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The Role of Haptoglobin in the Immune Response to Gram-Positive Bacteria

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by

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled

"The Role of Haptoglobin in the Immune Response to Gram-Positive Bacteria" is based on work conducted by the author at the University of Leicester mainly during the period between 14 January 2013 and 31 December 2016. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

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Abstract

The Role of Haptoglobin in the Immune Response to Gram-Positive Bacteria

Mutaib Mashraqi

Haptoglobin (HP) is a positive acute-phase serum protein. It is upregulated during infection and is a valuable marker for many inflammatory-related diseases. In serum it is present as a disulphide-linked homodimer, with each subunit being composed of two domains, a complement control protein domain (CCP-domain) and a serine-protease-like domain. During biosynthesis the polypeptides are cleaved into α- (the CCP-domain) and β-chains (the SP domain) and both remain linked together by a disulphide bond. A crucial function of HP is to act as a scavenger of free haemoglobin from plasma, since high quantities of free haemoglobin can be deleterious for the host. More recent work indicates that HP also interacts with lipoteichoic acid (LTA) of Gram-positive bacteria, an important virulence factor, and this is the focus of my thesis.

The results of this work demonstrate that HP binds to LTA directly as well as to LTA on a wide range of Gram-positive bacteria (including LTA from *S. aureus* and *S. pneumoniae*). The IC50 of the interaction is ~40 nM in competition experiments to immobilised *S. aureus*. My work has shown that the LTA interaction site of HP is located on the β-chain and that LTA competes for binding with haemoglobin indicating that the binding sites for LTA and haemoglobin overlap.

Surprisingly, *in vivo* studies showed that C57BL/6J $HP^{-/-}$ mice show a significant degree of protection from experimental *S. pneumoniae* infection. Over the course of the experiments, approximately 90% of $HP^{-/-}$ mice were resistance to the pneumococcal infection compared to 40% in strain, age and sex matched control wild-type mice infected in parallel. In line with the infection study, wild-type mice showed significantly higher levels of bacteraemia than $HP^{-/-}$ mice and the bacterial load in the lung, liver, kidney was significantly higher.

These findings demonstrate that HP interacts with bacteria and plays an important role during infection. Surprisingly, the presence of HP appears to give *S. pneumoniae* a so far unknown advantage during infection.

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α	Alpha
AP	Alkaline Phosphate
APPs	Acute-phase proteins
APR	Acute-phase reaction
$Å^2$	Angstrom
ADCC	Antibody-dependent cellular cytotoxicity
AP	Alternative Pathway
CP	Classical Pathway
LP	Lectin Pathway
bp	Base Pair
BHI	Brian Heart Infusion
BSA	Bovine Serum Albumin
CSF	cerebrospinal fluid
CR3	Complement receptor 3
CFUs	Colony forming units
CHIPS	Chemotaxis inhibitory protein

CCP	Complement control repeat
CXCR	C-X-C chemokine receptor
CCL	Chemokine C-C Motif Ligand
CCR	Chemokine Receptor
CRP	C-reactive protein
ClfA	clumping factor A
C4bp	C4 Binding Protein
cDNA	Complementary DNA
dATP	Deoxyadinosine triphosphate
dNTPs	Deoxyribo nucleotide PCR grade
DMSO	Dimethyl sulfoxide
D.D.T	Dichlorodiphenyltrichloroethane
DCs	Dendritic cells
ELISA	Enzyme immunosorbent assays
E.Coli	*Escherichia Coli*
EGF	Epidermal Growth Factor
EDTA	Ethylenediaminetetra Acetic Acid

FCS	Fetal calf serum
FcR	Fc receptors
FH	Factor H
FCS	Foetal Calf Serum
FCN	Ficolin
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GIcNAc	N-acetylated glucosamine
G.phosphate	Glycerophosphate
gDNA	Genomic DNA
GSH	Glutathione
GSSG	Glutathione disulfide
G-CSF	Granulocyte colony-stimulating factor
g	Grams
HP	Haptoglobin
HPr	Haptoglobin-related protein
HarA	Staphylococcal haptoglobin receptor A
HB	Haemoglobin

HPr	Haptoglobin-related protein
HGF	Hepatocyte growth factor
ICAM-1	Intercellular adhesion molecule-1
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IFNs	Interferons
IFN-γ	Interferon-gamma
ITAM	Immunoreceptor tyrosine based activation motifs
IsdA	Iron-regulated surface determinant protein A
IP	Intraperitoneal
IN	Intranasal
IPTG	Isopropyl thiogalactoside, Isopropyl beta-D-thiogalactopyranoside
IPD	Invasive pneumococcal disease
IC50	Median inhibition concentration

iC3b	Inactivated C3b
JAM	Junctional adhesion molecule
KO	Knockout
KDa,	Kilodaltons
KD	Dissociation constant
LDL	Low density lipoprotein
LPS	Lipopolysaccharides
LRR	Leucine rich-repeats
LTA	Lipotechoic acid
LP	Lectin Pathway
LFA-1	Leukocyte function-associated antigen 1
LBP	Lipopolysaccharide binding protein
LB	Luria broth medium
MAC	Membrane Attack Complex
MBL	Mannose Binding Lectin
MPO	Myeloperoxidase
MMP9	Matrix metalloproteinase 9

MALDI-TOF-MS	Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
M2BP	Mac-2 binding protein
Mac-1	Macrophage-1 antigen
MASP	Mannose binding lectin associated serine protease
MIP-1α	Macrophage inflammatory protein 1 alpha
MCP	Monocyte chemoattractant protein
MMP	Matrix metalloproteinases
MBL	Mannose binding lectin
MHC	Major histocompatibility complex
Map-19	Mannose-binding lectin associated protein 19
MIP-1	Macrophage inhibitory protein-1
MRSA	Methicillin-resistant *Staphylococcus aureus*
mHP	Mouse Haptoglobin
µ	Micro
Mins	Minutes
NHS	Normal Human Serum

NF-KB	Nuclear factor Kappa B
NK cells	Natural killer cells
NETs	neutrophil extracellular traps
NOD receptors	nucleotide-binding oligomerisation domain receptors
OD	Optical Density
PAGE	Poly-acrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PMNs	Polymorphonuclear neutrophils
PRRs	Pathogen-recognition receptors
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
PCR	Polymerase chain reaction
PECAM	Platelet endothelial cell adhesion molecule
PHA	Phytohaemagglutinin
PVL	Panton-valentine leukocidin

PGN	Peptidoglycan
RT	Room Temperature
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SR	Scavenger receptors
SP	Serine protease
SLE	Systemic lupus erythematous
SAP	Serum amyloid P
SBA	Serum Bactericidal Assay
TAs	Teichoic acid
TLRs	Toll-like receptors
Tfb	Transformation buffer
TBS	Tris-buffer saline
TEMED	Tetramethylethylenediamine
TLF1	Trypanosome lytic factor-1
TCRs	T cell antigen receptors
TGF- β	Transforming growth factor β

TNF-α	Tumor necrosis factor-α
VRSA	Vancomycin-resistant *Staphylococcus aureus*
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen 4
WT	Wild-Type

Chapter 1

Introduction

1.1 Introduction

1.2 Immune system

The immune system is composed of two main effector arms, the innate and the adaptive immune system that complement the barrier defense of the body. Anatomical and physiological barriers are significantly important as they provide the first line of defense. Examples of these barriers include skin, lysozyme of salivary glands and low pH of the stomach. Therefore, individuals with severe burns, for instance, are highly susceptible to infections (Kuby and Immunology, 1992, Delves and Roitt, 2000).

Innate immunity, in addition to physical barriers (e.g. skin), involves a wide variety of mechanisms that respond to infections; it is rapid and does not possess memory functions. Adaptive immunity, involving immune memory cells, is, in certain circumstances, more efficient and specific, but an effective adaptive immune response takes time to become established following a primary infection with a pathogen (Kuby and Immunology, 1992, Borghesi and Milcarek, 2007). This project will investigate the role of haptoglobin, an acute phase protein, in the innate immune response to Gram-positive bacteria, which will be discussed in details below.

The two main categories of immunity are cellular immunity (i.e. cell-mediated immunity) and humoral immunity (i.e. immune systems present in the blood and other body fluids). Whilst the former includes phagocytes, natural killer cells, cytotoxic T lymphocytes, which rapidly respond to cytokine stimulation, the humoral immune system includes

specific immunoglobulins (i.e. antibodies) secreted by activated B cells (Turvey and Broide, 2010; Janeway and Medzhitov, 2002). Innate immunity was previously incorrectly described as a non-specific response of phagocytic cells (e.g. macrophages, dendritic cells (DCs), and neutrophils), or proteases or nucleases to eliminate pathogens (Kuby and Immunology, 1992). However, more recent research has shown that the innate immune response has the ability to distinguish between self and non-self-antigens, using pattern recognition receptors that can recognise pathogen-associated molecular patterns (PAMPs) present on the surface of microorganisms such as lipopolysaccharides (LPS) or lipoteichoic acid (LTA) (Fujita, Matsushita and Endo, 2004a).

Cells contribute to both the innate and the adaptive immune response and contribute to the stimulation of the factors that generate the pro- or anti-inflammatory responses. These responses stimulate either killing or clearance of microbes to clear infection or to repair injured tissue, minimising the damage. Some acute phase proteins, including haptoglobin (HP), fulfil critical functions with the innate and the adaptive immune responses, contributing to both effector arms of immunity. This role includes recruitment and migration of the leukocytes, prostaglandin synthesis, cytokines generation post infection and injury, and tissue repair (Quaye, 2008).

1.2.1 Receptors of the immune system

Immune cells possess receptors called pathogen-recognition receptors (PRRs), which can identify and bind pathogen-associated molecular patterns (PAMPs) enabling the host to

distinguish between self and non-self-antigens, and trigger the immune responses (Janeway and Medzhitov, 2002; Akira, Uematsu and Takeuchi, 2006). PRRs have been grouped into signaling PRRs and endocytic PRRs. While signaling PRRs include toll-like receptors and nucleotide-binding oligomerisation domain (NOD) receptors, endocytic PRRs involve scavenger receptors, mannose receptors and N-formyl methionine receptors. PRRs have been reported to be expressed on the surface of the immune cells such as the phagocytic cells (e.g. macrophages, dendritic cells (DCs), and neutrophils) (Akira, Uematsu and Takeuchi, 2006; Meylan, Tschopp and Karin, 2006).

Microorganisms (i.e. bacteria, viruses, fungi and parasites) possess a large variety of PAMPs. LPS is a characteristic PAMP of the Gram-negative bacteria while LTA is a characteristic for Gram-positive bacteria (Akira, Uematsu and Takeuchi, 2006). This project will also assess the interaction between LTA as a Gram-positive bacterial component and haptoglobin.

1.2.2 Cells of the innate immune system

Bone marrow-derived cells, including blood cells and tissue resident cells, are the main cells of the innate immune system. All blood cells leukocytes are generated in the bone marrow in a process called hematopoiesis (Palis and Yoder, 2001). According to the location where leukocytes mature, they can be divided into two groups, the myeloid lineage (which includes neutrophils, monocytes, macrophages and myeloid dendritic cells) and the lymphoid lineage, which involves lymphocytes and natural killer cells

(Hoang, 2004). Some circulating proteins, such as C- reactive protein (CRP) and haptoglobin (HP), can interact with immune cells via specific receptors. One example is the interaction between HP-Hb complex and a scavenger receptor (CD163) found on monocytes and macrophages (Kristiansen *et al*, 2001). The roles of HP in the immune system is discussed in more details in section 1.4.

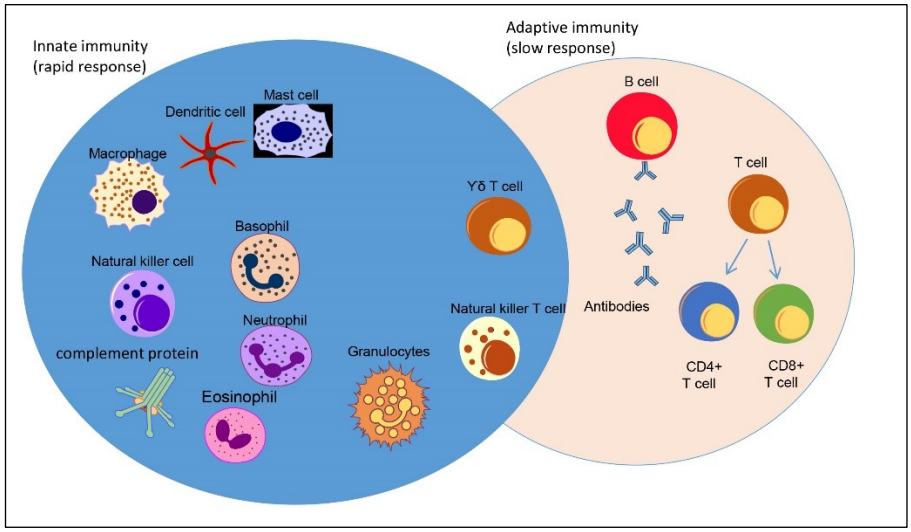

Figure 1-1: A Schematic representation of immune system arms and cell types. Reproduced from (Dranoff, G. 2004).

1.2.2.1 Neutrophils

Neutrophils belong to the bone marrow-derived cells of the myeloid lineage. They belong to the family of polymorphonuclear leukocytes, hence called polymorphonuclear neutrophils (PMNs). They are often the first cell type recruited at the site of inflammation and have the ability to eliminate pathogens through phagocytosis, which defines their fundamental role in the immune response (Kolaczkowska and Kubes, 2013). PMNs are granulocytic cells containing granules in their cytoplasm. Biosynthesis of these granules is controlled by granulocyte colony-stimulating factor (G-CSF) (Layton, 1992).

Neutrophil granules are categorised into three subtypes; azurophilic granules, comprising myeloperoxidase (MPO), specific granules containing lactoferrin, and gelatinase granules, which contain matrix metalloproteinase 9 (MMP9) also known as gelatinase B. Neutrophils can use either intra- or extracellular components to eliminate pathogens. Upon encountering microorganisms, neutrophils phagocytose them and utilise killing mechanisms such as antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme released both intra and extra-cellularly) or NADPH oxygenase-dependent mechanisms (reactive oxygen species). In addition, neutrophils can release neutrophil extracellular traps (NETs) comprised of chromatin DNA, histones and granular proteins, which contribute to the elimination of extracellular microbes. Therefore, three mechanisms of pathogen elimination can be utilised by neutrophils, phagocytosis, degranulation and the release of NETs (Kolaczkowska and Kubes, 2013).

1.2.2.2 Monocytes

Monocytes originate from the bone marrow and they derive from the myeloid lineage. They can be found circulating in the blood, bone marrow and spleen (van Furth and Cohn, 1968). They have a half-life of approximately 3 days in humans and 1 day in the mouse. Approximately 10% of all human leukocytes are monocytes, while they comprise about 4% of the total leukocytes in the mouse. Two main subtypes have been identified according to their differential expression, classical ($CD14^{++}$ $CD16^{-}$) monocytes accounting for 90% of monocytes in the blood, and non-classical ($CD14^{+}$ $CD16^{++}$) making up for about 10% of the total monocytes in blood (Yona and Jung, 2010). As

effector immune cells, monocytes have a significant role in immunomodulation through the release of cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and interleukin-6 (IL-6) (Taylor *et al*, 1998). Monocytes are phagocytes and defined by their ability to ingest bacterial cells and toxic molecules, indicating their function in phagocytosis. As all mononuclear phagocytes, monocytes can also serve as antigen presenting cells (Dale, Boxer and Liles, 2008). During the pro-inflammatory phase, marrow resident monocytes and blood monocytes have the ability to migrate into tissues and differentiate into histocytes, which can develop into either macrophages or dendritic cells (Geissmann *et al*, 2010).

1.2.2.3 Macrophages

Macrophages are phagocytic cells that can be found in lymphoid and non-lymphoid tissues. Their specific histological names are often defined by their location, for example, Kupffur cells for resident leukocytes in the liver, microglial cells in the brain, osteoclasts in bone and alveolar macrophages in the pulmonary airways (van Furth and Cohn, 1968). Macrophages possess a variety of pathogen-associated recognition receptors, which control production and release of inflammatory cytokines and may regulate their phagocytic activity. Macrophages contribute to homeostasis through the production of growth factors and by clearing apoptotic cells (Geissmann *et al*, 2010). For example, CD14 located on the surface of macrophages can recognise LPS on the surface of Gram-negative bacteria, facilitating their clearance (Naito, 2008). Macrophages are actually involved in the immunological and inflammatory responses. Macrophage activation can

occasionally have negative effects on tissues leading to tissue damage. The immune system has control mechanisms in place to minimise this damage (Bogdan *et al*, 1997). Some cytokines (e.g. interleukin-10 (IL-10) and transforming growth factor β (TGF- β)) can for example induce macrophage apoptosis (Mangan, Mergenhagen and Wahl, 1993; Naito *et al*, 1996).

1.2.2.4 Dendritic cells (DCs)

Dendritic cells (DCs) are derived from the myeloid lineage of hematopoietic cells and may stay in tissues as immature DCs. They are professional antigen presenting cells and have a significant role in both the innate and the adaptive immunity. Immature DCs are categorised as phagocytes, while mature DCs have a reticuloendothelial phagocytic activity. One characteristic feature of DCs is defined by their membranous projections. DCs can be divided into mature and immature DCs, and they are predominantly found in lymphoid tissues, mucosal epithelium, and organ parenchyma. Capturing foreign antigens and presenting them to lymphocytes are the pivotal functions of the DCs. Once they capture a foreign antigen, they develop into their mature state and they begin the process of antigen presentation to lymphocytes in lymphoid organs. Other types of DCs are termed plasmacytoid DCs. They respond to viral infections via identifying their intracellular components, releasing antiviral soluble proteins such as type I interferons (Abbas, Lichtman and Pillai, 2012).

1.2.2.5 Natural killer cells

Natural killer (NK) cells are lymphocytes, which are distinctly different from T and B cells. They fulfill essential protective roles in the innate immune responses predominantly acting against intracellular bacteria and virally infected cells. NK cells develop from precursors in the bone marrow and appear as large lymphocytes with abundant cytoplasmic granules. The term natural killer comes from the fact that these cells can perform their killing function without prior stimuli. NK cells make up about 5% to 15% of the mononuclear cells in the blood and spleen. NK cells are able to secrete several chemokines and cytokines such as interferon-γ, tumour necrosis factor-alpha, IL-3, GM-CSF, IL-5, IL-13, IL-10, IL-8 and others. For example, they can contribute to the activation of other cells such as macrophages via secretion of IFN-γ. NK cells can express CD56, but not CD3, which makes them distinguishable in blood. Furthermore, some cytokines trigger the NK cell functions such as IL-12, IL-15, IL-18, and type I interferons. There are some cytokines reported to be important growth factors for NK cells such as IL-12 and IL-15. An important feature of NK cells is also the capability of differentiating between healthy cells and other cells such as infected or stressed cells (Abbas, Lichtman and Pillai, 2012).

Some receptors regulate the activation of NK cells including activating receptors and inhibitory receptors. While the activating receptors identify damaged and infected cells via specific ligands, inhibitory receptors identify ligands on healthy cells, resulting in either promoting or inhibiting the response of NK cells. For example, NK cells can mediate a process called antibody-dependent cellular cytotoxicity (ADCC). They express

a low affinity Fc receptor, which is a characteristic for NK cells and can identify and kill infected cells coated with antibodies. In contrast, inhibitory receptors can be expressed by NK cells, which identify class I major histocompatibility complex (MHCI) molecules (Abbas, Lichtman and Pillai, 2012). Therefore, the contribution of NK cells to the elimination of intracellular microbes is significant. As discussed earlier, in the response of the immune system to viral infections, released cytokines such (e.g. IL-12 and IL-15) activate NK cells to kill infected cells (Bottino *et al*, 2005; Lewis *et al*, 2002). Activated NK cells secrete IFN-γ cytokine, which in turn triggers macrophages activation to eliminate phagocytosed microbes. In this manner, intracellular infection can be contained for several days enabling the acquired immunity establishment to mount a secondary response against such infections (Unanue, 1996).

1.2.2.6 Mast cells, basophils and eosinophils

Mast cells, basophils, and eosinophils have specific and critical roles in the innate and adaptive immunity. They resemble each other in the cytoplasmic granules containing different antimicrobial and inflammatory mediators. These cells are involved in the immune response that results in allergy as well as in the defense against helminthes (Abbas, Lichtman and Pillai, 2012).

Mast cells are derived from the bone marrow and they are usually found in the skin and mucosal epithelium. They are characterised through specific cytoplasmic granules, which contain cytokines, histamine, and other pro-inflammatory mediators. Some receptors can

be expressed by mast cells, which can bind IgE and IgG antibodies. Because of this binding, the release of the contents of cytoplasmic granules (such as cytokins and histamines) into the extracellular sites is induced, promoting alterations in the blood vessels that cause inflammation. Mast cells can also respond to complement activation products, neuropeptides, and microbial products via some receptors on their surface. Mast cells play a critical protective role against helminths but their uncontrolled activation can also be causative for the symptoms of allergic diseases (Abbas, Lichtman and Pillai, 2012).

Basophils are blood granulocytes and derived from the bone marrow precursors (different from the lineage of mast cells). They develop in the bone marrow and circulate in the blood. Basophils account for 1% of the total number of blood leukocytes. They can migrate to inflammatory sites even from the blood circulation. Basophils, like mast cells, possess receptors for IgG and IgE (Abbas, Lichtman and Pillai, 2012).

Eosinophils are also blood-resident granulocytes derived from the bone marrow. Resembling other granulocytes, they produce cytoplasmic granules, which contain enzymes that are involved in defending against parasites, but these enzymes may also damage host tissues. Their production from myeloid precursors and maturation is promoted by GM-CSF, IL-3 and IL-5. Eosinophils can normally be found in peripheral tissues such as gastrointestinal and mucosal linings of the respiratory and genitourinary

tracts. During inflammation, the number of eosinophils in tissues increases because of recruitment to inflammatory sites (Abbas, Lichtman and Pillai, 2012).

1.2.3 Opsonisation

Opsonisation is a process in which specific host proteins (called opsonins, which include some complement components and immunoglobulins) facilitate phagocytosis via their deposition onto cells or foreign particles. Phagocytic cells (e.g. neutrophils and macrophages) have receptors that can recognise and bind opsonins, resulting in the initialisation of a process called phagocytosis (Underhill and Ozinsky, 2002). The process of opsonisation is a specific process, which targets cells, microbes and cellular debris for subsequent clearance by the immune system (Stuart and Ezekowitz, 2005). Therefore, opsonisation is significantly important process because of its contribution to the elimination and clearance of foreign materials and antigens including pathogens.

Table 1-1: Examples of opsonins. Summarised from (Male, 2012).

Opsonins type	Examples
Antibodies	IgG, IgM
Complement proteins	C3b, C4b, and C1q
Circulating Proteins	C-reactive proteins (CRP), Serum amyloid P (SAP), Collectins (e.g. mannose-binding lectin, MBL)

1.2.4 Leukocytes recruitment to sites of infection

Leukocytes of the myeloid lineage (mostly neutrophil and monocytes) move from their maturation site (the bone marrow) to tissues (especially when injured or infected) where they coordinate critical immune functions. They eliminate pathogens, clear dead (apoptotic) cells and repair damaged tissues. This movement of leukocytes from blood into tissues is called recruitment. Host tissues, in response to the damage or infection, release chemo-attractants, which attract certain type of leukocytes (i.e. neutrophils, monocytes and lymphocytes) to the targeted site. This recruitment of leukocytes is a multistep process, which involves various molecules including adhesion molecules and chemokines. For example, neutrophils and monocytes, when responding to the chemo-attractants, interact with adhesion molecules (e.g. selectins and integrins) located on endothelial cells as well as their ligands, secreting cytokines such as IL-1 and TNF. Cytokines stimulate endothelial cells to express selectins, integrins and their ligands. Selectins are subdivided into three subtypes, P-selectin (CD62P), E-selectin (CD62E) and L-selectin (CD62L). While P-selectin (CD62P) and E-selectin (CD62E) are expressed by endothelial cells, L-selectin is exclusively expressed on leukocytes. Selectins vary in their mode of response; P-selectins respond rapidly to infection-related signals like cytokines (e.g. TNF-α) and histamine from mast cells, while E-selectins require about 1-2 hours to respond to cytokines interleukin-1 (IL-1), tumour necrosis factor (TNF) and microbial products such as LPS. L-selectins serve as binding mediators between neutrophils and endothelial cells that have been activated by IL-1, TNF, and other cytokines secreted at inflammatory sites (Abbas, Lichtman and Pillai, 2012).

The integrin family induces several cell adhesion receptors composed of α- and β-subunits, which mediate critical roles and functions in cell-cell adhesion processes and to an extracellular matrix. They mediate binding the adhesion of cells to cellular receptors such as the vascular cell adhesion molecule-1 (VCAM-1) and the intercellular cell adhesion molecule (ICAM-1 or CD54) (Hynes, 2002). LFA-1 (leukocyte function-associated antigen 1 or CD11a/CD18) and VLA-4 (very late antigen 4 or CD49d/CD29) are the two most prominent integrins of the immune system. There are also other integrins, such as Mac-1 (CD11b/CD18 also known complement receptor 3, CR3), which play critical roles in the immune system. Whereas ICAM-1 has the capability of binding to LFA-1 and Mac-1, VCAM-1 serves as a ligand for VLA-4. Mac-1 (which is found on monocytes and neutrophils) can contribute to phagocytosis via its ability to bind the complement component iC3b, which serves as an opsonin (Abbas, Lichtman and Pillai, 2012).

During an inflammatory response to injury or infection, some cells (e.g. macrophages and endothelial cells) secrete cytokines and small polypeptides called chemokines. The induction and direction of migration of leukocytes from blood to tissues (known as chemotaxis) is probably the main function of the chemokines. Chemokine production is induced by inflammatory cytokines (mainly TNF and IL-1) released upon recognition of invading microbes. Upon their release at the infected sites, chemokines move to the luminal surface of the endothelial cells, binding to heparan sulfate glycosaminoglycans. Responses to chemokines are mediated by specific receptors on the surface of leukocytes,

enhancing the binding-avidity of leukocyte integrins to their receptors on the endothelial surface. In addition, integrin expression by the endothelium is also induced by cytokines (TNF and IL-1), which results in the tight binding of leukocytes to the endothelium and mediating a stable arrest of these cells (Abbas, Lichtman and Pillai, 2012). Leukocytes possess ligands on their surfaces (such as CD31 and CD99), which contribute to the transmigration of leukocytes through the endothelium (Liu *et al*, 2004). It has also been reported that leukocytes secrete enzymes, which facilitate their migration to the extravascular sites where tissues are infected (Yonekawa and Harlan, 2005).

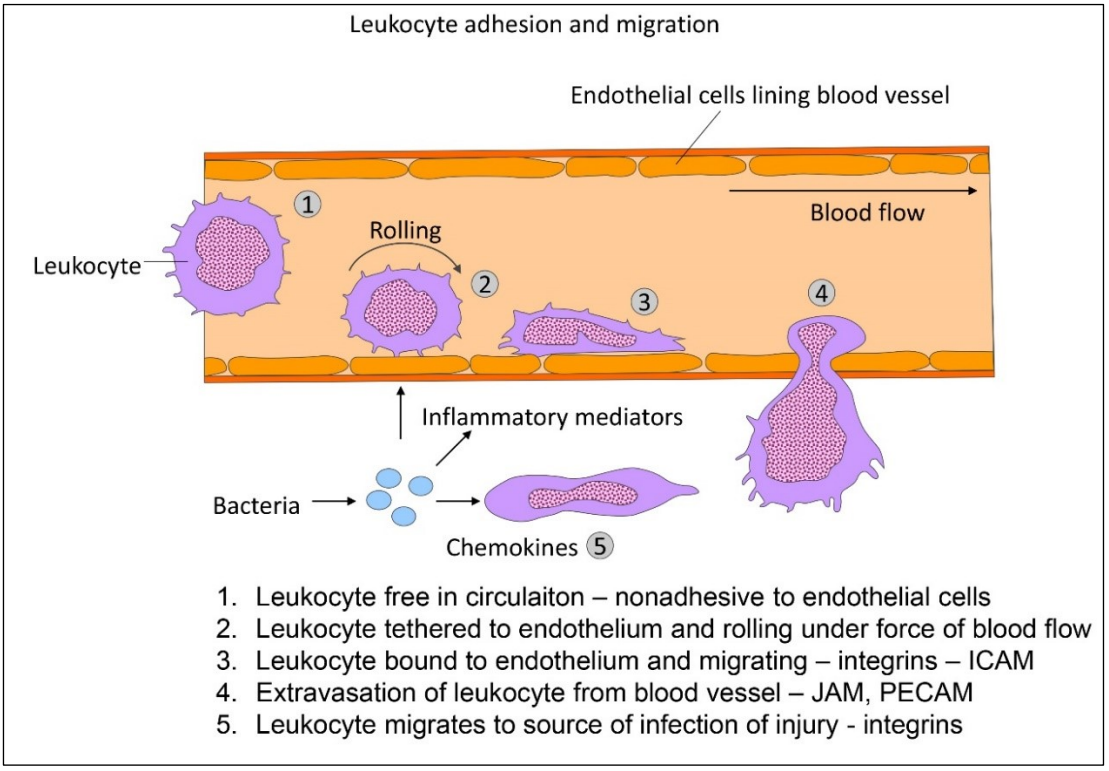

Figure 1-2: Chemotaxis process of leukocytes
Figure was modified and made based on the original materials purchased from Motifolio (www.motifolio.com).

Selectins, integrins, and chemokines work together to manage the interactions between leukocytes and endothelium, and this process is essential for the migration of leukocytes into tissues. This migration process of leukocytes has specificity depending on the expression of adhesion molecules and chemokine receptors on leukocytes. Because neutrophils and monocytes express different chemokine receptors and adhesion molecules, they migrate either to different sites or to the same site at different times. Neutrophils abundantly express chemokines receptors, CXCR1 and CXCR2, which specifically bind to their chemokine ligand, CXCL8. Monocytes express the chemokine receptor CCR2, which is the main receptor for the chemokine CCL2. The recruitment of neutrophils to the infection sites occurs usually earlier than the recruitment of monocytes. Upon their arrival to the infected sites, they perform many functions, of which the most obvious and prominent function is phagocytosis (Olson and Ley, 2002).

1.2.5 Phagocytosis

Phagocytosis, by phagocytic cells, is a process, which includes recognition, binding and then internalisation of material into a phagosome (Stuart and Ezekowitz, 2008). The initiation of phagocytosis can be mediated by different types of receptors, including PRRs, opsonic receptors and apoptotic corpse receptors (Flannagan, Jaumouillé and Grinstein, 2012). Phagocytic cells possess receptors (e,g, Fc-gamma receptor 1, FcγRI and also known CD64) on their surfaces which identify opsonins (e.g. antibodies or complement opsonins like iC3b) bound to foreign bodies (e.g. bacteria). The opsonin mediated adhesion of foreign particles to the surface of phagocytes initiates the process

of phagocytosis (Gordon, 2002). The phagocytes, for example, upon binding of opsonised foreign particles to their cellular receptors, engulf the foreign particles into formed organelle, termed the phagosome. The phagosome then undergoes a process of maturation by fusing with lysosomes to form a phagolysosome (Desjardins, 2003), in which the digestion of the engulfed pathogen is achieved by proteolytic enzymes contained in lysosomes (e.g. elastase and cathepsins contained inside lysosomes) (Korkmaz, Moreau and Gauthier, 2008).

Phagocytosis occurs via monocytes, macrophages, neutrophils and dendritic cells; hence, they are called "professional phagocytes". Some phagocytic cells (e.g. macrophages, dendritic cells) play a significant role in the adaptive immune response as antigen presenting cells. Professional phagocytes, after degradation of phagocytosed material, can present processed degraded antigens with or without their own MHC antigens to lymphoid cells. They can promote the release of pro-inflammatory cytokines, which in turn attract other lymphoid cells. They can also contribute to the release of anti-inflammatory mediators upon recognition of apoptotic cells, and clear these cells preventing inflammatory responses to damage tissues and thereby limiting inflammation mediated tissue damage. There are other non-professional phagocytic cells, such as fibroblasts, epithelial cells, and endothelial cells, which can help in the clearance of apoptotic cells and cellular debris. They cannot, however, internalise and eliminate microbes (Flannagan, Jaumouillé and Grinstein, 2012).

The process of phagocytosis has been well studied due to its significant contribution to the maintenance of the integrity of human body as an essential component of the immune response. Due to the involvement of numerous numbers of overlapping components to the process of phagocytosis, microscopical techniques and cell biology have been applied to assist studying the receptors and cells involved in phagocytosis. Investigators have resorted to use isolated cells as well as experimental animal models (mainly in mice) to identify new insights into phagocytosis. Using these models revealed a new understanding of the involvement of the relevant components involved in phagocytosis such as the role of Fc receptors (FcR) and complement receptors in phagocytosis (Stuart and Ezekowitz, 2008).

1.2.6 Complement system

The complement system has a major role in the immune defense system. It is composed of more than 30 components that are present in both plasma (soluble) and on the surface of cells (cell-bound) (Walport, 2001; Takahashi *et al*, 2010), including regulatory components (Frank, 2010). In 1890, the complement system was first described by Buchner and colleagues as heat labile components of blood plasma/serum that contributed to bacterial killing (Skarnes and Watson, 1957). The majority of the components of the complement system are present in plasma as inactive proenzymes (zymogens), which, upon complement activation, are cleaved and converted into their enzymatically active state (Sarma and Ward, 2011). It is widely accepted that the

complement system contributes to various immunological activities, including lysis of bacteria and cells, opsonisation, leukocytes activation, chemotaxis and clearance of antigen-antibody complexes. It is known that the complement system is either activated by the classical pathway (CP), lectin pathway (LP) or alternative pathway (AP).

The three distinct pathways that differ in their mode of recognition can mediate the activation of complement system, in which they all share one essential step, the activation of complement factor 3 (C3). As a result of C3 activation, a small chemoattractant molecule, called an anaphylatoxin C3a is released while the major fragment C3b attaches covalently to the surface of the pathogen following exposure of a reactive thioester bond. The resulting immobilized C3b molecule can be converted to the opsonin iC3b or contribute to the formation of more complex complement activation enzymes as described below. The activation of the three complement pathways generates the C3 convertases, surface-associated enzyme complexes that cleave C3. The C3 convertases of the classical and lectin pathway involve fragments of C4 and C2 that combine to form C4bC2a. The C3 convertase of the alternative pathway comprises C3bBb. C4b2a and C3bBb are instable complexes, so are rapidly inactivated in the absence of other complement components. However, upon C3b binding to C4b2a or C3bBb, a C5 convertase is generated which cleaves and activates C5, releasing C5a and C5b. C5a is an important anaphylatoxin and chemoattractant. Moreover, C5b is required for the commencement of the generation of the membrane-attack complex (MAC complex; C5b-

C6-C7-C8-$C9_n$) that can lyse certain bacteria by forming pores in their cell walls (Rooijakkers, van Kessel and van Strijp, 2005).

The complement system is responsible for many functional activities within the immune system and the main functions contribute towards the defense against pathogens (i.e., opsonisation and chemotaxis) and connects the two arms of the immune system (innate and adaptive immunity). The complement system also acts as a waste clearance (scavenger) system for the immune complexes, apoptotic cells and debris (Aoyagi *et al*, 2005; Markiewski and Lambris, 2007). C3b, C4b, iC3b and iC4b may be considered as the main mediators for opsonisation mediated by the complement system. Through its ability to bind to the surface of pathogens, C3b attracts leukocytes, which bind C3b opsonised pathogens via complement receptors 1 and 3 (CR1 and CR3) and thus facilitate phagocytosis (Aoyagi *et al*, 2005; Miyaike *et al*, 2002). Phagocytosis can also be initiated by complement recognition molecules such as MBL and L-ficolin through binding to collectin receptors present on phagocytic cells (Turner, 2003; Matsushita, 2010), although the identities of the receptors are not known.

Complement anaphylatoxins (C3a, C4a and C5a) are released by cleavage of C4, C3 and C5 during activation of the complement system, and initiate a potent pro-inflammatory reaction, attracting and activating inflammatory cells (i.e. leukocytes like neutrophils, mast cells and phagocytes) to the site of infection. Through the effects of complement anaphylatoxins, vascular permeability of endothelial cells is increased, allowing

leukocytes to extravagate towards inflammatory sites (adhesion process) and thus eliminating pathogens (Markiewski and Lambris, 2007). Furthermore, C5a was shown to trigger the production of some chemokines (such as tumour necrosis factor (TNF)) and interleukines (such as interleukine 1, IL-1) (Markiewski and Lambris, 2007; Schindler, Gelfand and Dinarello, 1990) and IL-8 (Hsu *et al*, 1999). Studies in mouse models have proven the role of C5a and C3a in stimulating the endothelial cells to produce macrophage inflammatory protein-2 and monocyte chemoattractant protein-1 (Laudes *et al*, 2002).

1.3 Inflammation and acute phase reaction

The acute phase response (APR) is a homeostatic process, which in mammalian species occurs upon counteracting either systemic or local disturbances due to tissue injury, infection, immunologic disorders and/or any abnormal growth of tissues (neoplastic growth) (Kushner and Rzewnicki, 1994). This results in the initiation of various responses in order to protect the integrity of the host organism and to isolate and destroy the invading foreign organism, limit tissue damage, initiate removal of harmful molecules, tissue repair, and eliminate possible trauma-associated infections.

Tissue injury and invading microorganisms are the main stimuli, which trigger a physiological response in the local tissue itself. This process defines inflammation in which a number of mediators such as cytokines, chemokines and growth factors, are employed at the site of damage. The release of cytokines is the first signal, followed by the activation of the inflammatory cells and vascular system. As a result, more cytokines

and other inflammatory mediators are produced, diffused into the extracellular fluid, and then circulated in the blood. These cytokines trigger receptors located on target cells, resulting in a systemic reaction. This reaction involves stimulation of hypothalamic-pituitary adrenal axis, and a decrease in the growth hormone secretion (Walton and Cronin, 1989). This situation can be characterised by clinical symptoms such as fever (Dinarello, 1983; Kushner, Gewurz and Benson, 1981; Kushner, 1982). Persistence of these signals may lead to detrimental consequences, starting with chronic APR and leading to damage of host organs (Harris *et al*, 1999). The degree of the APR can be measured in the laboratory using a number of markers for instance, reduction of the cholesterol and leukocytes in the blood plasma, initiation of the coagulation and activation of the complement system, and release of various acute phase proteins (Gruys *et al*, 2005).

1.4 Cytokines

Cytokines are typically secreted proteins, produced by leukocytes such as monocytes and macrophages that can trigger the APR at the site of inflammation. Cytokines have been categorised into three groups (Gruys *et al*, 2005) as summarised in Table 1-2.

Table 1-2: A summary of cytokines groups

Group 1 cytokines positive or negative growth factors for a variety of cells	IL-2, IL-3, IL-4, IL-7, IL-11, IL-12 and granulocyte-macrophage colony stimulating factor, GM-CSF
Group 2 cytokines with pro-inflammatory properties	TNF-α/β, IL-1α/β, IL-6, IFN-α/γ, IL-8, and macrophage inhibitory protein-1
Group 3 cytokines factors with anti-inflammatory activity	IL-1 receptor antagonists, soluble IL-1 receptors, TNF-α binding protein and IL-1 binding protein

Infection can cause a local inflammatory response at the site of the injured tissue. However, this is not the case with all individuals as infection can occur without inflammation for example in immunocompromised patients. One of the causes that lead to a strong APR is bacterial infection because of the rapid response of the cells of mononuclear-phagocytic system. When bacteria release endotoxins, the release of cytokines (e.g. TNF-α and IL-1β) is induced (Gruys *et al*, 2005).

The acute phase proteins (APPs) are mainly produced by the liver and then secreted into the circulation. They exert several functions including inhibition of protease activities and removal and clearance of harmful components generated by trauma, in order to contain and limit the enzyme-mediated tissue damage. APPs can help to restore the ability of blood to clot and re-establish homeostasis and assist wound healing (Moshage, 1997). The APPs can contribute to the inhibition of bacterial growth via the antimicrobial activity of some proteins and via targeting cellular components. This assists and complements the scavenging and opsonising activities to clear bacteria from the site of

infection (Gruys *et al*, 2005). Following inflammatory stimuli, APPs, due to the alteration in their production in the liver, can be categorised into two groups; positive APPs (which increase in their serum levels) and negative APPs (which decrease in their levels) (Gabay and Kushner, 1999). As previously mentioned, hepatic production of APPs is enhanced during inflammation. Nevertheless, APP biosynthesis can be reduced during phases of malnutrition and starvation (Gruys *et al*, 2005). Approximately 40 APPs have been described. Among these, C-reactive protein (CRP) was firstly discovered in 1930, detected in the blood of patients during the acute phase of pneumococcal infection. CRP concentrations, in some patients, were found to be 1000 fold enhanced compared to normal levels (Gabay and Kushner, 1999). Another important example is HP. The term "hapto" (classic Greek for: to bind to) indicates its main function, which is the binding of haemoglobin (Hb). In 1938, Jayle first described HP (Smithes, 1955). HP has recently been identified in our lab as a new binding partner for staphylococcal LTA, and the focus of this thesis will be on the role of HP in the immune response against infection by Gram-positive bacteria.

Table 1-3: Acute Phase Proteins (Gabay and Kushner, 1999; Heinrich et al, 1998)

Group	Individual proteins
Positive APRs	
Major APRs	Serum amyloid A, C-reactive protein, Serum amyloid P component
Complement proteins	C2, C3,C4, C5, C9, B, C1 inhibitor, C4 binding protein
Coagulation proteins	Fibrinogen, von Willebrand factor (vWF)
Proteinase inhibitors	Heparin cofactor II, plasminogen activator inhibitor I
Metal-binding proteins	Haptoglobin, haemopexin, ceruloplasmin, manganese superoxide dismutase
Other proteins	haeme oxygenase, mannose-binding protein, leukocyte protein I, lipoprotein (a), lipopolysaccharide-binding protein
Negative APRs	Albumin , pre-albumin, transferrin, apoAI , apoAII, histidine-rich glycoprotein

1.5 Haptoglobin

HP is an acute phase protein that is mainly synthesised and secreted in the liver. HP is composed of four chains, two alpha chains (approximately 9 kDa each) and two beta-chains (approximately 33 kDa each). It is synthesised as a single polypeptide precursor which undergoes a proteolytic cleavage forming an N-terminal light α-chain and a C-terminal heavy β-chain. The α-chain represents a complement-control repeat domain and the β-chain represents a serine protease domain (Nielsen *et al*, 2007). This cleavage is claimed to occur in the endoplasmic reticulum via a protein called C1r-like protein (C1r-

LP) (Wicher and Fries, 2004). The main portion of the β-chain is a serine protease domain (SP), however it lacks proteolytic activity. Instead, the β-chain serves as a mediator and assists the binding to binding partners such as Hb and CD163 the endocytic scavenger receptor on macrophages to which HP-Hb complexes bind prior to their elimination from serum (Alayash, 2011). In mammalian species, the two chains are attached together by a disulphide bond linking the α-chains of the two αβ subunits (named HP1-1 in humans). However, because of the existence of two HP gene alleles in humans (termed HP1 and HP2), three phenotypes are present (HP1-1, HP2-1 and HP2-2), and this due to an intragenic duplication of the HP1 allele generating the HP2 allele (Nielsen *et al*, 2007).

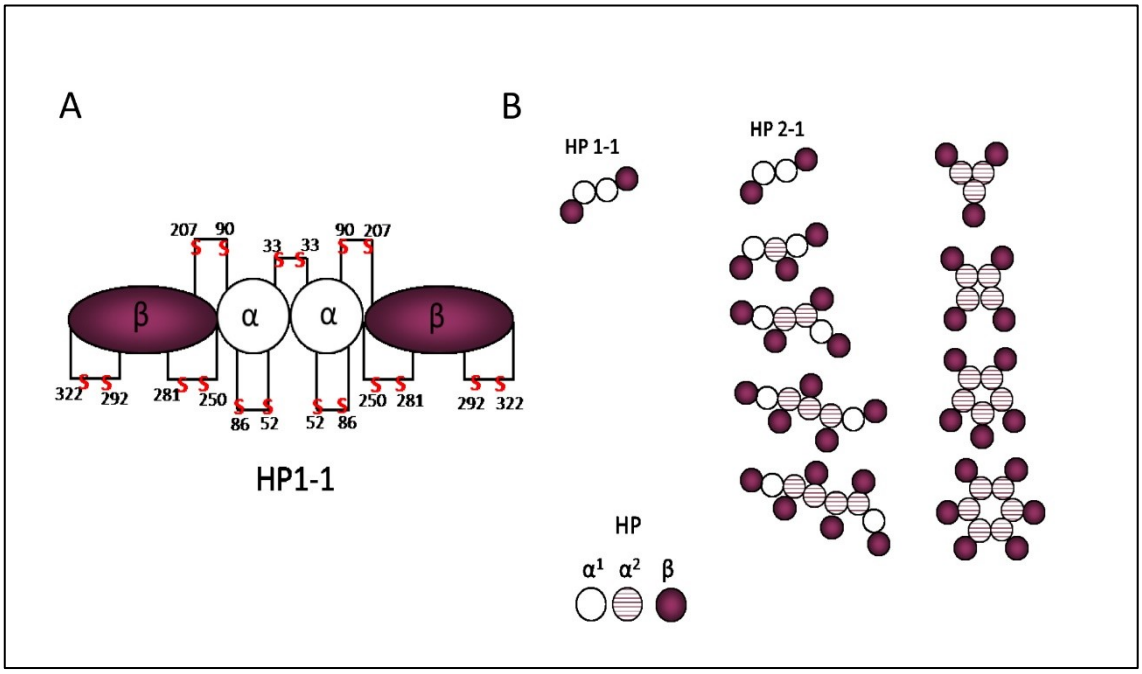

Figure 1-3: HP structural illustration

(A) A diagram showing the disulphide bonding pattern in HP, modified from (Nielsen *et al*, 2007). **(B)** A schematic representation of human HP phenotypes; reproduced from (Langlois and Delanghe, 1996).

Haptoglobin related protein (Hpr) is the result of a duplication event of HP. It is also termed as a trypanosome lytic factor-1 (TLF1) reflecting its capability of inducing lysis of *Trypanosoma brucei*. Hpr is produced with an approximate molecular weight of 45 kDa, which during maturation undergoes proteolytic cleavage forming α- and β-chain. Like HP, Hpr was found to bind Hb (forming a complex of Hpr-Hb) in a way that competes with that of HP. However, Hpr-Hb complexes, unlike HP-Hb complexes, do not bind with high affinity to CD163 reflecting structural differences between HP and Hpr (Nielsen *et al*, 2006).

HP is mainly synthesised in the liver but biosynthesis has also been shown in other organs, such as the kidney, lung, spleen and the skin (D'Armiento, Dalal and Chada, 1997; D'Armiento, Dalal and Chada, 1997; Friedrichs *et al*, 1995). HP can also be detected in other body fluids such as cerebrospinal fluid (CSF), urine and synovial fluid (Javid, 1978). Inflammatory mediators (e.g. bacterial endotoxins, growth hormones and cytokines TNF, IL-1 and IL-6-type cytokines) released during inflammation can induce the synthesis of HP (Raynes, Eagling and McAdam, 1991; Wang *et al*, 2001). HP concentration in plasma ranges between 0.45 and 3 mg/ml (Nielsen and Moestrup, 2009). The plasma levels are often utilised by clinicians as markers for the degree of inflammation-related disorders (e.g. in tuberculosis and hepatitis C). Plasma levels can greatly be affected by malnutrition and starvation (Gruys *et al*, 2005). In response to inflammation, these levels increase approximately 3-5 fold in humans and up to 50-fold in mice (Baumann and Jahreis, 1983). HP has been reported to have a half-life of 4.5 days

in normal healthy persons. However, this half-life is decreased to approximately 2.8 days in patients with acute burns due to the severe inflammatory response. The half-life of the HP-Hb complex is about 10 minutes because of the rapid clearance of these complexes from circulation via CD163 on macrophages (Dobryszycka *et al*, 1969; Engler, Moretti and Jayle, 1967).

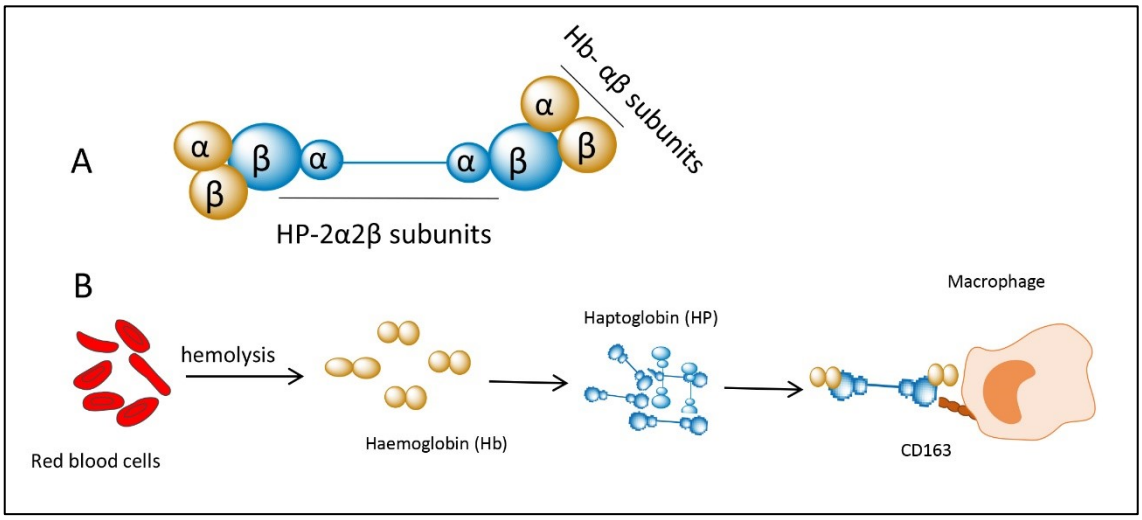

Figure 1-4: HP-Hb binding interaction and clearance via macrophage

Figure **A** illustrates the binding interaction between HP and Hb. The β subunits of HP interacts with α and β subunits of Hb. Figure **B** shows the clearance mechanism of HP-Hb complexes through CD163 scavenger receptor on macrophages. Figure was made with the help of ChemBioDraw application provided by the University of Leicester.

1.5.1 HP functional activities

The best known function of the HP is to target and bind free Hb that is released from erythrocytes during intravascular haemolysis leading to formation of HP-Hb complexes (Raijmakers *et al*, 2003; Sadrzadeh and Bozorgmehr, 2004). Both HP and Hb are composed of α and β chains. The β-chains of HP interact with α and β chains of Hb, forming HP-Hb complexes with a binding affinity (equilibrium dissociation constant,

Kd) of 10^{-12} - 10^{-15} M (Quaye, 2008). These complexes bind to the Hb scavenger receptor (CD163) found on monocytes and macrophages, resulting in Hb removal and clearance by the reticuloendothelial system in the liver (Kristiansen *et al*, 2001). The binding affinity of HP-Hb complex to CD163 is dependent on the HP phenotype. For instance, HP1-1 has the highest binding affinity followed by HP1-2 and then HP2-2 (Quaye, 2008). Since Hb contains an abundant level of iron, the release of Hb during vascular haemolysis could lead to significant detrimental effect on organs. Therefore, HP serves as an antioxidant by binding free Hb and thus inhibiting further tissue damage. During intravascular haemolysis, free Hb can also cause renal damage (Lim *et al*, 2001). However, HP protects the kidney by forming a complex with free Hb, because this large complex cannot pass through the glomeruli of the kidney. Thus, HP inhibits any possible oxidative damage that may occur to the renal parenchyma (Dobryszycka, 1997; Javid, 1978), and mediates the recycling of the iron stored in the released Hb (Moestrup and Moller, 2004). An *in vivo* study conducted using HP-deficient mice showed severe renal tissue injury in comparison to the wild-type mice, indicating the antioxidant role of HP against free Hb (Lim *et al*, 1998; Lim *et al*, 2000).

HP synthesis is elevated during inflammation. HP has recently been shown to have anti-inflammatory activities. Upon activation, macrophages release various inflammatory modulators such as platelet activation factor and prostaglandin (Saeed, Ahmad and Ahmed, 2007). HP can significantly inhibit prostaglandin biosynthesis by binding to Hb and restricting haem that is required for the enzymatic activity of prostaglandin

synthetase. Therefore, in the earlier stages of inflammation, HP may contribute to the reduction of the tissue injury (Jue, Shim and Kang, 1983).

HP has been described to inhibit the association of proteins induced during oxidation and heat thereby assisting in the preservation of the important cellular and extracellular function. HP levels increase as a result of anti-inflammatory responses, during such conditions as myocardial infarction, pregnancy and obesity. These conditions require tissue growth and repair, where HP may perform an important role via its ability to bind Mac-1 (complement receptor-3, CD11b/CD18) (Quaye, 2008). Furthermore, HP was shown to contribute to angiogenesis by promoting cell motility and endothelial cell growth differentiation during the formation of new blood vessels. HP may also participate in the development of tissue repair and collateral vessels as physiologically illustrated in chronic inflammatory disorders and chronic systemic vacuities (Cid *et al*, 1993; de Kleijn *et al*, 2002).

1.5.2 Immunomodulatory Role of HP

HP was reported to have immunomodulatory roles via the inhibition of calcium influx and formation of reactive oxygen species through binding to activated neutrophils (El Ghmati *et al*, 1996). In addition, HP has been shown to suppress macrophage functions (including tumor necrosis factor-α (TNF-α) production) and has been shown to suppress the production and proliferation of cytokines in T cells and B cells. This is probably because of that HP can interact with CD11b/CD18 receptors, which are located on

granulocytes, $CD8^+$ cells, natural killer cells and monocytes (El Ghmati *et al*, 1996). A study found that HP can bind to CD22, suggesting that it can be involved in the suppression of B cell differentiation (Yuan *et al*, 1996). Furthermore, HP can suppress lymphocyte functions during APR by inhibiting the phytohaemagglutinin (PHA)-induced blastogenesis of lymphocytes (Israel *et al*, 1981). A study demonstrated HP to have inhibitory effect on granulocyte chemotaxis, bactericidal activity and phagocytosis (Rossbacher, Wagner and Pasternack, 1999). HP can induce the inflammatory response of dendritic cells leading to production of IL-6 and TNF-α (Shen *et al*, 2012). HP can also involve in various inflammatory diseases such as arthritis (Cylwik *et al*, 2010), systemic lupus erythematous (SLE) (Pavon *et al*, 2006), suggesting an immunomodulatory role.

1.5.3 Use of HP as a biomarker

HP can be used as a bio-marker by clinicians. HP levels are low in various disease conditions including haemolytic anaemia, thalassemia, sickle cell anaemia and haemoglobin C disease. A haptoglobinaemia, which is a lack of HP in the serum, is observed in 80-90% of new-born children until they reach the age of ~three months. This may be because of the immature state of hepatocytes in the new-borns' liver which are not yet capable of synthesising HP (Langlois and Delanghe, 1996).

1.6 Bacteria

Bacteria have been categorised based on their susceptibility to Gram staining into Gram-positive and Gram-negative bacteria. This property in turn is dependent on their cell wall structure. The bacterial cell wall protects bacteria from the host's immune system. The cell wall of Gram-negative bacteria consists of a thin layer of peptidoglycan (PGN) and an outer membrane of LPS. In contrast, Gram-positive bacteria have a single wall membrane with a very thick multilayered peptidoglycan, teichoic acids and LTA (Neuhaus and Baddiley, 2003).

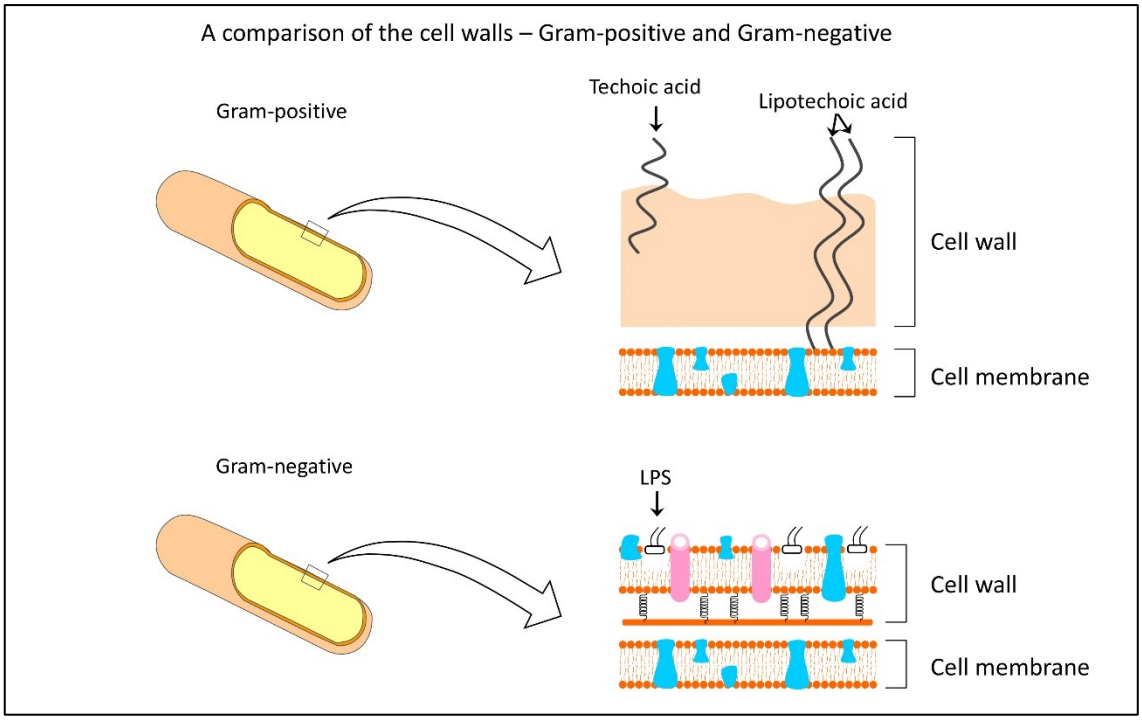

Figure 1-5: Structure of Gram-positive and negative bacteria.

Figure was made based on the original materials purchased from Motifolio (www.motifolio.com).

Despite all the improvement that the introduction of antibiotics have brought, bacterial infections remain one of the major threats to human health. One of the key targets of the immune system in Gram-negative bacteria is LPS, whereas major targets in Gram-positive bacteria include peptidoglycan and LTA. Although these targets are different, the host response to either bacteria is almost indistinguishable (Draing *et al*, 2008; Tietze *et al*, 2006). Activation of the immune system by LPS of Gram-negative bacteria has been extensively studied. However, the immune response to LTA, the major constituent of Gram-positive bacteria is relatively poorly understood (Draing *et al*, 2008). As well as triggering the immune system, LPS in Gram-negative bacteria and LTA and peptidoglycans in Gram-positive bacteria participate in the pathogenicity of infections. Some components in plasma have been reported to trigger lysis of Gram-positive bacteria such as neutrophil cationic agents or beta-lactam antibiotics, resulting in releasing these components, which are considered to act as virulence factors (van den Broek *et al*, 2007). The scope of this thesis will be on the interaction between HP and LTA of different Gram-positive bacteria, with a focus on *Staphylococcus aureus* and *Streptococcus pneumoniae.*

1.6.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive bacterium which is known to cause opportunistic infections such as toxic shock syndrome and septic arthritis. It produces a variety of virulence factors including chemotaxis-inhibitory protein of staphylococci (CHIPS) and protein A that interfere with the host's immune system. Over the years, multidrug-resistant strains of *S. aureus* have developed (known as methicillin-resistant

strains - MRSA, and vancomycin-resistant strains - VRSA). These multidrug-resistant strains cause roughly 65% of nosocomial staphylococcal infections (van den Broek *et al*, 2007). The cell wall of this bacterium consists of peptidoglycans, proteins, teichoic and teichuronic acids, and LTA (Neuhaus and Baddiley, 2003). These components stimulate the innate immune system and induce the inflammatory response. Receptors on human neutrophils such as CD14 and TLR2 can bind to LTA, inducing the secretion of the proinflammatory cytokines IL-8, TNF-α and granulocyte-colony stimulating factor (Aulock *et al*, 2006; Hattar *et al*, 2006). LTA can also target receptors on macrophages such as TLR2 and type I scavenger receptors, leading to the release of cytokines TNF-α, interferon-γ and interleukins (Bhakdi *et al*, 1991; Wang *et al*, 2000). Peptidoglycan binds TLR2 with high affinity (Fournier and Philpott, 2005).

S. aureus produces a variety of virulence factors that contribute to the development of diseases. Examples include protein A, clumping factor A (ClfA), chemotaxis-inhibitory protein of staphylococci (CHIPS) and the panton-valentine leukocidin (PVL). Protein A binds to the Fc region of IgG antibodies, preventing IgG-coated bacteria from being recognized by IgG receptors on phagocytes. CHIPS can also contribute to the prevention of neutrophil-recruitment to the inflammatory site via interacting with the C5a receptor on neutrophils. ClfA binds fibrinogen, which in turn gives the bacterium the ability to evade phagocytosis (Foster, 2005).

The peptidoglycans of *S. aureus* can stimulate the alternative pathway of complement system by augmenting the formation of the C3 convertase. It can also stimulate phagocytes (e.g. macrophages) to produce pro-inflammatory cytokines and chemokines (Travassos *et al*, 2004). Synergistic effects of LTA with peptidoglycan, induces cytokines production and promotes septic shock that can lead to multiple organ failure (Fournier and Philpott, 2005). Other virulence factors (e.g. α-,) produced by *S. aureus* include α-hemolysin and δ-hemolysins. α-hemolysin mediates cell lysis via interaction with the membrane of different cells including peripheral monocytes and endothelial cells (Bhakdi *et al*, 1989; Onogawa, 2002). They can also trigger neutrophils to release of TNF-α (Schmitz *et al*, 1997).

1.6.2 *Streptococcus pneumoniae*

S. pneumoniae (aka pneumococcus) is another pathogenic Gram-positive bacterium used in this study, which belongs to the Gram-positive bacteria. It is a facultative anaerobe that grows in 5-10 % CO2. It causes community-acquired pneumonia in both adults and children worldwide as well as causing otitis media (glue ear) in children, meningitis and septicaemia (Kadioglu and Andrew, 2012). *S. pneumoniae* causes invasive pneumococcal disease (IPD) when it breaches sterile sites such as the blood and brain. It can gain access to the circulation through binding to the type II alveolar cells (Cundell and Tuomanen, 1994). It annually accounts for approximately 1.6 million deaths, of which, children under the age of 5 years are mostly affected (Lynch and Zhanel, 2010). Despite mostly infecting the upper respiratory tract, pneumococcus can cause infection to almost all

human tissues. Consequences vary from minor infections to severe illness and death (Ferreira and Gordon, 2015).

S. pneumoniae colonises the nasopharynx by adhesion to the epithelium via a capsular polysaccharide. This polysaccharide coat also reduces the effects of complement. For further adhesion and attachment, pneumococcus employ neuraminidases to cleave sialic acids and glycoproteins of the host, exposing underlying binding sites (King, Hippe and Weiser, 2006). Pneumococci also utilise other virulence factors to evade immune defensive mechanisms and damage the host. Pneumolysin, for example, is a cytolytic toxin that can form pores in cells leading to cell lysis (Gratz, Loh and Tuomanen, 2015).

1.6.3 Lipoteichoic acids

The term "Teichoic Acid" describes two bacterial cell wall polymers found in Gram-positive bacteria, which are wall teichoic acid (TAs) and LTA. Based on the structural differences, there are so far five types of LTAs; type I, II, III, IV, and V. Although they are different, they all comprise of glycolipid moiety anchored in the cell membrane and a glycerophosphate moiety extended into the cell wall (Percy and Gründling, 2014; Cox *et al*, 2012). LTA types have been summerised from (Percy and Gründling, 2014) as in

Table 1-4.

Table 1-4: Types of Lipotechoic acids (LTAs).

Types of Lipotechoic Acids	Type I (e.g. *Bacillus subtilis, Staphylococcus aureus, and Listeria monocytogenes*)
	Type II (e.g. *Lactococcus garvieae*)
	Type III (e.g. *Clostridium innocuum*)
	Type IV (e.g. *Streptococcus pneumoniae*)
	Type V (e.g. *Clostridium difficile*)

LTA is the major macroamphiphile molecule of Gram-positive bacteria. Type I LTA, the well-described LTA, consists of polyglycerol phosphate linked to a diacylglycerolipid anchor. The hydrophilic polyglycerol phosphate chain is long enough to penetrate the peptidoglycan, and the lipid moiety attaches the polymer to the surface of the cytoplasmic membrane (Percy and Gründling, 2014).

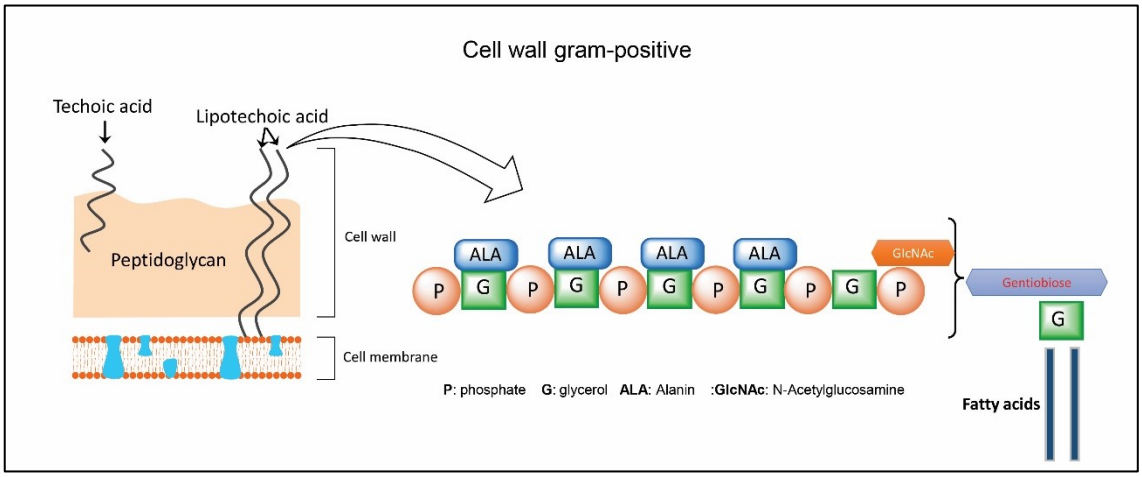

Figure 1-6: Schematic illustration of Gram-positive bacteria and Type I LTA structure

Figure illustrates the structure of Gram-positive bacteria and also indicates the structural components of Type I LTA containing; **P**:phosphate, **G**: glycerol, **ALA**: Alanin, **GlcNAc**: N-Acetylglucosamine. Figure was made based on the original matyerials purchased from Motifolio (www.motifolio.com).

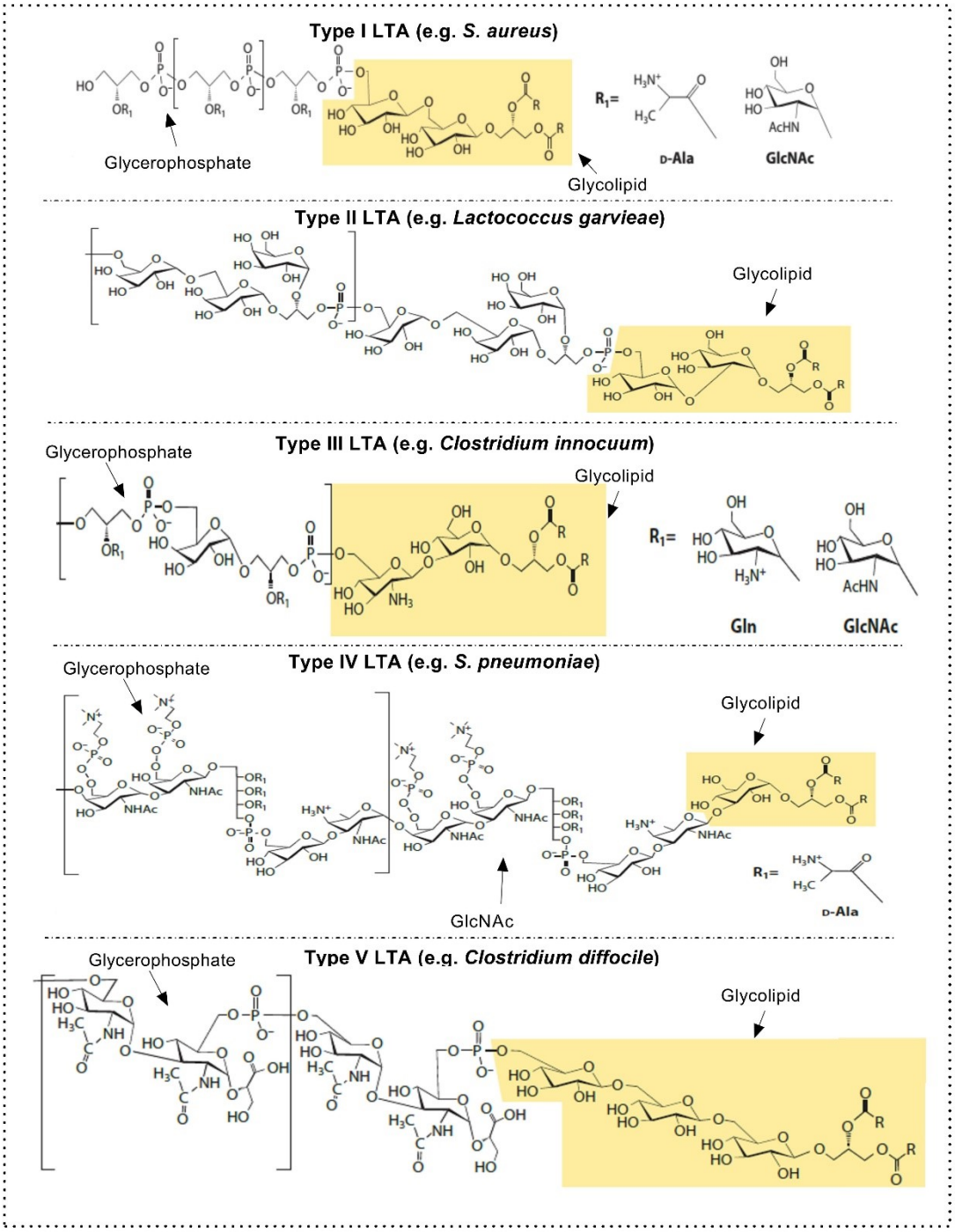

Figure 1-7 Chemical structures of LTA types.

Figure illustrates different types of LTA with examples of where these LTA are found. Glycolipid anchors indicated; R indicates a fatty acid, and R1 indicates backbone substitutions. Figure was modified from (Percy and Gründling, 2014).

Several ways have been evolved by pathogens to overcome host response. Gram-positive cocci for example can interact with cellular receptors and host proteins to favour their growth and colonisation. LTA as a cell wall component of the Gram-positive bacteria may not only function as a cytoskeletal component but could also contribute to bacterial interaction with host environment (Cray, 2012). LTA as a conserved bacterial molecular structure is known as one of PAMPs, which can be recognised by PRRs found on phagocytic cells. This subsequently results in the initiation of the inflammatory response (Buckley, Wang and Redmond, 2006). LTA was shown to enhance cytokines and chemoattractants secretion (TNF-α, IL-1β, IL-10, IL-12, IL-8, leukotriene B4, complement factor 5a and granulocyte colony-stimulating factor) from monocytes or macrophages (Fournier and Philpott, 2005). In addition, LTA can bind neutrophils via CD14, inducing them to release pro-inflammatory mediators such as IL-8 and TNF-α (Hattar *et al*, 2006). LTA has been claimed to be involved to sepsis, indicating its possible role in inflammatory conditions (Fournier and Philpott, 2005). Furthermore, LTA was found to be contributing to leukocytes recruitment to inflammatory sites. LTA can also participate in complement activation via binding to L-ficolin, a lectin pathway's recognition molecule (Lynch *et al*, 2004). It was shown that staphylococcal LTA could be recognised by TLR2, as a pattern recognition receptor, mediating the immune response to staphylococcal bacteria. TLR2 can be found on several effector cells of the immune system including monocytes/macrophages, neutrophils, dendritic cells and mast cells (Fournier and Philpott, 2005).

1.7 Interactions between HP and bacteria

HP acts as a bacteriostatic agent by preventing the release of iron from the free Hb, which could otherwise be used by the bacteria for growth (Eaton *et al*, 1982). HP also has a protective role against lung infections (Yang *et al*, 1995). HP2-2 can inhibit streptococcus bacteria from growth by agglutinating them via binding the T4 antigen located on the bacterial surface (Delanghe *et al*, 1998). In agreement with this bacteriostatic role, it has been reported that HP has the ability to bind *S. aureus* via a protein called staphylococcal surface protein HarA (Dryla *et al*, 2003). Moreover, a recent *in vitro* study conducted to characterise the interaction between *S. aureus* and HP showed binding between HP and LTA on the staphylococcal surface. A bactericidal activity of purified human monocytes, macrophages and polymorphonuclear leukocytes (PMNs) towards *S. aureus* in presence of normal human serum (NHS) and serum depleted of HP was also assessed. While all types of human phagocytes were high efficient in killing *S. aureus* within 60 minutes of incubation at 37°C in presence of 20% NHS, this killing of *S. aureus* was dramatically reduced (up to 90%) in presence of 20% HP-depleted NHS. Reconstitution of the HP-depleted NHS with pure HP, however, fully restored *S. aureus* killing in all of the three types of freshly prepared blood phagocytes. This work thus identified HP as an opsonin for the elimination of *S. aureus* during infection (Djebari B, University of Leicester, 2010).

HP can also have a negative impact during some parasitic infections. For example, *Trypanosome brucei* possesses a receptor that can bind to the HP-Hb complex and thereby utilise the haem to increase its growth rate. On the other hand, Hpr also binds Hb and associates with an innate immune protein called Trypanosome lytic factor (TLF). TLF penetrates and kill the parasite. The Hpr-Hb complex mediates this penetration. Interestingly, the two complexes Hpr-Hb and HP-Hb, are recognized by the same receptor on the trypanosomal surface (Pays and Vanhollebeke, 2009).

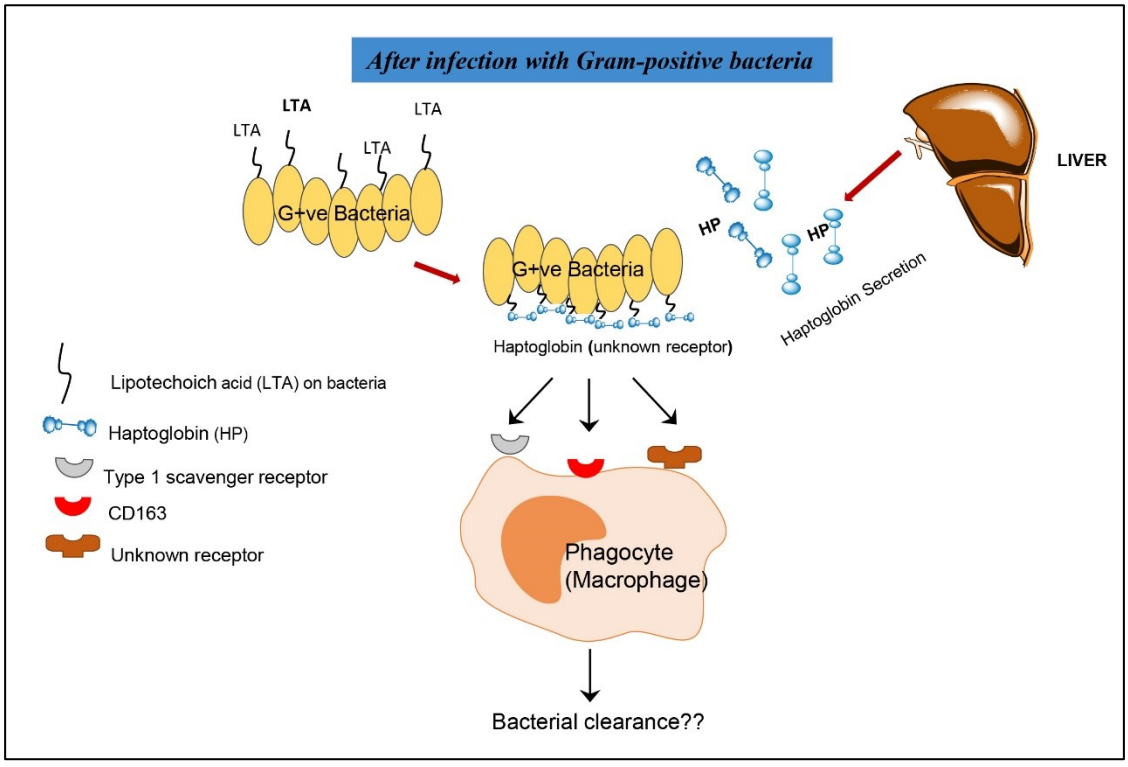

Figure 1-8: Hypothesised role of HP in the immune response to infection

Figure illustrates that after infection with Gram-positive bacteria, HP binds to LTA and then may mediate its clearance via an unknown receptor. Figure was made with the help of ChemBioDraw application and materials purchased from Motifolio (www.motifolio.com).

1.8 Aims of the thesis

Previous work in our laboratory has demonstrated that HP interacts with *Staphylococcus aureus* via its LTA. However, it is not yet clear how this interaction occurs. HP, as reported, could have a regulatory effect on the immune system as discussed above in section 1.5.2.

Therefore, the overall aim is to characterise HP/LTA interaction and to assess the role of HP in the immune system during Gram-positive infections.

The objectives to fulfil this aim are as follows:

- To produce recombinant HP in order to understand its properties,
- To characterise the interaction of HP with LTA of Gram-positive bacteria;
- To assess the possible contribution of HP in the immune response to infection.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and media

Material	Company
1kb plus DNA ladder	Invitrogen
Agarose, electrophoresis grade	Melford
Ampicillin	Sigma-Aldrich
Bromophenol blue	Sigma-Aldrich
Calcium chloride	Sigma-Aldrich
Ethanol	Fisher
Ethidium Bromide	Sigma-Aldrich
Glycerol	Sigma
High fidelity polymerase	New England Biolabs
L-Agar Bas	LAB M Limited
Magnesium chloride	Sigma-Aldrich
Potassium Acetate (K-acetate)	Sigma-Aldrich
potassium chloride (KCl)	Fisher
T4 ligase	New England Biolabs
T4 DNA ligase	New England Biolabs
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Bovine serum albumin (BSA)	New England Biolabs
Human and mouse liver cDNA	Dr.Youssif,University of Leicester
2-mercaptoethanol	Sigma-Aldrich
2-Propanol	Sigma-Aldrich
36.5% formaldehyde solution	Sigma-Aldrich
6X DNA loading dye	Sigma-Aldrich
Barbital	Sigma-Aldrich
Blood agar base	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich

Deoxyadinosine triphosphate (dATP)	Thermo scientific
Deoxyribo nucleotide PCR grade (dNTPs)	Promega
Ethidium bromide	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Gelatin	BDH
RNAse A solution	Promega
Sodium bicarbonate	Sigma-Aldrich
Sodium dodecyl sulphate (SDS	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Triton X-100	BDH laboratory
Trypsin from bovine pancreas	Sigma-Aldrich
Tween-20	Sigma-Aldrich
Zymosan	Sigma-Aldrich
GSH	Duchefa Biochemie
GSSH	Acros organics
D.D.T.	Sigma-Aldrich
Urea	Sigma-Aldrich
Bugbuster	Thermo Scientific
Protease inhibitor cocktail tablet	Roche
5-bromo-4-chloro-3-indolyl-beta D-galactopyranoside (Xgal)	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich

2.1.1.1 Extraction and purification of LTA

All LTAs were kindely provided by Thomas Hartung and Siegfried Morath (University of Konstanz, Biochemical Pharmacolog, Germany). LTA was purified from cell extracts of *Staphylococcus aureus*, *Bacillus subtilis*, *Streptococcus pyogenes*, *Listeria monocytogenes* and *Streptococcus pneumoniae* as previously described (Morath, Geyer and Hartung, 2001). According to the nuclear magnetic resonance (NMR) and mass spectrometry, the purity of the LTA was greater than 99%.

2.1.2 Buffers and solutions

	DNA Electrophoresis running buffer
Tris-Acetate-EDTA buffer	10 mM Tris-HCL 1 mM EDTA 10 mg/ml Ethidium bromide pH 8.4
	Competent cells preparation buffers
TfbI solution	3 mM K-acetate 50 mM $MnCl_2$ 100 mM KCl 10 mM $CaCl_2$ 15% glycerol pH 7.4
TfbII solution	10 mM Na-MOPS 75 mM $CaCl_2$ 10 mM KCl 15% glycerol pH 7.4
	Buffers used for protein purification and screening
Lysis buffer	25 mM Tris-HCL 150 mM NaCl 0.5 mg/ml lysozyme 1 mM EDTA 0.5% (v/v) Triton X-100 pH 8.0
Wash buffer 1	25 mM Tris-HCL 1 mM EDTA 0.5 M NaCl 0.5% (v/v) Triton X-100 pH 8.0
Wash buffer 2	25 mM Tris-HCL 0.5 M NaCl 1 mg/ml Sodium Deoxycholate , 1 M Urea pH 8.0
Solubilisation buffer	25 mM Tris-HCL 8 M Urea 5 mM D.D.T pH 7.5

Gel filtration buffer	20 mM Tris-HCL 25 mM NaCl pH 7.5
Refolding buffer	50 mM Tris-HCL 240 mM NaCl 10 mM KCL 2 mM $MgCl_2$ 2 mM $CaCl_2$ 0.4 M Sucrose pH 8.5
Dialysis buffer	20 mM Tris-HCL 20 mM NaCl 2 mM $CaCl_2$ pH 8.5
Stacking gel (5%)	3.4 ml dH_2O 0.83 ml of 30% Bis-acrylamide gel 0.63 ml of 1M Tris-HCl (pH 6.8) 0.05 ml of 10% (w/v) SDS 0.05 ml of 10% (w/v) ammonium per-sulphate 0.005 ml TEMED
Resolving gel (15%)	11.5 ml dH_2O 25 ml of 30% Bis-acrylamide gel 12.5 ml of 1M Tris-HCl (pH 8.8) 0.5 ml of 10% (w/v) SDS 500 µL of 10% (w/v) ammonium per-sulphate 20 µL TEMED
SDS loading dye	100 mM Tris-HCl 4 % SDS 10 % glycerol 0.2 % (w/v) Bromophenol blue pH 6.8
SDS-de-staining buffer	20 mM Tris base 150 mM glycine 0.038 % SDS 20% (v/v) methanol pH 8.3
SDS running buffer	20 mM Tris base 150 mM glycine 0.038 % SDS pH 8.3

ELISA buffers	
Coating Buffer	15 mM Na_2CO_3 35 mM $NaHCO_3$ pH 9.6
Tris-buffer saline (TBS)	10 mM Tris-HCL, pH 7.4 140 mM NaCl 2.5 mM $CaCl_2$ 2.5 mM $MgCl_2$
BSA-TBS Blocking Buffer	TBS with 1% (w/v) BSA pH 7.4
Phosphate Buffer	2.7 mM KCl 6.5 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4

2.1.3 Kits

QIAquick gel extraction kit	Promega
Wizard plus SV minipreps DNA purification system	Promega
QuickFold™ Protein Refolding Kit	Athena Enzyme systems
Bio-Rad Silver Stain	Bio-Rad

2.1.4 Oligonucleotides

All primers were purchased from Europhin MWG Biotechnology at 100 pmol/µl. And those primers were prepared at concentration 5 pmol/µl. 'm' stands for mouse primers; 'F' stands for forward, and 'R' for reverse. Primers used are detailed below;

Primer	Sequence (5'→3')
hHp_F_NdeI	TGTCAACATATGCATCACCATCACCATCACGTGGACTCAGGCAATGAT
hHp_R_EcoR1	GACATTGAATTC TTAGTTCTCAGCTATGGTCTT
mHp_F_NdeI	TGTCAACATATGCATCACCATCACCATCACGTGGAGTTGGGCAATGAT
mHp_R_EcoR1	GACATTGAATTCCTAGTTCTTGGCCATGGTTTC
Highlights	'h' stands for human primers 'm' stands for mouse primers; 'F' stands for forward, and 'R' for reverse; restriction sites *NdeI* (containing the start codon highlighted in *green*) and *EcoR1* are in red underlined; histidine tag sequence (*blue*), *stop* codons (red)

2.1.5 Vectors

pGEM-T Easy	Promega
pET-22b (+)	Dr Umakhanth, University of Leiceater

2.1.6 Bacterial growth medium

Brain heart infusion (BHI)	37 g of BHI dissolved in 1 litre of distilled water
Blood agar	4% blood agar base (w/v) , sterilised 1 liter of distilled water, 5% (v/v) horse blood

2.2 Methods

2.2.1 Molecular biology techniques

2.2.1.1 Polymerase chain reaction (PCR)

A normal PCR was used to amplify human/mouse haptoglobin (hHP and mHP respectively) from human/mouse cDNA libraries (provided by Dr Youssif Ali, University of Leicester) using two designed primers as detailed in the material section above (provided from Eurofins mwg Operon). The forward primer binds to a special sequence on the antisense strand of the cDNA, whereas, another sense of a specific strand is amplified via the reverse primer. The master mix consisted of a Phusion High-Fidelity DNA polymerase and a fixed molar concentration of $MgCl_2$ in high fidelity polymerase phusion buffer 5x and also dNTPs are needed for DNA template production. The PCR was run to amplify the target DNA sequence. PCR reaction mixture consisted of the following:

cDNA 10 ng	1 µl
High fidelity phusion buffer (5x)	5 µl
dNTP mix. (10 mM)	0.5 µl
Forward primer	2.5 µl
Reverse primer	2.5 µl
Phusion High fidelity-DNA polymerase (5U/µl)	0. 25 µl
Nanopure distilled water	13.25 µl

The cycling program was as follows:

Cycle	Temperature	Time	Cycles
Initial denaturation	98°C	1:30 minute	
Denaturation	98°C	15 second	
Annealing	70°C	30 second	15
Extending	72°C	40 second	
Denaturation	98°C	15 second	
Annealing	58°C	30 second	35
Extending	72°C	40 second	
Final extending	72°C	5 minutes	
Cooling down	4°C	∞	

2.2.1.2 DNA purification from agarose gel (band prep)

After cutting the desired DNA band under UV-light by sterile and sharp scalpel, all DNA fragments were purified from agarose gel using QIAquick Gel Extraction Kit (Promega). The sliced Gel were placed in 1.5 µl eppendorf tube and then weighted and dissolved in three volumes of QG buffer (1 volume of gel into 3 volumes of QG buffer) (provided by the kit) at 55C° for 5-10 minutes with shaking. The dissolved gel was transferred into the QIAquick column and then the column was centrifuged for 1 minute at 6000xg. The silica column is prepared to be sensitive for binding DNA. The column was then washed by adding 500 µl of QG buffer and centrifuged for 1 minute. Next, 750 µl of PE buffer (Ethanol) was added for washing purpose and then centrifuged for 1 minute. The

QIAquick column was centrifuged again to release the residual ethanol amount of PE buffer at maximum speed for 1 minute. The DNA was then eluted by adding 50ul of elution buffer EB into a clean 1.5µl eppendorf tube.

2.2.1.3 A-Tailing of PCR products

There are several enzymes can be used to provide Adenine (A) to the 3' terminus of PCR DNA product in order to make blunt ends. As a result of A-tailing, the T-overhang in pGEM-T Easy vector (Promega) can be ligated with the PCR product. Taq-polymerase (Thermo) enzyme was used. This enzyme has a dependency of 5'-3' exonuclease activity. The reaction mixture consisted of 10 µl containing 1 µl of DNA Taq-polymerase (Thermo), 1 µl of Taq-polymerase reaction buffer 10x (containing $MgCl_2$), 1 µl of 0.2 mM dATP (invitrogen), and 7 µl of the gel-purified DNA. The reaction mixture of A-tailing was incubated with the PCR product at 72 C° for 25 minutes.

2.2.1.4 Ligation and cloning PCR products into pGEM-T Easy vector

pGEM-T Easy Vector (Promega) was provided with a single thymidine (T) at 3' terminus in both ends to keep the vector linear. The T-overhang prevents vector recircularisation and keeps and provides compatible overhang for PCR products (Mezei and Storts, 1994 and Robles and Doers, 1994). The ligation reaction mixture was completed to a final volume of 10 µl with Nano pure water. The reaction mixture included 1 µl of pGEM-T Easy vector (50ng/µl) and then mixed with amount of insert to assess 3:1 molar ratio (insert:vector) (x) µl of the PCR product (x ng), 1 µl of T4 DNA ligase (5 units)

(Promega) and 5 µl of T4 DNA ligase buffer (2x) (Promega). After that, the reaction mixture was incubated for overnight at 4C°.

2.2.1.5 Chemically competent *E.coli* Top10 strain

In order to generate *E.coli* Top10 competent cells, the protocol of (Hanahan, 1983) was followed by treating the bacterium with ice cold $CaCl_2$ solution by which presumably renders of adherence the plasmid DNA into the cells. This method briefly depends on heat at 42C° or 37C° for 45 seconds or 5 minutes respectively, by which the plasmid DNA is up taken by bacterial cells.

2.2.1.6 Preparation of chemically competent *E.coli* Top10 strain

E.coli strain frozen stock was streaked onto LB agar plate in order to get one single colony. The plate was incubated for 16-18 hours at 37C°. Next day, one single colony was inoculated into 5 ml of non-selective medium (LB broth medium) containing 20 mM MgSO4 and then cells grew at 37C° for overnight incubation. After incubation, 100 ml of LB broth medium was incubated in shaker incubator with 1 ml of overnight culture at 37C° for 2-3 hours until the optical density (O.D.) reach 0.7 – 0.8 at 550 nm. Then, cells were harvested by centrifugation at 2000 xg for 10 minutes. After that, 30 ml of cold TfbI buffer (section 2.1.2) was used to re-suspend the cells pellet following incubation on ice for 30 minutes. Next, cells were centrifuged again at 2000 xg for 10 minutes at 4C° and then, 4 ml of TfbII buffer (section 2.1.2) was used to re-suspend the cells pellet in order

to distribute aliquot of 100 µl in each 0.5 µl of eppendorf tube to store them at -80C° or for immediate transformation.

2.2.1.7 Transformation of chemically competent *E.coli* Top10 Strain

2 µl of the ligation product was transformed into 50 µl of competent *E.coli* (Top10 strain) with gentle mixing and incubation in ice for 20 minutes and then, cells were heat shocked at 37C° for 5 minutes and then incubated on ice for 2 minutes. Cells were incubated with 450 µl of non-selective medium (LB broth) and incubated at 37C° for 1 hour with gentle shaking. Meanwhile, 30 µl of X-gal (20 mg/mL 5-bromo-4-chloro-3-indolyl-beta-Dgalactopyranoside dissolved in DMSO) and IPTG (0.1 M of isopropyl thiogalactoside, Isopropyl beta-D-thiogalactopyranoside in distilled water) were spread on the LB agar containing 100 µg/ml of ampicillin. Chemically transfected competent cells were spread into the LB agar plates and then incubated for overnight at 37C°. After overnight incubation, white single colonies were selected and cultured again into 5 ml of LB broth medium containing 100 µg/ml of ampicillin and then incubated for overnight at 37C° with gently shaking.

2.2.1.8 Mini scale isolation and purification of plasmid DNA vector (Miniprep)

Plasmid DNA was isolated and purified by using Wizard plus SV Miniprep DNA Purification System (Promega). The 5 ml of the overnight culture was centrifuged for 10 minutes at 13000 xg in order to re-suspend the pellet into 250 µl of cell re-suspension solution. Then, 250 µl of cell lysis was added and tubes were inverted gently for 4 times

and all tubes kept for 1-5 minutes at room temperature (RT). After that, 10 µl of alkaline protease solution was added and mixed and then kept at RT for 5 minutes. Next, 350 µl of neutralisation solution was added and then mixed well (vortex). All cell debris was removed by centrifugation at maximum speed of 13000 xg for 10 minutes. The clear supernatant containing the plasmid was transferred into a spin column (provided by the kit) and washed with 750 µl of washing buffer. The plasmid DNA was eluted with 75 µl of distilled water in clear tubes with centrifugation at maximum speed 13000 xg for 1 minute.

2.2.1.9 Restriction digestion of pGEM-T-Easy vector

The purified and isolated DNA fragment and DNA plasmid were digested as follow:

The purified DNA (1ug)	3 µl
BSA 10µg/µl	2 µl
Restriction enzyme buffer (2) 10X	2 µl
Restriction enzyme (*EcoR1*) 10µg/µl	1 µl
Restriction enzyme (*NdeI*) 10µg/µl	1 µl
Deionized distilled water	11 µl

The reaction mixture was incubated in the water bath at 37C° for 1.5-2 hours and then, was run on 1% agarose gel in order to determine the size of the expected bands.

2.2.1.10 Sub-cloning the Construct into the pET-22b(+) expression vector

The DNA constructs of HP in the pGEM-T easy vector (Promega) were digested by the same restriction enzymes and evaluated by agarose gel electrophoresis. Then, the purified DNA construct was ligated into pET-22b(+) vector (provided by Dr Umakhanth, University of Leicester) and then linearised using the restriction enzymes (*EcoR1* and *Nde1*) following the previous procedure described above in digestion of pGEM-T easy vector. After that, transformation into competent cells and Mini scale purification of plasmid DNA using (Minipreps) were done as previously mentioned.

2.2.2 Screening for HP deficiency in mice

2.2.2.1 Genomic DNA (gDNA) isolation from ear snips

Wizard gDNA Purification Kit (Promega) was used to isolate the gDNA from ear snips of mice. A mixture of 60 µL of 0.5 M EDTA and 250 µL of nuclei lysis solution (Promega) was added to an eppendorf tube containing a fresh mouse ear snip. 10 µL of 20 mg/mL of proteinase K (Qiagen) was added and the mixture was then incubated overnight at 55˚C after. The following day, after confirming that all tissues had been lysed and dissolved, the sample was vortex vigorously and then a 1.5 µL of RNAse A solution was added and then incubated for 15 minutes at 37˚C. Then, a 100 µL of protein precipitation solution (Promega) were added, and mixture was mixed well and then chilled on ice for 5 minutes. Chilled sample was then centrifuged at 13000 rpm for 4 minutes and the supernatant containing DNA was transferred into a fresh 1.5 mL eppendorf tube. After that, a 300 µL of 2-propanol was added and the reaction mixture

was centrifuged for 5 minutes. To wash the precipitated DNA, 750 µL of 70% ethanol was added and then centrifuged at 13000 rpm for 1 minute. Ethanol was then discarded and the purified DNA was re-hydrated by adding 50-70 µL of nuclease free water and sample was stored at 2-8˚C.

2.2.2.2 Genotyping of HP deficient mice with PCR

The gDNA Isolated from mouse ear snips was PCR amplified. A multiplex PCR was used for the identification of the homozygous, heterozygous and wild type mice, these primers were as follows:

Primer	Sequence
F HP-WT	5’ TCT ACG GGG AGA GGT GAG AA 3’
R HP-WT	5’ CTG GGT GTG CAC CAT CAT AC 3’
R Null PGK	5’ GGG GGA ACT TCC TGA CTA GG 3’

Each PCR reaction mixture consisted of the following:

DNA	1.5 µl
F HP-WT	1.5 µl
R HP-WT	1.5 µl
R Null PGK	1.5 µl
M.mix	12 µl
Nanopure distilled water	7 µl

The cycling program of the PCR used in the table below. PCR should yield a ~700 bp product for the wild-type and ~ 300 bp band for the mutant.

Cycle	Temperature	Time	Cycles
Initial denaturation	98°C	1:30 minute	
Denaturation	98°C	15 second	
Annealing	62°C	30 second	
Extending	72°C	40 second	35
Final extending	72°C	5 minutes	
Cooling down	4°C	∞	

The PCR products were run in 1% agarose gel for 45 minutes at 120V.

2.2.3 Protein expression and analysis methods

2.2.3.1 Transformation and protein expression

Transformation of competent *E. coli* (BL-21) (DE3), carrying the desired DNA constructs of pET22b(+) and the sequence of the hHP and mHP, was performed using chemical transformation. The cells were removed from -80°C and thawed slowly on ice. Then 2 µl of the ligation reaction were added to 50 µl of the competent cells and mixed gently, then incubated in ice for 20 minutes. After that cells were heat shocked in water bath at 37°C for 5 minutes and then transferred again into ice for another 2 minutes. 450 µl of LB broth was added to the tubes and incubated at 37°C for 1-2 hours with gentle shaking at 220 rpm. Two different volumes; 50 µl and 100 µl were placed on LA media plates containing ampicillin (100 µg/ml) and then incubated overnight at 37°C. The next day, one colony was picked up using a sterile yellow tip then inoculated into a universal container with 10 ml LB media, and incubated at 37°C overnight with shaking at 220 rpm. For protein expression, the growth culture was the next day transferred to baffled flasks containing 1000 ml of LB medium with 50 µg/ml ampicillin and the OD_{600} was measured. Flasks

were incubated at 37°C until the OD_{600} reached 0.7–0.9, at which time protein expression was induced by addition of IPTG to an end concentration of 1mM. After 4-5 hours incubation, bacteria were harvested by centrifugation at 4000 xg, 4ºC, for 20 minutes and the cell pellet was re-suspended in 40 ml phosphate-buffered saline (PBS) for washing. Then, cells were centrifuged again and pellet was kept at -80 ºC after collecting 1ml sample for protein expression screening on SDS-PAGE gel.

2.2.3.2 Inclusion bodies washing and processing protocol

Once the expression of the protein is confirmed, four main buffers are used to process the cell pellet (lysis buffer, wash buffer 1 (Triton X-100), wash buffer 2 (Urea) and BugBuster) (components of each buffer are detailed in the material section (2.1.2.). A 20 µl sample was collected after each wash for purity screening. Firstly, cell pellet was taken from -80 ºC and defrosted on ice. It was then dissolved in 40 ml of lysis buffer (25mM Tris-HCL pH 8.0, 150 mM NaCl, 0.5 mg/ml lysozyme, 1 mM EDTA) and one tablet of protease cocktail inhibitor (by Roche). Solution was incubated for 30 minutes at room temperature (RT) with shaking (viscous solution should be noticed due to the release of DNA). A mixture buffer (5 mM $MgCl_2$, 5 µg/ml DNAse) was added to the solution and incubated for 15 minutes at RT with shaking. This was followed by sonication (amplitude at 8, macro-probe; 8 to 9 pulses, 30 seconds per pulse with 1 minute break between each one) and then centrifugation 20000 g, 4ºC, for 20 minutes. The lysate was collected and supernatant was discarded. Pellet was then re-suspended in 40 ml of wash buffer 1 (25 mM Tris-HCL pH 8.0, 0.5 M NaCL, 0.5% Triton X-100, 1 mM EDTA) and then 2-3

pulses sonication were applied for further purification followed by centrifugation as the previous step. After discarding supernatant, wash buffer 2 was used (25 mM Tris-HCL pH 8.0, 0.5 M NaCl, 1 mg/ml Sodium DeoxyCholate, 1 M Urea) followed by 2-3 pulses sonication and then centrifugation as the previous steps. The pellet was after that washed with 40 ml of 1:10 protein extraction master mix reagent (BugBuster) and then sonicated and centrifuged. At this step, white powdery pellet was seen and the pellet was dissolved in 10 ml of 25 mM Tris-HCL pH 8.0. Finally, resuspension was distributed into eppendorf tubes 1ml each, centrifuged at 13,000 rpm for 10 minutes. Small pellets were then frozen immediately in liquid nitrogen and then retained at -80 ºC. To confirm the purity of the inclusion bodies, the 20 µl samples were then screened on SDS-PAGE gel.

2.2.3.3 Inclusion bodies solubilisation and refolding process

After confirming the washing and purification of the inclusion bodies, samples were dissolved in a 50 ml solubilisation buffer (25 mM Tris-HCL pH 7.5, 8 M Urea with 5 mM D.D.T reducing agent) and then incubated in the a water bath at 42 ºC for 15 minutes. Subsequently, suspension was centrifuged to remove any remaining insoluble cell debris and supernatant was collected and the concentration was adjusted to 1 mg/ml to be used for the refolding process (the buffer should be prepared at same day with refolding process).

Refolding process was firstly started by screening the optimal refolding buffer for the desired protein (proceeded only with mHP because hHP was not expressed efficiently and requires more optimisation) using QuickFold™ Protein Refolding Kit (by Athena

Enzyme systems). It contains 15 buffers which were all screened. Finally buffer number 15 (50 mM Tris-HCl pH 8.5, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose) was the optimum buffer which successfully refolded mHP. The volume of the refolding buffer should be 4 times more than the volume of the solubilised protein. Once the refolding buffer was determined, the 50 ml solution, containing soluble protein, was mixed slowly with refolding buffer at 4 ºC overnight, using drip technique (50 ml syringe) with gentle stirring. The refolded protein was then dialysed in a dialysis buffer (25 mM Tris-HCL pH 7.4, 150 mM NaCl) at 4 ºC with gentle stirring.

2.2.3.4 Purification with HisGravi Trap column (GE Healthcare)

To facilitate the purification process, the sequence of the DNA construct was designed with histidine-tag in the N-terminus. HisGravi Trap column (GE Healthcare) provides rapid purification of histidine-tagged proteins. After refolding the protein, to obtain the maximum purity of the protein, the solution containing the refolded protein was run through a HisGravi Trap column (GE Healthcare), allowing the solution to flow through the column by gravity. The column was then washed with 20 ml washing buffer (PBS buffer pH 7.4, containing 20 mM imidazole). Finally, the protein was eluted using 5 ml of the elution buffer (PBS buffer pH 7.4, containing 500 mM imidazole) divided into 1 ml fractions (5 fractions were collected). Fractions were analysed on SDS-PAGE.

2.2.3.5 Purification with gel filtration

Superdex 75 10 300 HiLoad column and Superdex 200 10 300 Hiload column from GE Healthcare were used. Each column was equilibrated with filtered buffer (20 mM Tris-HCL pH 7.5, 25 mM NaCl). When the equilibration process was completed, 5 ml of the concentrated protein was injected in the loading loop and then the sample was run over the column at a flow rate of 1 ml/min. Peaks folded protein were compared to an elution profile with standard molecular weights provided from the manufacturer. To determine where the sizes of the eluted fractions are, samples were analysed using SDS-PAGE gel.

2.2.3.6 SDS poly-acrylamide gel electrophoresis (SDS-PAGE)

Protein expression and purification were analysed by 15% SDS-polyacrylamide gel under reducing and non-reducing conditions. In the first case the protein was boiled for 4-5 minutes at 95 ^{o}C with loading dye containing SDS with or without reducing agent (β-Mercaptoethanol or D.D.T). The gel was run on 1x SDS-gel running buffer at 150 V for 80 minutes. The size of the fragments was estimated according to the standard molecular weight of the protein marker. Gel was stained using Coomassie brilliant blue R-250 (Serva) solution with gentle shaking for 30 minutes and then decolourised with de-staining buffer until bands are visible. In other experiment where the concentration of the protein was low a silver stain was used. Silver staining protocol as follows;

The oxidizer and silver reagent solutions (Bio-Rad silver stain) should be prepared on the same day of the experiment.

Silver stain steps and reagents	Time
1. Fixative: 40% methanol/10%	30 minutes minimum. Gel may be acetic acid (v/v) stored overnight at this step.
2. Oxidizer: gel should be immersed completely	5 minutes
3. Water Washes: large volumes of water and change the wash many times 6-7 times, particularly in the first 5 minutes. This flushes the oxidizer from the gel without removing it from the proteins	15 minutes maximum. It is recommended by manufacturer to proceed to step 4 after 15 minutes even if gel is still slightly yellow in colour.
4. Silver Reagent	20 minutes
5. Quick Water Rinse	30 seconds maximum
6. Developer	~ 30 seconds or until a brown or smokey precipitate appears. The solution be removed and fresh developer should be added until the bands are clear.
7. Stop: 5% acetic acid (v/v) gel should be completely immersed.	15 minutes

2.2.4 Crystallisation screen of mHP

The purified protein of mHP was concentrated to reach at least 3 mg/ml to be supersaturated to produce crystals. A precipitant was used to assist the protein to nucleate and grow. When the concentration of the protein and the precipitant are increased, this leads the protein to the supersaturation and precipitation zones. Crystals will grow if the concentration of the protein is decreasing; growth will continue until it reaches the limit between the two precipitation curves, Figure 2-1. High protein concentrations may lead to either precipitation or excessive nucleation (Asherie, 2004). mHP was concentrated to

a maximus possible concentration of 3.4 mg/ml. Then, crystallisation plates were set up using screens JCSG+ and PACT from Molecular dimensions.

Each plate was 96 wells representing different conditions. In an MRC96-well plate using crystallization robot, 0.1 µL of the protein (3.2 mg/ml) was mixed with 0.1 µL of crystallisation buffer. The plates were set up by the sitting drop method, in which the plate of mixed protein and buffer is kept in a closed chamber with a reservoir of crystallisation buffer. Then, the plates were incubated in two conditions at room temperature and 4ºC. Plates were examined for the expected crystals and further experiments were carried out with a larger drop by setting up 1.2 µL of protein and 1.2 µL of buffer from the most promising condition.

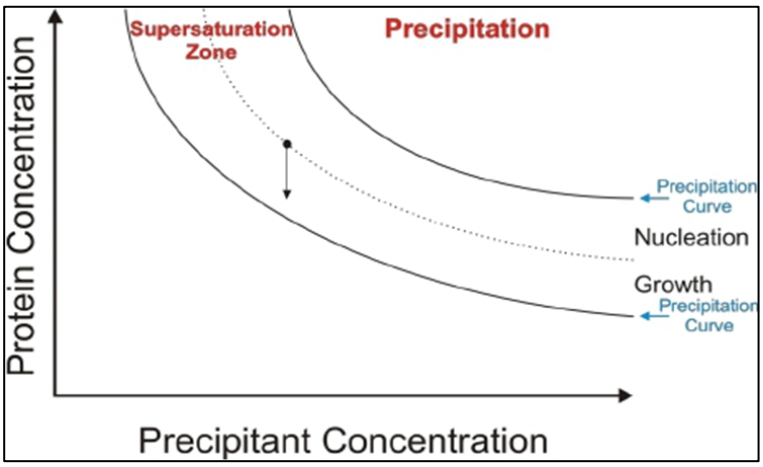

Figure 2-1: A diagram showing crystallisation of proteins

The black dotted line represents the optimal conditions for protein crystallisation. Sufficient protein and precipitant concentration is important to allow nucleation. The growth of the crystals reduces protein concentration and the growth will continue as long as it is not below the lower precipitation curve. The optimum concentration of the protein is important as high concentration may causes nucleation or aggregation and less concentration may not produce crystals (Asherie, 2004).

2.2.5 Trypsin digestion

Trypsin was used to investigate the stability of recombinant mHP. The principle behind this experiment is that folded proteins are relatively resistant to proteolysis, whereas unfolded proteins are sensitive. A serial dilution of trypsin was prepared in TBS-buffer starting at 1 mg/ml. Then, 9 µL of mHP at 3.2 mg/ml was incubated with 1 µL of each serial dilution of trypsin (the actual amount of proteins w/w is 0.08 µg of trypsin: 28.8 µg of mHP). Samples were then incubated for 1 hour at 37 ˚C and 5 µL of loading dye with DDT and heated 4-5 minutes in 95˚C and then immediately separated by SDS-polyacrylamide gel electrophoresis.

2.2.6 Preparation of human and mouse serum

Normal human and mouse wild type serum were kindly provided by Professor Wilhelm Schwaeble's laboratory at the University of Leicester. Serum deficient in some components (e.g haptoglobin) was used for certain assays. Mice were bled via cardiac puncture route under general anaesthesia. To avoid any detrimental effects (e.g complement activation), collected blood samples were kept on ice during samples preparation. Blood were collected in 1.5 ml eppendorf tubes and immediately kept on ice. Samples were kept on ice for 3-4 hours to clot and then were centrifuged using cooled centrifuge at 14,000 rpm for 10 minutes to separate serum. Samples were transferred into different tubes and then kept for further use at -80°C.

2.2.7 Binding experiments using enzyme immunosorbent assays (ELISA)

2.2.7.1 Bacterial fixation and preparation for ELISA assays

Bacteria were grown in BHI overnight at 37°C, with shaking and 37°C for *S. aureus* and under anaerobic conditions by static incubation overnight at 37°C for *S. pneumoniae*. The following day, bacterial growth was centrifuged for 10 minutes at 4000xg. Then, the supernatant was discarded and the cell pellet was washed three times in phosphate-buffered saline (PBS). Subsequently, 10 ml of 0.5% formalin in PBS was used for re-suspending the cell pellet, which was then kept for 30-60 minutes at room temperature and was followed by washing twice with PBS. To confirm fixation, fixed bacteria were plated and incubated at 37°C overnight, considering the growth conditions for each bacterium on brain heart infusion (BHI) agar. To ensure formalin fixation was successful, no growth should have been apparent following overnight incubation. After fixation, the bacterial suspension was spun down and then the coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% sodium azide, pH 9.6) was used for re-suspending the cell pellet. The re-suspension was used for certain *in-vitro* assays (Lynch *et al*., 2004).

2.2.7.2 Binding Assays of HP to Hb, LTA, *S. aureus* and *S. pneumoniae*

Micro-titre ELISA (Nunc) plates were coated with formalin-fixed bacteria (*S. pneumoniae* and/or *S. aureus*, with an adjusted OD_{550} value of approximately 0.6), LTA (10 µg/ml) and/or Hb (10 µg/ml). Coated plates were then incubated at 4°C overnight. Next day, the residual binding sites were blocked for two hours at room temperature by the addition of 250 µl of blocking buffer (1% w/v BSA, PBS pH 7.4) to the plates. Then,

250 µl of washing buffer (PBS with 0.05% tween-20) was added three times, for washing. The dilution of the samples of either serum (starting from 1/40), HP (10 µg/ml) and/or β-chain of HP (10 µg/ml) was prepared in the PBS buffer (pH 7.4) and 100 µl of each diluted sample was added to the wells in duplicates, followed by incubation for 1 hour at room temperature (RT) or 37°C according to the assay condition (differences are explained in Table 2.1). The wells, which only contained PBS buffer without any sample, were used as negative controls as well as using BSA as a double negative control. Following 1or 2 hours of incubation, the plates were washed three times, using washing buffer, and then the 100 µl/well of the primary antibodies (mouse monoclonal anti-HP antibody or Chicken polyclonal anti-HP antibody by Sigma-Aldrich) was added (diluted 1/1000 in PBS, with 0.05% tween20)**.** The addition of the primary antibodies was followed by incubation (incubation time differences are explained in Table 2.1) and then the plates were washed three times with washing buffer, as previously done. Next, a 100 µl/well of the secondary antibodies (an alkaline phosphatase-conjugated goat anti-mouse IgG antibody diluted 1/30000 or an alkaline phosphatase-conjugated goat anti-chicken IgG antibody diluted 1/10000) was added to the plates followed by incubation (incubation time differences are explained in Table 2-1). The penultimate step was washing the plates three times and then adding 100 µl/well of substrate solution (Fast pNPP tablet sets, Sigma), in order to detect the presence of alkaline phosphatase. Finally, absorbance was measured at 405 nm by a BioRad micro-titre ELISA plate reader.

Table 2-1: Table showing incubation times and antibodies used for HP Binding assays

Serial dilution of HP (starting at concentration 10 µg/ml) was incubated with the immobilised antigen.

Immobilised antigen	HP Incubation	Primary Ab's	Secondary Ab's
LTA (10µg/ml)	1 hour at RT	Chicken anti-HP (1/1000 for 1 hour at RT)	Goat anti-chicken IgG (1/10000 for 1 hour at RT)
Hb (10µg/ml)	1 hour at RT	Chicken anti-HP (1/1000 for 1 hour at RT)	Goat anti-chicken IgG (1/10000 for 1 hour at RT)
S. aureus (OD_{550} of 0.6)	2 hours at 37°C	Mouse anti-HP (mAb) (1/4000 for 2 hours at 37°C)	Goat anti-mouse IgG (1/30000 for 2 hours at 37°C)
S. pneumoniae (OD_{550} of 0.6)	1 hour at RT	Chicken anti-HP (1/1000 for 1 hour at RT)	Goat anti-chicken IgG (1/10000 for 1 hour at RT)

2.2.7.3 Binding Assays of LTA to HP and β-HP

Micro-titre ELISA (Nunc) plates were coated with HP and/or β-Hp (10 µg/ml). Coated plates were then blocked and washed as explained in section (2.2.7.2). The LTA samples (starting at 20 µg/ml) were diluted in PBS buffer (pH 7.4) and 100 µl of each diluted sample was added to the wells in duplicates, followed by incubation for 1 hour 37°C. The wells, which only contained PBS buffer without any protein, were used as a double negative controls with BSA as a negative control. Following 1 hour of incubation, the plates were washed three times, using washing buffer, and then a 100 µl/well of the primary antibodies (mouse monoclonal anti-LTA antibody diluted 1/1000 in PBS) was added. The addition of the primary antibodies was followed by incubation at 37°C for 1

hour and then the plates were washed as previously done. Next, a 100 µl/well of the secondary antibodies (an alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody diluted 1/10000) was added to the plates and then incubated at room temperature for 1 hour. Then experiment was completed as in section (2.2.7.2).

2.2.8 Competition inhibitory assays

Micro-titre ELISA (Nunc) plates were coated with Haemoglobin (Hb) (10 µg/ml), LTA (10 µg/ml) and/or formalin-fixed *S. aureus* (with an adjusted OD_{550} value of 0.6). Coated plates were then blocked and washed as explained in section (2.2.7.2). Constant concentration of HP was mixed with different concentration of the competitor material (soluble LTA or Hb or LTA structural components) and then the mixture was incubated with the immobilised antigen. The incubation times and the concentration of the inhibitors and antibodies used are explained in the table below (

Table 2-2 and Table 2-3). The rest of the experiment was completed the same as binding assays in section (2.2.7.2).

Table 2-2: Table showing inhibitors and antibodies used for HP competition assays

Constant concentration of HP (10 µg/ml) was mixed with increasing concentration of the inhibitor according to the condition of the assay.

Immobilised antigen	Inhibitor	Incubation Time (HP/Inhibitor mixture with the immobilised antigen)	Primary Ab's	Secondary Ab's
LTA (10µg/ml)	Soluble Hb (starting at 2 mg/ml)	1 hour at RT	Chicken anti-HP (1/1000 for 1 hour at RT)	Goat anti-chicken IgG (1/10000 for 1 hour at RT)
S. aureus (OD_{550} of 0.5)	Soluble LTA (starting at 500 µg/ml)	2 hours at 37°C	Mouse anti-HP (mAb) (1/4000 for 2 hours at 37°C)	Goat anti-mouse IgG (1/30000 for 2 hours at 37°C)
Hb (10µg/ml)	Soluble LTA (starting at 500 µg/ml)	1 hour at RT	Chicken anti-HP (1/1000 for 1 hour at RT)	Goat anti-chicken IgG (1/10000 for 1 hour at RT)

Table 2-3: LTA structural components as inhibitors for HP/LTA binding

Constant concentration of HP (10 µg/ml) was mixed with increasing concentration of the inhibitor according to the condition of the assay.

Immobilised antigen	Inhibitor (decreasing Concentration starts at 5 mM)	Incubation Time (HP/Inhibitor mixture with the immobilised antigen)	Primary Ab's	Secondary Ab's
LTA (10µg/ml)	-glycerophosphate - D.alanine -GlcNac - gentiobiose	1 hour at RT	Chicken anti-HP (1/1000 for 1 hour at RT)	Goat anti-chicken IgG (1/10000 for 1 hour at RT)

2.2.9 Ficolin-A binding to *S. pneumoniae*

Micro-titre ELISA (Nunc) plates were coated with formalin-fixed *S. pneumoniae* and blocked as explained in section (2.2.7.2). Serum (wild type serum and haptoglobin-deficient serum) samples were prepared in barbital buffered saline (BBS, 4 mM barbital, 145 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) to a maximum concentration of 5%. After washing three times with TBS buffer (plus 0.05% tween-20 and 5 mM $CaCl_2$), a 100 µl/well of serum was added to corresponding wells and wells received only buffer were used as a negative control (Celik *et al*., 2001; Takahashi *et al*., 2008; Schwaeble *et al*., 2011) followed by 1 hour incubation at 37°C. Plates were then washed as before and a 100 µl of rabbit anti-mouse Ficolin A antibodies (Prof. T. Fujita, School of Medicine, Fukushima, Japan; 0.7 mg/ml stock solution), and incubated for 90 minutes at 37°C. Subsequently, samples were washed as before and a 100 µl of secondary antibody diluted in wash buffer 1/10000 (alkaline phosphatase-conjugated goat anti-rabbit IgG, Sigma) was added followed by 60-90 minutes incubation at room temperature (RT). The rest of the experiment was completed the same as binding assays in section (2.2.7.2).

2.2.9.1 HP competition with ficolin-A for binding LTA on *S. pneumoniae* (D39)

The same experiment's protocol was followed as before but using only HP-deficient serum increasing concentration of recombinant mHP to test the inhibitory effect of mHP on ficolin/ *S. pneumoniae* interactions. A serial dilution of mHP (starting at 1 mg/ml) was added to fixed concentration of 10% of HP-deficient serum. The rest of the experiment was completed the same as binding assays in section (2.2.7.2). Wells received only BSA-

blocking buffer was used a negative control as before. HP-deficient serum was used as a positive control.

2.2.10 *In vivo* studies

All experiments performed *in vivo* were under the project license 60/4327 in agreement with standard operating procedures following the guidelines from the Animal Scientific Procedure Act 1986 of the UK Home Office.

2.2.10.1 Mice

C57BL/6J wild type mice were bought from Charles River Laboratories, UK. CD-1 mice purchased from Charles River Laboratories, UK, was utilised for passaging bacteria. Sperm of haptoglobin knockout mice on C57BL/6J background were provided by Dr Heinz Baumann (Department of Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo NY 14263, USA). Mice used in the study were in the age from 8 to 12 weeks old in all infection studies. Two mouse groups were used; haptoglobin deficient mice and their C57BL/6J wild type littermates (WT mice).

2.2.10.2 Bacterial preparation for infection studies

Two animal models, *Staphylococcus aureus* (Newman, SH1000 and EMRSA-16) and *Streptococcus pneumoniae* (D39), were studied to assess the interaction between the haptoglobin and the lipotechoic acid (LTA) of Gram-positive bacteria.

2.2.10.2.1 Preparation of *S. aureus* (Newman, SH1000 and EMRSA-16)

S. aureus was streaked from frozen stock on BHI agar plates overnight at 37˚C. Then, one colony of the growth was transferred into 10 mL of brain heart infusion (BHI) and incubated for overnight for at 37˚C. Bacterial culture was centrifuged for 10 minutes at 3000 rpm and pellet was then collected, re-suspended in 10 ml of BHI augmented with 20% of fetal calf serum (FBS). Suspension was then aliquoted and kept at -80˚C until used. To confirm the viability of the bacteria, next day an aliquot was plated onto BHI agar.

2.2.10.2.2 Preparation of bacterial passage of *Streptococcus pneumoniae* (D39)

With view modification of procedures used by Kadioglu *et al*. (2000), CD-1 mouse strain was used to passage *S. pneumoniae* D39 to gain the more virulent bacteria. Using blood agar *S. pneumoniae* were streaked and incubated anaerobically overnight at 37˚C. Few colonies next day were inoculated into 10 ml BHI broth followed by overnight static incubation at 37˚C. Then, bacterial growth was centrifuged for 10 minutes at 3000 rpm and pellet was then collected, re-suspended in 5 ml phosphate buffered saline (PBS). Subsequently, 5 CD-1 mice were injected with 100 µl into the peritoneal cavity. Under general anaesthesia by cardiac puncture blood samples were collected 24 hours post-infection when mice started showing signs of disease and then mice were culled by cervical dislocation. 50 µL of the blood samples collected was inoculated into 10 ml of BHI broth followed by static incubation at 37˚C. Next day, bacterial culture was centrifuged for 10 minutes at 3000 rpm and pellet was then collected, re-suspended in 1

ml of BHI augmented with 20% of fetal calf serum (FBS). 667 µl of the suspension was then pipetted into 10 ml of BHI with 20% of fetal calf serum (FBS) with an adjusted OD_{500} of 0.7. Suspension was then incubated until OD_{500} reached 1.6, which was then aliquot and kept at -80˚C to be used. To confirm the viability of the bacteria, next day an aliquot was plated onto blood agar.

To test the virulence of the passaged bacterial stock, one aliquot was thawed and centrifuged at 13000 rpm for 3 minutes. Pellet was then re-suspended in PBS to attain a final count of $5x10^5$ CFU/50 µL. CD-1 mice, under general anaesthesia (2.5% (v/v) of inhaled flurane, 2 litres O_2/minute), were then injected via the intranasal route. PBS used to adjust the bacterial dose was then cultured to confirm sterility. Mice were monitored regularly for 48-72 hours and 80% of the infected mice reached +/+ lethargic when they were culled by cervical dislocation.

2.2.10.3 Infection studies

2.2.10.3.1 *Staphylococcus aureus* (Newman) mouse infection model

Two female age-matched groups (Haptoglobin deficient mice, HP-/- mice, and their wild type littermates, WT mice) were injected via either intraperitoneal (IP) or intranasal (IN) route with *Staphylococcus aureus* 5 x 10^7 CFU, 1 x 10^8 CFU and 4 x 10^8 CFU in PBS under general anaesthesia (150 µL with the IP route and 50 µL with IN route). The viable count of the dose administered to the animals was checked by culturing on LA medium. The cages of the infected animals were placed into a specific infectious rack. Mice were

then monitored for signs of disease for 24 hours and those that reached +/+ lethargic were terminated by cervical dislocation in line with Home Office Regulations. The strains and dose used per each route is indicated in Figure 2-1 below.

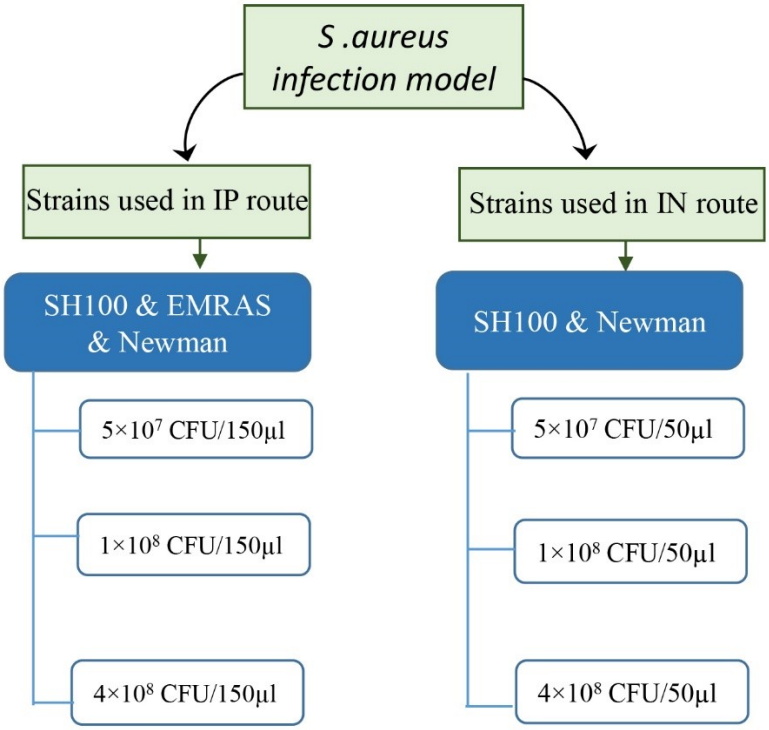

Figure 2-2: *S. aureus* infection model routs of infections and doses

2.2.10.3.2 *S. pneumoniae* (D39) mouse infection model

Using strain, sex and age-matched groups of HP deficient mice and wild type mice, mice were injected via the intranasal route with passaged bacteria of *S. pneumoniae* $5x10^5$ CFU/50 µL in PBS under general anaesthesia as explained above. The viable count of the dose administered to the animals was checked by culturing on blood agar medium. The

cages were treated as before with *S. aureus* and mice were then monitored as before for 72 hours and those that reached +/+ lethargic were terminated by cervical dislocation in line with Home Office Regulations.

2.2.10.3.3 Pain scoring method

The signs of illness were monitored and were scored based on scoring methods of the Division of Biomedical Services manages the Central Research Facility (CRF).

Table 2-4: Scores of disease severity associated with clinical signs in infected mice

Score	Signs
0	Normal
1	Hunched +
1-2	Hunched ++
3	Starry coat +
3-4	Starry coat ++
4-5	Lethargic +
5	Lethargic ++

2.2.10.3.4 Bacterial load determination of blood and organs

Bacterial viable count was also measured in the blood and organs (e.g. lung, kidney, liver and spleen) for two strain, sex and age-matched groups of HP deficient mice and wild type mice at pre-selected time points post infection (at 6, 12, and 18, 24 hours for *S. aureus* infection model, and 48, 72 hours for *S. pneumoniae* infection model). Mice under general anaesthesia underwent cardiac puncture to collect blood followed by mice

dissection and the collection of organs under aseptic condition. Twenty microliters of blood were 10 folds diluted in PBS and then 60 µL were dropped onto blood agar plates which then were incubated at 37^{o} overnight (*S. pneumoniae* were anaerobically incubated using candle jar). Following organs collection and weighing them with 5 ml PBS, they were homogenised using Ultra-Turrax T10 basic IKA-Werke homogenizer. Subsequently, bacterial burdens were counted and serially diluted in PBS as before, 10 folds of 20 µL of the homogenised organ and plated on blood agar. Equation utilised to calculate colony forming units CFU/mg as follow: CFU/mg = [(No. of colonies x dilution factor x 1000 x volume of homogenising solution) volume plated in µL] / organ weight in mg.

Chapter 3

Generation of Recombinant Mouse Haptoglobin (mHP)

3.1 Introduction

Haptoglobin (HP) is an acute phase protein found in serum at concentrations between 0.45 and 3 mg/ml (Neilsen and Moestrup 2009). HP is a tetramer composed of four chains, two alpha chains (approximately 9 kDa each) and two beta-chains (approximately 33 kDa each) linked by disulfide bonds (Langlois and Delanghe, 1996). It is synthesised as a single polypeptide precursor which undergoes a proteolytic cleavage (Thomsen *et al*, 2013) forming an N-terminal light α-chain (83 amino acids) and a C-terminal heavy β-chain (245 amino acids) (Jia *et al*, 2013). The α-chain consists of a complement control repeat domain (CCP) and the β-chain represents a serine protease domain (SP) (Neilsen M *et al*, 2007). In the mature polypeptide, the α- and β-chains are linked by a disulphide bond. The SP domain of HP, unlike other serine proteases such as complement proteases C1s and C1r, lacks catalytic activity, possessing only one of the three residues of the functional catalytic triad (Andersen *et al*, 2012). For example, in porcine haptoglobin the catalytic serine residue is replaced by an alanine and the histidine by a lysine residue.

Recently, a study done at the University of Leicester (Djebari B, University of Leicester, 2010) showed that HP binds to lipotechoic acid (LTA) from *S. aureus. In-vitro* studies supported this finding and showed that HP also bound to LTA on the staphylococcal surface. Nevertheless, this work did not address how HP interacts with LTA. In order to understand this process at the molecular level, it is crucial to investigate how the HP-LTA complex forms. Since this work requires large amounts of pure protein, it was proposed to produce a recombinant form of human and mouse haptoglobin (hHP and

mHP respectively). The use of recombinant protein has the added advantage that it is not contaminated with other serum proteins as may occur for serum-derived material. We chose to express human and mouse haptoglobin because they can be used in reconstitution experiments in animal studies or using serum.

To our knowledge, full length recombinant HP has not been produced before. A variety of systems can be utilised for cloning and expression of eukaryotic proteins. In this study, a bacterial system, *E. coli*, was implemented for the amplification and expression of the hHP and mHP. Using bacterial host is advantageous because it is relatively cheap, rapid and simple to culture; in addition to that expression is easily scalable. However, producing soluble proteins can be a challenge when using bacterial host, particularly secreted proteins with disulphide bonds such as HP that fold in the endoplasmic reticulum (Lilie, Schwarz and Rudolph, 1998).

3.1.1 HP Binding to Immobilised *S. aureus* and Staphylococcal LTA

Initial experiments were conducted to confirm that HP binds to LTA and *S. aureus*. An ELISA was established in which binding of HP from human serum was measured to immobilised LTA using a monoclonal anti-HP antibody to detect bound HP. BSA was used as a negative control. The results showed that serum-HP specifically binds to LTA, see Figure 3-1.

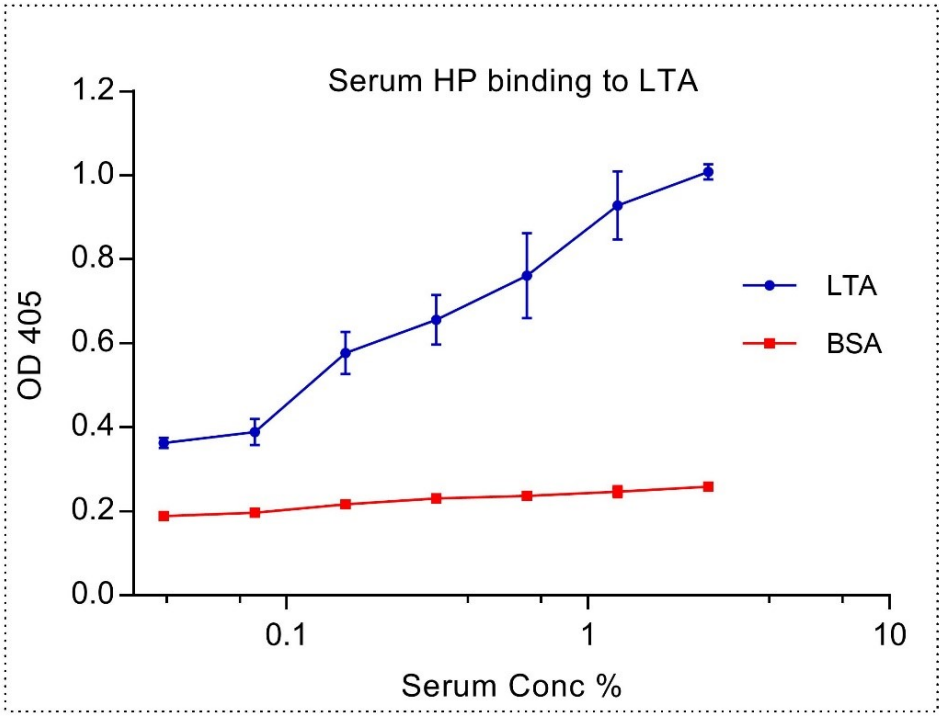

Figure 3-1: ELISA showing human serum HP binding to LTA

ELISA plates were coated with LTA**.** Subsequently, diluted human serum was incubated with LTA coated wells for two hours at 37 °C. Binding of human serum was detected using a mouse monoclonal anti-HP antibody, followed by an alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody. BSA was used as a negative control. Three independent experiments were carried out and results are expressed as mean ± SEM.

To confirm that HP also recognised the LTA on bacteria, the experiment was repeated by immobilising whole bacteria in the wells. As shown in Figure 3-1 specific HP binding was detected at similar dilutions of serum, see Figure 3-2.

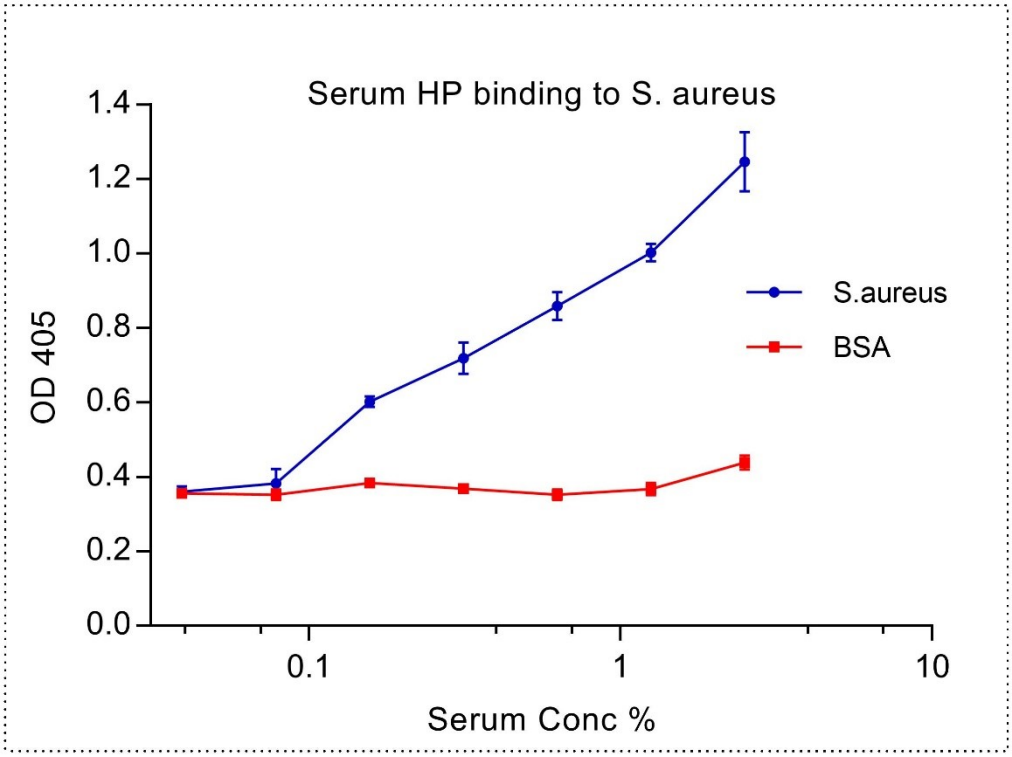

Figure 3-2: ELISA showing human serum HP binding to *S. aureus*

ELISA plates were coated with *S. aureus*. Subsequently, diluted human serum was incubated in *S. aureus* coated wells for two hours at 37 °C. Binding of human serum was detected using a mouse monoclonal anti-HP antibody and an alkaline phosphatase-conjugated goat anti-mouse antibody. BSA was used as a negative control. Results are expressed as means ± SEM from three independent experiments were done.

3.2 Cloning of human (hHP) and mouse (mHP)

To produce recombinant HP, the cDNA of human and mouse haptoglobin (hHP and mHP) were amplified from a mouse and human liver cDNA libraries.

- **Amino acids Sequence similariteis between mouse and human haptoglobin**

Mouse HP

MVDSGNDVTDIADDGCPKPPEIAHGYVEHSVRYQCKNYYKLRTEGDGVYTLNDKKQWINKAVGDKLPECEAVCGKPKN
PANPVQRILGGHLDAKGSFPWQAKMVSHHNLTTGATLINEQWLLTTAKNLFLNHSENATAKDIAPTLTLYVGKKQLVE
IEKVVLHPNYSQVDIGLIKLKQKVSVNERVMPICLPSKDYAEVGRVGYVSGWGRNANFKFTDHLKYVMLPVADQDQCI
RHYEGSTVPEKKTPKSPVGVQPILNEHTFCAGMSKYQEDTCYGDAGSAFAVHDLEEDTWYATGILSFDKSCAVAEYGV
YVKVTSIQDWVQKTIAEN

Human HP

MVELGNDAMDFEDDSCPKPPEIANGYVEHLVRYRCRQFYRLRAEGDGVYTLNDEKQWVNTVAGEKLPECEAVCGKPKH
PVDQVQRIIGGSMDAKGSFPWQAKMISRHGLTTGATLISDQWLLTTAKNLFLNHSETASAKDITPTLTLYVGKNQLVE
IEKVVLHPNHSVVDIGLIKLKQRVLVTERVMPICLPSKDYIAPGRVGYVSGWGRNANFRFTDRLKYVMLPVADQDKCV
VHYENSTVPEKKNLTSPVGVQPILNEHTFCAGLTKYQEDTCYGDAGSAFAIHDMEEDTWYAAGILSFDKSCAVAEYGV
YVRATDLKDWVQETMAKN

- Alignment of the amino acids sequence of mouse haptoglobin (mHP) and human haptoglobin (hHP) showed 263 identical amino acids out of 330 (80%) between the two sequences. Identical amino acids are shaded.

```
mHP     1    MVDSGNDVTDIADDGCPKPPEIAHGYVEHSVRYQCKNYYKLRTEGDGVYTLNDKKQWINK  60
             MV+ GND  D  DD CPKPPEIA+GYVEH VRY+C+ +Y+LR EGDGVYTLND+KQW+N
hHP     1    MVELGNDAMDFEDDSCPKPPEIANGYVEHLVRYRCRQFYRLRAEGDGVYTLNDEKQWVNT  60

mHP     61   AVGDKLPECEAVCGKPKNPANPVQRILGGHLDAKGSFPWQAKMVSHHNLTTGATLINEQW  120
               G+KLPECEAVCGKPK+P + VQRI+GG +DAKGSFPWQAKM+S H LTTGATLI++QW
hHP     61   VAGEKLPECEAVCGKPKHPVDQVQRIIGGSMDAKGSFPWQAKMISRHGLTTGATLISDQW  120

mHP     121  LLTTAKNLFLNHSENATAKDIAPTLTLYVGKKQLVEIEKVVLHPNYSQVDIGLIKLKQKV  180
             LLTTAKNLFLNHSE A+AKDI PTLTLYVGK QLVEIEKVVLHPN+S VDIGLIKLKQ+V
hHP     121  LLTTAKNLFLNHSETASAKDITPTLTLYVGKNQLVEIEKVVLHPNHSVVDIGLIKLKQRV  180

mHP     181  SVNERVMPICLPSKDYAEVGRVGYVSGWGRNANFKFTDHLKYVMLPVADQDQCIRHYEGS  240
              V ERVMPICLPSKDY   GRVGYVSGWGRNANF+FTD LKYVMLPVADQD+C+ HYE S
hHP     181  LVTERVMPICLPSKDYIAPGRVGYVSGWGRNANFRFTDRLKYVMLPVADQDKCVVHYENS  240

mHP     241  TVPEKKTPKSPVGVQPILNEHTFCAGMSKYQEDTCYGDAGSAFAVHDLEEDTWYATGILS  300
             TVPEKK   SPVGVQPILNEHTFCAG++KYQEDTCYGDAGSAFA+HD+EEDTWYA GILS
hHP     241  TVPEKKNLTSPVGVQPILNEHTFCAGLTKYQEDTCYGDAGSAFAIHDMEEDTWYAAGILS  300

mHP     301  FDKSCAVAEYGVYVKVTSIQDWVQKTIAEN  330
             FDKSCAVAEYGVYV+ T ++DWVQ+T+A+N
hHP     301  FDKSCAVAEYGVYVRATDLKDWVQETMAKN  330
```

It was primarily planned to clone the sequence of the full length human haptoglobin (HP) as well as its two chains α-HP and β-HP using pRSET B cloning vector. The primers used to amplify the PCR fragments needed to generate the all fragments of human HP (full length HP, α-HP and β-HP) encoding sequence were engineered to contain endonuclease restriction sites EcoR1, HindIII and Xho1. Unfortunately, these cloning attempts were not successful and had to change the strategy focus only on the full length HP of both human and mouse as it is explained below.

3.2.1 Amplification of hHP and mHP cDNA using polymerase chain reaction (PCR)

Human and mouse liver cDNA libraries (provided by Dr Youssif Ali, University of Leicester) were used as templates for the generation of cDNA of hHP and mHP. Primers, shown in Table 3-1 below, were designed to amplify the entire cDNA and PCR was carried out using Phusion High-Fidelity DNA polymerase to minimise the chances of introducing mutations during cloning (section 2.2.1.1). PCR products were then separated by electrophoresis on a 1% agarose gel. The expected size of the product was ~1 Kb. The DNA products of the PCR reaction are shown in Figure 3-3**.**

Table 3-1: Primers used for cloning

Primer	Sequence (5'→3')
hHp_F_NdeI	TGTCAACATATGCATCACCATCACCATCACGTGGACTCAGGCAATGAT
hHp_R_EcoR1	GACATTGAATTC TTAGTTCTCAGCTATGGTCTT
mHp_F_NdeI	TGTCAACATATGCATCACCATCACCATCACGTGGAGTTGGGCAATGAT
mHp_R_EcoR1	GACATTGAATTCCTAGTTCTTGGCCATGGTTTC
Highlights	'h' stands for human primers 'm' stands for mouse primers; 'F' stands for forward, and 'R' for reverse; restriction sites *NdeI* (containing the start codon highlighted in *green*) and *EcoR1* are in red underlined; histidine tag sequence (*blue*), *stop* codons (red)

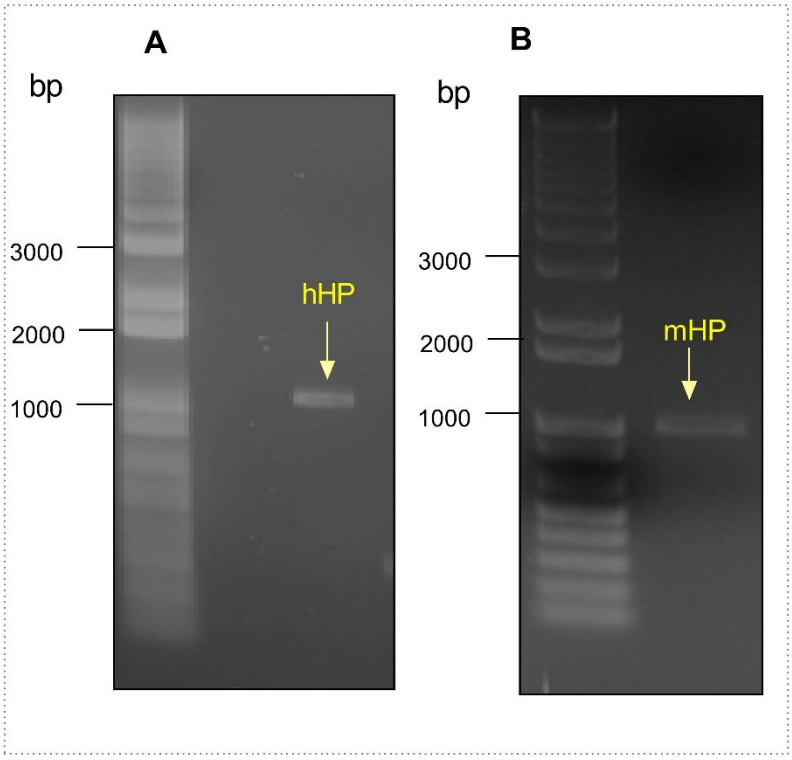

Figure 3-3: Agarorose gel electrophoresis of amplified hHP and mHP cDNA

The human (hHP) cDNA **(A)** and mouse HP (mHP) cDNA **(B)** amplified using the polymerase chain reaction (PCR) with a Phusion High-Fidelity DNA polymerase (Thermo Scientific, Cat# F-530S). The PCR products were ~1000 bp as expected. A 1 Kb+ ladder was run alongside the cDNA on a 1% agarose TAE-gel.

3.2.2 Cloning the cDNA of mHP and hHP into pGEM-T Easy vector and pET-22b(+) expression vector

The PCR products were initially cloned into pGEM-T easy cloning vector, which is sold as a linearised vector with single base T-overhangs to facilitate the sub-cloning of PCR products. The primers used to amplify the PCR fragments contained the endonuclease restriction sites NdeI, which contains the ATG start codon within its recognition sequence (CATATG), and an EcoR1 at the 3' end to enable cloning into the bacterial expression vector pET-22b(+).

After successful cloning and verification of the presence of an insert at the correct size, the 1 kb inserts of both hHP and mHP were excised from the pGEM-T easy vector using EcoR1 and NdeI restriction enzymes (see Figure 3-4) and was sub-cloned into the expression vector pET-22b(+). The resulting expression constructs were verified by restriction digestion as shown in **(**Figure 3-5**)**, and the sequence of the HP cDNA was confirmed by DNA sequencing (performed by the Protein Nucleic Acid Chemistry Laboratory University of Leicester; PNACL) to ensure no mutations were introduced during the PCR amplification steps.

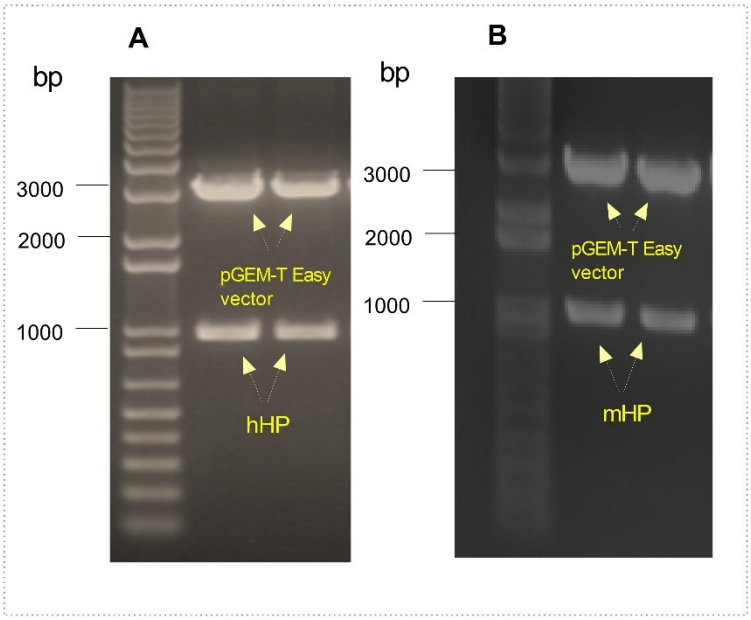

Figure 3-4: Agarose gel electrophoresis of hHP and mHP cDNA cloned into pGEM-T Easy vector

Restriction digests of hHP **(A)** and mHP cDNA **(B)** in pGEM-T Easy vector. The plasmid was digested using *EcoR1* and *NdeI* resulting in the release of the full length cDNA of both hHP and mHP (~1000 bp) and pGEM-T easy vector band (~3 kb). Two identical clones of both hHP and mHP are shown.

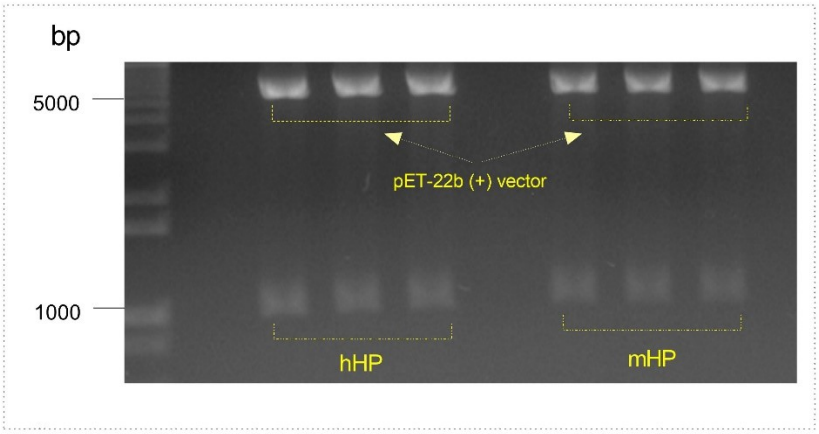

Figure 3-5: Agarose gel electrophoresis of hHP and mHP cDNA cloned into pET-22b (+) expression vector

The purified cDNA encoding hHP and mHP were sub-cloned into the expression vector pET-22b (+). Clones were verified by digestion with *EcoR*1 and *Nde*I resulting in the release of the HP cDNA (~1000 bp) and a band corresponding to the pET-22b (+) vector (~5.5 kb). Three identical clones of both hHP and mHP are shown.

3.3 Protein expression and purification

3.3.1 Protein production and purification

To express the recombinant HPs, BL21 (DE3) *E. coli* competent cells were transfected with the pET-22b (+) expression vector containing the mouse and human cDNAs. After conducting several optimisation experiments including growth at different temperatures and for different times: 20°C overnight, 25°C overnight, 37°C for 5 hours and 37°C overnight, mHP was successfully produced as inclusion bodies by growth in LB broth for 5 hours at 37°C. However, the protein was not detected at 37°C overnight, and this could indicate that the protein could have been degraded. No protein was detected for human HP, suggesting that it is either not expressed efficiently or is degraded following synthesis. Therefore I proceeded with mHP. Cultures were induced at an OD_{550} of 0.6 to 0.8 with 1 mM IPTG. An SDS-PAGE gel of expressed HP is shown Figure 3-6. Cells containing the inclusion bodies were lysed using 8 M urea lysis buffer as detailed in Chapter 2 (section 2.2.3.2), the inclusion bodies were then purified, and washed to reduce the contaminants. Samples were checked on 15% SDS-polyacrylamide gels as shown in Figure 3-7. The washing steps were effective in removing most of the contaminants leading to approximately 85% purity based on scans of the gel.

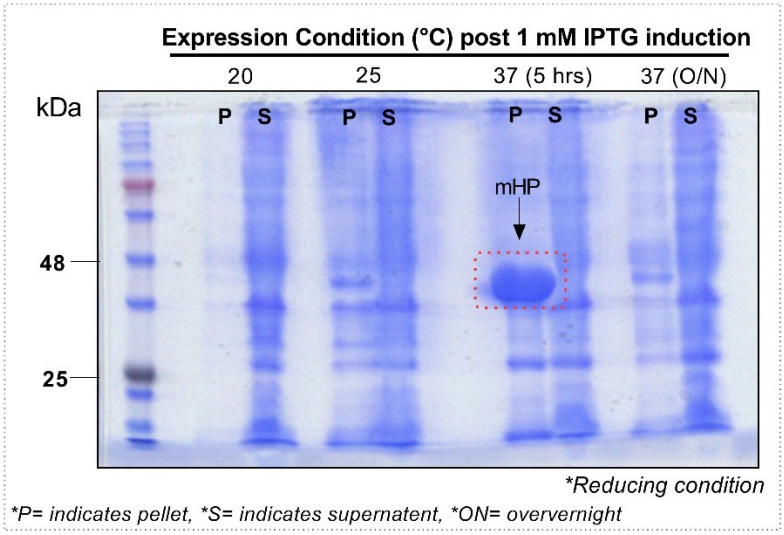

Figure 3-6: SDS polyacrylamide gel electrophoresis of the suspected HP bacterial expression as inclusion bodies

A 15% SDS-polyacrylamide gel run under reducing conditions was stained using Coomassie blue. The gel shows soluble (supernatant, 'S') and insoluble fractions (pellet, 'P') from the lysed cell pellets of culture grown under different growth conditions. A large band corresponding to the suspected mouse haptoglobin (mHP) is indicated. The expected molecular mass of mHP is ~44 kDa.

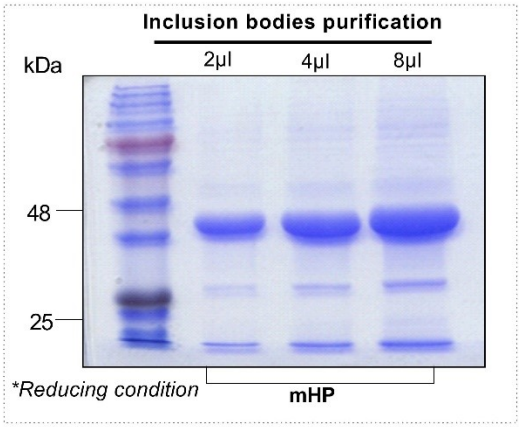

Figure 3-7: SDS polyacrylamide gel electrophoresis showing purification of inclusion bodies containing the putative mHP

A 15% SDS-polyacrylamide gel run under reducing conditions was stained by Coomassie blue. It shows three different loading amounts of the purified inclusion bodies after the wash steps to remove contaminants.

Protein was refolded by solubilising the inclusion bodies in 8 M urea and folding the protein by drop dilution into refolding buffer at 4ºC overnight. To aid purification, recombinant mHP was synthesised with an N-terminal histidine tag, allowing purification by affinity chromatography using a HisGravi Trap column (GE Healthcare). After washing in buffer containing 20 mM imidazole to remove weakly bound contaminants, recombinant mHP protein was eluted from the column with PBS buffer containing 500 mM imidazole. Fractions eluted from the column were analysed by SDS-PAGE as shown in Figure 3-8.

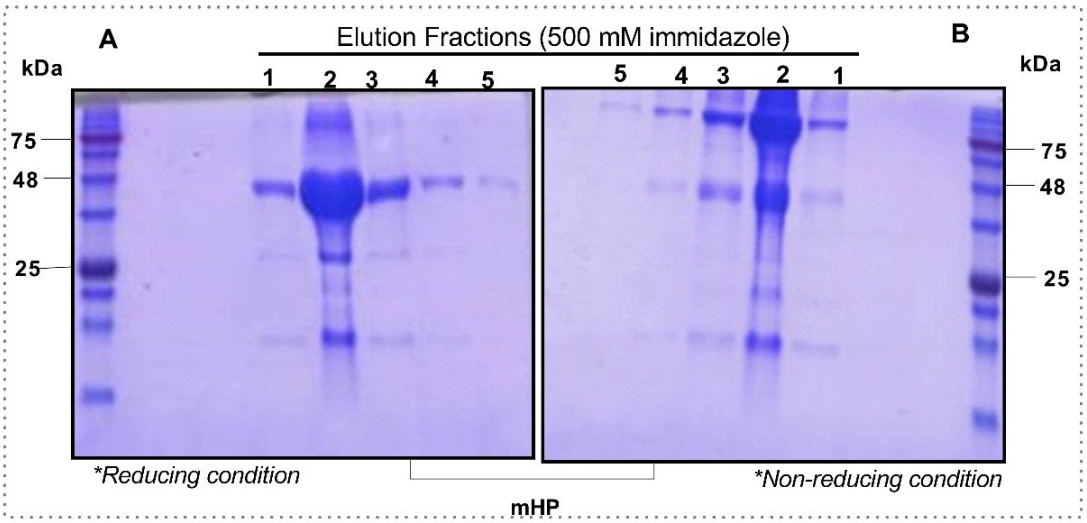

Figure 3-8: SDS polyacrylamide gel electrophoresis showing fractions after purification of the putative mHP on a nickel column

A 15% SDS-polyacrylamide gel was stained by Coomassie blue stain**.** Gels show bands of elution fractions collected after purification with HisGravi Trap Ni chelating column (GE Healthcare). Gel **A** shows fractions under reduced condition (~44kDa), whilst gel **B** shows fractions under non-reduced condition (~88kDa).

Native mHP is a disulphide-linked dimer. As seen in Figure 3-8, under reducing conditions recombinant protein migrated as a band of ~44 kDa, whereas under non-reducing conditions it migrated as ~88 kDa dimer, suggesting that refolding was successful and that correct disulphide bonds had formed during the refolding process. Although relatively little additional purification was achieved using the Ni2+-affinity purification step, it was useful for concentrating the protein after drop dialysis and for removing aggregates that did not pass through the column.

For further purification, fractions were pooled and run on a gel filtration column. Most of the protein eluted as a single peak from the gel filtration column at the expected molecular mass of a ~88 kDa dimer as in Figure 3-9. The fractions containing mHP were then pooled, concentrated, distributed into small aliquots (30 – 50 µL), snap-freezed in liquid nitrogen and stored at -80° C until needed.

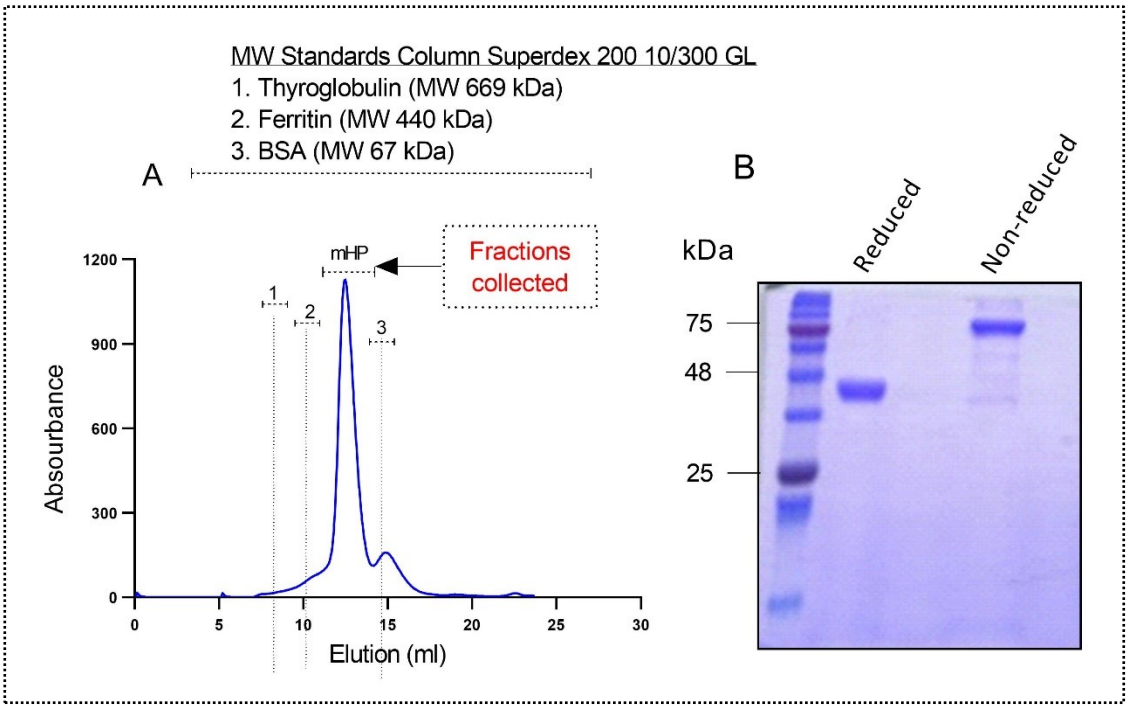

Figure 3-9: Purification using gel filtration column Superdex 200 10/300 GL

Gel filtration column results of refolded N-histagged mouse HP. Protein eluted from the gel filtration column as dimers. Analysis by SDS PAGE showed that mHP was dimers under non-reduced condition and monomer under reducing conditions. **A** shows elution profile of the refolded protein. The mHP peak eluted at the expected position for a dimer of polypeptides. **B** Fractions were collected, pooled, checked under reducing conditions (~44 kDa) and non-reducing condition by SDS-PAGE (~88 kDa).

Protein was analyzed by in gel trypsin digestion/MALDI-TOF-MS. Identity was confirmed as a mouse HP (for matching peptides see Table 3-2). Identity of the purified protein mHP was confirmed as a mouse haptoglobin by protein sequencing, see Appendix. Mass spectrometry data showed that mouse haptoglobin (mHP) is the top score of matching peptides.

Table 3-2: Matching peptide sequence of mHP. Protein on the SDS-PAGE gel was stained with Coomassie blue, and analyzed by in gel trypsin digestion/MALDI-TOF-MS.

Observed Mass (Da)	Mass(calc) (Da)	Matching peptide sequence
716.4090	715.4228	R.VLVTER.V
791.3880	790.3973	K.DYIAPGR.V
920.4540	919.4552	K.GSFPWQAK.M
978.5050	977.5043	K.HPVDQVQR.I
980.4860	979.4876	R.VGYVSGWGR.N
1131.5910	1130.5720	K.QWVNTVAGEK.L
1373.6530	1372.6445	K.SCAVAEYGVYVR.A
1387.6720	1386.6635	K.LPECEAVCGKPK.H
1431.7260	1430.7154	K.NLFLNHSETASAK.D
1635.8170	1634.7974	R.ATDLKDWVQETMAK.N
1679.8300	1678.8162	R.LRAEGDGVYTLNDEK.Q
1740.0170	1739.0094	K.VVLHPNHSVVDIGLIK.L
2127.1640	2126.1372	R.HGLTTGATLISDQWLLTTAK.N
2496.3030	2495.2843	K.NLTSPVGVQPILNEHTFCAGLTK.Y

- Matching amino acids sequence of mHP. The top score, in the table above, using Mass Spectometry was revealed to **Q61646**, as a mouse HP. The data were obtained from PNACL, Core Biotechnology Services, University of Leicester.

Mouse Haptoglobin amino acids sequence

MHHHHHHVEL GNDAMDFEDD SCPKPPEIAN GYVEHLVRYR CRQFYRLRAE GDGVYTLNDE

KQWVNTVAGE KLPECEAVCG KPKHPVDQVQ RIIGGSMDAK GSFPWQAKMI SRHGLTTGAT

LISDQWLLTT AKNLFLNHSE TASAKDITPT LTLYVGKNQL VEIEKVVLHP NHSVVDIGLI

KLKQRVLVTE RVMPICLPSK DYIAPGRVGY VSGWGRNANF RFTDRLKYVM LPVADQDKCV

VHYENSTVPE KKNLTSPVGV QPILNEHTFC AGLTKYQEDT CYGDAGSAFA IHDMEEDTWY

AAGILSFDKS CAVAEYGVYV RATDLKDWVQ ETMAKN

3.3.2 Binding activity of successfully purified recombinant mHP

To ensure that recombinant mouse HP functioned as the native protein, its activity was then tested using an ELISA to examine binding towards purified staphylococcal LTA. Binding of mHP to LTA was compared to that of the purified human serum-derived HP (Sigma). Mouse HP showed a very good binding in comparison to the human HP, as shown in Figure 3-10. Thus, recombinant mHP behaves as expected and can be used for further analysis to characterise its interaction with LTA.

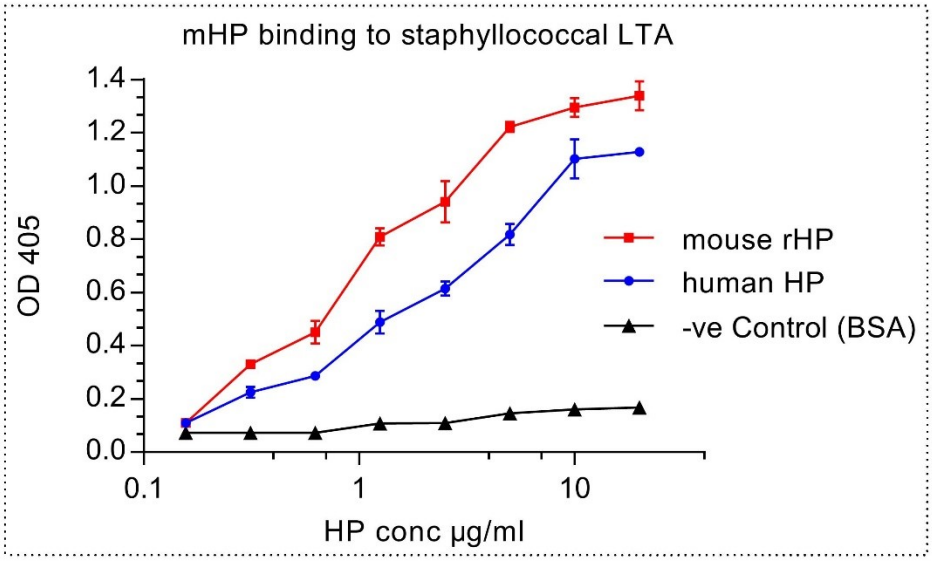

Figure 3-10: Recombinant mHP binding to LTA

ELISA plates were coated with staphylococcal LTA**.** Recombinant mHP was incubated with LTA coated wells for one hour at room temperature (RT). Human HP was included as a positive control. Binding was detected using chicken polyclonal anti-HP antibody, using an alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. Five independent experiments were carried out and the results are expressed as the mean ± SEM.

3.3.3 Crystallisation of recombinant mHP

Mouse HP has not been crystallised before, so crystallisation plates were set up using screens JCSG+ and PACT from Molecular dimensions. Small crystals (plates) were obtained in 0.1 M Bis-Tris propane pH 8.5 containing 0.2 M Sodium iodide and 20 % w/v PEG 3350. Unfortunately these crystal diffracted poorly >15 Å resolution (at Diamond Light Source synchrotron) and it was not possible to determine the structure.

3.4 Discussion

To generate enough protein to characterise the binding properties of HP, I developed an expression system in *E. coli*. Protein was synthesised with an N-terminal His tag to assist purification. HP was expressed as inclusion bodies and purified using a two-step protocol comprising nickel-affinity chromatography. Yields were typically ~3.5 mg/mL of bacterial culture. Gel filtration and SDS-PAGE confirmed that the recombinant mHP is a disulphide-linked dimer with a molecular weight of ~88 kDa under non-reducing conditions, but ~44 kDa under reducing conditions, confirming that it is folded correctly. Furthermore recombinant mHP bound to LTA from *S. aureus* with similar properties as human HP from serum (Sigma). Initially we planned to cleave the recombinant mHP into its α- and β-chains with limited digestion with a protease (e.g. trypsin). However, binding analysis showed that this was not necessary for binding to LTA.

No expression of human HP was detected in *E. coli*. This might be due to low levels of expression or degradation following synthesis. Poor expression could be caused by the differences in codon usage of humans and *E. coli*. In the future, codon optimisation or the use of modified *E. coli* for expression with additional tRNAs (e.g. Rosetta strains) might facilitate production.

Chapter 4

Characterisation of the Interaction between Haptoglobin and Lipoteichoic Acid

4.1 Introduction

HP is an acute phase protein comprising 2α and 2β chains linked by disulphide bonds. The β-chain comprises a serine protease (SP) domain and the α-chain consists of a complement control protein (CCP) domain (Andersen *et al*, 2012). It is well established that HP captures and binds to free haemoglobin, released in the circulation as a result of haemolysis, to form a stable non-covalent complex. This complex is then cleared from circulation via binding to a macrophage scavenger receptor, CD163, described in chapter one. While the CCP domain is responsible for dimerisation of HP, the SP domain forms extensive interactions with both the α- and β-subunits of haemoglobin.

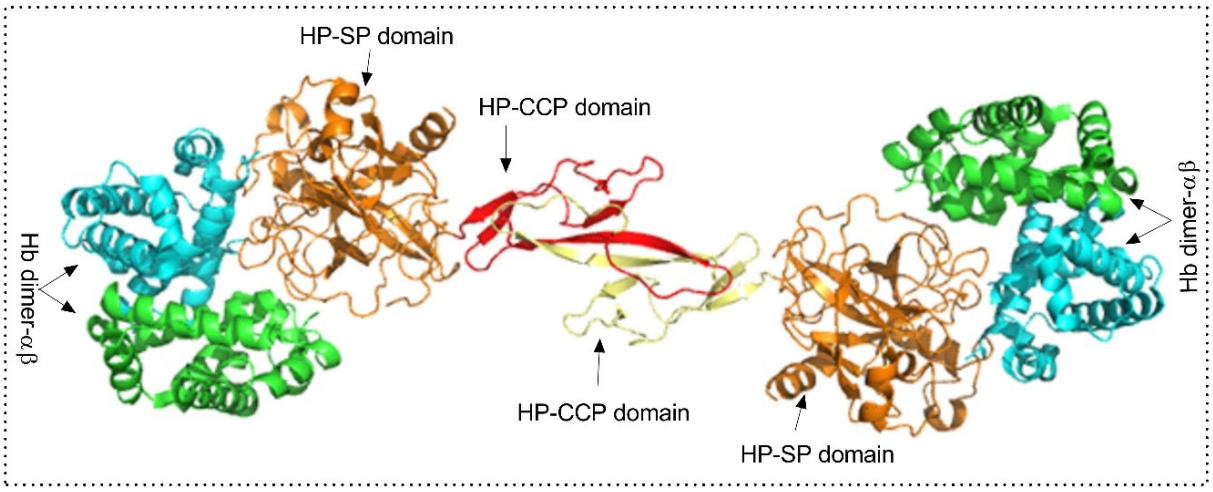

Figure 4-1: HP-Hb complex interactions

Previous work done at Leicester by Djebari B showed that lipotechoic acid (LTA) from *S. aureus* is a novel binding partner for HP. However, the mechanism of binding is poorly understood. The aim of the work described in this Chapter is to characterise the interaction between the HP and LTA using recombinant mouse HP (mHP), produced as described in Chapter 3.

In this chapter I will address the following questions:

- What is the specificity of HP for LTA? Does HP bind to LTA from other Gram-positive bacteria (e.g. *S. pneumoniae*) in addition to LTA from *S. aureus*?
- Which fragment/s of LTA are required for binding to HP
- Which fragments (α or β or both) of HP are required for binding to LTA?

4.2 Results

4.2.1 Binding of recombinant mHP to LTA from different Gram-positive bacteria

LTA is present on the cell membrane of most Gram-positive bacteria. Having demonstrated that HP binds directly to staphylococcal LTA, it was naturally interesting to explore whether LTA from other Gram-positive bacteria does the same. Binding of recombinant mHP to purified LTAs from different Gram-positive bacteria was tested using an ELISA in which immobilised LTA was incubated with soluble HP. HP bound to LTAs from all five Gram-positive bacteria tested: *Staphylococcus aureus* (control), *Listeria monocytogenes*, *Bacillus subtilis*, *Streptococcus pneumoniae* and *Streptococcus pyogenes* as shown in Figure 4-2. A similar degree of binding was detected in each case. Subsequent analysis was focused on the interaction between HP and staphylococcal LTA.

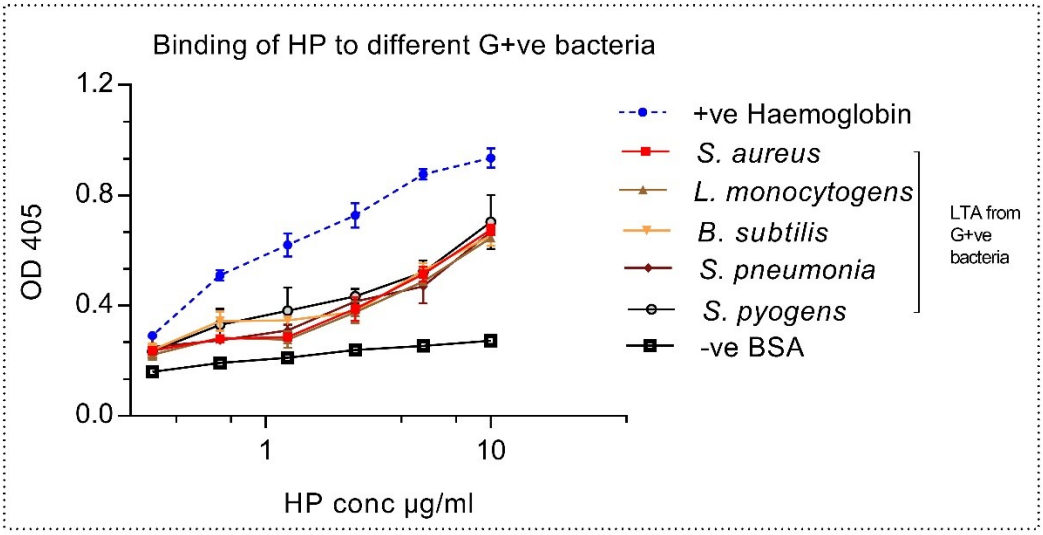

Figure 4-2: Recombinant mHP can bind to LTAs from different Gram-positive bacteria

ELISA plates were first coated with LTA and recombinant mHP was incubated for one hour at room temperature. Binding was detected using chicken polyclonal anti-HP antibody, followed by an alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. Wells were coated with Hb as a positive control and bovine serum albumin (BSA) as a negative control. The results are expressed as mean ± SEM from three independent experiments.

4.2.2 Localisation of the binding site of HP for LTA

Initially limited proteolysis was used to attempt to locate the binding site of LTA on HP. Unlike serum-derived material, recombinant mHP is not cleaved during biosynthesis, so is present as a single polypeptide of ~44 kDa. The CCP domain is ~9 kDa and the SP domain is ~33 kDa. The full-length haptoglobin dimer contains 9 disulphide bonds, a single disulphide within each CCP module, three in each SP domain and a single bond connecting the CCP module of one polypeptide with the CCP module of its partner.

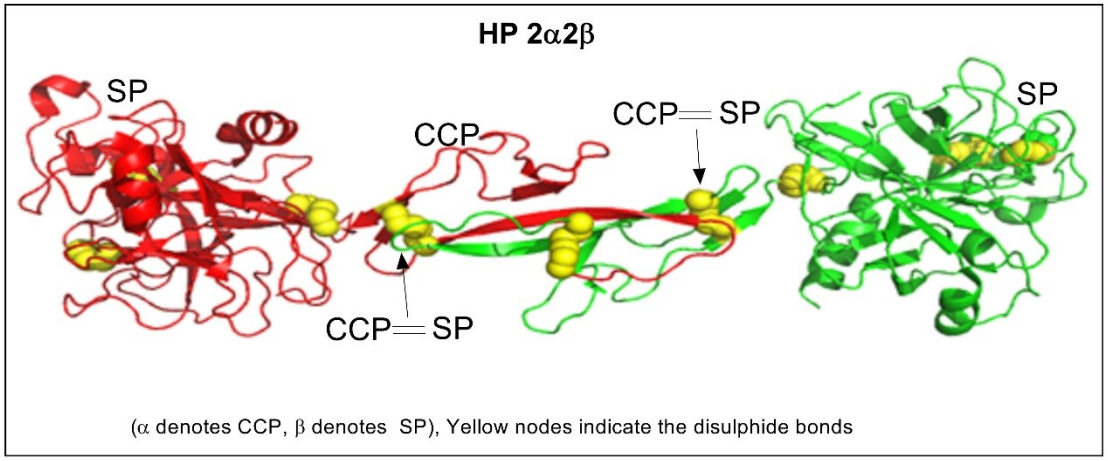

Figure 4-3: An illustration of HP structure

The figure illustrates the HP domains and the position of the disulphide bonds. The β-chain contains the SP domain and the α-chain the CCP domain.

Digestion of HP with trypsin was carried out by incubating a fixed amount of mHP (9µL at 3.2 mg/ml) with 1µL of trypsin which was serially diluted starting at 0.8 mg/ml (Trypsin: mHP, 0.08 µg: 28.8 µg w/w respectively). After incubation for 1 hour at 37^{o}C, samples were analysed by SDS-PAGE. Results showed that mHP is susceptible to trypsin treatment as can be seen in Figure 4-4. Multiple bands are observed on gels indicating that trypsin cleaves at multiple sites. A band is detected at ~30 kDa which probably corresponds to the intact SP domain. However this product is rapidly degraded into smaller fragments.

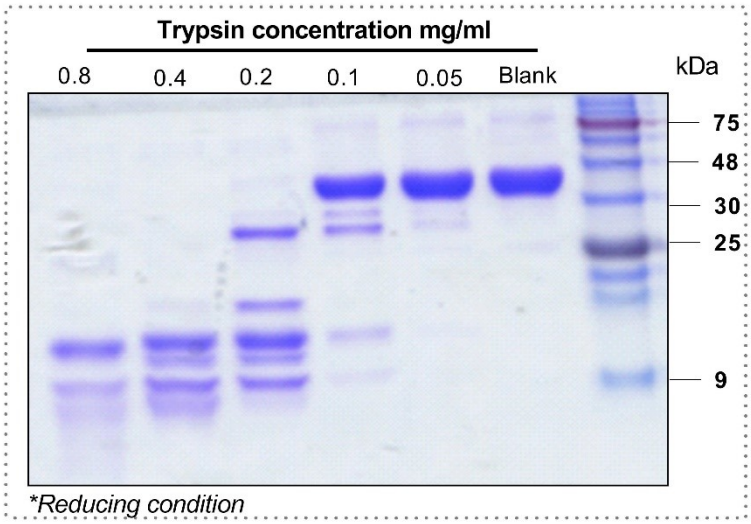

Figure 4-4: SDS polyacrylamide gel electrophoresis showing trypsin digestion of the recombinant mHP

Recombinant mHP was susceptible to trypsin digestion. Samples were treated with various concentration of trypsin starting at 0.8 mg/ml (Trypsin: mHP, 0.08 ug: 28.8 ug w/w respectively). Samples were run on %15 SDS-polyacrylamide gel under reduced stained by Coomassie blue stain.

Based on the results from the preliminary digests, I carried out a large scale trypsin digestion of mHP using 288 µg of mHP and 2 µg of Trypsin in a 100 µL reaction. The reaction mixture was incubated for 1 hour at 37ºC and stopped using a protease inhibitor Pefabloc® SC. (Roche). Subsequently, the protein fragments were separated by gel filtration on a Superdex 75 10/300 GL column (GE healthcare). Fractions were collected across the elution peaks and these were tested for LTA binding using an ELISA (see Figure 4-5A). Peaks were also analysed by SDS-PAGE and visualised by silver staining which is more sensitive than Coomassie blue stain (see Figure 4-5B).

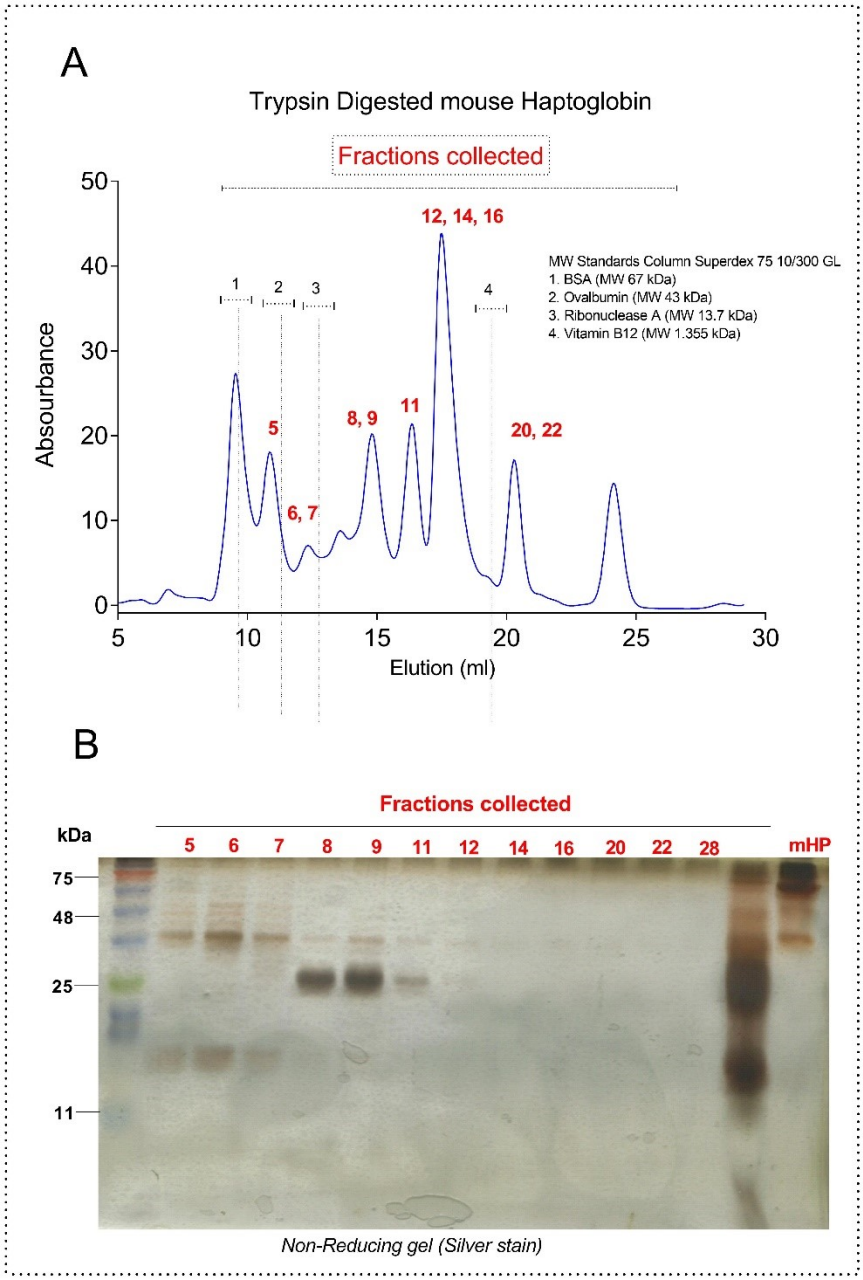

Figure 4-5: Separation of digested fragments by gel filtration column

Digested fragments were separated on a gel filtration column Superdex 75 10/300 GL**.** Graph **A** shows the elution profile of the trypsin-digested mHP. The MW standards for gel filtration column Superdex 75 10/300 GL are indicated as well. Graph **B** shows a 15% SDS-polyacrylamide gel of the collected fractions visualized using silver stain. The numbers correspond to peaks in A above.

Analysis by SDS-PAGE shows that fractions 5-7 contain appreciable amounts of undigested HP and fractions 5-11 consist largely of the ~30 kDa fragment. No fragments were detected in fractions 12-28. Because the concentration of fragments was low, the ELISA was modified so that the fragments were immobilised and binding to soluble LTA was measured. Surprisingly, all fractions displayed at least some LTA binding as shown in Figure 4-6. The result could indicate that there is a fragment and/or fragments in common in all fractions that binds to LTA or that there are multiple binding sites for LTA on HP. The latter possibility seems most likely particularly since LTA is a large polysaccharide with multiple sugar epitopes.

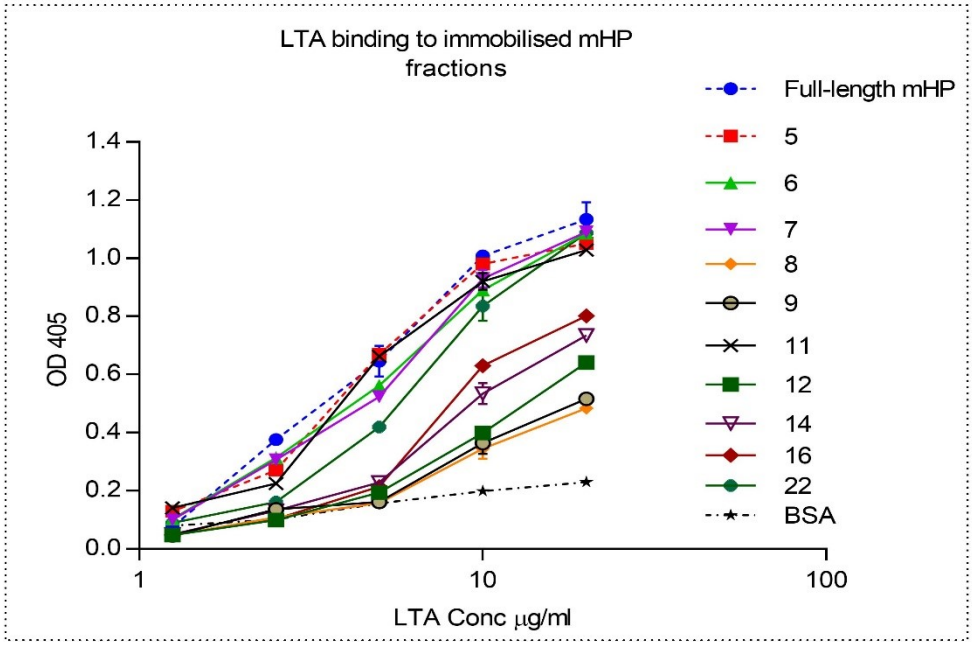

Figure 4-6: LTA binds to immobilised mHP digested fractions.

ELISA plates were coated with fractions from the gel filtration column (labelled as in Figure 4-5 above). Soluble LTA was incubated in the coated wells (numbers of the samples correspond to numbers in Figure (4-5) for one hour at 37°C. Binding was detected using anti-LTA monoclonal antibody produced in mouse, and followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG as a secondary antibody. BSA was used as a negative control. Results are expressed as means ± SEM from three separate experiments.

As an alternative strategy to identify the region of HP that binds to LTA, purified β-chain of human HP alone was used. This is a commercially available protein produced in *E. coli.* Binding to immobilised LTA was compared with binding by full-length human protein purified from serum. Some binding of the β-chain was detected but it was less than binding by full-length HP (see Figure 4-7). This result must be treated with caution, because only a proportion of anti-HP antibodies may recognise the β-chain. Nevertheless, it does show that the binding site for LTA is located at least in part on the β-chain. A similar experiment was carried out using whole *S. aureus.* This time, a monoclonal antibody was used to detect binding of HP or the β-chain, because the polyclonal antibody gave very high background binding. Similar results were seen with some binding by the β-chain but less than for full-length protein (see Figure 4-8). Overall, the results suggest that HP binds directly to LTA via its β-chain. Decreased binding compared to full-length protein may reflect 1) the difference in oligomeric state of full-length HP (dimer) compared to the β-chain (monomer), 2) that the α-chain of HP also binds to HP or 3) a difference in binding between the polyclonal antibody and full-length or truncated HP.

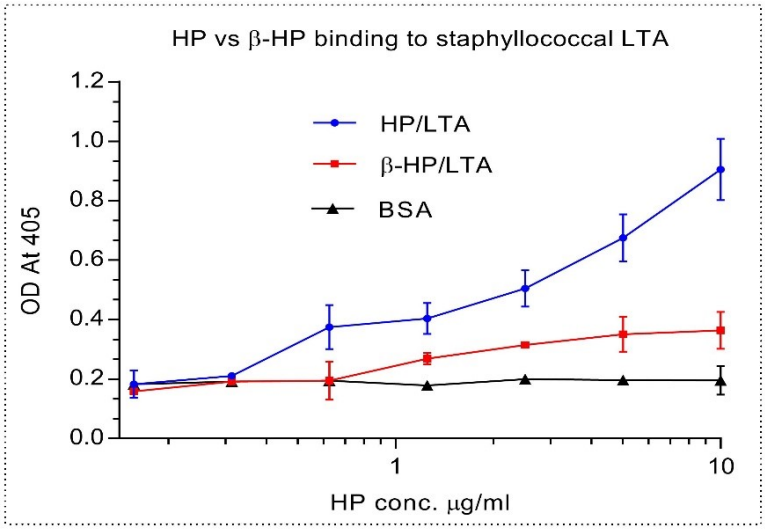

Figure 4-7: ELISA showing HP binding to LTA

ELISA plates were coated with LTA**.** Full-length HP or the β-chain were incubated with LTA-coated wells for one hour at room temperature (RT). Binding was detected using a polyclonal anti-HP antibody produced in chicken, and followed by alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. BSA was used as a negative control. Results are expressed as means ± SEM from four independent experiments.

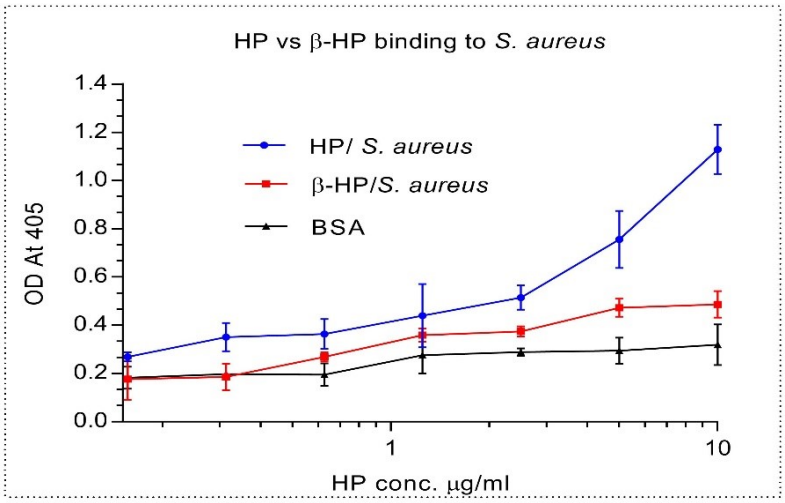

Figure 4-8: ELISA showing HP binding to *S. aureus*

ELISA plates were coated with *S. aureus***.** Full-length HP or the β-chain of HP were incubated with *S. aureus* coated wells for two hours at room temperature (RT). Binding was detected using a mouse monoclonal anti-HP antibody followed by alkaline phosphatase-conjugated goat anti-mouse antibody. BSA was used as a negative control. Results are expressed as means ± SEM from three independent experiments were done.

To overcome potential technical problems caused by anti-HP antibodies, HP or β-chain were immobilised and binding of LTA was measured using an anti-LTA antibody. The advantage of this approach is that the assay detects LTA rather than HP, so it is not dependent on how the antibody recognises its target.

Binding of LTA to immobilised human (hHP, Sigma), recombinant mouse HP (mHP) and the β-chain of the human HP (Randox) was therefore tested. The results demonstrate that LTA binds to both full-length human and mouse HP and the β-chain of hHP as shown in Figure 4-9**.** Similar binding was observed for all three proteins, suggesting that LTA binds predominantly to the β-chain of HP.

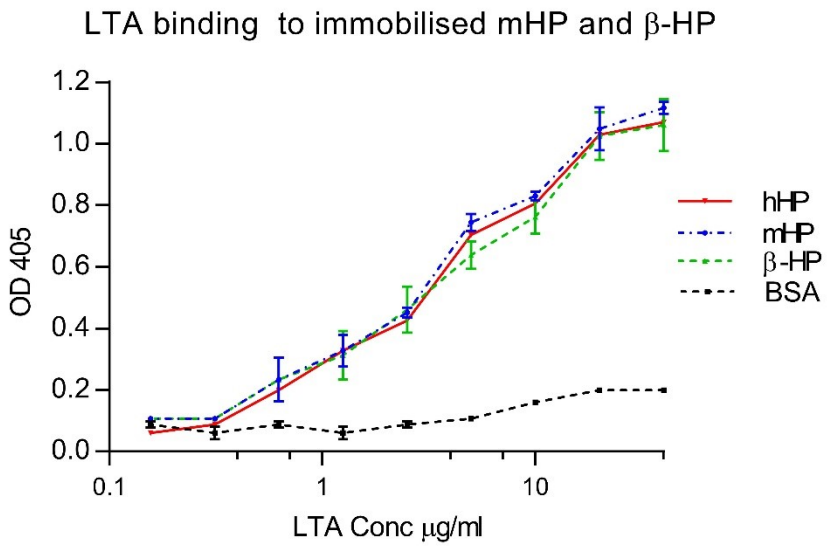

Figure 4-9: LTA binds to hHP, mHP and β-HP

ELISA plates were coated with mHP, hHP and β-chain of hHP. LTA was incubated with coated wells for one hour at 37ºC. Binding was detected using anti-LTA monoclonal antibody produced in mouse, and followed by alkaline phosphatase-conjugated rabbit anti-mouse IgG as a secondary antibody. BSA was used as a negative control. Results are expressed as means ± SEM from three independent experiments.

4.2.3 Affinity of binding between LTA and HP

4.2.3.1 Mobilised LTA Competes with Immobilised LTA and *S. aureus* for HP binding

To investigate binding between HP and LTA in more detail, a competition assay was developed. This was used to measure the binding affinity of HP to LTA and also to identify whether soluble LTA can inhibit HP binding to *S. aureus*. In this assay, increasing concentration of soluble LTA was used as a competitor for HP binding to immobilised LTA. Results showed inhibition of HP binding in a concentration dependent manner, i.e. as the concentration of soluble LTA was increased, less HP was bound to the immobilised LTA (see Figure 4-10)**.** The IC50 was 0.756 μg/ml from six independent experiments.

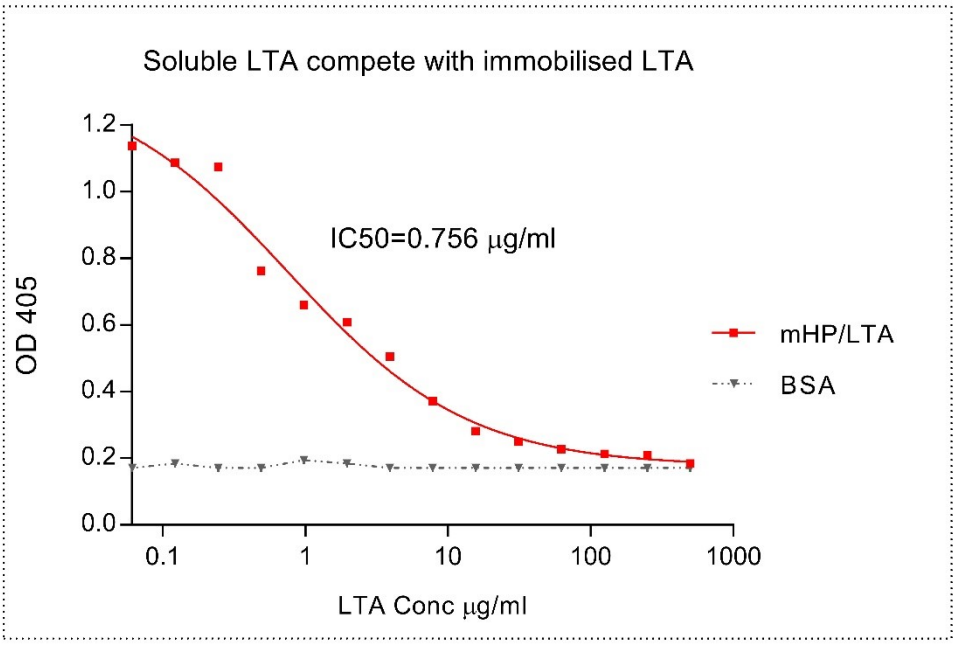

Figure 4-10: Soluble LTA competes with immobilised LTA for HP binding

ELISA plates were coated with LTA**.** A fixed amount of mHP (10 µg/ml) was mixed with increasing concentrations of soluble LTA and then the mixture was incubated with LTA-coated wells for one hour at room temperature (RT). Binding was detected using a chicken polyclonal anti-HP antibody and followed by goat anti-chicken alkaline phosphatase-conjugated IgG as a secondary antibody. HP only (blue line) indicates the positive control where no LTA was added to the mixture. BSA was used as a negative control. Results are expressed as means ± SEM from six independent experiments.

To see if soluble LTA could compete with whole *S. aureus* for binding to HP, the competition assay was conducted with immobilised *S. aureus* and soluble LTA. Soluble LTA inhibited the binding of HP to *S. aureus* bacteria as shown in Figure 4-11. The IC50 was ~22 µg/ml, approximately 20-fold higher than before. Thus, LTA blocks binding of HP to *S. aureus*, indicating it binds to the same site or an overlapping site on the HP molecule.

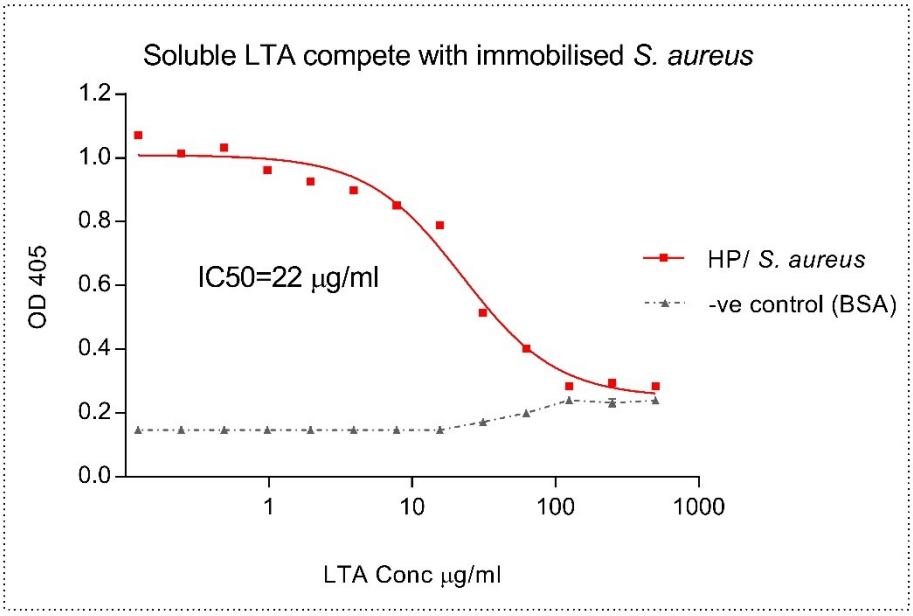

Figure 4-11: Soluble LTA competes with immobilised *S. aureus* for HP binding

ELISA plates were coated with *S. aureus*. A fixed concentration of mHP (10 µg/ml) was mixed with increasing concentration of soluble LTA and then the mixture was incubated with the *S. aureus* coated wells for two hours at 37°C. Binding was detected using mouse monoclonal anti-HP antibody, and followed by alkaline phosphatase-conjugated goat anti-mouse IgG as a secondary antibody. BSA was used as a negative control. Results are expressed as means ± SEM from three independent experiments.

4.2.4 Competition experiments with other ligands

Since the β-chain of HP binds to Hb as well as LTA, the HP/Hb interaction was used as a platform to characterise the interaction with LTA. Initially I confirmed that HP binds to Hb using a simple ELISA (see Figure 4-12). As expected HP bound to immobilised Hb.

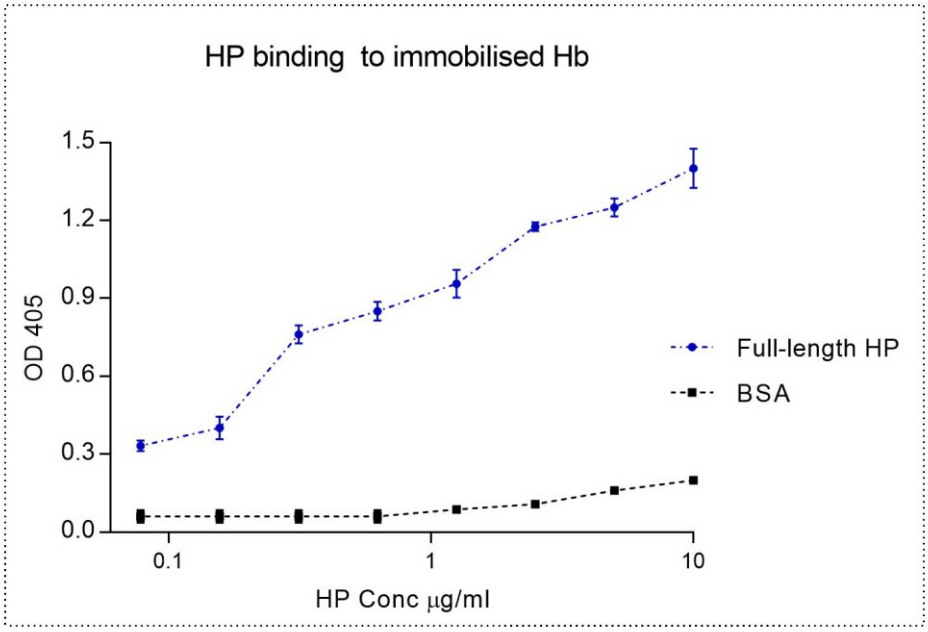

Figure 4-12: mHP binding to Hb

ELISA plates were coated with Hb. mHP was incubated with Hb coated wells for one hour at RT. Binding was detected using anti-HP polyclonal antibody produced in chicken, and followed by alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. BSA was used as a negative control. Four independent experiments were done and results are expressed as mean ± SEM.

I then used the competition assay using soluble LTA as a competitor for binding of HP to Hb. Significant inhibition of HP/Hb binding was detected in a concentration dependent manner (see Figure 4-13). As the LTA concentration increases, less HP binds to the immobilised Hb. Hb is known to bind exclusively to the β-subunit of HP (Andersen *et al*, 2012). Therefore, these data are consistent with LTA also binding to the β-subunit as shown earlier in Figure 4-9 and indicate that the LTA and Hb-binding sites overlap on the HP molecule. The IC50 was ~3.5 µg/ml, broadly similar to that measured before.

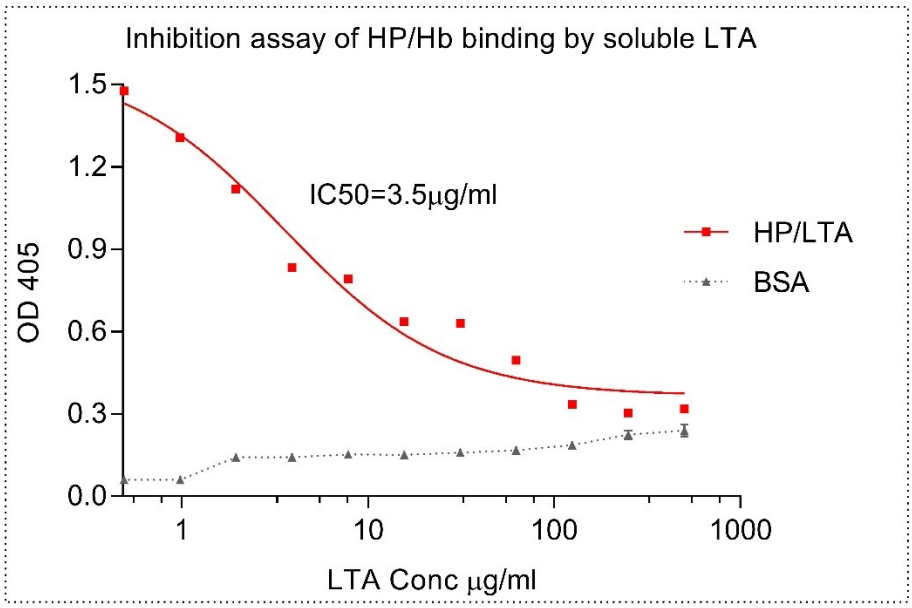

Figure 4-13: Soluble LTA competes with immobilised Hb for HP binding

ELISA plates were coated with Hb. A fixed concentration of mHP was mixed with increasing concentrations of soluble LTA and the mixture was incubated with Hb coated wells for one hour at room temperature (RT). Binding was detected using a chicken polyclonal anti-HP antibody, and followed by an alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. BSA was used as a negative control. Three independent experiments were carried out and results are expressed as means ± SEM.

4.2.4.1 Mobilised Hb competes with LTA and *S. aureus* for HP binding

A similar assay was carried out but using Hb as the competitor for HP binding to immobilised LTA. As shown in Figure 4-14, Hb competed with LTA for mHP binding in a concentration dependent manner (IC50 ~ 13 µg/ml). Hb also inhibited the binding of HP to the immobilised *S. aureus* as shown in Figure 4-15**,** although a somewhat higher concentration of Hb was required for inhibition (IC50 ~ 34 µg/ml). Hb

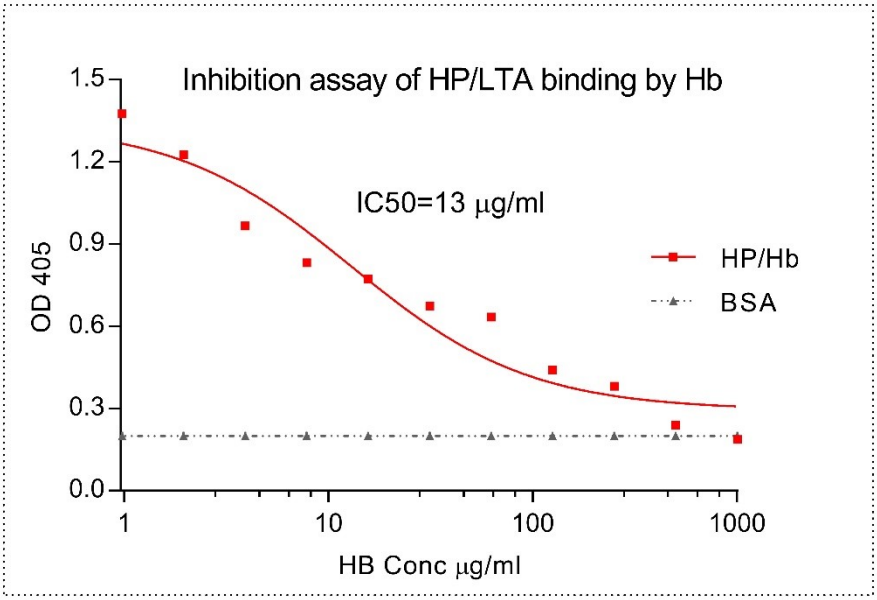

Figure 4-14: Hb competes with immobilised LTA for HP binding

ELISA plates were coated with LTA**.** A fixed concentration of mHP (10 µg/ml) was mixed with increasing concentrations of Hb and then the mixture was incubated with Hb coated wells for one hour at room temperature (RT). Binding was detected using chicken polyclonal anti-HP antibody, and followed by alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. BSA was used as a negative control. Results are expressed as means ± SEM from three independent experiments were done.

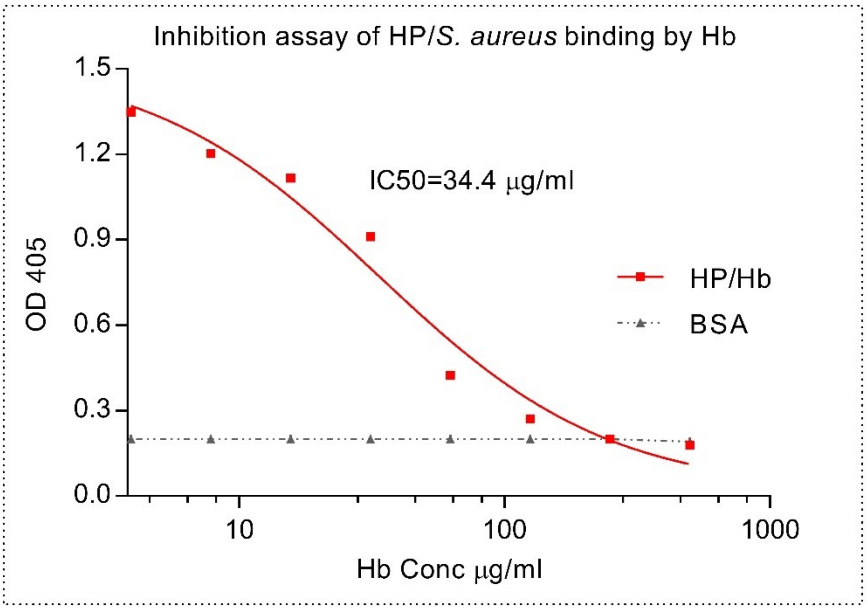

Figure 4-15: Hb competes with immobilised *S. aureus* for HP binding

ELISA plates were coated with *S. aureus*. A fixed amount of HP (10 μg/ml) was mixed with increasing concentration of Hb and then the mixture was incubated with *S. aureus* coated wells for two hours at 37°C. Binding was detected using a mouse monoclonal anti-HP antibody, and followed by an alkaline phosphatase-conjugated goat anti-mouse IgG. BSA was the negative control. Two independent experiments were done and results are expressed as means ± SEM.

4.2.4.2 Structural Aspects of the LTA/HP interactions

To identify the part of LTA that binds to HP a series of competition experiments were carried out using components of LTA as soluble ligands. Five types of LTA have been reported (Percy and Gründling, 2014) and LTAs from *S. aureus* and *S. pneumoniae* belong to type I and type IV respectively. Type I LTA is well described and comprises glycerophosphate, D.alanine, a glycosyl group, GlcNac, gentiobiose and fatty acids (Percy and Gründling, 2014). The inhibitory effects of glycerophosphate, D.alanine, GlcNac and gentiobiose were tested on LTA/HP binding. None of these components showed any inhibition on LTA for HP binding at concentrations up to 5 mM. This suggests that binding probably involves multiple components of LTA (see Figure 4-16).

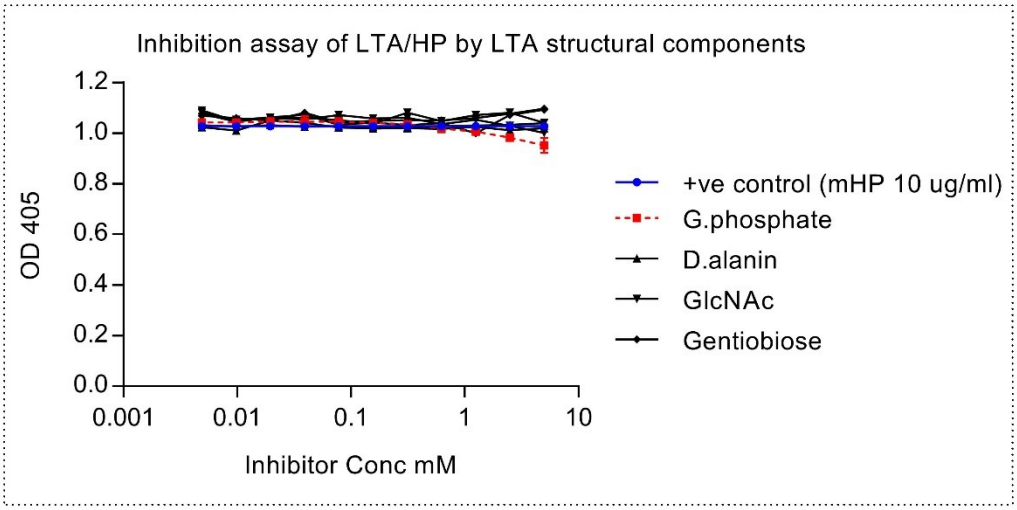

Figure 4-16: Inhibition assay of LTA/HP by LTA components

ELISA plates were coated with LTA**.** A fixed concentration of mHP (10 µg/ml) was mixed with increasing concentrations of an LTA fragment (glycerophosphate or D.alanine or GlcNac or gentiobiose) and then the mixture was incubated with LTA coated wells for one hour at room temperature (RT). Binding was detected using chicken polyclonal anti-HP antibody, and followed by alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. BSA was used as a negative control. Results are expressed as means ± SEM from three independent experiments were done.

4.3 Discussion

The results described here show that LTA binds to the β-chain of HP. The IC50 of the interaction is ~3.5 µg /ml. This interaction was confirmed using different approaches including binding and competition experiments.

We initially attempted to use trypsin digestion to isolate a LTA-binding fragment from HP. However, all of the fragments bound to LTA to some extent (Figure 4-6). This result could indicate that there is a fragment and/or fragments in common in all fractions that bind to LTA or that there are multiple binding sites for LTA on HP. However, the analysis was complicated because there are multiple disulphide bonds in HP and because HP is a dimer, making it particularly difficult to isolate single fragments. Therefore, it was not possible to make clear conclusions from this experiment.

Subsequent analysis was focused on the interaction between HP and staphylococcal LTA. Binding of commercially available full-length human HP (Sigma) and the β-subunit of human HP (Randox) to both immobilised LTA and *S. aureus* was compared. Results indicate a clear specific binding of the full-length HP but much weaker binding of the β-subunit of HP (Figure 4-7). These observed binding could possibly be as a result of the anti-haptoglobin antibody because the β-HP was not recognised as well as the full-length HP. Consequently further investigation testing the LTA binding to the immobilised full-length and the β-subunit of HP was important. Interestingly, results demonstrated that LTA bound to both the immobilised full-length and the β-subunit of HP (Figure 4-9), suggesting that β-HP is interacting with LTA.

The results show that LTA binds to the β-chain of HP. Previous analysis has shown that Hb also binds to the β-chain of HP and the results described here indicate that the binding sites are overlapping. Recently the crystal structure of the human Hb-HP complex has been determined (the original structure was of porcine HP) (Andersen *et al*, 2012). The β-chain (SP domain) of HP interacts with both the α- and β-chains of haemoglobin, via contacts involving loops in the HP (see Figure 4-17). The total buried surface of HP comprises 780 $Å^2$ (23 residues at the interface) with the α-chain of Hb and 754 $Å^2$ with the β-chain (21 residues at the interface). LTA bound to most of the fragments of HP generated by trypsin digestion (Figure 4-6), so LTA might also interact with the α-chain to some extent, but most of the binding is via the β-chain.

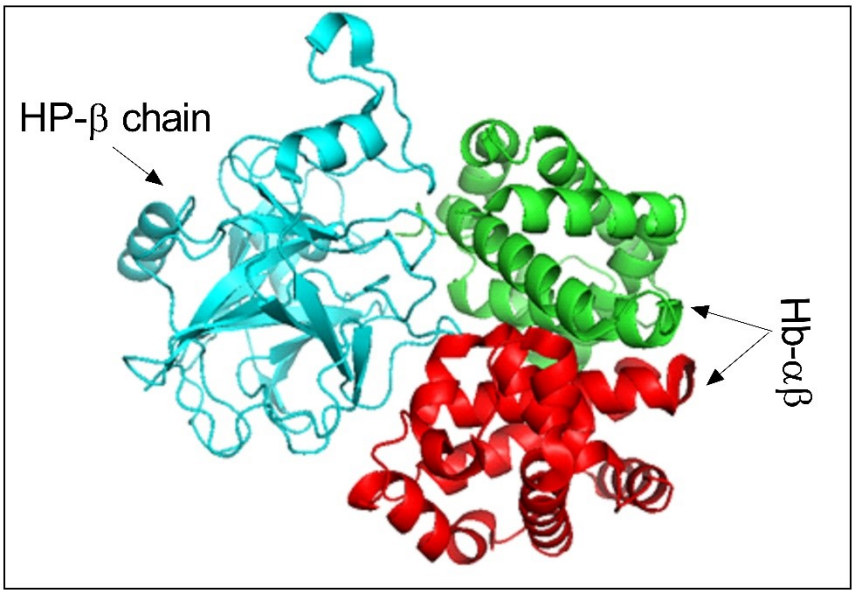

Figure 4-17: The interaction of HP chain with Hb chains
This figure illustrates the structural interaction between the the β-chains of HP with the α- and β-chains of Hb (Andersen *et al*, 2012).

It has also been reported that HP has the ability to bind *S. aureus* via a protein called staphylococcal surface protein HarA (Dryla *et al*, 2003). However, the finding that soluble LTA competes for binding of HP to *S. aureus,* suggests that HP is binding predominantly to LTA.

The data also show that HP binds to LTA from a wide variety of bacteria, including *Streptococcus pneumoniae, Streptococcus pyogenes, Bacillus subtilis* and *Listeria monocytogenes*. HP binding to LTAs from these bacteria was remarkably similar to that observed with staphylococcal LTA. LTA is categorised into 5 types I-V based on the structure of the hydrophilic backbone as discussed in Chapter 1 (Percy & Grandling 2014). In our study, most of the LTA tested was type I, except LTA from *S. pneumoniae* which belongs to type IV. As in Figure 4-18 below, both type I and type IV LTAs contain common features including GlcNac, glycerophosphate, D-alanine and a glycolipid anchor (Percy & Grandling 2014), so it is likely that HP is binding to similar structures on the LTAs from different species. No individual component inhibited binding of LTA to HP (glycerophosphate, D-alanine, GlcNac or gentiobiose) suggesting that HP binds to multiple components of the LTA (Figure 4-16). The glycolipid anchor can probably be excluded as a ligand for HP because it is not accessible unless the LTA is released from the bacteria. Binding of HP to whole *S. aureus*, thus excludes this possibility. It is possible that synthetic LTA may help in identifying binding epitopes. However, previous study on LTA/ficolin interactions conducted in our laboratory by Dr Lynch showed that none of the synthetic LTA was able activate complement.

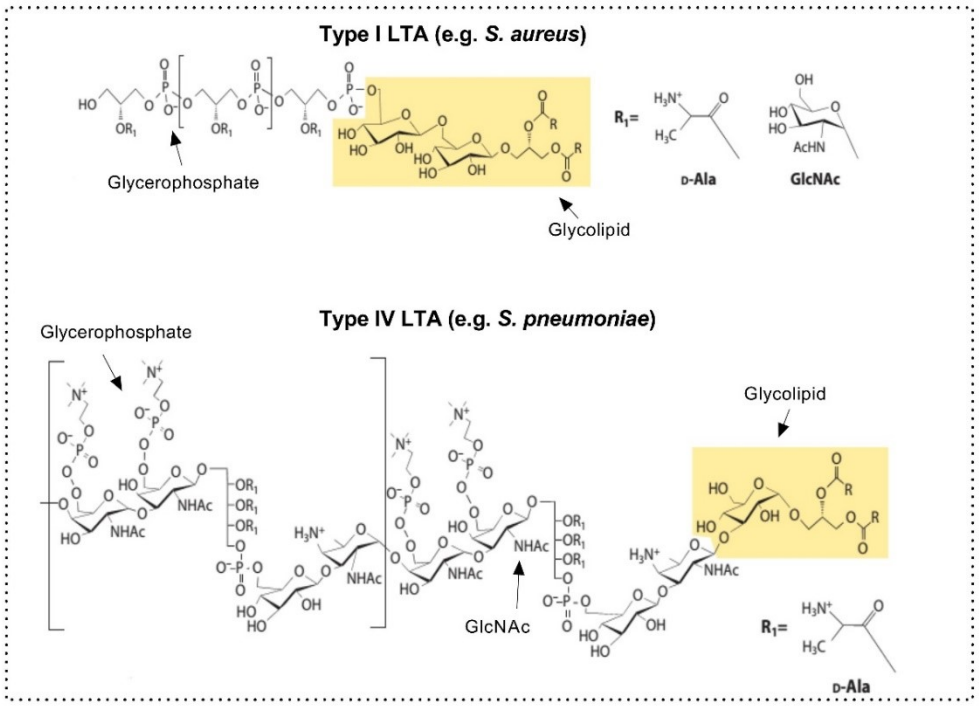

Figure 4-18: A diagram illustrating structural components of LTA Type I and Type IV.

Type I and type IV LTAs similar structural features such as GlcNAc, glycerophosphate, D-alanine and a glycolipid anchor. Picture has been modified from (Percy & Grandling 2014).

4.3.1 The Interaction between HP, Hb and LTA

The well-known partner for HP is Hb. HP and Hb, form a complex which is subsequently cleared from circulation by the endocytosis. HP interacts with Hb via the β-subunit of HP (Andersen *et al*, 2012) and LTA inhibits this process in a concentration dependent manner (Figure 4-13). This competition might be relevant in pneumococcal infections in particular (see Chapter 5), because pneumolysin (the toxin produced by pneumococcus) causes significant haemolysis. In this case, there would be appreciable amounts of Hb, HP and LTA in serum and the free Hb may prevent any protective effect mediated via HP.

Chapter 5

Assessment of the Role of HP Using *in vivo* Mouse Models of Infection

5.1 Introduction

My previous work has shown that HP binds to LTA from a variety of Gram-positive bacteria. The aim of this chapter is to examine the role of HP in the immune system *in vivo*. Previous work in our laboratory has shown that HP facilitates phagocytosis by macrophages and neutrophils, suggesting that it has a possible protective role in infection. To assess the role of HP during infection, I chose two disease models: *S. aureus* and *S. pneumoniae.* These were selected because they are serious pathogens globally, causing fatal diseases in both children and adults (Woodford and Livermore, 2009). *S. aureus*, for instance, causes diseases ranging from minor skin infections to life-threatening conditions including toxic shock syndrome and bacteraemia (von Köckritz-Blickwede *et al*, 2008). There are multi-drug resistant forms of *S. aureus* such as MRSA and VRSA. Thus, *S. aureus* has very limited therapeutic options in severe infections (von Köckritz-Blickwede *et al*, 2008). *S. pneumoniae* is the leading cause of bacterial pneumonia as well as causing other invasive life-threatening diseases, such as meningitis and septicaemia (Kadioglu and Andrew, 2012). There are well-established models of infection of *S. pneumoniae* in mice. These reasons prompted us to select these two models and examine the possible role of HP in the immune response to Gram-positive infections *in vivo*.

To assess the effects of HP in the immune response, I used C57BL/6J HP-deficient mice and C57BL/6J wild type mice as a control. Previous studies on C57BL/6J HP-deficient mice confirmed the important role of HP in the immune system at various levels of immune function and cell development. For example, a study investigating the effect of

acute haemolysis showed increased renal susceptibility of HP-deficient mice compared to C57BL/6J wild type mice (Lim *et al*, 1998). Another study conducted to assess the anti-inflammatory and immune-modulatory activities of HP demonstrated that the absence of HP is associated with reduced T and B cell responses, suggesting a regulatory effect of HP on adaptive immunity (Huntoon *et al*, 2008). Moreover, a recent animal study revealed that HP can enhance the acute transplant rejection of skin graft in mice. This role occurs through the initiation of MyD88-dependent inflammatory process. MyD88 is known to induce the inflammatory mediators (e.g. TNF-α and IL-6) as well as dendritic cells maturation (Shen *et al*, 2012). Therefore, the development of C57BL/6J HP-deficient mice provided a better understanding of the relevant role of HP during the immune response. However, to date, nobody has studied the role of haptoglobin in the immune response to bacterial infections.

To assess the severity of disease in the infected animals, I measured the role of HP via infection studies using C57BL/6J HP-deficient mice in comparison to their wild type littermates. I also measured bacterial counts in the blood and certain organs by determining the numbers of colony forming units (CFUs) in infected mice. These measurements assess the capacity of mice to control bacterial growth.

5.2 Results

5.2.1 Genotyping of HP-knockout mice

I used HP knockout (HP $^{-/-}$) mice to assess the role of HP in the immune response to Gram-positive infections *in vivo*. Dr Heinze Baumann (Russell Park institute, NY, USA) kindly provided sperm of C57BL/6J HP-deficient mice, which was imported to the University of Leicester Biomedical services facility. The sperms were then used to re-derive the mouse line in a specific pathogen-free environment. The re-derivation of the mouse line using C57BL/6J wild type oocytes produced the first generation of heterozygous HP$^{+/-}$ mice. Mice were then intercrossed to produce homozygous HP $^{-/-}$ mice.

The HP gene in mouse is located on chromosome 8 (Yang *et al*, 1993). It is comprised of 5 exons. In C57BL/6J HP $^{-/-}$ mice used in our study, exons 2-4 were replaced with a neomycin resistance cassette (Lim *et al*, 1998). Genotyping by PCR confirmed the homozygous presence of the gene disruption in the targeted allele. A multiplex PCR shown in Table 5-1 below was used to amplify PCR products. A forward and reverse primer detect the wild type allele after exon 1 and amplify a band of 700 bp. The forward primer and a neomycin specific reverse primer amplify the disrupted allele of HP $^{-/-}$ carrying the neomycin cassette, giving a band of 300 bp. Heterozygotes could easily be distinguished from homozygotes as shown in Figure 5-1.

Table 5-1: Primers used for mouse PCR genotyping

Primer	Sequence -5'-3
F_HP-WT	5' TCT ACG GGG AGA GGT GAG AA 3'
R_ HP-WT	5' CTG GGT GTG CAC CAT CAT AC 3'
R_Null-HP	5' GGG GGA ACT TCC TGA CTA GG 3'

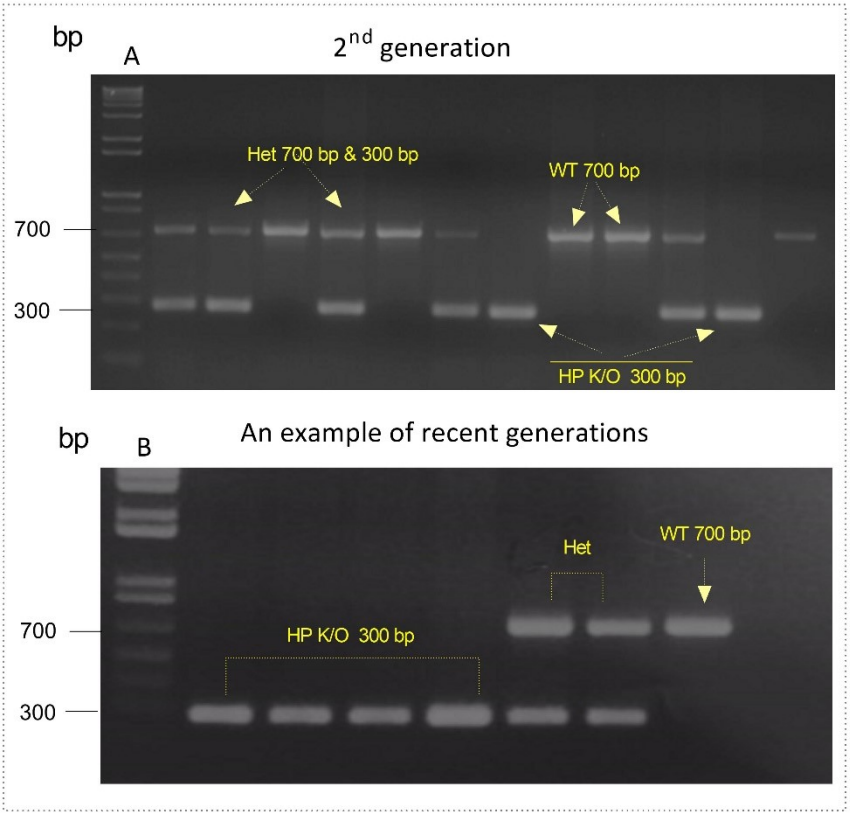

Figure 5-1: Agarose gel electrophoresis of PCR amplification showing screening for HP-deficient mice

Ploymerase chain reaction (PCR) of purified DNA from ear snips taken from breading mice. Samples of HP-deficient mice show bands of approximately 300 bp while wild-type mice show bands of 700 bp. Heterozygous (Het) mice show bands of both 300 bp and 700 bp. Samples of the second generation are shown in graph (A), while graph B shows an example following generations.

5.2.2 *S. aureus* mouse infection model

Human isolate strains belonging to *S. aureus* were tested to establish a mouse model of staphylococcal infection which included *S. aureus* Newman (Kindly provided by Dr Vitor Fernandes, University of Leicester), *S. aureus* SH1000 and *S. aureus* EMRSA-16 (Kindly provided by Dr Julie Morriessy, University of Leicester).

5.2.2.1 *S. aureus* mouse infection model using HP knockout mice

A number of attempts were made to establish an infectious model of *S. aureus*, using different strains. Based on studies done on C57BL/6J wild type mice using Newman strain of *S. aureus* (Rauch *et al*, 2012; Bubeck Wardenburg, Patel and Schneewind, 2007), initially *S. aureus* Newman was tested on C57BL/6J wild type mice through the intraperitoneal route (IP) to determine the optimal infective dose. For each strain, three groups of mice were challenged with three different inoculums of 5 x 10^7 CFU, 1 x 10^8 CFU and 4 x 10^8 CFU of *S. aureus* Newman. In contrast to our expectations, all mice resisted the infection. Even though mice infected with the higher dose of 4 x 10^8 CFU showed signs of disease of disease, they recovered within 10 hours. In another attempt to establish this model of infection, I selected more virulent human isolate strains of *S. aureus* (*S. aureus* SH1000 and *S. aureus* EMRSA). Mice were IP injected with 1 x 10^8 CFU and 4 x 10^8 CFU of *S. aureus* SH1000 and EMRSA. Similar to our previous observation with the Newman *S. aureus*, mice showed remarkable resistance to infection. The severity of infection corresponding with each dose is indicated in Figure 5-2.

I therefore attempted the intranasal route as another approach to establish the infection. For each strain of *S. aureus* (Newman, SH1000 and EMRSA-16), two groups of mice were challenged with two different inoculums of 1 x 10^8 CFU and 4 x 10^8 CFU of *S. aureus* Newman. While mice inoculated with 1 x 10^8 CFU showed moderate signs of disease, severe signs of disease were observed in mice challenged with 4 x 10^8 CFU. However, both groups recovered from infections. The severity limit of the project license is ++lethargic. Therefore, in order not to breach this regulation, it was not possible to increase the dose because the mice had already shown signs of disease reaching +lethargic status.

To assess the role of HP, mice were inoculated with 4 x 10^8 CFU of *S. aureus* Newman. HP-deficient mice were compared with C57BL/6J wild type mice. Unexpectedly, although both sets of mice showed signs of disease as before, they were resistant to *S. aureus* infection and all mice survived (see Figure 5-4). All mice were monitored regularly by measuring the average illness score. Both groups of the C57BL/6J HP-deficient and wild type mice showed signs of disease of acute infection within the first 6 to 12 hours as shown in Figure 5-3, but all mice managed to clear the bacteria and recovered from infection within 20-24 hours. Because there were severe signs of disease, it was important to measure the blood CFUs of the C57BL/6J HP-deficient and wild type mice. Presence of bacteraemia was observed within the first 6 to 12 hours in both groups of HP deficient mice and their wild type littermates. However, all mice cleared the bacteria 18 hours after infection (see Figure 5-5).

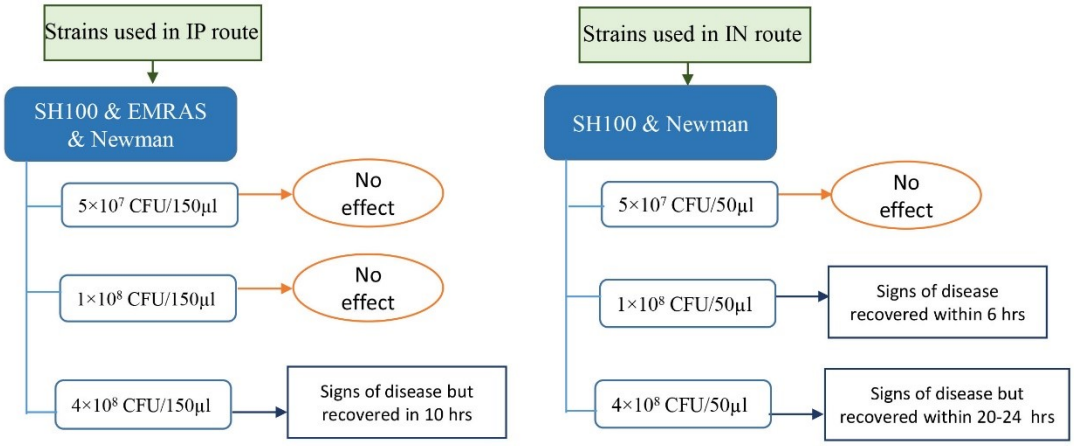

Figure 5-2: A diagram illustrating *S. aureus* infection models trials

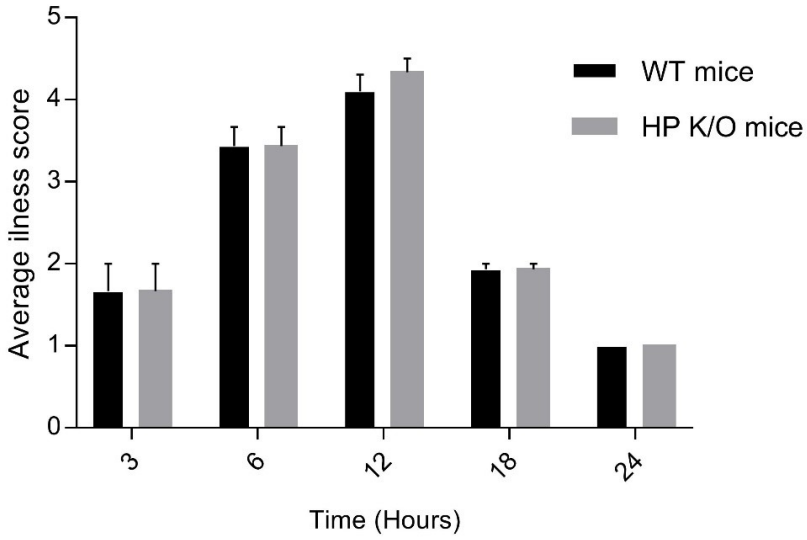

Figure 5-3: Average illness score of the *S. aureus* infected mice

Severity of the disease were compared between wild-type mice and HP-deficient mice following I.N. injection with $4x10^8$ CFU of *S. aureus* (Newman).
Score=0 “normal”, Score=1 “hunched +”, Score=1-2 “hunched ++”, Score=3 “Starry coat +”, Score= 3-4“Starry coat ++”, Score=4-5 “Lethargic +”, Score=5 “Lethargic ++”.

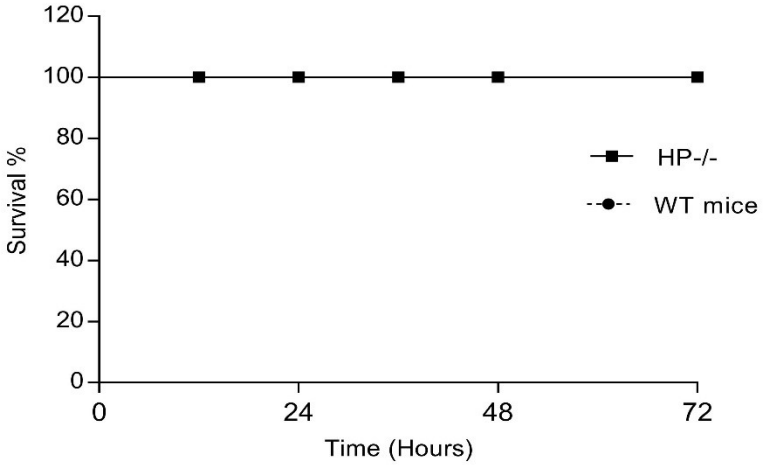

Figure 5-4: Comparison between HP-deficient mice and their wild-type littermates post intranasal infection with of *S. aureus* (Newman)

HP-deficient mice and their wild-type littermates (9-12 weeks old) were challenged with $4x10^8$ CFU of *S. aureus* (Newman). Both resisted the infection and showed same observations.

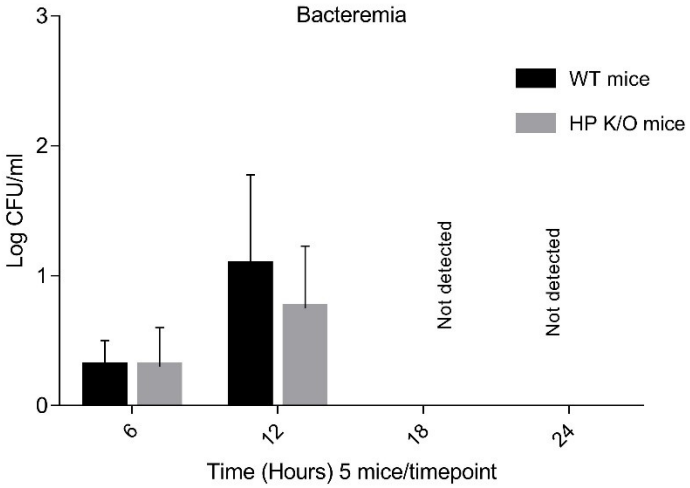

Figure 5-5: Bacterial load in blood of HP-deficient mice and their wild-type littermates post intranasal infection with of *S. aureus*

CFU counts in blood for HP deficient mice and their wild-type littermates post intranasal infection with $4x10^8$ CFU of *S. aureus* (Newman). Results are means (means±SEM). n=5 at times, 6, 12, 18 and 24 hours for both mice groups.

5.2.2.2 Viable bacterial burden in lung, kidney, liver and spleen post intranasal infection with *S. aureus* (Newman)

Two age-matched groups of the wild type and HP-deficient mice were infected with $4x10^8$ CFU of *S. aureus* via the intranasal route and mice were euthanised at different times post infection 6, 12, 18 and 24 hours to collect lung, kidney, liver and spleen to assess the bacterial load.

Results showed slightly higher bacterial load in wild type mice controls than the HP-deficient mice. When analysing the data of the bacterial viable count in the lung tissue of both groups, there was a significant increase in the bacterial count at 18 hours of the wild type mice in comparison to the HP-deficient mice as illustrated in Figure 5-6-A. In contrast, statistical analysis revealed no significant difference in the bacterial burden between the two groups in kidneys, livers and spleens (see Figure 5-6-B, C and D).

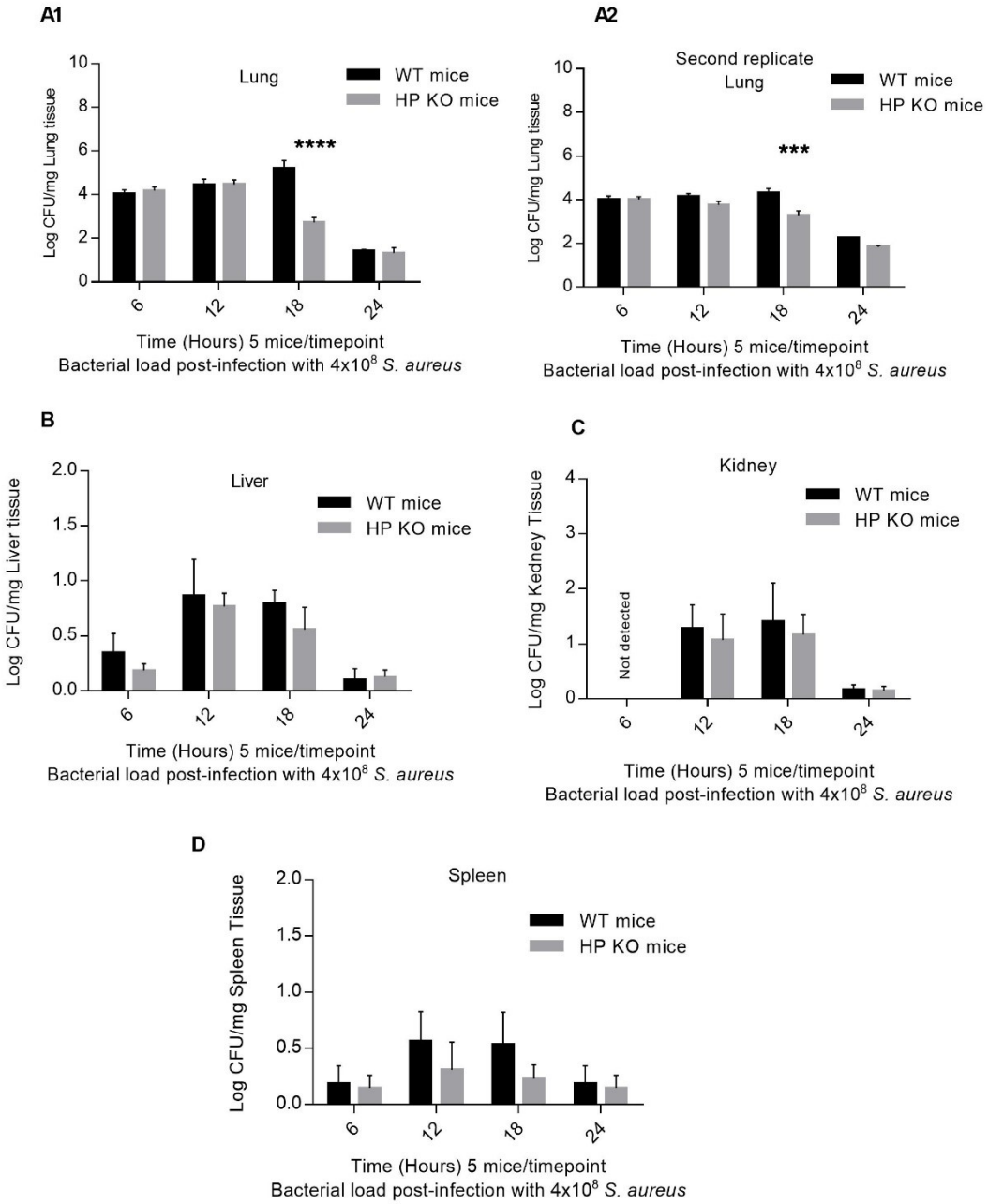

Figure 5-6: Bacterial load in organs of HP deficient mice and their wild-type littermates post intranasal infection with of *S. aureus*

CFU counts of lung (A), kidney (B), liver (C) and spleen (D) for HP deficient mice and their wild-type littermates post intranasal infection with $4x10^8$ CFU of *S. aureus* (Newman). Results are expressed as means (means ± SEM). n=5 at times, 6, 12, 18 and 24 hours for both mice groups. A 2Way ANOVA multiple comparisons test was used to analyse the data. CFU counts in lungs as shown in graph A at 18 hours showed significant difference between the two groups, (A1=****P<0.0001, A2=***P<0.0004).

In conclusion, the C57BL/6J mouse strain (which is the strain of our HP-deficient mice) was resistance to *S. aureus* at the highest possible titre tested. Very little difference was observed in parameters tested. In the second model system, I used *S. pneumoniae*. *S. pneumoniae* research is well established in our department.

5.2.3 *S. pneumoniae* (D39) infection model

5.2.3.1 HP-knockout mice are protective against intranasal infection with *S. pneumoniae* (D39)

In these experiments, *S. pneumoniae* (serotype D39) was utilised as a tool to assess the role of HP in the immune system. Female $HP^{-/-}$ mice and their wild-type littermate controls were challenged with an intranasal infection of (5×10^5 CFU/mouse) *S. pneumoniae* (D39). Mice were monitored for 5 days. Surprisingly, the experiment showed HP-deficient mice to be more protected than wild type mice, with only around 10% susceptibility of the HP-deficient mice to infection, compared to 60% of the wild type mice as demonstrated in Figure 5-7.

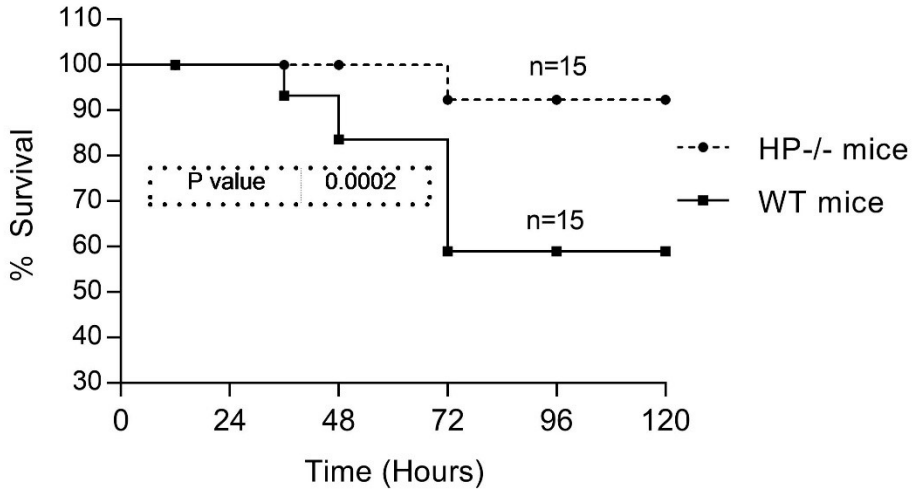

Figure 5-7: Comparison between HP-deficient mice and their wild-type littermates post intranasal infection with of *S. pneumoniae* (D39)

Female HP deficient mice and their wild-type littermates (9-12 weeks old) post intranasal infection with $5x10^5$ CFU of *S. pneumoniae* (D39). By GraphPad Prism, statistical analysis using Mantel-Cox test revealed significant difference between the two groups (***P<0.0002).

Events of humanely culling ill mice once the severity limit was reached (moderate signs of disease in line with Home Office Regulation, ++lethargic stage) was also analysed. While moderate signs of disease of the $HP^{-/-}$ mice were delayed up to 72 hours with approximately10% susceptibility, the first mouse of the wild type ones had to be euthanised at 36 hours. A significant number of the wild type mice were also humanely culled when progressed to ++lethargic stage at 48, 60, and 72 hours reaching a total percentage of 60%. When monitoring the health of mice post infection, there was clearly significant difference between the two groups, in line with the infection study results. As in Figure 5-8, HP-deficient mice showed a strong ability to withstand the infection when

compared to their wild type littermates, which progressed earlier to moderate signs of disease.

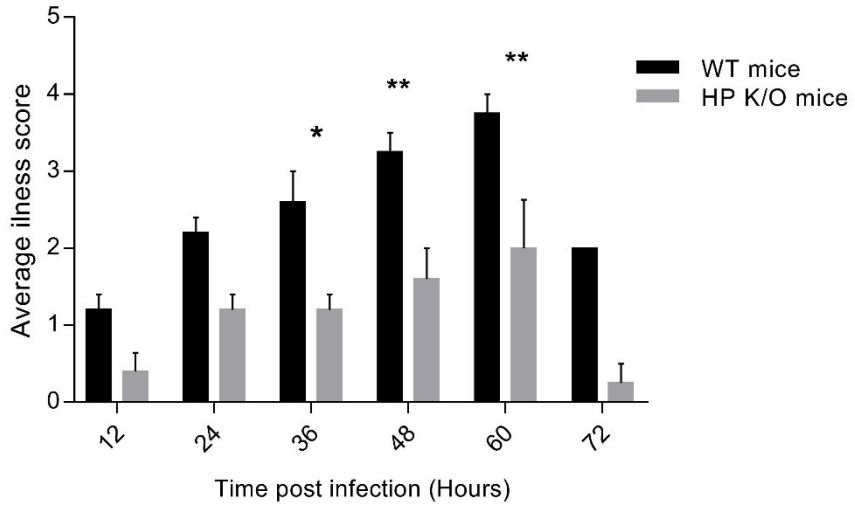

Figure 5-8: Average illness score of the *S. pneumoniae* infected mice

Severity of the disease were compared between wild-type mice and HP deficient mice following I.N. injection with with $5x10^5$ CFU of *S. pneumoniae* (D39). A 2Way ANOVA multiple comparisons test was used to analyse the data. Significant difference between groups at 36 hrs (*P<0.02), 48 hrs (**P<0.0083), 60 hrs (**P<0.0045).
Score=0 "normal", Score=1 "hunched +", Score=1-2 "hunched ++", Score=3 "Starry coat +", Score= 3-4"Starry coat ++", Score=4-5 "Lethargic +", Score=5 "Lethargic ++".

5.2.3.2 Viable bacterial burden in blood post intranasal infection with *S. pneumoniae* (D39)

Bacterial viable count was analysed in the blood for two age-matched female groups of HP-deficient mice and wild type mice at 24, 48, and 72 hours post infection. Both groups showed bacteraemia after 24 hour with slightly higher (but not statistically significant) CFUs in the $HP^{-/-}$ group. However, at 48 hours onwards HP-deficient mice started clearing the bacteria from the blood while the wild type ones showed a significant

increase in the bacterial load (see Figure 5-9). This result is consistent with the infection study that showed significant susceptibility of the wild type mice to pneumococcus infection as previously illustrated in Figure 5-7.

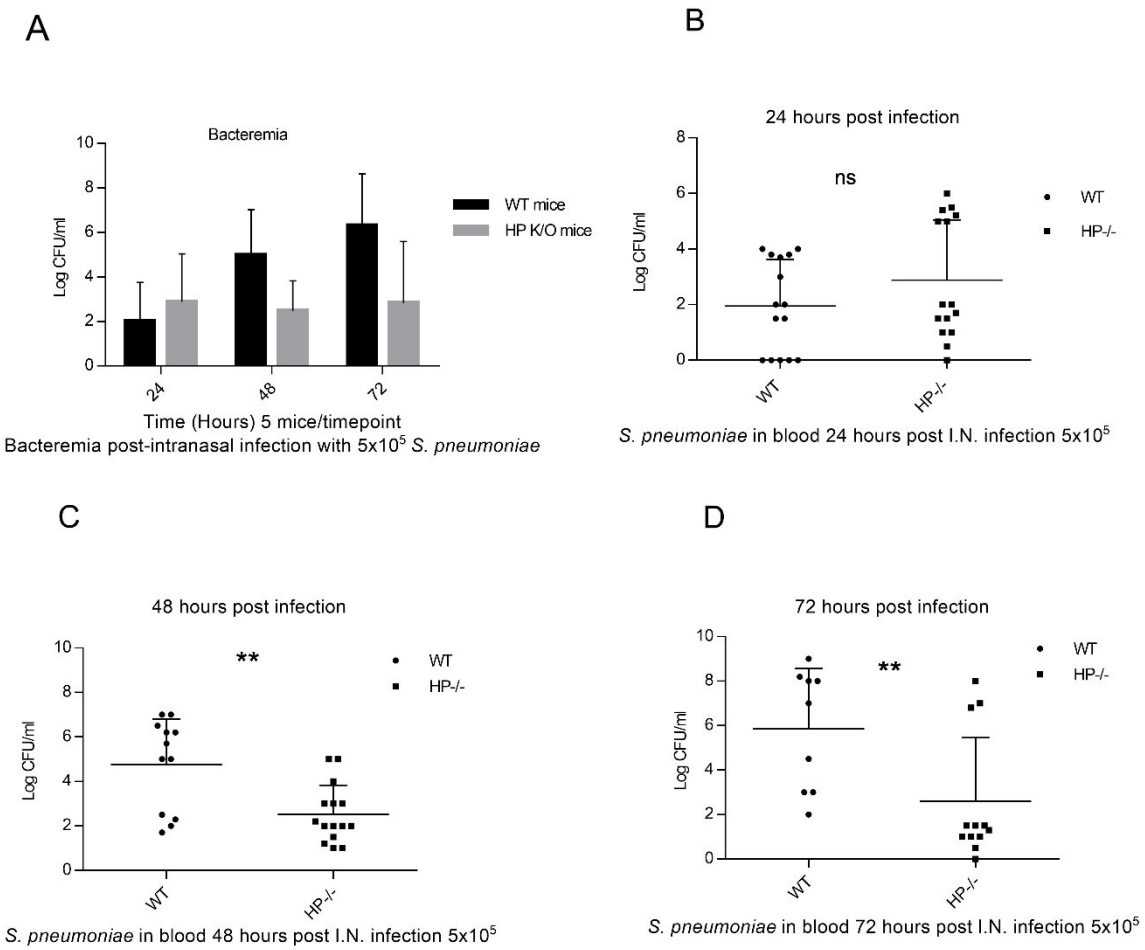

Figure 5-9: Bacterial load in blood of HP-deficient mice and their wild-type littermates post intranasal infection with of *S. pneumoniae*

CFU counts in blood for HP deficient mice and their wild-type littermates post intranasal infection with $5x10^5$ CFU of *S. pneumoniae* (D39). Results are shown as means of three independent experiments (mean with SD). By GraphPad Prism, statistical analysis by *Unpaired t test* revealed significant difference between the two groups at time points 48 hrs (**P<0.002) and 72 hrs (**P<0.0048).

5.2.3.3 Viable bacterial burden in lung, kidney, liver and spleen post intranasal infection with *S. pneumoniae* (D39)

Bacterial burdens in the organs were also measured post infection. Two groups of HP-deficient mice and wild type mice mice were humanely culled at 24, 48 hours. Then, lungs, kidneys, livers and spleens were collected and homogenised. Consistently with the bacterial load in the blood, while HP-deficient mice started clearing the bacteria from blood 24 hours post infection with very mild signs of disease, bacteraemia increased dramatically in the wild type mice. Hence, wild type mice showed signs of infection 48 hours post infection due to that bacteria managed to gain access to tissues. Therefore, the number of bacteria in lung, kidney, liver and spleen in the wild type mice was more than their HP-deficient littermates almost at all time points (see Figure 5 11).

Observing the bacterial load in lung tissues of mice after infection, results showed significantly higher bacterial counts in the wild type mice compared to HP-deficient mice over the experiment time points (24 and 48 hours). It was also noticed that the bacterial load in HP-deficient mice was relatively modest during the experiment. However, wild type mice were compromised in clearing bacteria and as a result bacterial counts increased significantly until the end of observation (see Figure 5-11-A).

Furthermore, bacterial burden in the kidney and liver tissues revealed a relatively low number of bacteria at 24 hours post infection in both groups (HP-deficient and wild type mice). However, there was considerable increase in bacterial viable counts in the wild type mice after 48 hours of infection in kidney and liver tissues (see Figure 5-11-B & C). This dramatic increase explains the severity of the illness in mice of the same group,

which had to be culled at 36 hours post infection. Measurements in the spleen showed that the bacterial burden was also higher in the wild type mice when compared to HP-deficient mice. Nevertheless, statistical analysis did not show a significant difference between the two groups (Figure 5-11-D).

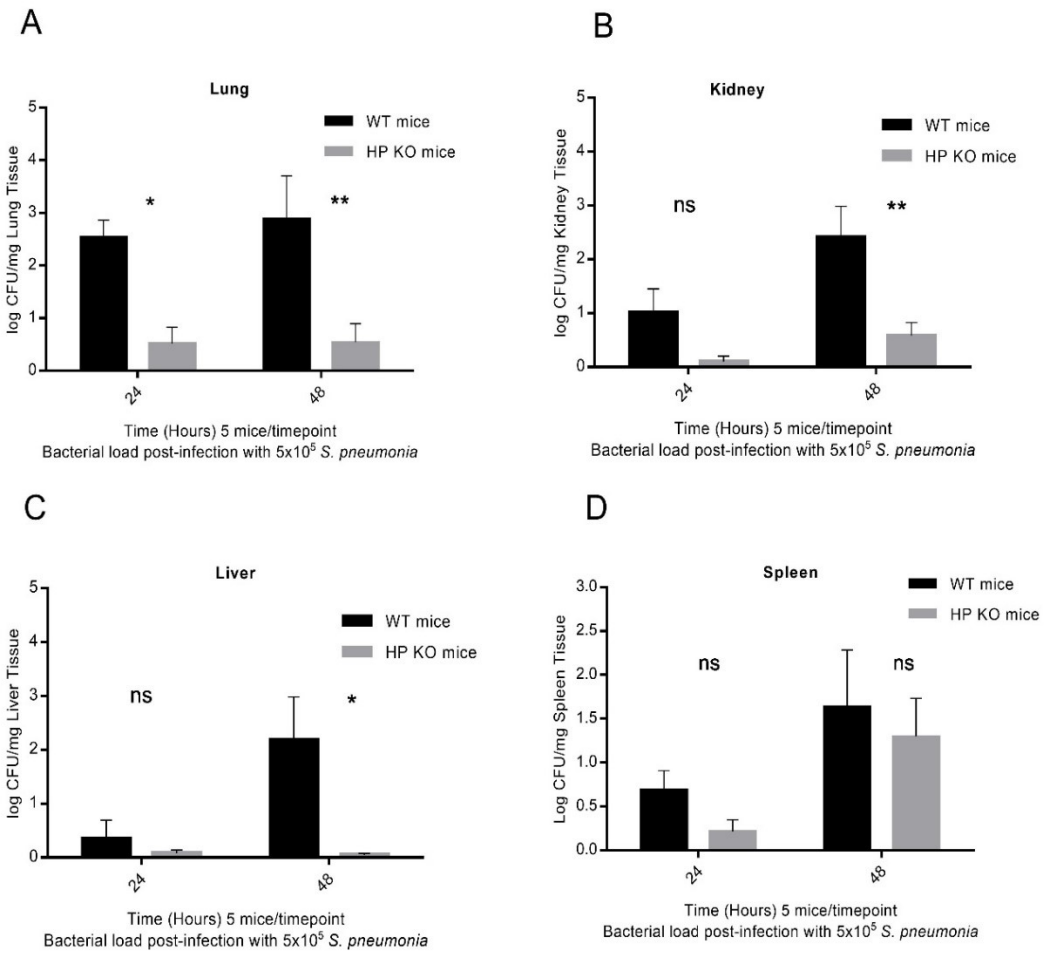

Figure 5-10: Bacterial load in organs of HP deficient mice and their wild-type littermates post intranasal infection with of *S. pneumoniae*

CFU counts lung (A), kidney (B), liver (C) and spleen (D) for HP-deficient mice and their wild-type littermates post intranasal infection with $5x10^5$ CFU of *S. pneumoniae* (D39). Results are means (means±SEM). n=5 at time 24 and 48 hours for both mice groups. A 2Way ANOVA multiple comparisons test was used to analyse the data. Significant CFU counts in WT group in lungs as graph A at 24 hrs (*P<0.0018), 48 hrs (**P<0.024), and kidney 48 hrs (**P<0.018) as in graph B, and in liver 48 hrs (*P<0.016) as in graph C.

5.2.3.4 HP level increases post infection

HP concentration in mice increases as a result of infection (Cray, 2012). Therefore, it was important to test serum levels of HP in the mice post-infection. Wild type mice contained significantly higher serum levels than serum from uninfected wild type mice, consistent with HP being an acute phase protein (see Figure 5-11). As expected no HP was detected in serum of HP-deficient mice.

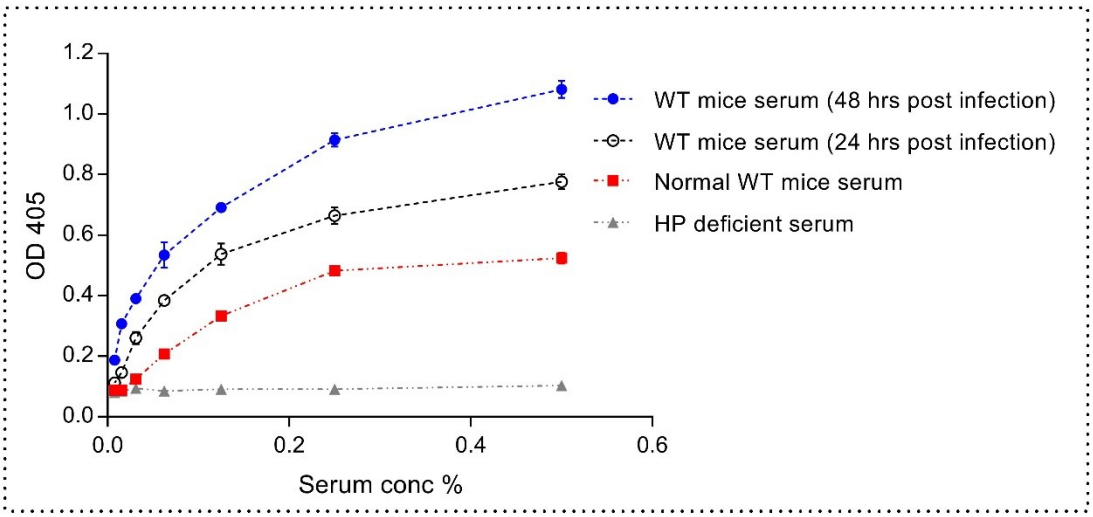

Figure 5-11: ELISA showing serum-levels of HP post infection with *S. pneumoniae*

ELISA plates were coated with LTA**.** Diluted mouse sera was incubated in LTA coated wells at 37 °C for 1 hour. HP was detected using chicken polyclonal anti-HP antibody, and followed by alkaline phosphatase-conjugated goat anti-chicken IgG as a secondary antibody. WT sera was used as a positive control while bovine serum albumin (BSA) was used as a negative control. Three independent experiments were done and results are expressed as means±SEM

5.2.3.5 HP does not inhibit Ficolins binding to LTA

A previous study reported that L-ficolin can specifically bind to LTA, a binding that can lead to complement activation via the lectin activation pathway (Lynch *et al*, 2004). Ficolin-A and ficolin-B are essential components in the immune defence against *S. pneumoniae* infections (Ali *et al*, 2012; Endo *et al*, 2012). This raised the possibility that HP may interfere with ficolins binding to *S. pneumoniae*. To test this hypothesis, ficolin binding to *S. pneumoniae* was assessed using wild type serum and HP-deficient serum. Results showed no difference in binding towards *S. pneumoniae* indicating that HP does not compromise ficolin binding as in Figure 5-13-A. Competition assays also show no competitive inhibition of ficolin binding indicating that HP (here I used recombinant mouse haptoglobin, mHP) must bind to a site on LTA that is different from the ficolin-A binding site (see Figure 5-13-B).

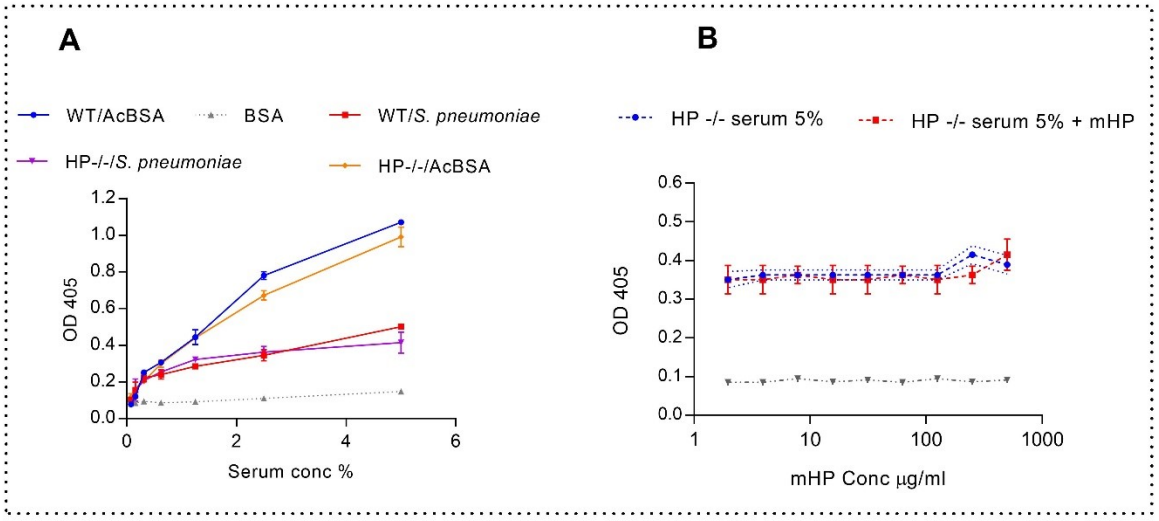

Figure 5-12: HP does not interfere with FCN for LTA binding

ELISA to assess ficolin A binding to D39 , microtiter plates were coated with N-acetyl BSA (+ve control) and formalin fixed bacteria, then plates were incubated for 2 hours at room temperature with normal mouse sera and HP-deficient sera. Binding of ficolin-A was detected using a specific rabbit anti-mouse ficolin A (provided by T. Fujita) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG as a secondary antibody (Sigma). Results are means of three independent experiments (±SEM).

5.3 Discussion

The aim of this Chapter was to assess the possible role of HP in the immune response to Gram-positive bacteria by comparing the susceptibility and severity of infectious disease between HP-deficient mice and their respective HP sufficient WT- controls on C57BL/6J background. The use of a *Staphylococcus aureus* infection model did not reveal reproducible results since mice on C57BL/6J background are remarkably resistant to *S. aureus* infection, even when using extremely high infectious CFUs. Since this model could not be established beyond the point of acute infection we could not observe any differences between HP-deficient mice and their wild type littermates. My result are consistent with previous exploratory studies of *S. aureus* infection, which showed that the C57BL/6J mouse strain is resistant to *S. aureus* infection (von Köckritz-Blickwede *et al*, 2008). Moreover, all indications pointed to the conclusion that the innate immune response is responsible for this high degree of resistance towards *S. aureus* infection because even C57BL/6J mice deficient in B cells, or T cells, or NK cells also showed to be highly resistant towards *S. aureus* infection (von Köckritz-Blickwede *et al*, 2008). Using high infectious doses of *S. aureus* ($4x10^8$ CFU) achieved clear signs of acute infection and bacteremia. Consequently, we further investigated bacterial load in lung, liver, spleen and kidney. In the HP-sufficient wild type mice, the bacterial burden in liver, spleen and kidney was slightly (but not significantly) higher than that in HP-deficient mice. Interestingly, 18 hours post infection we observed a significant bacterial load in the lungs of HP-sufficient mice when compared to that of HP-deficient mice. However, most of the bacteria were cleared 3-6 hours later indicating the remarkable capacity of the

C57BL/6J strain to control bacterial growth and eliminate *S. aureus* infection (section 5.2.2).

Using *Streptococcus pneumoniae* (D39) as an infectious agent proved to produce a reliable and reproducible infection model. To our surprise the HP-deficient mice were more resistant to pneumococcal infection with limited signs of infectious disease resulting in approximately 10% mortality overall, in comparison to 60% mortality achieved when using their strain, age and sex-matched wild-type control littermates. Looking at the time course of the death events of both groups (humanely culling mice with signs of infectious disease when reaching ++ lethergic), severe signs of disease appeared in HP-sufficient wild type mice as early as 36 hours post infection while these signs where significantly delayed in the HP-deficient mice up to 72 hours post infection. The presence of HP also affected the level of bacteraemia in the challenged mice, whereby the $HP^{+/+}$ wild type mice showed significantly higher levels of bacteraemia than $HP^{-/-}$ mice (section 5.2.3).

The bacterial load of lungs, livers, spleens and kidneys was also investigated. Results were consistent with the mortality and illness scores with a relatively high bacterial load in the organs of HP-sufficient mice indicating compromised bacterial clearance.

To investigate further the underlying mechanism behind these unexpected results, serum HP levels of the infected mice were analysed. Consistent with previous findings with Cray (2012), HP concentrations in mouse serum increased as a result of infection, with

infected wild type mice showed much higher serum HP levels than uninfected wild type controls as early as 24 hours post infection. Furthermore, HP levels were even higher after 48 hours post infection. These findings are consistent with HP being an acute-phase protein and that the increase of HP concentration in serum is caused by the infection.

Differences in infection in HP-sufficient and -deficient animals occurred very early during infection suggesting that HP may promote bacterial survival and facilitate growth of the pneumococcus. Similar observation have been observed for other proteins. For example, Thrombospondin-1, a glycoprotein that is secreted by cells upon injury or infection, binds to Gram-positive bacteria and mediates their cellular adherence and colonisation (Rennemeier *et al*, 2007).

It is well established that the complement system, for example via ficolin binding to *S. pneumoniae*, has a crucial role in the innate immune defence against *S. pneumoniae* (Ali *et al*, 2012; Ali *et al*, 2014; Endo *et al*, 2012). As reported by (Lynch *et al*, 2004), L-ficolin binds specifically to LTA leading to complement activation. We therefore speculated that based on our observation with *S. pneumoniae* infection model, HP might have interfered with complement activation on the bacterial surface inhibiting ficolin from binding LTA. My analysis (see Figure 5-12), however, demonstrated that this was not the case, HP did not act as a competitive inhibitor of ficolin/*S. pneumoniae* binding interactions. My results indicate that HP must bind to a different site on LTA than ficolins.

Clearly the results reported here indicate that HP impedes the clearance of *S. pneumoniae* and thus the survival from infectious disease. While this is an interesting result, the mechanism underlying this phenomenon remains to be determined. It may involve one and/or more of the following;

- A previous study demonstrated that HP had an inhibitory effect on granulocyte chemotaxis, bactericidal activity and phagocytosis (Rossbacher, Wagner and Pasternack, 1999). This result is consistent with my data showing that the presence of HP promoted bacterial survival.

- HP is a positive acute phase protein in many species such as human and mouse. The hepatic expression of HP post infection increases significantly as this increase was described to be moderate in humans and major in mice (Cray, 2012). The increase in the concentration of HP might result in one of the following two possibilities;

    A- HP might suppress the immune reaction. HP has been shown to suppress macrophage functions (including production of tumor necrosis factor-α, TNF-α) and has been shown to suppress the production and proliferation of cytokines in T cells and B cells. This is probably because of that HP can interacts with CD11b/CD18 receptors, which are located on granulocytes, $CD8^+$ cells, natural killer cells and monocytes (El Ghmati *et al*, 1996). A study found that HP can also bind to CD22 on B cells, suggesting that it might be involved in the suppression of B cell differentiation (Yuan *et al*, 1996). Furthermore, HP can suppress lymphocyte functions during APR by

inhibiting the phytohaemagglutinin (PHA)-induced blastogenesis of lymphocytes (Israel *et al*, 1981). The suppression of the effect of HP on immune cells and/or receptors could explain our observation that the presence of HP in the HP-sufficient wild type mice contributed to the increased mortality in these mice post pneumococcal infection.

B- In contrast to A above, it has been reported that HP can enhance the inflammatory response in a model of skin transplantation leading to production of IL-6 and TNF-α by dendritic cells (Shen *et al*, 2012). Thus, the HP-sufficient mice might have deteriorated due to the severe inflammatory response mediated by HP, resulting in multi-organ failure caused by inflammation. It could also be that the presence of HP at the site of infection enhanced inflammation and hence HP-sufficient mice may have had an increased burden of apoptotic macrophages. This could also explain why wild type HP-sufficient mice were more susceptible to the infection than the HP deficient mice.

- Studies so far reported that a number of serum proteins recognise LTA including mannose binding lectin (MBL) (previously called mannose-binding protein (MBP)), lipopolysaccharide-binding protein (LBP), CD14, soluble TLR2 (Jang *et al*, 2012), and L-ficolin (Lynch *et al*, 2004). These LTA binding proteins can either contribute towards the immune response or contribute to LTA neutralisation as a scavenger (Jang *et al*, 2012). Another study done on LTA binding serum proteins highlighted

that the high concentration of neutralising serum proteins could impair the ability of LTA to interact with its cellular targets and trigger the inflammatory response (Triantafilou *et al*, 2012). We have demonstrated that HP-deficient mice, during pneumococcal infection, are more protective than HP-sufficient wild type mice. Therefore, it could be that HP via binding to LTA on bacterial surfaces, rather than mediating immune system activation, covers immune receptors on bacteria.

- HP may assist bacteria to acquire iron and augment bacterial growth. Thus, *S. pneumoniae* might bind HP-Hb complexes, to acquire haem, providing iron for the bacterium. A similar mechanism is employed by *Trypanosome brucei,* which possesses a receptor that can bind to the HP-Hb complexes and hence can utilise haem to increase the growth rate of the parasite. (Pays and Vanhollebeke, 2009).

Chapter 6

Conclusion and Future Directions

6.1 Conclusion

The data presented in this thesis highlights the importance of HP in the immune response to Gram-positive infections. It also increases our understanding of the HP interaction with LTA from certain Gram-positive bacteria *S. pneumoniae* and *S. aureus*.

I have demonstrated that HP interacts with LTA via the β-chain of HP. Several independent lines of evidences confirmed that HP binds to all so far tested LTAs (type I and type IV) via the β-chain. The specificity of binding has been assessed through direct binding of LTA to the β-chain of HP and in competition binding assays where Hb was used to compete for the binding of LTA to the HP β-chain. I have also shown that multiple components of LTA may be required for HP binding, but localisation of these components requires more research. Therefore, HP is now confirmed to be a ligand for LTA in addition to previously reported ligands for LTA including mannose-binding lectin (MBL, previously called MBP), lipopolysaccharide-binding protein (LBP), CD14, soluble TLR2 (Jang *et al*, 2012) , and L-ficolin (Lynch *et al*, 2004).

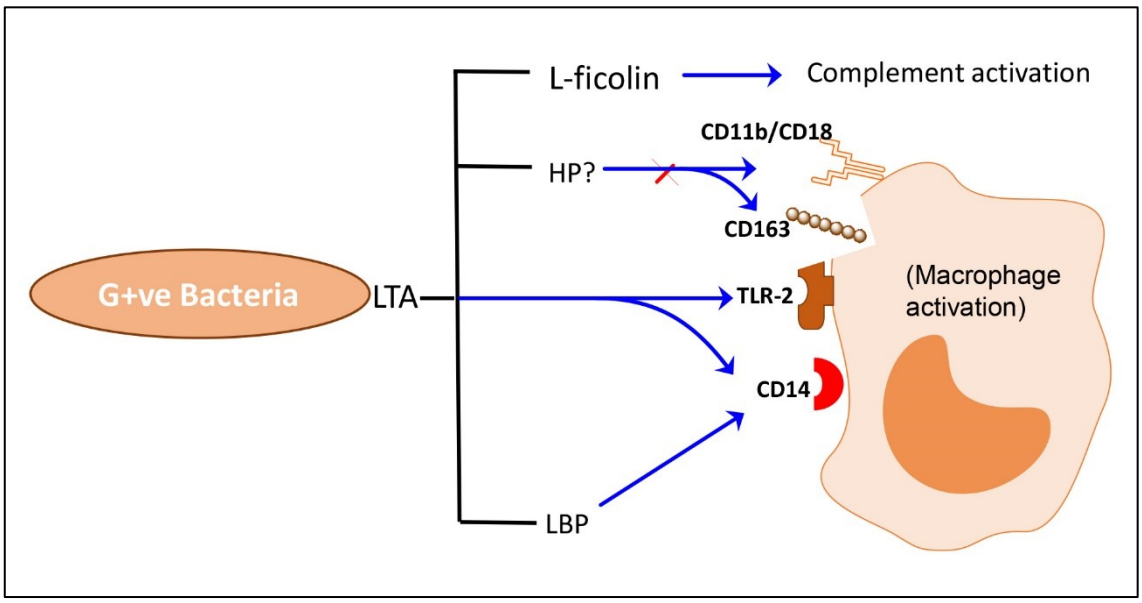

Figure 6-1: Summary of receptors and LTA binding proteins identified so far

LTA from Gram-positive bacteria (e.g. *S. pneumoniae*) can bind to macrophages via LBP, TLR-2, CD14. It is still unknown to what extent HP contributes to the modulation of LTA mediated activation of macrophages. Figure was made with the help of some materials purchased from (www.motifolio.com).

One of the most surprising findings of my work is that HP-deficient mice show a significant degree of protection from pneumococcal infections, indicating that HP may contribute to the survival of this pathogen or impede its elimination mechanism. This conclusion is based on my observation that in the presence of HP (i.e. in HP-sufficient wild type), a significantly higher number of CFUs were recovered from blood and assessed organs in contrast to strain, sex and age-matched HP deficient mice (see Figure 5-10). Interestingly, a suppressive effect of HP on phagocytic activity has previously been described that HP inhibited phagocytosis and bactericidal activity of *E. coli* (El Ghmati *et al*, 1996).

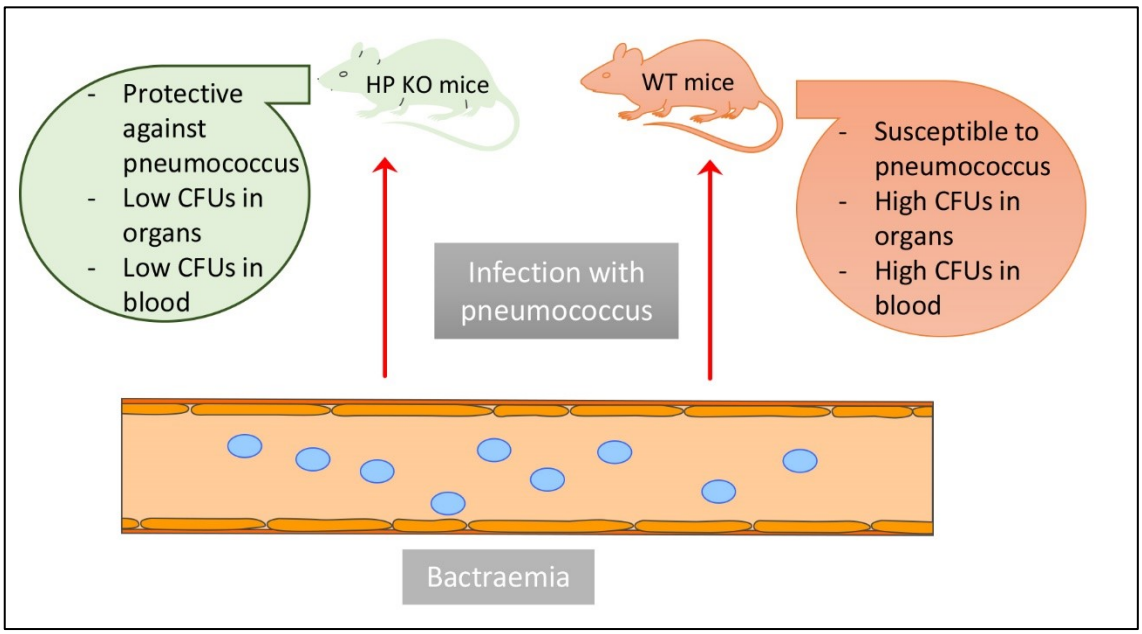

Figure 6-2: A diagram showing the observation with *S. pneumoniae* infection model

Absence of HP enhances the immune response to *S. pneumoniae* while presence of HP makes mice more susceptible to *S. pneumoniae* infection. Figure was made with the help of some materials purchased from (www.motifolio.com).

Iron acquisition by bacteria is essential for bacterial survival and promotes bacterial growth. In order to explain the positive effect that HP was shown to have on *S. pneumoniae* during infection, I hypothesise that *S. pneumoniae* may use HP to enhance iron uptake. Pneumolysin, a toxin released by *S. pneumoniae* during infection could also facilitate iron uptake by *S. pneumoniae* through the lytic activity of pneumolysin towards red blood cells (Gratz *et al* 2015). As a consequence, the pneumococcus can cause haemolysis and Hb release into the serum. As a professional scavenger of Hb, HP may facilitate bacterial iron acquisition by the bacteria.

The primary function of the immune system is to prevent and eliminate infections. However, an increased inflammatory response can be deleterious and cause damage to

the host. A complex sequence of events including cytokines, cell receptors and serum proteins regulate the immune response. The results achieved in our pneumococcus model indicate that HP has an important role in shifting the balance between bacterial growth and bactericidal activity with the immune response, particularly during the early acute inflammatory response, in which wild type mice showed severe signs of infection at an early time point (36 hours) after infection. In contrast, severe signs of infection were seen at a much later time point (72 hours) in HP-deficient mice. This implies that HP cannot just favour bacterial growth, but that HP has a negative regulatory activity on the onset of inflammation. During the inflammatory response, HP production is dramatically increased as an acute phase reactant. There are many other examples where the course of pneumococcal infections is worsened by the anti-inflammatory activity of modulators of the immune response such as factor H (FH) and C4-binding protein (C4BP). Pneumococcal virulence factors PspA and PspC were reported to recruit FH and C4BP from host plasma, which both accelerate C4b breakdown into C4dg by factor I (Ali *et al* 2012). Furthermore, properdin-deficient mice, challenged with *S. pneumoniae*, showed increased resistance to infection, displaying enhanced protection compared to their wild type littermates (Dupont *et al* 2014). Properdin is a positive regulator of complement by stabilising the alternative pathway C3 convertase and promotes complement activation and the downstream effects on immune and inflammatory processes. The phenotype of properdin-deficient mice and HP-deficient mice is thus similar implying that certain immune processes initiated by properdin and HP are deleterious during pneumococcal infection.

In conclusion, the acute phase proteins (e.g. HP) either inhibit or enhance inflammatory response in manners dependent on the microbial ligand and immune cell receptors. We demonstrated that the presence of HP during pneumococcal infection favours the bacteria rather than the host, so it is detrimental to the body in this infection model. We speculate that this observation is explained by the possible negative regulation of the innate immune cells particularly phagocytes, but the precise mechanism requires more research. Understanding the precise role of the innate immunity as mediated by serum proteins (e.g. HP) will develop the treatments of infectious and inflammatory diseases.

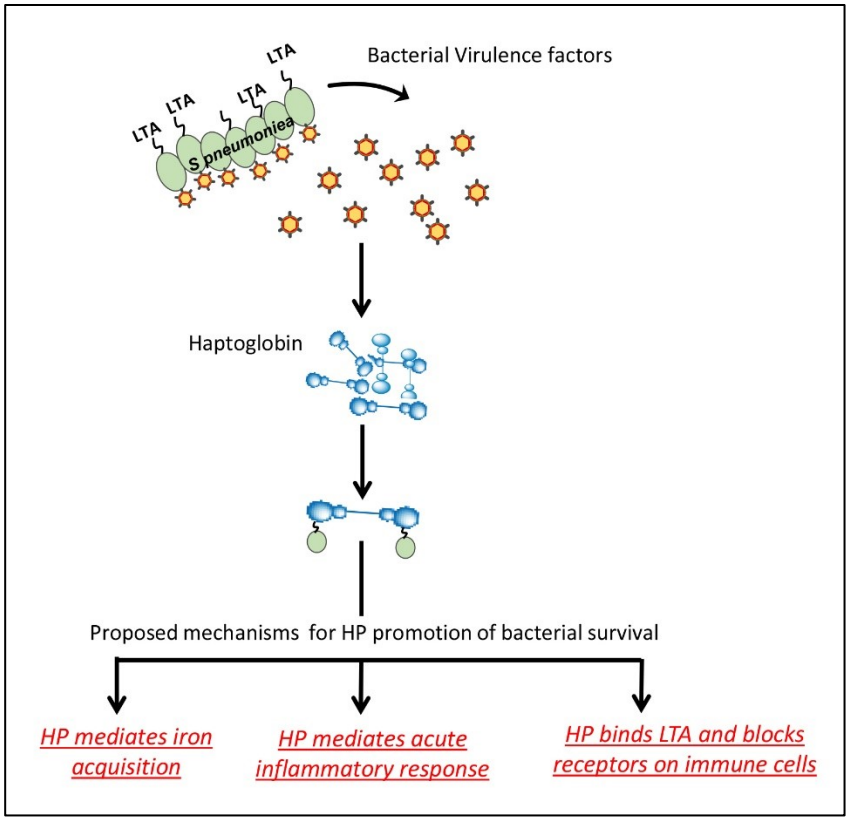

Figure 6-3: Summary of the proposed roles of HP during pneumococcal infection

HP may mediate and block the binding of LTA to phagocytic receptors. Figure was made the help of ChemBioDraw application.

6.2 Future directions

6.2.1 HP crystallisation

HP screens on JCSG+ and PACT from molecular dimensions for mouse HP showed small crystals. However, these crystals diffracted poorly and require further optimisation. It is possible that increasing the concentration of the protein, may improve crystallisation but due to the time limitations we were unable to do test this possibility. Other groups have crystallised HP-Hb complex but using porcine HP (Andersen *et al*, 2012).

6.2.2 LTA structural component/s mediating HP/LTA interaction

There are five different types of LTA, i.e. LTA I-V. Examples of LTAs from Type I and Type IV were tested and all displayed similar binding towards HP. Four LTA structural components (glycerophosphate, D.alanine, GlcNac and gentiobiose) were tested for their binding activity to HP but none of them bound to HP at the concentrations tested. Thus, it would be useful to investigate this further to identify which component/s are responsible for this binding, perhaps by using larger synthetic fragments. One strategy could be through forming complexes of these components and examine their interaction with HP.

6.2.3 Reconstitution of HP-deficient mice with recombinant HP

One of the unexpected finding of this work is the negative impact of HP presence in the wild type mice during pneumococcus infection. It would be interesting to test if addition of recombinant HP would have a detrimental effect in HP-deficient mice.

6.2.4 Supplementation of HP-deficient mice with iron

Iron is a very important source for microbial growth and survival. Bacteria have developed several mechanisms to acquire iron from host. Surprisingly, HP-deficient mice were protected against *S. pneumoniae* infections. Considering that HP facilitated the iron uptake and caused increased mortality rate observed in wild type mice. Therefore, it is important to further investigate this phenomenon by supplementing HP-deficient mice with iron before infection with *S. pneumoniae* and validate this speculation.

6.2.5 HP interaction with complement

Preliminary results revealed that serum deficient of HP has significantly reduced activity of the alternative pathway of the complement system activation on the surface of *S. pneumoniae* (data not shown). Our *S. pneumoniae* infection study on HP deficient mice showed high amount of protection against pneumococcus infection. Previous studies showed that mice deficient with properdin, a positive regulator of the complement system, displayed similar amount of protection against *S. pneumoniae* infections to that observed with HP-deficient mice. This indicates that HP and complement system may have a linked role during pneumococcus infection. Thus, identifying the linkage between HP and the complement system would be important.

6.2.6 HP role during infection with other Gram-positive pathogens using HP-deficient mice

Two models of Gram-positive bacterial infection (*S. pneumoniae* and *S. aureus*) were assessed during this project. I would be very much interested to test and compare the role

of HP in the immune response to other Gram-positive bacteria such as *Listeria monocytogenes*. This model has been tested before on properdin-deficient mice, which surprisingly showed high mortality rate in properdin-deficient mice in contrast to the protection capacity of the same mice to pneumococcal infections.

Chapter 7. Appendix

7.1 Nucleotides sequence alignment between human and mouse HP

Human HP

ATGGTGGACTCAGGCAATGATGTCACGGATATCGCAGATGACGGCTGCCCGAAGCCCCCCGAGATTGCACATGGCTAT
GTGGAGCACTCGGTTCGCTACCAGTGTAAGAACTACTACAAACTGCGCACAGAAGGAGATGGAGTATACACCTTAAAT
GATAAGAAGCAGTGGATAAATAAGGCTGTTGGAGATAAACTTCCTGAATGTGAAGCAGTATGTGGGAAGCCCAAGAAT
CCGGCAAACCCAGTGCAGCGGATCCTGGGTGGACACCTGGATGCCAAAGGCAGCTTTCCCTGGCAGGCTAAGATGGTT
TCCCACCATAATCTCACCACAGGTGCCACGCTGATCAATGAACAATGGCTGCTGACCACGGCTAAAAATCTCTTCCTG
AACCATTCAGAAAATGCAACAGCGAAAGACATTGCCCCTACTTTAACACTCTATGTGGGGAAAAAGCAGCTTGTAGAG
ATTGAGAAGGTTGTTCTACACCCTAACTACTCCCAGGTAGATATTGGGCTCATCAAACTCAAACAGAAGGTGTCTGTT
AATGAGAGAGTGATGCCCATCTGCCTACCTTCAAAGGATTATGCAGAAGTAGGGCGTGTGGGTTATGTTTCTGGCTGG
GGGCGAAATGCCAATTTTAAATTTACTGACCATCTGAAGTATGTCATGCTGCCTGTGGCTGACCAAGACCAATGCATA
AGGCATTATGAAGGCAGCACAGTCCCCGAAAAGAAGACACCGAAGAGCCCTGTAGGGGTGCAGCCCATACTGAATGAA
CACACCTTCTGTGCTGGCATGTCTAAGTACCAAGAAGACACCTGCTATGGCGATGCGGGCAGTGCCTTTGCCGTTCAC
GACCTGGAGGAGGACACCTGGTATGCGACTGGGATCTTAAGCTTTGATAAGAGCTGTGCTGTGGCTGAGTATGGTGTG
TATGTGAAGGTGACTTCCATCCAGGACTGGGTTCAGAAGACCATAGCTGAGAACTAA

Mouse HP

ATGGTGGAGTTGGGCAATGATGCCATGGACTTTGAAGATGACAGCTGCCCAAAGCCCCCAGAGATTGCAAACGGCTAT
GTGGAGCACTTGGTTCGCTATCGCTGCCGACAGTTCTACAGACTACGGGCCGAAGGAGATGGGGTGTACACCTTAAAC
GACGAGAAGCAATGGGTGAACACAGTCGCTGGAGAGAAACTCCCCGAATGTGAGGCAGTGTGTGGGAAGCCCAAGCAC
CCTGTGGACCAGGTGCAGCGCATCATCGGTGGCTCTATGGATGCCAAAGGCAGCTTTCCTTGGCAGGCCAAGATGATC
TCCCGCCACGGACTCACCACCGGGGCCACGTTGATCAGTGACCAGTGGCTGCTGACCACGGCCAAAAACCTCTTCCTG
AACCACAGCGAGACGGCGTCAGCCAAGGACATCACCCCCACCCTAACGCTCTACGTGGGGAAAAACCAGCTGGTGGAG
ATTGAGAAGGTCGTTCTCCACCCCAACCACTCCGTGGTGGATATCGGGCTAATCAAACTCAAGCAGAGGGTGCTTGTA
ACCGAGAGAGTCATGCCTATCTGCCTGCCTTCCAAAGACTACATAGCACCAGGCCGTGTGGGCTACGTGTCTGGCTGG
GGGCGGAACGCCAACTTTAGATTTACCGATCGTCTCAAGTATGTCATGCTGCCTGTGGCCGACCAGGACAAGTGTGTG
GTGCACTATGAGAATAGTACAGTGCCCGAGAAGAAAAACTTGACGAGTCCCGTTGGGGTCCAGCCTATCTTGAACGAG
CACACCTTCTGTGCTGGCCTCACCAAGTACCAGGAAGACACCTGCTACGGTGACGCCGGCAGTGCCTTTGCCATTCAT
GACATGGAGGAGGACACCTGGTACGCAGCTGGGATCCTGAGCTTTGACAAGAGCTGCGCTGTCGCTGAGTATGGTGTG
TACGTGAGGGCGACCGACCTGAAGGACTGGGTTCAGGAAACCATGGCCAAGAACTAG

- **Sequence similariteis between mouse and human haptoglobin**

Alignment of the nucleotides sequence of mouse haptoglobin (mHP) and human haptoglobin (hHP) showed 783 identical nucleotides out of 992 (79%) between the two sequences. Identical nucleotides are shaded.

```
Human  1     ATGGTGGACTCAGGCAATGATGTCACGGATATCGCAGATGACGGCTGCCCGAAGCCCCCC  60
             |||||||| |  |||||||||| || |||  | | ||||||| ||||||| |||||||| 
Mouse  1     ATGGTGGAGTTGGGCAATGATGCCATGGACTTTGAAGATGACAGCTGCCCAAAGCCCCCA  60

Human  61    GAGATTGCACATGGCTATGTGGAGCACTCGGTTCGCTACCAGTGTAAGAACTACTACAAA  120
             ||||||||| | |||||||||||||||| ||||||||| |  ||     | | ||||| |
Mouse  61    GAGATTGCAAACGGCTATGTGGAGCACTTGGTTCGCTATCGCTGCCGACAGTTCTACAGA  120

Human  121   CTGCGCACAGAAGGAGATGGAGTATACACCTTAAATGATAAGAAGCAGTGGATAAATAAG  180
             || ||  | ||||||||||| || ||||||||||| ||  ||||||| ||| | || |  
Mouse  121   CTACGGGCCGAAGGAGATGGGGTGTACACCTTAAACGACGAGAAGCAATGGGTGAACACA  180

Human  181   GCTGTTGGAGATAAACTTCCTGAATGTGAAGCAGTATGTGGGAAGCCCAAGAATCCGGCA  240
             |  | |||||| ||||| || |||||||| ||||| ||||||||||||||| | || |  
Mouse  181   GTCGCTGGAGAGAAACTCCCCGAATGTGAGGCAGTGTGTGGGAAGCCCAAGCACCCTGTG  240

Human  241   AACCCAGTGCAGCGGATCCTGGGTGGACACCTGGATGCCAAAGGCAGCTTTCCCTGGCAG  300
              |||  |||||||| ||| | |||||     ||||||||||||||||||| ||| ||||||
Mouse  241   GACCAGGTGCAGCGCATCATCGGTGGCTCTATGGATGCCAAAGGCAGCTTTCCTTGGCAG  300

Human  301   GCTAAGATGGTTTCCCACCATAATCTCACCACAGGTGCCACGCTGATCAATGAACAATGG  360
             || |||||| | |||| |||    |||||||| || |||||| |||||| ||| || |||
Mouse  301   GCCAAGATGATCTCCCGCCACGGACTCACCACCGGGGCCACGTTGATCAGTGACCAGTGG  360

Human  361   CTGCTGACCACGGCTAAAAATCTCTTCCTGAACCATTCAGAAAATGCAACAGCGAAAGAC  420
             |||||||||||||| ||||| ||||||||||||||    || |  ||  |||| || |||
Mouse  361   CTGCTGACCACGGCCAAAAACCTCTTCCTGAACCACAGCGAGACGGCGTCAGCCAAGGAC  420

Human  421   ATTGCCCCTACTTTAACACTCTATGTGGGGAAAAAGCAGCTTGTAGAGATTGAGAAGGTT  480
             ||  |||| ||  |||| ||||| ||||||||||| ||||| || |||||||||||||| 
Mouse  421   ATCACCCCCACCCTAACGCTCTACGTGGGGAAAAACCAGCTGGTGGAGATTGAGAAGGTC  480

Human  481   GTTCTACACCCTAACTACTCCCAGGTAGATATTGGGCTCATCAAACTCAAACAGAAGGTG  540
             ||||| ||||| ||| |||||  ||| ||||| ||||| ||||||||||| |||| ||||
Mouse  481   GTTCTCCACCCCAACCACTCCGTGGTGGATATCGGGCTAATCAAACTCAAGCAGAGGGTG  540

Human  541   TCTGTTAATGAGAGAGTGATGCCCATCTGCCTACCTTCAAAGGATTATGCAGAAGTAGGG  600
               ||| |  ||||||| |||||| |||||||| ||||| || || ||   || |  ||| 
Mouse  541   CTTGTAACCGAGAGAGTCATGCCTATCTGCCTGCCTTCCAAAGACTACATAGCACCAGGC  600

Human  601   CGTGTGGGTTATGTTTCTGGCTGGGGGCGAAATGCCAATTTTAAATTTACTGACCATCTG  660
             |||||||| || || |||||||||||||| || ||||| |||| |||||| || | ||| 
Mouse  601   CGTGTGGGCTACGTGTCTGGCTGGGGGCGGAACGCCAACTTTAGATTTACCGATCGTCTC  660

Human  661   AAGTATGTCATGCTGCCTGTGGCTGACCAAGACCAATGCATAAGGCATTATGAAGGCAGC  720
             ||||||||||||||||||||||| ||||| ||| | ||  |   ||| |||||    || 
Mouse  661   AAGTATGTCATGCTGCCTGTGGCCGACCAGGACAAGTGTGTGGTGCACTATGAGAATAGT  720

Human  721   ACAGTCCCCGAAAAGAAGACACCGAAGAGCCCTGTAGGGGTGCAGCCCATACTGAATGAA  780
             ||||| ||||| ||||| |    || ||| || || ||||| ||||| ||  |||| || 
Mouse  721   ACAGTGCCCGAGAAGAAAAACTTGACGAGTCCCGTTGGGGTCCAGCCTATCTTGAACGAG  780

Human  781   CACACCTTCTGTGCTGGCATGTCTAAGTACCAAGAAGACACCTGCTATGGCGATGCGGGC  840
             |||||||||||||||||| |  | |||||||| |||||||||||||| || || || |||
Mouse  781   CACACCTTCTGTGCTGGCCTCACCAAGTACCAGGAAGACACCTGCTACGGTGACGCCGGC  840

Human  841   AGTGCCTTTGCCGTTCACGACCTGGAGGAGGACACCTGGTATGCGACTGGGATCTTAAGC  900
             |||||||||||| |||| ||| ||||||||||||||||||| ||  |||||||| | |||
Mouse  841   AGTGCCTTTGCCATTCATGACATGGAGGAGGACACCTGGTACGCAGCTGGGATCCTGAGC  900

Human  901   TTTGATAAGAGCTGTGCTGTGGCTGAGTATGGTGTGTATGTGAAGGTGACTTCCATCCAG  960
             ||||| |||||||| ||||| ||||||||||||||||| |||| || |||   | |  ||
Mouse  901   TTTGACAAGAGCTGCGCTGTCGCTGAGTATGGTGTGTACGTGAGGGCGACCGACCTGAAG  960

Human  961   GACTGGGTTCAGAAGACCATAGCTGAGAACTA  992
             |||||||||||| | ||||| ||  |||||||
Mouse  961   GACTGGGTTCAGGAAACCATGGCCAAGAACTA  992
```

7.2 Peptide sequencing of recombinant mouse HP

The recombinant mouse HP sequenced (MRC Toxicology Unit, Leicester, UK). Protein was analyzed by in gel trypsin digestion/MALDI-TOF-MS. Identity was confirmed as a mouse HP.

University of Leicester Mascot Search Results

Search title : MM_01_0001.dat - Sample Info, F:\160811\MM_01_0001.dat submitted from Mascot Wizard on HT-4575
Database : UniProtKB-SwissProt 2014_11 (547085 sequences; 194742747 residues)
Timestamp : 11 Aug 2016 at 14:00:54 GMT
Top Score : 215 for Q61646, Haptoglobin OS=Mus musculus GN=Hp PE=1 SV=1
Mass: 39241 Score: 215 Expect: 1.7e-016 Queries matched: 14

Observed Mass (Da)	Mass(calc) (Da)	Matching peptide sequence
716.4090	715.4228	R.VLVTER.V
791.3880	790.3973	K.DYIAPGR.V
920.4540	919.4552	K.GSFPWQAK.M
978.5050	977.5043	K.HPVDQVQR.I
980.4860	979.4876	R.VGYVSGWGR.N
1131.5910	1130.5720	K.QWVNTVAGEK.L
1373.6530	1372.6445	K.SCAVAEYGVYVR.A
1387.6720	1386.6635	K.LPECEAVCGKPK.H
1431.7260	1430.7154	K.NLFLNHSETASAK.D
1635.8170	1634.7974	R.ATDLKDWVQETMAK.N
1679.8300	1678.8162	R.LRAEGDGVYTLNDEK.Q
1740.0170	1739.0094	K.VVLHPNHSVVDIGLIK.L
2127.1640	2126.1372	R.HGLTTGATLISDQWLLTTAK.N
2496.3030	2495.2843	K.NLTSPVGVQPILNEHTFCAGLTK.Y

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