The role of haptoglobin in phagocyte-mediated killing of *Staphylococcus aureus*

A thesis submitted to the Faculty of Medicine and Biological Sciences, University of Leicester, in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Spring term, 2010

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Abstract

Haptoglobin is a positive acute-phase serum protein. It is upregulated during infection and is a valuable marker for many inflammatory-related diseases. Its primary known function is to eliminate haemoglobin from plasma to prevent loss of iron through the kidneys, thereby protecting the kidneys from damage and sequestering iron, a key target for invading bacteria. In this thesis, I show that haptoglobin also interacts with lipoteichoic acid (LTA) of *Staphylococcus aureus*, an important virulence factor. Bacteria are subsequently eliminated by neutrophil-, monocyte- and macrophage-mediated killing.

S. aureus is an extremely common human pathogen associated with various diseases including septic arthritis and toxic-shock syndrome. A current major concern is the emergence of multidrug-resistant strains such as methicillin-resistant *S. aureus* (MRSA) that cause severe infections in hospitals and in the community. To increase our understanding of the defence mechanisms employed by the host against *S. aureus*, LTA was used to capture LTA-binding proteins from human serum and these were identified using a proteomics-based strategy. A variety of targets were captured including complement proteins, lipid-transport proteins, coagulation-cascade proteins and acute-phase proteins. Haptoglobin was selected for further study, because its expression is known to be upregulated in response to infection, and it interacts with phagocytic cells to stimulate phagocytosis. ELISA and column chromatography studies confirmed that haptoglobin binds LTA directly as well as *S. aureus* and suggest that the α-chain of haptoglobin is critical for this interaction.

The role of haptoglobin in promoting the killing of *S. aureus* by neutrophils, monocytes and macrophages was evaluated by comparing *in vitro* bacterial-killing using serum depleted of haptoglobin. For each cell type haptoglobin was found to be a key mediator in phagocyte-mediated bacterial killing. This work highlights an important new role for haptoglobin in immune defence, and explains its upregulation during infection.

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List of contents

Abbreviations

1D-PAGE	1 dimensional polyacrylamide gel electrophoresis
2D-PAGE	2 dimensional polyacrylamide gel electrophoresis
ApoE	Apolipoprotein E
APPs	Acute-phase proteins
APR	Acute-phase reaction
AT III	Antithrombin III
BSA	Bovine serum albumin
C1-Inh	C1-inhibitor
CIfA	Clumping factor A
CHIPS	Chemotaxis inhibitory protein of staphylococci
CPD	Carboxypeptidase D
CRP	C-reactive protein
CRDs	Carbohydrate-recognition domains
CUB	C1r/C1s-Uegf-bone morphogenetic protein
Cul-3	Cullin-3
DIC	Disseminated intravascular coagulation
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal growth factor
G-CSF	Granulocyte colony-stimulating factor
HarA	Staphylococcal haptoglobin receptor A
HB	Haemoglobin
HDL	High-density lipids
HGF	Hepatocyte growth factor
HP	Haptoglobin
HPr	Haptoglobin-related protein
HPV	Human papillomavirus

HTS	Heme transporting system
HuPON1	Human serum paraoxanase
ICAM-1	Intercellular adhesion molecule-1
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-	Interleukin
IFN-γ	Interferon-gamma
IFNs	Interferons
ITAM	Immunoreceptor tyrosine-based activation motifs
KDa, kd	Kilo Dalton
K_D	Dissociation constant
KIRs	Killer-cell immunoglobulin-like receptors
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GIcNAc	N-acetylated glucosamine
LDL	Low density lipoprotein
LPS	Lipopolysaccharides
LRR	Leucine rich-repeats
LTA	Lipoteichoic acid
MALDI-TOF-MS	Matrix assisted laser desorption ionisation time-of-flight mass spectrometry
M2BP	Mac 2 binding protein
MASP	Mannose-binding lectin associated serine protease
MIP-1α	Macrophage inflammatory protein 1 alpha
MMP	Matrix metalloproteinases
Map 19	19kDa – mannose-binding lectin associated protein
MBL	Mannose-binding lectin
NF-KB	Nuclear factor Kappa B
NK cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain

PAGE	Poly-acrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate Buffered Saline
PMNs	Polymorphonuclear neutrophils
pkd2	Polyccytin-2
PRRs	Pathogen-recognition receptors
PspC	Pneumococcal surface protein C
pNPP	p-nitrophenyl phosphate
PVDF	Polyvinylidene Fluoride
PVL	Panton-valentine leukocidin
RTK	Receptor tyrosine kinases
SAA	Serum amyloid A
SAP	Serum amyloid P-component
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SR	Scavenger receptors
SP-A	Pulmonary-surfactant protein-A
SP-D	Pulmonary-surfactant protein-D
StbA	Staphylococcal transferrin binding protein A
IsdA	Iron-regulated surface determinant protein A
TCRs	T cell antigen receptors
TGF- β	Transforming growth factor β
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TPCK	N-tosyl-L-phenylalanyl chloromethyl ketone
TRP	Transient receptor potential
VCAM-1	Vascular cell adhesion molecule-1

Chapter 1
General Introduction

Bacterial infections can cause severe tissue damage and organ malfunction. Multiple synergetic activities between the bacterial cell wall components, such as lipopolysaccharides (LPS), lipoteichoic acids (LTA), peptidoglycans, superantigens and many host-derived agents of the immune system are believed to be associated with such abnormalities (Ginsburg, 1999, Ginsburg, 2001, Ginsburg and Kohen, 1995, Periti, 2000, Glauser, 2000). LTA, a cell wall component of Gram-positive bacteria, triggers immune and inflammatory responses in the mammalian host. Different plasma proteins have been found to interact with LTA to initiate such responses. For example, LTA binds specifically to L-ficolin to initiate the lectin pathway of the complement system (Lynch et al., 2004). In the following studies, novel host plasma proteins capable of interacting with LTA have been identified and studied to further unlock the immune and inflammatory mechanisms initiated by Gram-positive bacteria. Moreover, the role of one of these proteins, haptoglobin, in host defence will be assessed.

1.1 The human immune system

The main function of the immune system is to protect the host against infectious microorganisms in its milieu. Different protective mechanisms are employed to contain and eliminate these pathogens. Distinctive structural features of pathogens are

recognized by the immune system to remove them before excessive damage is caused to host tissues (Chaplin, 2006). Two general responses are employed to detect invading pathogenic structures: **innate and adaptive immune responses.** This project focuses on innate immunity, which is discussed in more detail below.

1.2 Innate immune responses (innate immunity)

Innate immunity is phylogenetically the oldest machinery for protection against invading pathogens, and co-evolved alongside these pathogens to provide protection from infection. The innate machinery is present in the host prior to any microbial encounter and is the first response against microbes to prevent, control and eliminate infection of the host. The innate immune system is composed of epithelial barriers, circulating and tissue cells, and secreted proteins. Properties and functions of such key components of innate immunity are described in the following sections.

1.3 Epithelial barriers

Intact epithelial surfaces form physical barriers between invading pathogens in the external milieu and host tissues. The skin and the mucosal surfaces of the gastrointestinal and respiratory tracts are the main physical barriers between the host and the environment. In each case, they are protected by continuous epithelia that block the entrance of microorganisms. Epithelia and some leukocytes secrete peptides with antimicrobial properties such as defensins and cathelicidins (Bulet et al., 2004, Dommett et al., 2005, Selsted and Ouellette, 2005).

Defensins are cationic peptides with 29 to 34 amino acids and contain three intrachain disulfide bonds. The positions of these bonds characterize three different types of defensins; α, β, and Φ. Defensins are expressed by epithelial cells of mucosal surfaces and some leukocytes such as neutrophils, cytotoxic T lymphocytes and natural killer

(NK) cells. α-Defensins are mainly produced by Paneth cells within the crypts of the small bowel and secreted in response to cholinergic stimulation or when exposed to bacterial antigens. Mitotically active crypt cells that renew the epithelial cell monolayer are subsequently protected from colonization by potentially pathogenic microbes. α-Defensins may also bind to bacteria in the intestinal lumen above the crypt–villus boundary and modify the composition of the enteric microbial flora (Ouellette, 2004). The other defensins are produced by respiratory mucosal cells and in the skin. All defensins are directly toxic to microbes such as bacteria and fungi and activate cells of the inflammatory response to target the invading pathogens, although the mechanism underlying this toxicity is yet to be characterized (Bulet et al., 2004, Dommett et al., 2005, Ouellette, 2004, Selsted and Ouellette, 2005).

Cathelicidins are produced by neutrophils and epithelial cells of mucosal surfaces such as skin, gastrointestinal mucosal cells and respiratory mucosal cells. They are synthesized as two-domain precursor proteins that are proteolytically cleaved into two peptides, each of which possesses antimicrobial activity. The C-terminal fragment known as LL-37 has two leucine residues at its N-terminus that are important for toxicity against a range of pathogens. LL-37 can target and neutralize toxic membrane components of Gram-negative bacteria such as LPS and induce monocyte, neutrophil and mast cell chemotaxis (Gudmundsson and Agerberth, 1999, Agerberth et al., 2000, Ayabe et al., 2000, Niyonsaba et al., 2002). The antimicrobial role of the other cathelicidin precursor fragment is yet to be well defined (Bulet et al., 2004, Dommett et al., 2005).

As microorganisms cross epithelial barriers, they can also be attacked by certain types of lymphocytes such as intraepithelial T lymphocytes and the B-1 subset of B

lymphocytes found in barrier epithelia and serosal cavities respectively (Agerberth et al., 2000, Dommett et al., 2005, Nogueira-Martins and Mariano, 2009, Komori et al., 2006). The majority of T and B lymphocytes (T and B cells) have a highly diverse range of specificities for different antigens arising through somatic recombination of germline DNA segments and variation of nucleotide sequences at the junction between the recombined segments. However, these subsets show relatively little diversity, because the same DNA segments are recombined in each clone and there is little or no variation of junctional sequences, and target common molecular features of invading pathogens often called pathogen-associated molecular patterns (PAMPs) on the surface of pathogens. Like other T and B cells, they display antigen receptors on their cell surfaces and have similar effector activities. However, the limited nature of the specificities of their antigen receptors classifies them into a separate group that is more similar to effectors cells of innate immunity than adaptive immunity.

Intraepithelial T lymphocytes are found in mucosal epithelia and in the epidermis of the skin and are characterized by their expression of distinguishable T cell antigen receptors (TCRs). For example, some T cells in epithelia produce an antigen receptor known as the γδ TCR that targets peptide and non-peptide antigens (Komori et al., 2006). Intraepithelial lymphocytes may also participate in host defence by producing cytokines to activate phagocytes and eliminate infected cells (Komori et al., 2006). B-1 lymphocytes are found in the peritoneal cavity, and similar to all B lymphocytes, carry immunoglobulin molecules as antigen receptors. However, like the intraepithelial T lymphocytes, these are limited with regard to their diversity compared to other classes of B cells. Many produce immunoglobulin M (IgM) antibodies specific for polysaccharide and lipid antigens including phosphorylcholine and LPS found in Gram-negative bacteria (Paciorkowski et al., 2000, Nogueira-Martins and Mariano, 2009).

These antibodies are known as natural antibodies and provide protection against microorganisms that have succeeded in penetrating epithelial barriers (Nogueira-Martins and Mariano, 2009).

Mast cells can also be found under epithelia and in serosal cavities. These cells express cytokines and lipid mediators to stimulate inflammation and eliminate invading pathogens. Figure 1.1 shows a schematic representation of the defence mechanisms mediated by epithelial barriers.

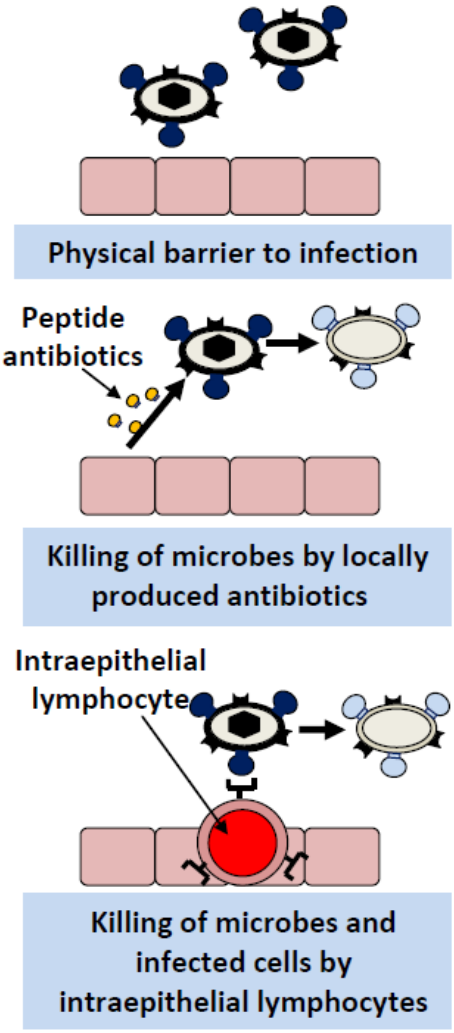

Figure 1.1 Epithelial barriers. Epithelia provide physical barriers that block microbial entry into the host. Antimicrobial chemicals are secreted, and intraepithelial lymphocytes are stimulated to eliminate invading microbes and infected cells. Figure was reproduced from Hooper (2009).

1.4 Receptors of the innate immune system

Components of the innate immune response are encoded by genes in the germline of the host and detect PAMPs. These molecular patterns are not present in the mammalian host so provide a means for distinguishing self from non-self (Janeway and Medzhitov, 2002, Akira et al., 2006). The following figure exemplifies the innate immune response specificity for PAMPs.

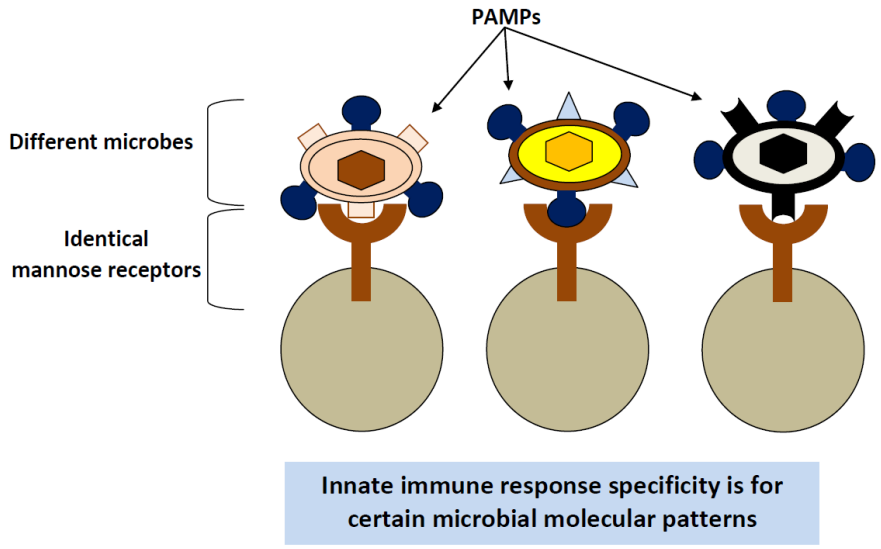

Figure 1.2 The specificity of innate immunity. Components of the innate immune response detect common molecular features of invading pathogens known as pathogen-associated molecular patterns (PAMPs). The mannose receptor is an example of an endocytic pathogen-recognition receptor (PRR). The receptor typically recognizes multiple sites on the pathogen. Figure was modified from Brown (2006).

Different pathogens (including viruses, Gram-negative bacteria, Gram-positive bacteria and fungi) display different PAMPs. PAMPs include microbial nucleic acids, such as double-stranded RNA found in replicating viruses or unmethylated CpG DNA sequences found in bacteria, complex lipids and carbohydrates including LPS in Gram-negative bacteria and teichoic acids in Gram-positive bacteria (Akira et al., 2006, Janeway and Medzhitov, 2002, Meylan et al., 2006, Kawai and Akira, 2006). The innate immune machinery identifies microbial products that are fundamental for microbial survival, thereby preventing the invading pathogen from discarding these targets in an

attempt to escape recognition by the host. For example, LPS and teichoic acid are essential structural components for bacterial survival, so cannot be discarded or easily modified (Akira et al., 2006). The following table lists some of the molecular components of microbial organisms that are recognized by the innate immune response.

Molecular component	Type of organism
Nucleic acids	
Double-stranded RNA (dsRNA)	Viruses
CpG-containing DNA (CpG DNA)	Bacteria, fungi, viruses
Cell wall components	
Lipopolysaccharide	Gram-negative bacteria
Lipoteichoic acids	Gram-positive bacteria
Peptidoglycan	Bacteria
Flagellin	Gram-negative bacteria
Lipoproteins	Bacteria
Β-glucans	Bacteria, fungi
Mannose-, fucose-rich polysaccharides	Bacteria, fungi
Membrane properties	
Exposed acidic phospholipids	Bacteria, fungi (apoptotic cells)
Biosynthetic components	
N-formylated methionyl peptides	Bacteria

Table 1.1 Molecules recognized by the innate immune system. Components of the innate immune response detect common molecular features of invading pathogens (PAMPS). Selected examples of these molecular patterns are listed above. Table was modified from Aderem and Ulevitch (2000).

Host receptors called pathogen-recognition receptors (PRRs) are able to bind PAMPs on invading bacteria to trigger immune responses (Beutler, 2003, Beutler et al., 2003, Chaplin, 2006). Examples of PRRs include endocytic PRRs such as mannose receptors, scavenger receptors and N-formyl methionine receptors and signaling PRRs including toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors (Figure 1.3).

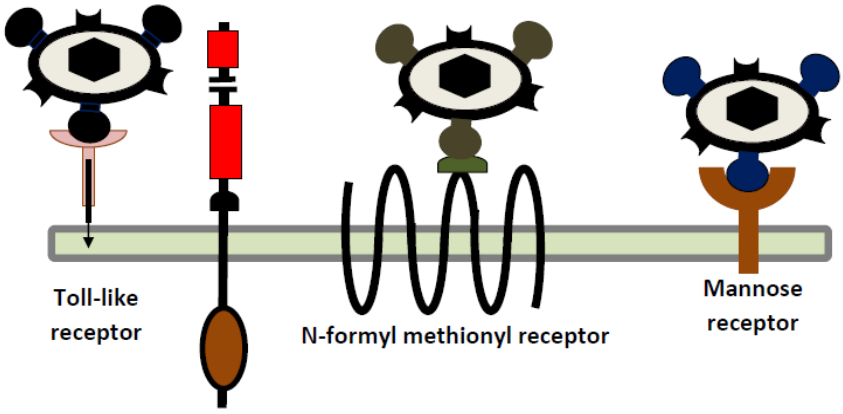

Figure 1.3 Examples of pathogen recognition receptors (PRRs). Endocytic PRRs include mannose receptors and N-formyl methionine receptors and signalling PRRs include toll-like receptors. Figure was modified from Trinchieri and Sher (2007).

PRRs are expressed by a variety of host cells including neutrophils, macrophages and dendritic cells, discussed in more detail later in this chapter. They are generally located on the cell surface, in endosomal membranes or in the cytoplasm, and target pathogens in these locations (Janeway and Medzhitov, 2002, Akira et al., 2006, Meylan et al., 2006, Kawai and Akira, 2006). Figure 1.4 illustrates some of the PRRs in the innate immune system.

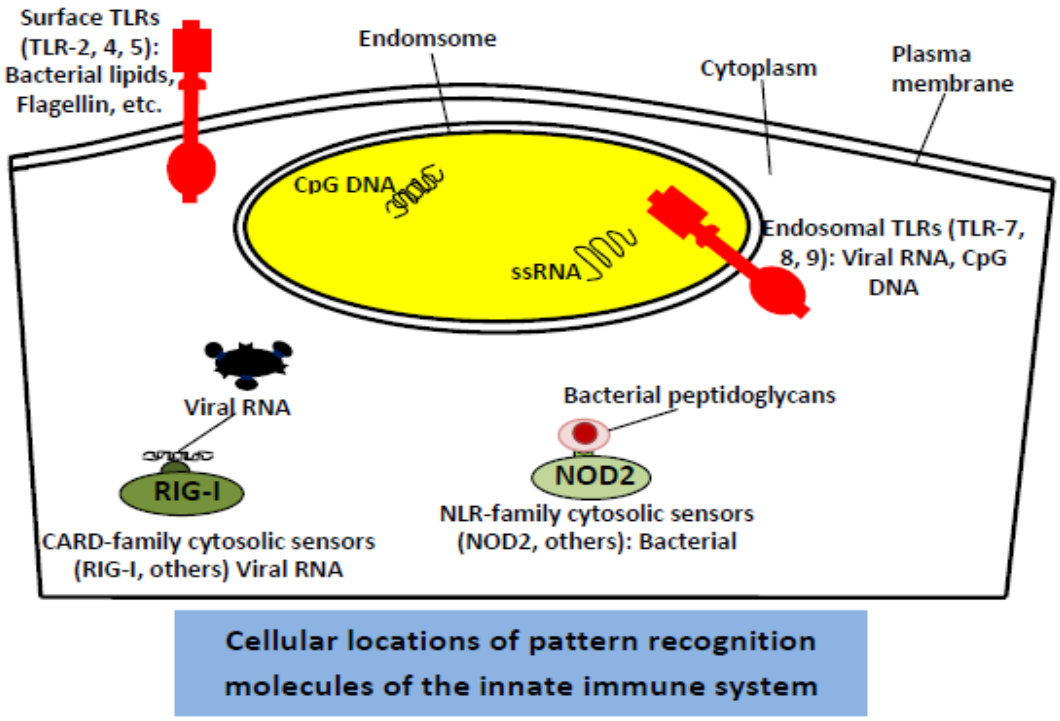

Figure 1.4 Cellular locations of pathogen recognition receptors (PRRs) of the innate immune system. PRRs of the TLR family (TLRs 2, 4 and 5) are expressed on cell surfaces, where they bind PAMPs. Other TLRs (TLRs 3, 7, 8 and 9) are located on endosomal membranes and recognize the nucleic acids of phagocytosed microbes. Cytoplasmic sensors of microbial infections such as, the NLR family of proteins recognize bacterial peptidoglycans and a subset of CARD family proteins binding viral RNA, are found in the cytoplasm of the cell. Figure was modified from Trinchieri and Sher (2007).

1.5 Effector cells of the innate immune system

The most common effector cells of the innate immune system are bone marrow-derived cells that are found in tissues or circulating in blood. The bone marrow is the site generating almost all the circulating blood cells by a process called hematopoiesis. Hematopoiesis is initiated in blood islands of the yolk and the paraaortic mesenchyme and expands to the liver and spleen at later stages (Palis and Yoder, 2001). The following diagram illustrates the development of the different lineages of blood cells by hematopoiesis.

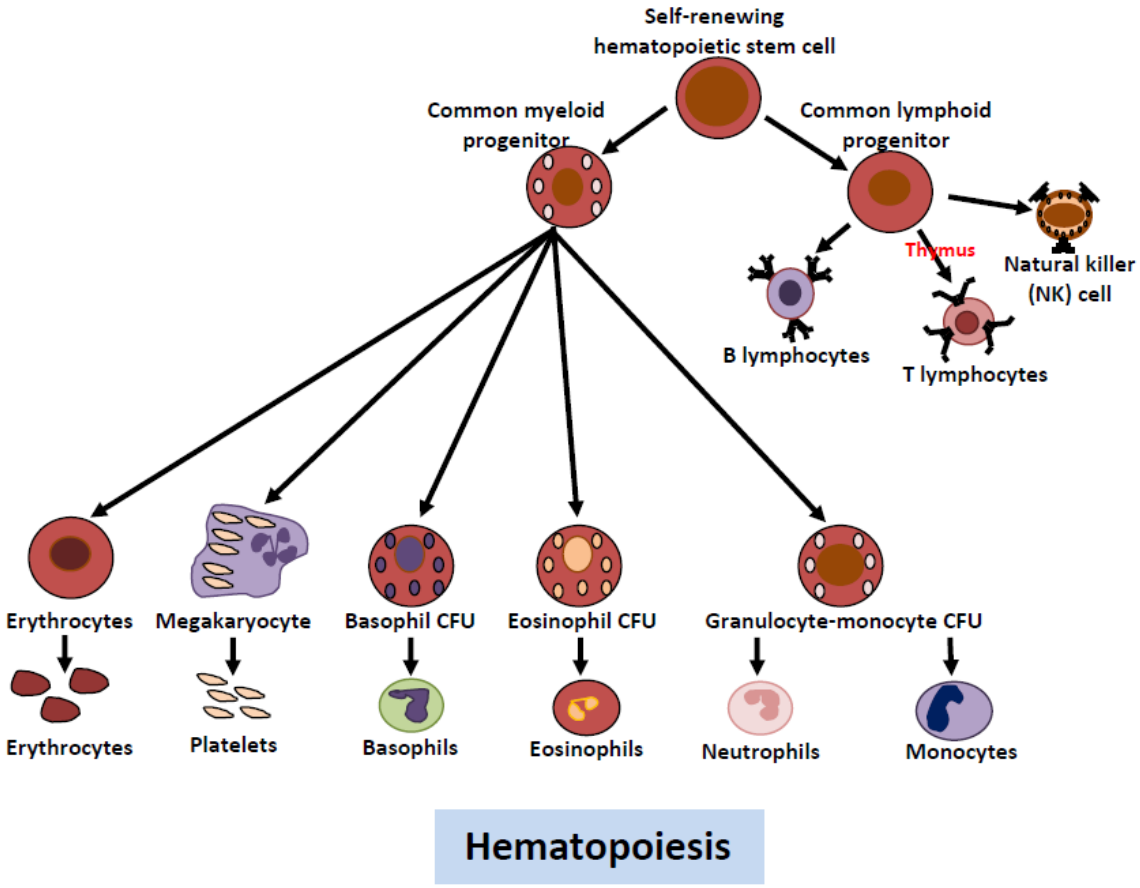

Figure 1.5 Hematopoiesis. Hematopoiesis generates all the different blood cells. These cells are derived from myeloid or lymphoid progenitors. Figure was modified from Hoang (2004).

Effector cells can be myeloid lineage such as neutrophils, monocytes, macrophages and myeloid dendritic cells or lymphocyte lineage such as NK cells. The number of each cell type can vary dramatically in healthy individuals (Orfanakis et al., 1970, Simmons et al., 1974, Weissman and Shizuru, 2008, England and Bain, 1976). Table 1.2 summarizes this variability, showing the typical range of cell numbers in the blood of healthy donors.

Cell Type	Normal Range per microliter
White blood cells (leukocytes)	4000-11,000
Neutrophils	1500-8000
Eosinophils	0-500
Basophils	0-250
Lymphocytes	1000-5000
Monocytes	0-900

Normal blood cell counts

Table 1.2 Normal blood cell counts. The number of cells varies dramatically in blood from a cell type to another.

In the next section, the properties and functions of the most common innate effector cells are discussed.

1.6 Neutrophils (polymorphonuclear neutrophils or PMNs)

PMNs are fundamental for an effective host response to bacteria as demonstrated by the morbidity and mortality rates of individuals carrying reduced numbers of healthy PMNs. For example in one study, the clinical morbidity rate (corresponding to the number of new cases per unit of person-time at risk) caused by infection, increased dramatically in patients with peripheral blood counts of <500 granulocytes/ml compared to control groups. Such patients experienced an average of 43 episodes of severe infection for every 1000 days examined (Bodey et al., 1966). Furthermore, patients with inherited disorders leading to low levels of PMNs tend to experience regular bacterial infections due to the absence of sufficient PMN numbers to eliminate such invading pathogens (Malech and Nauseef, 1997).

PMNs are produced in the bone marrow from a lineage common with mononuclear phagocytes (Figure 1.5). Production is stimulated by granulocyte colony-stimulating

factor (G-CSF) (Layton, 1992). Neutrophil recruitment and activation is one of the first responses to infection. PMNs are the most abundant type of granulocytes, which are leukocytes with granules in their cytoplasm. Figure 1.6 shows the key features of PMNs. The granules of mature neutrophils carry a wide range of anti-bacterial host defence proteins (Borregaard and Cowland, 1997) such as β2 integrins (Sengelov et al., 1993), the formyl-peptide receptor (Sengelov et al., 1994) and a variety of different proteases (eg, elastase and cathepsins) (Nauseef, 2007). During neutrophil stimulation, the granules fuse with nascent phagosomes. As a result, their toxic contents are released setting off a complex series of dynamic events between components of the host phagosome, the released toxic proteins, and other granule proteins eventually leading to destruction of the ingested bacteria (Nauseef, 2007).

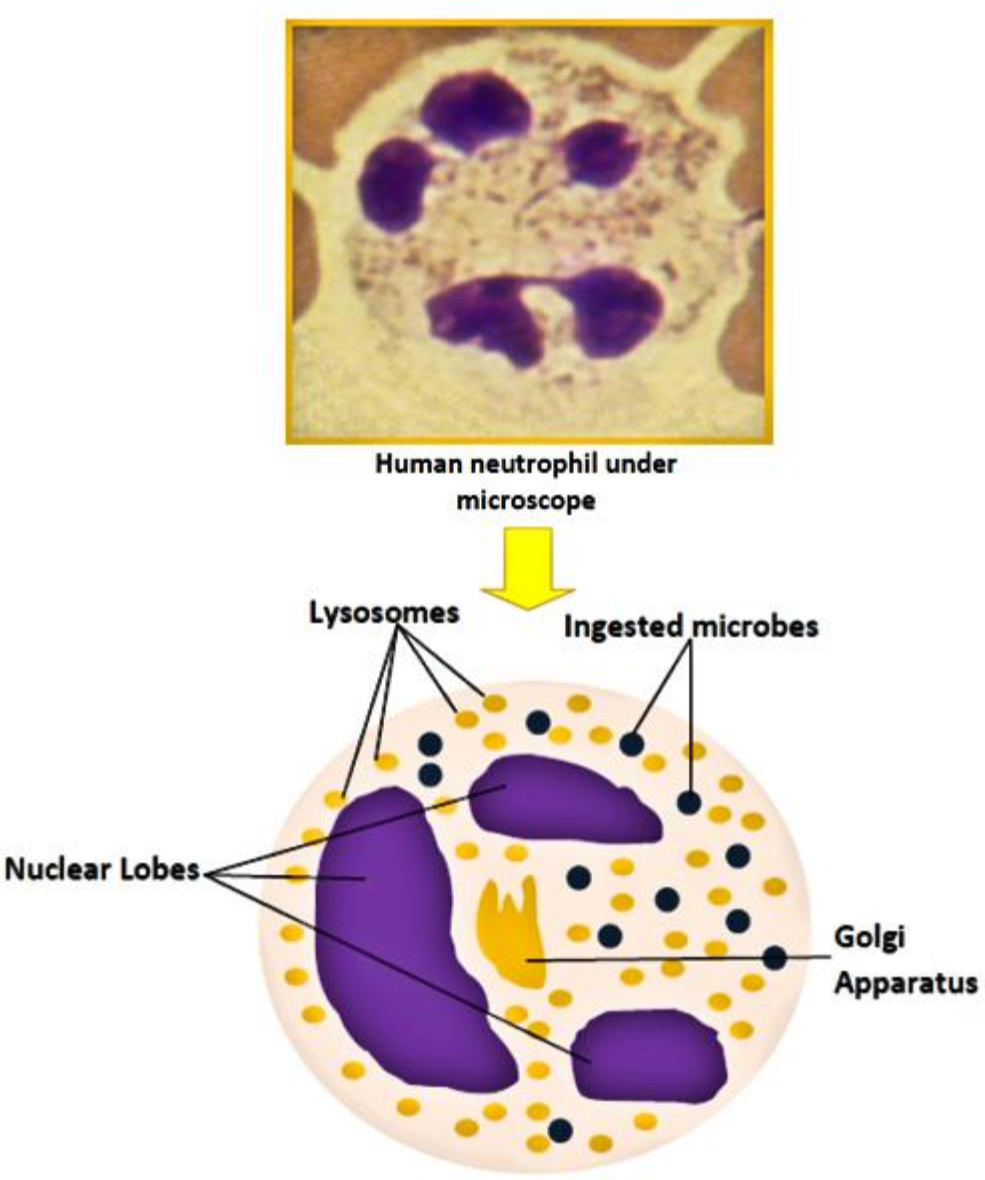

Figure 1.6 Neutrophils. Neutrophils are one of the first lines of defence in the immune response. They carry granules (nuclear lobes) within their cytoplasm. They also contain a Golgi apparatus and lysosomes that participate in the digestion of invading microbes. Microscopic image was acquired using optical microscopy (100X).

1.7 Monocytes

The term "monocyte" describes a particular, homogenous population of cells with the same physiology (Strauss-Ayali et al., 2007). Monocytes count for around 5-10% of human peripheral blood leukocytes (Tacke and Randolph, 2006). They are produced by a myeloid precursor in the bone marrow before circulating in the blood, and enter different tissues upon activation where they become macrophages (Fogg et al., 2006, van Furth and Cohn, 1968). They can survive up to 3 days in human blood (Whitelaw, 1972). Human monocytes are classified according to their differential expression of CD14 and CD16 receptors into two major subsets: classical ($CD14^{+}CD16^{-}$) and non-classical ($CD14^{low}CD16^{+}$). Classical ($CD14^{+}CD16^{-}$) monocytes represent around 95% of the total number of monocytes in a healthy individual whereas non-classical count for just 5% (Passlick et al., 1989, Ziegler-Heitbrock et al., 1993). Monocytes increase in numbers more gradually and remain at the site of infection for a longer period of time compared to neutrophils (Dale et al., 2008). They are known to have three main functions: antigen presentation, immunomodulation and phagocytosis. Immunomodulation describes the release of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) to contribute in the regulation of hematopoiesis (Taylor et al., 1998). Phagocytosis is performed by monocytes to ingest and destroy invading bacteria and to remove waste products (Dale et al., 2008). For example, insoluble haemoglobin inclusion bodies from red blood cells are filtered by the spleen and are removed by mononuclear phagocytes including monocytes (Kashimura and Fujita, 1987, Geske et al., 2002). Figure 1.7 shows the common structural features of a human monocyte.

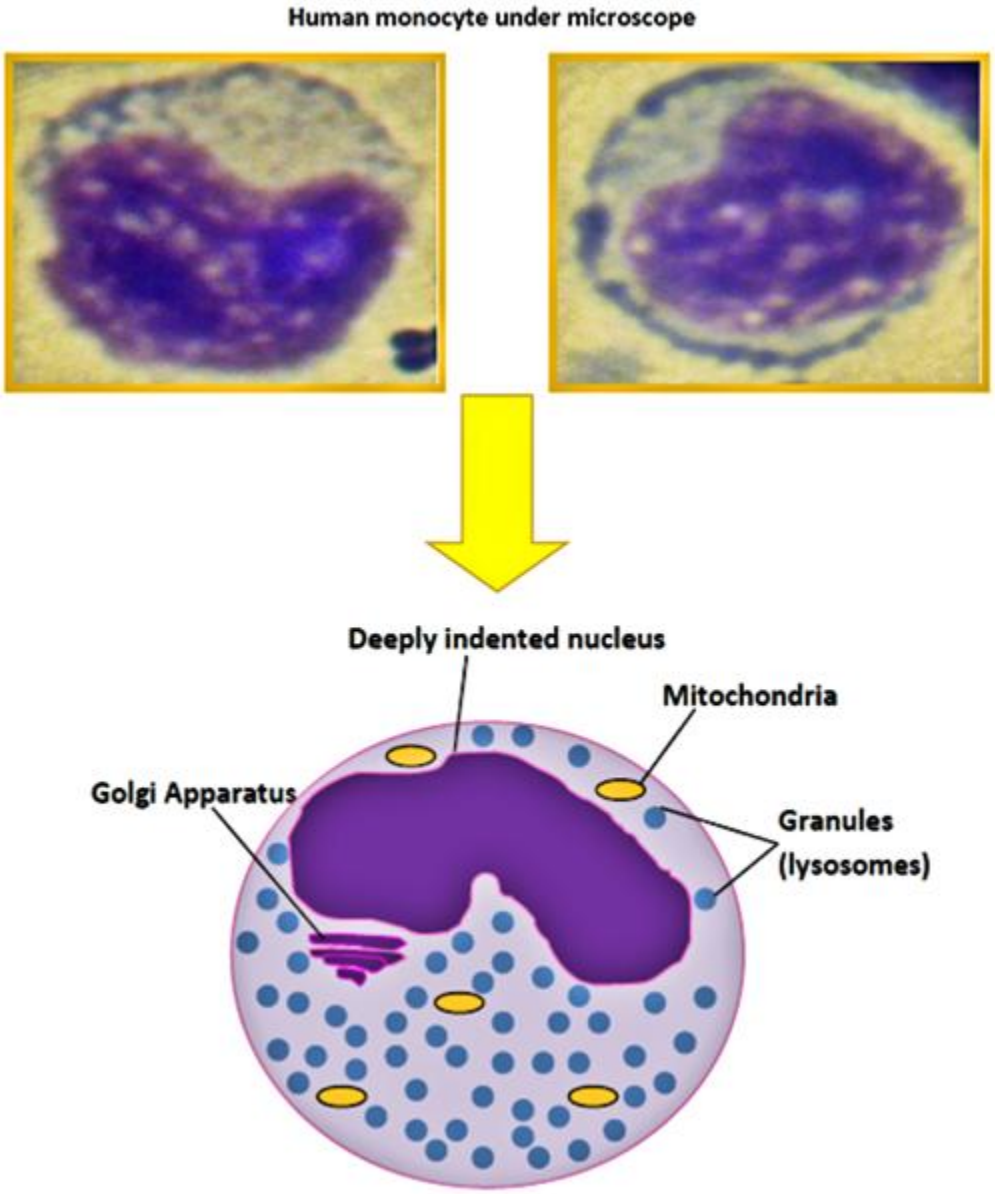

Figure 1.7 Monocytes. Monocytes count for around 5-10% of human peripheral blood leukocytes. The nucleus is kidney shaped with a deep indentation. The cytoplasm has a ground glass appearance due to the presence of fine granules. Microscopic images were acquired using optical microscopy (100X).

1.8 Macrophages

Marrow and blood monocytes can differentiate into tissue-localized phagocytic cells known as histiocytes in the spleen, liver, lung and other tissues (Dale et al., 2008, Leonarduzzi et al., 2005). Histiocytes include macrophages and dendritic cells. Macrophages may emerge in different morphological shapes. For example, they can develop an abundant cytoplasm as in epithelioid cells which resemble epithelial cells from the skin. Activated macrophages can combine to form multi-nucleate giant cells (fused macrophages). The following figure shows common structural features of human macrophages.

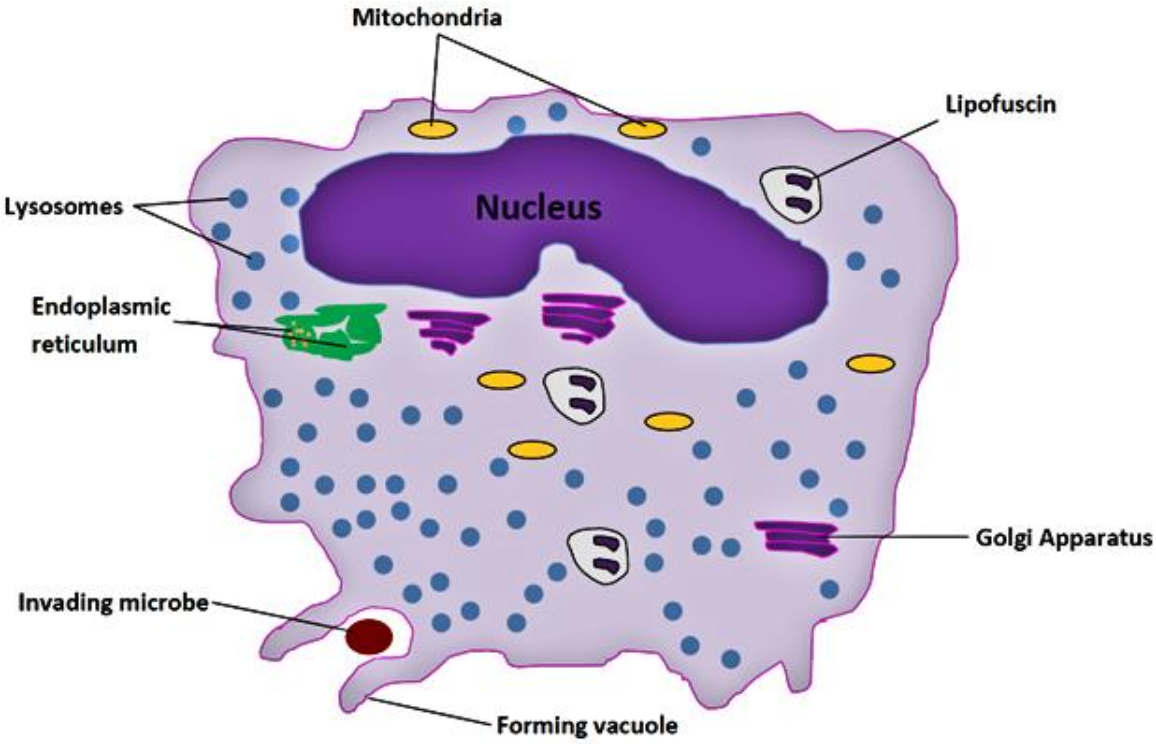

Figure 1.8 Macrophages. Macrophages are derived from monocytes. They are found in organs and tissues. They are mostly large with a nucleus, and abundant cytoplasm. Figure was modified from Rosenberger and Finlay (2003).

Macrophages are found in various tissues where they are often given different names. For example, macrophages in the central nervous system are known as microglial cells; whereas they are called Kupffer cells in the vascular sinusoids of the liver; alveolar

macrophages in pulmonary airways and osteoclasts in bone (van Furth et al., 1972). They contribute metabolic, immunological and inflammatory responses (Naito, 2008) as shown in Figure. 1.9.

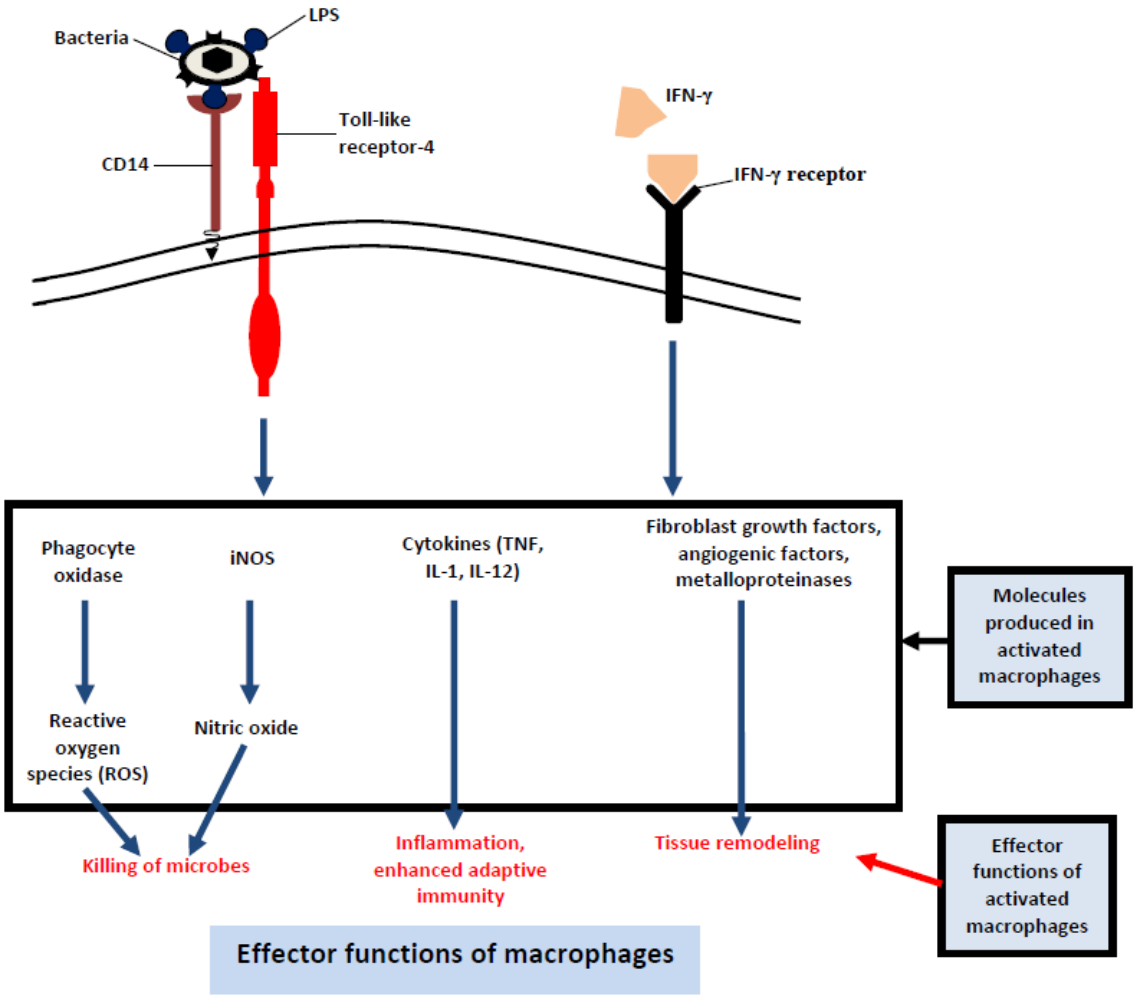

Figure 1.9 Effector functions of macrophages. Macrophages are activated by microbial components such as LPS and NK cell-derived Interferon-gamma (IFN-γ). Consequently, the transcription factors of various genes are activated and proteins mediating the functions of macrophages cells are produced. Figure was modified from Rosenberger and Finlay (2003).

Macrophages are major participants in the elimination of pathogens and of apoptotic cells during infection (Lopes et al., 2000). For example, receptors on the surface of macrophages such as CD14 facilitate the removal of Gram-negative bacteria by recognizing LPS molecules on the surface of these pathogens (Naito, 2008).

Macrophages react to microbes almost as rapidly as neutrophils do, but they tend to survive longer and undergo cell division at sites of inflammation. Macrophage responses can sometimes cause tissue damage, which challenges the immune response to contain and minimise it (Bogdan et al., 1997). For example, regulatory cytokines such as interleukin-10 (IL-10) and transforming growth factor β (TGF- β) are employed to reduce the effects of macrophage-induced inflammatory cytokines, interleukin-12 (IL-12), TNF-α and interferon-gamma (IFN-γ) (Mosmann and Sad, 1996, Gazzinelli, 1996). In addition, interleukin 4 (IL-4) and interleukin 6 (IL-6) are released to induce macrophage apoptosis (Naito et al., 1996, Mangan et al., 1993, Mangan et al., 1992).

1.9 Opsonization

As described above, PRRs-mediated recognition of PAMPs is one of the major ways that macrophages target and eliminate pathogens (Taylor et al., 2005). Opsonization presents an alternative platform for recognition (Stuart and Ezekowitz, 2005). Opsonins are specific host proteins that coat microbes or endogenous macromolecules targeted for elimination and are subsequently recognized by phagocytic receptors. They include immunoglobins, collectins and complement components. Binding of immobilized opsonins to receptors on phagocytes enhances phagocytosis (Aderem and Underhill, 1999, Underhill and Ozinsky, 2002). For example, elevated haemolysis releases free haemoglobin in plasma where it is captured by the acute-phase protein haptoglobin. The resulting complex subsequently binds to the macrophage receptor CD163 after which it is removed by phagocytosis (Kristiansen et al., 2001). Complement proteins deposited on bacteria, such as C3b, also engage receptors on macrophages as well as a variety of other cell types to stimulate phagocytosis (van Lookeren Campagne et al., 2007). Thus, opsonization is a crucial process by which host-recognition molecules contribute to

pathogen elimination. Some of the opsonic receptors on macrophages and other phagocytes are shown in Table 1.3.

Receptor	Type	Expression	Ligands
CR3 (αMβ2, CD11b/CD18)	Integrin	Macrophages, neutrophils	IC3b, β-glucans, ICAM- 1/2
CR4 (αxβ2, CD11C/CD18)	Integrin	Macrophages, neutrophils, dendritic cells	IC3b, fibrinogen
FcγRI (CD64)	Ig, ITAM	Macrophages, neutrophils	IgG, CRP, SAP
FcγRII (CD32)	Ig, ITAM	Macrophages, neutrophils	IgG
FcγRIII (CD16)	Ig, ITAM	Macrophages, neutrophils, natural killer cells	IgG, SAP
FcγRIV	Ig, ITAM	Macrophages, neutrophils, dendritic cells	IgG2A, IGg2b
FcαR (CD89)	Ig, ITAM	Macrophages, neutrophils, eosinophils	IgA
Integrin αvβ3	Integrin	Macrophages	Thrombospondin-opsonised cells
Mer	RTK	Macrophages	Apoptotic cells

Opsonic receptors

Table 1.3 Opsonic receptors. Phagocytes express different type of opsonic receptors that initiate phagocytosis. These receptors can be C-type lectin receptors, integrins, Fc receptors (ITAM; immunoreceptor tyrosine-based activation motifs, Ig; immunoglobulin) and receptor tyrosine kinases (RTK). Table was modified from Aderem and Ulevitch (2000).

1.10 Dendritic cells

Dendritic cells contribute significantly to innate immune responses and help to connect innate and adaptive immune responses during infection (McKenna et al., 2005). These cells are characterized by their phagocytic abilities and their extended membranous projections (Reis e Sousa et al., 1993). They can be divided into immature and mature populations. Immature dendritic cells are present within epithelia where their main function is to capture and transport antigens to lymphoid organs. They become mature after encountering microbes and make vital contributions to the adaptive immune system by interacting with and activating T and B cells in lymphoid organs to initiate and shape the immune response. Lymphoid and myeloid dendritic cells are generated from lymphoid and myeloid precursors respectively and thus are of haemopoetic origin. By contrast follicular dendritic cells are possibly of mesenchymal origin. Dendritic cells carry pattern recognition receptors on their surface such as TLR family (TLRs 3, 4, 5, 7,

8 and 9), CD8 and CD11, and secrete cytokines in response to infection such as IL-6, IL-12 and type I interferons (IFNs) (see Cytokines of the innate immune system, section 1.21). For example, dendritic cells respond to endocytosed viruses by producing IFNs that directly inhibit viral replication in infected cells (Kadowaki et al., 2000, Fonteneau et al., 2003, Gary-Gouy et al., 2002).

1.11 Natural Killer (NK) cells

NK cells count for 5% to 20% of the mononuclear cells in the blood and spleen, and are rarely found in other lymphoid organs. They are called natural killer cells because of their ability to kill target cells without prior stimulation. Besides their ability to kill infected cells directly, they secrete IFN-γ that activates macrophages to eliminate invading pathogens. NK cells are produced in the bone marrow and emerge as large lymphocytes containing cytoplasmic granules. NK cell recruitment and responses are regulated by activating and inhibitory receptors on their surfaces. These receptors contain ligand-binding subunits that bind molecules on the surface of other cells and signalling subunits that transform activating or inhibitory signals to the cell. Inhibitory signals block the activating signals and prevent NK cells from killing uninfected cells (Biron et al., 1999). NK receptors mainly interact with class I MHC molecules or with proteins that are structurally homologous to class I MHC molecules such as UL18 encoded by Cytomegalovirus. Activating receptors bind class I MHC-peptide ligands typically presented on stressed cells, virally infected cells, malignantly transformed cells or cells containing intracellular microbes. NKG2D is one of the better characterized activating receptors that interacts with proteins that are structurally homologous to class I MHC molecules on virally infected cells and tumour cells. Other activating receptors such as CD16 (also known as FcγRIIIa) are low affinity receptors that target the Fc parts of IgG1 and IgG3 antibodies and enable NK cells to kill cells

coated with antibody molecules (Lewis et al., 2002). On the other hand, inhibitory receptors interact with class I MHC molecules that are regularly expressed on uninfected cells. These receptors can then trigger phosphatase-dependent signalling cascades that inhibit the effect of kinases activated by activating receptors. Moreover, inhibitory receptors are also characterized by their specificity for self-class I MHC, which prevents the killing of uninfected host cells. However, when a host cell becomes infected, the inhibitory response decreases as a result of reduced production of class I MHC molecules. As a consequence, NK cells become active and eliminate the infected cell (Lewis et al., 2002). A variety of different inhibitory receptors have been characterized including the killer-cell immunoglobulin-like receptors (KIRs) and CD94/NKG2A; KIRs are known to interact with various class I MHC molecules whereas CD94/NKG2A bind a specific class I MHC molecule known as HLA-E (Lewis et al., 2002). NK cell stimulation can also be regulated by cytokines such as IL-15 and IL-12. IL-15 is mainly secreted by macrophages and acts as a growth factor for NK cells whereas IL-12 is also derived from macrophages and enhances NK cell activity to kill cells coated with antibodies (Lewis et al., 2002, Bottino et al., 2005).

The main functions of NK cells are to eliminate infected cells and stimulate macrophages to destroy ingested microorganisms. Figure 1.10 illustrates these two functions.

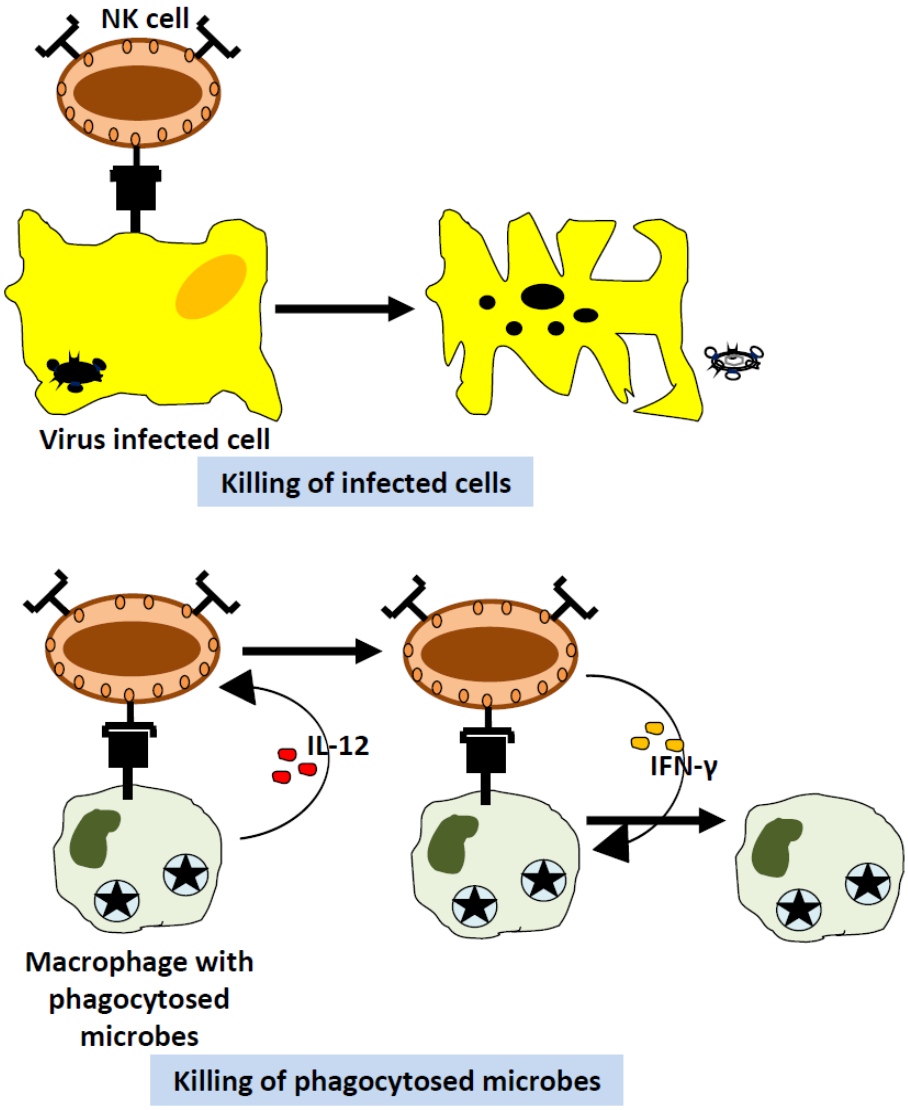

Figure 1.10 Functions of NK cells. Killing of infected cells: NK cells bind ligands on infected cells and destroy the host cells. Thus, NK cells succeed to remove infection and dysfunctional cells. **Killing of phagocytosed microbes:** NK cells are stimulated to produce IFN-γ by IL-12 derived from macrophages. IFN-γ activates macrophages to destroy phagocytosed microbes. Figure was modified from Moretta (2002).

NK cells kill target cells via the action of granules carrying an array of bactericidal proteins. These proteins are released next to target cells when NK cells become activated. One of the granule proteins known as perforin supports the access of other granule proteins, known as granzymes, into the cytoplasm of target cells to activate apoptosis. NK cells contribute significantly in the elimination of intracellular microbes. They are activated at the early stage of viral infections by cytokines of the innate

immune response such as IL-12 and IL-15 to kill infected cells (Bottino et al., 2005, Lewis et al., 2002). As mentioned previously, NK cells release IFN-γ cytokine that stimulates macrophages to destroy ingested microbes. This response is able to contain infections by intracellular bacteria such as *Listeria monocytogenes* for many days or even weeks and provides the adaptive immune system with an adequate amount of time to develop and effective response (Unanue, 1996, Jin et al., 2001).

1.12 Mast cells and Basophils

Mast cells are generated from progenitors in the bone marrow and mature after migrating into the peripheral tissues (see Table 1.4). They are found throughout the body particularly underneath epithelia, in lymphoid organs and in the vicinity of blood vessels and nerves (Galli et al., 2005, Metz et al., 2007, Pulendran and Ono, 2008). Human mast cells appear in different shapes and carry membrane-bound granules and lipid bodies in their cytoplasm. They are classified according to their anatomical locations, granule contents and activities into two main subsets: mucosal and connective tissue mast cells. Mucosal mast cells are regularly found in the mucosa of the gastrointestinal tract and carry relatively low-levels of histamine and abundant amounts of chondroitin sulfate in their granules. The growth of these mast cells is commonly regulated by the cytokine IL-3 secreted by T lymphocytes (Haig et al., 1988). Connective tissue mast cells are found in the lung and in the serosa of body cavities. They are known to secrete large amounts of histamine and contain mainly heparin (rather than chondroitin sulphate) in their granules (Nakano et al., 1985). Heparin and chondroitin sulphate function as storage matrices for positively charged biogenic amines and proteases within the granules of mast cells, and prevent their accessibility to the rest of the cell. Moreover, connective tissue mast cells are less dependent on T lymphocytes than mucosal mast cells (Kashiwakura et al., 2004). The different subsets

of mast cells are suggested to contribute significantly to different sets of diseases, but their distinctive functions are yet to be understood. For example, connective tissue mast cells are likely to initiate the immediate-hypersensitivity reactions associated with allergies. Environmental antigens activate mast cells to release mediators that increase vascular permeability, vasodilation and bronchial and visceral smooth muscle contraction (Sertl et al., 1988, Martin et al., 1993, Oshiba et al., 1996, Kobayashi et al., 2000, Fujita et al., 2001).

Basophils are blood granulocytes characterized by structural and functional features similar to mast cells. They are also produced in the bone marrow but from a different progenitor lineage to mast cells. After differentiating in the bone marrow, they circulate in the blood and are rarely recruited to inflammatory sites. Basophils represent less than 1% of blood leukocytes and secrete similar mediators to mast cells but rarely produce immediate-hypersensitivity reactions (see Table 1.4) (Prussin and Metcalfe, 2006).

IgE molecules specific to environmental antigens are captured on mast cells via FcεRI and basophils and trigger their activation during allergic reactions (Prussin and Metcalfe, 2006). Activation produces three responses: release of granular contents, synthesis and secretion of cytokines. Granules contain mainly serine proteases such as tryptase and chymase, which contribute to tissue damage in immediate-hypersensitivity reactions. Since tryptase can only be found in human mast cells, its presence in human biologic fluids is considered to be a marker for mast cell activation in general (Prussin and Metcalfe, 2006). Chymase is found only in certain subsets of human mast cells (connective tissue mast cells) and its presence is used as a marker for these subsets (Nagata et al., 2003). Activated mast cells and basophils also express a wide range of cytokines such as IL-1, IL-4, IL-5, IL-6, IL-13, MIP-1α and MIP-1β. Some of these cytokines induce immune responses against viral infections. For example, mast cells

and basophils are activated by HIV-1 glycoprotein 120 and secrete IL-4 and IL-13 that induce IgE production and the differentiation of naïve T helper cells (Florio et al., 2000, Marone et al., 2001, Prussin and Metcalfe, 2006). The characteristics of human mast cells, basophils and eosinophils are summarized in Table 1.4.

Characteristic	Mast cells	Basophils	Eosinophils
Major site of maturation	Connective tissue	Bone marrow	Bone marrow
Major cells in ciculation	No	Yes (0.5% of blood leukocytes)	Yes (-2% of blood leukocytes)
Mature cells recruited into tissues from circulation	No	Yes	Yes
Mature cells residing in connective tissue	Yes	No	Yes
Proliferative ability of mature cells	Yes	No	No
Life span	Weeks to months	Days	Days to weeks
Major develpment factor (cytokine)	Stem cell factor, IL-3	IL-3	IL-5
Expression of FcεRI	High levels	High levels	Low levels (function unclear)
Major granule contents	Histamine, heparin and/ or chondroitin sulfate, proteases	Histamine, chondroitin sulfate, protease	Major basic protein, eosinophil cationic protein, peroxidases, hydrolases, lysophospholipase

FcεRI, Fcε receptor type I; IL-3, interleukin-3

Table 1.4 Properties of mast cells, basophils and eosinophils.

1.13 Eosinophils

Eosinophils are derived from the bone marrow and circulate in the blood after maturation (see Table 1.4). They are normally found in peripheral tissues such as gastrointestinal and genitourinary tracts. The recruitment of eosinophils into tissues relies mainly on the chemokine eotaxin (CCL11), which is expressed by epithelial cells at sites of allergic reactions and targets the chemokine receptor CCR3 of eosinophils (chemokines are small polypeptide cytokines) (Prussin and Metcalfe, 2006). Eosinophils release toxic proteins from their granules that eliminate invading microbes.

Examples include lysosomal hydrolases and eosinophil-specific proteins which are toxic to helminth microbes. Moreover, eosinophilic granules produce peroxidises to stimulate the secretion of hypochlorous or hypobromous acids, which are toxic to helminths, protozoa and host cells (Prussin and Metcalfe, 2006, Aldridge et al., 2002, Klion and Nutman, 2004).

Activation of eosinophils also stimulates the release of lipid mediators such as prostaglandins and leukotrienes. The role of these lipid mediators is not completely characterized in immediate-hypersensitivity responses, but they are likely to participate in the development of allergic diseases (Klion and Nutman, 2004, Kay, 2005, Prussin and Metcalfe, 2006). Moreover, activated eosinophils produce a wide range of cytokines such as IL-3 and IL-4 that trigger different inflammatory responses, although the biological importance of these cytokines is yet to be fully characterized (Prussin and Metcalfe, 2006, Bochner, 2000).

1.14 Recruitment of leukocytes to sites of infection

Neutrophils and monocytes migrate from the blood to infected sites by interacting with adhesion molecules (such as selectins and integrins) on endothelial cells in response to chemoattractants released by host tissues following microbial invasion. Selectins, integrins, and chemokines orchestrate different steps in the recruitment of leukocytes to the sites of infection, as shown in Figure 1.11.

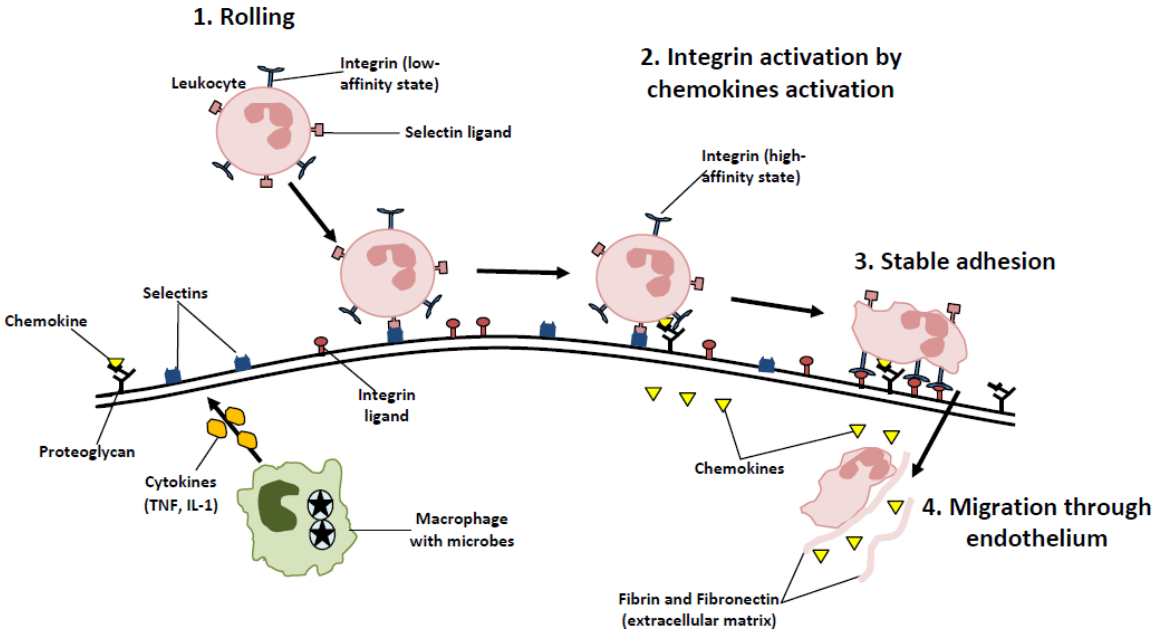

Figure 1.11 Recruitment of leukocytes. Macrophages encounter microbes and secrete cytokines (such as TNF and IL-1) that stimulate the endothelial cells of nearby venules to express selectins, integrin ligands and chemokines. Selectins generate weak tethering and rolling of blood leukocytes such as neutrophils on the endothelium and integrins create a more stable adhesion. Chemokines enhance the affinity of neutrophil-integrin interactions by binding to heparin-sulface glycosaminoglycans (polysaccharide components of cell-surface and extracellular-matrix proteoglycans) and specific receptors on these cells, and assist their migration through the endothelium to the infected sites. Blood monocytes and activated T lymphocytes use similar mechanisms to migrate to infected sites. Figure was modified from Kunkel and Butcher (2003).

Endothelial cells coating post-capillary venules stimulate the surface expression of selectins in response to microbes and leukocyte secreted cytokines, IL-1 and TNF (Tedder et al., 1995). There are three different types of selectins: P-, E-and L-. P-selectins are stored in cytoplasmic granules and migrate to the surface during infection, whereas E-selectins appear within 1 to 2 hours of microbial encounter. L-selectins (CD62L) are generated on the surface of the leukocytes (such as neutrophils) and mediate the binding of neutrophils to endothelial cells activated by cytokines (TNF, IL-1 and IFN-γ) (Tedder et al., 1995).

Chemokines are small polypeptide cytokines secreted by cells such as tissue macrophages and endothelial cells in response to microbial encounter and inflammatory cytokines, TNF and IL-1. Their main activity is to enhance leukocyte chemotaxis (the

movement of cells into tissues) (Witt and Lander, 1994, Ley, 2002). Chemokines expressed at sites of infection are carried to the luminal surface of the endothelial cells where they are targeted by heparan sulface glycosaminoglycans (polysaccharide components of cell-surface and extracellular-matrix proteoglycans) and then chemokine receptors on the surface of the rolling leukocytes (Sadir et al., 1998, Witt and Lander, 1994). As a result, the avidity of integrin-mediated binding of the leukocytes to the endothelial surface is enhanced (Witt and Lander, 1994, Ley, 2002).

TNF and IL-1 cytokines stimulate endothelial production of integrin ligands especially vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1). Whilst VCAM-1 binds VLA-4 integrins, ICAM-1 binds LFA-1 and Mac-1 integrins. Consequently, leukocytes bind tightly to the endothelium and stretch their cytoskeleton on the endothelial surface in preparation for migration (Hogg and Leitinger, 2001, Ley, 2002, Yonekawa and Harlan, 2005, Wu, 2007).

Chemokines enhance the migration of the adherent leukocytes through interendothelial spaces towards the infected sites. The adherent leukocytes express proteins on their surface such as CD31 and CD99 that contribute to their migration by forming homophilic interactions with the same molecules on endothelium (Liu et al., 2004). Migrating cells are also thought to produce enzymes that facilitate their passage through the vessel wall and eventually gather in the extravascular tissue surrounding the invading pathogen (Yonekawa and Harlan, 2005).

The accumulation of leukocytes in tissues is a significant indication of inflammation. It is regularly caused by microorganisms and occasionally by non-infectious agents. The migration of leukocytes to the sites of infection is a process distinguished by its specificity because different leukocytes produce different adhesion molecules and

chemokine receptors. For instance, the migration of neutrophils is mostly based on specific interactions employed uniquely by these cells such as the binding of CXCL8 chemokines to CXCR1 and CXCR2 chemokines receptors, while monocyte migrations relies on other interactions such as CLL2 chemokines binding to CCR2 chemokines receptors (Proost et al., 2008, Maus et al., 2005). The temporary expression of different adhesion molecules, chemokines and chemokines receptors in the infected sites frequently leads to an early enrolment of neutrophils (hours to days) and a later enrolment of monocytes (days to weeks) to these sites (Maus et al., 2005). When these phagocytes arrive at the sites of infection, they ingest microbes into vesicles by a process called phagocytosis (Stuart and Ezekowitz, 2008, Stuart and Ezekowitz, 2005), which is discussed in the next section.

1.15 Phagocytosis

Phagocytosis is a mechanism by which molecules or particles are bound to the surface of cells and internalized into a phagosome (the organelle forming around the engulfed molecule). Phagocytic receptors start this cascade of events by specifically recognizing microbes or self-structures targeted for degradation. Some of these receptors are pathogen-recognition molecules (PRRs) such as C-type lectins and scavenger receptors. In this case, PRRs participate in phagocytosis only when microbes express specific molecular patterns on their surface such as carbohydrates (Gordon, 2002). In addition, phagocytes express another group of receptors that recognize host opsonins such as antibodies, complement proteins and lectins, which coat invading microbes and label them for phagocytosis. For example, phagocytes expressing high-affinity Fc receptors known as FcγRI, specifically bind to IgG antibodies attached to microbes to initiate phagocytosis (Gordon, 2002). Figure 1.12 shows a schematic diagram illustrating some of the different types of phagocytic receptors.

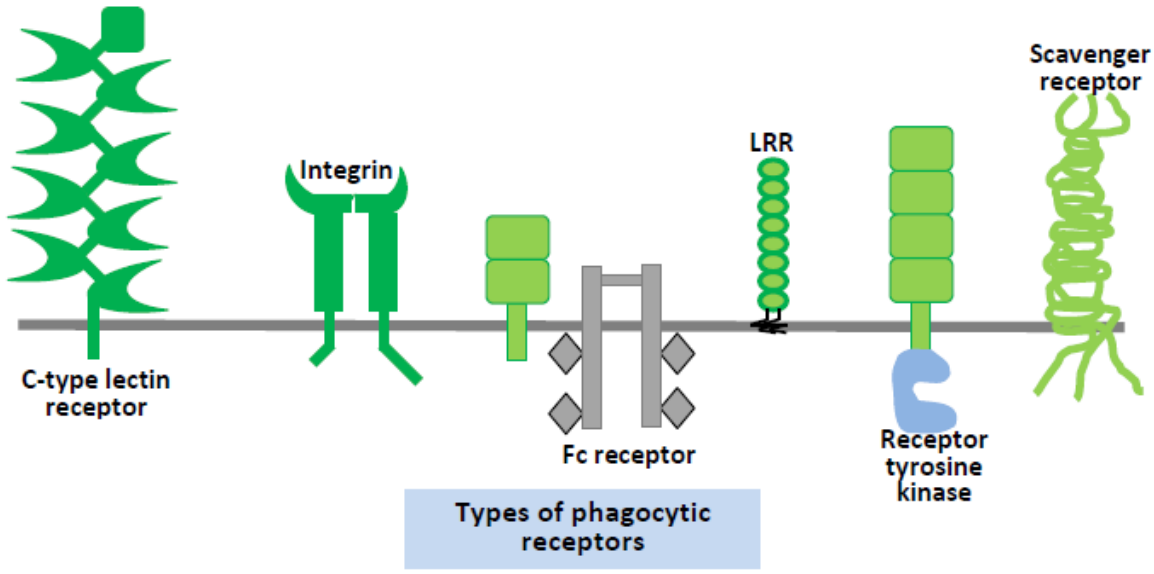

Figure 1.12 Types of phagocytic receptors. Phagocytes express different type of receptors that initiate phagocytosis. These receptors include C-type lectin receptors, integrins, Fc receptors, receptor tyrosine kinases, Leucine rich-repeats (LRR) or scavenger receptors. Figure was modified from Stuart and Ezekowitz (2008).

The main classes of phagocytic receptors are listing in more detail in Table 1.5.

Receptor	Type	Expression	Ligands
Innate Immune receptors			
Mannose receptor	C-type lectin	Macrophages, dendritic cells	Mannans
DEC 205	C-type lectin	Dendritic cells	Mannans
Dectin-1	C-type lectin	Macrophages	Glucose-rich polysaccharides
CD14	Leucine-rich repeats	Macrphages, neutrophils	Apoptotic cells, LPS
MARCO	SR-A	MZ macrophages	Bacterial cell walls
Scavenger receptor A I	SR-A	Macrophages	Apoptotic cells; LPS, LTA
CD36	SR-B	Macrophages	Apoptotic cells; parasitized RBCs
Opsonin receptors			
CR3 (αMβ2, CD11b/CD18)	Integrin	Macrophages, neutrophils	IC3b, β-glucans, ICAM- 1/2
CR4 (αxβ2, CD11C/CD18)	Integrin	Macrophages, neutrophils, dendritic cells	IC3b, fibrinogen
FcγRI (CD64)	Ig, ITAM	Macrophages, neutrophils	IgG, CRP, SAP
FcγRII (CD32)	Ig, ITAM	Macrophages, neutrophils	IgG
FcγRIII (CD16)	Ig, ITAM	Macrophages, neutrophils, natural killer cells	IgG, SAP
FcγRIV	Ig, ITAM	Macrophages, neutrophils, dendritic cells	IgG2A, IGg2b
FcαR (CD89)	Ig, ITAM	Macrophages, neutrophils, eosinophils	IgA
Integrin αvβ3	Integrin	Macrophages, platelets	Thrombospondin-opsonised cells
Mer	RTK	Macrophages	Apoptotic cells

Phagocytic receptors

Table 1.5 Phagocytic receptors. Phagocytes express different type of receptors that initiate phagocytosis. These receptors can be C-type lectin receptors, integrins, Fc receptors (ITAM; immunoreceptor tyrosine-based activation motifs, Ig; immunoglobulin), receptor tyrosine kinases (RTK), Leucine rich-repeats (LRR) or scavenger receptors (SR). These receptors can be classified into innate immune receptors and opsonin receptors. Both different types of receptors initiate microbial phagocytosis. Table was modified from Aderem and Ulevitch (2000).

After the recognition of microbes by phagocytic receptors, an organized progression of changes is orchestrated by the phagocyte, called phagocytosis, leading to elimination of the pathogen. The following figure summarizes the events involved in phagocytosis.

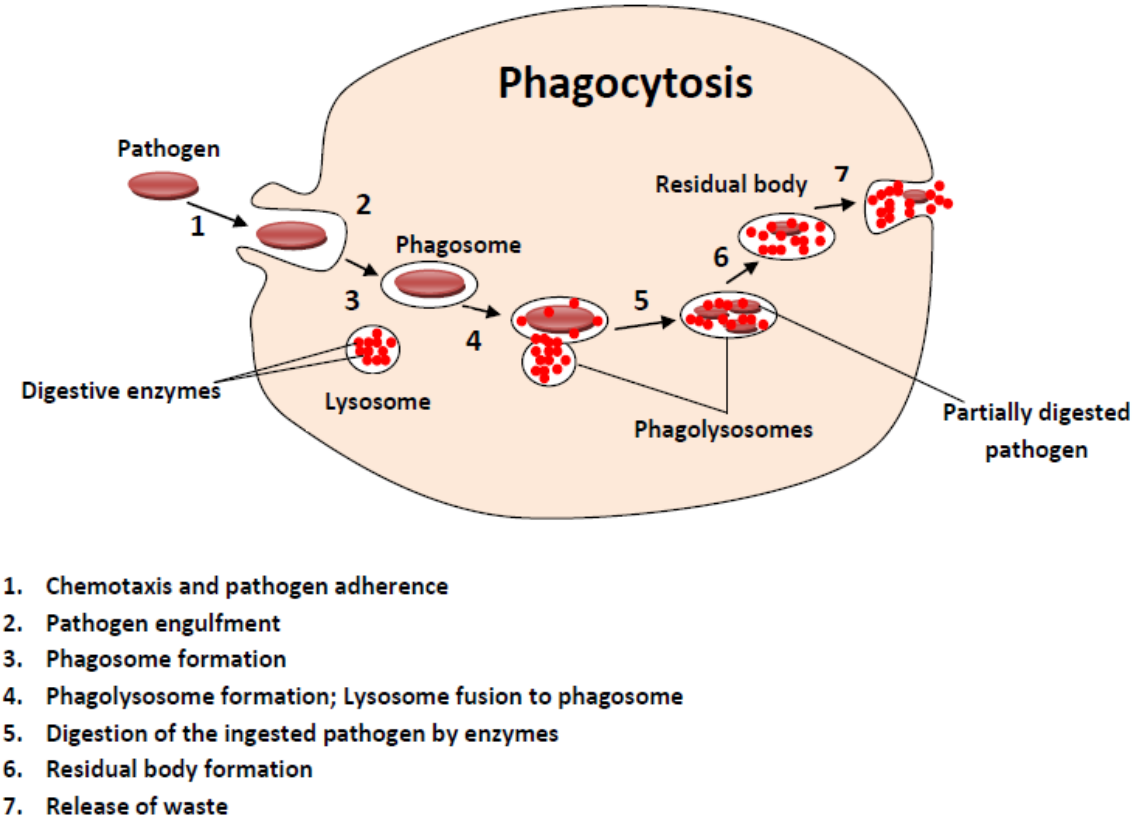

Figure 1.13 Phagocytosis. Phagocytosis is a mechanism by which pathogens or host molecules targeted for destruction are removed by phagocytes. Once ingested by the phagocytic cell, lysosomes fuse with phagosomes to form phagolysosomes, leading to digestion and destruction of the microbe. Figure was modified from Desjardins (2003).

Once the phagocytic receptors have bound to an invading pathogen, the plasma membrane in the area of these receptors starts to relocate to form cup-shaped extensions around the pathogen. These extensions then form into a vesicle (called a phagosome) carrying the ingested pathogen inside it. The phagosome migrates away from the plasma membrane and combines with lysosomes, which carry concentrated levels of microbicidal molecules, to form phagolysosomes (Desjardins, 2003). Microbicidal molecules include proteolytic enzymes such as elastase (a broad-spectrum serine protease) and cathepsins that break down the ingested pathogen (Korkmaz et al., 2008).

Phagocytosis is a unique characteristic of cells known as professional phagocytes including neutrophils, monocytes, macrophages and dendritic cells (see above). These cells have the ability to engulf molecules even larger than their own surface area. Microorganisms and apoptotic cells are major targets for phagocytosis. Apoptotic cells are engulfed during tissue remodelling and during embryogenesis, where surplus cells endure programmed cell death and elimination (Stuart and Ezekowitz, 2008). Microorganisms are targeted at early stages of the innate immune response (Hoffmann et al., 1999). Phagocytes can also act as fundamental antigen-presenting cells to help produce an effective adaptive immune response (Stuart and Ezekowitz, 2008).

Many studies have been dedicated to unlock the complicated machinery of phagocytosis and numerous cell surface receptors and serum components have been identified (see Table 1.5). However, studies have frequently been complicated by the apparent overlapping functions of several key components. In addition, primary mammalian phagocytes are generally not amenable to genetic manipulations making their study difficult (Stuart and Ezekowitz, 2008). Consequently, it has been difficult to evaluate the relative input of the components of this machinery. As a result, cell biology and microscopy methods have been employed by researchers to investigate phagocytosis and to identify potential signalling pathways involved in this process (Aderem and Underhill, 1999, Greenberg and Grinstein, 2002, Palmer, 2007, Stuart and Ezekowitz, 2008). For example, one of the best characterized mammalian systems is Fc receptor-mediated phagocytosis (see Figure 1.12 and Table 1.5). Fc receptors bind IgG antibody-coated microbes and activate a number of signalling events including tyrosine phosphorylation, which eventually induce to the recruitment of actin and actin-associated proteins to form the phagocytic cup (Beningo and Wang, 2002, Palmer, 2007). Neutrophils, monocytes and macrophages have been the main phagocytes

employed to identify and characterize the cell-surface receptors and serum components involved in phagocytosis, and for this reason I have focussed on these cell types in my research project.

1.16 Humoral immune system

In addition to cell-associated molecules, the innate immune system also employs a wide range of soluble proteins located in plasma and extracellular fluids that can target pathogen-associated molecular patterns (PAMPs) and eliminate invading microbes. Other soluble proteins can behave as opsonins, binding microbes and stimulating their phagocytosis by neutrophils or macrophages. The soluble pattern-recognition proteins and opsonin proteins are commonly known as the humoral branch of the innate immune response. Key components of the humoral innate immune system include the complement system, the collectins, the pentraxins, the ficolins and cytokines (Manfredi et al., 2008, Thiel and Gadjeva, 2009).

1.17 The complement system

The complement system contributes significantly to the innate immune system by providing an immediate defence against microbes and infected cells (Muller-Eberhard, 1988, Reid, 1983). It comprises circulating blood proteins and enzymes which activate on contact with pathogens to initiate a cascade of reactions culminating in destruction of the microbe. The response also stimulates a wide range of immune and inflammatory responses. For example, microbial cells are tagged by complement components which act as opsonins for host leukocytes.

Complement activation occurs via three different pathways: the classical, alternative and lectin pathways. The following diagram summarizes the complement pathway and the main routes of activation.

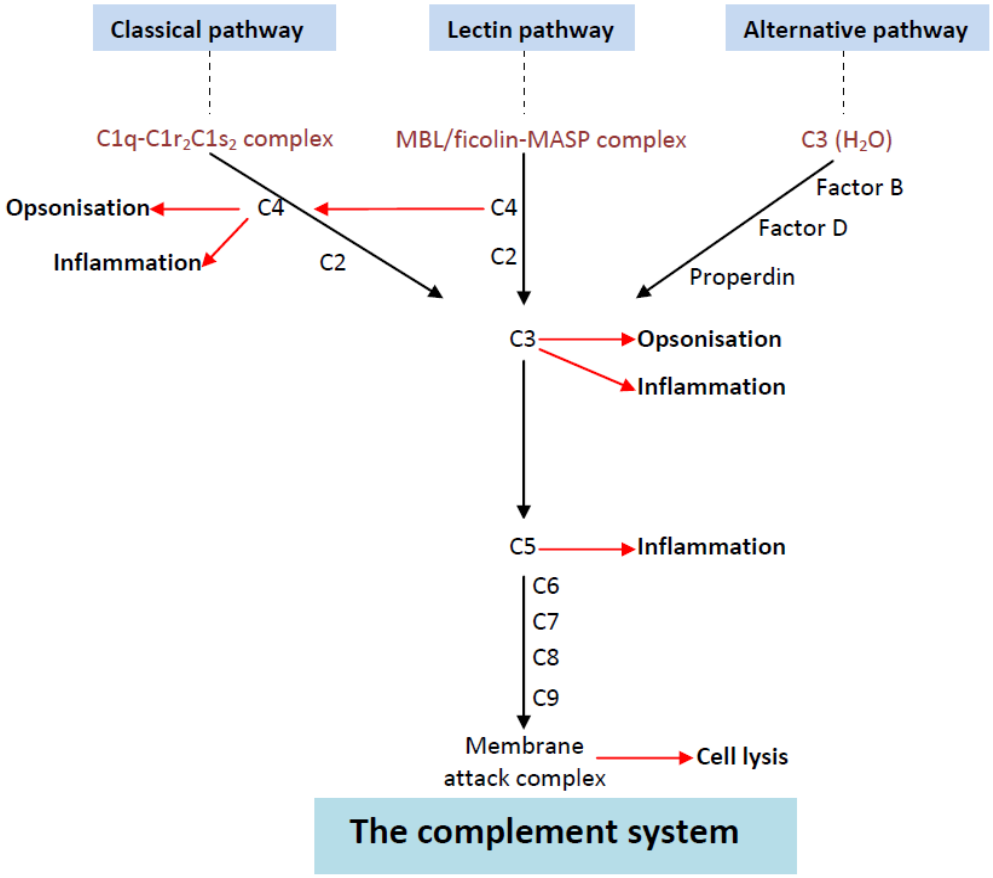

Figure 1.14 The complement system. Three different pathways activate the complement system: the classical, lectin and alternative pathways. Complement activation leads to a wide range of immune and inflammatory responses as well as direct cell lysis.

All three pathways can activate directly on the surfaces of pathogens and help to stimulate an effective adaptive immune response, via receptors on host leukocytes. Complement can also become activated via immobilized antibodies and other host immune proteins eg. C-reactive protein (Burton et al., 1980, Duncan and Winter, 1988, Perkins et al., 1991, Sim and Reid, 1991, Roos et al., 2001).

1.17.1 The classical pathway

The first component of the classical pathway of complement is called the C1 complex, which consists of the recognition molecule C1q and its associated serine proteases C1r and C1s (Arlaud and Colomb, 1987). C1q is a multimeric protein composed of hexamers of a subunit containing three homologous polypeptide chains (A, B and C).

The proteases of the C1 complex combine to form heterotetramers containing two molecules of C1r and two of C1s (C1s-C1r-C1r-C1s).

The C1 complex undergoes conformational changes when it binds to the surface of pathogens or to immobilized immune complexes involving IgG or IgM, leading to autoactivation of C1r, which subsequently activates C1s. C1s in turn cleaves C4 to produce C4a and C4b. C4a is a peptide anaphylatoxin released into the blood stream where it stimulates inflammation, whereas C4b binds covalently to the surface of target cells via an exposed thioester bond (Dodds et al., 1996). C4b then recruits C2, which is also cleaved by C1s to release C2b. The remaining C4b2a complex is the C3 convertase, which cleaves C3 molecules leading to attachment of C3b onto the surrounding cell surfaces (also via a thioester bond). As the cascade continues, the C5 convertase (C42a3b) is formed and cleaves C5 producing C5a and C5b to activate the terminal pathway. Ultimately a membrane attack complex assembles in the cell membrane of microbial cells leading to lysis and death (Muller-Eberhard, 1985). Many regulatory components control complement activation thereby preventing self damage. For example, spontaneous activation of the C1 complex results in immediate inactivation by C1-inhibitor, which blocks uncontrolled complement activation in the serum.

The most well-known ligands of the classical pathway are antibody-antigen complexes involving IgG or IgM, and in this way the classical pathway connects innate and adaptive immunity. In addition, C1q has been shown to bind to a wide range of bacterial cell wall components such as lipid A, nucleic acids, ligand-bound C-reactive protein (CRP) and amyloids in an antibody independent manner (Blanquet-Grossard et al., 2005, Mitchell et al., 2007, Sim and Malhotra, 1994), thus it is also a pattern-recognition receptor.

1.17.2 The lectin pathway

The activation mechanism of the lectin pathway of complement has only recently been characterized compared to the classical and alternative pathways. The recognition molecules, MBLs and ficolins form heterogeneous complexes with three different zymogen MASPs and at least two non-enzymatic protein components known as MAp19 or sMAp and Map44 (Schwaeble et al., 2002, Wallis and Dodd, 2000). MASP-2 is believed to be the main activating enzyme of the lectin pathway. The binding of MBL-MASP-2 and ficolin-MASP-2 complexes to the surface of microbes leads to the activation of MASP-2 by autolysis, followed by cleavage of C4 into C4a and C4b, and C4b2 into C4b2a (the C3 convertase) and C2b, and thus follows a similar activation path as in the classical pathway. Subsequent reactions are identical to those of the classical pathway. Activation of the lectin pathway of complement is antibody-independent and thus facilitates early elimination of pathogens even without an effective adaptive immune response.

1.17.3 The alternative pathway

The alternative pathway is activated by a different mechanism from that of the classical and lectin pathways. Instead of target recognition molecules, spontaneous low-level hydrolysis of C3 in plasma initiates this pathway by forming C3(H_2O) that has a similar structure and function to C3b. Factor B attaches to C3(H_2O) and is cleaved by factor D into two fragments: Ba and Bb. Only Bb remains bound to C3(H_2O) to form the C3Bb complex, a fluid-phase form of the C3 convertase that cleaves other C3 molecules. The deposition of C3b molecules subsequently occurs randomly onto the surface of host cells as well as any pathogens. Factor B binds to membrane-bound C3b fragments and generates the membrane-phase C3 convertase (C3bBb) after its cleavage by factor D, which is stabilised by properdin (Farries et al., 1988, Fearon and Austen, 1975).

Moreover, C3bBb can interact with another C3b molecule to assemble the C5 convertase and initiate the downstream cascade.

Because C3 deposition is non-specific, the alternative pathway is incapable of differentiating between self and non-self in the early phases of activation. However, host cells express a wide range of regulators and inhibitors that prevent activation from progressing thereby avoiding self destruction. On the other hand, the absence of these regulators on invading pathogens exposes them to complement mediated neutralisation. Thus, the complement system is an important tool to battle invading microbes directly. In addition to eliminating invading pathogens, it depletes circulating immune complexes and facilitates the removal of necrotic or apoptotic cells (Whaley and Ahmed, 1989). It also participates in regulating the adaptive immune system via arrays of complement receptors on immune cells (Dempsey et al., 1996).

1.18 Pentraxins

Many plasma proteins bind microbial structures and contribute in innate immunity. Some of these proteins are members of the pentraxin family, which is phylogenetically an old group of structurally homologous pentameric proteins. Members of this family include C-reactive protein (CRP) and serum amyloid P-component (SAP). Circulating levels of CRP and SAP increase significantly in individuals during infection and target a variety of different microbial species through cell surface patterns. For example, CRP is an opsonin that interacts directly with IgG Fc receptors as well as C1q and where it is targeted for elimination by phagocytic C1q receptors (Manfredi et al., 2008, Black et al., 2004).

1.19 Collectins

Collectins, including MBL, the first component of the lectin-pathway of complement, and pulmonary-surfactant proteins (SP-A and SP-D) are a group of proteins characterized by a collagen-like N-terminal region linked by an alpha-helical neck region to a calcium-dependent (C-type) carbohydrate-recognition domain. The quaternary structure of collectins resembles that of C1q in overall appearance. However, collectin subunits are composed of three identical polypeptide chains, whereas three different chains combine to form C1q. Collectins can be divided into two main types based on their architectures: bouquet and cruciform-like structures. While MBL and SP-A form bouquet-like structures, SP-D, conglutinin and CL-46 are cruciform (Lu et al., 2002, Gupta and Surolia, 2007). Collectins are soluble pattern recognition molecules (Gupta and Surolia, 2007). MBL is the only member of the collectin family that activates complement. It also functions as an opsonin that targets carbohydrates with terminal mannose, fucose and N-acetyl glucosamine on the surface of microbial pathogens, although it is unclear which phagocytic receptors are involved in this process (Drickamer, 1992, Lee et al., 2002, Gupta and Surolia, 2007). SP-A and SP-D are present in the alveoli of the lungs and are characterized by their lipophilic properties. Their main function is to modulate the innate immune response in the lung and bind microorganisms to facilitate their ingestions by alveolar macrophages. SP-A and SP-D can directly inhibit bacterial growth and stimulate their phagocytosis by activated macrophages (Gupta and Surolia, 2007). In addition to their innate immune function, they carry out important endogenous functions within the lung such as lipid homeostasis. Other collectins, including conglutinin and CL-43 have been identified only in Bovidae, where they are believed to function as PAMPs to bind and aggregate pathogens (Gupta and Surolia, 2007).

1.20 Ficolins

Ficolins, like MBLs serve as initiating components of the lectin pathway of the complement system. They bind and neutralise pathogens via antibody-independent mechanisms through their association with MASPs. They have similar structures to MBL and collectins and are multidomain, oligomeric proteins made from many copies of a subunit, which is characterized by its three identical polypeptide chains as shown in Figure 1.15 (Drickamer and Taylor, 1993, Fujita et al., 2004). The major difference from MBL is the recognition domain, which is a fibrinogen-like domain rather than a carbohydrate-recognition domain (CRD). They bind carbohydrates and N-acetyl groups on the surface of the invading pathogen to activate complement leading to neutralization via lysis or phagocytosis (Lynch et al., 2004, Krarup et al., 2004, Carroll, 2004, Runza et al., 2008).

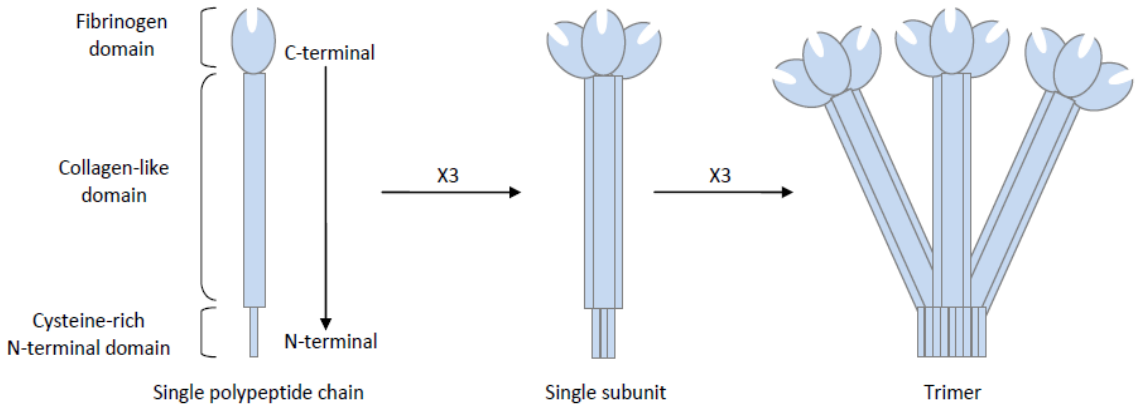

Figure 1.15 Ficolin structural features. Assembly of three ficolin polypeptide chains forms a subunit. Subunits subsequently oligomerise to form large structures that resemble bouquets (a trimer of subunits is shown on the right).

There are three known human ficolins; L-ficolin, H-ficolin and M-ficolin. They are encoded by the FCN2, FCN3 and FCN1 genes positioned on chromosomes 9q34 (L-ficolin and M-ficolin) and 1p35.3 (H-ficolin) (Fujita et al., 2004). L-ficolin and M-ficolin are 79% identical whereas H-ficolin is only 45% identical to L-ficolin and

M-ficolin (Thiel, 2007). L-ficolin is synthesized in the liver and its estimated serum concentration is between 3.7-5.0 µg/ml in Caucasians and around 13.7 µg/ml in the Japanese population (Taira et al., 2000, Kilpatrick et al., 1999). The variation of L-ficolin concentration is associated with three polymorphisms in the promoter region and a polymorphism in the structural gene (exon8) (Hummelshoj et al., 2005). Such variations have not been associated with any infectious diseases as yet.

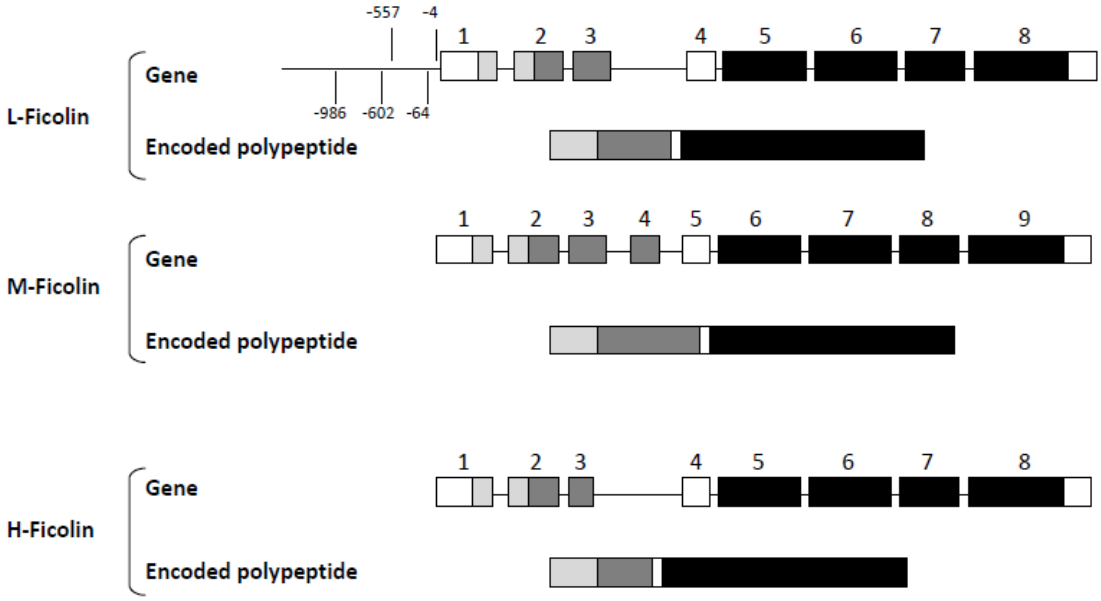

Figure 1.16 Human ficolin gene organizations (L-, M- and H-ficolins). Polypeptide chains are numbered (the leader sequences are included in the numbering). Exons are illustrated as boxes, and linked by introns (not shown to scale). The cysteine-rich, collagen and pathogen-recognition domains are represented in light, medium and dark grey colours, respectively. The exons encoding these domains are represented correspondingly. The short connector region between collagen and pathogen-recognition domains is shown in white (exon 4 in L- and H-ficolins and exon 5 in M-ficolin). Figure was modified from Thiel (2007).

L-ficolin has been shown to play key roles in immune defence. It interacts and activates complement on the surface of *Salmonella typhimurium* TV119 (Taira et al., 2000). It also stimulates opsonophagocytosis of group B streptococci (Aoyagi et al., 2005). Ficolins bind specifically to the bacterial capsule constituents of some *Staphylococcus aureus* and *Streptococcus pneumonia* serotypes (Krarup et al., 2005). Furthermore, they

contribute in the elimination of apoptotic and dead host cells (Jensen et al., 2007, Kuraya et al., 2005), and bind C-reactive protein to recognize bacteria (Ng et al., 2007).

Relatively few microbial ligands have been reported for H- or M-ficolins. M-ficolin can interact with *S. aureus* and *Escherichia coli* and induce the uptake of these bacteria by phagocytosis (Liu et al., 2005, Teh et al., 2000). H-ficolin binds specifically to a polysaccharide (polysaccharide *A. viridians*) present on *Aerococcus viridians* (Krarup et al., 2005, Matsushita et al., 2002). Moreover, recombinant H-ficolin aggregates leukocytes coated with LPS from *S. typhimurium*, *Salmonella Minnesota* and *E. coli* (O111) and this effect was inhibited by GaINAc, GIcNAc and D-fucose (Sugimoto et al., 1998).

1.21 Cytokines of the innate immune system

Cytokines participate in the recruitment and stimulation of leukocytes. They can also generate systemic alterations and enhance the recruitment of effector cells and proteins with antimicrobial properties. Macrophages, neutrophils and NK cells are the main producers of cytokines during innate immunity. Cytokines enable communication between inflammatory cells and between inflammatory cells and tissue cells such as vascular endothelial cells and leukocytes (Mantovani and Dejana, 1989).

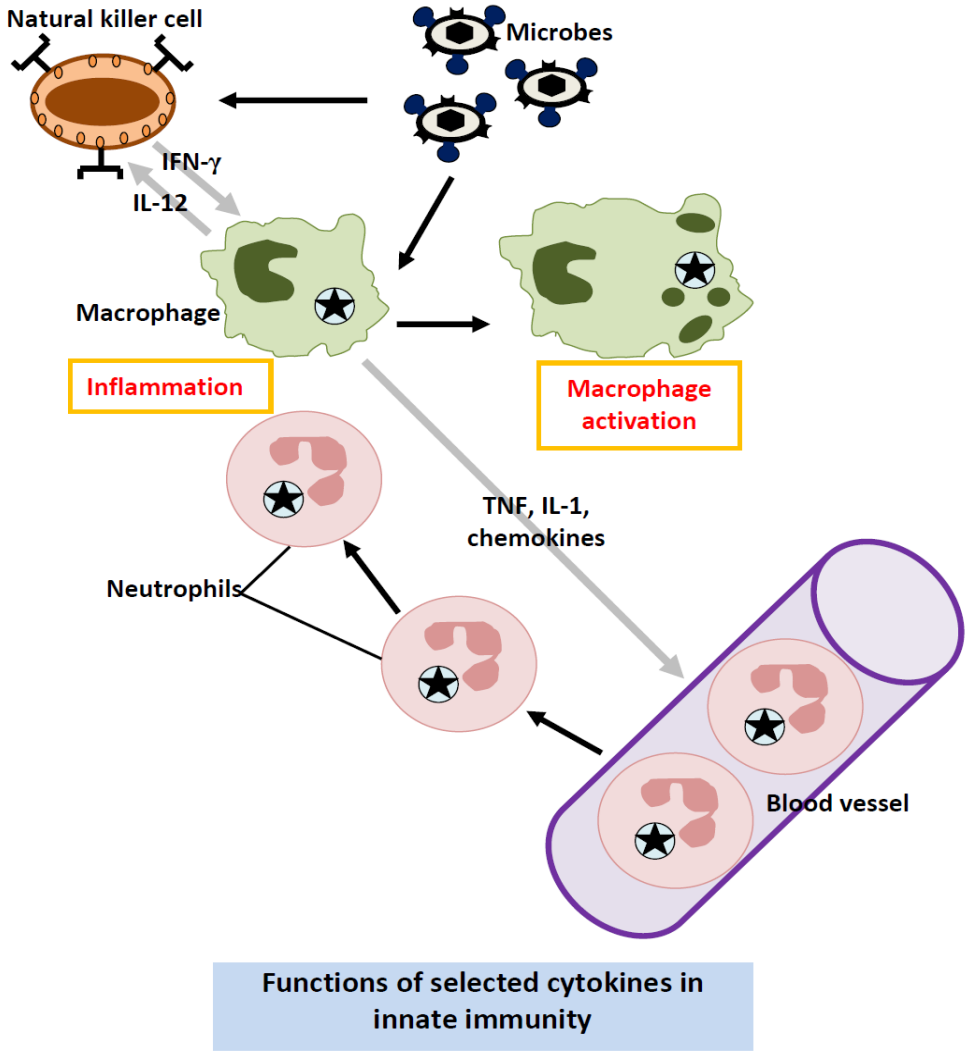

Figure 1.17 Functions of selected cytokines in innate immunity. Cytokines generated by macrophages and NK cells regulate the early inflammatory responses to microbes and stimulates the elimination of microbes. Figure was modified from Trinchieri (2003).

Cytokines of the innate immune system are also produced to contain and fight viral infections (IFN-α and IFN-β), mediate inflammation (TNF and IL-1), enhance the proliferation and activity of NK cells (IL-15 and IL-2), trigger macrophages (IFN-γ) and maintain macrophage activation (IL-10) (Medzhitov and Janeway, 2000, Mantovani et al., 2009). Furthermore, other cytokines of the innate immune system such as, IL-10 stimulate the production of neutrophils by the bone marrow and the secretion of different proteins involved in the immune response such as, CRP (de Vree et al., 1997, Moshage et al., 1988) (Table 1.6).

Cytokine	Size (kD)	Principal cell source	Principal cellular targets and biologic effects
Tumor necrosis factor (TNF)	17kd; 51kd homotrimer	Macrophages, T cells	Endothelial cells: activation (inflammation. Coagulation) Neutrophils: activation Hypothalamus: fever Liver: synthesis of acute phase proteins Muscle, Fat: catabolism (cachexia) Many cell types: apoptosis
Interleukin-1 (IL-1)	17kd mature form: 33kd precursors	Macrophages, endothelial cells, some epithelial cells	Endothelial cells: activation (inflammation coagulation) Hypothalamus: fever Liver: synthesis of acute phase proteins
Chemokines	8-12kd	Macrophages, endothelial cells, T cells, fibroblasts, platelets	Leukocytes: chemotaxis, activation: migration into tissues
Interleukin-12 (IL-12)	Heterodimer of 35kd+40Kd subunits	Macrophages, dendritic cells	T cells: T_H1 differentiation NK cells and T cells: IFN-γ synthesis, increased cytotoxic activity
Type I IFNs (IFN-α, IFN-β)	IFN-α: 15-21 kd IFN-β:20-25 kd	IFN-α: macrophages IFN-β: fibroblasts	All cells: antiviral state, increased class I MHC expression NK cells: activation
Interleukin-10 (IL-10)	Homodimer of 34-40kd: 18kd subunits	Macrophages, T cells (mainly regulatory cells)	Macrophages, dendritic cells: inhibition of IL-12 production and expression of co-stimulators and class II MHC molecules
Interleukin-6 (IL-6)	19-26 kd	Macrophages, endothelial cells, T cells	Liver: synthesis of acute phase proteins B cells: proliferation of antibody-producing cells
Interleukin-15 (IL-15)	13kd	Macrophages, others	NK cells: proliferation T cells: proflieration (memory CD8+ Cells)
Interleukin-18 (IL-18)	17kd	Macrophages and dendritic cells	T cells: maintenance of IL-17 producing T cells
Interleukin-23 (IL-23)	Heterodimer of unique 19kd subunit and 40kd subunit of IL-12	Macrophages and dendritic cells	T cells: maintenance of IL-17 producing T cells
Interleukin-27 (IL-27)	Heterodimer of 28kd and 13kd subunits	Macrophages and dendritic cells	T cells: inhibition of T_H1 Cells: role in T_H1 differentiation? NK cells: IFN-γ synthesis

IFN, interferon; kd, kilodalton; MHC, major histocompatibility complex; NK, natural killer

Table 1.6 Features of cytokines of the innate immune system. Table was modified from Medzhitov and Janeway (1997)**.**

1.22 Acute-phase reaction and acute-phase proteins

The acute-phase reaction (APR) is a systemic response of the organism to local or systemic changes in its homeostasis. It is initiated by infection, tissue injury, trauma or surgery and by neoplastic growth or immunological disorders (Gruys et al., 2005). Several acute-phase reactions including serum amyloid A (SAA) commence at the site of infection or tissue injury to induce the release of pro-inflammatory cytokines such as

IL-1, IL-6 and IL-17 that can activate the vascular system and inflammatory cells. Such reactions are also associated with the generation of other cytokines including TNF and inflammatory mediators such as chemokines, which circulate in the blood after their release into the extracellular fluid partition (Gruys et al., 2005). Consequently, cytokines including TNF can stimulate receptors on various target cells such as hypothalamic cells and can cause a systemic reaction to activate hypothalamic-pituitary-adrenal axis, which decreases the secretion of growth hormones (Gruys et al., 2005). Different physical symptoms can clinically be noticed such as fever, anorexia, negative nitrogen balance and catabolism of muscle cells (Gruys et al., 2005). Moreover, Laboratory examinations can quantify several other changes such as activation of the complement system and alterations in the concentration of a number of plasma proteins (acute-phase proteins). Table 1.7 below, gives some examples of acute-phase proteins.

The concentration of acute-phase proteins (APPs) such as haptoglobin and albumin is significantly changed shortly after infection due to changes in protein synthesis by liver. Whilst a dramatic increase of some proteins occurs, known as positive APPs (eg, haptoglobin), the synthesis of other blood proteins is decreased, negative APPs (eg, transferrin and albumin) (Gruys et al., 2005).

Acute-phase proteins
Positive acute-phase proteins
Opsonins
C-reactive protein
Serum Amyloid P component
Mannose-binding protein
Complement proteins
C2
C3
C4
C5
C9
Factor B
C1 inhibitor
C4-bining protein
Coagulation proteins
Fibrinogen α, β, γ
Von Willebrand factor
Protease inhibitors
α_1-antitrypsin
α_1-antichymotrypsin
α_2-antiplasmin
Plasminogen activator inhibitor I
Heparin cofactor II
Scavenging proteins
Haptoglobin
Ceruloplasmin
Hemopexin
Serum Amyloid A
Negative acute-phase proteins
Albumin
Transferin
Transthyretin
Transcortin
Retinol-binding proteins

Table 1.7 Acute-phase proteins (Heinrich et al., 1998).

The positive APPs are generally proteins secreted by hepatocytes following cytokine stimulation (Table 1.7) (Heinrich et al., 1998). However, hepatic protein production can also be reduced as a consequence of starvation and malnutrition (Gruys et al., 2005). The change in the concentrations of APPs is considered to be valuable to the host

because it stops bacterial growth through production of antimicrobial proteins and contributes to restore homeostasis. APPs are employed to target cellular remnants and free radicals for scavenging, opsonise invading bacteria, stimulate the complement pathway, and neutralise proteolytic enzymes (Gruys et al., 2005). The work presented in this thesis focuses on the role of the positive APP, haptoglobin, in neutralization of *S. aureus*. Different studies have suggested that haptoglobin contributes in regulating the immune response, autoimmune diseases and inflammatory diseases although the mechanisms underlying these processes have not been characterized, as will be discussed in the next section.

1.23 Haptoglobin (HP)

The normal serum concentration of haptoglobin (HP) ranges between 0.2 and 1.4 mg/ml, and clinically it is a useful marker for several inflammation-related disorders such as Hepatitis C, HIV, myocardial infarction, retinal hemorrhage and tuberculosis (Raijmakers et al., 2003, Engstrom et al., 2003, Palma et al., 2005, Sadrzadeh and Bozorgmehr, 2004). The primary known function of HP is to target free haemoglobin (HB) released from erythrocytes by intravascular haemolysis to form stable HP-HB complexes (Okazaki and Nagai, 1997, Okazaki et al., 1997). Complexes bind to a HB scavenger receptor CD163 located on the outer envelope of monocyte and macrophages (Kristiansen et al., 2001). As a result, they are rapidly removed by the reticuloendothelial system in the liver (Graversen et al., 2002). The scavenging mechanism is essential in the inhibition of HB glomerular filtration, which can cause renal failure (Gburek and Osada, 2000, Gburek et al., 2002). HP-HB complexes also bind directly to receptors on hepatocytes and hepatoma cell lines (Polticelli et al., 2008), for internalization into the liver parenchymal cells where organelles carry them into the

microsome fraction where they are dissociated and degraded into smaller subunits (Kino et al., 1982, Zuwala-Jagiello and Osada, 1998).

HP synthesis is stimulated by several cytokines during inflammation (Wang et al., 2001). For instance, IL-6 mediates HP gene expression and protein synthesis in the liver of most species that have been studied, including human and rat (Quaye, 2008, Baumann et al., 1990). HP is initially synthesized as a single chain that is cleaved into two chains; an N-terminal light α-chain (complement-control repeat) and a C-terminal heavy β-chain (serine protease-like domain) (Gordon et al., 1968, Langlois and Delanghe, 1996, Delanghe and Langlois, 2002). The α- and β-chains are covalently attached by an intermolecular disulfide bond made between Cys131 and Cys248 (Wejman et al., 1984). The following figure shows the molecular structure of HP.

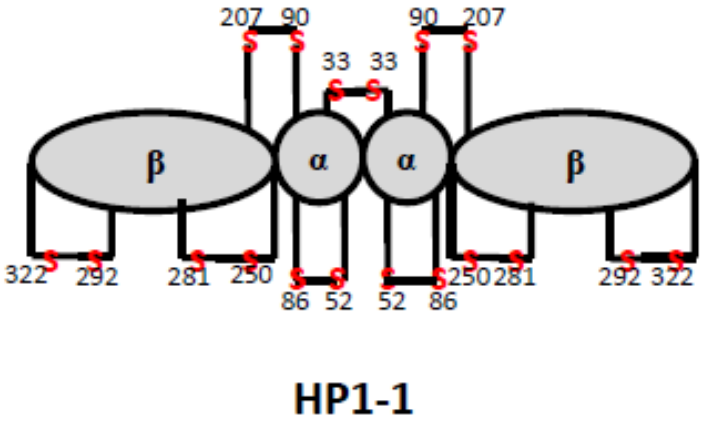

Figure 1.18 HP structure. Schematic drawing represents the molecular arrangements of human HP1-1 structure indicating the disulfide patterns. Figure was reproduced from Nielsen et al. (2007).

In most mammals, HP is formed by two (α-β) monomers linked together covalently by a disulfide bond between the two α-chains creating an $(\alpha\text{-}\beta)_2$ structure. This form is known as HP1-1 in humans (Langlois and Delanghe, 1996, Delanghe and Langlois, 2002). However, three different HP phenotypes are found in humans because of the existence of two HP gene alleles, known as HP1 and HP2. An intragenic duplication of

the HP1 allele forms the HP2 allele, which carries a bigger α-chain with a duplicated cysteine residue linking the α-chains. Consequently, other phenotypes of HP such as HP2-1 and HP2-2 present a range of different HP structures (Langlois and Delanghe, 1996, Delanghe and Langlois, 2002) as shown in Figure 1.19.

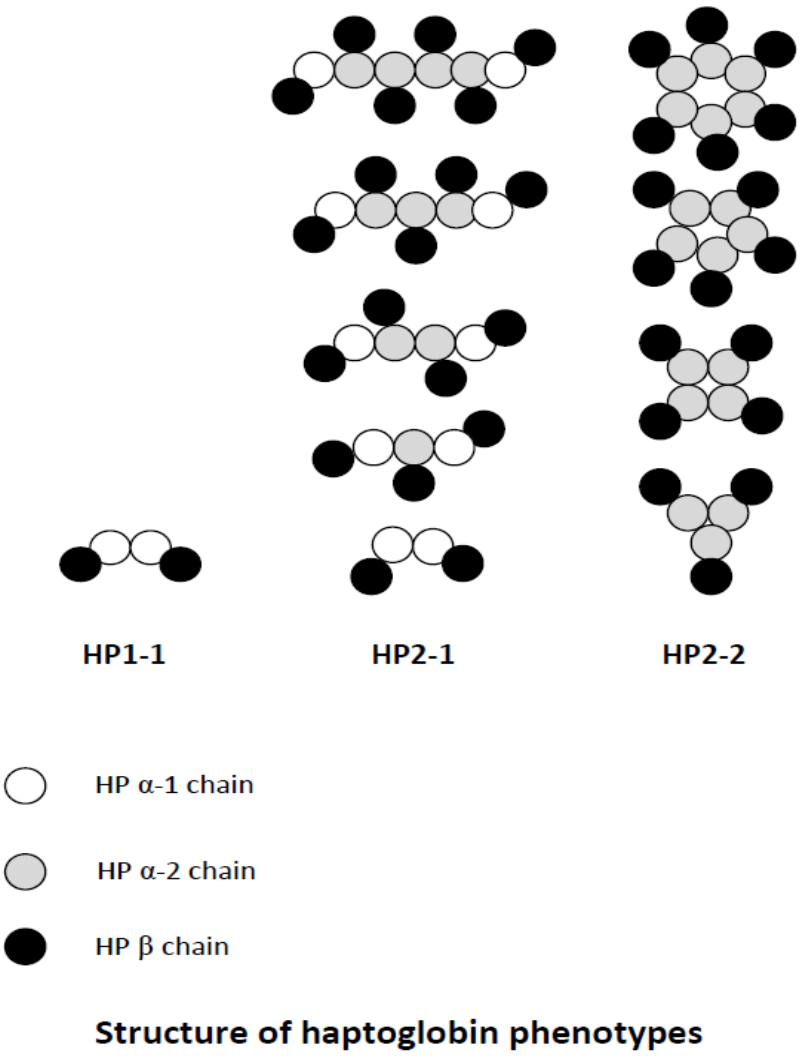

Figure 1.19 HP structural phenotypes. Schematic drawing showing the molecular arrangement of human HP phenotypes. Figure was reproduced from Gast et al. (2008).

Duplication of one of the HP alleles has resulted in a HP-related protein (HPr) in higher eukaryotes, which is 91% identical to HP1 (Bensi et al., 1985, Maeda, 1985). HPr is found in plasma as a constituent of two complexes, called trypanosome lytic factor 1 (containing high-density lipoprotein particles) and trypanosome lytic factor 2 (which are

predominantly protein complexes with less lipid components) (Hajduk et al., 1989, Raper et al., 1999). Trypanosome lytic factor 1 is toxic to *Trypanosoma brucei brucei* and provides protection against this African parasite by stimulating its lysis (Drain et al., 2001). Purified HPrs are also capable of targeting trypanosomes and trigger their elimination by displacing them to lysosomes through an unknown mechanism (Drain et al., 2001). Moreover, HPr also binds to HB, although HPr-HB complexes do not enhance removal of HB by CD163 as observed with HP-HB complexes (Nielsen et al., 2006).

HP has been suggested to contribute towards regulating the immune response, and play roles in autoimmune diseases and major inflammatory diseases (Delanghe et al., 1999, Eaton et al., 1982, Levy, 2004, Oh et al., 1990, Quaye et al., 2006). For example, it has been suggested to behave as an innate phenotype-dependent antioxidant to sustain cellular redox homeostasis and avoid cellular damage during inflammation. Chinese hamster ovary K1 cells transfected with HP1-1 showed 2-fold increased resistance to hydrogen peroxide exposure compared to control cells (Tseng et al., 2004). Moreover, both *in vitro* and *in vivo* studies have shown that HP1-1 phenotype is more resistant to cellular oxidative stress than HP2-2 and HP2-1 phenotypes (Tseng et al., 2004). HP was also shown to have a more effective role as an innate phenotype-dependent antioxidant at extravascular sites than that of vitamin C in neutrophil activation, preservation of reverse cholesterol transport, cyclooxygenase inhibition and lipooxygenase (Tseng et al., 2004). Primary pro-inflammatory cytokines IL-1β and TNF-α are released by cells targeted during infection, trauma, exercise and injury in order to activate endothelial cells, recruit neutrophils and other inflammatory cells (Kushner, 1993). HP is produced and stored throughout granulocyte differentiation to be released upon the activation of neutrophils (Theilgaard-Monch et al., 2006). HP was also demonstrated to target

ApoA-1 (a form of high-density lipoprotein) to prevent its damage by free radicals and its interaction with other lipoprotein substrates. As a result, the transport of cholesterol for its degradation in the liver is maintained and its storage in macrophages to form foam cells is prevented (Theilgaard-Monch et al., 2006). In addition, HP is considered to be a major extracellular inhibitor of protein misfolding by blocking inappropriate self-association of proteins caused by heat or oxidation (Yerbury et al., 2005). HP levels are significantly increased for the duration of pregnancy, myocardial infarction and obesity (Berkova et al., 2001, Blum et al., 2007, Chiellini et al., 2004). These conditions demand intensive anti-inflammatory responses to overcome tissue growth and repair (Quaye, 2008). HP blocks matrix metalloproteinases (MMP-2 and MMP-9) function to prevent gelatine degradation and initiates the required fibroblast migration for tissue regeneration (de Kleijn et al., 2002). It is also involved in various anti-inflammatory responses to sustain redox homeostasis throughout tissue repair enhancement (de Kleijn et al., 2002). HP also participates in a variety of different cellular and humoral processes within the innate and adaptive immune systems including prostaglandin production, recruitment and migration of leukocytes, the release of cytokines after injury, infection and tissue repair (Quaye, 2008).

HP knockout C57BL/6J mice were recently produced to help better understand the role of HP in the immune response. The adaptive immune response has been the primary focus of initial studies and mice showed reduced development of lymphoid organs and low numbers of mature T and B cells in blood. Moreover, these mice had impaired immune responses, which failed to effectively protect against *S. typhimurium* infection, suggesting a regulatory function for HP in lymphocyte immune responses (Huntoon et al., 2008). Interestingly, HP-deficient mice show no apparent defects associated with accumulation of haemoglobin. Thus, HP-associated clearance of haemoglobin may not

be the only function of HP. The research presented in this thesis focuses on a novel function of HP, in innate immunity, to enhance human neutrophil, monocyte and macrophage-mediated killing of the Gram-positive bacteria *S. aureus*. The next section summarizes the surface structure of bacteria focusing on the main antigenic components.

1.24 Gram-negative bacteria

The bacterial cell wall components of Gram-negative (such as, *E. coli*) and Gram-positive bacteria (such as, *S. aureus*) are different. The cell walls of Gram-negative bacteria comprise two lipid membranes separated by a periplasmic space containing thin peptidoglycan layers (20% of the cell wall thickness) attached to lipopolysaccharide layers by lipoproteins (Figure 1.20).

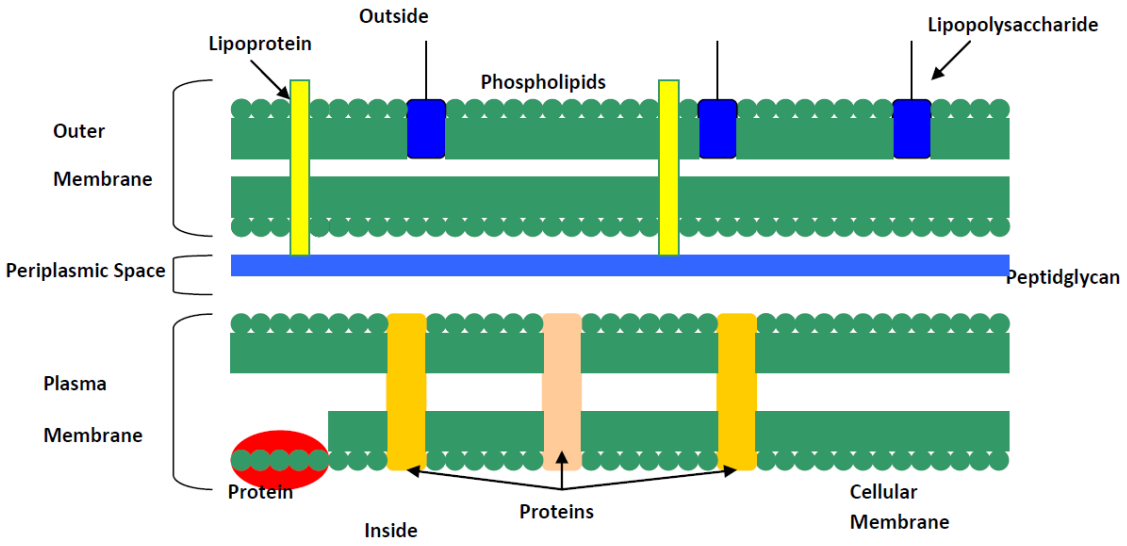

Figure 1.20 Structure of the cell wall of Gram-negative bacteria. The wall is relatively thin and contains 20% peptidoglycan (by thickness). The outer membrane is attached to the peptidoglycan by lipoprotein molecules. LPS is found on the surface anchored to the outer membrane. Figure was modified from Cabeen and Jacobs-Wagner (2005).

LPS is composed of polysaccharide moieties and lipid components; the lipids are effective stimulators of innate immunity whilst polysaccharide moieties are the major

antigens targeted by adaptive immunity. When LPS is released into the blood following bacterial infection, it can cause disseminated intravascular coagulation (DIC) following elevated secretion of pro-coagulant proteins (eg, tissue factor) and decreased anti-coagulant activity on endothelial cells via the action of TNF (Kume et al., 2003, Chen et al., 2004, Stief, 2009). Moreover, several organs such as liver and kidney can show inflammation and intravascular thrombosis leading to malfunction (Stief, 2009, Chen et al., 2004).

Peptidolgycan is a highly conserved polymer that supports the bacterial structure and counters the osmotic pressure of the cell. It also enhances the secretion of proinflammatory cytokines (eg, IL-1 and IL-12) by binding to receptors on host cells, such as Toll-like receptor 2 (TLR2), although binding is weak compared to LPS (Travassos et al., 2004).

Lipoproteins are essential for bacterial survival and pathogenesis but their pathogenic function is yet to be determined (Godlewska et al., 2009). They are targeted by Toll-like receptors (eg, TLR-2) that stimulate the secretion of cytokines such as IL-1. They are also suggested to participate in the transport of compounds through the cell membrane. For example, lipoprotein mutant strains of *Pseudomonas putida*, *Pseudomonas aeruginosa* and *E. coli* showed insignificant growth in media containing compounds such as arginine, fructose, saccharose and glycerol as the sole carbon source, probably as a result of defects in the transport of these compounds (Llamas et al., 2003).

1.25 Gram-positive bacteria

The cell wall of Gram-positive bacteria comprises a single lipid membrane and is mainly composed of thick peptidoglycan polymer layers (90% of the cell wall thickness) linked by amino acid bridges. Teichoic acid polymers intersperse the layers

(see *Staphylococcus aureus*, section 1.26) and occasionally anchor into the plasma membrane to form LTA (see Lipoteichoic acid, section 1.27) (Figure 1.21).

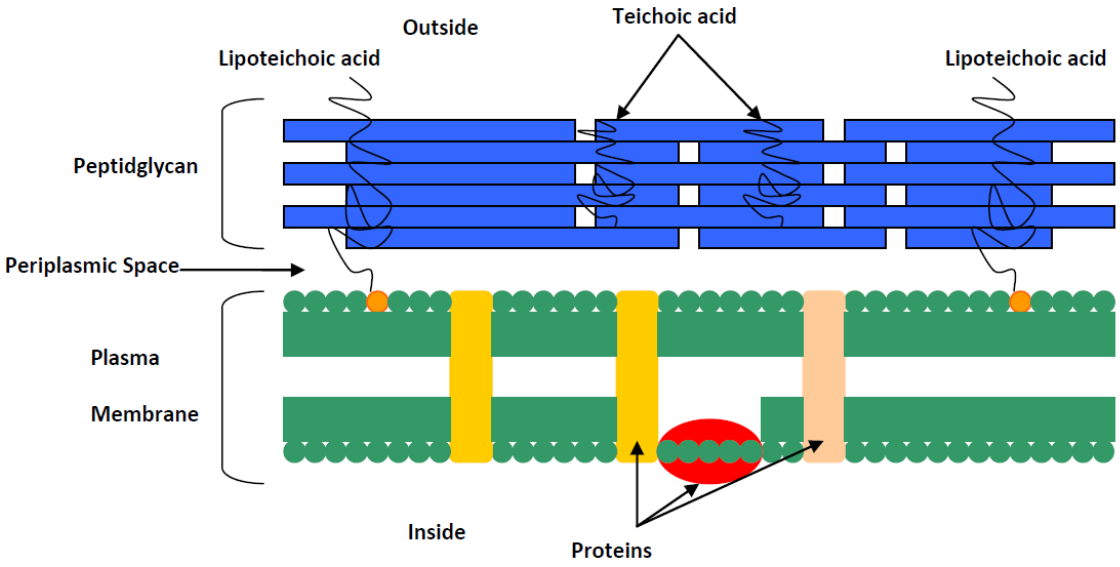

Figure 1.21 Structure of the Gram-positive bacteria cell wall. The wall is thick and contains 90% peptidoglycan (by thickness). It is interspersed with teichoic acids and lipoteichoic acids. Figure was modified from Cabeen and Jacobs-Wagner (2005).

Teichoic acids confer a high density of negative charge on the bacterial surface and attract cations such as Ca^{2+}, Fe^{3+}, and Mg^{2+}, which contribute towards the rigidity of the cell wall (Thwaite et al., 2002). They also facilitate the adherence of Gram-positive bacteria such as *S. aureus* to epithelial cells by targeting glycoprotein fibronectins on the surface of these cells (Aly and Levit, 1987).

1.26 *Staphylococcus aureus*

Around half million cases of sepsis with multiple organ dysfunction syndrome are annually recorded in the United States (Increase in National Hospital Discharge Survey rates for septicemia-United States, 1979-1987. *MMWR Morb Mortal Wkly Rep* 39:31, (1990). They contribute 35% towards the mortality rate in surgical intensive care units (Balk and Bone, 1989, Pinner et al., 1996). Despite the introduction of antibiotics, sepsis is still a major source of such high mortality rates. Gram-positive bacteria are the

cause of around 50% of all cases of sepsis (Wenzel, 1988, Pinner et al., 1996, Solomkin, 2001) and of these, *S. aureus* is most common pathogen (Solomkin, 2001, Richardson et al., 1999).

S. aureus is a common human pathogen associated with a variety of different diseases ranging from local infections (eg, skin infections including cellulitis and impetigo) to systemic dissemination and metastatic infections eg, septic arthritis, where *S. aureus* invades a joint to produce arthritis. Local and systemic effects of toxins produced by *S. aureus* include toxic shock syndrome and scalded skin syndrome (Archer, 1998, Lowy, 1998). The Staphylococcal outer envelope is formed from peptidoglycans, proteins, teichoic and teichuronic acids and LTA (Ghuysen et al., 1965, Robbins and Schneerson, 1990, Fischer, 1994, Labischinski et al., 1998, Sriskandan and Cohen, 1999, Neuhaus and Baddiley, 2003). *S. aureus* is able to cause so many different diseases mainly because of its capability to produce multiple virulence factors (Table 1.8).

***Staphylococcus aureus* virulence factors**	**Abbreviation**
Protein A	Spa
Clumping factor A	ClfA
Capsular polysaccharide serotypes 5 and 8	Cps
Chemotaxis inhibitory protein	CHIPS
Staphylokinase	Sak
MHC Class II analogous protein/extracellular adherence protein	Map/Eap
Extracellular fibrinogen binding protein	Efb
Aureolysin	Aur
Panton-Valentine leukocidin	PVL
Leukocidin E-D	LukED
γ-haemolysin	HIg
Enterotoxin A	Sea
Enterotoxin B	Seb
Enterotoxin C	Sec
Enterotoxin D	Sed
Enterotoxin G	Seg
Enterotoxin H	Seh
Toxic shock syndrome toxin-1	TSST-1
Staphylococcal response AB	SrrAB
Staphylococcus aureus type 8 capsular polysaccharide	CP8

Table 1.8 Selected *S. aureus* virulence factors (Foster, 2005).

Virulence factors such as protein A, chemotaxis-inhibitory protein of staphylococci (CHIPS), the panton-valentine leukocidin (PVL) and clumping factor A (CIfA) weaken the host immune response. Protein A is a surface protein anchored to the cell wall that binds to the Fc region of IgG. Binding coats the bacterial surface with IgG molecules oriented to prevent interactions with Fc receptors of phagocytes (Foster, 2005). CHIPS is a chemotaxis inhibitory protein that interacts with the formyl peptide receptor and the C5a receptor on the surface of neutrophils and inhibits the migration of these phagocytes to the site of inflammation (Foster, 2005). PVL lyses leukocytes and is most commonly associated with severe skin infections (Foster, 2005). CIfA is a fibrinogen-binding protein located on the surface of *S. aureus* and coats it with fibrinogen to provide protection against phagocytosis by human neutrophils (Foster, 2005). Despite the identification of all these virulence factors, the molecular pathology of the immune response to *S. aureus* is still poorly understood. However, it is known that staphylococcal secreted proteins and cell wall components contribute towards multiple inflammatory responses by host cells.

1.26.1 Capsule

Most *S. aureus* clinical isolates, including serotype 5, serotype 8 and serotype 336, possess a thin microcapsular layer made of different serotype capsular polysaccharides. An elevated virulence level in animal infection models is associated with the production of type 5 and type 8 capsules (Luong and Lee, 2002, Baddour et al., 1992). Moreover, *in vitro* studies have shown that the presence of the capsule may inhibit the binding of many serum opsonins, significantly decreasing phagocytosis by neutrophils (Thakker et al., 1998, Nilsson et al., 1997). However, elevated levels of particular anti-capsular antibodies mediate opsonophagocytosis and provide limited protection against infection (O'Riordan and Lee, 2004, Lee et al., 1997).

1.26.2 Peptidoglycan

The *S. aureus* peptidoglycan is a highly conserved polymer in the bacterial cell wall that triggers the alternative pathway of the complement system by enhancing the formation of C3 convertase. It also induces the release of proinflammatory cytokines (eg, IL-1 and IL-12) and chemokines (eg, IL-8) in phagocytes (eg, macrophages). Induction of these cytokines by peptidoglycan is weak compared to LPS (Travassos et al., 2004). However, synergetic effects with LTA promote septic shock and organ failure in rats (De Kimpe et al., 1995, Kengatharan et al., 1998, Thiemermann, 2002).

1.26.3 Hemolysins

S. aureus produce other virulence factors such as α-, and δ-hemolysins. α-hemolysin is a self-assembling, channel-forming toxin, which interacts with the membrane of different cells including peripheral blood monocytes and endothelial cells (Bhakdi et al., 1989, Bhakdi and Tranum-Jensen, 1988), where it forms pores and mediates cell lysis (Bhakdi et al., 1989, Bhakdi and Tranum-Jensen, 1988, Dragneva et al., 2001, Onogawa, 2002). δ-hemolysin is a 26 amino acid hemolytic peptide toxin that can rapidly cross the host lipid membrane to induce red blood cell lysis (Pokorny et al., 2002). It also interacts with human neutrophils, stimulating their oxidative burst and release of TNF-α (Schmitz et al., 1997).

1.27 Lipoteichoic acid (LTA)

LTA is a bacterial cell wall component found uniquely in Gram-positive bacteria. It is an amphiphile composed of a hydrophilic backbone with glycerophosphate and D-alanine (or hexose substituents) repeats in addition to lipophilic glycolipids (Figure 1.22).

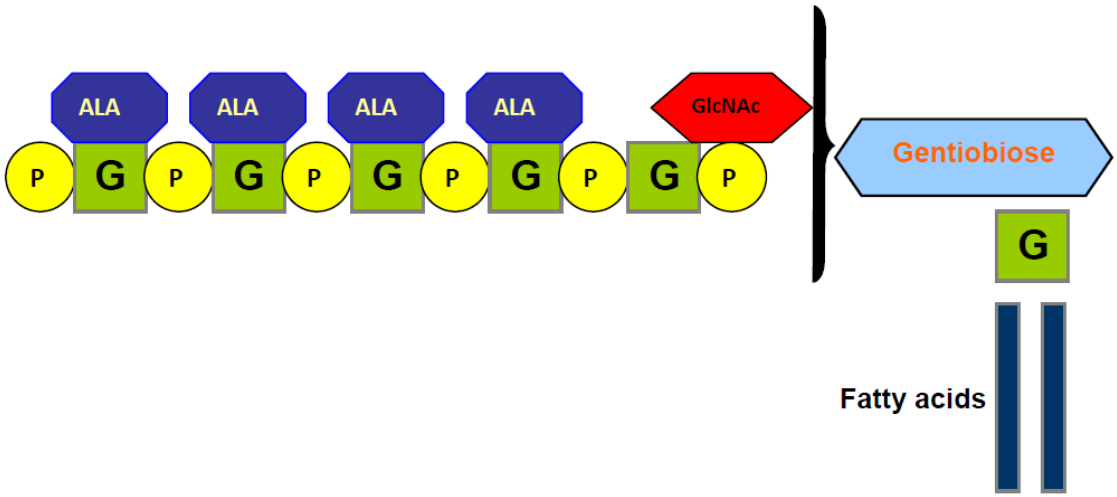

Figure 1.22 Structure of lipoteichoic acid. LTA is an amphiphile composed of a hydrophilic backbone with glycerophosphate (G: glycerol, P: phosphate) and D-alanine (ALA) repeats in addition to lipophilic glycolipids. Figure was modified from Xia et al.(2010).

Gram-positive bacteria release LTA following bacteriolysis triggered by components in plasma and by neutrophil cationic agents (Ginsburg, 1987, Ginsburg, 1998, Sriskandan and Cohen, 1999) or beta-lactam antibiotics (Sela et al., 1977, Alkan and Beachey, 1978, Horne and Tomasz, 1979, Ginsburg, 1987, Ginsburg, 1998, van Langevelde et al., 1998, Periti and Mazzei, 1998, Heer et al., 2000). LTA is also an adhesion molecule by which Gram-positive bacteria can interact with host cells. *S. aureus* binds fatty acids carried by albumin via LTA (Beachey et al., 1988). Moreover, *in vitro* studies have suggested that LTA targets a receptor on human neutrophils known as CD14 to induce the release of proinflammatory mediators such as IL-8 and TNF-α (Hattar et al., 2006).

LTA is considered to be a virulence factor because it induces the release of proinflammatory agonists. It may have a role in cell damage in inflammatory conditions and in postinfectious sequelae (eg, sepsis and septic shock) (Ginsburg, 2002). For example, LTA from group-A streptococci stimulated human polymorphonuclear cells to produce superoxide and secrete lysosomal enzymes when treated with antistreptococcal serum (Ginsburg et al., 1988). Moreover, macrophages released lysosomal hydrolases once sensitised with LTA in the absence of antibodies (Harrop et al., 1980).

LTA can contribute to the regulation, recruitment and activation of leukocytes during inflammation (Ginsburg, 2002). For instance, LTA purified from *S. aureus* induces the release of hepatocyte growth factor (HGF) from human dermal fibroblasts and the secretion of the macrophage activating and chemotactic cytokine MIP-1α (Baroni et al., 1998, Danforth et al., 1995). In addition, LTA was shown to inhibit chemotaxis of human polymorphonuclear leukocytes and phagocytosis of *S. aureu*s by these leukocytes (Ginsburg and Quie, 1980, Raynor et al., 1981). LTA was also demonstrated to activate the lectin pathway of the complement system by specific interactions with L-ficolin (Lynch et al., 2004).

LTA can behave as an adaptable immunomodulator capable of regulating cell responses in inflammatory conditions (Ginsburg, 2002). For example, LTA purified from *S. aureus* induces a blastogenic response in T lymphocytes (transformation of small T lymphocytes into larger cells undergoing mitosis) isolated from healthy individuals (Aasjord et al., 1986). Moreover, LTA binds TLR2 on macrophages to stimulate the activation of nuclear factor-kappa B (NF-kB; a transcription factor contributes to cytokine secretion) and blocks proliferation of fibroblast (cells of connective tissues) by unknown mechanisms (Ginsburg, 2002, Elgavish, 2000). LTA also induces mononuclear phagocytes to release cytokines such as TNF-α, interferon-γ, interleukins; (IL-1, IL-5, IL-6 and IL-8) and anti inflammatory interleukins; (IL-10 and IL-12) (Bhakdi et al., 1991, Wang et al., 2000). These cytokines play an essential role in the host defence mechanism against different bacterial infections especially in sepsis and other post infectious sequelae (Arndt and Abraham, 2001, Carlet, 2001).

1.28 General aims of this thesis

LTA is an important inflammatory component of *S. aureus*. However, important questions remain unanswered with regard the interactions of LTA with human serum components and human phagocytes and the mechanisms employed by the host to target LTA and thereby eliminate *S. aureus*. In order to provide new insights into these processes, this thesis aims to address the following areas:

- Identify human serum proteins capable of binding to LTA from *S. aureus*
- Study the interactions of selected binding proteins with LTA and *S. aureus*
- Evaluate the role of these proteins in the elimination of *S. aureus* by isolated human neutrophils, monocytes and macrophages

Chapter 2
Identifying human serum LTA-binding proteins

2.1 Introduction and Objectives

LTA is a distinctive bacterial cell wall constituent of Gram-positive bacteria that can facilitate the interaction of microorganisms with host cells. It is also a virulence factor, which stimulates the release of proinflammatory agonists and the recruitment and activation of leukocytes during inflammation (Ginsburg, 2002). For example, LTA induces mononuclear phagocytes to release cytokines such as TNF-α, interferon-γ and different interleukins essential for the defence mechanism of the host against infection (Bhakdi et al., 1991, Wang et al., 2000). LTA is increasingly considered as the Gram-positive counterpart of LPS, although its potent immunostimulatory functions are relatively poorly characterized. In a recent study in our laboratory, LTA was shown to bind directly to human L-ficolin and promote activation of the lectin pathway of complement (Lynch et al., 2004). Based on this work, it was hypothesized that LTA might interact with additional serum proteins to initiate alternative immune responses and assist in the elimination of invading bacteria. This chapter focuses on identifying novel LTA-binding proteins from human serum using a highly purified LTA preparation from *S. aureus*, and evaluating whether these interactions are likely to have significant immunological importance.

Purification methods for LTA have developed considerably over the past years to produce biologically active material that is free from contamination by other bacterial components. Early methods for extraction employed hot phenol extraction followed by hydrophobic interaction chromatography (Wang et al., 2003). LTA purified from *Bacillus Subtilis*, *Listeria monocytogenes*, *Streptococcus pygoenes* and several *Enterococci* strains stimulated adherent human monocytes to release inflammatory cytokines such as IL-1β, IL-6, and TNF-α (Bhakdi et al., 1991). However, LTA purified from *S. aureus* and *S. pneumoniae* using the same method did not stimulate cytokine release (Bhakdi et al., 1991). As a consequence, LTA from *S. aureus* was considered to be not very potent relative to other species (Wang et al., 2003). Nevertheless, cells could be stimulated by higher concentrations of LTA. For instance, relatively high doses of LTA from *S. aureus* stimulated the expression of iNOS in vascular smooth muscle cells, which may contribute to the severe hypotension induced by sepsis (Auguet et al., 1992). This study was one of the first to suggest that LTA may have similar properties to LPS, the most potent known microbial structure at that time, but only at significantly elevated concentrations (Wang et al., 2003).

Commercial preparations of LTA purified from *S. aureus*, *B. subtilis* and *Streptococcus sanguis* have been shown to carry significant amounts of contaminants capable of stimulating inflammatory responses, and thus are unsuitable for immunological analysis (Gao et al., 2001, Morath et al., 2002). For example, hydrophobic interaction chromatography demonstrated the presence of significant amounts of LPS contaminants (Gao et al., 2001). The potency of these preparations to induce production of NO in RAW 264.7 mouse macrophages was remarkably reduced by the LPS-inhibitor polymyxin B, implying that much of the activity was LPS mediated (Gao et al., 2001). Moreover, nuclear magnetic resonance spectroscopy and hydrophobic interaction

chromatography showed significant decomposition of the LTA characterized by a loss of glycerol-phosphate units, D-alanine and N-acetylglucosamine substituents (Morath et al., 2001).

The original phenol extraction method was therefore modified using n-butanol extraction at room temperature, in place of the phenol step (Morath et al., 2001). Using this strategy, highly pure, biologically active LTA was produced from *S. aureus* (Morath et al., 2001). Nuclear magnetic resonance and mass spectroscopy structural analysis demonstrated the presence of intact D-alanine substituents (Morath et al., 2001). Moreover these preparations were found to have increased biological activity by several orders of magnitude (Morath et al., 2001). Thus, LTA was re-established to be an important inflammatory component of Gram-positive bacteria (Wang et al., 2003). All experiments in this thesis were conducted with LTA purified using this protocol.

2.2 Materials and Methods

2.2.1 Materials

Octyl-Sepharose CL-4B was purchased from Amersham and n-butanol was from Merck. Sepharose 6B, divinyl sulfone, Protein A agarose fast flow were procured from Sigma-Aldrich. SilverQuest staining kit and protein molecular weight markers for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Invitrogen. Silver staining, Gradient gels and 2 dimensional-PAGE (2D-PAGE) kits were obtained from Bio-Rad. VisPRO 5 minutes protein staining kit was from Avegene and Colloidal Coomassie was from Calbiochem.

2.2.2 Purification of LTA from *S. aureus*

LTA was purified from *S. aureus* (DSM 20233) at Konstanz University using the procedure described by Morath et al. (2001). *S. aureus* was grown aerobically in a 42-liter fermentor at 37 ºC and harvested at an estimated OD_{578} of 15 using a constant flow centrifuge. The bacterial pellet was then resuspended in 0.1 M sodium citrate buffer, pH 4.7, and broken with glass beads in a Braun disintegrator. The disrupted bacteria were mixed with an equal volume of n-butanol under shaking for 30 minutes at room temperature (RT). Following centrifugation at 13,000 g for 20 minutes, the aquous phase was lyophilized overnight, resuspended with 15% n-propanol in 0.1 M ammonium acetate, pH 4.7 (chromatography start buffer) and centrifuged at 45,000 g for 15 minutes. The supernatant was then applied to an octyl-Sepharose CL-4B column (1.5 by 29 cm) equilibrated with 15% n-propanol in 0.1 M ammonium acetate; pH 4.7. The column was washed with 4 column volumes of equilibration buffer at 15 ml/h and LTA was eluted using a linear gradient (15% to 60% n-propanol) in 0.1 M ammonium acetate, pH 4.7 at 15 ml/h and aliquots of 4 ml were collected. The purity of LTA was

tested and determined to be greater than 99% pure by our collaborator Dr. Corinna Hermann (Department of Biology, University of Konstanz, Germany), using nuclear magnetic resonance, mass spectrometry and cytokine induction assays as described by Morath et al. (2001).

2.2.3 Coupling LTA to Sepharose beads

Sepharose 6B (2 ml) was activated in 0.5 M NaHCO3, pH 11 (3 ml) containing divinyl sulfone (0.3 ml) for 70 minutes at RT with mixing. LTA (1.09 mg) was then mixed with the activated Sepharose 6B in 0.5 M NaHCO3, pH 10 (1 ml), with stirring overnight at RT. After coupling the LTA to the Sepharose beads, any remaining reactive groups were blocked by mixing with 0.5 M NaHCO3, pH 8.5 (1 ml), and 2-mercaptethanol (0.02 ml) for two hours at RT. The affinity matrix was washed extensively in water prior to use.

2.2.4 Separation of serum from healthy human blood

Human blood was extracted from healthy volunteers and was left to clot overnight at 4 ºC. The clot was removed by centrifugation for 30 minutes at 3500 g and at 4 ºC, and the serum was isolated and stored at -20 ºC.

2.2.5 Depletion of IgG antibodies from serum

Protein A agarose fast flow was used to deplete IgG antibodies from human serum (its binding capacity for IgG is 37-43 mg/ml). It was packed in a chromatography column (2 ml), equilibrated with ice cold TBS buffer (10 mM Tris/HCl and 140 mM NaCl), pH 7.4, and loaded with serum (5 ml), and fractions depleted of IgG were collected. After washing the column with 50 mM Glycine, pH 2.7, and ice cold TBS buffer to remove the bound IgG, the serum was re-loaded onto the same column to further deplete it of IgG.

2.2.6 Removal of Sepharose-binding proteins from serum

To remove Sepharose-binding proteins from the IgG-depleted serum, serum (6 ml) was loaded onto a Sepharose 6B (2 ml) equilibrated with ice cold TBS. Fractions containing serum were collected and the procedure was repeated one additional time using fresh Sepharose.

2.2.7 Identifying serum LTA-binding proteins

Equivalent volumes (6ml) of pretreated human serum were loaded onto the LTA-Sepharose column and a control column, in parallel, containing Sepharose 6B (activated with divinyl sulfone and blocked with β-mercaptoethanol as above but without LTA). Both columns were washed with 10 column volumes of TBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ and 1.5 ml fractions were collected until the absorbance at 280 nm < 0.05. To remove any metal ion-dependent binding components, the column was then washed with TBS containing 1 mM EDTA and finally was eluted with TBS containing 1% SDS. Columns were further washed with 50 column volumes of TBS containing 1% SDS and were stored in TBS containing 0.02% sodium azide at 4 ºC for further use. Fractions were stored at -20 ºC. Proteins eluted from the column were analyzed by SDS-PAGE; using 1 dimensional (1D) or 2 dimensional (2D) gels. Different staining methods were utilized to detect LTA-binding proteins, including Coomassie blue, Colloidal Coomassie, Silver stain, SilverQuest silver stain and VisPRO 5 minutes protein stain. Proteins of interest were identified by in-gel trypsin digestion and MALDI-TOF-MS (matrix assisted laser desorption ionisation time-of-flight mass spectrometry) by the sequencing service at Leicester University (PNACL).

2.3 Results

In order to capture human serum LTA-binding proteins, highly pure LTA from *S. aureus* was chemically coupled to Sepharose beads using divinyl sulfone. This method targets hydroxyl groups, so LTA is likely to be bound in a variety of different conformations. Before loading (Sepharose 6B coupled with LTA column and the control column), the serum was first depleted of IgG using protein A-agarose to prevent saturation of the LTA-Sepharose with IgG, which could hamper binding and detection of other LTA-binding proteins. Serum was then loaded onto the columns and proteins were first eluted with EDTA to capture any metal ion-dependent binding proteins followed by 1% SDS to denature and elute the more-tightly bound proteins. Fractions were analyzed by SDS-PAGE under reducing condition and serum proteins were detected by a variety of different staining methods.

2.3.1 Coomassie blue staining

Coomassie blue staining was the first method used. The Coomassie stain is a disulfonated triphenylmethane dye that interacts with protonated basic amino acids such as lysine, arginine and histidine via electrostatic interactions, and binds aromatic residues via hydrophobic interactions. Consequently, protein bands are stained bright blue (Steinberg, 2009). This method is relatively insensitive and can detect around 50 ng protein. However it is non-covalent, reversible and compatible with identification by in gel trypsin digestion/MALDI-TOF-MS, so was used as an initial screen. A typical gel is shown in Figure 2.1.

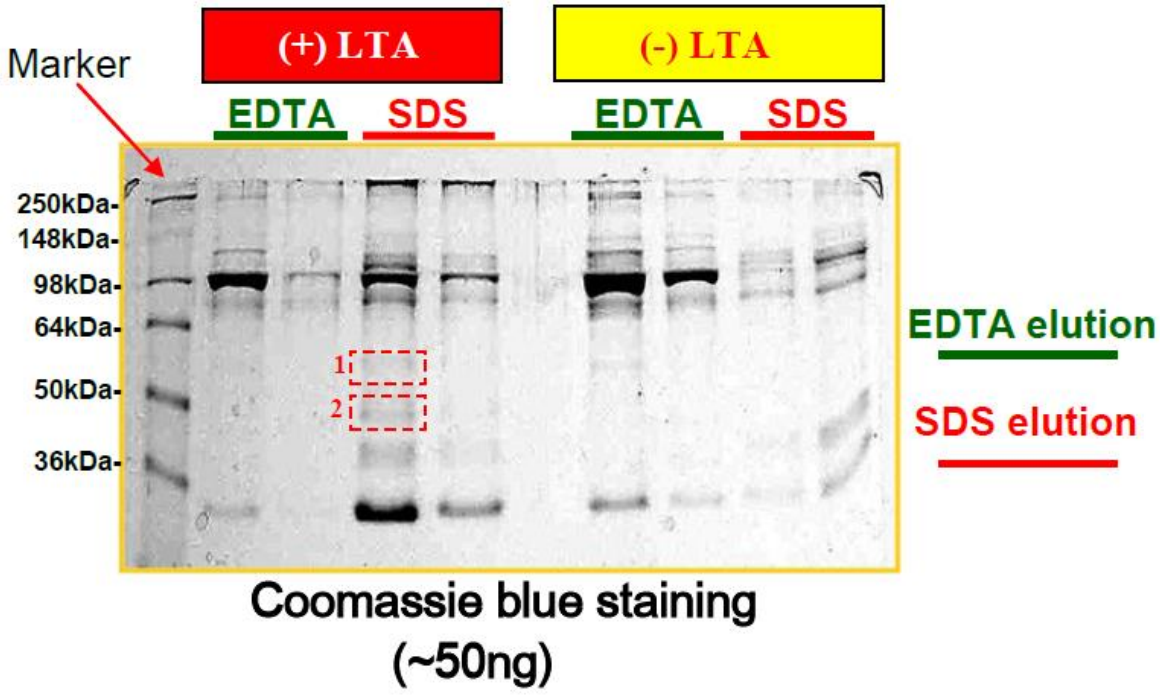

Figure 2.1 SDS-PAGE gel stained with Coomassie blue to identify serum proteins capable of interacting with LTA. SDS-PAGE gel under reducing conditions (12%), the first two elution fractions using EDTA and SDS are shown. Fractions from LTA-Sepharose and control columns were analyzed in parallel. After staining with Coomassie blue, two protein bands (1 and 2) were identified as putative LTA-binding proteins.

Two unique protein bands (1 and 2) were identified in SDS-elution fraction 1 as putative LTA-binding proteins. These bands were not seen in the corresponding elution fraction from the control column. Both bands were extracted and analyzed by in gel trypsin digestion/MALDI-TOF-MS.

Mass spectrometry data showed that the first band contains apolipoprotein A-IV and haptoglobin (HP) whereas the second band is apolipoprotein E3/E3K (Table 2.1). Seven peptides that match sequences in apolipoproteins A-IV were detected (Table 2.1 and Figure 2.2). In addition, five other peptides were identified to match peptide sequences in HP (Table 2.1 and Figure 2.2). In the second band, twenty peptides were found to match peptide sequences in apolipoprotein E3/E3K (Table 2.1 and Figure 2.2).

Importantly, all peptides correspond to those that would be expected to be generated by trypsin digestion, supporting their predicted identities. For example, the preceding residue of peptide 113-123 of apolipoprotein A-IV would be an arginine residue, corresponding to the cleavage specificity of trypsin. Furthermore the C-terminal residues of peptides are invariably lysine or arginine residues, as expected.

Bands	Proteins	Matching peptide sequences		Observed Mass (Da)	Calculated Mass (Da)
1	apolipoprotein A-IV	113 - 123	LLPHANEVSQK	1235.5305	1234.6670
		135 - 143	LEPYADQLR	1103.4743	1103.5611
		144 - 154	TQVSTQAEQLR	1259.6178	1259.6470
		201 - 209	LTPYADEFK	1082.4404	1082.5284
		222 - 233	SLAPYAQDTQEK	1349.6619	1349.6463
		256 - 264	ISASAEELR	974.5118	974.5032
		317 - 326	ALVQQMEQLR	1230.6113	1230.6390
	haptoglobin	112 - 119	GSFPWQAK	919.4123	919.4552
		157 - 168	DIAPTLTLYVGK	1289.6894	1289.7231
		170 - 176	QLVEIEK	857.4614	857.4858
		321 - 332	SCAVAEYGVYVK	1344.5728	1344.6384
		333 - 342	VTSIQDWVQK	1202.6068	1202.6295
2	apolipoprotein E3/E3K	34 - 43	QQTEWQSGQR	1246.5336	1246.5691
		44 - 50	WELALGR	843.3848	843.4603
		51 - 56	FWDYLR	898.3855	898.4337
		80 - 87	ALMDETMK	969.3755	969.4147
		94 - 108	SELEEQLTPVAEETR	1729.5614	1729.8370
		94 - 108	SELEEQLTPVAEETR	1729.8826	1729.8370
		122 - 132	LGADMEDVCGR	1237.4978	1237.5067
		138 - 152	GEVQAMLGQSTEELR	1662.7743	1662.7883
		138 - 152	GEVQAMLGQSTEELR	1662.8849	1662.7883
		177 - 185	LAVYQAGAR	947.4698	947.5188
		199 - 207	LGPLVEQGR	967.4593	967.5451
		210 - 224	AATVGSLAGQPLQER	1496.7949	1496.7947
		225 - 231	AQAWGER	816.3371	816.3878
		252 - 258	EQVAEVR	829.4831	829.4294
		261 - 269	LEEQAQQIR	1113.5431	1113.5778
		270 - 278	LQAEAFQAR	1032.4717	1032.5352
		281 - 292	SWFEPLVEDMQR	1535.7217	1535.7079
		281 - 292	SWFEPLVEDMQR	1551.6556	1551.7028
		293 - 300	QWAGLVEK	929.4857	929.4971
		301 - 317	VQAAVGTSAAPVPSDNH	1619.7824	1619.7904

Table 2.1 Putative LTA-binding proteins identified following staining with Coomassie blue. Protein bands were extracted from the SDS-PAGE gel, stained with Coomassie blue, and analyzed by in gel trypsin digestion/MALDI-TOF-MS.

Apolipoprotein A-IV

```
  1 MFLKAVVLTL ALVAVAGARA EVSADQVATV MWDYFSQLSN NAKEAVEHLQ
 51 KSELTQQLNA LFQDKLGEVN TYAGDLQKKL VPFATELHER LAKDSEKLKE
101 EIGKELEELR ARLLPHANEV SQKIGDNLRE LQQRLEPYAD QLRTQVSTQA
151 EQLRRQLTPY AQRMERVLRE NADSLQASLR PHADELKAKI DQNVEELKGR
201 LTPYADEFKV KIDQTVEELR RSLAPYAQDT QEKLNHQLEG LTFQMKKNAE
251 ELKARISASA EELRQRLAPL AEDVRGNLRG NTEGLQKSLA ELGGHLDQQV
301 EEFRRRVEPY GENFNKALVQ QMEQLRQKLG PHAGDVEGHL SFLEKDLRDK
351 VNSFFSTFKE KESQDKTLSL PELEQQQEQQ QEQQQEQVQM LAPLES
```

Haptoglobin (HP)

```
  1 MSALGAVIAL LLWGQLFAVD SGNDVTDIAD DGCPKPPEIA HGYVEHSVRY
 51 QCKNYYKLRT EGDGVYTLND KKQWINKAVG DKLPECEAVC GKPKNPANPV
101 QRILGGHLDA KGSFPWQAKM VSHHNLTTGA TLINEQWLLT TAKNLFLNHS
151 ENATAKDIAP TLTLYVGKKQ LVEIEKVVLH PNYSQVDIGL IKLKQKVSVN
201 ERVMPICLPS KDYAEVGRVG YVSGWGRNAN FKFTDHLKYV MLPVADQDQC
251 IRHYEGSTVP EKKTPKSPVG VQPILNEHTF CAGMSKYQED TCYGDAGSAF
301 AVHDLEEDTW YATGILSFDK SCAVAEYGVY VKVTSIQDWV QKTIAEN
```

Apolipoprotein E3/E3K

```
  1 MKVLWAALLV TFLAGCQAKV KQAVETEPEP ELRQQTEWQS GQRWELALGR
 51 FWDYLRWVQT LSEQVQEELL SSQVTQELRA LMDETMKELK AYKSELEEQL
101 TPVAEETRAR LSKELQAAQA RLGADMEDVC GRLVQYRGEV QAMLGQSTEE
151 LRVRLASHLR KLRKRLLRDA DDLQKRLAVY QAGAREGAER GLSAIRERLG
201 PLVEQGRVRA ATVGSLAGQP LQERAQAWGE RLRARMEEMG SRTRDRLDEV
251 KEQVAEVRAK LEEQAQQIRL QAEAFQARLK SWFEPLVEDM QRQWAGLVEK
301 VQAAVGTSAA PVPSDNH
```

Matched peptides shown in **bold red**

Figure 2.2 Protein sequences of apolipoprotein A-IV, haptoglobin and apolipoprotein E3/E3K showing the matched tryptic peptides. The peptide coverage was 17% of apolipoprotein IV, 14% of HP and 54% of apolipoprotein E3/E3K (matched peptides are shown in *bold red*).

Although Coomassie blue staining was effective enabling detection and identification of some serum proteins, sensitivity is relatively low compared to other staining methods, so only the most abundant proteins are likely to be identified. To detect less-abundant proteins, more sensitive staining methods were used as discussed below.

2.3.2 Silver staining

Silver staining is a very sensitive tool for protein visualizations on SDS gels. The staining mechanism is relatively complex and involves the reduction of protein-bound silver ions to metallic silver. Glutaraldehyde is used to bind amino groups in proteins and enhance the binding of silver ions. Following saturation, protein is visualized by reduction of bound silver ions to insoluble, visible metallic silver (Steinberg, 2009). This method can detect very low amounts of protein, with as little as 2.5 ng protein per band. However, identification by mass spectrometry can be problematic because proteins become cross-linked to the gel matrix, preventing the elution of peptides.

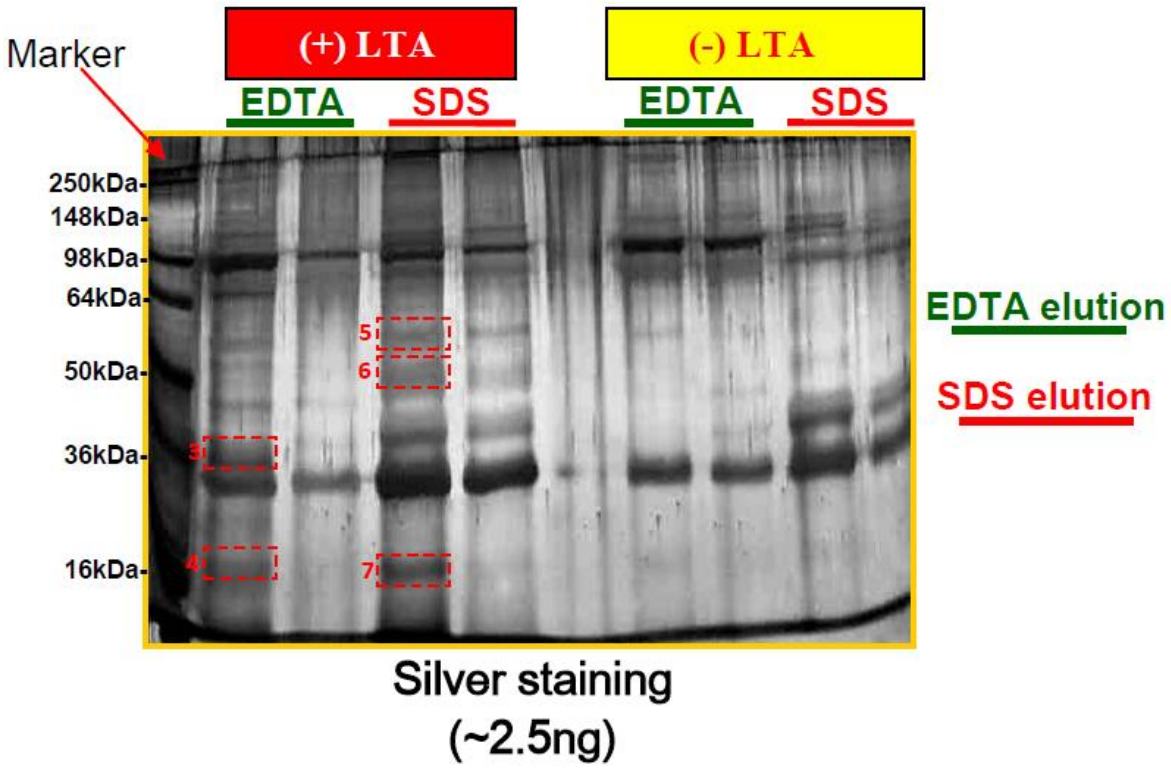

Figure 2.3 SDS-PAGE gel stained using Silver stain to identify LTA-binding proteins from human serum. Reducing SDS-PAGE gel (12%) of EDTA and SDS elution fractions equivalent to those in Figure 2.1. Five putative LTA-binding protein bands (3, 4, 5, 6 and 7) were identified in the first EDTA and SDS elution fractions.

Five protein bands (3, 4, 5, 6 and 7) were extracted from the gel shown in Figure 2.3, which were unique to elution fractions from the LTA-Sepharose column. These were identified as before (see Table 2.2 and Scheme 1 in Appendix 1).

Bands	Proteins	Number of matched peptide sequences	Peptides sequence coverage (%)
3	unidentified	None	None
4	unidentified	None	None
5	apolipoprotein A-IV	15	36
	haptoglobin	4	16
	serum paraoxanase	3	10
6	apolipoprotein E3	13	38
	apolipoprotein J	5	15
	haptoglobin related protein	4	10
7	unidentified	None	None

Table 2.2 Identification of proteins stained by Silver stain. Protein bands were extracted from the SDS-PAGE gel stained with Silver stain and analyzed by in-gel trypsin digestion/MALDI-TOF-MS. Although clearly detectable on the gel, only some of the proteins could be identified by mass spectrometry.

Band 5 contained at least three proteins: apolipoprotein A-IV, HP and serum paraoxanase and band 6 comprised apolipoprotein E3, apolipoprotein J and haptoglobin-related protein (HPr). Proteins in the other bands (3, 4 and 7) were not identified. As described above, this is a common problem with silver-stained gels and is thought to be caused by poor elution of peptides from the gel following trypsin digestion as a result of formaldehyde and glutaraldehyde cross-links, hampering detection of low abundant protein species.

To overcome this problem, other staining methods such as SilverQuest silver stain and Colloidal Coomassie were tested.

2.3.3 SilverQuest silver stain

The SilverQuest silver staining method is more sensitive than standard silver staining, and can detect as little as 0.3 ng protein per band. As in the standard silver staining method, it is also based on chemical reduction of silver ions to metallic silver on protein bands. However a specially formulated sensitizer solution is utilised to improve sensitivity and compatibility with mass spectrometry analysis. Although it still forms glutaraldehyde bonds with the gel matrix, it does not form formaldehyde crosslinks, so more proteins can be identified.

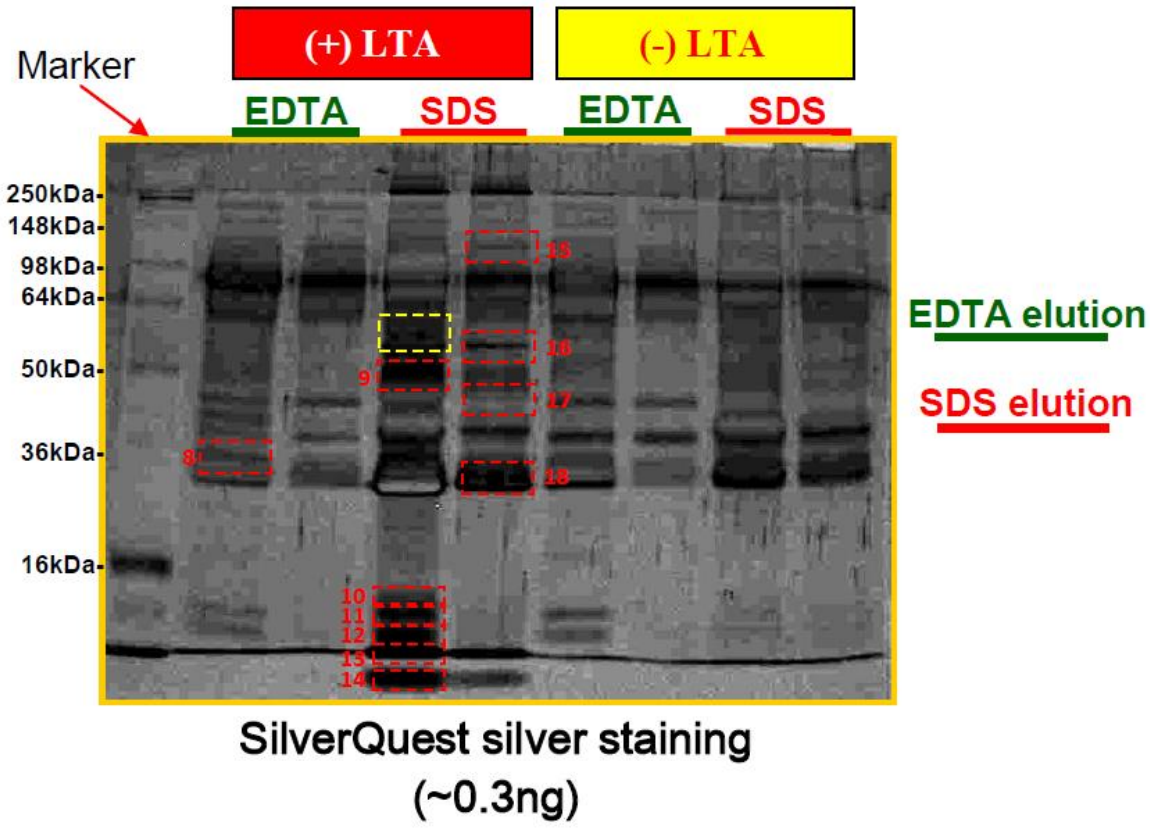

Figure 2.4 SDS-PAGE gel stained with SilverQuest. Reducing SDS-PAGE gel (12%), showing EDTA and SDS elution fractions as in Figures 2.1 and 2.3. Eleven putative LTA-binding protein bands (8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18) were identified.

Eleven protein bands (8, 9, 10, 11, 12, 13, 14, 15, 16, 17 and 18), specific to the LTA column were identified (see Table 2.3 and Scheme 2 in Appendix 1).

Bands	Proteins	Number of matched peptide sequences	Peptides sequence coverage (%)
8	pre-serum amyloid P component	9	33
10	carboxypeptidase D	4	3
9,11,12,13,14,16	keratins	multiple (eg, 12 for band 9)	(eg, 16 for band 9)
15	collagen	2	6
17	apolipoprotein E	5	16
18	plasminogen	2	1
	lipoprotein GIn I	7	25

Table 2.3 Identification of LTA binding proteins stained by SilverQuest silver stain. Protein bands were extracted from SDS-PAGE gel stained with SilverQuest silver stain and sent for identification by mass spectrometry. The listed proteins in this table are the identity of the extracted protein bands, provided after mass spectrometry analysis.

Novel putative LTA-binding proteins including pre-serum amyloid P component (band 8) and carboxypeptidase D (band10) were identified using this method. On the other hand, other bands represented a common contaminant from human hair, called keratin were detected (bands 9, 11, 12, 13, 14 and 16; Table 2.3 and Scheme 2 in Appendix 1). A band corresponding to the migration positions of HP and apolipoprotein A-IV was also seen on the gel (yellow box in Figure 2.4), but was not analyzed to avoid repetition and keep down the cost of the analysis.

2.3.4 Colloidal Coomassie

Colloidal Coomassie is a sensitive protein stain that can detect ~5 ng protein per band. It is similar to the standard Coomassie blue staining method described above, but the colloidal stain does not penetrate the gel matrix entirely but still interacts specifically with proteins, allowing reduced background staining and improved sensitivity (Steinberg, 2009). It is also fully compatible with mass spectrometry.

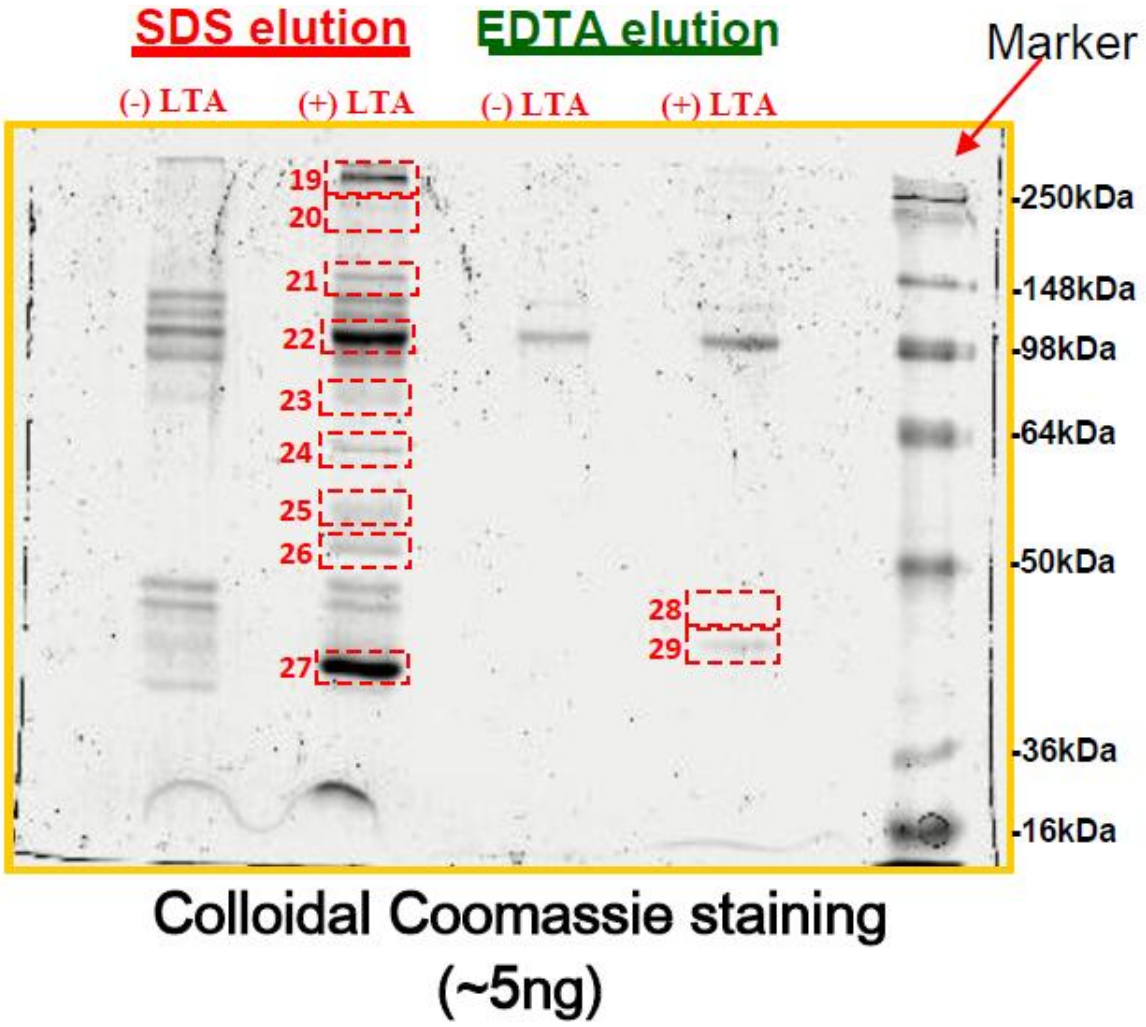

Figure 2.5 SDS-PAGE gel stained with Colloidal Coomassie to identify putative LTA-binding proteins. Reducing SDS-PAGE gel (12%) of EDTA and SDS elution fractions, eleven putative LTA-binding protein bands (19, 20, 21, 22, 23, 24, 25, 26, 27, 28 and 29) were identified.

Eleven protein bands (19, 20, 21, 22, 23, 24, 25, 26, 27, 28 and 29), were analyzed by mass spectrometry and the results are shown in Table 2.4 and Scheme 3 in Appendix 1.

Bands	Proteins	Number of matched peptide sequences	Peptides sequence coverage (%)
19	apolipoprotein B-100 precursor	4	0
20	cul-3	2	5
21	centromere protein	2	2
22	antithrombin III	3	37
23	human serum albumin	9	14
	plasminogen	3	1
24	OMM protein	8	31
25	apolipoprotein A-IV precursor	6	15
	complement component C3	3	1
	plasminogen	2	1
26	apolipoprotein J precursor	7	15
	apolipoprotein E	12	37
27	apolipoprotein E3	6	31
28	pre-serum amyloid P component	7	31
29	pro-apolipoprotein	2	9

Table 2.4 MALDI-TOF-MS identification of putative LTA-binding proteins stained with Colloidal Coomassie stain. Protein bands were identified by in gel trypsin digestion MALDI-TOF-MS.

2.3.5 VisPRO 5 minutes protein staining method

VisPRO 5 minutes protein staining is a highly sensitive and speedy method to detect proteins on SDS-PAGE gel (around 1 ng protein per band is detected within 5 minutes). It utilises a negative stain by forming a zinc-imidazole complex on the gel which turns the gel white. Proteins resist staining and remain transparent as shown in Figure 2.6. This staining method is also compatible with mass spectrometry.

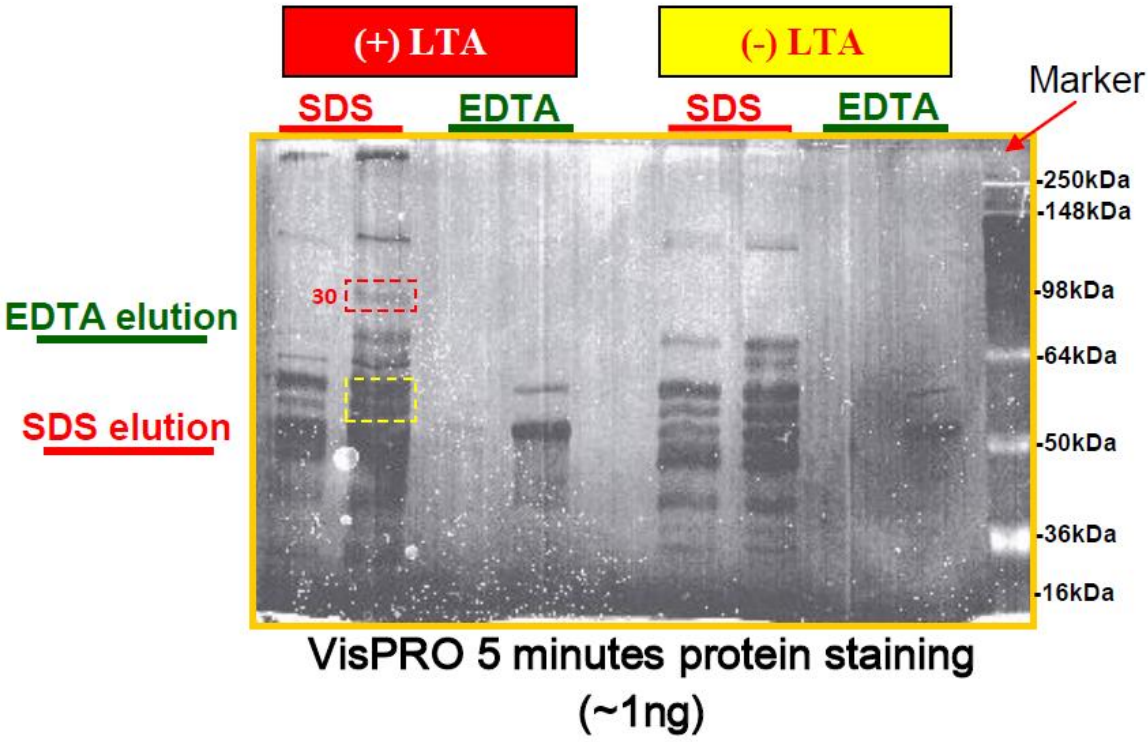

Figure 2.6 SDS-PAGE gel stained with VisPRO 5. Reducing SDS-PAGE gel (12%), showing EDTA and SDS elution fractions. A single protein band (band 30) was analysed.

Using this method, antithrombin was identified by MALDI-TOF-MS (Scheme 4 in Appendix 1). Once again, bands containing proteins already identified, such as HP and apolipoproteins (yellow box in Figure 2.6), were also observed but were not re-analyzed.

2.3.6 Gradient SDS-PAGE gel

As well as using a variety of different staining methods to identify putative LTA-binding proteins, I also used different gel types. Gradient SDS-PAGE gels were used to give better separation of proteins. These gels are characterized by a low percentage of acrylamide at the top of the gel, which gradually increases to a high percentage at the bottom. Consequently, a wider range of protein sizes can be separated compared to fixed-concentration gels. Gradient gels were stained with Colloidal Coomassie.

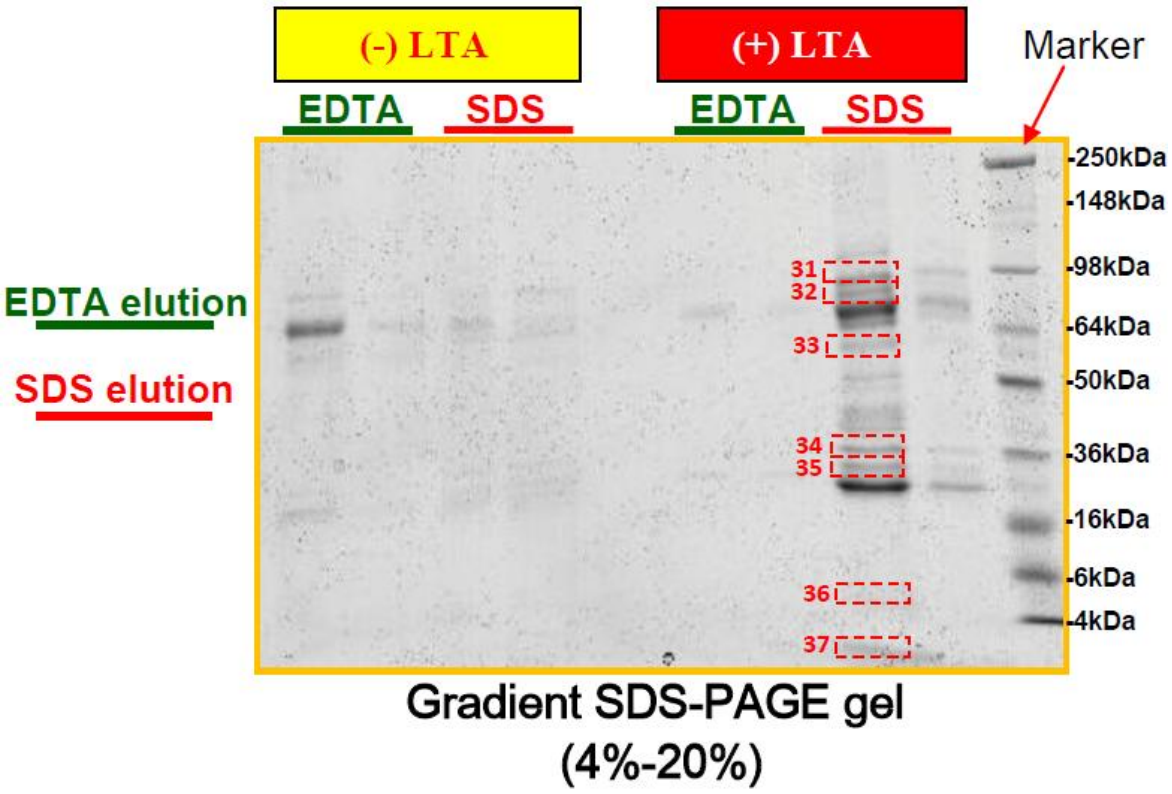

Figure 2.7 Separation of LTA-binding proteins by SDS-PAGE using a gradient gel (4-20%) stained with Colloidal Coomassie. Seven LTA-specific protein bands (31, 32, 33, 34, 35, 36 and 37) were identified.

Using this approach seven additional LTA-specific protein bands (21, 32, 33, 34, 35, 36 and 37) were detected and identified (see Table 2.5 and Scheme 5 in Appendix 1).

Bands	Proteins	Number of matched peptide sequences	Peptides sequence coverage (%)
31	immunoglobin heavy chain variable region	4	19
32	human serum albumin	9	15
	complement component 4 binding protein	6	10
	human complement component C1r	3	4
	plasminogen	1	1
33	keratin	7	11
	hypothetical protein (gi|51476390)	9	14
	plasminogen	1	1
34	keratin	3	4
	apolipoprotein D	2	6
35	T cell receptor beta chain variable region	2	22
	sialic acid-binding Ig-like lectin 10	2	5
	M4 protein	2	4
36	lipoprotein CIII	2	20
37	plasminogen	1	1

Table 2.5 Separation of LTA-binding proteins using a gradient SDS-PAGE gel. Protein bands were extracted from a 4-20% gradient gel stained with Colloidal Coomassie stain and analyzed by MALDI-TOF-MS.

2.3.7 Two dimensional-PAGE gel (2D-PAGE)

2D-PAGE was used to improve separation of proteins. The first dimension separates according to the isoelectric point of proteins, using isoelectric focusing and the second dimension employs regular SDS-PAGE and separates proteins according to their mass. 2D-PAGE is a useful method in proteomic research because of its ability to separate protein of similar sizes, facilitating their analysis. A ReadyPrep 2D starter kit from BIO-RAD was utilized and the 2D-PAGE gel was stained using the SilverQuest.

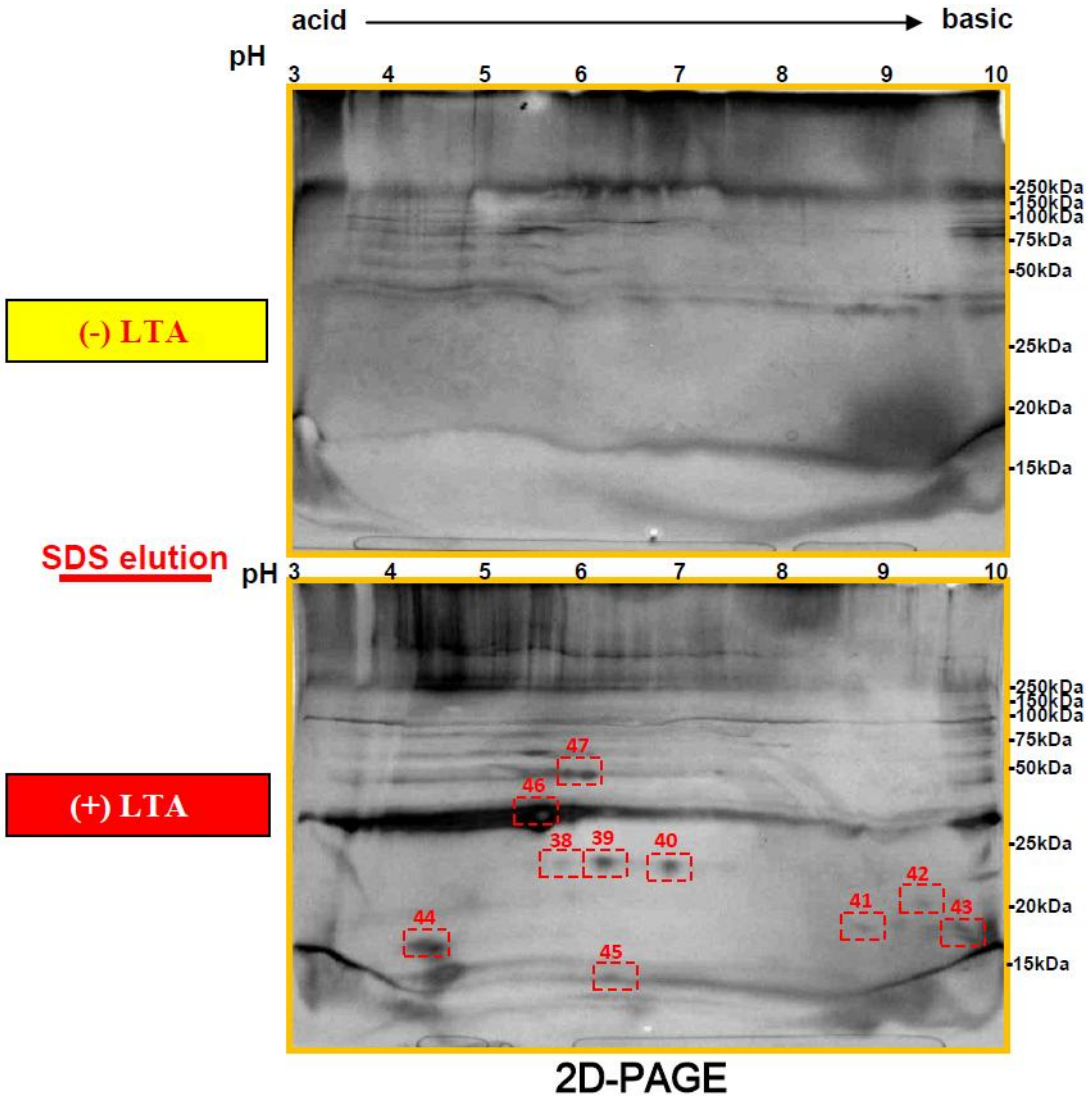

Figure 2.8 2D-PAGE gel stained with SilverQuest. SDS elution fractions from the LTA-Sepharose and control columns were analyzed. After staining the 2D-PAGE gel with SilverQuest silver stain, ten protein spots (38, 39, 40, 41, 42, 43, 44, 45, 46 and 47) were analyzed by mass spectrometry.

Figure 2.7 illustrates a 2D-PAGE stained with the SilverQuest silver stain. Ten additional LTA-specific proteins were sequenced (see Table 2.6 and Scheme 6 in Appendix 1).

Spots	Proteins	Number of matched peptide sequences	Peptides sequence coverage (%)
38	hypothetical protein (gi|34365470)	3	4
39	Polycystin-2	2	2
	carboxypeptidase D	2	3
40	Polycystin-2	4	2
41	KIAA0626 protein	2	5
42	trypsin cationic trypsinogen	1	11
43	plasminogen	1	1
	carboxypeptidase D	2	3
44,45,46,47	unidentified	None	None

Table 2.6 Identification of putative LTA-binding proteins from a 2D-PAGE gel. Protein spots were extracted from a 2D-PAGE gel stained with SilverQuest silver stain and analyzed by in gel trypsin digestion/MALDI-TOF-MS.

Using this approach novel serum proteins were identified as putative LTA-binding proteins such as polycystin-2 (spot 39 and 40). However, there was insufficient material to enable all of the novel features to be identified (eg. spots 44, 45, 46 and 47).

2.3.8 Other elution methods (eluting with LTA)

As an alternative method for eluting LTA-binding proteins from the affinity column, LTA itself was used to elute by competing for binding sites on the protein targets (see Figure 2.9, Table 2.7 and Scheme 7 in Appendix 1).

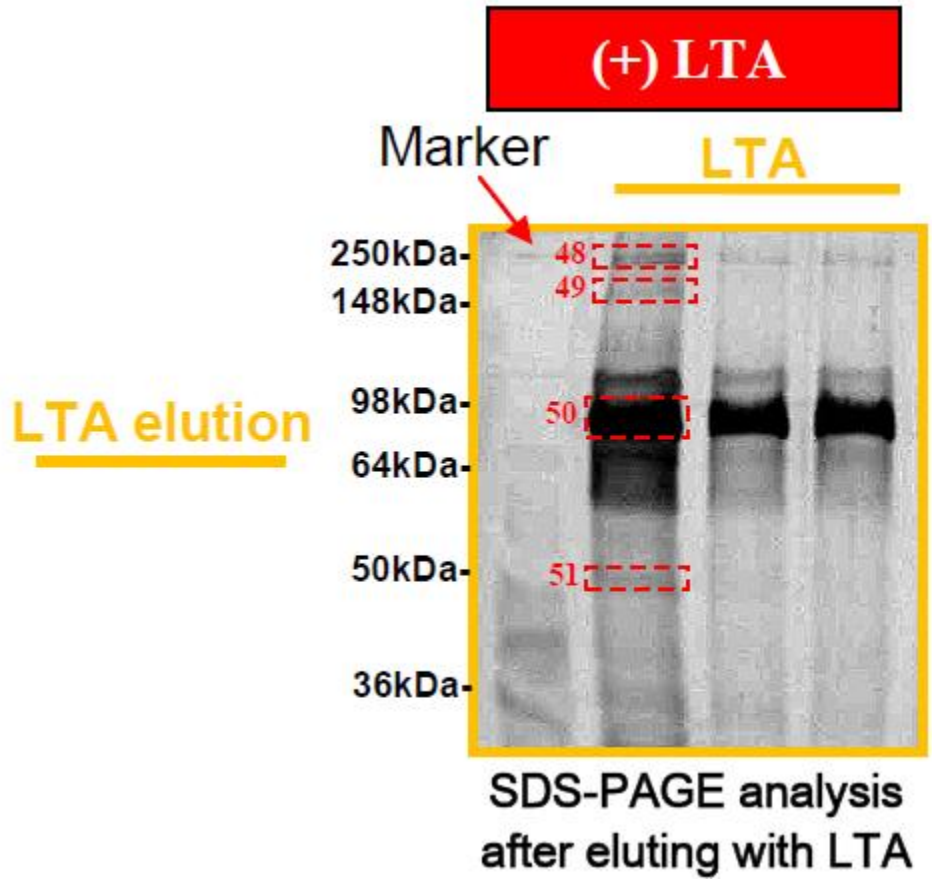

Figure 2.9 Fractions from the LTA-affinity column eluted using LTA and separated by SDS-PAGE. Proteins were eluted using 0.4 mM LTA in TBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and were separated under reducing conditions on a 12% gel and stained with SilverQuest silver stain. Four bands (48, 49, 50 and 51) were analyzed by in gel trypsin digestion/MALDI-TOF-MS.

Bands	Proteins	Number of matched peptide sequences	Peptides sequence coverage (%)
48	keratin	4	5
	centrosomal protein	1	0
49	epidermal growth factor	3	43
50	human serum albumin	12	19
51	keratin	8	13
	plasminogen	3	1

Table 2.7 Serum proteins stained with SilverQuest silver stain on SDS-PAGE.

To summarize, using a variety of different methods a large number of candidate LTA-binding proteins were identified and are listed in the following table.

Proteins detected from the TBS, 1 mM EDTA elution fraction	Protein detected from the TBS, 1% SDS elution fraction	Proteins detected from the TBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.4 mM LTA elution fraction
1. Lipid-transport proteins: Apolipoprotein A-IV precursor Apolipoprotein E Apolipoprotein E3 2. Complement proteins: Complement C1r subcomponent precursor Mannose-binding lectin Complement factor H isoform a precursor 3. Coagulation-cascade proteins: Plasminogen 4. Other proteins Fibulin isoform D precursor Fibulin 1C Haptoglobin related protein precursor Haptoglobin Hypothetical proteins Immunoglobulin kappa light chain VLG region Immunoglobulin kappa light chain VLJ IgG Ig kappa chain C region Pre-serum amyloid P component Keratins Serum albumin Serum paraoxonase/arylesterase 1 (PON 1) (Serum aryldialkylphosphatase 1) (A-esterase 1) (Aromatic esterase 1) (K-45) Protein Rei,Bence-Jones Protein Len,Bence-Jones HCG1798436 Plasma protease inhibitor	1. Lipid-transport proteins: APOB protein Apolipoprotein E- Apolipoprotein A-IV Apolipoprotein A-I Apolipoprotein A-IV precursor Apolipoprotein J Apolipoprotein J precursor Apolipoprotein A-IV precursor Apolipoprotein E3/E3K Apolipoprotein E3 Apolipoprotein C-III Apolipoprotein D Apolipoprotein B-100 precursor Apolipoprotein A-I Lipoprotein Gln I Lipoprotein CIII 2. Complement proteins: C1 inhibitor Complement factor H related protein 4A Complement component 4 binding protein Complement system protein C1q Complement component C1r Human complement component C3 Complement component C4A Complement component 4 binding protein Factor H 3. Coagulation-cascade proteins Antithrombin III Alpha-thrombin Antithrombin Plasminogen 4. Other proteins: Galectin 3 binding protein Haptoglobin Haptoglobin-related protein Immunoglobulin heavy chain variable region Binding protein-Ig gamma 3 Chain IGM Serum albumin Keratins Pre-serum amyloid P component	1. Complement proteins: Complement component C4 2. Coagulation-cascade proteins: Plasminogen 3. Other proteins Serum albumin Transferrin Cardiotrophin-like cytokine factor 1 Centrosomal protein Keratins Glutathione s-transferase fused with the nuclear matrix targeting signal of the transcription factor Aml-1 Epidermal growth factor

	IKK interacting protein isoform 1 **Serum paraoxanase** **Polycystin-2** **Carboxypeptidase D** **Cul-3** **TPR repeat-containing protein** **Trypsin cationic trypsinogen** **KIAA1043 protein** **KIAA0626 protein** **Centromere protein** **Pleckstrin homology-like domain** **Lipopolysaccharide binding protein** **OMM Protein** **Protein tro alpha1 H** **Kininogen 1** **Heat shock 70kDa protein 9B** **Alpha2-HS glycoprotein** **PENK protein** **Proapolipoprotein** **Globular glutathione s-transferase fused with the nuclear matrix targeting signal of the transcription factor Aml-1** **Truncation mutant 165 transmembrane protein 27** **Vitamin K dependent protein S precursor** **Collagen** **Hypothetical proteins** **T cell receptor beta chain variable region** **Sialic acid-binding Ig-like lectin 10** **M4 protein**	

Table 2.8 Putative LTA-binding proteins identified by in gel trypsin digestion/MALDI-TOF-MS.

2.4 Discussion

The serum components identified as putative LTA-binding proteins are listed in Table 2.8. In order to assess their potential involvement in immune responses against *S. aureus*, the literature was scanned to evaluate the known properties of these proteins.

2.4.1 Haptoglobin (HP)

HP was one of the first proteins to be identified as a putative LTA-binding protein in this study and was seen by Coomassie staining, indicating its high abundance. Although HP is an acute-phase protein, its main known function is to interact with free haemoglobin (HB), released from erythrocytes by intravascular haemolysis (Okazaki et al., 1997, Okazaki and Nagai, 1997). HP-HB complexes subsequently bind to the scavenger receptor CD163 on monocytes and macrophages promoting endocytosis and clearance from the serum (Kristiansen et al., 2001, Graversen et al., 2002). Interestingly, HP is also stored during granulocyte differentiation and is released upon neutrophil activation, suggesting a role in immunity (Theilgaard-Monch et al., 2006). However, such a role has not been explored previously. HP has many of the hallmarks of an innate immune molecule; it is an acute-phase protein and is known to associate with phagocytic cells to promote elimination of bound HB. Thus, binding of LTA by HP may have considerable immunological importance in the killing and the clearance of *S. aureus* by the immune system. It can be hypothesized that HP binds to *S. aureus* via cell wall components including LTA to mediate activation of phagocytic cells such as neutrophils, monocytes and macrophages and promote neutralization by phagocytosis. For these reasons HP was prioritized for further work.

2.4.2 Galectin-3 binding protein (Mac-2 binding protein)

Galectin-3 binding protein or Mac-2 binding protein (M2BP) is a secreted glycoprotein found in the extracellular matrix of many tissues including epithelial and gastrointestinal tissues (Sasaki et al., 1998, Ullrich et al., 1994), and in extracellular fluids such as serum and milk (Koths et al., 1993). Increased concentrations of M2BP are observed in certain tumors and viral infections (Iacobelli et al., 1988, Longo et al., 1993). In addition, human M2BP stimulates the production of cytokines such as IL-1 and IL-6 and the activation of NK-cells (Ullrich et al., 1994, Powell et al., 1995). M2BP can also interact with a macrophage-associated lectin known as galectin-3 (Mac-2) and other proteins such as collagens IV, V and VI, fibronectins and nidogen (Sasaki et al., 1998, Koths et al., 1993, Rosenberg et al., 1991, Inohara and Raz, 1994). Furthermore, M2BP facilitates the binding of LPS to CD14, which performs a major role in mediating cellular responses (Yu and Wright, 1995). Thus, M2BP is another excellent candidate for a LTA-binding immune molecule. For example, like HP, it may bind LTA on the staphylococcal surface and activate the killing through phagocytic receptors. Although, further studies were not continued in this thesis because of time constraints, M2BP is a high priority target for future study.

2.4.3 Apolipoproteins

Apolipoproteins are lipid binding proteins that play essential roles in the transportation of dietary lipids through the blood stream from the intestine to the liver by forming lipoprotein complexes. They are synthesized in the liver and the intestine and can be stored in adipocytes, metabolized by heart, muscle or lung tissues or secreted by breast tissues. They are found to perform many important functions in the immune system. Apolipoprotein E (apoE), a member of the apolipoprotein family has been reported to play key roles in the innate immune response (Feingold et al., 1995, Laskowitz et al.,

2000, Van Oosten et al., 2001, Berbee et al., 2005). For instance, apoE can prevent endotoxic shock by interacting with LPS and apoE-deficient knockout mice were found to be more susceptible to bacterial infections (Berbee et al., 2005, Van Oosten et al., 2001, Laskowitz et al., 2000, Feingold et al., 1995). Furthermore, apolipoproteins including apoE are subcomponents of low density lipoprotein (LDL), which can stimulate immune responses such as initiating phagocytosis by macrophages (Carvalho et al., 2000) and trypanosome lysis (Hajduk et al., 1989). Apolipophorin III, a key exchangeable lipid transport molecule found in blood, is also composed of apolipoproteins and may also participate significantly in the innate immune response. It interacts with both Gram-positive bacteria and LTA (Halwani et al., 2000, Halwani and Dunphy, 1999), binds and detoxifies LPS (Halwani and Dunphy, 1997, Kato et al., 1994) and promotes phagocytosis (Gotz et al., 1997). Thus, apolipoproteins have been suggested to participate in different immune responses mainly as components of complexes such as LDL and apolipophorin. Interactions of various apolipoproteins with LTA observed in this study may reflect important immunological functions. However, unlike the hydrophilic backbone, the lipophilic glycolipids of LTA are not normally exposed on the surface of Gram-positive bacteria, so apolipoproteins probably target LTA that has been shed from the bacterial surface, rather than as part of the living bacteria. Nevertheless, investigating the immunological importance of LTA-lipoprotein interactions represents another exciting future project.

2.4.4 Transferrin

Transferrin is a high-affinity iron-binding glycoprotein, which transports iron to various tissues in the human body. It is a monomeric protein with N-terminal and C-terminal lobes, where ferric and bicarbonate ions bind (Baker and Lindley, 1992). *S. aureus* targets transferrin to acquire iron, which is an essential nutrient for its growth within the

host. Different staphylococcal cell wall proteins are responsible for this interaction including, Staphylococcal transferring-binding protein A (StbA) (Taylor and Heinrichs, 2002). The interaction between transferrin and LTA described here (Table 2.8) may be another route for *S. aureus* to target transferrin and acquire iron. Thus, it is likely that transferrin is targeted to facilitate bacterial survival within the host, rather than as a mechanism for elimination by the immune system. Consequently, although interesting, the LTA-transferrin interaction was not pursued further in this project.

2.4.5 Serum paraoxanase

Human serum paraoxanase (HuPON1) is a calcium-dependent enzyme that binds high-density lipids (HDL) in serum. This interaction contributes towards protection of HDL against LDL oxidation (Banka, 1996, Mackness et al., 1991, Mackness et al., 1993b, Mackness et al., 1993a, Mackness and Durrington, 1995, Navab et al., 1994, Hayek et al., 1995). HuPON1 is also reported to prevent macrophage cholesterol biosynthesis and stimulate macrophage cholesterol efflux (Fuhrman et al., 2002, Rosenblat et al., 2005). Moreover, mice deficient of HuPON1 were found to be susceptible to organophosphate toxicity and lose their anti-inflammatory properties during acute influenza A infection (Van Lenten et al., 2001). It is likely that HuPON1 bound to the lipophilic glycolipids of LTA, so additional studies would need to be performed to assess the physiological importance of these interactions.

2.4.6 Clotting cascade proteins, antithrombin III and plasminogen

The clotting cascade proteins antithrombin III and plasminogen were identified in this study. This cascade is activated as soon as blood is taken from the body and components tend to attach to a wide variety of surfaces either directly or as complexes. Thus, these interactions may not be specific for LTA. Antithrombin III (AT III) is a

glycoprotein characterized by serum concentrations of 15-20 mg/L. It is a serine protease inhibitor and a member of the serpin family of human plasma proteinase inhibitors. It is expressed in the liver and functions as an anticoagulant mainly by inhibiting thrombin factor Xa, Ixa, Xia, XIIa and plasmin particularly in the presence of heparin, when its activity is significantly increased (Ranucci, 2002). It can also have anti-inflammatory effects mostly in the absence of circulating thrombins, when it can bind to endothelial surfaces and induce the release of prostacyclin (Horie et al., 1990, Uchiba et al., 1995), which prevents activation of leukocytes by blocking expression of TNF-α and restricting the activation of neutrophils and their adhesion to endothelial cells (Okajima and Uchiba, 1998). Heparin inhibits AT III binding to endothelial cells because of the formation of thrombin-heparin complexes, which target AT III (Uchiba et al., 1995).

Plasminogens are serum proteases released into the circulation where they degrade plasma proteins including fibrin clots. They are activated by tissue plasminogen activator, urokinase plasminogen activator, thrombin and Hageman factor (factor XII) and are inactivated by a serine protease inhibitor called alpha2-antiplasmin. Plasminogens can bind to *S. aureus* in the presence of staphylokinase to enhance the release of a protecting protease on the staphylococcal surface (Molkanen et al., 2002). This protease facilitates spreading of bacteria by assisting them to directly remove components of the extracellular matrix (Molkanen et al., 2002).

2.4.7 Complement proteins, C1r, C1q, MBL, C1-inhibitor and factor H

As described in the introduction complement proteins are key components of the innate immune system and form complexes on the surfaces of foreign material, so it is unsurprising that a variety of complement components were identified in this study. The

classical pathway of the complement system is initiated by the interaction of C1 complex constituents, C1r, C1s and C1q with immune complexes or other activators such as, C-reactive protein (CRP) complexes and pathogens (Volanakis, 1982, Loos et al., 1981). C1 complexes bind directly to Gram-positive bacteria and may bind to LTA (Loos et al., 1981).

C1-inhibitor (C1-Inh) is a serpin, which regulates the complement system by limiting activation (Emonts et al., 2007). C1-Inh is a serpin that binds to C1r and C1s inactivating them and pulling apart the C1 complex (Bos et al., 2002). C1-Inh also inhibits proteases of the contact system such as kallikrein and blood coagulation factor XII and contributes to the binding of leukocytes to endothelium (Matsushita et al., 2000, Kaplan et al., 2002, Cai et al., 2005). Furthermore, it is suggested to be involved in facilitating the escape of *S. aureus* from complement attack by unknown mechanisms and therefore help staphylococcal survival within the host (Emonts et al., 2007). Thus, C1-Inh might interact with staphylococcal LTA directly as a protection mechanism. Interestingly, L-ficolin, previously shown to bind LTA, was not detected. Serum concentrations of L-ficolin are quite low (~2.5 μg/ml) compared to many of the proteins that were captured and identified, so it is possible that amounts were too low to detect.

2.4.7.1 Complement factor H

Factor H was identified by Nilsson and Mueller-Eberhard (1965) as β1H globulin. It is a plasma glycoprotein consisting of a single polypeptide chain that stimulates the decay of C3-convertase of the alternative pathway and functions as a cofactor for factor I-mediated proteolytic inactivation of C3b (Weiler et al., 1976, Whaley and Ruddy, 1976, Pangburn et al., 1977). Factor H regulates the complement system both in fluid-phase or cellular surfaces. Whilst it rapidly targets and inactivates C3b in

fluid-phase, it inactivates surface-bound C3b in the presence of sialic acids or other polyanionic molecules such as glycosaminoglycans and heparin on the chemical composition of the surface where C3b is bound (Cordoba et al., 2004, Fearon, 1978). Thus, the interaction of factor H with polyanions protects the host cellular surfaces from complement activation (Cordoba et al., 2004). Furthermore, the binding of factor H to surface components of tumour cells or invading bacteria protects these cells from complement activation and facilitates their survival. For example, cell surface proteins bone-sialoprotein and osteopontin are significantly upregulated in several tumours and bind tightly to factor H (Fedarko et al., 2001, Fedarko et al., 2000, Cordoba et al., 2004). Similarly, several microbes including *S. pneumoniae* express surface components such as pneumococcal surface protein C (PspC), which bind to factor H and protect against complement activation (Cordoba et al., 2004, Lu et al., 2006, Quin et al., 2007). Thus, factor H might also interact with staphylococcal LTA directly as a protection mechanism by the bacterium.

2.4.7.2 Mannose-binding lectin (MBL)

MBL is a member of the collectin family, and the first component of the lectin pathway of the complement system (Drickamer and Taylor, 1993). It targets microorganism surfaces to activate the lectin pathway, leading to an early destruction of invading pathogens (see Chapter 1). MBL can bind to a wide range of bacteria including *S. aureus*, *S. enterica* and *E. coli* (Levitz et al., 1993, Neth et al., 2000). However, in previous studies, recombinant human MBL did not bind to LTA preparations from *S. aureus* (Polotsky et al., 1996) but instead targeted other surface components of *S. aureus* such as mannose-rich peptidoglycan (Lynch et al., 2004). Thus, the interaction of serum MBL and the LTA in this study was somewhat surprising. It is possible that

binding is indirect via other serum components that target MBL and LTA simultaneously.

2.4.8 Other putative LTA-binding proteins

A number of other proteins were identified as putative LTA-binding proteins, some of which are described below. However, the significance of their apparent interactions with LTA is not known.

2.4.8.1 Bence-Jones proteins

Bence-Jones proteins are monoclonal immunoglobulin light chains that are found in high concentrations in the urine of multiple myeloma patients, a cancer of the bone marrow (Sanders, 1994) They are soluble molecules also found in the serum of cancer patients (Solomon et al., 1991). Proteolytic degradation of Bence-Jones proteins can lead to their polymerization into fibrils in various tissues, where they form structured arrays (Makino et al., 2005).

2.4.8.2 Carboxypeptidase D

Carboxypeptidase D (CPD) is a member of the mammalian regulatory B-type carboxypeptidase, which plays various key cellular functions such as prohormone processing and modifying protein-cell interactions (Skidgel et al., 1996, Skidgel and Erdos, 1998). CPD can be found in different cells and tissues and including monocytes and macrophages. Its expression is also elevated in rat brain, lung and kidney (McGwire et al., 1997, Song and Fricker, 1995). Bovine CPD is a mammalian homologue of duck gp180, a hepatitis B virus-binding protein, but not characterized as a carboxypeptidase (Kuroki et al., 1995). Avian hepadnaviruses bind gp180 to enter host hepatocytes (Breiner and Schaller, 2000, Breiner et al., 1998, Ishikawa et al., 1994, Kuroki et al., 1994, Kuroki et al., 1995, Urban and Tyrrell, 2000, Tong et al., 1995).

2.4.8.3 Cullin-3 (Cul-3)

Cul-3 is a member of the cullin family of E3 ubiquitin-protein ligases that specifically degrade short-lived proteins in eukaryotic cells, which are employed in the male reproductive system (Singer et al., 1999, Furukawa et al., 2003, Baarends et al., 1999). Cul-3 binds to Cyclin E, which is an essential protein for the promotion of the cell cycle transition from G_1 to S phase, and regulates Cyclin E ubiquitination (Knoblich et al., 1994, Ohtsubo et al., 1995, Singer et al., 1999). Ubiquitination is an important mechanism for regulating protein degradation and key cellular processes such as cell cycle, DNA repair, signal transduction and antigen presentation (Pickart, 2001, Weissman, 2001). Many viruses such as Poxviruses modulate the ubiquitin protein modification pathway to facilitate their survival (Banks et al., 2003, Shackelford and Pagano, 2005, Wilton et al., 2008).

2.4.8.4 Fibulins

Fibulins are a group of five extracellular glycoproteins, which are related to basement membranes and elastic fibers in vertebrates (Timpl et al., 2003, Argraves et al., 2003). Fibulin deficiency causes disorders in connective tissues such as macular degenerative diseases and Cutis Laxa (Chu and Tsuda, 2004). Fibulin-1, a member of the fibulin family and a component of the skin, lung and cardiovascular tissue performs an important function in the morphology of endothelial cells to arrange capillary walls and maintain the integrity of small blood vessels (Kostka et al., 2001). The existence of conserved alternate splice forms in the arrangement of vertebrate fibulin-1 gene lead to synthesis of fibulin-1C and fibulin-1D; Fibulin 1C is reported to play a key role in tumour progression and fibuilin-1D is critical for tumour suppression, synpolydactyly and giant platelet syndrome (Qing et al., 1997, Debeer et al., 2002, Toren et al., 2003). For example, fibulin-1 can be used as an early biomarker for breast cancer since it can

enhance specific B-cell and T-cell-mediated responses in breast cancer patients (Forti et al., 2002, Pupa et al., 2004, Pupa et al., 2002) and can suppress cancer related human papillomavirus (HPV) infection by specifically binding to E6 proteins (E6 proteins promote the degradation of the tumor suppressor p53) and inhibiting their transformation (Du et al., 2002).

2.4.8.5 Polycystin-2

Polycystin-2 (pkd2) is a large membrane related protein participates in signal transduction and Ca^{2+} regulation (Wilson, 2001, Arnaout, 2001, Boletta and Germino, 2003, Igarashi and Somlo, 2002). It is characterized by structural features that belong to the transient receptor potential (TRP) superfamily of ion channels, which span plasma membranes six times with their N- and C-termini positioned within the cell (Tsiokas et al., 1999, Montell et al., 2002). TRP channels have been associated with various cellular functions such as osmosensing and Mg^{2+} homeostasis (Montell et al., 2002, Ramsey et al., 2006). Naturally occurring mutations in pkd2 gene cause autosomal dominant polycystic kidney disease (ADPKD), a genetic disease that leads to the formation of large cysts in the kidney, liver and pancreas, which may eventually cause kidney failure (Mochizuki et al., 1996, Gabow, 1993, Grantham, 1996, Tsiokas et al., 2007).

Chapter 3
Characterizing HP interactions with LTA and *S. aureus*

3.1 Introduction and Objectives

In Chapter 2, haptoglobin (HP) was identified as a putative LTA-binding protein. This is a novel observation and is explored in more detail in this chapter. Earlier studies have shown that haemoglobin (HB), HP-HB complexes and to a lesser extent HP are targeted by a staphylococcal membrane-associated protein known as HarA (Dryla et al., 2003) as a means of scavenging iron from the host. However, none of this work suggested a possible interaction of HP with LTA of *S. aureus*. Thus, it is important to evaluate this interaction further before assessing its possible relevance in the immune response. The aims of this part of the project were to:

1. Determine if HP binds directly to LTA or whether binding occurs via the intervention of other serum proteins
2. Estimate the affinity of this interaction
3. Evaluate the interaction of HP with *S. aureus*
4. Identify the parts of HP that bind to LTA

3.2 Materials and Methods

3.2.1 Materials

Materials for coupling LTA and protein detection were procured as mentioned in Chapter 2 (section 2.2.1). Purified human HP (a mix of HP1-1, HP2-2 and HP2-1, product number H3536) and HB (product number H7379), mouse monoclonal antibody against HP (product number H6395), alkaline phosphatase-conjugated rabbit antibody against mouse IgG (product number A4312), TPCK-heated trypsin, formalin, bovine serum albumin (BSA) and p-nitrophenyl phosphate (pNPP) substrate were purchased from Sigma-Aldrich. Nunc Maxisorb microtiter plate was obtained from Thermo Fisher Scientific. Phosphate buffered saline (PBS), pH 7.4 was from Invitrogen. *S. aureus* DSM 20233 was provided by Dr. Corinna Hermann (Department of Biology, University of Konstanz, Germany).

3.2.2 The interaction of HP with LTA by column chromatography

Purified HP from human serum (50 µg) was loaded onto an LTA-Sepharose column (2 ml) or an identical column (containing Sepharose 6B prepared using the same coupling procedure but without LTA). Columns were washed with 20 ml TBS and eluted with TBS containing 1% SDS and 0.5 ml fractions were collected. Fractions were stored at -20 ºC and analyzed by SDS-PAGE. Gels were stained using Silver or Colloidal Coomassie staining kits.

3.2.3 The interaction of HP with LTA by ELISA

ELISA was modified from the procedure described by Lynch et al. (2004). Nunc Maxisorb microtiter plate was coated with per well, 1 µg LTA in 100 µl of coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). After an overnight incubation at 4 ºC, the plate was washed with PBS, pH 7.4 (wash buffer) and blocked with 2% BSA

in PBS, pH 7.4 for two hours at 37 ºC, to saturate residual protein-binding sites on the plate. Following another wash step, wells were incubated with two-fold dilutions of HP in PBS and incubated for one hour at 37 ºC. The plate was then washed thoroughly with wash buffer. Bound HP was detected using a mouse monoclonal anti-HP antibody (primary antibody; 1:4000 dilution in wash buffer and two hours incubation with wells at 37 ºC) and an alkaline phosphatase-conjugated rabbit anti-mouse IgG antibody (secondary antibody; 1:30000 dilution in wash buffer and two hours incubation with wells at 37 ºC), using the colorimetric substrate for alkaline phosphatase pNPP.

3.2.4 Interaction of HP with *S. aureus* by ELISA

ELISA was modified from the procedure described by Lynch et al. (2004). The wells of a Nunc Maxisorb microtiter plate were coated with 100 µl of formalin-fixed *S. aureus* DSM 20233 (OD_{550}= 0.5) in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6). After overnight incubation at 4 ºC, the wells were washed with PBS, pH 7.4 (wash buffer) and blocked with 2% BSA in PBS, pH 7.4 for two hours at 37 ºC. After another wash step, HP was diluted in PBS, pH 7.4 and two-fold dilutions were added to the plate and incubated for two hours at 37 ºC. The plate was then carefully washed with wash buffer. Bound HP was detected using mouse monoclonal antibody against HP as described above.

3.2.5 The interaction of HP-HB complexes and HB with LTA by column chromatography

The columns described in section 3.2.2 were also utilized to look at binding of HP-HB complexes to LTA. In this procedure, a mixture of 50 µg HB (MW~ 68,000) and 100 µg HP (MW~ 400,000) at a HP, HB molar ratio of 1:3 was incubated in TBS for one hour at RT to form HP-HB complexes. The LTA-Sepharose and control columns

were both prewashed with TBS prior loading HP-HB complexes. Columns were washed with TBS (20 ml) and eluted in TBS containing 1% SDS, and 0.5 ml fractions were collected.

As an additional control, purified HB (50 µg) from human serum was loaded onto the LTA-Sepharose and control columns in the absence of HP. Columns were washed and eluted as before, and fractions were collected and stored at -20 ºC for analysis by SDS-PAGE.

3.2.6 Trypsin digestion of HP and analysis of LTA-binding by affinity chromatography

Limited trypsin digestion of HP was carried out using five-fold serial dilutions of TPCK-heated trypsin at a starting concentration of 1 mg/ml. HP (1 mg) was dissolved in TBS (1 ml). Trypsin (1 mg) was also dissolved in TBS (1 ml) and dilutions were prepared immediately before mixing with HP. The mixtures of HP (10 µl) and trypsin (1 µl) were incubated for an hour at 37 ºC and then analyzed immediately by SDS-PAGE. Identical samples of trypsin-digested HP (200 µg HP/20 µg trypsin) were loaded onto the LTA-Sepharose and control columns and columns were washed and eluted as before. Fractions were immediately analyzed by SDS-PAGE.

3.3 Results

3.3.1 Interaction of HP with LTA analyzed by column chromatography

Although HP was identified as a putative LTA-binding protein in Chapter 2, it may have bound to the affinity column indirectly via another serum component or components. In order to study binding in more detail, purified HP was loaded onto the LTA-Sepharose column in the absence of any other serum components. Fractions were collected and analyzed by SDS-PAGE (Figure 3.1).

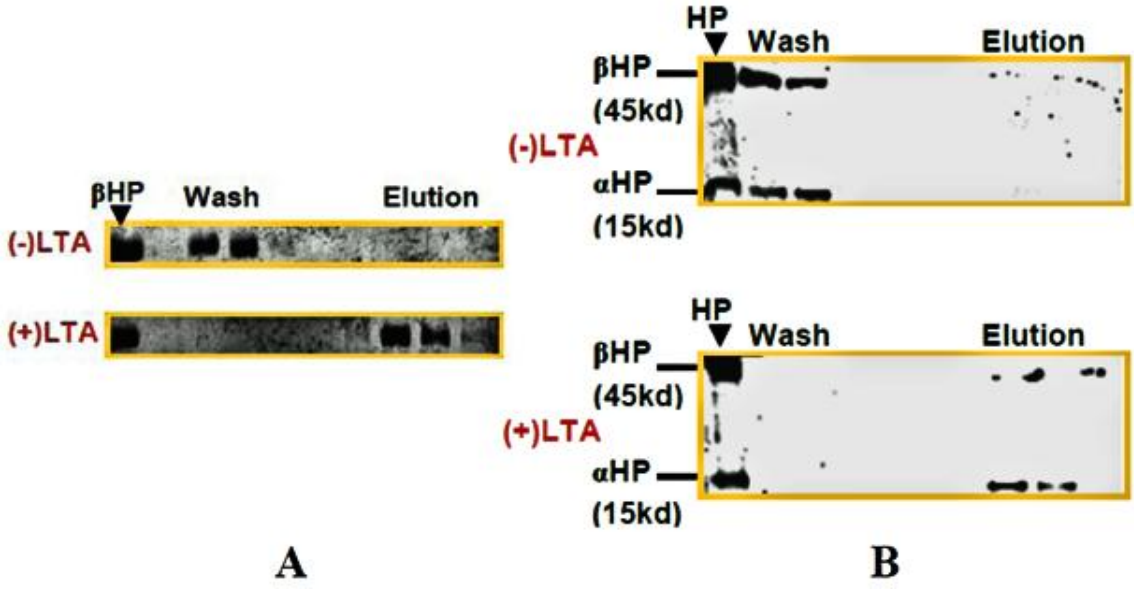

Figure 3.1 HP binds directly to LTA coupled to Sepharose beads. Purified HP (50 µg) was loaded onto LTA-Sepharose and control columns and fractions were collected after washing with TBS and eluting with TBS containing 1% SDS. (A) Silver stained gels (B) Colloidal Coomassie-stained gels under reducing conditions. Only the β-chain of HP is shown in A.

HP (HP1-1) is composed of two disulfide-linked polypeptides which associate to form αβ dimers $(\alpha\beta)_2$. The α- and β-chains migrate with apparent molecular masses of 15 kd and 45 kd by SDS-PAGE, respectively, and are separated under reducing conditions. Purified HP binds to LTA-Sepharose and elutes only in the presence of 1% SDS. However, in the absence of LTA, it elutes in the wash fractions, so does not bind to Sepharose, implying that it is binding to the immobilized LTA directly.

3.3.2 Studying the interaction of HP with LTA using ELISA

To confirm that HP binds to LTA and to estimate the affinity of the interaction, an ELISA was developed in which LTA was coated onto the surface of a microtiter plate and incubated with HP. After wash steps, bound HP was detected using a monoclonal antibody against HP.

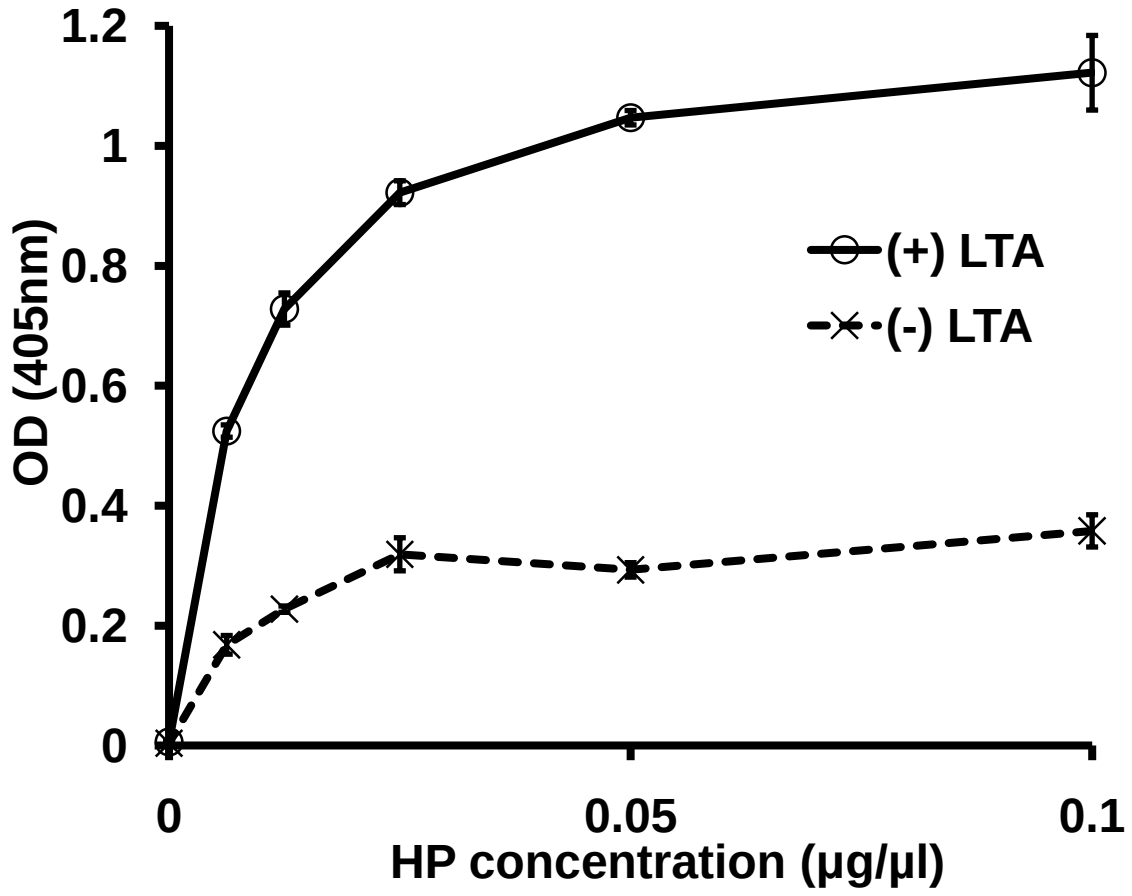

Figure 3.2 ELISA showing HP binding to LTA of *S. aureus*. LTA-coated microtiter wells were incubated with HP for one hour at 37 °C. Bound HP was detected using a mouse monoclonal anti-HP antibody, with alkaline phosphatase-conjugated rabbit anti-mouse IgG as a secondary antibody. Data are the means of duplicate measurements and are representative of two independent experiments.

As shown in Figure 3.2, although there is some non-specific binding to the plate in the absence of LTA, the signal is much greater in the presence of LTA indicating specific binding. Binding was concentration dependent and the apparent dissociation constant,

K_D (HP concentration at half maximal binding) was ~20 nM (0.01 µg/µl), confirming that LTA and HP form stable complexes.

3.3.3 The interaction of HP with *S. aureus* by ELISA

ELISA was also used to measure binding of HP to *S. aureus*. Formalin-fixed *S. aureus* was immobilized onto the microtiter plates and specific binding was detected using anti-HP monoclonal antibody, indicating that HP binds to *S. aureus*.

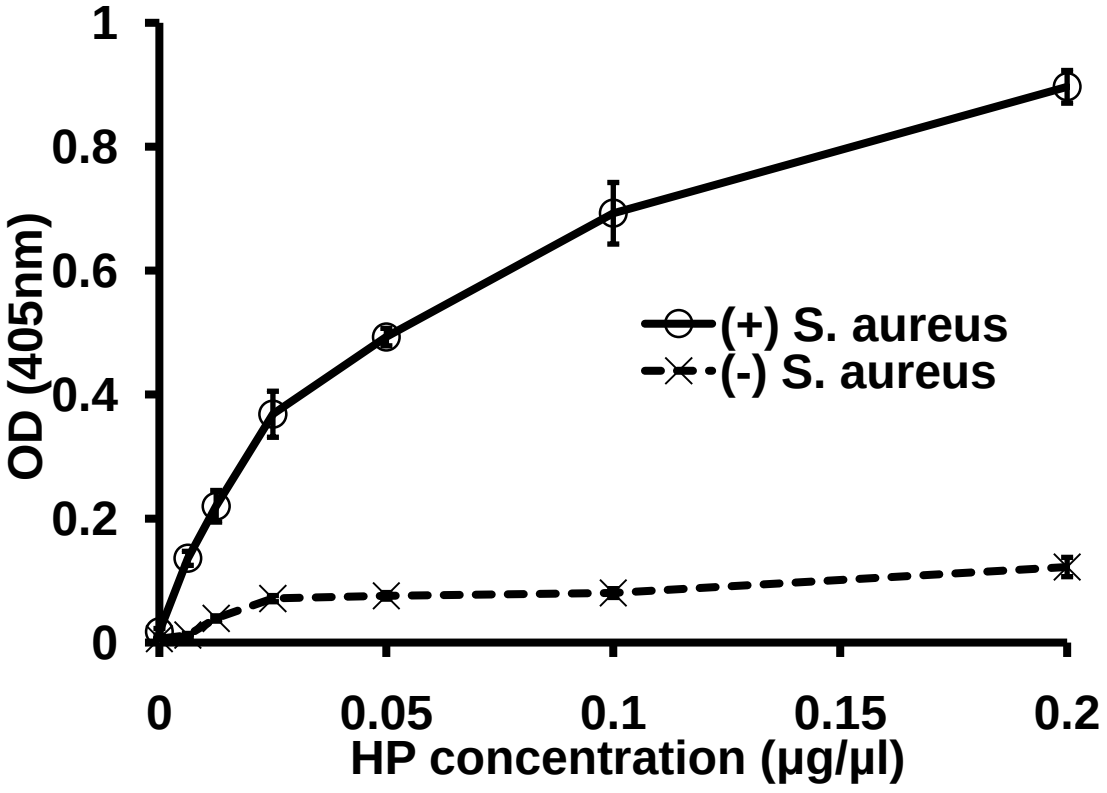

Figure 3.3 ELISA showing HP binding to *S. aureus* coated microtiter wells. Formalin-fixed *S. aureus* was incubated with increasing concentrations of HP for 2 hours at 37 °C. HP binding was detected using a mouse monoclonal antibody against HP, with alkaline phosphatase-conjugated rabbit anti-mouse IgG as a secondary antibody. Data are the means of duplicate measurements and are representative of two independent experiments.

This binding was also concentration dependent and the apparent K_D was ~160 nM (0.06 µg/µl). Thus, the apparent K_Ds for LTA and *S. aureus* are broadly similar, as would be expected if the HP was binding to the LTA on the bacterial surface.

3.3.4 Interaction of HB with LTA by affinity chromatography

In order to further characterize the binding of HP to LTA, HP-HB complexes were formed, by pre-mixing purified components, and were applied to the LTA-Sepharose affinity column, as before. As an additional control, HB was also applied to the column in the absence of HP. These experiments were designed to identify which parts of the HP molecule interact with LTA; i.e. binding of HP-HB complexes to LTA would suggest separate binding sites for LTA and HB on HP, whereas, loss of binding could be caused by overlapping binding sites or by conformational changes in HP resulting in disruption of the LTA binding sites.

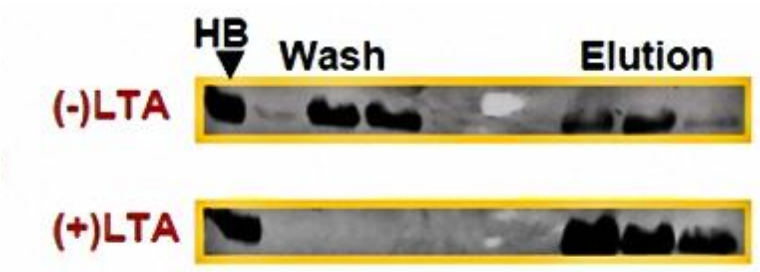

Figure 3.4 Studying the interaction of HB with LTA. HB alone bound to the LTA-Sepharose and more weakly to Sepharose alone. Gels were stained using Silver stain.

As shown in Figure 3.4, HB alone bound to the LTA-Sepharose and more weakly to the Sepharose matrix itself, so it was not possible to obtain information about the relative binding sites for LTA and HB on HP.

3.3.5 Characterizing the interaction of HP with LTA by trypsin digestion and column chromatography

As an alternative strategy to localize the binding site of HP for LTA, HP was digested with trypsin prior to application on the LTA-affinity column. The trypsin preparation had been pretreated with N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) to block the activity of contaminating chymotrypsin, which is difficult to remove from trypsin

preparations. Trypsin is a serine protease that cleaves polypeptides C-terminal to lysine or arginine residues. Exposed regions of proteins, such as interdomain linkers tend to be cleaved preferentially. To assess cleavage of HP with limiting amounts of trypsin, HP was mixed with different dilutions of trypsin and incubated for an hour at 37 ºC. Mixtures were then analyzed by SDS-PAGE to identify cleavage products. As shown in Figure 3.5A, the β-chain is relatively sensitive to trypsin digestion compared to the α-chain, which is resistant even at the highest amount of trypsin used (1 µg trypsin/10 µg HP).

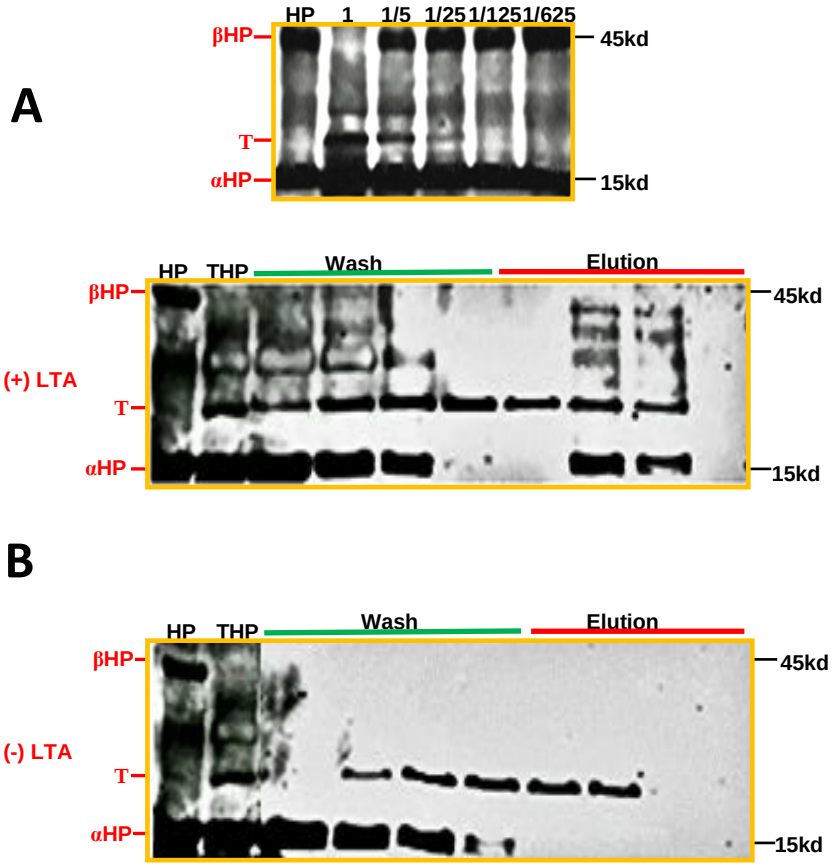

Figure 3.5 Characterizing the interaction of HP with LTA. (A) HP (10 µg) was digested with different amounts of trypsin (T) starting from 1 µg (represented by number 1). (B) 200 µg HP digested with 20 µg trypsin (THP; represented by number 1 in Figure 3.5.A) was loaded onto Sepharose 6B column coupled to LTA. Protein fragments were analyzed on a 15% SDS-PAGE gel and detected by Silver-staining. In the presence of LTA (*upper gel*), some of the α-chain remain bound to the affinity column. In the absence of LTA (*lower gel*), the α-chain eluted in the wash fractions.

To see if the α-chain alone is able to bind to LTA, HP digested with trypsin to remove the β-chain (200 µg HP/20 µg trypsin) was loaded onto the LTA-Sepharose column. As shown in Figure 3.5B, although some of the α-chain eluted in the wash fractions, a significant proportion was retained (~30%) and came off in the elution fractions (with SDS). By contrast, all of the α-chain eluted in the wash fractions from the control column lacking LTA, indicating that the α-chain alone binds to LTA. Although not quantified, binding was probably weaker than for full-length HP, because some of the α-chain eluted in the wash fractions, whereas, full-length HP did not (see Figure 3.1B). Thus the β-chain, probably also binds to LTA to strengthen the interaction, or alternatively facilitates binding by the α-chain.

3.4 Discussion

3.4.1 HP binds directly to LTA and *S. aureus*

The data shown here indicate that HP binds to LTA. Binding is direct and does not require any other serum components. As expected, HP also binds to *S. aureus*. The apparent affinities are broadly comparable (20 nM for HP-LTA and 160 nM for HP-bacteria) as would be expected if HP was targeting the LTA on the bacterial surface. Because of the nature of the ELISA assays, the apparent K_D values are likely to underestimate the true affinities (because they also depend on the antibody steps used for detection), suggesting that HP-LTA complexes are stable, as would be expected for PAMP-PRR binding. Previous studies have shown that HP also binds to the staphylococcal surface protein HarA (Dryla et al., 2003). However, this interaction is weak (5 μM) compared to HP-LTA complexes (20 nM), so probably contributes relatively little to bacterial binding. Interestingly, HarA binds much more tightly to HB (30 nM) or HB-HP complexes (5 nM), and is probably used by the bacteria to target iron from heme during the early stages of infection (Dryla et al., 2003, Skaar et al., 2004, Skaar and Schneewind, 2004, Dryla et al., 2007). Heme iron is believed to be the preferred iron source for *S. aureus* during the start of infection, and for this reason the bacteria possess a heme-transporting system (HTS) that promotes heme-iron uptake (Skaar et al., 2004, Skaar and Schneewind, 2004). An interesting but unexpected finding in my experiments was that HB also binds directly to LTA. Thus, the heme-iron acquisition system via HP-HB or directly via HB could be a potential trap employed by the immune system to target bacteria. From this perspective, it would be interesting to see if LTA-HP, LTA-HB and/or LTA-HP-HB interactions promote phagocytosis and the killing of *S. aureus*.

Other staphylococcal proteins such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and StbA/IsdA are also used to acquire iron from the host. In this case they target another host protein known as transferrin, which acts as an iron storage/delivery system in the serum (Dryla et al., 2003, Taylor and Heinrichs, 2002, Modun and Williams, 1999). It is notable that transferrin was also identified as a potential LTA-binding protein in Chapter 2, so the immune system might use transferrin as bait to target bacteria, as well as HP and HB.

3.4.2 Characterization of the direct interaction between HP and LTA

Affinity chromatography and trypsin digestion were employed to identify which parts of HP interact with LTA. However, because both HP and HB bound to the LTA-Sepharose beads, it was not possible to gain information about the relative binding sites for LTA and HB on HP. Nevertheless, trypsin digestion of HP followed by affinity chromatography showed that the α-chain of HP alone bound to LTA, although more weakly than for full-length HP. The two α-chains are disulfide linked (see Figure 3.6), so the loss of affinity is not caused by a change in the oligomeric state of the HP fragments. Thus, it is likely that the β-chain of HP also contributes to recognition of LTA, either directly through an additional binding site or by modulating the binding activity of the α-chains.

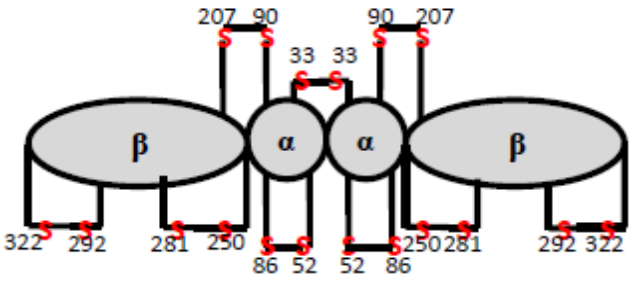

Figure 3.6 HP structure. Schematic drawing showing the molecular arrangements of human HP1-1 indicating the disulfide bonding pattern

Chapter 4
Evaluating the role of HP in phagocyte-mediated killing of *S. aureus*

4.1 Introduction and Objectives

S. aureus is an important human pathogen associated with diseases including impetigo, septic arthritis, toxic shock syndrome and scalded skin syndrome (Archer, 1998, Lowy, 1998). Multidrug-resistant strains, eg. methicillin- (MRSA) and vancomycin-resistant strains cause severe infections in hospitals because they are not eliminated by existing antibiotics and cause up to 65% of nosocomial staphylococcal infections (van den Broek et al., 2007). Consequentially, the natural immune response against *S. aureus* needs to be further characterized to help identify new targets for therapeutics. Several *S. aureus* cell wall components, including LTA stimulate the innate machinery of eukaryotic defence and induce inflammatory responses. For instance, LTA targets receptors on human neutrophils such as CD14 and TLR2 to induce the release of proinflammatory cytokines IL-8, TNF-α and granulocyte-colony stimulating factor (Aulock et al., 2006, Lotz et al., 2004, Hattar et al., 2006). LTA also interacts with receptors on macrophages such as TLR2 and type I scavenger receptor to stimulate the release of cytokines TNF-α, interferon-γ and interleukins (Dunne et al., 1994, Edds et al., 2000, Bhakdi et al., 1991, Wang et al., 2000). In my research, I have shown that the

acute-phase protein HP interacts directly with LTA and *S. aureus* and have hypothesized an essential function for this protein in the killing of these Gram-positive bacteria by the immune response. The work presented in this chapter tests this hypothesis by evaluating the role of HP in killing *S. aureus* by human neutrophils, monocytes and macrophages. The following objectives have been addressed:

4.1.1 The role of HP in neutrophil-mediated killing of *S. aureus*

Previous findings indicated that HP is generated and stored throughout granulocyte differentiation and released when neutrophils are activated (Theilgaard-Monch et al., 2006). LTA interacts with receptors on the surface of neutrophils to recruit these immune cells to the site of infection and stimulate the release of cytokines (Aulock et al., 2006, Lotz et al., 2004, Hattar et al., 2006). Interestingly, exogenously added LTA was also found to reduce the ingestion and elimination of *S. aureus* by neutrophils but not when the bacteria were pretreated with serum, implying that LTA on the staphylococcal cell wall may interact with serum components to promote bacterial killing (Raynor et al., 1981). However, no previous study has reported a role for HP in the killing of *S. aureus* by neutrophils, or the significance of HP interaction with LTA in bacterial killing. The strategy used in the work presented in this chapter was to deplete HP from human serum or use HP-deficient serum from C57BL/6 HP knockout mice and then compare Staphylococcal killing using complete and depleted/deficient serum.

4.1.2 The role of HP in monocyte- and macrophage-mediated killing of *S. aureus*

Earlier studies have shown that the CD163 receptor on macrophages is essential for the clearance of HB and HP-HB complexes (Graversen et al., 2002). CD163 is also suggested to be involved in host defence as a receptor for *S. aureus* and its expression in monocytes enhances bacterial-induced proinflammatory cytokine production (Fabriek et

al., 2009). In addition, LTA of *S. aureus* interacts with other receptors on these phagocytes such as TLR2 and type I scavenger receptor and enhances the release of cytokines such as TNF-α, interferon-γ and interleukins (eg. IL-1 and IL-5), which are important in the host immune response (Dunne et al., 1994, Edds et al., 2000, Elgavish, 2000, Bhakdi et al., 1991, Wang et al., 2000). However, as with neutrophils, none of these studies have suggested a role for HP in the elimination of *S. aureus* by monocytes and macrophages. The data presented in this chapter confirm that HP is a key mediator in the elimination of *S. aureus* by monocytes, macrophages as well as neutrophils, thus identifying a novel innate defence mechanism against infection.

4.2 Materials and Methods

4.2.1 Materials

Purified human HP (product number H3536) and HB (product number H7379), BCIP/NBT substrate system for alkaline phosphatase detection, Histopaque™ 1077 and 1119 solutions for isolating neutrophils and Iscove's modified Dulbecco medium and Corning cell culture ultra low attachment flasks for culturing isolated monocytes into macrophages were purchased from Sigma-Aldrich. Ficoll-Paque PLUS™ for isolating monocytes was from GE Healthcare and alkaline phosphatase-conjugated sheep antibody against human HP (product number ab35307) was from ABcam. Hanks balanced salt solution (HBSS) containing calcium and magnesium, phosphate buffered saline (PBS), pH 7.4 and penicillin streptomycin (product number 15070-063) were procured from Invitrogen. Thermo-spin coated plates and DPX mountant for cytospin and microscopy preparations were from Thermo Fisher Scientific and Reagena Giemsa stains for staining cytospin slides was from Reagena LTD, Finland. Affinity gel 10 and nitrocellulose membrane 0.2 µm were purchased from Bio-Rad and 0.22 µM GV PVDF membranes were from Millipore. 24-well tissue culture Nunc plates utilized for bacterial killing assays were procured from Nunc, Denmark. Serum extracted from C57BL/6 wild type mice was provided by Professor Wilhelm Schwaeble (University of Leicester, UK) and HP-deficient serum from C57BL/6 HP knockout mice was kindly supplied by Professor Jan Ceuppens (Section of Clinical Immunology, University of Leuven, Belgium) and Professor Heinz Bauman (Roswell Park Cancer Institution, USA). *S. aureus* DSM 20233 was provided by Dr. Corinna Hermann (Department of Biology, University of Konstanz, Germany) and LPS from *Salmonella abortus equii* and heparin were kind gifts from Dr. Bernard Burke (University of Leicester, UK). Whole blood was withdrawn from healthy and sickle cell anaemia carrier human

volunteers within the Department of Infection, Immunity and Inflammation at the University of Leicester.

4.2.2 Coupling HB to affinity gel 10

Affinity gel 10 couples neutral or basic proteins with pIs in the range 6.5 to 11. It is an N-hydroxysuccinimide ester of a derivatized crosslinked agarose gel-bead support that couples ligands with free alkyl amino groups in aqueous or non-aqueous solution. The addition of ligands displaces the N-hydroxysuccinimide and allows a stable amide bond to be formed. In this study, HB was coupled to affinity gel 10 in order to deplete HP from human serum. All the following procedures were undertaken at 4 ºC. Affinity gel 10 (25 ml) was filtered using a 0.22 µM GV PVDF membranes and was washed with sterile nano-pure water to remove the storage solution. HB (0.6 g) in 10 mM Mes, pH 6 was then mixed with affinity gel 10 with shaking overnight. The resulting HB-affinity resin was then washed extensively with PBS pH 7.4, to remove any uncoupled HB and was subsequently stored at 4 ºC in PBS, 0.02% (v/v) sodium azide for future use.

4.2.3 HP depletion from human serum

Human blood was withdrawn from healthy volunteers and was left to clot overnight at 4 ºC. After centrifuging the clotted blood for 30 minutes at 3500 g and 4 ºC, serum (0.5 ml) was isolated and mixed with the HB affinity resin (0.5 ml), which had been pre-equilibrated in PBS, pH 7.4 under shaking for two hours at 4 ºC before forming a column and collecting the HP-depleted serum flow-through. This procedure was then repeated two more times using two fresh HB-affinity columns. HP-depleted serum was subsequently collected and the level of HP was measured by Western blotting.

4.2.4 Western and Dot-blot experiments to measure HP depletion from human serum

4.2.4.1 Dot blot

Two-fold dilutions of 10% (v/v) healthy human serum, sickle cell anaemia-carrier serum and HP-depleted serum were prepared in PBS, pH 7.4 and were blotted on to a nitrocellulose membrane 0.2 μm. The membrane was then blocked with 5% (v/v) semi-skimmed milk in PBS (block buffer) with shaking for 30 minutes at RT. Alkaline phosphatase-conjugated sheep anti-human HP antibody (1:1000 dilution in block buffer) was subsequently added to detect the level of serum HP. After washing with 0.5% (v/v) Tween in PBS, pH 7.4, bound antibody was detected using alkaline phosphatase chromogen BCIP/NBT solution. The intensities of the dot blots were compared against known dilutions of human serum to evaluate the level of HP in the depleted serum sample.

4.2.4.2 Western blot

Serum samples were separated by 12% (v/v) SDS-PAGE under reducing conditions and transferred to nitrocellulose membrane 0.2 μm. The membrane was then blocked with 5% (v/v) semi-skimmed milk in PBS under shaking for 30 minutes at RT and probed with antibody as above. After washes with 0.5% (v/v) Tween in PBS, pH 7.4, HP was detected by alkaline phosphatase chromogen BCIP/NBT solution.

4.2.5 Quantitative analysis of *S. aureus*

S. aureus colony forming units were quantified by the standard viable count method (Holms, 1968) and by spectrophotometric analysis as described below. *S. aureus* DSM 20233 was grown in 10 ml nutrient broth overnight at 37 ºC. To determine the number of colony forming units (CFUs), the culture was diluted in PBS, pH 7.4 and 100-fold

serial dilutions were plated on to agar plates and incubated overnight at 37 ºC. CFUs per ml = number of colonies x dilution factor / volume plated in ml. In addition, two fold dilutions of the culture were also made and absorbance readings were measured at 550 nm for each dilution.

4.2.6 Evaluating HP role in phagocyte-mediated killing of *S. aureus*

4.2.6.1 Quantifying *S. aureus*

S. aureus DSM 20233 was grown in 10 ml nutrient broth overnight at 37 ºC, washed three times with HBSS buffer containing calcium and magnesium (HBSS/$Ca^{2+}Mg^{2+}$) and the final bacterial pellet was also resuspended in this buffer. The OD_{550} of the washed *S. aureus* was measured and CFUs/ml were determined as described above.

4.2.6.2 Isolation of human phagocytes (neutrophils, monocytes and macrophages)

4.2.6.2.1 Isolation of human neutrophils

Neutrophils were isolated using Histopaque™ 1077 and 1119 according to the manufacturers' instructions as follows; 12 ml of Histopaque™1077 was gently layered over 12 ml Histopaque™1119 in a 50 ml centrifuge tube. Healthy human blood (~20 ml) was collected in a tube containing 400 units of heparin as an anticoagulant and carefully layered over the Histopaque™ layers taking care not to mix the components. The sample was then centrifuged at 700 g for 20 minutes at RT in a swing-out rotor. After centrifugation, the upper layers were discarded and the first layer above the red blood cell pellet was isolated using a Pasteur pipette. This layer containing neutrophils was washed three times with HBSS/$Ca^{2+}Mg^{2+}$ buffer and the cells were resuspended. The number of cells was determined using a haemocytometer.

4.2.6.2.2 Isolation of human monocytes

Monocytes were isolated using Ficoll-Paque PLUS™ according to the manufacturers' instructions as follows; 20 ml of human blood was collected in a tube containing 400 units of heparin as an anticoagulant. The blood was diluted with an equal volume of HBSS/$Ca^{2+}Mg^{2+}$ buffer and 35 ml of the diluted blood was carefully layered over 15 ml Ficoll-Paque PLUS™ in a 50 ml centrifuge tube. The sample was centrifuged at 400 g for 30 minutes at RT using a swing-out rotor. Following centrifugation, the distinct band at the sample/medium interface was isolated using a Pasteur pipette without removing the upper layer. The isolated mononuclear cells were washed three times with HBSS/$Ca^{2+}Mg^{2+}$ buffer, resuspended and counted using a haemocytometer.

4.2.6.2.3 Isolation of human macrophages

Mononuclear cells were isolated as described above except that PBS, pH 7.4 was used instead of HBSS/$Ca^{2+}Mg^{2+}$ to wash the cells. The mononuclear cell pellet was resuspended in Iscove's modified Dulbecco medium supplemented with 2 mM glutamine, 10X penicillin streptomycin (5 ml) and 2.5% (v/v) human AB serum (Human AB serum is sterile, and tested negative for Hepatitis B, Hepatitis C, HIV-1 and HIV-2). Mononuclear cells were then transferred into Corning cell culture ultra low attachment flasks where the monocytes differentiate into macrophages after six days incubation at 37 ºC with 4% (v/v) CO_2. The medium was removed and the adhered macrophages were released from the flask by incubation with 10 mM EDTA in PBS, pH 7.4 for 30 minutes in a 37 ºC incubator. The isolated macrophages were washed and resuspended in HBSS/$Ca^{2+}Mg^{2+}$ buffer, and counted using a haemocytometer.

4.2.6.3 Neutrophil-, monocyte- and macrophage-mediated killing of *S. aureus*

S. aureus (~10^8 CFUs) were incubated in each well of a 24-well tissue culture Nunc plate with 20% (v/v) serum, either complete or HP-depleted, in HBSS/$Ca^{2+}Mg^{2+}$ buffer, under slow shaking for 30 minutes at 37 ºC. After incubation, immune cells (neutrophils, monocytes or macrophages) were added to the assay and the final serum concentration was adjusted to 10% (v/v) with a ratio of staphylococcal CFUs to immune cells of 10 to 1 (i.e. 10 CFUs for each isolated immune cell). Aliquots were taken from each well after 0, 20, 40 and 60 minutes following incubation at 37 ºC in a 4% (v/v) CO_2 incubator with shaking, and left on ice for 5 minutes before diluting in PBS, pH 7.4 and plating out on agar plates. CFUs were counted the following day and the bacterial survival (%) was determined. Negative controls were also set up in which serum, immune cells or both were omitted and replaced by an equivalent volume of HBSS/$Ca^{2+}Mg^{2+}$.

4.2.6.4 Neutrophil- and monocyte-mediated phagocytosis of *S. aureus*

Two methods were employed to detect the internalization of *S. aureus* by isolated human neutrophils and monocytes:

4.2.6.4.1 Neutrophil cell lysis

A killing assay was performed as described above using isolated human neutrophils. Aliquots from the incubated assays at 0, 20, 40 and 60 minute were centrifuged for 2 minutes at 120 g and the neutrophil pellet was washed twice in HBSS/$Ca^{2+}Mg^{2+}$ buffer followed by centrifugation for 2 minutes at 120 g. The washed pellet was then resuspended in a hypotonic lysis buffer (0.01% w/v BSA in HPLC-quality water) and vortexed vigorously in order to lyse the neutrophils. The mixture was then diluted in

PBS, pH 7.4 and plated out on to agar plates. Staphylococcal CFUs were counted the following day and internal live bacteria (%) was determined and plotted accordingly.

4.2.6.4.2 Cytospin and microscopy

As well as measuring internal live bacteria, human neutrophils and monocytes were analyzed by microscopy to visualize the internalized bacteria. Samples were diluted in PBS, pH 7.4 and placed in Thermo-spin coated plates and centrifuged in a Cytometer for 3 minutes at maximum speed (350 g). The plates were then allowed to dry and Reagena stains were applied according to the manufacturers' instructions. The plates were afterwards examined for *S. aureus* directly.

4.2.6.5 LPS effect in HP-mediated killing of *S. aureus*

A killing assay was performed as described above using isolated human neutrophils, monocytes and macrophages. HP-depleted serum was supplemented with tenfold serial dilution of LPS (from 10^{-4} up to 10^{9} pg/ml).

4.2.7 Statistical analysis:

Statistical analyses were performed using unpaired t-test two-tailed, assuming the null hypothesis, i.e. no significant difference between samples. Differences were considered to be significant when $P \leq 0.05$.

4.3 Results

4.3.1 HP depletion from human serum

HP was depleted from human serum using a HB-affinity column, prepared by coupling HB to an affinity resin. After coupling and copious washing, the resin remained dark red confirming that coupling was successful. The resulting HB-affinity resin was used to deplete HP from serum, through the stable HB-HP interaction. Western and Dot blot analysis confirmed that HP is effectively depleted from whole serum and contained <0.001 of the amount of HP in whole serum (Figure 4.1).

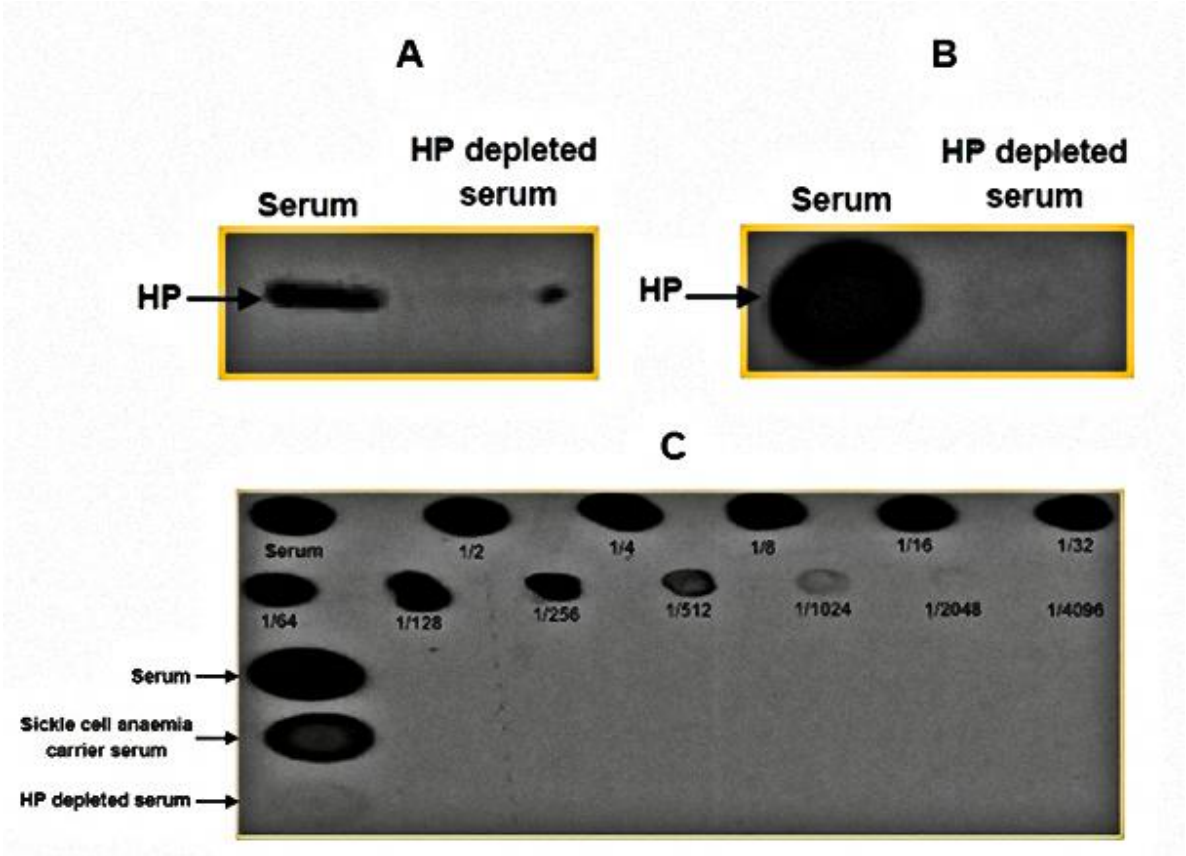

Figure 4.1 HP is efficiently depleted from human serum using HB-affinity column. (A) Western blot and (B) Dot blot analysis of whole serum (10%) and HP-depleted serum (10%). HP was depleted by affinity chromatography on an immobilized HB column and was detected using anti HP-antibody and a secondary alkaline phosphatase-conjugated sheep anti-human antibody. (C) Comparison of intensities of dots reveals that HP is reduced by at least 1024-fold in depleted serum compared to whole serum.

4.3.2 Quantitative analysis of *S. aureus*

To confirm that the methods used to quantify *S. aureus* were reliable and compatible, and to calibrate the two systems, test experiments were performed. Bacteria were quantified by determining the number of colony forming units per ml (CFUs/ml) and in parallel by spectrophotometric analysis. As shown in Figure 4.2, a plot of CFUs/ml against OD_{550} was approximately linear over the range $0 – 10^9$ CFUs/ml confirming the compatibility of the approaches. Based on this calibration curve, absorbance was subsequently used to provide a rapid and estimate of staphylococcal numbers prior to setting up the killing assays.

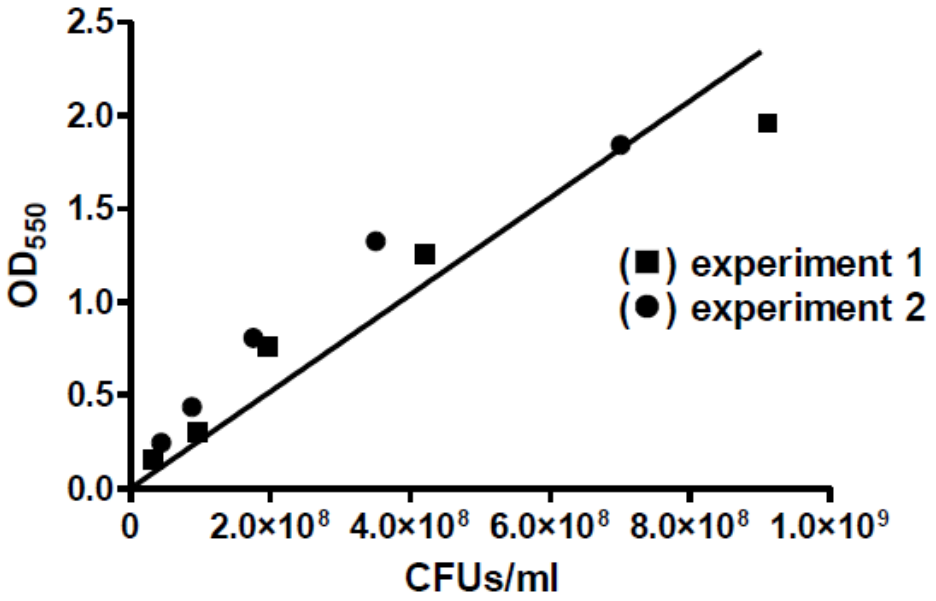

Figure 4.2 Quantification of *S. aureus*. (A) A linear graph represents staphylococcal CFUs/ml against OD_{550}. Staphylococcal CFUs/ml was quantified simultaneously by the standard viable count method and by spectrophotometric analysis. Data were fitted by linear regression after five measures were taken. Data are combined from two independent experiments.

4.3.3 HP-mediated killing of *S. aureus* by neutrophils, monocytes and macrophages

Initially, the role of HP in phagocyte-mediated killing of *S. aureus* was evaluated using human serum depleted of HP. *S. aureus* survival was compared using whole human serum and serum depleted of HP. Staphylococcal survival was also studied in the absence of phagocytes or serum.

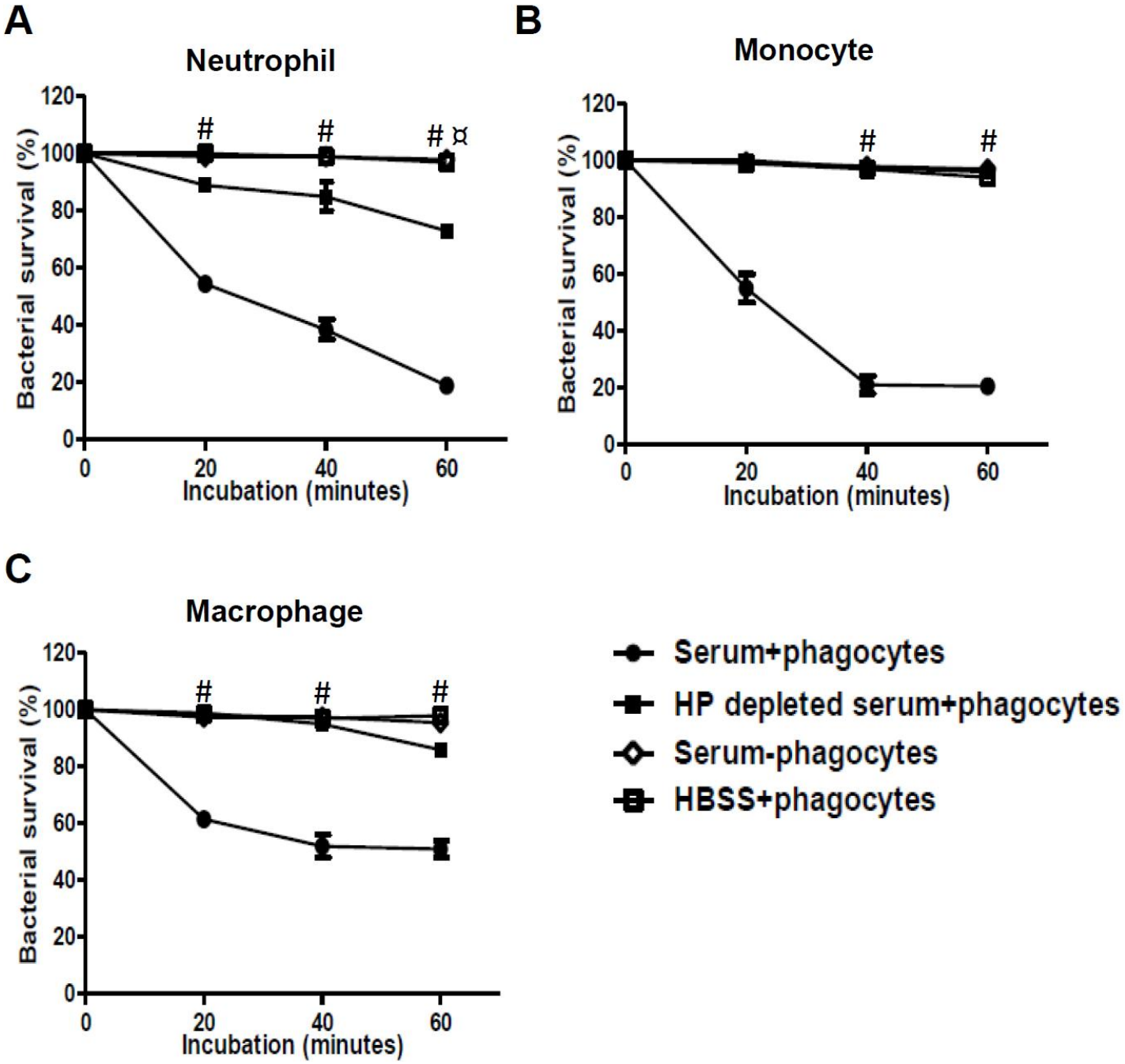

Figure 4.3 Neutrophil-, monocyte- and macrophage-mediated killing of *S. aureus*. *S. aureus* survival was measured in the presence of whole serum or HP-depleted serum for one hour incubation with human (A) neutrophils, (B) monocytes and (C) macrophages. *S. aureus* survival was also measured in the absence of phagocytes or serum. Results and errors are shown from two independent experiments. Statistical analysis: paired comparisons of staphylococcal survival for each cell type in HP-depleted and whole serum or in HBSS (unpaired t-test two-tailed, differences were considered to be significant when $P \leq 0.05$): (A) Neutrophils: overall (all time points combined) differences in survival in HP-depleted and whole serum ($p = 0.021$), or HBSS ($p = 0.014$) were significant. In addition, differences between whole and HP-depleted serum were significant (#) at twenty ($p = 0.024$), forty ($p = 0.022$) and sixty minutes ($p = 0.0007$). A significant difference (¤) in survival between HP-depleted

serum and HBSS was seen only at sixty minutes (p = 0.026) (B) Monocytes: the overall difference in survival was significant between HP-depleted serum and serum (p = 0.005), significant differences (#) in survival between whole and HP-depleted serum were seen at forty (p = 0.021) and sixty minutes (p = 0.008). (C) Macrophages: the overall difference in survival was significant between whole and HP-depleted serum (p = 0.006), significant differences (#) in survival between whole and HP-depleted serum were detected at twenty (p = 0.004), forty (p = 0.046) and sixty minutes (p=0.035). All other comparisons were statistically insignificant.

The survival of *S. aureus* decreased significantly when incubated with serum containing neutrophils, monocytes or macrophages. The most dramatic killing was observed by neutrophils and monocytes where only 20% of *S. aureus* survived after an hour (Figure 4.3.A and 4.3.B), but appreciable killing was also measured with macrophages where 60% of bacteria were viable (Figure 4.3.C). However, when HP-depleted serum was used, survival of *S. aureus* was much higher even in the presence of phagocytic cells. For example, almost all staphylococci survived in the assays which included monocytes or macrophages (Figure 4.3.B and 4.3.C), whereas 80% survival was observed with neutrophils (Figure 4.3.A), so depletion of HP leads to decreased killing of *S. aureus* by neutrophils, monocytes and macrophages. Thus, HP probably plays a critical role in the killing of *S. aureus* by phagocytes. Nevertheless, it is also possible that the process used to deplete HP leads to loss or activation of other serum components. For example, the affinity chromatography steps used to remove HP are likely to induce complement activation, so might deplete complement from serum, thus indirectly reducing its cytotoxic activity. Therefore, the possible role of HP in phagocyte-mediated killing of *S. aureus* was further investigated.

4.3.4 Purified human HP restores phagocyte-mediated killing of *S. aureus*

In order to test whether loss of killing activity of *S. aureus* in HP-depleted serum could be restored by exogenous HP, the survival assays were repeated using HP-depleted serum and depleted serum supplemented with 0.02 mg/ml (final concentration in 10%

serum) of purified HP. This concentration represents the lower limit of the normal physiological concentration of HP in serum (typical concentrations range from 0.2-1.4 mg/ml). Levels are elevated significantly following acute-phase upregulation.

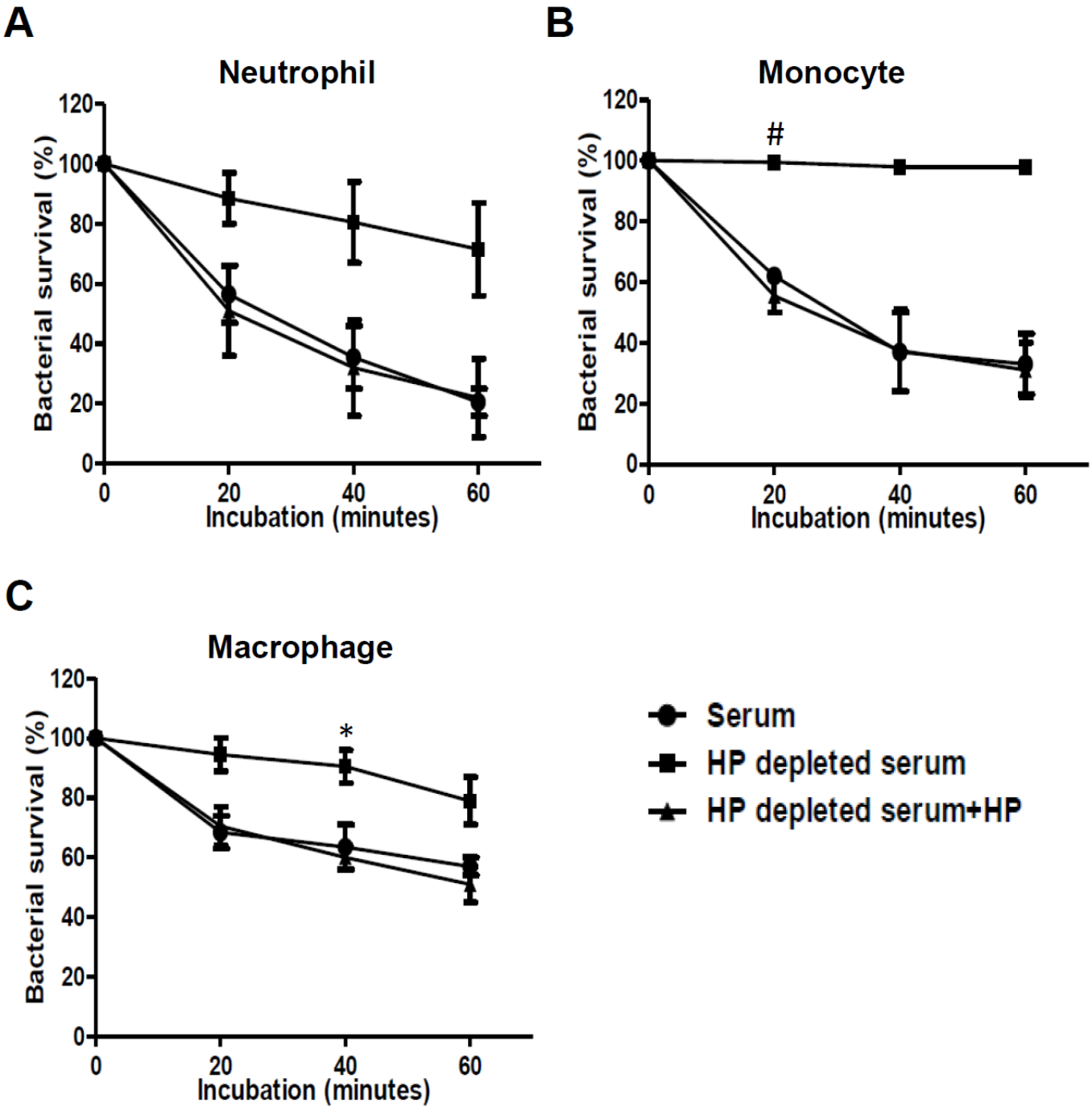

Figure 4.4 Exogenous HP restores killing of *S. aureus* by phagocytes. The role of HP in the killing of *S. aureus* was measured in the presence of whole serum, HP-depleted serum and HP-depleted serum supplemented with purified HP (0.02 mg/ml). Survival curves are shown for human neutrophils (A), monocytes (B) and macrophages (C). Results and errors are shown from two independent experiments. (A) Neutrophils: overall (all time points combined) differences in survival in HP-depleted and whole serum (p = 0.033), or HP-depleted serum + HP (p = 0.032) were significant

(B) Monocytes: overall differences in survival were significant between HP-depleted and whole serum (p = 0.006) or HP-depleted serum + HP (p = 0.005), a significant difference (#) in survival between whole and HP-depleted serum were seen after twenty minutes (p = 0.025) (C) Macrophages: overall differences in survival were significant between HP-depleted and whole serum (p = 0.028) or HP-depleted serum + HP (p = 0.027), a significant difference (∗) in survival between HP-depleted serum and HP-depleted serum + HP was detected at forty minutes (p = 0.05). All other comparisons were statistically insignificant

As seen before, *S. aureus* killing was much greater in the presence of whole serum compared with HP-depleted serum for all three phagocytic cell types (cells were taken from two different individuals for each cell type). Remarkably, killing was almost completely restored by adding purified HP to the HP-depleted serum. Similar results were also observed for HP concentration of 0.82 mg/ml (Figure 4.5), which represents HP levels in serum during infection (whole infected serum contains up to 8.2 mg/ml HP). Thus, depletion of HP did not reduce the capacity of the serum to kill bacteria indirectly eg. by depleting complement or other defence components, rather the data suggest that HP itself plays a significant role in the killing of *S. aureus* by phagocytes.

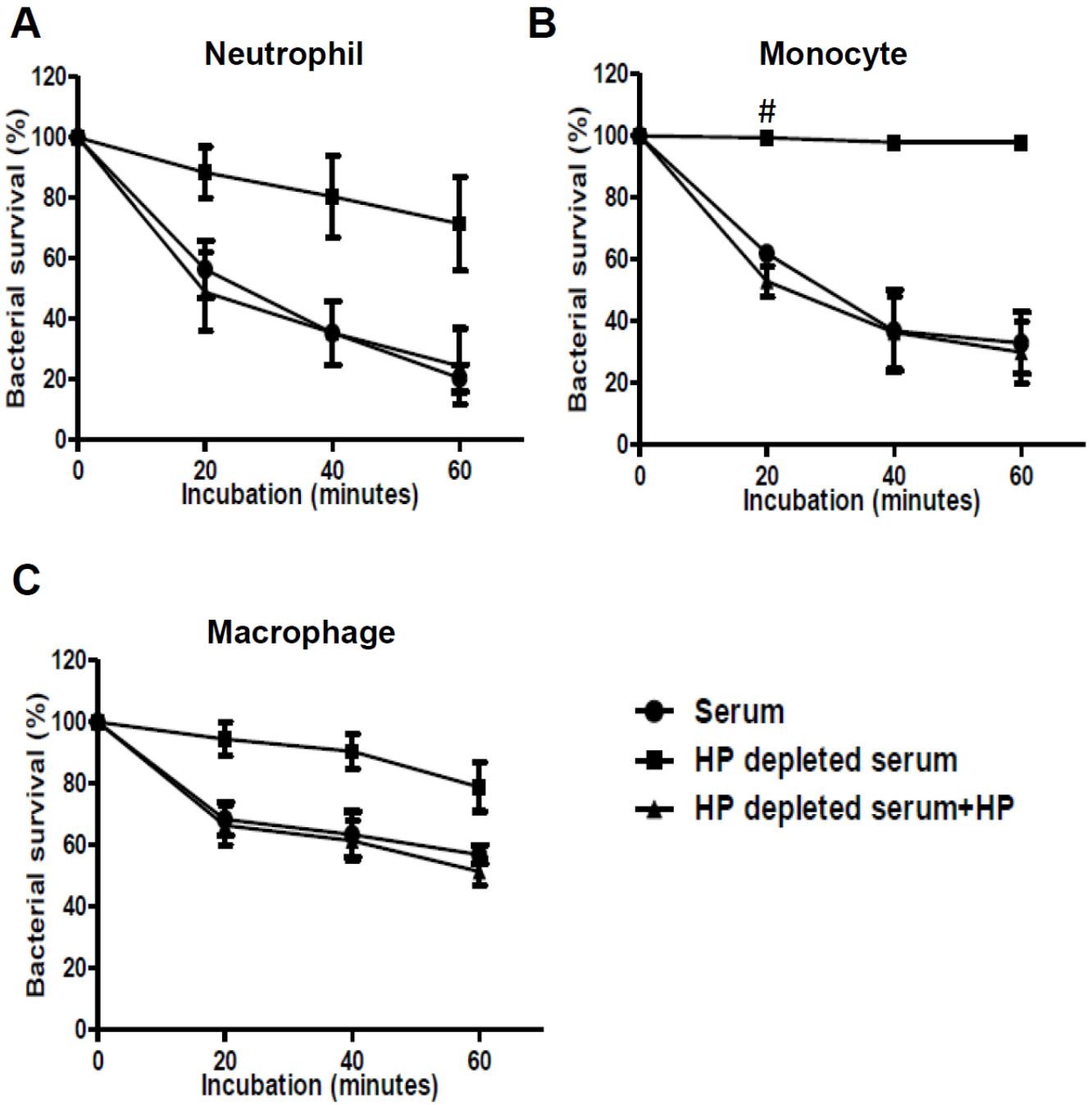

Figure 4.5 Exogenous HP restores killing of *S. aureus* by phagocytes. The role of HP in the killing of *S. aureus* was measured in the presence of serum, HP-depleted serum and HP-depleted serum supplemented with purified HP (0.82 mg/ml). Survival curves are shown for human neutrophils (A), monocytes (B) and macrophages (C). Results and errors are shown from two independent experiments. (A) Neutrophils: overall differences (all time points combined) were significant in survival in HP-depleted and whole serum (p = 0.033), or HP-depleted serum + HP (p = 0.029) (B) Monocytes: overall differences were significant in survival between HP-depleted and whole serum (p = 0.006), or HP-depleted serum + HP (p = 0.004). A significant difference (#) in survival between whole and HP-depleted serum was seen only after twenty minutes (p = 0.025) (C) Macrophages: overall differences were significant in survival between HP-depleted and whole serum (p = 0.028), or HP-depleted serum + HP (p = 0.024). All other comparisons were statistically insignificant.

4.3.5 HP-mediated phagocytosis of *S. aureus* by neutrophils and monocytes

While the above experiments show that HP is important for phagocyte-mediated killing of bacteria, they do not show how killing occurs i.e. by phagocytosis or some other phagocyte-mediated process. Therefore, a method was devised to capture and measure internalized but still viable bacteria from phagocytic cells. In this method, neutrophils were isolated following incubation with bacteria, washed and then lysed in buffer that will disrupt the neutrophil membranes and release their contents, but will not kill the bacteria. Numbers of viable cells could then be determined from CFUs.

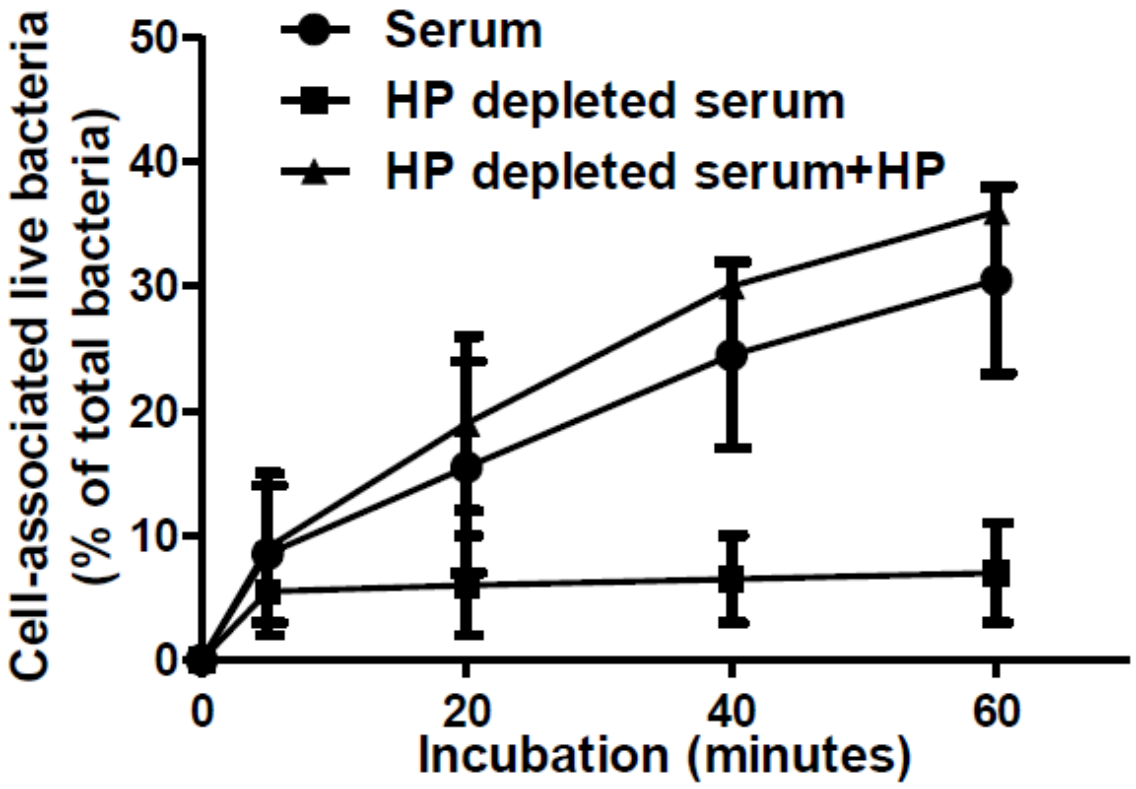

Figure 4.6 HP-mediated phagocytosis of *S. aureus* by neutrophils. Live *S. aureus* were extracted from neutrophils following incubation in whole serum, HP-depleted serum and HP-depleted serum supplemented with purified HP (0.02 mg/ml). Results and errors are shown from two independent experiments. Live bacteria are expressed as a percentage of total *S. aureus*. Significant overall differences (all time points combined) were observed for HP-depleted serum vs whole serum (p = 0.041) and HP-depleted serum vs HP-depleted serum + HP (p = 0.018).

As shown in Figure. 4.6, *S. aureus* were sequestered by the phagocytes and could be rescued by lysis of the neutrophils, indicating that bacteria are either phagocytosed or tightly associated to the surface of the neutrophil. The number of bound/phagocytosed bacteria increased appreciably when incubated with serum or HP-depleted serum supplemented with HP (~30% after an hour) but not with HP-depleted serum confirming that HP is an important factor in phagocyte-mediated killing.

To further examine the interactions of bacteria with phagocytes, neutrophils and monocytes were investigated by light microscopy after incubation with HP-containing serum. As shown in Figures 4.7 and 4.8, the bacteria are localized in clusters, rather than being evenly distributed around the phagocytic cell, consistent with their presence in internal vacuoles. Furthermore, bacteria are abundant when incubated with whole serum or HP-depleted serum + exogenous HP, but are relatively scarce in HP-depleted serum, consistent with HP-being a key mediator of phagocytosis.

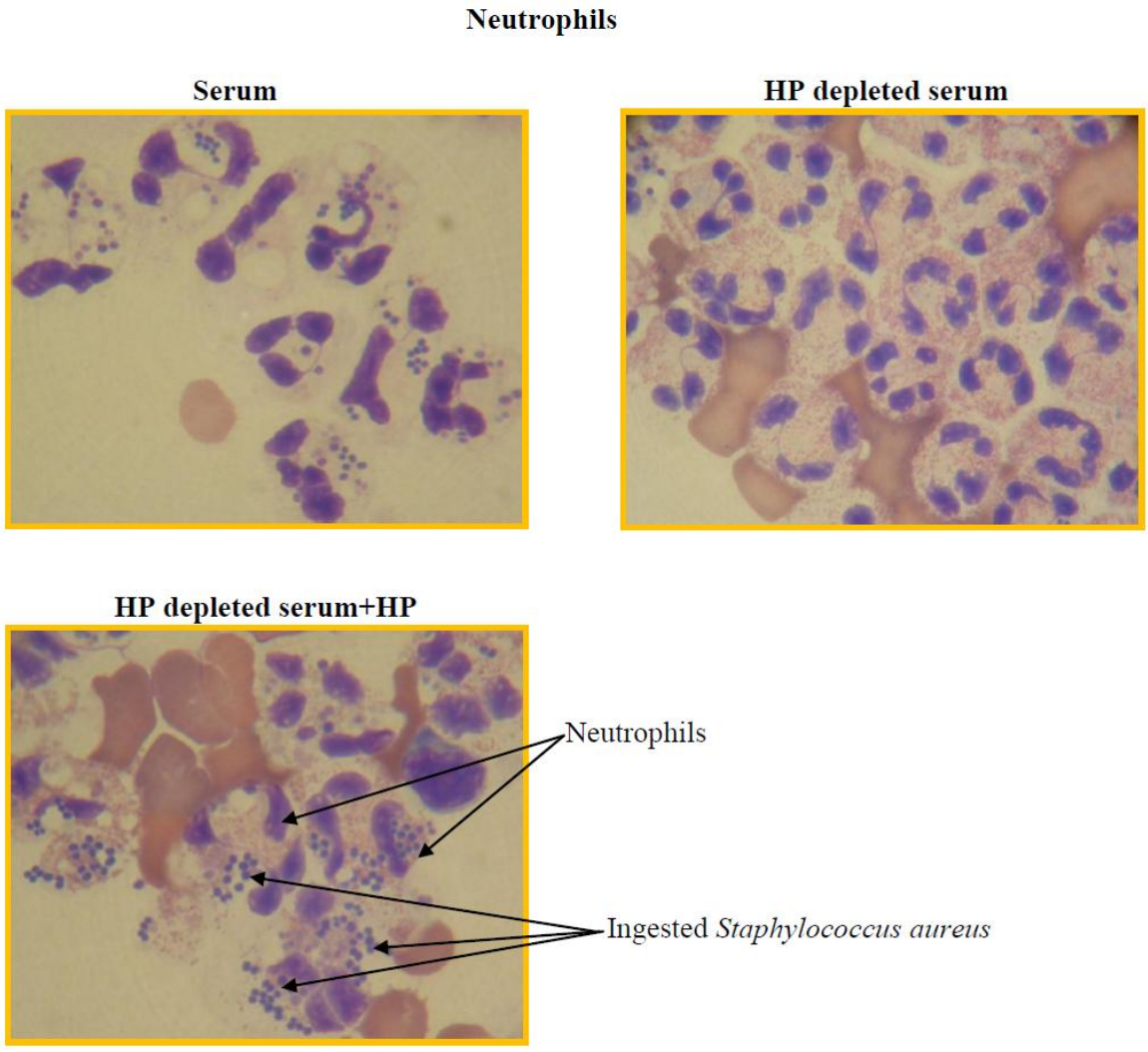

Figure 4.7 Phagocytosis of *S. aureus* by neutrophils. Neutrophils incubated with *S. aureus* in the presence of whole, HP-depleted or reconstituted serum were examined by optical microscopy (100X). Many engulfed *S. aureus* were observed in whole serum and HP-depleted serum reconstituted with purified HP (0.02 mg/ml), compared to HP-depleted serum.

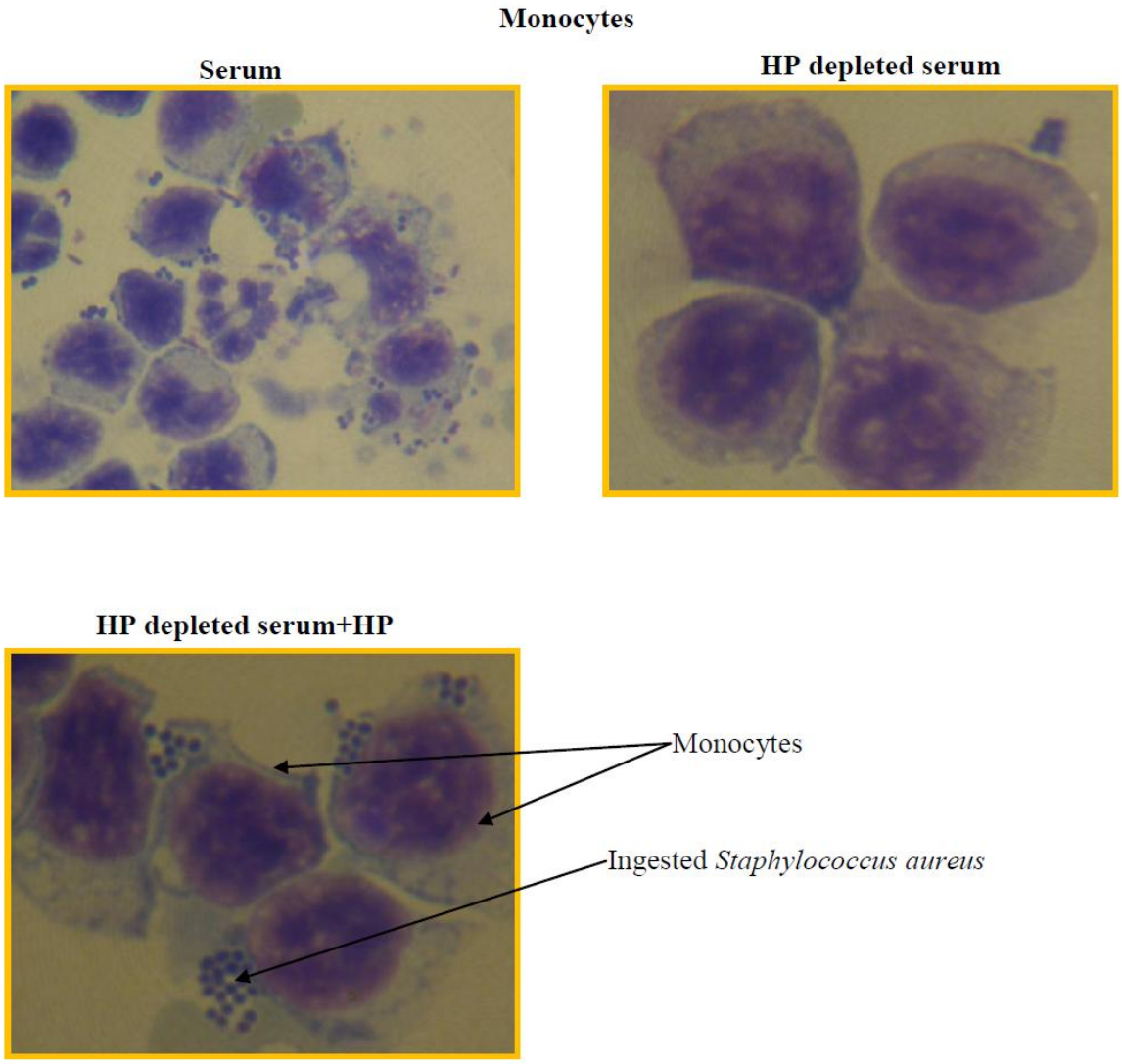

Figure 4.8 Phagocytosis of *S. aureus* by monocytes. Monocytes incubated with *S. aureus* in the presence of whole, HP-depleted or reconstituted serum were examined by optical microscopy (100X). Many engulfed *S. aureus* were observed in whole serum and HP-depleted serum reconstituted with purified HP (0.02 mg/ml), compared to HP-depleted serum.

4.3.6 LPS effect in HP-mediated killing of *S. aureus*

The inflammatory mediator LPS is often a contaminant of commercial-grade reagents including plasma-derived proteins (Weinstein et al., 2008). Because of its potent activity in stimulating an immune response, it was important to verify that HP preparations were not contaminated by LPS. The commercial preparation of purified HP used in the above survival assays was tested for LPS by Dr. Bernard Burke (University

of Leicester) using the Endotoxin Portable Testing System (supplied by Charles River Laboratories). LPS levels were found to be ≤0.68 and 28 pg/ml for 0.02 mg/ml and 0.82 mg/ml preparations of HP respectively. Although low, it was essential to confirm whether these trace amounts of LPS could be responsible for stimulating the observed phagocyte-mediated killing of *S. aureus*. Therefore, killing assays were carried out as before but in the presence of increasing amounts of LPS (from 10^{-4} to 10^{9} pg/ml) using HP-depleted serum (Figure 4.9).

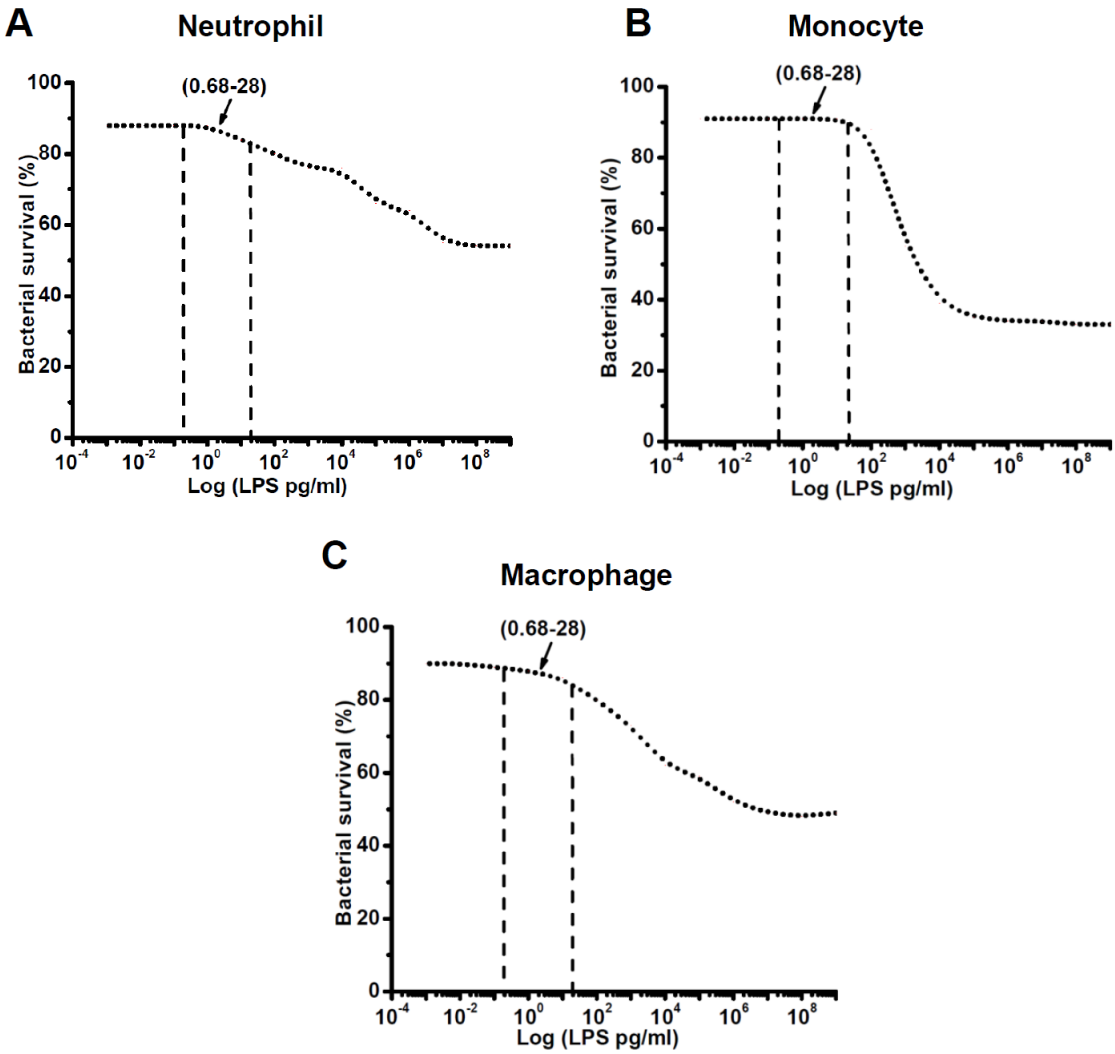

Figure 4.9 Modulation of phagocyte-mediated killing of *S. aureus* by LPS. The effect of LPS in the killing of *S. aureus* was measured in the presence of HP-depleted serum supplemented with tenfold serial dilution of LPS after an hour incubation with human neutrophils (A), monocytes (B) and macrophages (C).

The survival of *S. aureus* decreased with increasing LPS concentration until reaching a plateau at ~10^4 pg/ml for monocytes and ~10^6 pg/ml for neutrophils and macrophages. Importantly however LPS concentrations between 0.68 and 28 pg/ml had little effect on the survival of *S. aureus*, with <10% killing for neutrophils and macrophages and no detectable killing for monocytes. Thus, killing was mediated by HP rather than any contaminating LPS in the protein preparations.

All the data so far were obtained using human serum depleted of HP. However, it was also interesting to study whether serum completely deficient in HP would provide similar results. For this reason, serum was obtained from C57BL/6 HP knockout mice, with serum from wild type C57BL/6 as a control. The advantage of this system is that the HP-deficient serum can be compared directly with HP-sufficient serum, without any additional pre-treatment. This enables us to exclude any indirect effects caused by mechanical depletion of HP from the human serum. Human and mouse HP are very similar in sequence, sharing ≥80% identity (Maeda, 1985). Thus, it was considered likely that HP from humans could substitute for mouse HP and *vice versa*.

4.3.7 HP-mediated killing of *S. aureus* using mouse HP-deficient serum

The role of HP in phagocyte-mediated killing of *S. aureus* was evaluated using wild type (WT) and HP-deficient serum ($HP^{-/-}$). As before staphylococcal survival was also measured in the absence of phagocytes or serum as additional controls.

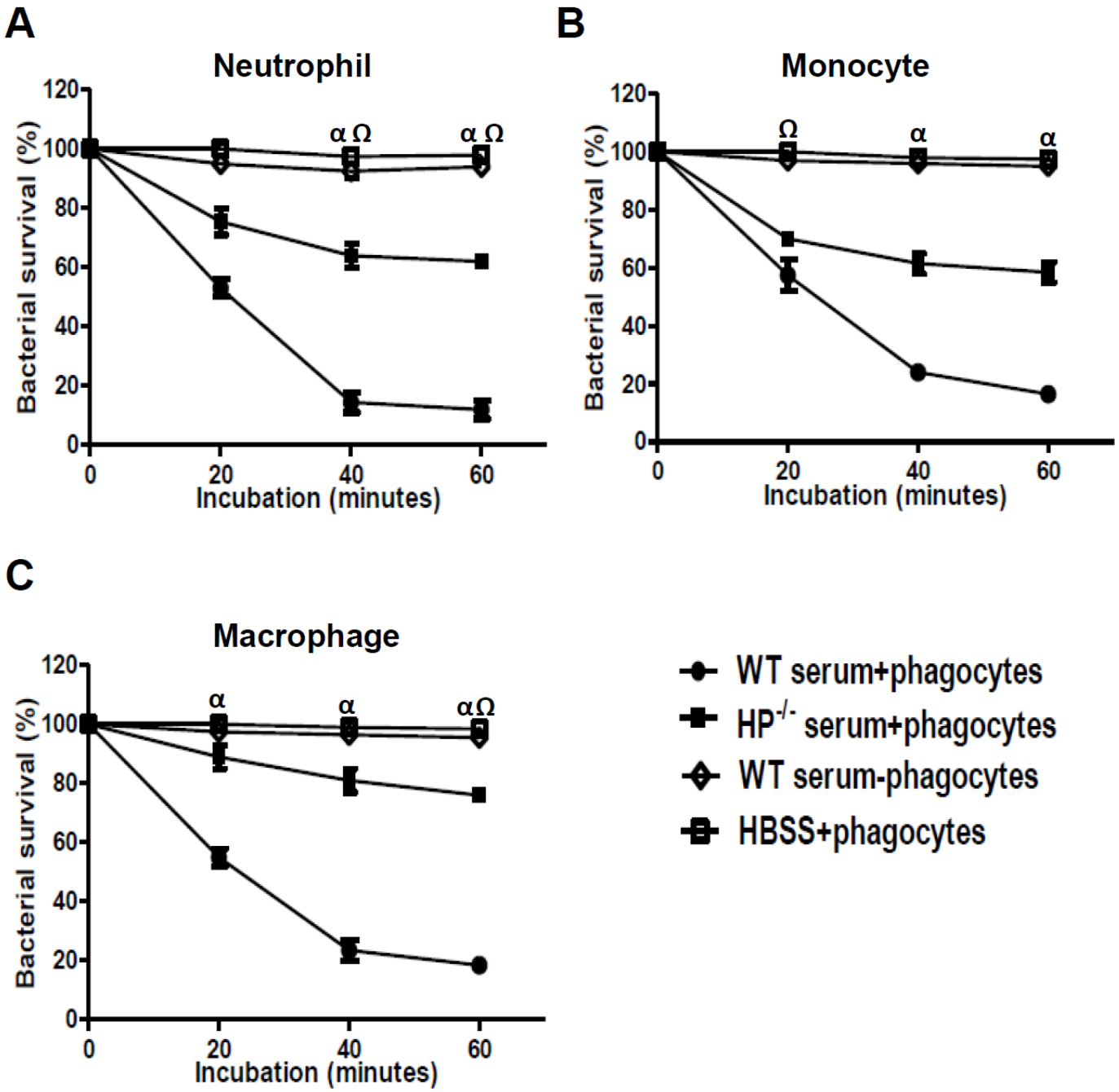

Figure 4.10 Neutrophil-, monocyte- and macrophage-mediated killing of *S. aureus* using HP-deficient serum. *S. aureus* survival was measured in the presence of WT serum or $HP^{-/-}$ serum for one hour incubation with human (A) neutrophils, (B) monocytes and (C) macrophages. *S. aureus* survival was also measured in the absence of phagocytes or serum. Results and errors are shown from two independent experiments. (A) Neutrophils: the overall (all time points combined) difference in survival in $HP^{-/-}$ serum and HBSS (p = 0.005) was significant, significant differences (Ω) in survival were also detected for this comparison at forty (p = 0.030) and sixty minutes (p = 0.035). Significant differences (α) in survival in $HP^{-/-}$ and WT serum were seen at forty (p = 0.011) and sixty minutes (p = 0.008) (B) Monocytes: the overall difference in survival was significant in $HP^{-/-}$ serum and HBSS (p = 0.004), a significant difference (Ω) in survival was also detected for this comparison at twenty minutes (p = 0.042). Significant differences (α) in survival in $HP^{-/-}$ and WT serum were seen at forty

(p = 0.022) and sixty minutes (p = 0.027) (C) Macrophages: the overall difference in survival was significant in $HP^{-/-}$ and WT serum (p = 0.019), significant differences (α) in survival were also detected for this comparison at twenty (p = 0.025), forty (p = 0.008) and sixty minutes (p = 0.002). The overall difference in survival in $HP^{-/-}$ serum and HBSS (p = 0.008) was significant, a significant difference (Ω) in survival was also seen for this comparison at sixty minutes (p = 0.008). All other comparisons were statistically insignificant.

Similar data were obtained using mouse serum as previously obtained from human depleted serum. Survival of *S. aureus* decreased significantly in WT serum containing neutrophils, monocytes or macrophages. The greatest level of killing was observed by neutrophils where only 10% of *S. aureus* survived after an hour (Figure 4.10.A), but appreciable killing by monocytes and macrophages was also measured where ~20% of bacteria were viable at the end of the study (Figure 4.10.B and Figure 4.10.C). Survival of *S. aureus* was much higher in $HP^{-/-}$ serum for all three types of phagocytic cells. 60% of the staphylococci survived in assays containing neutrophils or monocytes (Figure 4.10.A and 4.10.B), whereas 80% survived with macrophages (Figure 4.10.C). These data confirm that HP is vital for efficient killing of *S. aureus* by neutrophils, monocytes and macrophages, supporting the human studies. Moreover, loss of killing activity in $HP^{-/-}$ serum was restored by exogenous HP (0.02 mg/ml) as shown in figure 4.11.

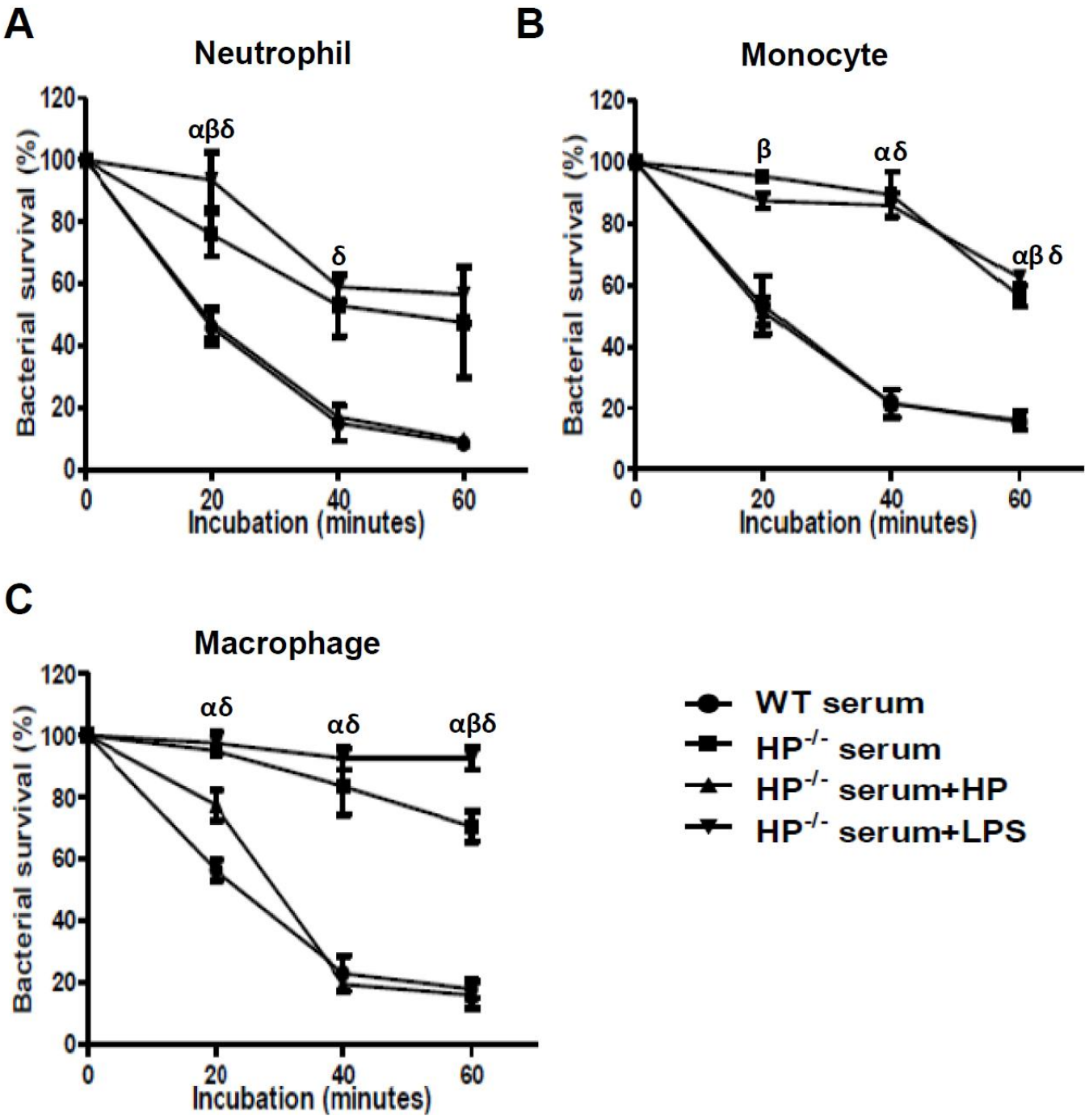

Figure 4.11 Exogenous HP restores killing of *S. aureus* by phagocytes using HP-deficient serum. The role of HP in the killing of *S. aureus* was measured in the presence of WT serum, $HP^{-/-}$ serum and $HP^{-/-}$ serum supplemented with purified HP (0.02 mg/ml) or LPS (28 pg/ml). Survival curves are shown for human neutrophils (A), monocytes (B) and macrophages (C). Results and errors are shown from two independent experiments. (A) Neutrophils: the overall (all time points combined) difference in survival in WT and $HP^{-/-}$ serum + LPS (p = 0.048) was significant, significant differences (δ) were also detected for this comparison at twenty (p = 0.038) and forty minutes (p = 0.016). Significant differences were also seen at twenty minutes in survival between $HP^{-/-}$ and WT serum (α; p = 0.046), or $HP^{-/-}$ serum + HP (β; p = 0.05) (B) Monocytes: the overall difference in survival in WT and $HP^{-/-}$ serum + LPS (p = 0.026) was significant, significant differences (δ) in survival were also detected for

this comparison at forty (p = 0.009) and sixty minutes (p = 0.027). Overall differences in survival in $HP^{-/-}$ and WT serum (p = 0.024) or $HP^{-/-}$ serum + HP (p = 0.022) were significant, significant differences (α) in survival in $HP^{-/-}$ serum and WT serum were detected after forty (p = 0.027) and sixty minutes (p = 0.014). Significant differences (β) in survival in $HP^{-/-}$ and $HP^{-/-}$ serum + HP were also seen at twenty (p = 0.044) and sixty minutes (p = 0.013) (C) Macrophages: the overall difference in survival in WT and $HP^{-/-}$ serum + LPS (p = 0.007) was significant, significant differences (δ) in survival were also detected for this comparison at twenty (p = 0.007), forty (p = 0.008) and sixty minutes (p = 0.002). Overall differences in survival in $HP^{-/-}$ and WT serum (p = 0.018) or $HP^{-/-}$ serum + HP (p = 0.045) were significant. Significant differences (α) in survival in $HP^{-/-}$ and WT serum were also detected at twenty (p = 0.041), forty (p = 0.025) and sixty minutes (p = 0.013). A significant difference (β) in survival in $HP^{-/-}$ and $HP^{-/-}$ serum + HP was also seen at sixty minutes (p = 0.007). All other comparisons were statistically insignificant.

Remarkably, killing was almost completely restored by adding purified HP to the $HP^{-/-}$ serum. Addition of LPS to $HP^{-/-}$ serum did not affect killing significantly. Thus, HP plays a key role in the phagocyte-mediated killing of *S. aureus*.

4.3.8 HP-LTA-mediated killing of *S. aureus* by neutrophils

Given that the interactions between HP and *S. aureus* are likely to be complex (i.e. involving, LTA-HP and HarA-HP). It was interesting to see if the addition of exogenous LTA would inhibit HP-mediated killing of *S. aureus* by phagocytes. Unfortunately, LTA was limiting in this study, so only a single concentration was used. Survival assays were repeated using mouse HP-deficient serum and deficient serum pre-incubated with a mix of HP and purified LTA.

As shown in Figure 4.12, killing by reconstituted serum ($HP^{-/-}$ serum + HP; *orange*) was partially inhibited by exogenous LTA (*blue*), implying that LTA competes with bacteria for HP binding. As expected addition of LTA alone (without HP) or trace amounts of LPS did not affect killing.

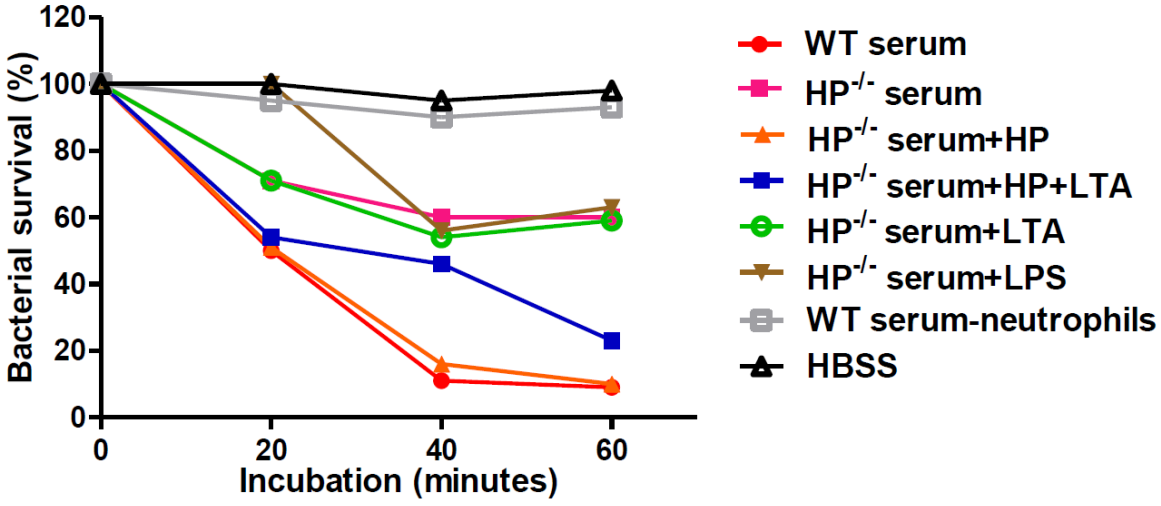

Figure 4.12 HP-LTA mediated killing of *S. aureus* by neutrophils. The role of HP-LTA interaction in the killing of *S. aureus* was measured in the presence of WT serum, $HP^{-/-}$ serum and $HP^{-/-}$ serum supplemented with purified HP (0.02 mg/ml), or LTA (0.25 mg/ml), or LPS (28 pg/ml), or a mix of HP (0.02 mg/ml) and LTA (0.25 mg/ml). *S. aureus* survival was also measured in the absence of neutrophils or serum.

The use of mouse serum instead of human serum in the above experiments can be limiting particularly that serum was acquired from mice with no prior exposure to *S. aureus* infection (i.e. naive adaptive immunity) and did not have IgG specific for *S. aureus* in contrast to human serum. However, both mouse and human innate immune responses were previously reported to be fundamental for protection against *S. aureus* infection by an efficient and rapid recruitment of neutrophils to the site of infection. For example, depletion of neutrophils in C57BL/6 mice increased staphylococcal numbers in the kidney during the early stages of infection, whereas neutrophil deficiency causes recurrent and often life-threatening *S. aureus* infection amongst humans (von Kockritz-Blickwede et al., 2008).

4.4 Discussion

The data described here show that HP is a key mediator in the phagocytosis and killing of *S. aureus* by neutrophils, monocytes and macrophages. The killing activity of these phagocytes is severely impaired when HP is depleted from serum or in serum lacking HP through gene disruption. Furthermore, this loss of activity can be almost completely restored by addition of exogenous HP. The activities observed were not due to contamination of HP preparations by LPS or by differential treatment of serum preparations.

Analysis of phagocytes by microscopy indicated that *S. aureus* were engulfed by the phagocytes (in the case of neutrophils and monocytes) consistent with their internalization into vesicles by phagocytosis. Interestingly, however, many of the bacteria were still viable despite being inside the defence cells, and could be rescued by lysing the phagocytes, thereby releasing their contents. *S. aureus* can survive inside neutrophils, monocyte and macrophages for long periods of time (eg. up to 5 days within macrophages), this ability is modulated by virulence factor regulator genes such as *sar* (Gresham et al., 2000, Kubica et al., 2008). Internalized bacteria inhabit incompetent endosomal vacuoles and lyse their membranes, and therefore escape into the cytoplasm where they may eventually kill phagocytes by secreting α-toxin, or through the induction of apoptosis (Kubica et al., 2008, Gresham et al., 2000).

Although only preliminary experiments were conducted due to limiting amounts of LTA, killing of *S. aureus* by neutrophils could be partially inhibited by addition of purified LTA. This finding implies that the exogenous LTA is competing with LTA (and possibly with the staphylococcal protein HarA) on the staphylococcal surface for HP, thereby reducing the number of bacteria that are targeted and internalized by

phagocytosis. Inhibition was only partial in this experiment, probably because the amount of LTA was limiting. However it is also possible that interaction of HarA with HP might not be affected by LTA, so is not inhibited. Interestingly, and consistent with this work, previous studies by Raynor et al. (1981) also showed that LTA reduces the ingestion of *S. aureus* by neutrophils, and it was suggested that it may interact with serum components to promote bacterial killing. The results described here confirm these studies and show that HP is the missing serum component.

As mentioned previously, activated neutrophils release HP and also interact with LTA via CD14 and TLR2 during recruitment to the site of infection (Theilgaard-Monch et al., 2006, Aulock et al., 2006, Lotz et al., 2004, Hattar et al., 2006). Consequently, granule proteins and chromatin are released and together form neutrophil extracellular traps (NETs) that degrade virulence factors and facilitate killing of Gram-Positive bacteria including *S. aureus* (Brinkmann et al., 2004). Activated neutrophils probably release HP, which then binds to LTA on the surface of *S. aureus* to facilitate additional bacterial recognition by phagocytic receptors such as CD14 and TLR2. In this way, it is likely that multiple host-pathogen interactions mediate the killing of *S. aureus* by phagocytosis or other phagocyte-mediated killing mechanisms.

As with neutrophils, the killing of *S. aureus* by monocytes or macrophages is also increased by HP. CD163 on these cells removes HP-HB complexes and may also be a receptor for *S. aureus* itself (Graversen et al., 2002, Fabriek et al., 2009). Another possibility is that HP-bacteria complexes (facilitated by HP-LTA interactions) are also internalized by CD163, through their interaction with HP, although non-complexed HP displays low affinity towards CD163 (Kristiansen et al., 2001, Moestrup and Moller, 2004). Moreover, other monocyte and macrophage receptors including TLR2 and type I scavenger receptor bind LTA and induce the release of critical cytokines for the host

immune response such as TNF-α and interleukins, so may also be involved in bacterial clearance (Dunne et al., 1994, Edds et al., 2000, Elgavish, 2000, Wang et al., 2000). Nevertheless, the data presented here suggest that HP-mediated phagocytosis is a major mechanism for the elimination of *S. aureus* by the mammalian immune system.

Chapter 5
General Discussion

The work described in this thesis has identified important novel functions of HP in the innate immune response and has provided new insights into molecular interactions between serum proteins, immune cell receptors and bacterial cell wall components. The results obtained, the methods employed and the outcomes observed in this study will form a basis for future work to further characterize the host immune response against *S. aureus* and provide novel targets for potential future therapeutics aimed at modifying the immune response.

5.1 The role of HP in innate immunity

Many human serum proteins including complement proteins, lipid-transport proteins, coagulation-cascade proteins and acute-phase proteins were identified to interact with highly purified LTA from *S. aureus,* either directly or indirectly. Further investigation revealed several of these proteins as potential innate immune components. HP, the focus of this study, has many of the hallmarks of an innate immune molecule: it is an acute-phase protein and is known to associate with phagocytic cells to promote elimination of bound HB. My work demonstrated that HP binds directly to LTA, and that the α-chain

is likely to contribute to this binding. The β-chain appears to enhance binding and might also bind to LTA, or modulate the binding activity of the α-chains.

HP was also shown to interact with *S. aureus*. Unlike the HP-LTA interaction, this has been observed before and was attributed to the staphylococcal surface component HarA. However, the HP-HarA interaction is relatively weak compared to HP-LTA or HP-staphylococcus binding, implying that it is unlikely to contribute appreciably. The staphylococcal physiological targets are more likely to be HB and HP-HB complexes, which bind much more tightly and would provide a vital source of iron, necessary for bacterial growth. As discussed in Chapter 3, the heme iron acquisition system via HP and other iron-containing targets such as transferrin may serve as traps employed by the immune system for invading bacteria, so there is likely to be a fine balance between the advantages gained from sequestering iron and the disadvantage resulting from detection and targeting by the immune system.

The major finding of this work is that HP is a key mediator of phagocytosis and the killing of *S. aureus* by neutrophils, monocytes and macrophages. Killing of *S. aureus* by all three phagocytes decreased dramatically in the presence of serum deficient in HP and was restored almost completely when purified HP was added back to deficient serum. Moreover, killing could be partially inhibited by exogenous LTA, implying that LTA competes for HP binding with LTA on the bacterial surface.

Receptors on phagocytic cells must target HP-*S. aureus* complexes to initiate phagocytosis, and the next major challenge will be to identify these receptors and characterize their interactions with HP. Neutrophils possess surface receptors including CD14 and TLR2 that recognize LTA and mediate bacterial killing by phagocytosis, and also employ alternative killing mechanisms, such as NETs (Brinkmann et al., 2004,

Theilgaard-Monch et al., 2006, Aulock et al., 2006, Hattar et al., 2006). Macrophage receptors such as CD163, TLR2 and type I scavenger also recognize either LTA or *S. aureus* and induce cytokines release (such as TNF-α and interleukins) and play key roles in immune responses against infections (Dunne et al., 1994, Edds et al., 2000, Elgavish, 2000, Bhakdi et al., 1991, Wang et al., 2000, Arndt and Abraham, 2001, Carlet, 2001, Fabriek et al., 2009). At this stage it is unclear whether HP-mediated killing of *S. aureus* is mediated by different phagocytic receptors or whether the difference cell types possess common recognition mechanisms.

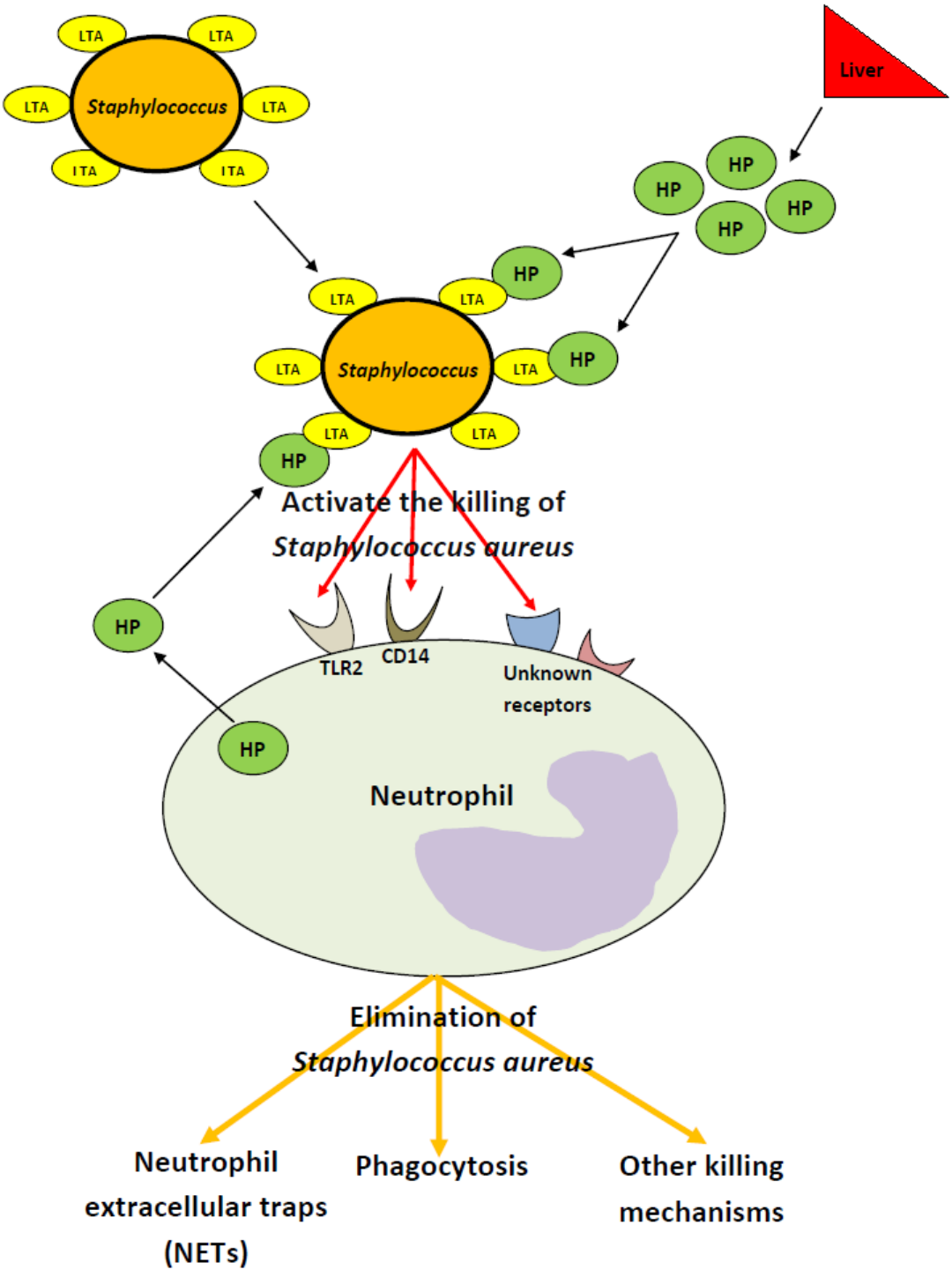

Figure 5.1 Putative receptors, interactions and mechanisms for HP-mediated killing of *S. aureus* by neutrophils. Neutrophils, the first line of defence, recruit circulating HP in complex with invading *S. aureus*, via surface LTA. Upon activation they secrete additional HP. Binding to surface receptors (such as TLR2 and CD14) promotes HP-mediated killing of *S. aureus* by phagocytosis and possibly by additional killing mechanisms (such as NETs).

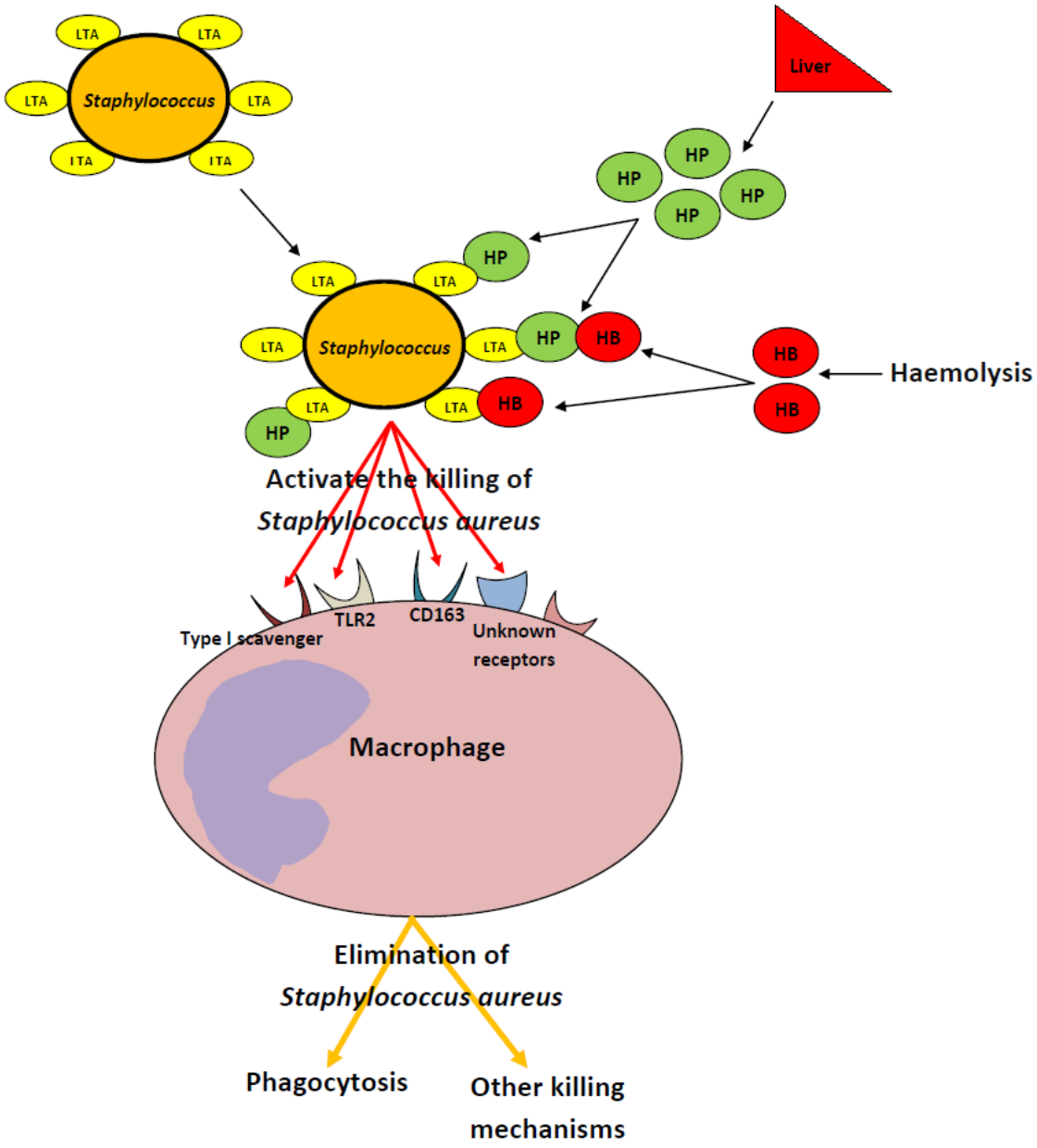

Figure 5.2 Putative receptors, interactions and mechanisms for HP-mediated killing of *S. aureus* by monocytes and macrophages. Monocytes are usually found circulating in the blood and differentiate into macrophages upon entering tissues. Both phagocytes bind HP-bacteria complexes via surface receptors (such as CD163, TLR2 and type I scavenger) to promote HP-mediated killing by phagocytosis and/or other killing mechanisms.

5.2 Future work

5.2.1 The possible role of HB in phagocyte-mediated killing of *S. aureus*

One of the unexpected and potentially interesting finding of this thesis is that free HB also binds to LTA, so could potentially induce the killing of *S. aureus* in association with HP via CD163 on monocytes and macrophages. This possibility could be tested using modified survival assays in which serum is supplemented with HB or HP-HB complexes in place of HP.

5.2.2 Cytokines regulating HP-mediated killing of *S. aureus* by phagocytes

Cytokines are likely to be released by neutrophils, monocytes and macrophages during HP-mediated killing of *S. aureus* to enable cell-cell communication and help coordinate an effective immune response. Thus, the identification of these cytokines is critical to help understand the events leading to killing. Cytokines can be detected in supernatants collected from the survival assays using specific antibodies. Preliminary work is already underway and supernatants have been collected following incubation of phagocytes with bacteria, and will be assayed in the near future.

5.2.3 Identifying phagocytic receptors for HP-mediated killing of *S. aureus*

A major focus of future work will be the identification of HP receptors on the surface of neutrophils, monocytes and macrophages. One way that this can be achieved is using antibodies to specifically block individual receptors and monitor bacterial survival. Once putative receptors have been identified, interactions with HP can be further characterized either using purified receptors or by expressing receptors in deficient cell lines and studying their interaction with *S. aureus*, LTA and HP using binding assays. Alternatively, receptors could be identified by preparing a HP-affinity matrix and probing with membrane extracts from phagocytic cells. In this way, CD163 was first

identified as a scavenger receptor for HP-HB (Kristiansen et al., 2001). The identification of HP receptors would significantly improve our understanding of the mechanisms and interactions employed by phagocytes to eliminate invading *S. aureus*.

5.2.4 HP-mediated killing of *S. aureus* using *in vivo* infection models

HP-mediated killing of *S. aureus* could also be investigated using HP-knockout mice, by comparing survival and immune responses of HP-deficient and -sufficient strains, challenged with *S. aureus*. Such a study has already been carried out using *S. typhimurium* (Huntoon et al., 2008). However in this case, the adaptive immune response was the primary focus rather than the innate immune response. HP deficiency was found to reduce T and B cell mediated immune responses and HP was suggested to have an immune regulatory function (Huntoon et al., 2008). My results would suggest that these may be indirect effects and the principal function of HP is in innate responses to infection. Nevertheless, this infection model could be used to study the role of HP role in immune responses against other pathogens such as *S. pneumoniae* and would also be useful to evaluate potential novel treatments for infection.

5.2.5 HP-mediating killing of other bacteria by phagocytes

The killing of other bacteria via HP interactions would be another interesting project for the future. Both Gram-positive bacteria such as *S. pneumoniae* and Gram-negative such as *E. coli* and *Salmonella* could be studied to assess overall role of HP in battling infection.

5.2.6 The role of other LTA-binding proteins in phagocyte-mediated killing of *S. aureus*

A number of other LTA-binding proteins were identified as potential immune components in this work. One of the most promising proteins was galectin-3 binding

protein or Mac-2 binding protein (M2BP), which has previously been reported to have an important role in the immune response (Chapter 2), including stimulating the release of cytokines IL-1 and IL-6 by monocytes and activating NK-cells (Ullrich et al., 1994, Powell et al., 1995). Like HP, M2BP was identified in thesis as a potential LTA-binding immune molecule, which might bind LTA on the staphylococcal surface and activate killing through phagocytic receptors. Its role could be investigated in a similar way as HP. Recombinant M2BP has already been produced (Hellstern et al., 2002) and M2BP deficient mice have been generated (Muller et al., 1999), so M2BP-deficient mice could be used as infection models to evaluate M2BP contribution in the immune system.

Appendix 1
MALDI-TOF-MS analysis of putative LTA-binding proteins

- **Scheme 1**

Band 5

Apolipoprotein A-IV (peptides sequence coverage: 36%)

Start - End	Mr(expt)	Mr(calc)	Peptides sequence
52 - 65	1633.8193	1633.8311	SELTQQLNALFQDK
52 - 65	1633.8374	1633.8311	SELTQQLNALFQDK
66 - 78	1406.6881	1406.7041	LGEVNTYAGDLQK
144 - 154	1286.6432	1286.6579	TQVNTQAEQLR
156 - 163	975.4401	975.5138	QLTPYAQR
190 - 198	1086.4935	1086.5557	IDQNVEELK
201 - 209	1082.4462	1082.5284	LTPYADEFK
212 - 221	1257.5973	1257.6677	IDQTVEELRR
222 - 233	1349.6092	1349.6463	SLAPYAQDTQEK
234 - 246	1558.8014	1557.7973	LNHQLEGLTFQMK
256 - 264	974.4359	974.5032	ISASAEELR
267 - 275	982.4214	982.5447	LAPLAEDVR
288 - 304	1926.9187	1926.9435	SLAELGGHLDQQVEEFR
317 - 326	1214.5614	1214.6441	ALVQQMEQLR
317 - 326	1230.6161	1230.6390	ALVQQMEQLR

```
  1 MFLKAVVLTL ALVAVAGARA EVSADQVATV MWDYFSQLSN NAKEAVEHLQ
 51 KSELTQQLNA LFQDKLGEVN TYAGDLQKKL VPFATELHER LAKDSEKLKE
101 EIGKELEELR ARLLPHANEV SQKIGDNLRE LQQRLEPYAD QLRTQVNTQA
151 EQLRRQLTPY AQRMERVLRE NADSLQASLR PHADELKAKI DQNVEELKGR
201 LTPYADEFKV KIDQTVEELR RSLAPYAQDT QEKLNHQLEG LTFQMKKNAE
251 ELKARISASA EELRQRLAPL AEDVRGNLRG NTEGLQKSLA ELGGHLDQQV
301 EEFRRRVEPY GENFNKALVQ QMEQLRTKLG PHAGDVEGHL SFLEKDLRDK
351 VNSFFSTFKE KESQDKTLSL PELEQQQEQH QEQQQEQVQM LAPLES
```

Haptoglobin (peptides sequence coverage: 16%)

Start - End	Mr(expt)	Mr(calc)	Peptides sequence
38 - 49	1289.6385	1289.7231	DIAPTLTLYVGK
51 - 57	857.4485	857.4858	QLVEIEK
100 - 108	979.4458	979.4876	VGYVSGWGR
214 - 223	1202.5661	1202.6295	VTSIQDWVQK

```
  1 MVSHHNLTTG ATLINEQWLL TTAKNLFLNH SENATAKDIA PTLTLYVGKK
 51 QLVEIEKVVL HPNYSQVDIG LIKLKQKVSV NERVMPICLP SKDYAEVGRV
101 GYVSGWGRNA NFKFTDHLKY VMLPVADQDQ CIRHYEGSTV PEKKTPKSPV
151 GVQPILNEHT FCAGMSKYQE DTCYGDAGSA FAVHDLEEDT WYATGILSFD
201 KSCAVAEYGV YVKVTSIQDW VQKTIAEN
```

Serum paraoxanase (peptides sequence coverage: 10%)

Start - End	Mr(expt)	Mr(calc)	Peptides sequence
234 - 244	1318.7980	1318.7285	YVYIAELLAHK
291 - 306	1882.9091	1882.9101	IFFYDSENPPASEVLR
307 - 316	1183.6358	1183.6448	IQNILTEEPK

```
  1 MAKLIALTLL GMGLALFRNH QSSYQTRLNA LREVQPVELP NCNLVKGIET
 51 GSEDMEILPN GLAFISSGLK YPGIKSFNPN SPGKILLMDL NEEDPTVLEL
101 GITGSKFDVS SFNPHGISTF TDEDNAMYLL VVNHPDAKST VELFKFQEEE
151 KSLLHLKTIR HKLLPNLNDI VAVGPEHFYG TNDHYFLDPY LQSWEMYLGL
201 AWSYVVYYSP SEVRVVAEGF DFANGINISP DGKYVYIAEL LAHKIHVYEK
251 HANWTLTPLK SLDFNTLVDN ISVDPETGDL WVGCHPNGMK IFFYDSENPP
301 ASEVLRIQNI LTEEPKVTQV YAENGTVLQG STVASVYKGK LLIGTVFHKA
351 LYCEL
```

Band 6

Apolipoprotein E3 (peptides sequence coverage: 38%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
44 - 50	843.3916	843.4603	WELALGR
94 - 108	1729.8331	1729.8370	SELEEQLTPVAEETR
114 - 121	885.3824	885.4668	ELQAAQAR
138 - 152	1646.8215	1646.7934	GEVQAMLGQSTEELR
138 - 152	1662.8743	1662.7883	GEVQAMLGQSTEELR
177 - 185	947.5029	947.5188	LAVYQAGAR
199 - 207	967.5377	967.5451	LGPLVEQGR
210 - 224	1496.7714	1496.7947	AATVGSLAGQPLQER
236 - 242	838.2826	838.3313	MEEMGSR
261 - 269	1113.5621	1113.5778	LEEQAQQIR
270 - 278	1032.5492	1032.5352	LQAEAFQAR
281 - 292	1535.7556	1535.7079	SWFEPLVEDMQR
293 - 300	929.4643	929.4971	QWAGLVEK

```
  1 MKVLWAALLV TFLAGCQAKV EQAVETEPEP ELRQQTEWQS GQRWELALGR
 51 FWDYLRWVQT LSEQVQEELL SSQVTQELRA LMDETMKELK AYKSELEEQL
101 TPVAEETRAR LSKELQAAQA RLGADMEDVR GRLVQYRGEV QAMLGQSTEE
151 LRVRLASHLR KLRKRLLRDA DDLQKRLAVY QAGAREGAER GLSAIRERLG
201 PLVEQGRVRA ATVGSLAGQP LQERAQAWGE RLRARMEEMG SRTRDRLDEV
251 KEQVAEVRAK LEEQAQQIRL QAEAFQARLK SWFEPLVEDM QRQWAGLVEK
301 VQAAVGTSAA PVPSDNH
```

Apolipoprotein J (peptides sequence coverage: 15%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
36 - 45	1116.5958	1116.6026	TLLSNLEEAK
126 - 134	1073.4827	1073.5353	IDSLLENDR
150 - 161	1392.6784	1392.6885	ASSIIDELFQDR
293 - 303	1287.5673	1287.6306	ELDESLQVAER
353 - 375	2313.4205	2313.1700	VTTVASHTSDSDVPSGVTEVVVK

```
  1 MSNQGSKYVN KEIQNAVNGV KQIKTLIEKT NEERKTLLSN LEEAKKKKED
 51 ALNETRESET KLKELPGVCN ETMMALWEEC KPCLKQTCMK FYARVCRSGS
101 GLVGRQLEEF LNQSSPFYFW MNGDRIDSLL ENDRQQTHML DVMQDHFSRA
```

```
151 SSIIDELFQD RFFTREPQDT YHYLPFSLPH RRPHFFFPKS RIVRSLMPFS
201 PYEPLNFHAM FQPFLEMIHE AQQAMDIHFH SPAFQHPPTE FIREGDDDRT
251 VCREIRHNST GCLRMKDQCD KCREILSVDC STNNPSQAKL RRELDESLQV
301 AERLTRKYNE LLKSYQWKML NTSSLLEQLN EQFNWVSRLA NLTQGEDQYY
351 LRVTTVASHT SDSDVPSGVT EVVVKLFDSD PITVTVPVEV SRKNPKFMET
401 VAEKALQEYR KKHREE
```

Haptoglobin related protein (peptides sequence coverage: 10%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
150 - 157	919.4071	919.4552	GSFPWQAK
241 - 249	1043.4748	1043.5508	VMPICLPSK
359 - 370	1344.5986	1344.6384	SCAVAEYGVYVK
371 - 380	1202.5880	1202.6295	VTSIQDWVQK

```
  1 MHVCVCVCVC VYMPVCVDAC MCCEAGRPAF RSFLFSLCSD LGAVISLLLW
 51 GRQLFALYSG NDVTDISDDR FPKPPEIANG YVEHLFRYQC KNYYRLRTEG
101 DGVYTLNDKK QWINKAVGDK LPECEAVCGK PKNPANPVQR ILGGHLDAKG
151 SFPWQAKMVS HHNLTTGATL INEQWLLTTA KNLFLNHSEN ATAKDIAPTL
201 TLYVGKKQLV EIEKVVLHPN YHQVDIGLIK LKQKVLVNER VMPICLPSKN
251 YAEVGRVGYV SGWGQSDNFK LTDHLKYVML PVADQYDCIT HYEGSTCPKW
301 KAPKSPVGVQ PILNEHTFCV GMSKYQEDTC YGDAGSAFAV HDLEEDTWYA
351 AGILSFDKSC AVAEYGVYVK VTSIQDWVQK TIAEN
```

Mr (expt) is the experimentally observed mass (Da)

Mr (calc) is the calculated mass (Da)

Matched peptides shown in *bold red*

- **Scheme 2**

Band 8

Pre-serum amyloid P component (peptides sequence coverage: 33%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
58 - 64	810.3627	810.3872	AYSDLSR
65 - 76	1405.7434	1405.6626	AYSLFSYNTQGR
77 - 84	992.6923	992.5178	DNELLVYK
87 - 96	1155.6287	1155.5924	VGEYSLYIGR
140 - 149	1165.6473	1165.5768	QGYFVEAQPK
150 - 162	1392.6488	1392.6885	IVLGQEQDSYGGK
150 - 162	1392.7040	1392.6885	IVLGQEQDSYGGK
150 - 165	1810.8764	1810.8850	IVLGQEQDSYGGKFDR
213 - 223	1285.7648	1285.7798	GYVIIKPLVWV

```
  1 MNKPLLWISV LTSLLEAFAH TDLSGKVFVF PRESVTDHVN LITPLEKPLQ
 51 NFTLCFRAYS DLSRAYSLFS YNTQGRDNEL LVYKERVGEY SLYIGRHKVT
101 PKVIEKFPAP VHICVSWESS SGIAEFWING TPLVKKGLRQ GYFVEAQPKI
151 VLGQEQDSYG GKFDRSQSFV GEIGDLYMWD SVLPPENILS AYQGTPLPAN
201 ILDWQALNYE IRGYVIIKPL VWV
```

Band 9,11,12,13,14,16

Keratin

eg, band 9 (peptides sequence coverage: 16%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
14 - 29	1231.8218	1231.5906	SGGGGGGGLGSGGSIR
47 - 59	1234.7187	1234.5215	FSSSSGYGGGSSR
155 - 163	1064.6853	1064.4920	STMQELNSR
155 - 163	1080.6412	1080.4870	STMQELNSR
164 - 170	808.5557	808.4330	LASYLDK
164 - 184	2376.7766	2376.1808	LASYLDKVQALEEANNDLENK
171 - 184	1586.0547	1585.7583	VQALEEANNDLENK
225 - 233	1059.7968	1059.5560	TLLDIDNTR
234 - 240	896.6102	896.4062	MTLDDFR
241 - 250	1323.9015	1322.6652	IKFEMEQNLR
262 - 271	1205.8402	1205.5962	QVLDNLTMEK
328 - 336	1120.8541	1120.5764	QEYEQLIAK

```
  1 MSCRQFSSSY LSRSGGGGGG GLGSGGSIRS SYSRFSSSGG GGGGGRFSSS
 51 SGYGGGSSRV CGRGGGGSFG YSYGGGSGGG FSASSLGGGF GGGSRGFGGA
101 SGGGYSSSGG FGGGFGGGSG GGFGGGYGSG FGGFGGFGGG AGGGDGGILT
151 ANEKSTMQEL NSRLASYLDK VQALEEANND LENKIQDWYD KKGPAAIQKN
201 YSPYYNTIDD LKDQIVDLTV GNNKTLLDID NTRMTLDDFR IKFEMEQNLR
251 QGVDADINGL RQVLDNLTME KSDLEMQYET LQEELMALKK NHKEEMSQLT
301 GQNSGDVNVE INVAPGKDLT KTLNDMRQEY EQLIAKNRKD IENQYETQIT
351 QIEHEVSSSG QEVQSSAKEV TQLRHGVQEL EIELQSQLSK KAALEKSLED
401 TKNRYCGQLQ MIQEQISNLE AQITDVRQEI ECQNQEYSLL LSIKMRLEKE
451 IETYHNLLEG GQEDFESSGA GKIGLGGRGG SGGSYGRGSR GGSGGSYGGG
501 GSGGGYGGGS GSRGGSGGSY GGGSGSGGGS GGGYGGGSGG GHSGGSGGGH
551 SGGSGGNYGG GSGSGGGSGG GYGGGSGSRG GSGGSHGGGS GFGGESGGSY
601 GGGEEASGSG GGYGGGSGKS SHS
```

Band 10

Carboxypeptidase D (peptides sequence coverage: 3%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
36 - 57	2210.4265	2212.0244	KAEATTTTTSAGAEAAEGQFDR
36 - 57	2210.5085	2212.0244	KAEATTTTTSAGAEAAEGQFDR
36 - 57	2210.5566	2212.0244	KAEATTTTTSAGAEAAEGQFDR
886 - 907	2298.6101	2298.0863	GASSSTNDASDPTTKEFETLIK

```
   1 MASGRDERPH WRLGRLLLLM CLLLLGSSAR AAHIKKAEAT TTTTSAGAEA
  51 AEGQFDRYYH EEELESALRE AAAAGLPGLA RLFSIGRSVE GRPLWVLRLT
 101 AGLGSLIPEG DAGPDAAGPD AAGPLLPGRP QVKLVGNMHG DETVSRQVLI
 151 YLARELAAGY RRGDPRLVRL LNTTDVYLLP SLNPDGFERA REGDCGFGDG
 201 GPSGASGRDN SRGRDLNRSF PDQFSTGEPP ALDEVPEVRA LIEWIRRNKF
 251 VLSGNLHGGS VVASYPFDDS PEHKATGIYS KTSDDEVFKY LAKAYASNHP
 301 IMKTGEPHCP GDEDETFKDG ITNGAHWYDV EGGMQDYNYV WANCFEITLE
 351 LSCCKYPPAS QLRQEWENNR ESLITLIEKV HIGVKGFVKD SITGSGLENA
 401 TISVAGINHN ITTGRFGDFY RLLVPGTYNL TVVLTGYMPL TVTNVVVKEG
 451 PATEVDFSLR PTVTSVIPDT TEAVSTASTV AIPNILSGTS SSYQPIQPKD
 501 FHHHHFPDME IFLRRFANEY PNITRLYSLG KSVESRELYV MEISDNPGVH
 551 EPGEPEFKYI GNMHGNEVVG RELLLNLIEY LCKNFGTDPE VTDLVHNTRI
 601 HLMPSMNPDG YEKSQEGDSI SVIGRNNSNN FDLNRNFPDQ FVQITDPTQP
 651 ETIAVMSWMK SYPFVLSANL HGGSLVVNYP FDDDEQGLAT YSKSPDDAVF
 701 QQIALSYSKE NSQMFQGRPC KNMYPNEYFP HGITNGASWY NVPGGMQDWN
 751 YLQTNCFEVT IELGCVKYPL EKELPNFWEQ NRRSLIQFMK QVHQGVRGFV
 801 LDATDGRGIL NATISVAEIN HPVTTYKTGD YWRLLVPGTY KITASARGYN
 851 PVTKNVTVKS EGAIQVNFTL VRSSTDSNNE SKKGKGASSS TNDASDPTTK
 901 EFETLIKDLS AENGLESLML RSSSNLALAL YRYHSYKDLS EFLRGLVMNY
 951 PHITNLTNLG QSTEYRHIWS LEISNKPNVS EPEEPKIRFV AGIHGNAPVG
1001 TELLLALAEF LCLNYKKNPA VTQLVDRTRI VIVPSLNPDG RERAQEKDCT
1051 SKIGQTNARG KDLDTDFTNN ASQPETKAII ENLIQKQNFS LSVALDGGSM
1101 LVTYPYDKPV QTVENKETLK HLASLYANNH PSMHMGQPSC PNKSDENIPG
1151 GVMRGAEWHS HLGSMKDYSV TYGHCPEITV YTSCCYFPSA ARLPSLWADN
1201 KRSLLSMLVE VHKGVHGFVK DKTGKPISKA VIVLNEGIKV QTKEGGYFHV
1251 LLAPGVHNII AIADGYQQQH SQVFVHHDAA SSVVIVFDTD NRIFGLPREL
1301 VVTVSGATMS ALILTACIIW CICSIKSNRH KDGFHRLRQH HDEYEDEIRM
1351 MSTGSKKSLL SHEFQDETDT EEETLYSSKH
```

Band 15

Collagen (peptides sequence coverage: 6%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
239 - 267	2788.8064	2787.4338	GVPGGRGLPGEDGEKGEMGLPGIIGPLGR
441 - 464	2210.3842	2210.2899	GKKGAPGPSGKPGIPGLQGLLGPK

```
   1 KITVFATLYS FLTGRSRRSR KYWKIGETGP VGLPGEVGMT GSIGEKGERG
  51 SPGPLGPQGE KGVMGYPGPP GVPGPIGPLG LPGHVGARGP PGSQGPKGQR
 101 GSRGPDGLLG EQGIQGAKGE KGDQGKRGPH GLIGKTGNPG ERGFQGKPGL
 151 QGLPGSTGDR GLPGEPGLRG LQGDVGPPGE MGMEGPPGTE GESGLQGEPG
 201 AKGDVGTAGS VGGTGEPGLR GEPGAPGEEG LQGKDGLKGV PGGRGLPGED
 251 GEKGEMGLPG IIGPLGRSGQ TGLPGPEGIV GIPGQRGRPG KKGDKGQIGP
 301 TGEVGSRGPP GKIGKSGPKG ARGTRGAVGH LGLMGPDGEP GIPGYRGHQG
 351 QPGPSGLPGP KGEKGYPGED STVLGPPGPR GEPGPVGDQG ERGEPGAEGY
 401 KGHVGVPGLR GATGQQGPPG EPGDQGEQGL KGERGSEGNK GKKGAPGPSG
 451 KPGIPGLQGL LGPKGIQGYH GADGISGNPG KIGPPGKQGL PGIRGGPGRT
 501 GLAGAPGPPG VKGSSGLPGS PGIQGPKGEQ GLPGQPGIQG KRGHRGAQGD
```

```
 551 QGPCGDPGLK GQPGEYGVQG LTGFQGFPGP KGPEGDAGIV GISGPKGPIG
 601 HRGNTGPLGR EGIIGPTGRT GPRGEKGFRG ETKQMDINAA IQALIESNTA
 651 LQMESYQNTE VTLIDHSEEI FKTLNYLSNL LHSIKNPLGT RDNPARICKD
 701 LLNCEQKVSD GKYWIDPNLG CPSDAIEVFC NFSAGGQTCL PPVSVTKLEF
 751 GVGKVQMNFL HLLSSEATHI ITIHCLNTPR WTSTQTSGPG LPIGFKGWNG
 801 QIFKVNTLLE PKVLSDDCKI QDGSWHKATF LFHTQEPNQL PVIEVQKLPH
 851 LKTERKYYID SSSVCFL
```

Band 17

Apolipoprotein E (peptides sequence coverage: 16%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
80 - 90	1339.8963	1339.6363	ALMDETMKELK
199 - 207	967.6883	967.5451	LGPLVEQGR
210 - 224	1496.9806	1496.7947	AATVGSLAGQPLQER
270 - 278	1032.7014	1032.5352	LQAEAFQAR
293 - 300	929.5947	929.4971	QWAGLVEK

```
   1 MKVLWAALLV TFLAGCQAKV EQAVETEPEP ELRQQTEWQS GQRWELALGR
  51 FWDYLRWVQT LSEQVQEELL SSQVTQELRA LMDETMKELK AYKSELEEQL
 101 TPVAEETRAR LSKELQTAQA RLGADMEDVC GRLVQYRGEV QAMLGQSTEE
 151 LRVRLASHLR KLRKRLLRDP DDLQKRLAVY QAGAREGAER GLSAIRERLG
 201 PLVEQGRVRA ATVGSLAGQP LQERAQAWGE RLRARMEEMG SRTRDRLDEV
 251 KEQVAEVRAK LEEQAQQIRL QAEAFQARLK SWFEPLVEDM QRQWAGLVEK
 301 VQAAVGTSAA PVPSDNH
```

Band 18

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
671 - 680	1044.7206	1045.5291	LSSPADITDK
671 - 680	1045.7707	1045.5291	LSSPADITDK

```
   1 MEHKEVVLLL LLFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
  51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
 101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
 151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
 201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
 251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
 301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
 351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
 401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
 451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
 501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
 551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
 601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
 651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
 701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
 751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
 801 TWIEGVMRNN
```

Lipoprotein GIn I (peptides sequence coverage: 25%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
1 - 10	1225.7054	1225.5364	DEPPQSPWDR
101 - 110	1251.7966	1251.6136	VQPYLDDFQK
111 - 119	1298.7292	1297.6125	KWQEMELYR
122 - 134	1466.9447	1466.7841	VEPLRAELQEGAR
127 - 134	872.6084	872.4352	AELQEGAR
144 - 152	1030.6727	1030.5117	LSPLGEEMR
199 - 209	1214.8635	1214.6143	ATEHLSTLSEK

```
  1 DEPPQSPWDR VKDLATVYVD VLKDSGRDYV SQFQGSALGK QLNLKLLWDD
 51 VTSTFSKLRQ ELGPVTEEWF NDLQEKLNLE KETGELRQEM SKDLEEVKAK
101 VQPYLDDFQK KWQEMELYRQ KVEPLRAELQ EGARQKLHEL QEKLSPLGEE
151 MRDRARAHVD ALRTHLAPYS DELRQRLAAR LEALKENGAG RLAEYHAKAT
201 EHLSTLSEKA KPALEDLRQG LLPVLESFKV SFLSALEEYT KLNTQ
```

Mr (expt) is the experimentally observed mass (Da)

Mr (calc) is the calculated mass (Da)

Matched peptides shown in *bold red*

- **Scheme 3**

Band 19

Apolipoprotein B-100 precursor (peptides sequence coverage: 0%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
116 - 128	1442.4532	1440.6667	TKNSEEFAAAMSR
220 - 229	1011.6419	1011.6328	TGISPLALIK
306 - 314	998.4041	998.4743	MGLAFESTK
642 - 654	1270.6610	1270.6769	SVSIPSLDPASAK

```
   1 MDPPRPALLA LLALPALLLL LLAGARAEEE MLENVSLVCP KDATRFKHLR
  51 KYTYNYEAES SSGVPGTADS RSATRINCKV ELEVPQLCSF ILKTSQCILK
 101 EVYGFNPEGK ALLKKTKNSE EFAAAMSRYE LKLAIPEGKQ VFLYPEKDEP
 151 TYILNIKRGI ISALLVPPET EEAKQVLFLD TVYGNCSTHF TVKTRKGNVA
 201 TEISTERDLG QCDRFKPIRT GISPLALIKG MTRPLSTLIS SSQSCQYTLD
 251 AKRKHVAEAI CKEQHLFLPF SYKNKYGMVA QVTQTLKLED TPKINSRFFG
 301 EGTKKMGLAF ESTKSTSPPK QAEAVLKTVQ ELKKLTISEQ NIQRANLFNK
 351 LVTELRGLSD EAVTSLLPQL IEVSSPITLQ ALVQCGQPQC STHILQWLKR
 401 VHANPLLIDV VTYLVALIPE PSAQQLREIF NMARDQRSRA TLYALSHAVN
 451 NYHKTNPTGT QELLDIANYL MEQIQDDCTG DEDYTYLILR VIGNMGQTME
 501 QLTPELKSSI LKCVQSTKPS LMIQKAAIQA LRKMEPKDKD QEVLLQTFLD
 551 DASPGDKRLA AYLMLMRSPS QADINKIVQI LPWEQNEQVK NFVASHIANI
 601 LNSEELDIQD LKKLVKEVLK ESQLPTVMDF RKFSRNYQLY KSVSIPSLDP
 651 ASAKIEGNLI FDPNNYLPKE SMLKTTLTAF GFASADLIEI GLEGKGFEPT
 701 LEALFGKQGF FPDSVNKALY WVNGQVPDGV SKVLVDHFGY TKDDKHEQDM
 751 VNGIMLSVEK LIKDLKSKEV PEARAYLRIL GEELGFASLH DLQLLGKLLL
 801 MGARTLQGIP QMIGEVIRKG SKNDFFLHYI FMENAFELPT GAGLQLQISS
 851 SGVIAPGAKA GVKLEVANMQ AELVAKPSVS VEFVTNMGII IPDFARSGVQ
 901 MNTNFFHESG LEAHVALKPG KLKFIIPSPK RPVKLLSGGN TLHLVSTTKT
 951 EVIPPLIENR QSWSVCKQVF PGLNYCTSGA YSNASSTDSA SYYPLTGDTR
1001 LELELRPTGE IEQYSVSATY ELQREDRALV DTLKFVTQAE GAKQTEATMT
1051 FKYNRQSMTL SSEVQIPDFD VDLGTILRVN DESTEGKTSY RLTLDIQNKK
1101 ITEVALMGHL SCDTKEERKI KGVISIPRLQ AEARSEILAH WSPAKLLLQM
1151 DSSATAYGST VSKRVAWHYD EEKIEFEWNT GTNVDTKKMT SNFPVDLSDY
1201 PKSLHMYANR LLDHRVPQTD MTFRHVGSKL IVAMSSWLQK ASGSLPYTQT
1251 LQDHLNSLKE FNLQNMGLPD FHIPENLFLK SDGRVKYTLN KNSLKIEIPL
1301 PFGGKSSRDL KMLETVRTPA LHFKSVGFHL PSREFQVPTF TIPKLYQLQV
1351 PLLGVLDLST NVYSNLYNWS ASYSGGNTST DHFSLRARYH MKADSVVDLL
1401 SYNVQGSGET TYDHKNTFTL SCDGSLRHKF LDSNIKFSHV EKLGNNPVSK
1451 GLLIFDASSS WGPQMSASVH LDSKKKQHLF VKEVKIDGQF RVSSFYAKGT
1501 YGLSCQRDPN TGRLNGESNL RFNSSYLQGT NQITGRYEDG TLSLTSTSDL
1551 QSGIIKNTAS LKYENYELTL KSDTNGKYKN FATSNKMDMT FSKQNALLRS
1601 EYQADYESLR FFSLLSGSLN SHGLELNADI LGTDKINSGA HKATLRIGQD
1651 GISTSATTNL KCSLLVLENE LNAELGLSGA SMKLTTNGRF REHNAKFSLD
1701 GKAALTELSL GSAYQAMILG VDSKNIFNFK VSQEGLKLSN DMMGSYAEMK
1751 FDHTNSLNIA GLSLDFSSKL DNIYSSDKFY KQTVNLQLQP YSLVTTLNSD
1801 LKYNALDLTN NGKLRLEPLK LHVAGNLKGA YQNNEIKHIY AISSAALSAS
1851 YKADTVAKVQ GVEFSHRLNT DIAGLASAID MSTNYNSDSL HFSNVFRSVM
1901 APFTMTIDAH TNGNGKLALW GEHTGQLYSK FLLKAEPLAF TFSHDYKGST
1951 SHHLVSRKSI SAALEHKVSA LLTPAEQTGT WKLKTQFNNN EYSQDLDAYN
2001 TKDKIGVELT GRTLADLTLL DSPIKVPLLL SEPINIIDAL EMRDAVEKPQ
2051 EFTIVAFVKY DKNQDVHSIN LPFFETLQEY FERNRQTIIV VLENVQRNLK
2101 HINIDQFVRK YRAALGKLPQ QANDYLNSFN WERQVSHAKE KLTALTKKYR
2151 ITENDIQIAL DDAKINFNEK LSQLQTYMIQ FDQYIKDSYD LHDLKIAIAN
2201 IIDEIIEKLK SLDEHYHIRV NLVKTIHDLH LFIENIDFNK SGSSTASWIQ
2251 NVDTKYQIRI QIQEKLQQLK RHIQNIDIQH LAGKLKQHIE AIDVRVLLDQ
2301 LGTTISFERI NDVLEHVKHF VINLIGDFEV AEKINAFRAK VHELIERYEV
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2351 DQQIQVLMDK LVELAHQYKL KETIQKLSNV LQQVKIKDYF EKLVGFIDDA
2401 VKKLNELSFK TFIEDVNKFL DMLIKKLKSF DYHQFVDETN DKIREVTQRL
2451 NGEIQALELP QKAEALKLFL EETKATVAVY LESLQDTKIT LIINWLQEAL
2501 SSASLAHMKA KFRETLEDTR DRMYQMDIQQ ELQRYLSLVG QVYSTLVTYI
2551 SDWWTLAAKN LTDFAEQYSI QDWAKRMKAL VEQGFTVPEI KTILGTMPAF
2601 EVSLQALQKA TFQTPDFIVP LTDLRIPSVQ INFKDLKNIK IPSRFSTPEF
2651 TILNTFHIPS FTIDFVEMKV KIIRTIDQML NSELQWPVPD IYLRDLKVED
2701 IPLARITLPD FRLPEIAIPE FIIPTLNLND FQVPDLHIPE FQLPHISHTI
2751 EVPTFGKLYS ILKIQSPLFT LDANADIGNG TTSANEAGIA ASITAKGESK
2801 LEVLNFDFQA NAQLSNPKIN PLALKESVKF SSKYLRTEHG SEMLFFGNAI
2851 EGKSNTVASL HTEKNTLELS NGVIVKINNQ LTLDSNTKYF HKLNIPKLDF
2901 SSQADLRNEI KTLLKAGHIA WTSSGKGSWK WACPRFSDEG THESQISFTI
2951 EGPLTSFGLS NKINSKHLRV NQNLVYESGS LNFSKLEIQS QVDSQHVGHS
3001 VLTAKGMALF GEGKAEFTGR HDAHLNGKVI GTLKNSLFFS AQPFEITAST
3051 NNEGNLKVRF PLRLTGKIDF LNNYALFLSP SAQQASWQVS ARFNQYKYNQ
3101 NFSAGNNENI MEAHVGINGE ANLDFLNIPL TIPEMRLPYT IITTPPLKDF
3151 SLWEKTGLKE FLKTTKQSFD LSVKAQYKKN KHRHSITNPL AVLCEFISQS
3201 IKSFDRHFEK NRNNALDFVT KSYNETKIKF DKYKAEKSHD ELPRTFQIPG
3251 YTVPVVNVEV SPFTIEMSAF GYVFPKAVSM PSFSILGSDV RVPSYTLILP
3301 SLELPVLHVP RNLKLSLPDF KELCTISHIF IPAMGNITYD FSFKSSVITL
3351 NTNAELFNQS DIVAHLLSSS SSVIDALQYK LEGTTRLTRK RGLKLATALS
3401 LSNKFVEGSH NSTVSLTTKN MEVSVATTTK AQIPILRMNF KQELNGNTKS
3451 KPTVSSSMEF KYDFNSSMLY STAKGAVDHK LSLESLTSYF SIESSTKGDV
3501 KGSVLSREYS GTIASEANTY LNSKSTRSSV KLQGTSKIDD IWNLEVKENF
3551 AGEATLQRIY SLWEHSTKNH LQLEGLFFTN GEHTSKATLE LSPWQMSALV
3601 QVHASQPSSF HDFPDLGQEV ALNANTKNQK IRWKNEVRIH SGSFQSQVEL
3651 SNDQEKAHLD IAGSLEGHLR FLKNIILPVY DKSLWDFLKL DVTTSIGRRQ
3701 HLRVSTAFVY TKNPNGYSFS IPVKVLADKF IIPGLKLNDL NSVLVMPTFH
3751 VPFTDLQVPS CKLDFREIQI YKKLRTSSFA LNLPTLPEVK FPEVDVLTKY
3801 SQPEDSLIPF FEITVPESQL TVSQFTLPKS VSDGIAALDL NAVANKIADF
3851 ELPTIIVPEQ TIEIPSIKFS VPAGIAIPSF QALTARFEVD SPVYNATWSA
3901 SLKNKADYVE TVLDSTCSST VQFLEYELNV LGTHKIEDGT LASKTKGTFA
3951 HRDFSAEYEE DGKYEGLQEW EGKAHLNIKS PAFTDLHLRY QKDKKGISTS
4001 AASPAVGTVG MDMDEDDDFS KWNFYYSPQS SPDKKLTIFK TELRVRESDE
4051 ETQIKVNWEE EAASGLLTSL KDNVPKATGV LYDYVNKYHW EHTGLTLREV
4101 SSKLRRNLQD HAEWVYQGAI REIDDIDERF QKGASGTTGT YQEWKDKAQN
4151 LYQELLTQEG QASFQGLKDN VFDGLVRVTQ EFHMKVKHLI DSLIDFLNFP
4201 RFQFPGKPGI YTREELCTMF IREVGTVLSQ VYSKVHNGSE ILFSYFQDLV
4251 ITLPFELRKH KLIDVISMYR ELLKDLSKEA QEVFKAIQSL KTTEVLRNLQ
4301 DLLQFIFQLI EDNIKQLKEM KFTYLINYIQ DEINTIFNDY IPYVFKLLKE
4351 NLCLNLHKFN EFIQNELQEA SQELQQIHQY IMALREEYFD PSIVGWTVKY
4401 YELEEKIVSL IKNLLVALKD FHSEYIVSAS NFTSQLSSQV EQFLHRNIQE
4451 YLSILTDPDG KGKEKIAELS ATAQEIIKSQ AIATKKIISD YHQQFRYKLQ
4501 DFSDQLSDYY EKFIAESKRL IDLSIQNYHT FLIYITELLK KLQSTTVMNP
4551 YMKLAPGELT IIL
```

Band 20

Cul-3 (peptides sequence coverage: 5%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
1 - 19	2283.5811	2283.1358	MYLNVIINNTWQGDFSQIK
1 - 19	2283.6210	2283.1358	MYLNVIINNTWQGDFSQIK

```
   1 MYLNVIINNT WQGDFSQIKV FLMTLKKNMI SKLKTECGCQ FTSKLEGMFR
  51 DMSISNTTMD EFRQHLQATG VSLGGVDLTV RVLTTGYWPT QSATPKCNIP
 101 PAPRHAFEIF RRFYLAKHSG RQLTLQHHMG SADLNATFYG PVKKEDGSEV
 151 GVGGAQVTGS NTRKHILQVS TFQMTILMLF NNREKYTFEE IQQETDIPER
 201 ELVRALQSLA CGKPTQRVLT KEPKSKEIEN GHIFTVNDQF TSKLHRVKIQ
 251 TVAAKQGESD PERKETRQKV DDDRKHEIEA AIVRIMKSRK KMQHNVLVAE
```

```
301 VTQQLKARFL PSPVVIKKRI EGLIEREYLA RTPEDRKVYT YVA
```

Band 21

Centromere protein (peptides sequence coverage: 2%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
89 - 99	1288.8854	1290.7116	RKSRSSQLSSRR
655 - 663	1178.7892	1178.2	RHQELLQKKK

```
  1 MGTTAPGPIH LLELCDQKLM EFLCNMDNKD LVWLEEIQEE AERMFTREFS
 51 KEPELMPKTP SQKNRRKKRR ISYVQDENRD PIRRRLSRRK SRSSQLSSRR
101 LRSKDSVEKL ATVVGENGSV LRRVTRAAAA AAAATMALAA PSSPTPESPT
151 MLTKKPEDNH TQCQLVPVVE IGISERQNAE QHVTQLMSTE PLPRTLSPTP
201 ASATAPTSQG IPTSDEESTP KKSKARILES ITVSSLMATP QDPKGQGVGT
251 GRSASKLRIA QVSPGPRDSP AFPDSPWRER VLAPILPDNF STPTGSRTDS
301 QSVRHSPIAP SSPSPQVLAQ KYSLVAKQES VVRRASRRLA KKTAEEPAAS
351 GRIICHSYLE RLLNVEVPQK VGSEQKEPPE EAEPVAAAEP EVPENNGNNS
401 WPHNDTEIAN STPNPKPAAS SPETPSAGQQ EAKTDQADGP REPPQSARRK
451 RSYKQAVSEL DEEQHLEDEE LQPPRSKTPS SPCPASKVVR PLRTFLHTVQ
501 RNQMLMTPTS APRSVMKSFI KRNTPLRMDP KEKERQRLEN LRRKEEAEQL
551 RRQKVEEDKR RRLEEVKLKR EERLRKVLQA RERVEQMKEE KKKQIEQKFA
601 QIDEKTEKAK EERLAEEKAK KKAAAKKMEE VEARRKQEED ARRLRWLQQE
651 EEERRHQELL QKKKEEEQER LRKAAEAKRL AEQREQERRE QERREQERRE
701 QERREQERRE QERREQERQL AEQERRREQE RLQAERELQE REKALRLQKE
751 QLQRELEEKK KKEEQQRLAE RQLQEEQEKK AKEAAGASKA LNVTVDVQSP
801 ACTSSPITPQ GHKAPPQINP HNYGMDLNSD DSTDDEAHPR KPIPTWARGT
851 PLSQAIIHQY YQPPNLLELF GTILPLDLED IFKKSKPRYH KRTSSAVWNS
901 PPLQGARVPS SLAYSLKKH
```

Band 22

Antithrombin III (peptides sequence coverage: 37%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
30 - 39	1233.8305	1231.7189	ANRPFLVFIR
40 - 51	1388.9395	1388.7486	EVPLNTIIFMGR
40 - 51	1404.9284	1404.7435	EVPLNTIIFMGR

```
  1 VNEEGSEAAA STAVVIAGRS LNPNRVTFKA NRPFLVFIRE VPLNTIIFMG
 51 RVANPCVK
```

Band 23

Human serum albumin (peptides sequence coverage: 14%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
35 - 44	1225.6497	1225.5979	FKDLGEENFK
66 - 75	1148.5701	1148.6077	LVNEVTEFAK
76 - 88	1497.5755	1497.5712	TCVADESAENCDK
98 - 105	932.4366	932.5113	LCTVATLR
162 - 168	926.4270	926.4861	YLYEIAR
187 - 198	1370.5437	1370.5595	AAFTECCQAADK
287 - 298	1442.6601	1442.6347	YICENQDSISSK
376 - 383	983.4690	982.5335	TYKTTLEK
427 - 434	959.5574	959.5552	FQNALLVR

```
  1 MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA
 51 FAQYLQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
101 VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA
151 FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
201 CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV ARLSQRFPKA
251 EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
301 ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
351 LGMFLYEYAR RHPDYSVVLL LRLAKTYKTT LEKCCAAADP HECYAKVFDE
401 FKPLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV
451 SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV LNQLCVLHEK TPVSDRVTKC
501 CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
551 TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
601 AASRAALGL
```

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
671 - 680	1044.7118	1045.5291	LSSPADITDK
671 - 680	1044.7395	1045.5291	LSSPADITDK
671 - 680	1045.7699	1045.5291	LSSPADITDK

```
  1 MEHKEVVLLL LLFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
 51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
801 TWIEGVMRNN
```

Band 24

OMM protein (peptides sequence coverage: 31%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
2 - 15	1760.8778	1759.0337	KWLWFFLLLVAAPR
38 - 49	1354.6650	1354.6300	TPLGDTTHTCPR
55 - 64	1185.4410	1185.4907	SCDTPPPCPR
115 - 121	850.4465	850.4218	DTLMISR
122 - 142	2413.1327	2413.1472	TPEVTCVVVDVSHEDPEVQFK
168 - 183	1807.0700	1806.9992	VVSVLTVLHQDWLNGK
193 - 200	837.2574	837.4960	ALPAPIEK
227 - 236	1160.5838	1160.6223	NQVSLTCLVK

```
  1 MKXLWFFLLL VAAPRWVLSQ VHLQESGPGL GKPPELKTPL GDTTHTCPRC
 51 PEPKSCDTPP PCPRCPEPKS CDTPPPCPRC PEPKSCDTPP PCPXCPAPEL
101 LGGPSVFLFP PKPKDTLMIS RTPEVTCVVV DVSHEDPXVQ FKWYVDGVEV
151 HNAKTKLREE QYNSTFRVVS VLTVLHQDWL NGKEYKCKVS NKALPAPIEK
201 TISKAKGQPX XXXXXXXXXX XEEMTKNQVS LTCLVKGFYP SDIAVEWESN
251 GQPENNYNTT PPMLDSDGSF FLYSKLTVDK SRWQQGNIFS CSVMHEALHN
```

```
 301 RYTQKSLSLS PGK
```

Band 25

Apolipoprotein A-IV precursor (peptides sequence coverage: 15%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
66 - 78	1406.6708	1406.7041	LGEVNTYAGDLQK
135 - 143	1103.4649	1103.5611	LEPYADQLR
190 - 200	1299.7480	1299.6783	IDQNVEELKGR
201 - 209	1082.4346	1082.5284	LTPYADEFK
256 - 264	974.4489	974.5032	ISASAEELR
267 - 275	982.5074	982.5447	LAPLAEDVR

```
   1 MFLKAVVLTL ALVAVAGARA EVSADQVATV MWDYFSQLSN NAKEAVEHLQ
  51 KSELTQQLNA LFQDKLGEVN TYAGDLQKKL VPFATELHER LAKDSEKLKE
 101 EIGKELEELR ARLLPHANEV SQKIGDNLRE LQQRLEPYAD QLRTQVNTQA
 151 EQLRRQLTPY AQRMERVLRE NADSLQASLR PHADELKAKI DQNVEELKGR
 201 LTPYADEFKV KIDQTVEELR RSLAPYAQDT QEKLNHQLEG LTFQMKKNAE
 251 ELKARISASA EELRQRLAPL AEDVRGNLRG NTEGLQKSLA ELGGHLDQQV
 301 EEFRRRVEPY GENFNKALVQ QMEQLRTKLG PHAGDVEGHL SFLEKDLRDK
 351 VNSFFSTFKE KESQDKTLSL PELEQQQEQH QEQQQEQVQM LAPLES
```

Complement component C3 (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
1442 - 1450	1091.6496	1091.6226	NTLIIYLDK
1451 - 1462	1407.4333	1406.6136	VSHSEDDCLAFK
1513 - 1522	1297.5111	1297.5431	CAEENCFIQK

```
   1 MGPTSGPSLL LLLLTHLPLA LGSPMYSIIT PNILRLESEE TMVLEAHDAQ
  51 GDVPVTVTVH DFPGKKLVLS SEKTVLTPAT NHMGNVTFTI PANREFKSEK
 101 GRNKFVTVQA TFGTQVVEKV VLVSLQSGYL FIQTDKTIYT PGSTVLYRIF
 151 TVNHKLLPVG RTVMVNIENP EGIPVKQDSL SSQNQLGVLP LSWDIPELVN
 201 MGQWKIRAYY ENSPQQVFST EFEVKEYVLP SFEVIVEPTE KFYYIYNEKG
 251 LEVTITARFL YGKKVEGTAF VIFGIQDGEQ RISLPESLKR IPIEDGSGEV
 301 VLSRKVLLDG VQNLRAEDLV GKSLYVSATV ILHSGSDMVQ AERSGIPIVT
 351 SPYQIHFTKT PKYFKPGMPF DLMVFVTNPD GSPAYRVPVA VQGEDTVQSL
 401 TQGDGVAKLS INTHPSQKPL SITVRTKKQE LSEAEQATRT MQALPYSTVG
 451 NSNNYLHLSV LRTELRPGET LNVNFLLRMD RAHEAKIRYY TYLIMNKGRL
 501 LKAGRQVREP GQDLVVLPLS ITTDFIPSFR LVAYYTLIGA SGQREVVADS
 551 VWVDVKDSCV GSLVVKSGQS EDRQPVPGQQ MTLKIEGDHG ARVVLVAVDK
 601 GVFVLNKKNK LTQSKIWDVV EKADIGCTPG SGKDYAGVFS DAGLTFTSSS
 651 GQQTAQRAEL QCPQPAARRR RSVQLTEKRM DKVGKYPKEL RKCCEDGMRE
 701 NPMRFSCQRR TRFISLGEAC KKVFLDCCNY ITELRRQHAR ASHLGLARSN
 751 LDEDIIAEEN IVSRSEFPES WLWNVEDLKE PPKNGISTKL MNIFLKDSIT
 801 TWEILAVSMS DKKGICVADP FEVTVMQDFF IDLRLPYSVV RNEQVEIRAV
 851 LYNYRQNQEL KVRVELLHNP AFCSLATTKR RHQQTVTIPP KSSLSVPYVI
 901 VPLKTGLQEV EVKAAVYHHF ISDGVRKSLK VVPEGIRMNK TVAVRTLDPE
 951 RLGREGVQKE DIPPADLSDQ VPDTESETRI LLQGTPVAQM TEDAVDAERL
1001 KHLIVTPSGC GEQNMIGMTP TVIAVHYLDE TEQWEKFGLE KRQGALELIK
1051 KGYTQQLAFR QPSSAFAAFV KRAPSTWLTA YVVKVFSLAV NLIAIDSQVL
1101 CGAVKWLILE KQKPDGVFQE DAPVIHQEMI GGLRNNNEKD MALTAFVLIS
1151 LQEAKDICEE QVNSLPGSIT KAGDFLEANY MNLQRSYTVA IAGYALAQMG
1201 RLKGPLLNKF LTTAKDKNRW EDPGKQLYNV EATSYALLAL LQLKDFDFVP
1251 PVVRWLNEQR YYGGGYGSTQ ATFMVFQALA QYQKDAPDHQ ELNLDVSLQL
1301 PSRSSKITHR IHWESASLLR SEETKENEGF TVTAEGKGQG TLSVVTMYHA
```

```
1351 KAKDQLTCNK FDLKVTIKPA PETEKRPQDA KNTMILEICT RYRGDQDATM
1401 SILDISMMTG FAPDTDDLKQ LANGVDRYIS KYELDKAFSD RNTLIIYLDK
1451 VSHSEDDCLA FKVHQYFNVE LIQPGAVKVY AYYNLEESCT RFYHPEKEDG
1501 KLNKLCRDEL CRCAEENCFI QKSDDKVTLE ERLDKACEPG VDYVYKTRLV
1551 KVQLSNDFDE YIMAIEQTIK SGSDEVQVGQ QRTFISPIKC REALKLEEKK
1601 HYLMWGLSSD FWGEKPNLSY IIGKDTWVEH WPEEDECQDE ENQKQCQDLG
1651 AFTESMVVFG CPN
```

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
671 - 680	1044.7206	1045.5291	LSSPADITDK
671 - 680	1045.7707	1045.5291	LSSPADITDK

```
   1 MEHKEVVLLL LFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
  51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
 101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
 151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
 201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
 251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
 301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
 351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
 401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
 451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
 501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
 551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
 601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
 651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
 701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
 751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
 801 TWIEGVMRNN
```

Band 26

Apolipoprotein J precursor (peptides sequence coverage: 15%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
12 - 21	1070.5769	1070.5720	EIQNAVNGVK
12 - 21	1071.5581	1070.5720	EIQNAVNGVK
36 - 45	1116.6153	1116.6026	TLLSNLEEAK
126 - 134	1073.5374	1073.5353	IDSLLENDR
274 - 289	1761.8981	1761.8203	EILSVDCSTNNPSQAK
293 - 303	1287.6126	1287.6306	ELDESLQVAER
397 - 404	969.4374	969.4477	FMETVAEK

```
   1 MSNQGSKYVN KEIQNAVNGV KQIKTLIEKT NEERKTLLSN LEEAKKKKED
  51 ALNETRESET KLKELPGVCN ETMMALWEEC KPCLKQTCMK FYARVCRSGS
 101 GLVGRQLEEF LNQSSPFYFW MNGDRIDSLL ENDRQQTHML DVMQDHFSRA
 151 SSIIDELFQD RFFTREPQDT YHYLPFSLPH RRPHFFFPKS RIVRSLMPFS
 201 PYEPLNFHAM FQPFLEMIHE AQQAMDIHFH SPAFQHPPTE FIREGDDDRT
 251 VCREIRHNST GCLRMKDQCD KCREILSVDC STNNPSQAKL RRELDESLQV
 301 AERLTRKYNE LLKSYQWKML NTSSLLEQLN EQFNWVSRLA NLTQGEDQYY
 351 LRVTTVASHT SDSDVPSGVT EVVVKLFDSD PITVTVPVEV SRKNPKFMET
 401 VAEKALQEYR KKHREE
```

Apolipoprotein E (peptides sequence coverage: 37%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
34 - 43	1246.4827	1246.5691	QQTEWQSGQR
44 - 50	843.4240	843.4603	WELALGR
122 - 132	1237.4355	1237.5067	LGADMEDVCGR
177 - 185	947.4739	947.5188	LAVYQAGAR
199 - 207	967.5246	967.5451	LGPLVEQGR
210 - 224	1496.8324	1496.7947	AATVGSLAGQPLQER
247 - 258	1413.8016	1413.7463	LDEVKEQVAEVR
259 - 269	1312.6800	1312.7099	AKLEEQAQQIR
270 - 278	1032.5277	1032.5352	LQAEAFQAR
293 - 300	929.5063	929.4971	QWAGLVEK
301 - 317	540.9309	1619.7709	VQAAVGTSAAPVPSDNH
301 - 317	810.9163	1619.8180	VQAAVGTSAAPVPSDNH

```
  1 MKVLWAALLV TFLAGCQAKV EQAVETEPEP ELRQQTEWQS GQRWELALGR
 51 FWDYLRWVQT LSEQVQEELL SSQVTQELRA LMDETMKELK AYKSELEEQL
101 TPVAEETRAR LSKELQAAQA RLGADMEDVC GRLVQYRGEV QAMLGQSTEE
151 LRVRLASHLR KLRKRLLRDA DDLQKRLAVY QAGAREGAER GLSAIRERLG
201 PLVEQGRVRA ATVGSLAGQP LQERAQAWGE RLRARMEEMG SRTRDRLDEV
251 KEQVAEVRAK LEEQAQQIRL QAEAFQARLK SWFEPLVEDM QRQWAGLVEK
301 VQAAVGTSAA PVPSDNH
```

Band 27

Apolipoprotein E3 (peptides sequence coverage: 31%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
1 - 15	1752.8556	1752.8894	KVEQAVETEPEPELR
16 - 25	1246.4827	1246.5691	QQTEWQSGQR
26 - 32	843.4240	843.4603	WELALGR
104 - 114	1237.4355	1237.5067	LGADMEDVCGR
159 - 167	947.4739	947.5188	LAVYQAGAR
181 - 189	967.5246	967.5451	LGPLVEQGR

```
  1 KVEQAVETEP EPELRQQTEW QSGQRWELAL GRFWDYLRWV QTLSEQVQEE
 51 LLSSQVTQEL RALMDETMKE LKAYKSELEE QLTPVAEETR ARLSKELQAA
101 QARLGADMED VCGRLVQYRG EVQAMLGQST EELRVRLASH LRKLRQRLLR
151 DADDLQKRLA VYQAGAREGA ERGLSAIRER LGPLVEQGRV R
```

Band 28

Pre-serum amyloid P component (peptides sequence coverage: 31%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
58 - 64	810.3801	810.3872	AYSDLSR
65 - 76	1405.7002	1405.6626	AYSLFSYNTQGR
77 - 84	992.5535	992.5178	DNELLVYK
87 - 96	1155.5954	1155.5924	VGEYSLYIGR
140 - 149	1165.6847	1165.5768	QGYFVEAQPK
150 - 162	1392.7068	1392.6885	IVLGQEQDSYGGK
213 - 223	1285.7426	1285.7798	GYVIIKPLVWV

```
  1 MNKPLLWISV LTSLLEAFAH TDLSGKVFVF PRESVTDHVN LITPLEKPLQ
```

```
  51 NFTLCFRAYS DLSRAYSLFS YNTQGRDNEL LVYKERVGEY SLYIGRHKVT
 101 SKVIEKFPAP VHICVSWESS SGIAEFWING TPLVKKGLRQ GYFVEAQPKI
 151 VLGQEQDSYG GKFDRSQSFV GEIGDLYMWD SVLPPENILS AYQGTPLPAN
 201 ILDWQALNYE IRGYVIIKPL VWV
```

Band 29

Pro-apolipoprotein (peptides sequence coverage: 9%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
52 - 65	1611.9110	1611.7781	LLDNWDSVTSTFSK
103 - 112	1251.4712	1251.6136	VQPYLDDFQK.K

```
   1 RHFWQQDEPP QSPWDRVKDL ATVYVDVLKD SGRDYVSQFE GSALGKQLNL
  51 KLLDNWDSVT STFSKLREQL GPVTQEFWDN LEKETEGLRQ EMSKDLEEVK
 101 AKVQPYLDDF QKKWQEEMEL YRQKVEPLRA ELQEGARQKL HELQEKLSPL
 151 GEEMRDRARA HVDALRTHLA PYSDELRQRL AARLEALKEN GGARLAEYHA
 201 KATEHLSTLS EKAKPALEDL RQGLLPVLES FKVSFLSALE EYTKKLNTQ
```

Mr (expt) is the experimentally observed mass (Da)

Mr (calc) is the calculated Mass (Da)

Matched peptides shown in *bold red*

- **Scheme 4**

Band 30

Antithrombin (peptides sequence coverage: 12%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
147 - 157	1339.6291	1339.6561	TSDQIHFFFAK
202 - 208	859.3772	859.4803	LQPLDFK
202 - 215	1673.8707	1673.8373	LQPLDFKENAEQSR
261 - 267	849.4923	849.3981	FSPENTR
355 - 364	1210.4789	1210.6346	FRIEDGFSLK
365 - 380	1847.9332	1847.8975	EQLQDMGLVDLFSPEK

```
  1 MYSNVIGTVT SGKRKVYLLS LLLIGFWDCV TCHGSPVDIC TAKPRDIPMN
 51 PMCIYRSPEK KATEDEGSEQ KIPEATNRRV WELSKANSRF ATTFYQHLAD
101 SKNDNDNIFL SPLSISTAFA MTKLGACNDT LQQLMEVFKF DTISEKTSDQ
151 IHFFFAKLNC RLYRKANKSS KLVSANRLFG DKSLTFNETY QDISELVYGA
201 KLQPLDFKEN AEQSRAAINK WVSNKTEGRI TDVIPSEAIN ELTVLVLVNT
251 IYFKGLWKSK FSPENTRKEL FYKADGESCS ASMMYQEGKF RYRRVAEGTQ
301 VLELPFKGDD ITMVLILPKP EKSLAKVEKE LTPEVLQEWL DELEEMMLVV
351 HMPRFRIEDG FSLKEQLQDM GLVDLFSPEK SKLPGIVAEG RDDLYVSDAF
401 HKAFLEVNEE GSEAAASTAV VIAGRSLNPN RVTFKANRLF LVFIREVPLN
451 TIIFMGRVAN PCVK
```

Mr (expt) is the experimentally observed mass (Da)

Mr (calc) is the calculated mass (Da)

Matched peptides shown in *bold red*

- **Scheme 5**

Band 31

Immunoglobin heavy chain variable region (peptides sequence coverage: 19%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
89 - 101	1302.5115	1302.6092	FSGSGSGTDFTLK
178 - 197	2136.0027	2134.9614	VDNALQSGNSQESVTEQDSK
219 - 235	1888.9583	1888.9353	LYACEVTHQGLSSPVTK
219 - 235	1889.0164	1888.9353	LYACEVTHQGLSSPVTK

```
  1 MKYLLPTAAA GLLLLAAQPA MADVVMTQSP LSLPVTLGQP ASISCRSSQS
 51 LVYSDGNTYL NWFQQRPGQS PRRLIYKVSN RDSGVPDRFS GSGSGTDFTL
101 KISRVEAEDV GVYYCMQGTH WPPGTFGQGT KVEIKRTVAA PSVFIFPPSD
151 EQLKSGTASV VCLLNNFYPR EAKVQWKVDN ALQSGNSQES VTEQDSKDST
201 YSLSSTLTLS KADYEKHKLY ACEVTHQGLS SPVTKSFNRG ECSARQSTPF
251 VCEYQGQSSD LP
```

Band 32

Human serum albumin (peptides sequence coverage: 15%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
11 - 20	1225.6497	1225.5979	FKDLGEENFK
42 - 51	1148.5701	1148.6077	LVNEVTEFAK
52 - 64	1497.5755	1497.5712	TCVADESAENCDK
74 - 81	932.4366	932.5113	LCTVATLR
138 - 144	926.4270	926.4861	YLYEIAR
163 - 174	1370.5437	1370.5595	AAFTECCQAADK
263 - 274	1442.6601	1442.6347	YICENQDSISSK
352 - 359	983.4690	983.4811	TYETTLEK
403 - 410	959.5574	959.5552	FQNALLVR

```
  1 DAHKSEVAHR FKDLGEENFK ALVLIAFAQY LQQCPFEDHV KLVNEVTEFA
 51 KTCVADESAE NCDKSLHTLF GDKLCTVATL RETYGEMADC CAKQEPERNE
101 CFLQHKDDNP NLPRLVRPEV DVMCTAFHDN EETFLKKYLY EIARRHPYFY
151 APELLFFAKR YKAAFTECCQ AADKAACLLP KLDELRDEGK ASSAKQRLKC
201 ASLQKFGERA FKAWAVARLS QRFPKAEFAE VSKLVTDLTK VHTECCHGDL
251 LECADDRADL AKYICENQDS ISSKLKECCE KPLLEKSHCI AEVENDEMPA
301 DLPSLAADFV ESKDVCKNYA EAKDVFLGMF LYEYARRHPD YSVVLLLRLA
351 KTYETTLEKC CAAADPHECY AKVFDEFKPL VEEPQNLIKQ NCELFEQLGE
401 YKFQNALLVR YTKKVPQVST PTLVEVSRNL GKVGSKCCKH PEAKRMPCAE
451 DYLSVVLNQL CVLHEKTPVS DRVTKCCTES LVNRRPCFSA LEVDETYVPK
501 EFNAETFTFH ADICTLSEKE EQIKKQTALV ELVKHKPKAT KEQLKAVMDD
551 FAAFVEKCCK ADDKETCFAE EGKKLVAASQ AALGL
```

Complement component 4 binding protein (peptides sequence coverage: 10%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
79 - 87	1127.5913	1127.5434	YTCLPGYVR
319 - 329	1249.6956	1248.6714	EDVYVVGTVLR
405 - 418	1580.7146	1580.7042	FSAICQGDGTWSPR
555 - 566	1442.6960	1442.6898	LMQCLPNPEDVK
567 - 573	868.2813	868.4364	MALEVYK

574 - 585	1469.8102	1469.8089	LSLEIEQLELQR

```
  1 MHPPKTPSGA LHRKRKMAAW PFSRLWKVSD PILFQMTLIA ALLPAVLGNC
 51 GPPPTLSFAA PMDITLTETR FKTGTTLKYT CLPGYVRSHS TQTLTCNSDG
101 EWVYNTFCIY KRCRHPGELR NGQVEIKTDL SFGSQIEFSC SEGFFLIGST
151 TSRCEVQDRG VGWSHPLPQC EIVKCKPPPD IRNGRHSGEE NFYAYGFSVT
201 YSCDPRFSLL GHASISCTVE NETIGVWRPS PPTCEKITCR KPDVSHGEMV
251 SGFGPIYNYK DTIVFKCQKG FVLRGSSVIH CDADSKWNPS PPACEPNSCI
301 NLPDIPHASW ETYPRPTKED VYVVGTVLRY RCHPGYKPTT DEPTTVICQK
351 NLRWTPYQGC EALCCPEPKL NNGEITQHRK SRPANHCVYF YGDEISFSCH
401 ETSRFSAICQ GDGTWSPRTP SCGDICNFPP KIAHGHYKQS SSYSFFKEEI
451 IYECDKGYIL VGQAKLSCSY SHWSAPAPQC KALCRKPELV NGRLSVDKDQ
501 YVEPENVTIQ CDSGYGVVGP QSITCSGNRT WYPEVPKCEW ETPEGCEQVL
551 TGKRLMQCLP NPEDVKMALE VYKLSLEIEQ LELQRDSARQ STLDKEL
```

Human complement component C1r (peptides sequence coverage: 4%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
647 - 661	1565.8133	1565.6893	QDACQGDSGGVFAVR
683 - 689	834.3402	834.3912	GYGFYTK
690 - 698	1148.5606	1148.6230	VLNYVDWIK

```
  1 MWLLYLLVPA LFCRAGGSIP IPQKLFGEVT SPLFPKPYPN NFETTTVITV
 51 PTGYRVKLVF QQFDLEPSEG CFYDYVKISA DKKSLGRFCG QLGSPLGNPP
101 GKKEFMSQGN KMLLTFHTDF SNEEDGTIMF YKGFLAYYQA VDLDECASRS
151 KLGEEDPQPQ CQHLCHNYVG GYFCSCRPGY ELQEDRHSCQ AECSSELYTE
201 ASGYISSLEY PRSYPPDLRC NYSIRVERGL TLHLKFLEPF DIDDHQQVHC
251 PYDQLQIYAN RKNIGEFCGK QRPPDLDTSS NAVDLLFFTD ESGDSRGWKL
301 RYTTEIIKCP QPKTLDEFTI IQNLQPQYQF RDYFIATCKQ GYQLIEGNQV
351 LHSFTAVCQD DGTWHRAMPR CKIKDCGQPR NLPNGDFRYT TTMGVNTYKA
401 RIQYYCHEPY YKMQTRAGSR ESEQGVYTCT AQGIWKNEQK GEKIPRCLPV
451 CGKPVNPVEQ RQRIIGGQKA KMGNFPWQVF TNIHGRGGGA LLGDRWILTA
501 AHTLYPKEHE AQSNASLDVF LGHTNVEELM KLGNHPIRRV SVHPDYRQDE
551 SYNFEGDIAL LELENSVTLG PNLLPICLPD NDTFYDLGLM GYVSGFGVME
601 EKIAHDLRFV RLPVANPQAC ENWLRGKNRM DVFSQNMFCA GHPSLKQDAC
651 QGDSGGVFAV RDPNTDRWVA TGIVSWGIGC SRGYGFYTKV LNYVDWIKKE
701 MEEED
```

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
671 - 680	1045.5415	1045.5291	LSSPADITDK

```
  1 MEHKEVVLLL LLFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
 51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
```

```
751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
801 TWIEGVMRNN
```

Band 33

Keratin (peptides sequence coverage: 11%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
186 - 197	1382.7050	1382.6830	SLNNQFASFIDK
200 - 211	1474.7266	1474.7780	FLEQQNQVLQTK
278 - 289	1392.6570	1392.7249	TNAENEFVTIKK
344 - 355	1301.6667	1301.7078	SLDLDSIIAEVK
356 - 364	1064.4168	1064.5138	AQYEDIAQK
396 - 403	972.4752	972.5240	IEISELNR
484 - 492	1032.4232	1032.5087	TLLEGEESR

```
  1 MSRQFSSRSG YRSGGGFSSG SAGIINYQRR TTSSSTRRSG GGGGRFSSCG
 51 GGGGSFGAGG GFGSRSLVNL GGSKSISISV ARGGGRGSGF GGGYGGGGFG
101 GGGFGGGGFG GGGIGGGGFG GFGSGGGGFG GGGFGGGGYG GGYGPVCPPG
151 GIQEVTINQS LLQPLNVEID PEIQKVKSRE REQIKSLNNQ FASFIDKVRF
201 LEQQNQVLQT KWELLQQVDT STRTHNLEPY FESFINNLRR RVDQLKSDQS
251 RLDSELKNMQ DMVEDYRNKY EDEINKRTNA ENEFVTIKKD VDGAYMTKVD
301 LQAKLDNLQQ EIDFLTALYQ AELSQMQTQI SETNVILSMD NNRSLDLDSI
351 IAEVKAQYED IAQKSKAEAE SLYQSKYEEL QITAGRHGDS VRNSKIEISE
401 LNRVIQRLRS EIDNVKKQIS NLQQSISDAE QRGENALKDA KNKLNDLEDA
451 LQQAKEDLAR LLRDYQELMN TKLALDLEIA TYRTLLEGEE SRMSGECAPN
501 VSVSVSTSHT TISGGGSRGG GGGGYGSGGS SYGSGGGSYG SGGGGGGGRG
551 SYGSGGSSYG SGGGSYGSGG GGGGHGSYGS GSSSGGYRGG SGGGGGGSSG
601 GRGSGGGSSG GSIGGRGSSS GGVKSSGGSS SVRFVSTTYS GVTR
```

Hypothetical protein, gi|51476390 (peptides sequence coverage: 14%)

Start - End	Mr(expt)	Mr(calc)	Peptide Sequences
35 - 44	1225.6497	1225.5979	FKDLGEENFK
66 - 75	1148.5701	1148.6077	LVNEVTEFAK
76 - 88	1497.5755	1497.5712	TCVADESAENCDK
98 - 105	932.4366	932.5113	LCTVATLR
162 - 168	926.4270	926.4861	YLYEIAR
187 - 198	1370.5437	1370.5595	AAFTECCQAADK
287 - 298	1442.6601	1442.6347	YICENQDSISSK
376 - 383	983.4690	983.4811	TYETTLEK
427 - 434	959.5574	959.5552	FQNALLVR

```
  1 MKWVTFISLL FLFSSAYSRG VFRRDAYKSE VAHRFKDLGE ENFKALVLIA
 51 FAQYPQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
101 VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA
151 FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
201 CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV ARLSQRFPKA
251 EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
301 ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
351 LGMFLYEYAR RHPDYSVVLL LRLAKTYETT LEKCCAAADP HECYAKVFDE
401 FKPLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV
451 SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV LNQLCVLHER TPVSDRVTKC
501 CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
551 AALVELVKHR PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
601 AASQAALGL
```

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
671 - 680	1045.4914	1045.5291	LSSPADITDK

```
  1 MEHKEVVLLL LLFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
 51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
801 TWIEGVMRNN
```

Band 34

Keratin (peptides sequence coverage: 4%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
189 - 195	826.3674	826.4225	FASFIDK
198 - 209	1474.7266	1474.7780	FLEQQNQVLQTK
394 - 401	972.4752	972.5240	IEISELNR

```
  1 MSCQISCKSR GRGGGGGGFR GFSSGSAVVS GGSRRSTSSF SCLSRHGGGG
 51 GGFGGGGFGS RSLVGLGGTK SISISVAGGG GGFGAAGGFG GRGGGFGGGS
101 SFGGGSGFSG GGFGGGGFGG GRFGGFGGPG GVGGLGGPGG FGPGGYPGGI
151 HEVSVNQSLL QPLNVKVDPE IQNVKAQERE QIKTLNNKFA SFIDKVRFLE
201 QQNQVLQTKW ELLQQMNVGT RPINLEPIFQ GYIDSLKRYL DGLTAERTSQ
251 NSELNNMQDL VEDYKKKYED EINKRTAAEN DFVTLKKDVD NAYMIKVELQ
301 SKVDLLNQEI EFLKVLYDAE ISQIHQSVTD TNVILSMDNS RNLDLDSIIA
351 EVKAQYEEIA QRSKEEAEAL YHSKYEELQV TVGRHGDSLK EIKIEISELN
401 RVIQRLQGEI AHVKKQCKNV QDAIADAEQR GEHALKDARN KLNDLEEALQ
451 QAKEDLARLL RDYQELMNVK LALDVEIATY RKLLEGEECR MSGDLSSNVT
501 VSVTSSTISS NVASKAAFGG SGGRGSSSGG GYSSGSSSYG SGGRQSGSRG
551 GSGGGGSISG GGYGSGGGSG GRYGSGGGSK GGSISGGGYG SGGGKHSSGG
601 GSRGGSSSGG GYGSGGGGSS SVKGSSGEAF GSSVTFSFR
```

Apolipoprotein D (peptides sequence coverage: 6%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
200 - 208	971.6407	971.4923	SPELQAEAK.S
216 - 223	942.1231	940.5593	EQLTPLIK.K

```
  1 QAFHLGKCPN PPVQENFDVN KYLGRWYEIE KIPTTFENGR CIQANYSLME
 51 NGKIKVLNQE LRADGTVNQI EGEATPVNLT EPAKLEVKFS WFMPSAPYWI
101 LATDYENYAL VYSCTCIIQL FHVDFAWILA RNPNLPPETV DSLKNILTSN
151 NIDVKKMTVT DQVNCPKLSQ AKEPCVESLV SQYFQTVTDY GKDLMEKVKS
201 PELQAEAKSY FEKSKEQLTP LIKKAGTELV NFLSYFVELG TQPATQ
```

Band 35

T cell receptor beta chain variable region (sequence coverage: 22%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
1 - 13	1588.2089	1585.8868	LGLRLIYFSYDVK
1 - 17	2117.6119	2118.1547	LGLRLIYFSYDVKMKEK

```
   1 LGLRLIYFSY DVKMKEKGDI PEGYSVSREK KERFSLILES ASTNQTSMYL
  51 CASRDSPSYE QYFGPGTRLT VTED
```

Sialic acid-binding Ig-like lectin 10 (peptides sequence coverage: 5%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
2 - 20	1940.2600	1940.0765	LLPLLLSSLLGGSQAMDGR
527 - 540	1393.0823	1394.7405	TPLSPGAPSPESKK.N

```
   1 MLLPLLLSSL LGGSQAMDGR FWIRVQESVM VPEGLCISVP CSFSYPRQDW
  51 TGSTPAYGYW FKAVTETTKG APVATNHQSR EVEMSTRGRF QLTGDPAKGN
 101 CSLVIRDAQM QDESQYFFRV ERGSYVRYNF MNDGFFLKVT ALTQKPDVYI
 151 PETLEPGQPV TVICVFNWAF EECPPPSFSW TGAALSSQGT KPTTSHFSVL
 201 SFTPRPQDHN TDLTCHVDFS RKGVSVQRTV RLRVAYAPRD LVISISRDNT
 251 PALEPQPQGN VPYLEAQKGQ FLRLLCAADS QPPATLSWVL QNRVLSSSHP
 301 WGPRPLGLEL PGVKAGDSGR YTCRAENRLG SQQRALDLSV QYPSENLRVM
 351 VSQANRTVLE NLGNGTSLPV LEGQSLCLVC VTHSSPPARL SWTQRGQVLS
 401 PSQPSDPGVL ELPRVQVEHE GEFTCHARHP LGSQHVSLSL SVHYKKGLIS
 451 TAFSNGAFLG IGITALLFLC LALIIMKILP KRRTQTETPR PKFSRHSTIL
 501 DYINVVPTAG PLAQKRNQKA TPNSPRTPLS PGAPSPESKK NQKKQYQLPS
 551 FPEPKSSTQA PESQESQEEL HYATLNFPGV RPRPEARMPK GTQADYAEVK
 601 FQ
```

M4 protein (peptides sequence coverage: 4%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
126 - 133	869.7147	871.5127	KAAEVLNK
322 - 344	2210.3549	2209.0330	GIGMGNIGPAGMGMEGIGFGINK

```
   1 MAAGVEAAAE VAATEIKMEE ESGGPACERQ RGSGPKGEGE RPAQNEKRKV
  51 KNIKRGGNRF EPYANPTKRY RAFITNIPFD VKWQSLKDLV KEKVGEVTYV
 101 ELLMDAEGKS RGCAVVEFKM EESMKKAAEV LNKHSLSGRP LKVKEDPDGE
 151 HARRAMQKVM ATTGGMGMGP GGPGMITIPP SILNNPNIPN EIIHALQAGR
 201 LGSTVFVANL DYKVGWKKLK EVFSMAGVVV RADILEDKDG KSRGIGTVTF
 251 EQSIEAVQAI SMFNGQLLFD RPMHVKMDER ALPKGDFFPP ERPQQLPHGL
 301 GGIGMGLGPG GQPIDANHLN KGIGMGNIGP AGMGMEGIGF GINKMGGMEG
 351 PFGGGMENMG RFGSGMNMGR INEILSNALK RGEIIAKQGG GGGGGSVPGI
 401 ERMGPGIDRL GGAGMERMGA GLGHGMDRVG SEIERMGLVM DRMGSVERMG
 451 SGIERMGPLG LDHMASSIER MGQTMERIGS GVERMGAGMG FGLERMAAPI
 501 DRVGQTIERM GSGVERMGPA IERMGPSMER MVPAGMGAGL ERMGPVMDRM
 551 ATGLERMGAN NLERMGLERM GANSLERMGL ERMGANSLER MGPAMGPALG
 601 AGIERMGLAM GGGGGASFDR AIEMERGNFG GSFAGSFGGA GGHAPGVARK
 651 ACQIFVRNLP FDFTWKMLKD KFNECGHVLY ADIKMENGKS KGCGVVKFES
 701 PEVAERACRM MNGMKLSGRE IDVRIDRNA
```

Band 36

Lipoprotein CIII (peptides sequence coverage: 20%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
25 - 40	1716.1230	1715.8438	DALSSVQESQVAQQAR
25 - 40	1717.6135	1715.8438	DALSSVQESQVAQQAR

```
  1 SEAEDASLLS FMQGYMKHAT KTAKDALSSV QESQVAQQAR GWVTDGFSSL
 51 KDYWSTVKDK FSEFWDLDPE VRPTSAVAA
```

Band 37

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
671 - 680	1045.4911	1045.5291	LSSPADITDK

```
  1 MEHKEVVLLL LLFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
 51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
801 TWIEGVMRNN
```

Mr (expt) is the experimentally observed mass (Da)

Mr (calc) is the calculated mass (Da)

Matched peptides shown in *bold red*

- **Scheme 6**

Spot 38

Hypothetical protein, gi|34365470 (peptides sequence coverage: 4%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
1 - 8	890.4628	889.5055	ALVIMATR
191 - 209	2357.6909	2358.1300	EDKIEAEAEVELYYMILER
427 - 439	1559.8117	1559.8130	LLGLYHTMDKNQK

```
  1 ALVIMATRGH VQDPNDRRLR PIYDYLDNGN NKMAIQQADK LLKKHKDLHC
 51 AKVLKAIGLQ RTGKQEEAFT LAQEVAALEP TDDNSLQALT ILYREMHRPE
101 LVTKLYEAAV KKVPNSEEYH SHLFMAYARV GEYKKMQQAG MALYKIVPKN
151 PYYFWSVMSL IMQSISAQDE NLSKTMFLPL AERMVEKMVK EDKIEAEAEV
201 ELYYMILERL GKYQEALDVI RGKLGEKLTS EIQSRENKCM AMYKKLSRWP
251 ECNALSRRLL LKNSDDWQFY LTYFDSVFRL IEEAWSPPAE GEHSLEGEVH
301 YSAEKAVKFI EDRITEESKS SRHLRGPHLA KLELIRRLRS QGCNDEYKLG
351 DPEELMFQYF KKFGDKPCCF TDLKVFVDLL PATQCTKFIN QLLGVVPLST
401 PTEDKLALPA DIRALQQHLC VVQLTRLLGL YHTMDKNQKL SVVRELMLRY
451 QHGLEFGKTC LKTELQFSDY YCLLAVHALI DVWRETGDET TVWQALTLLE
501 EGLTHSPSNA QFKLLLVRIY CMLGAFEPVV DLYSSLDAKH IQHDTIGYLL
551 TRYAESLGQY AAASQSCNFA LRFFHSNQKD TSEYIIQAYK YGAFEKIPEF
601 IAFRNRLNNS LHFAQVRTER MLLDLLLEAN ISTSLAESIK SMNLRPEEDD
651 IPWEDLRDNR DLNVFFSWDP KDRDVSEEHK KLSLEEETLW LRIRSLTLRL
701 ISGLPSLNHP VEPKNSEKTA ENGVSSRIDI LRLLLQQLEA TLETGKRFIE
751 KDIQYPFLGP VPTRMGGFFN SGCSQCQISS FYLVNDIYEL DTSGLEDTME
801 IQERIENSFK SLLDQLKDVF GKCKGDLLEV KDGNLKTHPT LLENLVFFVE
851 VSFCSLPKRH CCS
```

Spot 39

Polycystwin (peptides sequence coverage: 2%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
468 - 484	1856.1893	1855.9316	GSISSGVSYEEFQVLVR
468 - 484	1856.2653	1855.9316	GSISSGVSYEEFQVLVR

```
  1 RNGTAWIYTS EKDLNGSSHW GIIATYSGAG YYLDLSRTRE ETAAQVASLK
 51 KNVWLDRGTR ATFIDFSVYN ANINLFCVVR LLVEFPATVG VIPSWQFQPL
101 KLIRYVTTFD FFLAACEIIF CFFIFYYVVE EILEIRIHKL HYFRSFWNCL
151 DVVIVVLSVV AIGINIYRTS NVEVLLQFLE DQNTFPNFEH LAYWQIQFNN
201 IAAVTVFFVW IKLFKFINFN RTMSQLSTTM SRCAKDLFGF AIMFFIIFLA
251 YAQLAYLVFG TQVDDFSTFQ ECIFTQFRII LGDINFAEIE EANRVLGPIY
301 FTTFVFFMFF ILLNMFLAII NDTYSEVKSD LAQQKAEMEL SDLIRKGYHK
351 ALVKLKLKKN TVDDISESLR QGGGKLNFDE LRQDLKGKGH TDAEIEAIFT
401 KYDQDGDQEL TEHEHQQMRD DLEKEREDLD LDHSSLPRPM SSRSFPRSLD
451 DSEEDDDEDS GHSSRRRGSI SSGVSYEEFQ VLVRRVDRME HSIGSIVSKI
501 DAVIVKLEIM ERAKLKRREV LGRLLDGVAE DERLGRDSEI HREQMERLVR
551 EELERWESDD AASQISHGLG TPVGLNGQPR PRSSRPSSSQ STEGMEGAGG
601 NGSSNVHV
```

Carboxypeptidase D (peptides sequence coverage: 3%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
36 - 57	2212.4381	2212.0244	KAEATTTTTSAGAEAAEGQFDR
886 - 907	2298.6723	2298.0863	GASSSTNDASDPTTKEFETLIK

```
   1 MASGRDERPH WRLGRLLLLM CLLLLGSSAR AAHIKKAEAT TTTTSAGAEA
  51 AEGQFDRYYH EEELESALRE AAAAGLPGLA RLFSIGRSVE GRPLWVLRLT
 101 AGLGSLIPEG DAGPDAAGPD AAGPLLPGRP QVKLVGNMHG DETVSRQVLI
 151 YLARELAAGY RRGDPRLVRL LNTTDVYLLP SLNPDGFERA REGDCGFGDG
 201 GPSGASGRDN SRGRDLNRSF PDQFSTGEPP ALDEVPEVRA LIEWIRRNKF
 251 VLSGNLHGGS VVASYPFDDS PEHKATGIYS KTSDDEVFKY LAKAYASNHP
 301 IMKTGEPHCP GDEDETFKDG ITNGAHWYDV EGGMQDYNYV WANCFEITLE
 351 LSCCKYPPAS QLRQEWENNR ESLITLIEKV HIGVKGFVKD SITGSGLENA
 401 TISVAGINHN ITTGRFGDFY RLLVPGTYNL TVVLTGYMPL TVTNVVVKEG
 451 PATEVDFSLR PTVTSVIPDT TEAVSTASTV AIPNILSGTS SSYQPIQPKD
 501 FHHHHFPDME IFLRRFANEY PNITRLYSLG KSVESRELYV MEISDNPGVH
 551 EPGEPEFKYI GNMHGNEVVG RELLLNLIEY LCKNFGTDPE VTDLVHNTRI
 601 HLMPSMNPDG YEKSQEGDSI SVIGRNNSNN FDLNRNFPDQ FVQITDPTQP
 651 ETIAVMSWMK SYPFVLSANL HGGSLVVNYP FDDDEQGLAT YSKSPDDAVF
 701 QQIALSYSKE NSQMFQGRPC KNMYPNEYFP HGITNGASWY NVPGGMQDWN
 751 YLQTNCFEVT IELGCVKYPL EKELPNFWEQ NRRSLIQFMK QVHQGVRGFV
 801 LDATDGRGIL NATISVAEIN HPVTTYKTGD YWRLLVPGTY KITASARGYN
 851 PVTKNVTVKS EGAIQVNFTL VRSSTDSNNE SKKGKGASSS TNDASDPTTK
 901 EFETLIKDLS AENGLESLML RSSSNLALAL YRYHSYKDLS EFLRGLVMNY
 951 PHITNLTNLG QSTEYRHIWS LEISNKPNVS EPEEPKIRFV AGIHGNAPVG
1001 TELLLALAEF LCLNYKKNPA VTQLVDRTRI VIVPSLNPDG RERAQEKDCT
1051 SKIGQTNARG KDLDTDFTNN ASQPETKAII ENLIQKQNFS LSVALDGGSM
1101 LVTYPYDKPV QTVENKETLK HLASLYANNH PSMHMGQPSC PNKSDENIPG
1151 GVMRGAEWHS HLGSMKDYSV TYGHCPEITV YTSCCYFPSA ARLPSLWADN
1201 KRSLLSMLVE VHKGVHGFVK DKTGKPISKA VIVLNEGIKV QTKEGGYFHV
1251 LLAPGVHNII AIADGYQQQH SQVFVHHDAA SSVVIVFDTD NRIFGLPREL
1301 VVTVSGATMS ALILTACIIW CICSIKSNRH KDGFHRLRQH HDEYEDEIRM
1351 MSTGSKKSLL SHEFQDETDT EEETLYSSKH
```

Spot 40

Polycystwin (peptides sequence coverage: 2%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
468 - 484	1856.3078	1855.9316	GSISSGVSYEEFQVLVR
468 - 484	1856.3082	1855.9316	GSISSGVSYEEFQVLVR
468 - 484	1856.3091	1855.9316	GSISSGVSYEEFQVLVR
468 - 484	1857.4334	1855.9316	GSISSGVSYEEFQVLVR

```
   1 RNGTAWIYTS EKDLNGSSHW GIIATYSGAG YYLDLSRTRE ETAAQVASLK
  51 KNVWLDRGTR ATFIDFSVYN ANINLFCVVR LLVEFPATVG VIPSWQFQPL
 101 KLIRYVTTFD FFLAACEIIF CFFIFYYVVE EILEIRIHKL HYFRSFWNCL
 151 DVVIVVLSVV AIGINIYRTS NVEVLLQFLE DQNTFPNFEH LAYWQIQFNN
 201 IAAVTVFFVW IKLFKFINFN RTMSQLSTTM SRCAKDLFGF AIMFFIIFLA
 251 YAQLAYLVFG TQVDDFSTFQ ECIFTQFRII LGDINFAEIE EANRVLGPIY
 301 FTTFVFFMFF ILLNMFLAII NDTYSEVKSD LAQQKAEMEL SDLIRKGYHK
 351 ALVKLKLKKN TVDDISESLR QGGGKLNFDE LRQDLKGKGH TDAEIEAIFT
 401 KYDQDGDQEL TEHEHQQMRD DLEKEREDLD LDHSSLPRPM SSRSFPRSLD
 451 DSEEDDDEDS GHSSRRRGSI SSGVSYEEFQ VLVRRVDRME HSIGSIVSKI
 501 DAVIVKLEIM ERAKLKRREV LGRLLDGVAE DERLGRDSEI HREQMERLVR
 551 EELERWESDD AASQISHGLG TPVGLNGQPR PRSSRPSSSQ STEGMEGAGG
 601 NGSSNVHV
```

Spot 41

KIAA0626 protein (peptides sequence coverage: 5%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
11 - 28	1900.9668	1900.7898	CSSSSFSSSFFSSASSPR
67 - 77	1166.6054	1166.6044	TQQVEPGVPGR

```
   1 APARRGLGDR CSSSSFSSSF FSSASSPRRL ATAAARAGGA AVIPVPEEPA
  51 LPVPGGRGAG EAGPRRTQQV EPGVPGRAPP AHHAALCHLS RPQAKILSMM
 101 EDNKQLALRI DGAVQSASQE VTNLRAELTA TNRRLAELSG GGGPGPGPGA
 151 AASASAAGDS AATNMENPQL GAQVLLREEV SRLQEEVHLL RQMKEMLAKD
 201 LEESQGGKSS EVLSATELRV QLAQKEQELA RAKEALQAMK ADRKRLKGEK
 251 TDLVSQMQQL YATLESREEQ LRDFIRNYEQ HRKESEDAVK ALAKEKDLLE
 301 REKWELRRQA KEATDHATAL RSQLDLKDNR MKELEAELAM AKQSLATLTK
 351 DVPKRHSLAM PGETVLNGNQ EWVVQADLPL TAAIRQSQQT LYHSHPPHPA
 401 DRQAVRVSPC HSRQPSVISD ASAAEGDRSS TPSDINSPRH RTHSLCNVRP
 451 AAAGPGPLGP AQKLQGRGWR GEAILAVSSR PPREHSGECI SCSVLSFCKK
 501 RWMWGEKGMR PVCSLCPGG
```

Spot 42

Trypsin cationic trypsinogen (peptides sequence coverage: 11%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
36 - 45	1174.6479	1173.6427	TLNNDIMLIK

```
   1 RIQVRLGEHN IEVLEGNEQF INAAKIIRHP QYDRKTLNND IMLIKLSSRA
  51 VINAHVSTIS LPTAPPATGT KCLISGWGNT ASSG
```

Spot 43

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Sequence
671 - 680	1045.4997	1045.5291	LSSPADITDK

```
   1 MEHKEVVLLL LLFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
  51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
 101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
 151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
 201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
 251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
 301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
 351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
 401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
 451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
 501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
 551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
 601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
 651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
 701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
 751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
 801 TWIEGVMRNN
```

Carboxypeptidase D (peptides sequence coverage: 3%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
36 - 57	2211.5394	2212.0244	KAEATTTTTSAGAEAAEGQFDR
886 - 907	2298.7250	2298.0863	GASSSTNDASDPTTKEFETLIK

```
   1 MASGRDERPH WRLGRLLLLM CLLLLGSSAR AAHIKKAEAT TTTTSAGAEA
  51 AEGQFDRYYH EEELESALRE AAAAGLPGLA RLFSIGRSVE GRPLWVLRLT
 101 AGLGSLIPEG DAGPDAAGPD AAGPLLPGRP QVKLVGNMHG DETVSRQVLI
 151 YLARELAAGY RRGDPRLVRL LNTTDVYLLP SLNPDGFERA REGDCGFGDG
 201 GPSGASGRDN SRGRDLNRSF PDQFSTGEPP ALDEVPEVRA LIEWIRRNKF
 251 VLSGNLHGGS VVASYPFDDS PEHKATGIYS KTSDDEVFKY LAKAYASNHP
 301 IMKTGEPHCP GDEDETFKDG ITNGAHWYDV EGGMQDYNYV WANCFEITLE
 351 LSCCKYPPAS QLRQEWENNR ESLITLIEKV HIGVKGFVKD SITGSGLENA
 401 TISVAGINHN ITTGRFGDFY RLLVPGTYNL TVVLTGYMPL TVTNVVVKEG
 451 PATEVDFSLR PTVTSVIPDT TEAVSTASTV AIPNILSGTS SSYQPIQPKD
 501 FHHHHFPDME IFLRRFANEY PNITRLYSLG KSVESRELYV MEISDNPGVH
 551 EPGEPEFKYI GNMHGNEVVG RELLLNLIEY LCKNFGTDPE VTDLVHNTRI
 601 HLMPSMNPDG YEKSQEGDSI SVIGRNNSNN FDLNRNFPDQ FVQITDPTQP
 651 ETIAVMSWMK SYPFVLSANL HGGSLVVNYP FDDDEQGLAT YSKSPDDAVF
 701 QQIALSYSKE NSQMFQGRPC KNMYPNEYFP HGITNGASWY NVPGGMQDWN
 751 YLQTNCFEVT IELGCVKYPL EKELPNFWEQ NRRSLIQFMK QVHQGVRGFV
 801 LDATDGRGIL NATISVAEIN HPVTTYKTGD YWRLLVPGTY KITASARGYN
 851 PVTKNVTVKS EGAIQVNFTL VRSSTDSNNE SKKGKGASSS TNDASDPTTK
 901 EFETLIKDLS AENGLESLML RSSSNLALAL YRYHSYKDLS EFLRGLVMNY
 951 PHITNLTNLG QSTEYRHIWS LEISNKPNVS EPEEPKIRFV AGIHGNAPVG
1001 TELLLALAEF LCLNYKKNPA VTQLVDRTRI VIVPSLNPDG RERAQEKDCT
1051 SKIGQTNARG KDLDTDFTNN ASQPETKAII ENLIQKQNFS LSVALDGGSM
1101 LVTYPYDKPV QTVENKETLK HLASLYANNH PSMHMGQPSC PNKSDENIPG
1151 GVMRGAEWHS HLGSMKDYSV TYGHCPEITV YTSCCYFPSA ARLPSLWADN
1201 KRSLLSMLVE VHKGVHGFVK DKTGKPISKA VIVLNEGIKV QTKEGGYFHV
1251 LLAPGVHNII AIADGYQQQH SQVFVHHDAA SSVVIVFDTD NRIFGLPREL
1301 VVTVSGATMS ALILTACIIW CICSIKSNRH KDGFHRLRQH HDEYEDEIRM
1351 MSTGSKKSLL SHEFQDETDT EEETLYSSKH
```

Mr (expt) is the experimentally observed mass (Da)

Mr (calc) is the calculated mass (Da)

Matched peptides shown in *bold red*

- **Scheme 7**

Band 48

Keratin (peptides sequence coverage: 5%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
148 - 156	1089.5706	1089.5237	VTMQNLNDR
157 - 163	808.3358	808.4330	LASYLDK
229 - 235	806.2949	806.3923	LAADDFR
323 - 333	1364.5726	1364.6320	SQYEQLAEQNR

```
   1 MSVRYSSSKH YSSSRSGGGG GGGGCGGGGG VSSLRISSSK GSLGGGFSSG
  51 GFSGGSFSRG SSGGGCFGGS SGGYGGLGGF GGGSFRGSYG SSSFGGSYGG
 101 SFGGGSFGGG SFGGGSFGGG GFGGGGFGGG FGGGFGGDGG LLSGNEKVTM
 151 QNLNDRLASY LDKVRALEES NYELEGKIKE WYEKHGNSHQ GEPRDYSKYY
 201 KTIDDLKNQI LNLTTDNANI LLQIDNARLA ADDFRLKYEN EVALRQSVEA
 251 DINGLRRVLD ELTLTKADLE MQIESLTEEL AYLKKNHEEE MKDLRNVSTG
 301 DVNVEMNAAP GVDLTQLLNN MRSQYEQLAE QNRKDAEAWF NEKSKELTTE
 351 IDNNIEQISS YKSEITELRR NVQALEIELQ SQLALKQSLE ASLAETEGRY
 401 CVQLSQIQAQ ISALEEQLQQ IRAETECQNT EYQQLLDIKI RLENEIQTYR
 451 SLLEGEGSSG GGGRGGGSFG GGYGGGSSGG GSSGGGYGGG HGGSSGGGYG
 501 GGSSGGGSSG GGYGGGSSSG GHGGSSSGGY GGGSSGGGGG GYGGGSSGGG
 551 SSSGGGYGGG SSSGGHKSSS SGSVGESSSK GPRSAETSWD TNKTRVIKTI
 601 IEEVAPDGRV LSSMVESETK KHYY
```

Centrosomal protein (peptides sequence coverage: 0%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
315 - 326	1475.0497	1473.8191	FQELQLQLEKAK

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   1 MSWALEEWKE GLPTRALQKI QELEGQLDKL KKEKQQRQFQ LDSLEAALQK
  51 QKQKVENEKT EGTNLKRENQ RLMEICESLE KTKQKISHEL QVKESQVNFQ
 101 EGQLNSGKKQ IEKLEQELKR CKSELERSQQ AAQSADVSLN PCNTPQKIFT
 151 TPLTPSQYYS GSKYEDLKEK YNKEVEERKR LEAEVKALQA KKASQTLPQA
 201 TMNHRDIARH QASSSVFSWQ QEKTPSHLSS NSQRTPIRRD FSASYFSGEQ
 251 EVTPSRSTLQ IGKRDANSSF FDNSSSPHLL DQLKAQNQEL RNKINELELR
 301 LQGHEKEMKG QVNKFQELQL QLEKAKVELI EKEKVLNKCR DELVRTTAQY
 351 DQASTKYTAL EQKLKKLTED LSCQRQNAES ARCSLEQKIK EKEKEFQEEL
 401 SRQQRSFQTL DQECIQMKAR LTQELQQAKN MHNVLQAELD KLTSVKQQLE
 451 NNLEEFKQKL CRAEQAFQAS QIKENELRRS MEEMKKENNL LKSHSEQKAR
 501 EVCHLEAELK NIKQCLNQSQ NFAEEMKAKN TSQETMLRDL QEKINQQENS
 551 LTLEKLKLAV ADLEKQRDCS QDLLKKREHH IEQLNDKLSK TEKESKALLS
 601 ALELKKKEYE ELKEEKTLFS CWKSENEKLL TQMESEKENL QSKINHLETC
 651 LKTQQIKSHE YNERVRTLEM DRENLSVEIR NLHNVLDSKS VEVETQKLAY
 701 MELQQKAEFS DQKHQKEIEN MCLKTSQLTG QVEDLEHKLQ LLSNEIMDKD
 751 RCYQDLHAEY ESLRDLLKSK DASLVTNEDH QRSLLAFDQQ PAMHHSFANI
 801 IGEQGSMPSE RSECRLEADQ SPKNSAILQN RVDSLEFSLE SQKQMNSDLQ
 851 KQCEELVQIK GEIEENLMKA EQMHQSFVAE TSQRISKLQE DTSAHQNVVA
 901 ETLSALENKE KELQLLNDKV ETEQAEIQEL KKSNHLLEDS LKELQLLSET
 951 LSLEKKEMSS IISLNKREIE ELTQENGTLK EINASLNQEK MNLIQKSESF
1001 ANYIDEREKS ISELSDQYKQ EKLILLQRCE ETGNAYEDLS QKYKAAQEKN
1051 SKLECLLNEC TSLCENRKNE LEQLKEAFAK EHQEFLTKLA FAEERNQNLM
1101 LELETVQQAL RSEMTDNQNN SKSEAGGLKQ EIMTLKEEQN KMQKEVNDLL
1151 QENEQLMKVM KTKHECQNLE SEPIRNSVKE RESERNQCNF KPQMDLEVKE
1201 ISLDSYNAQL VQLEAMLRNK ELKLQESEKE KECLQHELQT IRGDLETSNL
1251 QDMQSQEISG LKDCEIDAEE KYISGPHELS TSQNDNAHLQ CSLQTTMNKL
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1301 NELEKICEIL QAEKYELVTE LNDSRSECIT ATRKMAEEVG KLLNEVKILN
1351 DDSGLLHGEL VEDIPGGEFG EQPNEQHPVS LAPLDESNSY EHLTLSDKEV
1401 QMHFAELQEK FLSLQSEHKI LHDQHCQMSS KMSELQTYVD SLKAENLVLS
1451 TNLRNFQGDL VKEMQLGLEE GLVPSLSSSC VPDSSSLSSL GDSSFYRALL
1501 EQTGDMSLLS NLEGAVSANQ CSVDEVFCSS LQEENLTRKE TPSAPAKGVE
1551 ELESLCEVYR QSLEKLEEKM ESQGIMKNKE IQELEQLLSS ERQELDCLRK
1601 QYLSENEQWQ QKLTSVTLEM ESKLAAEKKQ TEQLSLELEV ARLQLQGLDL
1651 SSRSLLGIDT EDAIQGRNES CDISKEHTSE TTERTPKHDV HQICDKDAQQ
1701 DLNLDIEKIT ETGAVKPTGE CSGEQSPDTN YEPPGEDKTQ GSSECISELS
1751 FSGPNALVPM DFLGNQEDIH NLQLRVKETS NENLRLLHVI EDRDRKVESL
1801 LNEMKELDSK LHLQEVQLMT KIEACIELEK IVGELKKENS DLSEKLEYFS
1851 CDHQELLQRV ETSEGLNSDL EMHADKSSRE DIGDNVAKVN DSWKERFLDV
1901 ENELSRIRSE KASIEHEALY LEADLEVVQT EKLCLEKDNE NKQKVIVCLE
1951 EELSVVTSER NQLRGELDTM SKKTTALDQL SEKMKEKTQE LESHQSECLH
2001 CIQVAEAEVK EKTELLQTLS SDVSELLKDK THLQEKLQSL EKDSQALSLT
2051 KCELENQIAQ LNKEKELLVK ESESLQARLS ESDYEKLNVS KALEAALVEK
2101 GEFALRLSST QEEVHQLRRG IEKLRVRIEA DEKKQLHIAE KLKERERERD
2151 SLKDKVENLE RELQMSEENQ ELVILDAENS KAEVETLKTQ IEEMARSLKV
2201 FELDLVTLRS EKENLTKQIQ EKQGQLSELD KLLSSFKSLL EEKEQAEIQI
2251 KEESKTAVEM LQNQLKELNE AVAALCGDQE IMKATEQSLD PPIEEEHQLR
2301 NSIEKLRARL EADEKKQLCV LQQLKESEHH ADLLKGRVEN LERELEIART
2351 NQEHAALEAE NSKGEVETLK AKIEGMTQSL RGLELDVVTI RSEKENLTNE
2401 LQKEQERISE LEIINSSFEN ILQEKEQEKV QMKEKSSTAM EMLQTQLKEL
2451 NERVAALHND QEACKAKEQN LSSQVECLEL EKAQLLQGLD EAKNNYIVLQ
2501 SSVNGLIQEV EDGKQKLEKK DEEISRLKNQ IQDQEQLVSK LSQVEGEHQL
2551 WKEQNLELRN LTVELEQKIQ VLQSKNASLQ DTLEVLQSSY KNLENELELT
2601 KMDKMSFVEK VNKMTAKETE LQREMHEMAQ KTAELQEELS GEKNRLAGEL
2651 QLLLEEIKSS KDQLKELTLE NSELKKSLDC MHKDQVEKEG KVREEIAEYQ
2701 LRLHEAEKKH QALLLDTNKQ YEVEIQTYRE KLTSKEECLS SQKLEIDLLK
2751 SSKEELNNSL KATTQILEEL KKTKMDNLKY VNQLKKENER AQGKMKLLIK
2801 SCKQLEEEKE ILQKELSQLQ AAQEKQKTGT VMDTKVDELT TEIKELKETL
2851 EEKTKEADEY LDKYCSLLIS HEKLEKAKEM LETQVAHLCS QQSKQDSRGS
2901 PLLGPVVPGP SPIPSVTEKR LSSGQNKASG KRQRSSGIWE NGRGPTPATP
2951 ESFSKKSKKA VMSGIHPAED TEGTEFEPEG LPEVVKKGFA DIPTGKTSPY
3001 ILRRTTMATR TSPRLAAQKL ALSPLSLGKE NLAESSKPTA GGSRSQKVKV
3051 AQRSPVDSGT ILREPTTKSV PVNNLPERSP TDSPREGLRV KRGRLVPSPK
3101 AGLESNGSEN CKVQ
```

Band 49

Epidermal growth factor (peptides sequence coverage: 43%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
1 - 23	2282.6978	2283.2191	MRPSGTAGAALLALLAALCPASR
1 - 23	2283.5100	2283.2191	MRPSGTAGAALLALLAALCPASR
29 - 36	886.0542	886.5461	KGKGVSRR

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   1 MRPSGTAGAA LLALLAALCP ASRALEEKKG KGVSRRLPRR PRIAPRTPQP
  51 AQPRTGAPAR ARAPARPFLF P
```

Band 50

Human serum albumin : (peptides sequence coverage 43%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
66 - 75	1148.6000	1148.6077	LVNEVTEFAK
98 - 105	932.5214	932.5113	LCTVATLR
162 - 168	926.4074	926.4861	YLYEIAR

187 - 198	1370.5696	1370.5595	AAFTECCQAADK
250 - 257	879.3689	879.4338	AEFAEVSK
287 - 298	1442.6756	1442.6347	YICENQDSISSK
376 - 383	983.4611	982.5335	TYKTTLEK
397 - 413	2044.2991	2044.0881	VFDEFKPLVEEPQNLIK
414 - 426	1656.7728	1656.7453	QNCELFEQLGEYK
427 - 434	959.5464	959.5552	FQNALLVR
500 - 508	1137.4049	1137.4907	CCTESLVNR
550 - 558	999.5263	999.5964	QTALVELVK

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  1 MKWVTFISLL FLFSSAYSRG VFRRDAHKSE VAHRFKDLGE ENFKALVLIA
 51 FAQYLQQCPF EDHVKLVNEV TEFAKTCVAD ESAENCDKSL HTLFGDKLCT
101 VATLRETYGE MADCCAKQEP ERNECFLQHK DDNPNLPRLV RPEVDVMCTA
151 FHDNEETFLK KYLYEIARRH PYFYAPELLF FAKRYKAAFT ECCQAADKAA
201 CLLPKLDELR DEGKASSAKQ RLKCASLQKF GERAFKAWAV ARLSQRFPKA
251 EFAEVSKLVT DLTKVHTECC HGDLLECADD RADLAKYICE NQDSISSKLK
301 ECCEKPLLEK SHCIAEVEND EMPADLPSLA ADFVESKDVC KNYAEAKDVF
351 LGMFLYEYAR RHPDYSVVLL LRLAKTYKTT LEKCCAAADP HECYAKVFDE
401 FKPLVEEPQN LIKQNCELFE QLGEYKFQNA LLVRYTKKVP QVSTPTLVEV
451 SRNLGKVGSK CCKHPEAKRM PCAEDYLSVV LNQLCVLHEK TPVSDRVTKC
501 CTESLVNRRP CFSALEVDET YVPKEFNAET FTFHADICTL SEKERQIKKQ
551 TALVELVKHK PKATKEQLKA VMDDFAAFVE KCCKADDKET CFAEEGKKLV
601 AASRAALGL
```

Band 51

Keratin (peptides sequence coverage: 13%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
14 - 29	1231.7753	1231.5906	SGGGGGGGLGSGGSIR
47 - 59	1234.6841	1234.5215	FSSSSGYGGGSSR
155 - 163	1064.6071	1064.4920	STMQELNSR
225 - 233	1059.7541	1059.5560	TLLDIDNTR
234 - 240	896.5121	896.4062	MTLDDFR
251 - 261	1156.8016	1156.5836	QGVDADINGLR
262 - 271	1189.7947	1189.6013	QVLDNLTMEK
328 - 336	1120.7156	1120.5764	QEYEQLIAK

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  1 MSCRQFSSSY LSRSGGGGGG GLGSGGSIRS SYSRFSSSGG GGGGGRFSSS
 51 SGYGGGSSRV CGRGGGGSFG YSYGGGSGGG FSASSLGGGF GGGSRGFGGA
101 SGGGYSSSGG FGGGFGGGSG GGFGGGYGSG FGGFGGFGGG AGGGDGGILT
151 ANEKSTMQEL NSRLASYLDK VQALEEANND LENKIQDWYD KKGPAAIQKN
201 YSPYYNTIDD LKDQIVDLTV GNNKTLLDID NTRMTLDDFR IKFEMEQNLR
251 QGVDADINGL RQVLDNLTME KSDLEMQYET LQEELMALKK NHKEEMSQLT
301 GQNSGDVNVE INVAPGKDLT KTLNDMRQEY EQLIAKNRKD IENQYETQIT
351 QIEHEVSSSG QEVQSSAKEV TQLRHGVQEL EIELQSQLSK KAALEKSLED
401 TKNRYCGQLQ MIQEQISNLE AQITDVRQEI ECQNQEYSLL LSIKMRLEKE
451 IETYHNLLEG GQEDFESSGA GKIGLGGRGG SGGSYGRGSR GGSGGSYGGG
501 GSGGGYGGGS GSRGGSGGSY GGGSGSGGGS GGGYGGGSGG GHSGGSGGGH
551 SGGSGGNYGG GSGSGGGSGG GYGGGSGSRG GSGGSHGGGS GFGGESGGSY
601 GGGEEASGSG GGYGGGSGKS SHS
```

Plasminogen (peptides sequence coverage: 1%)

Start - End	Mr(expt)	Mr(calc)	Peptides Sequence
671 - 680	1044.6761	1045.5291	LSSPADITDK
671 - 680	1044.7312	1045.5291	LSSPADITDK
671 - 680	1045.6644	1045.5291	LSSPADITDK

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  1 MEHKEVVLLL LLFLKSGQGE PLDDYVNTQG ASLFSVTKKQ LGAGSIEECA
 51 AKCEEDEEFT CRAFQYHSKE QQCVIMAENR KSSIIIRMRD VVLFEKKVYL
101 SECKTGNGKN YRGTMSKTKN GITCQKWSST SPHRPRFSPA THPSEGLEEN
151 YCRNPDNDPQ GPWCYTTDPE KRYDYCDILE CEEECMHCSG ENYDGKISKT
201 MSGLECQAWD SQSPHAHGYI PSKFPNKNLK KNYCRNPDRE LRPWCFTTDP
251 NKRWELCDIP RCTTPPPSSG PTYQCLKGTG ENYRGNVAVT VSGHTCQHWS
301 AQTPHTHNRT PENFPCKNLD ENYCRNPDGK RAPWCHTTNS QVRWEYCKIP
351 SCDSSPVSTE QLAPTAPPEL TPVVQDCYHG DGQSYRGTSS TTTTGKKCQS
401 WSSMTPHRHQ KTPENYPNAG LTMNYCRNPD ADKGPWCFTT DPSVRWEYCN
451 LKKCSGTEAS VVAPPPVVLL PDVETPSEED CMFGNGKGYR GKRATTVTGT
501 PCQDWAAQEP HRHSIFTPET NPRAGLEKNY CRNPDGDVGG PWCYTTNPRK
551 LYDYCDVPQC AAPSFDCGKP QVEPKKCPGR VVGGCVAHPH SWPWQVSLRT
601 RFGMHFCGGT LISPEWVLTA AHCLEKSPRP SSYKVILGAH QEVNLEPHVQ
651 EIEVSRLFLE PTRKDIALLK LSSPADITDK VIPACLPSPN YVVADRTECF
701 ITGWGETQGT FGAGLLKEAQ LPVIENKVCN RYEFLNGRVQ STELCAGHLA
751 GGTDSCQGDS GGPLVCFEKD KYILQGVTSW GLGCARPNKP GVYVRVSRFV
801 TWIEGVMRNN
```

Mr (expt) is the experimentally observed mass (Da)

Mr (calc) is the calculated mass (Da)

Matched peptides shown in *bold red*

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