disease outbreak, H584 struggled to drive an invasive infection in mice, and differences between the SpyCEP positive and negative isolates were negligible. These data are comparable to previous *in vivo* evaluation of H584 intramuscular infection that showed bacterial burden peaks at 3 hours in the lymphatics with a marked reduction in lymphatic *S. pyogenes* burden after 24 hours, however bacterial burden peaks at 24 hours in the systemic circulation (147). The work did not, however, assess the role of the SpyCEP deletion strain, H1464. Whilst not assessed in this chapter, it could be reasoned that without transient modulation of the CovR/S system, this bacterial infection may have been cleared over time and is therefore not an ideal model with which to evaluate a novel therapy.

To correct for this minimally invasive phenotype, we assessed the clinical infection of H1565, a CovR mutant and abundant SpyCEP producer and the relevant isogenic SpyCEP deficient mutant, H1567. In contrast to H584, mice infected with H1565 demonstrated a strong invasive disease phenotype; mice demonstrated features of systemic inflammation and reached pre-determined experimental end points by 24-hours of infection, see section 2.2.14. These pre-defined endpoints help limit experimental bias and reduce animal suffering. A high bacterial burden was found in all examined tissues and infection would have likely progressed to severe disease in all mice had the infection been allowed to progress. Infection with a SpyCEP

negative strain did result in a reduced bacterial burden in the blood, spleen, liver, and contralateral lymph nodes, however these differences were not significant in any tissue.

Previously published *in vivo* data using H1565 and H1567 infection, demonstrated a significant reduction in the inguinal and axillary lymphatic spread when infected with the SpyCEP deficient mutant over 3-hours (147). However, the effect of SpyCEP was not assessed at later 24-hour timepoint. As previously discussed in section 1.3.2, the CovR/S system regulates a wide variety of important virulence factors in addition to SpyCEP; Sda1, SpeB, SLO, SLS, Ska, EndoS, HA capsule for example (98-102). The marked change in the regulation of these proteins caused by mutations in the CovR/S system will strongly drive an invasive phenotype *in vivo* and is likely acting to mask measurable SpyCEP specific effect in our model.

These factors necessitated a change in experimental design and instigated a subsequent shift to the lactococcal infection model. In addition to changing model, the infection time course was also adjusted. Data from this thesis coupled with previously published work (30), suggested that SpyCEP-specific effects may be more easily measurable during earlier infection. Subsequent infections with SpyCEP-expressing or -deficient *L. lactis* demonstrated marked and significant differences in bacterial clearance from the site of infection and dissemination throughout all examined tissues, even when using small groups. These data corroborated previous work (30) and underlined the ability of SpyCEP to drive invasive infection; these lactococcal isolates only differed in SpyCEP expression and importantly expressed no other known virulence factors. As such, any effects can be directly attributed to SpyCEP action and are not influenced by any masking effects. This provided the perfect system to begin examining inhibitor efficacy.

Assessment of inhibitor 1 on lactococcal and streptococcal infection

Despite being well tolerated in humans and mice it was important to establish that administration of inhibitor 1 would not cause ‘off target’ effects that would influence bacterial dissemination. This was especially important as work earlier in the thesis showed that inhibitor 1 is capable of inhibiting multiple host proteases, section 5.2.2, in addition to previously published effects of the compound on p38 kinase activation and subsequent TNF- α translation. Mice were infected with a SpyCEP negative strain and treated with inhibitor 1 or DMSO vehicle control. There were no measurable effects on bacterial dissemination between groups and overall inhibitor 1 was well tolerated by the mice. Having established that inhibitor 1 did not cause measurable off target effects we assessed its efficacy against SpyCEP expressing *L. lactis* and *S. pyogenes*.

One dose of inhibitor 1 produced moderate effects when administered to mice infected with *L. lactis* expressing SpyCEP. The greatest effect was seen in the spleen, with over a 5.63-fold reduction with inhibitor 1 treatment. Burden in the thigh was reduced 2-fold. These data showed that the inhibitor is effective in reducing local and systemic bacterial loads. However, mice infected with the SpyCEP negative *L. lactis* exhibited a 2-log fold reduction in thigh burden, representing a 50-fold greater effect than inhibitor treatment suggesting that inhibition of SpyCEP by inhibitor-1 was not maximal or complete. Multiple dosing using inhibitor 1 was not significantly better than a single dose, indeed, one dose outperformed two doses in reducing local soft tissue burden. Two doses of inhibitor resulted in reductions of *L. lactis* in the systemic tissues examined, but with no statistical significance. These data support a clinical effect for the inhibitor that might be more apparent with increased power. Indeed, power calculations based on the spleen data demonstrate that n=14 mice per group, would be required to result in a significant result assuming similar magnitude of effect.

Chemical inhibition of SpyCEP using inhibitor 1 in *S. pyogenes* infection yielded a less clear effect, with no measurable effect on bacterial thigh burden. This is in contrast to the effect of genetic manipulation of SpyCEP expression which significantly impacted bacterial thigh load, as highlighted by the 5.55-fold reduction between H292 and H575. Interestingly, and in contrast to findings with *L. lactis*, one dose of inhibitor 1 did significantly reduce lymphatic dissemination to the draining and distal lymph nodes, although no effect was seen systemically. This may point to a particular role for SpyCEP in *S. pyogenes* survival in lymph nodes that was noted previously (30, 147) . As before, two doses did not significantly increase inhibitor 1 effect at the site of infection or systemically, although there was an interesting and significant effect on contralateral lymph nodes. Although the contralateral nodes were used as an indicator of systemic spread and were not previously believed to receive *S. pyogenes* directly from a hindlimb infection, recent data from other co-workers suggest that *S. pyogenes* may spread from the thigh to the lower abdominal wall, resulting in the contralateral inguinal lymph node receiving bacteria direct from the hindlimb infection. As such, the inhibitor 1-induced reduction in contralateral inguinal lymph node reproduces the findings in the ipsilateral node.

The results from our initial inhibitor assessment were moderate and more nuanced than expected, likely for multifaceted reasons. Initial dosing was set at 150 µg / dose, to match the highest published mouse dose. However as discussed above, this may be too low to elicit a high enough serum concentration for measurable SpyCEP inhibition. Due to the short inhibitor 1 half-life, dosing at 6-hour intervals is unlikely to raise the serum concentration appreciably above the initial dose. The dose administered could be increased to raise the C_{max} , however this would require toxicology studies, that lie outside the remit of our animal licence. The timing between dosing intervals could also be decreased, and indeed, in a clinical setting it is likely that an inhibitor would be administered as an infusion. Inhibitor 1 could also be given closer to

the time of infection instead of 2-hours prior to infection, a method that was not assessed in this thesis.

There are a number of other important caveats to the dosing of inhibitor 1 in our initial *in vivo* assessment. Firstly, inhibitor 1 was not administered alongside supplementary antibiotics; in patients with invasive disease, the inhibitor would not be expected to function alone, and it is possible that combined with a beta lactam antibiotic to counter the wider impacts of bacterial infection, a difference would have been seen using the inhibitor compared with control reagent. Synergistic or increased inhibitor effects may have been apparent if antibiotic treatment had cleared mouse bacteraemia and reduced soft tissue burden. Secondly, in all experiments inhibitor 1 was pre-administered to mice, in contrast to reactive treatment upon disease manifestation. This was an intentional choice for the pilot assessment of inhibitor 1, however future work to develop the therapeutic will require vital experiments examining coadministration of antibiotics and variation in the timing of inhibitor dosing. Additionally, whilst we did not detect off target activity from inhibitor 1 with regard to bacterial dissemination, we cannot rule out interference from other effects of inhibitor 1 *in vivo*. Inflammation is a key mechanism in the innate immune response to pathogens, but it would require a broad, systems wide, biology approach to quantify all downstream effects of blocking p38 MAP kinase activation. The wide acting anti-inflammatory profile of inhibitor 1, with effects on pleiotropic master cytokines and regulatory proteins such as TNF- α and NF- κ B, will likely affect pathophysiological response to infection.

Due to the inflammatory modulation and previous reports showing that inhibitor 1 confers protection against polymicrobial sepsis, it would be of interest to undertake survival studies of *S. pyogenes* infection following treatment with inhibitor 1. Indeed, in both lactococcal and streptococcal infections, mice treated with inhibitor 1 were more responsive and showed less

visible markers of systemic illness, although this did not translate to any measurable effect on bacterial counts. In such studies, histopathology of infected thigh tissue, and analysis of serum inflammatory biomarkers coupled with systemic bacterial burden could provide comparative markers to assess wider inhibitor efficacy. In studies conducted in this thesis, there was a focus on bacterial clearance as the primary metric of inhibitor efficacy. Blood was not collected or analysed for cytokine analysis. Furthermore, histopathological analysis of neutrophil influx to muscle was not evaluated. It is also of note that a subcutaneous infection may provide a more effective model in assessing inhibitor 1 action, a delivery route which was not assessed in this thesis.

In summary, in the final results chapter of this thesis, the impact of inhibitor 1 on the progression of lactococcal and streptococcal disease *in vivo* has been evaluated. By inference from bacterial quantification, Inhibitor 1 appears capable of penetrating infected tissue and, in a lactococcal infection model, reduced bacterial burden; data from both lactococcal and streptococcal models suggest inhibitor 1 can affect systemic bacterial load. While these initial data suggest there may be therapeutic potential of inhibitor 1, *in vivo* assessment is still at a preliminary stage. Further research with antibiotic administration, streamlined dosing schedules and concentrations, and assessment across a broader array of clinically relevant *S. pyogenes* backgrounds, will be vital in the progression of inhibitor 1 development.

Noting the potentially deleterious effects of inhibitor 1 on other aspects of inflammation, it is possible that any inhibitory activity directed at SpyCEP is offset by the inhibition of other aspects of innate immunity. Hence, although biochemically inhibitor 2 was inferior to inhibitor 1 and is less well characterised, this may become an important molecule to explore.

7 Summary and future work

7.1 Summary of findings

During this thesis I have advanced the understanding of SpyCEP by demonstrating:

7.1.1 Chapter 3

1. Bioactive SpyCEP can be detected and accurately quantified in both tissue samples from *S. pyogenes* infected mice and patient samples from clinical *S. pyogenes* infection.
2. Bioactivity of SpyCEP is independent of *S. pyogenes* viability and persists after antibiotic killing. Dead bacteria can cleave CXCL8 to the same extent as live bacteria.

7.1.2 Chapter 4

1. The C-terminus of SpyCEP, containing the catalytic residues H279 and S617, is sufficient to catalyse the proteolysis of CXCL1, CXCL6 and CXCL8. This activity is independent of the SpyCEP N-terminus.
2. The C-terminus of SpyCEP is equally capable of binding CXCL8 as the full-length enzyme.
3. The C-terminus of SpyCEP is 1,500-fold less efficient at cleaving CXCL8 than the full-length enzyme.
4. Identification of two novel small molecule inhibitors of SpyCEP.

7.1.3 Chapter 5

1. Inhibitor 1 is more effective at inhibiting SpyCEP than inhibitor 2, however both are more effective than Pefabloc.
2. Inhibitor 1 can additionally inhibit the streptococcal C5a peptidase and both inhibitor 1 and inhibitor 2 demonstrate activity against archetypical serine proteases, chymotrypsin, subtilisin and elastase.
3. Inhibitor 1 actively binds SpyCEP during inhibition, however, it does not induce the dimerization of the enzyme.
4. Treatment of SpyCEP with inhibitor 1 successfully restores downstream CXCL8-mediated neutrophil chemotaxis.

7.1.4 Chapter 6

1. Inhibitor 1 can successfully penetrate soft tissue and reduce SpyCEP-expressing *L. lactis* burden during a necrotizing infection.
2. Inhibitor 1 can reduce the systemic spread of SpyCEP-expressing *L. lactis* to the spleen during a necrotizing infection.
3. Inhibitor 1 can reduce the spread of bacteria from the site of infection to the draining lymph nodes during an invasive *S. pyogenes* infection.

7.2 Discussion and future work

Work conducted in this thesis has furthered our understanding of SpyCEP, its biochemical activity and its relation to streptococcal disease.

Prior to this study, the only accurate quantification of SpyCEP was obtained from a heterologous SpyCEP producing *L. lactis* strain, H487, and a high *S. pyogenes* SpyCEP producing strain, H292. However, these data were limited to measuring enzyme released into rich growth media and did not quantify cell-associated enzyme. This work has furthered these results, quantifying SpyCEP across a range of *S. pyogenes* isolates with different CovR/S backgrounds. We have demonstrated that the majority of SpyCEP is in fact cell wall-associated, although soluble released enzyme is quantifiable in high SpyCEP-expressing isolates. Infection of mice with *S. pyogenes* strains expressing high levels of SpyCEP demonstrated SpyCEP bioactivity in infected serum, although we were unable to elucidate whether this activity was due to soluble enzyme, owing to mice being bacteraemic.

Little work has been done to establish the differential effects of soluble SpyCEP compared to cell wall-associated enzyme. CXC chemokines are vitally important during multiple stages of the neutrophil life cycle, including bone marrow release, neutrophil extravasation into tissues and neutrophil activation and chemotaxis (208). As such, the effects of soluble circulating SpyCEP, distant from the infection site, may compound the effects of locally produced SpyCEP and provide further escape from the neutrophil response. Future work to elucidate the systemic function of soluble SpyCEP, without the confounding effects of bacteria, are now possible due to the production of recombinant SpyCEP. This work has specifically quantified the concentration of SpyCEP present in stored samples from infected human tissues early in the infection course. Utilising these results to give accurate dosing, recombinant SpyCEP could be intramuscularly injected, and local and systemic chemokines levels measured in models where

chemokines are normally induced, perhaps including other bacterial infections or LPS-treatment. In addition to the absolute quantification of chemokines in tissues and systemically, the bioactivity assay developed in this thesis could be used to visualise the bioactivity of SpyCEP activity in these sites.

This availability of recombinant SpyCEP has also furthered accurate quantification of chemokine cleavage. Previous data have demonstrated differential cleavage ability across the CXC substrate range, however accurate quantification has been limited to using fixed concentrations of bacteria or specific volume of growth supernatant as a source of SpyCEP. In this work we have leveraged recombinant SpyCEP to accurately quantify CXCL1 and CXCL8 cleavage, demonstrating that CXCL8 cleavage occurs at an increased rate. These data confirmed previous results and demonstrated that CXCL8 is cleaved more efficiently than CXCL1, which in turn, is cleaved more efficiently than CXCL6. The increased activity against CXCL8 is perhaps unsurprising, as CXCL8 is the most potent neutrophil chemokine at inducing chemotaxis (209). However, the availability of recombinant SpyCEP can now facilitate future experiments to derive accurate cleavage kinetics for all SpyCEP substrate and create a true comparative list.

One surprising result from our SpyCEP quantification studies was the finding that SpyCEP present on dead bacteria can cleave CXCL8 to the same extent as live bacteria. These data support and explain the observation that neutrophils are often ‘held back’ from the infection site despite the patient receiving antibiotic treatment that results in bacterial killing (115). This raises the question of whether antibiotic treatment regimens could be adapted to limit the effect of SpyCEP, post-streptococcal death. However, patients with invasive diseases already receive broad spectrum antibiotics, including bactericidal and protein synthesis inhibitor-based antibiotics. It is unlikely, therefore, that altering treatment regimens would impede SpyCEP

bioactivity, since such drugs act on live bacterial only. However, this does not preclude future experiments using other inhibitors to assess impact on SpyCEP activity. The work highlights a possible role for antibody-based therapy although our results also suggest that IVIG penetration into such tissues may be insufficient, though inhibition studies were not carried out. Taken together the data underline a definite need for adjunctive treatment, that can block or inhibit SpyCEP activity in severe *S. pyogenes* disease, to allow neutrophil influx and subsequent clearance of antibiotic-killed bacteria.

SpyCEP activity post-streptococcal death was not the only novel aspect of SpyCEP function identified during this work. Analysis of multiple recombinant SpyCEP constructs demonstrated that proteolytic activity against CXCL1, CXCL6 and CXCL8 could be conferred by the C-terminal domain of SpyCEP alone, independent of the N-terminus. These were surprising results, since SpyCEP has been described as a classical subtilisin like serine protease, but the C-terminus comprises just two of the classical triad of residues normally required for serine protease activity. Furthermore, previous work attributed no enzyme activity to the C-terminus domain of SpyCEP (135). This was an interesting finding and provided evidence for a key role of the SpyCEP C-terminus in binding and processing chemokine substrates. The importance of this finding *in vivo* was not expounded upon in this thesis. It is likely that the extremely slow rate of proteolysis we observed with the C-terminal SpyCEP fragment would not cause a measurable effect on neutrophil function. Indeed, there are no reports of C-terminal SpyCEP mutants being isolated from invasive disease clinical samples, however, future work could assess the effect of the C-terminus *in vivo*. Heterologous expression of the C-terminal of SpyCEP in *L. lactis* may provide a convenient tool to examine any biologically relevant activity attributable to the C-terminal domain alone.

A major output of this thesis is the identification of two novel SpyCEP inhibitors, designated inhibitor 1 and inhibitor 2. These molecules were observed to reduce the production of the 13 amino acid C-terminal α -helix released after incubation of CXCL8 with SpyCEP. We subsequently confirmed that inhibitor 1 was actively binding SpyCEP and not subverting the assay by binding and sequestering CXCL8. Binding characteristics were not assessed for inhibitor 2, as such, future experiments will be required to confirm a direct interaction of SpyCEP with inhibitor 2.

Assessment of inhibitor 1 in assays of neutrophil chemotaxis confirmed that inhibitor-based ablation of CXCL8 cleavage successfully restores downstream CXCL8 neutrophil chemotaxis. Whilst this was not a surprising result, it was important to assess CXCL8 mediated effector functions before the use of inhibitor 1 *in vivo*. As such, future experimentation with inhibitor 2 will be required to ensure downstream effects are conserved after inhibition of cleavage.

Both inhibitor 1 and inhibitor 2 demonstrated reproducible inhibition of SpyCEP activity, with a greater efficacy than that of the only previously identified SpyCEP inhibitor, Pefabloc. However, much like Pefabloc, their action was not limited to SpyCEP. Both inhibitors demonstrated activity against the archetypal serine proteases, chymotrypsin, elastase and subtilisin, with inhibitor 1 also showing action against the streptococcal C5a peptidase. Initial activity against C5a peptidase was encouraging; the ability of 1 inhibitor to negate the action of two streptococcal proteases involved in subverting the innate immune system and neutrophil recruitment would be advantageous for an adjunctive treatment. However, the broad action of both inhibitors against multiple serine proteases could provide serious obstacles to therapeutic use. As mentioned in 5.3, there are currently 184 described human serine proteases. These are not just involved in the innate immune system but in multiple aspects of host cell function. Indeed, the human genomes encodes 30 active host serine protease inhibitors which are tightly

regulated to control cellular functions, the dysfunction of which is associated with human disease (210-212). In the case of inhibitor 1, this broad serine protease inhibitory action is compounded by its published effects on c-Raf and p38-MAP kinase activation and its stimulation of the anti-inflammatory reflex, increasing the chances of off target effects when given therapeutically. Future experiments to precisely define the inhibitory profile of inhibitor 1 and inhibitor 2 will be important before their implementation as therapeutics in infection.

Despite these concerns, inhibitor 1 has been proven to be safe and well tolerated in both toxicological studies and clinical trials (182, 184-188). Reactions have been limited to mild infection site phlebitis and some nephrotic abnormalities in rats when administered with the highest dose over multiple days during dose escalation studies (184). Whilst *in vivo* work carried out in this thesis was not designed to assess off target and systemic effects of inhibitor 1 administration, we did not detect any untoward reactions to inhibitor 1.

Inhibitor 1 demonstrated mild therapeutic effects on local soft tissue bacterial burden and dissemination when administered during *in vivo* infections caused by SpyCEP expressing *L. lactis* or high SpyCEP producing *S. pyogenes* isolates. Inhibitor 1 was able to significantly reduce the soft tissue bacterial burden in a *L. lactis* infection and the dissemination of bacteria to the draining lymphatics in *S. pyogenes* infection. Whilst in most cases the administration of inhibitor 1 did result in a reduction of systemic burden during infection, few results were significant compared to controls. The difference in observed efficacy of inhibitor 1 between *L. lactis* and *S. pyogenes* is most likely because of the many other (non-SpyCEP) virulence factors expressed by *S. pyogenes* that contribute to bacterial survival and disease progression.

The exploratory work carried out in this thesis is only the start of characterising inhibitor 1. Future experiments will be vital to developing its potential use as an adjunctive treatment in invasive streptococcal disease. Most vital will be the assessment of inhibitor 1 in conjunction

with antibiotic treatment, to align with routine patient treatment. Combined with antibiotic treatment, inhibitor 1 may display a greater measurable therapeutic effect, even in *S. pyogenes* experimental infections. Experiments to assess the correct dose and administration schedule will also be vital for future *in vivo* experiments. In addition to monitoring the effects on bacterial spread, future experiments should also assess the effect of inhibitor 1 on circulating chemokines and other biomarkers of disease severity such as IL-6. Had mouse KC levels been measured, it may have been possible to demonstrate an effect of the inhibitor 1 on chemokine cleavage in the local soft tissue and systemically. Histopathology should also be further assessed, as infected tissues may show a greater influx of neutrophils after inhibitor treatment for example, as seen following IVIG treatment (162).

Importantly, a second SpyCEP inhibitor was identified, that has not yet been assessed *in vivo*. While inhibitor 2 is not as effective at inhibiting SpyCEP as inhibitor 1, is it still more effective than Pefabloc. There is currently a limited supply and no commercial source for more inhibitor 2, so chemical synthesis would be required to enable further testing. However, due to the potential off target effects of inhibitor 1, continued experimental analysis of inhibitor 2 would be beneficial and it may result in a more effective molecule for *in vivo* therapy.

Future work on either inhibitor may also necessitate chemical modification. Structural changes could be introduced to modify or increase SpyCEP specific binding and inhibition or to minimise off target effects. These changes would require a much deeper understanding of both inhibitor action and binding to SpyCEP and may be necessary during late-stage therapeutic development. Indeed, new salt forms of inhibitor 1 are currently under development to allow for higher oral dosing and to address infection site phlebitis (187).

In conclusion, this thesis has for the first time, quantified SpyCEP in human clinical samples, quantified SpyCEP activity against the potent chemoattractant CXCL8, identified novel

domains necessary for enzymatic function and identified two novel SpyCEP inhibitors for use as an adjunctive therapeutic in invasive streptococcal infections. This work has furthered our understanding of SpyCEP function and will provide a platform for future SpyCEP-specific targeted therapies.

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9 Appendix

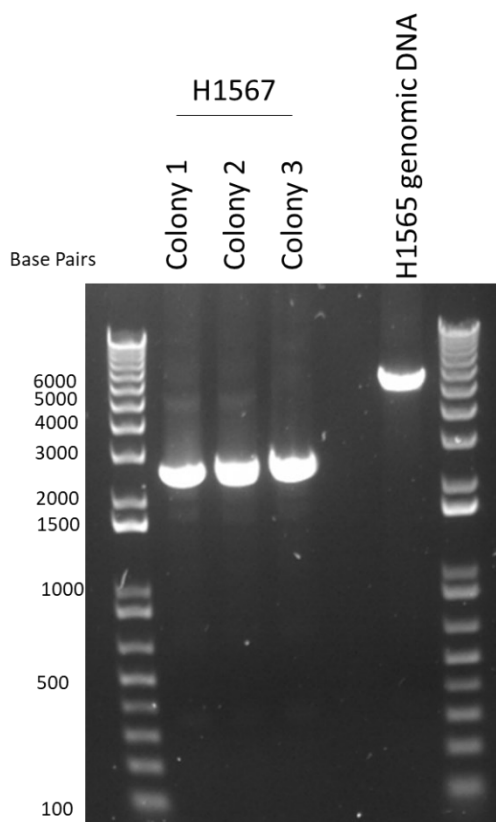


Figure 9-1. Confirmation of *cepA* deletion in *S. pyogenes* H1565

1% agarose DNA electrophoresis gel of PCR products from genomic DNA using the LCT0 – HYPO (*Spy0421*) primers 5'CTTCGCCACAATCCGTATGA3' and 5' TCGTATTTGTCGAGACGCCT 3'. Colonies 1-3 represent three successful electroporations of PUCMUT*cepA* into *S. pyogenes* isolate H1565 to create SpyCEP deletion mutant H1567. Successful gene replacement of *CepA* with *AphA3* gene is indicated by a 2.5 kb band compared to wild type 5.5 kb band in parent strain H1565, indicative of the 3 kb shorter *AphA3* gene compared to *cepA*. The figure is representative of PCR confirmation of H584 SpyCEP deletion mutant H1464. For a homologous recombination map and gene arrangement see Figure 3-1.

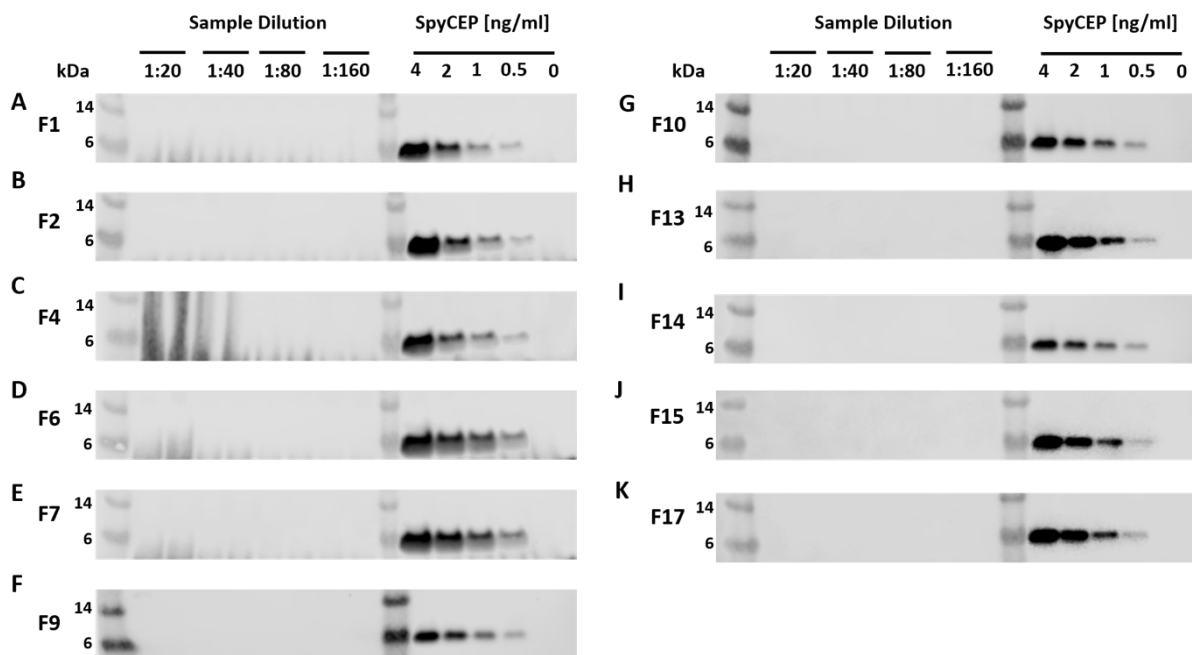


Figure 9-2. Clinical samples that lacked detectable SpyCEP activity from patients with confirmed *S. pyogenes* infections.

Of the 17 fluid samples tested (F1-F17) in section 3.2.5, 11/17 lacked detectable SpyCEP activity. Depicted here (A-K) are results from fluids F1, F2, F4, F6, F7, F9, F10, F13, F14, F15, and F17. The negative immunoblots show the lack of cleaved CXCL8 (6 kDa) after a 20-minute, 37°C incubation of 150 ng exogenous CXCL8 with human clinical samples from confirmed *S. pyogenes* infections from Table 3-1. N=2 experimental duplicates for each of the dilutions 1:20 – 1:160. Standard concentrations of SpyCEP (0-4 ng/mL) were also co-incubated with 150 ng CXCL8 and the resultant generation of cleaved CXCL8 is shown on the right side of each panel. Densitometry was used to calculate the amount of SpyCEP in each setting.

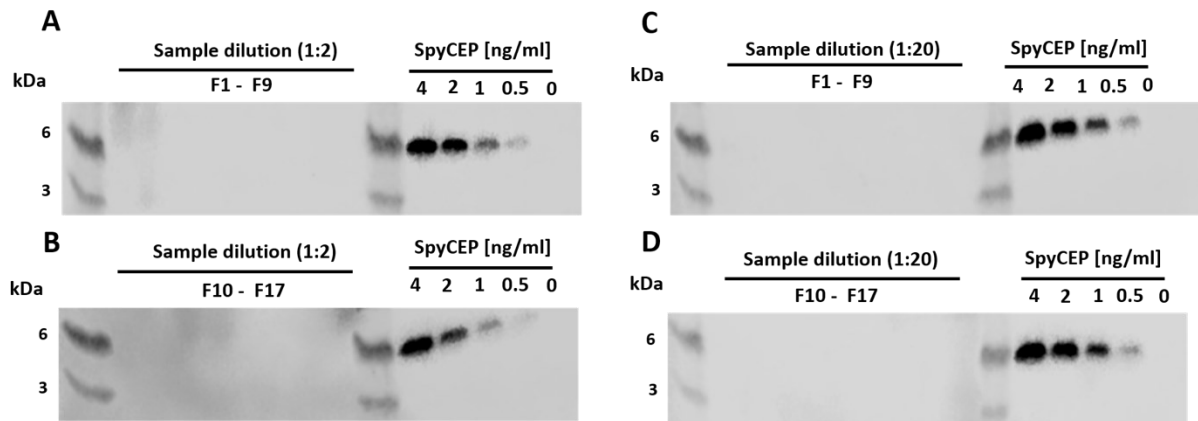


Figure 9-3 Lack of pre-existing cleaved CXCL8 in *S. pyogenes* infected human clinical samples

Negative immunoblots showing the absence of pre-existing cleaved endogenous CXCL8 (6 kDa) in fluid samples F1-F17. No exogenous CXCL8 was added to these samples. Samples were tested after a 20-minute, 37°C incubation of human clinical samples from confirmed *S. pyogenes* infections from Table 3-1, **A-B**. Fluids 1 - 17. n=1 experimental replicate at a 1:2 dilution of fluid. **C-D**. Fluids 1 - 17. n=1 experimental replicate at a 1:20 dilution of fluid. For comparison, standard concentrations of SpyCEP (0-4 ng/mL) were co-incubated with 150ng CXCL8 and the resultant generation of cleaved CXCL8 is shown on the right side of each panel.

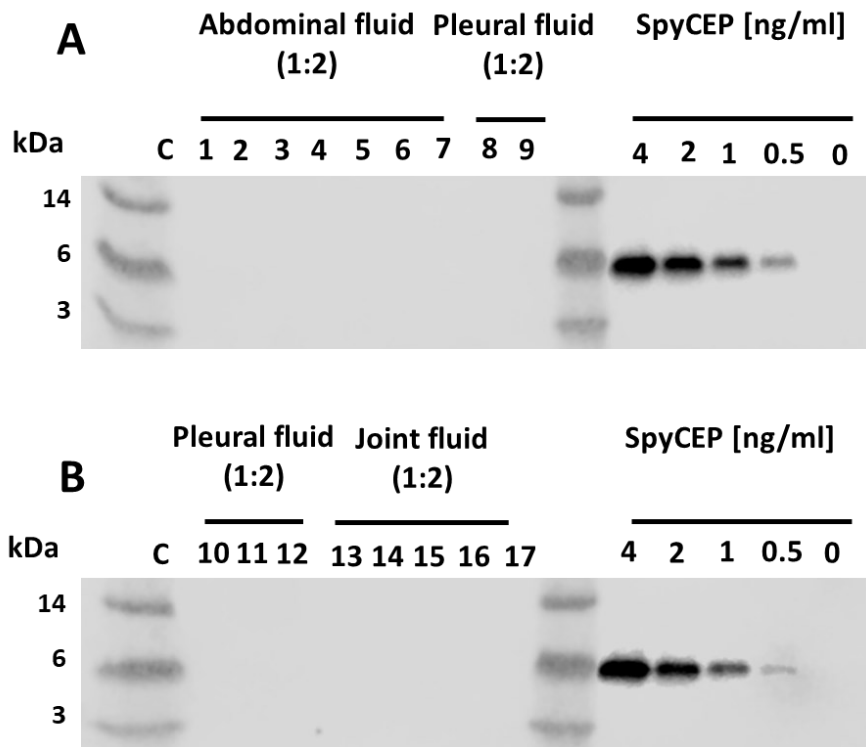


Figure 9-4. Lack of detectable SpyCEP activity in control fluid samples from non-*S. pyogenes* infections

Seventeen control fluid samples submitted for microbiological culture from patients without *S. pyogenes* infections were tested for SpyCEP bioactivity. The negative immunoblots show the lack of cleaved CXCL8 (6 kDa) after a 20-minute, 37°C incubation of 150 ng exogenous CXCL8 with clinical samples from confirmed non - *S. pyogenes* infections, see section 3.2.5, **A**. Seven abdominal fluids (C1-7) and two pleural fluids (C8-9). n=1 experimental replicate at a 1:2 dilution of fluid. **B**. Three pleural fluids (C10-13) and 5 joint fluids (C4-8). n=1 experimental replicate at a 1:2 dilution of fluid. Standard concentrations of SpyCEP (0-4 ng/mL) were also co-incubated with 150 ng CXCL8 and the resultant generation of cleaved CXCL8 is shown on the right side of each panel.