The Role of Extracellular Histones in Complement Activation and Phagocytosis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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Abstract

Complement activation leads to membrane-attack complex (MAC) formation which can lyse not only pathogens but also host cells. Histones can be released from the lysed or damaged cells and serve as a major type of damage-associate molecular pattern (DAMP), but their effects on the complement system are not clear.

In this study, we pulled down two major proteins from human serum using histone-conjugated beads, one was C-reactive protein and the other was complement component C4 as identified by mass spectrometry. In Surface Plasmon Resonance (SPR) analysis, histone H3 and H4 showed stronger binding to C4 than other histones with KD around 1.0 nM. The interaction did not affect C4 cleavage to C4a and C4b. Since histones bind to C4b, a component of C3 and C5 convertases, their activities were significantly inhibited in the presence of histones. Although it is not clear whether the inhibition was achieved through blocking C3 and C5 convertase assembly or just through reducing their activity, the outcome was that both classical and mannan-binding lectin (MBL) pathways were dramatically inhibited. Using a high concentration of C4 protein, histone-suppressed complement activity could not be fully restored, indicating C4 is not the only target of histones in those pathways. In contrast, the alternative pathway was almost spared but the overall complement activity activated by zymosan was inhibited by histones. Therefore, we believe that histones inhibiting complement activation is a natural feedback mechanism to prevent the excessive injury of host cells.

In addition, the effect of extracellular histones on phagocytosis has been investigated. Surprisingly, histone-induced inhibition of complement activation did not reduce phagocytosis because histones themselves are able to enhance phagocytosis. The underlying molecular mechanism is not clear. Blocking toll-like receptors 2 and 4
only partially reduced the phagocytic activity. Further study is required.

In summary, histone release not only damages cells contacted but also initiates protective mechanisms, including limiting excess complement activation and facilitating bacterial clearance through phagocytosis.
Acknowledgement

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<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ahscFv</td>
<td>Anti-histone scFv</td>
</tr>
<tr>
<td>aHUS</td>
<td>Atypical haemolytic-uremic syndrome</td>
</tr>
<tr>
<td>ALF</td>
<td>Acute liver failure</td>
</tr>
<tr>
<td>ALI</td>
<td>Acute lung injury</td>
</tr>
<tr>
<td>AMD</td>
<td>Age related macular degeneration</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOI</td>
<td>Acute organ injury</td>
</tr>
<tr>
<td>AP</td>
<td>Alternative pathway</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BALF</td>
<td>Bronchoalveolar lavage fluid</td>
</tr>
<tr>
<td>bp</td>
<td>Base-pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C1INH</td>
<td>C1 inhibitor</td>
</tr>
<tr>
<td>C4bp</td>
<td>C4b-binding protein</td>
</tr>
<tr>
<td>CFHR1</td>
<td>Complement factor H-related protein 1</td>
</tr>
<tr>
<td>CIP</td>
<td>Complement interfering protein</td>
</tr>
<tr>
<td>CL-10</td>
<td>Collectin-10</td>
</tr>
<tr>
<td>CL-11</td>
<td>Collectin-11</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>CP</td>
<td>Classical pathway</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor type 1</td>
</tr>
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<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CRs</td>
<td>Complement receptors</td>
</tr>
<tr>
<td>DAF</td>
<td>Decay-accelerating factor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EA</td>
<td>Antibody-sensitized sheep erythrocytes</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescent</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPCR</td>
<td>Endothelial protein C receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>fB</td>
<td>Factor B</td>
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<tr>
<td>fD</td>
<td>Factor D</td>
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<td>FHLL1</td>
<td>Factor H-like protein 1</td>
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<td>fI</td>
<td>Factor I</td>
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<tr>
<td>FXa</td>
<td>Factor Xa</td>
</tr>
<tr>
<td>GBS</td>
<td>Group B Streptococcus</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GpE</td>
<td>Guinea pig erythrocytes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term</td>
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<tr>
<td>PRMs</td>
<td>Pattern-recognition molecules</td>
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<tr>
<td>ProT</td>
<td>Prothrombin</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>RbE</td>
<td>Rabbit erythrocyte</td>
</tr>
<tr>
<td>RD</td>
<td>Retinal detachment</td>
</tr>
<tr>
<td>RHB</td>
<td>Rehydration buffer</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptors</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>rTM</td>
<td>Thrombomodulin</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>SRBCs</td>
<td>Sheep red blood cells</td>
</tr>
<tr>
<td>TAT</td>
<td>Thrombin-antithrombin</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TM</td>
<td>Thrombomodulin</td>
</tr>
<tr>
<td>TMA</td>
<td>Thrombotic microangiopathy</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3,5,5-Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRALI</td>
<td>Transfusion-associated lung injury</td>
</tr>
<tr>
<td>VWF</td>
<td>Von Willebrand factor</td>
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Chapter 1: General Introduction

1.1 Danger signals: DAMPs & PAMPs

In recent decades, the danger model of the immune system has been predicted, it was proposed that the host immunity prioritises to damage rather than foreignness in the sense of the immune response (1). Moreover, this approach assumed that the body’s immunological recognition is not confined to differentiation between self and non-self but extends to safe-unsafe structure discrimination (2).

Thus, the danger model has been widely expanded to incorporate numerous patterns that can elicit the immune response which can be pathogen related or result from tissue injury, necrosis or cells that undergo non-programmed death (1).

The inflammation cascade is stimulated by either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Intriguingly, similar inflammatory responses can be triggered by these two types (3).

DAMPs are endogenous components that mostly exist inside cells and are secreted as a result of cell injury, activation or irregular death; they comprise various types of molecules such as those that can modulate coagulation like histones, high mobility group box 1 (HMGB1), polyphosphates, DNA, RNA and small molecules such as ATP (4). Moreover, some DAMPs can be part of the extracellular or degraded matrix like hyaluronan (5), numerous leaderless secretory proteins LSPs can be released by dying cells such as HMGB1 which is able to elicit inflammation and act as a DAMP when secreted, in addition to its role as a nuclear factor (6).

Following cell injury, DAMPs and some intracellular substances like oxido-reductases and non-protein thiols can be released, this modifies the extracellular redox state in a way that simulates the reducing intracellular environment (7). Such extracellular redox alteration
protects translocated DAMPs from being inactivated by oxidation and enhances their pathological potency (7).

Sepsis is a life-threatening organ dysfunction due to a disordered host response to infection which may be remarkably augmented through endogenous constituents (8), like extracellular RNA, DNA, and histones (9). Thus it is one of the outstanding clinical manifestations that shows the synergistic role of DAMPs and PAMPs in a host immune response, where endogenous alarmins or DAMPs are released passively after cell death such as histones, or actively like HMGB1(10), whereupon these DAMPs are recognised by the innate immune system receptors, leading to an early activation of different serine proteases systems such as coagulation and complement cascade (11). Together with the pathogenic immune response, this results in a systemic immune response (10). Recently, it was postulated that sepsis is characterized with both pro- and anti-inflammatory early activation (12), alongside significant modifications in non-immunologic pathways such as cardiovascular, neuronal, autonomic, hormonal, bio-energetic, metabolic, and coagulation (8), (Fig. 1.1).

During infections and through their molecular patterns, microorganisms are recognized by Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs), thus commences a host response which thereafter develops to inflammation (13-15). When this infection is accompanied by tissue injury and release of DAMPs from damaged or dying cells, an inflammatory reinforcement can occur, which due to DAMP interaction with pattern recognition receptors (PRRs) up-regulates the inflammatory events (7). Unlike the infectious events, even in the absence of pathogen, stressed and damaged cells can still initiate an inflammatory response and recruit the related cells after DAMP liberation (7).

Some non-infectious inflammatory responses can lead to extensive cell death, these include early phase of acute pancreatitis, acetaminophen-induced liver injury, ischaemia-reperfusion-induced
brain injury, myocardial infarction and blunt trauma, where high levels of cytokines and chemokines (16, 17) in addition to intracellular contents release events occur (18-20).

DAMPs and PAMPs use several overlapping receptors such as TLR4, TLR3, TLR7-9 and both NLRs and RLRs (21), on this basis of receptor similarity, it would not be surprising to find a similarity between infectious and sterile inflammatory responses.

![Diagram of the host response in sepsis](image)

**Figure 1.1 The host response in sepsis.** Pro-inflammatory responses and anti-inflammatory immune suppressive responses both occur in sepsis. Inflammatory responses are elicited via interaction between PAMPs or DAMPs and pattern recognition receptors (PRRs). Excessive inflammation with subsequent tissue damage and necrotic cell death lead to release of DAMPs that can sustain ongoing inflammation. The pro-inflammatory response is promoted by activation of leukocytes, complement, and the coagulation system. The anti-inflammatory immune suppressive response relies on impaired function of immune cells, neuroendocrine
regulation, and suppression of pro-inflammatory gene transcription. Substantially, direction, extent, and duration of the septic response are determined by both host and pathogen factors. Adapted from Wiersinga et al (22).

1.2 Histones

Histones were first found in goose erythrocytes by Albrecht Kossel in 1884 (23). Normally, they are highly alkaline intra-nuclear proteins that are present in eukaryotic cells. However, recent studies found some histones are homologous in certain Archaea (20). The basic task of these nuclear proteins is to pack and sort the DNA into functional units (Nucleosomes) as well as gene regulation (24).

Histones are also liberated through neutrophil extracellular traps (NETs), these traps have a significant protection role against exogenous pathogens (25, 26). Basically, histones are divided into two functional groups which are core histones that includes H2A, H2B, H3 and H4, while the other group is linker histones which includes H1 and H5 (H1 variant in Avian erythrocytes (20). The four core histones possess analogous structural characteristics, composed of a central helix flanked by two helix-strand-helix motifs (27). Core histones form a “nucleosome” when two turns of 147 base-paired DNA strand wraps around an octamer of core histones complex (28) (Fig. 1.2).

It has been found that whilst H3-H4 heterodimers further arrange to form a tetramer structure that is essential for commencing nucleosome construction, H2A-H2B homodimers do not submit to these structural changes and they are stable only in this dimeric form (20). Furthermore, core histones organize gene structure and expression through their N-terminals tails’ post transitional modifications PTMs (29), these modifications include acetylation, methylation, phosphorylation, ubiquitination, citrullination, sumoylation, biotinylation or ADP ribosylation (20, 30). On the other hand, linker
histones attach two neighbouring nucleosomes together and control DNA accessibility to other intra-nuclear proteins, and so regulate the epigenetic modifications of transcription as well as the replication and repair processes of DNA (31). In the case of histone scarcity, genomic DNA would be in a disordered and non-functional form (32). Yet, Singh et al has found that excessive histone production in budding yeast has a cytotoxic effect which reflects the importance of many mechanisms that regulate their synthesis inside cells (33).

![Figure 1.2 Structure of histones and nucleosome assembly](image)

**Figure 1.2 Structure of histones and nucleosome assembly.** A nucleosome has octameric histone molecules. An octamer is composed of two H2A-H2B homodimer and one (H3-H4) tetramer, adapted from Silk et al (31).

### 1.2.1 Extracellular histones, translocation and pathology

In the face of their role in nucleosome construction and chromatin stability, misplaced histones in the extracellular space can display toxic and pro-inflammatory properties both *in vitro* and *in vivo* (34),
this happens when they are released passively or actively by dead or triggered cells (20, 35). Furthermore, extracellular histones represent a cell death alarm that stimulates the immunological response as well as the body repair program (36).

These outcomes qualify extracellular histones to be one of the novel damage associate molecular patterns (20). Thus, extensive research is ongoing to interpret their distressing role in various pathological circumstances, where my research group was one of the outstanding examples in this field (35, 37-39).

Mainly, histones are released extracellularly in response to various stress conditions such as infection, trauma and tissue injury (40), hence they are secreted to extracellular spaces through different routes including extracellular traps of active immune cells, cellular apoptosis and necrosis (20).

During sepsis, histones can be released into the blood stream and act as DAMPs due to cell death and prolonged inflammation (37). Hereby, it has been found that they are toxic both in vitro and in vivo and play a role in sepsis-induced endothelial dysfunction, organ failure and death (34). The cytotoxic effect was assigned mainly to H3 and H4 translocation which binds to cellular membranes, accompanied by elevated calcium influx in endothelial cells in vitro (34). In vivo experiment showed that intravenous histone administration to mice caused neutrophil accumulation within the microcirculation, serious endothelial injury, dysfunction and coagulation which resulted in animal death (34).

Peritonitis is an inflammation of the peritoneum which is also initiated by a microbial origin such as bacteria or fungi (20). Histone release from necrotized cells during peritonitis can stimulate sterile inflammation in an NLRP3 ASC-caspase1 inflammasome dependent mechanism, mice administration with H4 abs and activated protein C (APC) restricted the histone dependent peritonitis significantly (41). Another peritonitis model showed histone-induced distant organ
injury to the heart, which led to mechanical and electrophysiological dysfunction that was reduced by anti-histone antibody treatment (37).

Activated neutrophils can be another source of histones, as they expelled in the form of NETs, to ensnare bacteria in sepsis (42). NETs are large extracellular web-like structures which are stuffed with abundance of nuclear and intracellular components such as DNA, core histones and anti-microbial proteins (e.g. myeloperoxidase and elastase), that are discharged out of the neutrophils through a process termed “NETosis” (20, 42-44), these traps can bind and kill pathogens extracellularly and independently (10). However, dysregulation and excessive NETosis have deleterious consequences to the host and can cause adjacent cell death (27, 43).

Furthermore, non-conserved forms of histones within NETs can result in cell damage and vascular thrombosis (40). Although DNase administration can decompose NETs and eliminate DNA in vivo, it does not decrease the tissue injury to a significant extent as it does not affect histones (45), hereby it clarifies the cytotoxic side of extracellular histones (40). Histones released through NETosis can also mediate inflammation in several autoimmunity associated diseases, e.g. rheumatoid arthritis (46) and systemic lupus (47). Extracellular histone autoantigens can also bind with DNA and form histone-DNA complex and prohibit DNA degradation (20, 46).

Another source of extracellular histone release can be extensive cell death associated with non-infectious conditions, such as blunt trauma (35), ischaemia-reperfusion-induced brain injury (48), myocardial infarction (49), liver injury (50) and acute pancreatitis (39). In these cases, a heightened release of cellular content surpasses the neutralizing capacity (51) in addition to the secretion of many inflammatory mediators (31).

Trauma is usually associated with extensive tissue necrosis and cellular death, which elicits a systemic inflammatory response syndrome that clinically resembles sepsis. It has been found that
Histones were cytotoxic when trauma patients' serum that contains >50µg/ml of histones were incubated with endothelial cells (35). Furthermore, circulating histone concentrations have been shown to be significantly raised in patient serum after severe non-thoracic blunt trauma (35). Likewise Johansson et al showed that extracellular histones tend to be associated with anti-coagulant activity in traumatic injury patients (52). Taken together, these observations highlight the pathological role that circulating histones may play in trauma.

In the same context, extracellular histone levels were found to be significantly raised in animal models with other sterile inflammatory conditions such as liver (53), kidney (54), lung (55) and brain injury (48), revealing their role in such types of inflammation. Moreover, these histone DAMPs mediate tissue injury through cytotoxic and pro-inflammatory mechanisms, as they bind to TLRs in the presence of myeloid differentiation factor 88 (MyD88), to release pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), to produce a robust inflammation (20).

Another non-infectious inflammatory disorder that may lead to cell death is acute pancreatitis (20), in which severe complications of necrosis and/or multiple organ failure (MOF) can occur in critical cases (56). Histones can be released from necrotized cells and elicit a sterile inflammation (35). In the same vein, Xelong et al clarified that circulating histone levels rise significantly in a mouse pancreatitis model and correlate with disease severity and distant organ failure (57). Furthermore, a recent study has revealed that measuring circulating histones in pancreatitis patients with abdominal pain can predict future organ failure and mortality (39).

Extracellular histones play a role in ocular disease pathology like retinal detachment (RD); Kwano et al has demonstrated that histone concentration was increased in RD patients (24). In a rat RD model, histones have pro-inflammatory and cytotoxic effects at concentrations of >10 and >20µg/ml respectively (24); histone
modification also associates with some ocular diseases e.g. retinal degeneration, neovascular response, retinitis pigmentosa and spinocerebellar ataxia type (58). On the other hand, it has been found that extracellular histones as well as their epigenetic modification participate in some central nervous system illness like Huntington’s disease (59, 60), (Fig.1.3).

![Figure 1.3 Histone related tissue injury, Chen et al (20).](image)

1.2.2 Extracellular histone toxicity mechanisms

Histones can cause a multiple organ dysfunction (MOD) through their tripartite synergetic effects including inflammatory, pro-coagulant and cytotoxic effects (38).

1.2.2.1 Pro-inflammatory influence of extracellular histones

Several TLRs have been found to be associated with recognition of both DAMPs and PAMPs and the subsequent immune response (21), so this makes the infectious and non-infectious inflammatory response share many characteristics (3).

The activation of TLR2/TLR4 by histones leads to release of many pro-inflammatory cytokines e.g. IL-6, IL-10 and TNF-α through MyD88 associated mechanism as well as platelet activation (6, 61-64), which in turn controls the provoked immune response in non-
infectious acute organ injury (AOI) (31). Moreover, knocking out TLR2 and TLR4 genes in mice models was found to be protective against lethal histone dosages (53, 62) and TLR2 and TLR4 monoclonal abs blocked the corresponding receptors and protected wild type animals (31). Nevertheless, TLR9 may also participate in the non-infectious inflammatory response during hepatic IR injury of animal models (65), in which exogenous histones activate TLR9 and result in cytokine release then massive tissue necrosis, whereas TLR9 null mice have no response to histones (66). Additionally, TLR9 stimulation may play another role in the sterile inflammation cascade through NLRP3 inflammasome-caspase1 activation (67), leading to cytokine secretion and leucocyte recruitment (68). Histone binding to TLR9 activates these receptors, thus it triggers mitochondrial reactive oxygen species (ROS) generation, which in turn prompts the NLRP3 inflammatory pathway of pro-inflammatory Kupffer cells (KCs) during liver injury after hepatic ischaemia/reperfusion (67).

1.2.2.2 Extracellular histones and impact on coagulopathy

Conventionally, coagulation is an organised process through which platelets and coagulation proteins synergise to form a blood clot and so culminates in haemostasis; disorders in such mechanism may lead to haemorrhage or thrombosis (69). There is an association between thrombosis and inflammation, they share a variety of mechanisms in this pattern of crosstalk (70), whereby pro-inflammatory histones can have a role when they are released extracellularly in some pathological conditions (6). Abrams et al (35) has found that thrombin–anti-thrombin complexes (TAT) were significantly increased in the circulation of trauma patients’ and correlate significantly with histone concentrations. In vivo, exogenous histones have been shown to demonstrate vacuolated endothelium, intra-alveolar haemorrhage and macro and microvascular thrombosis, which culminate in organ failure in mice models (34). Among all histone types, H4 was the most potent in inducing platelet aggregation and platelet-dependent thrombin generation in mice in vivo and in
Sameraro et al showed that extracellular histones activate platelets through their TLR2/TLR4 receptors which leads to enhanced platelet-mediated thrombin generation in humans (61). TLR2 and TLR4 induce platelet activation through several signalling pathways (e.g., ERK, Akt, p38, and NF-κB), induction of calcium influx and fibrinogen recruitment (16). By interacting with TLR2 and TLR4, histones stimulate tissue factor expression (TF) in human endothelial cells as a result of NF-κB and AP-1 transcription factor up-regulation (6).

However, histones may adopt other mechanisms to promote thromboembolism such as suppressing thrombomodulin (TM)-dependent protein C activation where H3 and H4 were the most active effectors (71). This effect could be neutralized with recombinant TM which binds to histones and protects mice against Histone H3-induced lethal thrombosis (72). Circulating histones play a role in the crosstalk between coagulation, inflammation and innate immunity (Fig. 1.4) which qualifies them to be interesting therapeutic targets in haematological disorders and critical illness (40). Another study demonstrated that H4 can bind to prothrombin and generate thrombin independently of the coagulation cascade via auto-activation (73). Histones cause endothelial damage via Willebrand factor–mediated leucocyte recruitment, platelet activation, inhibition of the protein C anticoagulant pathway, and prothrombin auto-activation thus enhancing the microvascular thrombosis (34, 38, 62, 73).
Figure 1.4 Histones are the cornerstone of interaction between coagulation, innate immunity and inflammatory pathways, adapted from Alhamdi et al (40).

1.2.2.3 Nonspecific TLR independent cytotoxicity of free histones

Extracellular histones may have direct cytotoxic effects to epithelial and endothelial tissue, herewith administrating exogenous histones results in significant reduction in cell viability (31). A high binding avidity was first found between histones and anionic phospholipids (Cardiolipin and phosphatidylserine) in 1994 (1). In the same vein, recent researches have suggested that extracellular histones bind to phospholipid–phosphodiester bonds, in the same manner as they bind to DNA, which causes increase of transmembrane conductance, cell swelling, calcium ion influx and cytolysis (34, 35, 74). Consequently, intracellular components will be discharged, culminating in cell death due to cellular functions deteriorating (31).

Abrams et al has found that this histone-membranous phospholipids binding and the consequent calcium influx associates with vascular endothelial permeability increase, myeloperoxidase and cytokine release, coagulation activation and NETosis (35). Blocking TLR2/4 receptors did not alter the endothelial cell calcium influx, while controlling the extracellular Ca$^{+2}$ or treating with histone abs has cytoprotective effects. (35).

Interestingly, the inflammatory mediator C-reactive protein has been found to compete with histones in opposition to their phospholipids binding (75), thus this interaction protects against histone-phospholipid mediated cytotoxicity by disrupting histones binding to phospholipids, preventing histone association with cellular membranes and so blocking the calcium influx cytotoxicity mechanism of histones (75).
Besides, intracellular excess of Ca\(^{+2}\) ions possibly correlates with cardiac injury which results from cardiomyocyte injury and death (38, 76, 77). In human MCF7 cancer cells, Ganapathy et al has revealed that H1 promotes extracellular Ca\(^{+2}\) influx and intracellular release, taking into account the cytotoxic impact due to the higher levels mainly from the extracellular influx (78). Histone mediated-calcium influx events have been observed in other tissues like platelets (63), urinary bladder epithelium (79) and thymocytes (80).

Altogether, histone-enhanced inflammation, coagulation, and cytotoxicity effects may synergistically result in multiple organ dysfunctions that are common in critical illness (38).

1.2.3 Extracellular histones and sterile acute organ injury

1.2.3.1 Lung

Acute lung injury (ALI) has a high mortality rate which is around 40% (81) and is mostly attributed to other primary factors like trauma or ischemia (31). ALI causes alveolar and lung endothelial cell functional alteration, which in turn leads to improper pulmonary circulation, protein permeability increase, cell death and inflammatory response that culminates in permanent lung dysfunction, MOF and late mortality (23, 25, 31, 35).

In mouse models mortality is observed within 4 hours after histone infusion (75mg/kg), as histone treatment led to cyanosis and dyspnoea which indicates both lung and cardiac failure (35). Furthermore, histological microscopy has shown clear alteration in lung tissue such as oedema, neutrophil-obstructed alveolar capillaries and pulmonary haemorrhage, while liver and kidney displayed mild pathological alteration like vacuole formation (35). On the other hand, anti-histone abs (ahscFv) co-infusion diminished histone-mediated lung injury significantly (35).
In humans, severe blunt trauma patients showed significant rapid increase of circulating histone levels, which passed the toxicity threshold and correlated with ALI severity, endothelial damage and coagulation (35). Serum histones over 50µg/ml were also toxic to cultured endothelial tissue (35). In the same study, it was concluded that histones may exhibit their cytotoxicity effects through calcium influx and coagulation mechanisms in acute lung injury (35). With regard to the mechanism of histone release, an ALI mouse model clarified that complement receptors C5aR and C5L2 are contributors in such a process (55).

Animal models of transfusion-associated lung injury (TRALI) displayed an obvious NET-mediated inflammation (82), where platelet activation stimulates NETosis from neutrophils leading to oedema and vascular endothelial permeability in lung tissues (27), which could be reduced with anti-histones abs (82).

Altogether, these data indicate that lungs are the most susceptible organs to circulating histone toxicity, this may due to the dense nature of the capillary network in the pulmonary microcirculation (35).

1.2.3.2 Heart

Generally, ischemia/reperfusion (I/R) is the main cause for cardiac injury in which shortage of the oxygen supply to cardiomyocytes led to necrosis and discharge of immunogenic intracellular constituents and a cascade of inflammation and repair (31, 49).

Soon after myocardial infarction (MI), uncontrolled histone release from necrotized cells occurs, and then histones aggregate within the myocardium and promote dose dependent cytotoxicity to cardiac myocytes (49). Furthermore, treating the mouse model with DNase protected myocardiocytes from histone-mediated cell death and left ventricular dysfunction after IR. It was supposed that DNAse disseminated extracellular chromatin by linker DNA degradation which reduced local histone concentrations and thus cytotoxicity (49).
On the other hand, Savchenko et al. has demonstrated that NETs are also incorporated in the inflammatory cascade and exacerbate cardiac ischemia injury in a mouse model (83). Peptidylarginine deiminase 4 (PAD4) is essential enzyme for histone citrullination, which is a posttranslational histone process for decondensation of chromatin during NETosis, Martinod et al. has shown that venous thrombosis can result from NET formation via PAD4 (84).

It should also be mentioned that PAD4 knock-out mice showed no NETs formation (85), lack of PAD4 decreased leucocyte infiltration and NETosis, hereby having a cardio-protective impact in a mouse model of acute myocardial infarction (AMI) (83).

1.2.3.3 Liver

Non-viral hepatitis can result from liver ischemia and drug toxicity, this type of sterile inflammation may lead to hepatic enzyme and synthetic function disorder (86, 87). This dysregulation brings out cardiovascular instability, cerebral edema, coagulopathy, renal failure, metabolic disturbance, hemodynamic instability, and susceptibility to infection which are the main clinical characteristics of acute liver failure (ALF) (31, 59, 86).

Histone release after hepatic sterile inflammation can activate TLR2/TRL4/TLR9 on KCs, amplify the inflammation and cause a robust release of a number of cytokines (53, 62, 65, 66). Whilst H1/H4 have proved to play a major role in alveolar cell death in ALI (82), H3 which displayed inert properties in ALI (27, 55) has shown to have significant effects on hepatic damage that can be reduced with anti-H3 abs which minimized the mortality, and serum TNF-α and IL-6 in ALF animal models (66).

In humans, ALF patients’ sera had significantly increased histone level which can trigger hepatocyte death and up-regulate U937 (Monocytes) cytokine release activity (50). Using histone neutralizing agents like non-anticoagulant heparin prevented the histones’ pro-
inflammatory effect, which demonstrates that circulating histones are vital mediators of ALF patients’ systemic inflammation and cell death (50).

Furthermore, levels of the endothelial tissue dysfunction marker Angiopoietin-2 increased notably in ALF patients, which may be a response to the cytotoxic impact of extracellular histones (59).

NETs also contribute to hepatocytotoxicity (31, 44). NETosis is initiated upon activation of neutrophil receptors TL4 and TLR9-MyD88 by injured hepatocyte histones, which stimulate KCs to release pro-inflammatory mediators (31). This process could be suppressed in mice by treatment with NETosis inhibitors e.g. peptidyl-arginine-deiminase 4 PAD4i or DNAse1, which in turn has led to the suppression of NET formation as well as reduction in HMGB1 and histone mediated hepatic injury (31, 44).

In addition, it has been recently shown that there is an association between acute pancreatitis and liver inflammation, with the liver being implicated as a main source of DAMPs histones in animal models of pancreatitis-associated liver injury (88).

**1.2.3.4 Kidney**

Acute ischemic or any toxic insult can lead to acute renal failure, which is characterized by a sudden reduction in glomerular filtration function and consequently results in azotemia (89). Acute ischemic kidney injury associates with high rate of morbidity and mortality (90). Frequent insult to the renal parenchyma may cause enduring necrosis and renal failure, which can develop to a chronic kidney disease if untreated (91). Cell death that attributes to apoptosis and necrosis is a main manifestation of ischemic renal failure which is prolonged via cytokine release and neutrophil invasion (92).

Histones are released extracellularly from necrotic tubular epithelial cells in acute renal ischemia (54, 93). Those free histones exhibit a
dose-dependent cytotoxic effect for both renal endothelium and tubular epithelium (93).

Furthermore, extracellular histones aggravate leukocyte adhesion and enhance the vascular permeability of the renal arteries (93). However, histones-mediated pro-inflammatory effects could be reduced by blocking with H4 monoclonal abs (31, 93).

TLR2/TLR4 receptors have been shown to mediate the inflammatory cascade in acute kidney injury via stimulating MyD88-NFkB and the mitogen-activated protein kinase (MAPK) signaling mechanisms, while null-MyD88 and TLR mice have shown alleviated pro-inflammatory manifestations (31, 64, 94).

### 1.3 Relief of adverse effects of histones: Therapeutic approaches

As histones are considered potential mediators in the interaction between different host systems including inflammation, coagulation and innate immunity (40), so they represent a useful therapeutic target in histone-associated clinical conditions (40). From this direction comes the importance of finding therapeutic strategies to reduce the harmful effects of extracellular histones, which may be based on three main elements including blocking histone release, extracellular histone neutralisation and blocking of histone-induced signalling (31).

#### 1.3.1 Histone release blocking

Targeting NETosis had a successful impact in animal models of AOI and sepsis (31). Various PAD inhibitors can decrease the pathological manifestation of many diseases models e.g. collagen-induced arthritis, colitis, atherosclerosis, lupus, kidney injury, hypoxic ischemia and cancer (95). Specifically screened PAD4 suppressors GSK199 and GSK484 were able to reduce histone citrullination and diminish NET formation (96), which had greater protective effects than DNase treatment, against tissue damage (45). In addition, Kusunoki et al (97) has clarified that the pan-PAD inhibitor C1-amedine was able to
suppress NETosis both in vitro with human neutrophils and in vivo in a mouse model. However, DNase through breaking down nucleosomes is still able to alleviate NETs and extruded intracellular histones toxicity, due to extracellular chromatin dispersion by linker DNA degradation, that reduced local histone concentrations and significantly ameliorated MI associated left ventricular remodelling in mice (31, 49). Moreover, a recent study showed that treating mice with PAD4 inhibitor (YW3-56, YW4-03) or DNase1, suppressed NET generation and decreased HMGB1 and histone-mediated liver I/R injury (44). Another inhibitor for NETosis was found to be APC (98), where a recent study has revealed that APC can bind to human leukocytes and prevent phorbol 12-myristate 13-acetate (PMA) or activated platelets from stimulating NETosis, APC at therapeutic concentrations may result in NETosis inhibition via EPCR-, PAR3-, and Mac-1-dependent pathways (98). Developing APC mutants with cytoprotective and anti-inflammatory but not anticoagulant activity is interesting for therapeutic use in certain inflammatory diseases, without compromising the haemostasis (98-100).

1.3.2 Released histones neutralisation

The acute phase protein CRP associates with both innate and adaptive immunity, it participates in infections, non-infectious, autoimmune and apoptotic scenarios (75, 101-104). Histone-CRP complexes have been demonstrated in ICU patients’ serum with high levels of both CRP and histones (75). When this serum is incubated with endothelial cells, CRP has shown a cytoprotective effect due to preventing histones from integrating into plasma membranes and causing the consequent calcium influx; this protective role is due to competition for binding with histones to membranous phospholipids (75, 101). In a mouse model treated with exogenous histones, it has been demonstrated that infused CRP neutralised the cytotoxic effects of histones via decreasing vascular permeability, suppressing histone-mediated-coagulation and endothelial damage (75). Histological examination showed that CRP infusion prolonged mouse viability in a
dose dependent manner, due to significantly reduced lung oedema, haemorrhage and thrombosis in mice challenged by a lethal dose of histones (75).

Disseminated intravascular coagulation (DIC) is associated with increased levels of circulating histones (40, 72). Recombinant thrombomodulin (rTM) treatment for DIC patients has been validated in Japan (20); it was demonstrated that rTM binds directly to histones and rescues mice from lethal thrombosis; this protective impact is organised via APC-dependent and independent pathway (71, 72, 105). APC have been shown to cleave histones both in vitro and in vivo; such cleavage yields fragments that are unable to cause endothelial cell damage and dampen the cytotoxicity (106). However, developing APC mutants with cytoprotective and anti-inflammatory but not anticoagulant activity should be the targeted approach, since administration with conventional APC may cause serious complications to homeostasis (98).

Heparin has been found to neutralize histone toxicity and histone-mediated platelet activation both in vitro and in vivo (20). In the same vein, Wildhagen et al (74) demonstrated that non-anticoagulant heparin binds to histones and inhibits their toxic effects in vitro; the same study also clarified that heparin decreased the mortality in mouse models of septic and sterile inflammation. In another study (63), heparin was also able to prevent extracellular histone toxicity in vitro and upon infusion into mice, since it prohibits histones from platelet activation and prevents histone-induced thrombocytopenia, tissue damage, and animal death (63). Intriguingly, treatment with non-anticoagulant heparin excludes the risk of haemorrhage and makes it a promising strategy for treating septic patients (74).

Substantially, anti-histones abs have demonstrated protective effects when used in many AOI animal models (27, 34, 35, 53-55, 62, 66, 67, 82), which is reflected by inflammation amelioration, functional score and overall survival (31).
Anti-histone antibody protected mice against transfusion-related acute lung injury (82) and also attenuated acute lung injury in a murine model of trauma and in a histone-infusion mouse model, as well as protecting the mice from histone-associated lethality (35). Moreover, antibodies to histones neutralized the immunostimulatory effects of histones, inhibited intra-renal inflammation, neutrophil infiltration and ameliorate the kidney function (54), and reduced neutrophil recruitment by necrotic cells in vitro and in vivo (107). In addition, histones-mediated cardiotoxicity as well as cardiac dysfunction has been abrogated using anti-histones abs (38).

1.3.2.1 Others

Lam et al (108) indicated that albumin can bind to H4 and prevent H4-mediated platelet aggregation; the study attributed this binding affinity to charge variance between the histone and albumin, since neutralized albumin exposed both less H4 affinity as well as reduced inhibitory effect. Yet, the study’s findings have not been further confirmed, and lack in vivo experiments and were applied in a serum or plasma-histone only environment, which did not include any other cell signalling and incorporation. Furthermore, the concentration of H4 was substantially below the toxic value (10µg/ml) which should be tackled by the immune system, taking into consideration that platelet activation is one of many mechanisms that histones adopts to up-regulate the host response.

A shotgun proteomics study has pointed out that exogenous histones precipitate numerous plasma proteins like lipoproteins, proteinase inhibitors, complement proteins, coagulation factors and immunoglobulins, suggesting a possible protective effect of histone-protein insoluble copolymer formation (109). However, the study has stated that the exact pattern of interaction between alike individual histones and plasma proteins was beyond its scope (109).

Regarding complement subunits that aggregate with histones, this study has also stated that comprehensive work would be important to
assign whether this represents simple protein deposition, or whether it can be attributed to complement activation (109). In addition, the cytotoxicity of circulating histones has been well reviewed (20, 31) and established in a clinical context by our group (35, 37-40).

1.3.3 Histone-TLR signalling blocking

Extracellular histones have been shown to interact with many TLR receptors comprising TLR2, TLR4 as well as TLR9 (54, 61, 62, 66). Activating TLRs via binding with histones leads to stimulating different inflammatory, coagulopathy and histone-mediated cytotoxic effects in various clinical conditions (110).

TLR4 null mice were protected against histone-related lethal effects upon exogenous histone infusion (62). In addition, Ekanet et al showed that blocking TLR4 reduced histone-associated toxicity to endothelial cells that were infused with a toxic concentration of histones (50µg/ml) (111). Blocking platelets’ TLR/TLR4 receptors with monoclonal abs resulted in suppression of both platelet activation as well as thrombin generation (61). Moreover, histones showed no effect in TLR9 inhibitor treated mice (66). Nevertheless, blocking TLR2/TLR4 has not revealed obvious protective impact according to Abrams et al (35). This seems logical if we take into consideration that histones have other mechanisms to cause deleterious toxicity effects, like calcium influx and up-regulating the coagulation cascade (38).

Treatment with TLR blockers may ameliorate the clinical conditions in several cases despite not being the only distinctive mechanism that histones mediate to cause cell damage. However, as TLRs are important in innate immune defence after infection, so blocking this arm of the immune system may lead to significant immune system impairment (31).
1.4 Novel innate immunity-histone release strategies

The body’s defence strategy comprises three main chronological lines including anatomical and physiological barrier, innate, and adaptive immune response (Fig. 1.5). Innate immunity propagates the physical barrier protection and stimulates the later adaptive response (112).

In general, the innate immune response involves cellular and humoral components which are interacting between each other to develop a protective inflammatory cascade. The cellular part includes haematopoietic cells like neutrophils, macrophages and natural killer cells (NKs) (113). While the humoral events are orchestrated by a distinct array of mediators, such as the contact system, naturally existing abs, complement, pentraxins (CRP) and microbicides peptides, which synergise to up-regulate the cellular events and provide the required protection (112, 114). Based on the similarity between PAMP and DAMP receptors (21) as well as the subsequent inflammation (3), a recent approach has included tackling of DAMPs as one of the innate immune response functions (112).

![Figure 1.5 Integral Human Immune System, Turvey et al (112).](image-url)
In addition to the initial overlapping between conventional PAMPs and DAMPs immune response, many studies have referred recently to the role of complement in the induction of NETosis (31). Bosman et al (55) showed that C5a receptors C5aR and C5L2 mediate NETosis and exacerbate the severity of ALI in a mouse model. Furthermore, another study has found that both TLRs and complement C3 are essential to stimulate NETosis, where no NET-related extracellular histones or even DNA have been found in TLR2/C3 null mice despite normal neutrophil recruitment (115). In a caecal ligation and puncture (CLP) animal study, Kalbitz et al and Grailer et al have stated that existence of histones in plasma was due to the interaction between three innate immune system arms, which are the complement system (C5a and its receptors), PMNs, and the NLRP3 inflammasome (116, 117).

In a review, Peter et al indicated that immune complexes stimulate NETosis via C5a receptors on neutrophils during ALI. Interestingly, neutrophil depletion in mice with C5a induced ALI decreased the H4 levels dramatically in the bronchoalveolar lavage fluid (BALF) (55); silencing C5aR and C5L2 receptors in mice results in a sharp reduction in extracellular histone levels, which highlights that these free histones were released in a neutrophil-C5a dependent mechanism (55). Surprisingly, C3 null mice were still able to develop ALI and this ameliorated after anti-C5a treatment; the study has demonstrated that C5a generation was attributed to an unusual pathway via thrombin mediated C5-cleavage and only thrombin blockading terminated the C5a-PMNs associated ALI pathology in C3-/- mice (118).

These outcomes revealed that extracellular histone release is also linked to a novel approach that incorporates complement components and innate immunity (Fig. 1.6), which raises a question about any other probable interaction that histones may have with complement proteins whether directly or indirectly.
Figure 1.6 Early initiating events in sepsis are complement activation with C5a/receptor incorporation which leads to death, Kalbitz et al (116).
1.5. The complement System

1.5.1 Overview

The complement system represents one of the main arms of innate immunity that bridges innate and acquired immune responses (119), it supplies the body with a pivotal guard against pathogenic invasion (120), as well as a crucial participation in the pathogenesis of many autoimmune and inflammatory diseases (121) and a novel non-inflammatory role in tissue regeneration and repair (122, 123). Initially, complement was considered as a heat-labile source in serum which “complements” antibodies in bactericidal function (124, 125). In terms of number of components, the complement system is composed of over than 50 proteins in plasma and on the surfaces of cells (126), these proteins comprise more than 3g/l, where they amount to around 15% of the globulins (124, 125, 127).

Basically, the complement system can be activated via three pathways, the classical pathway (CP) that tackles antibody-antigen complex, gram-negative bacteria, apoptotic cells and some viruses (123, 124). The second pathway is via mannose binding lectin (MBL), which is directed to kill pathogens that expose mannose in their structure (123, 124).

Finally, the alternative pathway (AP) destroys different sorts of contagious factors such as viruses, bacteria and fungi, as well as having a role in immune surveillance against tumours (123, 124). These pathways are linked into a common termination lane, leading to the formation of the membrane attack complex (MAC) (Fig. 1.7), this complex is amphipathic and composed of C5b, C6, C7, C8 and several C9 molecules and causes lysis of the pathogen (123, 128, 129).

In addition, many of the products generated during complement activation are able to opsonize damaged cells or pathogens to facilitate phagocytosis (130, 131). Altogether, complement activation enhances
the ability of antibodies and phagocytic cells to clear invading pathogens and cellular debris from the circulation (132).

Induction of the complement cascade through DAMPs and PAMPs or antibodies is a fundamental for pathogen elimination, humoral immunity development, homeostatic clearance of immune complexes and damaged cells, and cellular inflammatory event regulation (120, 133). To be mentioned, a clear concept about complement interaction with some DAMPs like histones while clearing damaged cells has not been demonstrated yet.

Figure 1.7 The complement pathways converge into a common termination lane, leading to the formation of C5b-9 MAC.

Complement activation steps are monitored by many inhibitors, in order to achieve a balance between active detection and destruction of pathogens and reduction of bystander tissue damage (125). This
regulation occurs mainly at the convertase level, and through the assembly of MAC (134).

In the face of the protective role that the complement system undertakes, it has been demonstrated that dysregulation or insufficiency of complement proteins can lead to many autoimmune and non-autoimmune diseases (123, 135)

1.5.2 The development of Complementology

More than one century ago, Hans Buchner first characterized the complement system as a heat-labile bactericidal component that exists in blood (136). Thereafter, Jules Bordet indicated that the bactericidal activity of serum can be reinstated upon the thermal activity destruction by adding fresh serum (135). Paul Ehrlich replaced the term “Alexine” system that was given by Buchner and Bordet with “Complement” (119, 137). Bordet and Gengou then designed the complement fixation test in 1901, a haemolytic assay that incorporates the erythrocyte lysis to determine the complement activation termination point; this fixation assay has been used potentially for many decades to discover the activation cascade and to unravel the protein-protein and protein-membrane interactions which occur in the complement system (124, 138, 139).

In the following decades, pioneer complementologists like Louis Pillemer and Hans Müller-Eberhard recognized the key factors that are engaged in the classical (140, 141), alternative (142), and terminal pathways of complement (119, 143, 144). In the 1970s and 1980s, progress in complement studies started on a genomic basis, this included complement protein isolation and sequencing, cDNA and genomic clones and sequence yield (138, 139, 145, 146). In addition, work progressed in identification of cell-surface complement receptors as well as soluble and superficial complement regulators
Furthermore, the bridging role of complement to the adaptive immune response development was demonstrated (135, 149).

The last 20 years has seen a great development in different aspects of the complement system, e.g. the role of complement in homeostasis and clearance of apoptotic cells has been disclosed (150). Moreover, the association between genetic variance (e.g. Factor H) of complement proteins and disease predisposition like age related macular degeneration (AMD) has been clarified. Despite the previous observation of MBL (151) and AP (142) pathways, better comprehension of those pathways took place recently. The exploration of the functions of the new MBL-associated proteases and ficolins (152), and proof of the direct role of properdin in AP activation (153) together have disclosed many aspects of the interaction patterns in those pathways.

Although the complement factors in circulation are generally produced in the liver, it is now understood that most tissues and inflammatory cells like neutrophils and macrophages, are effective origins of complement factors (154-158). On the other hand, recent studies have shown the essential impact of complement deposition and anaphylatoxin-mediated signalling on active surfaces, which play a part in adaptive immune response initiation (159-161). Clinically, modification of complement activity for therapeutic purposes has developed, for example a monoclonal antibody, eculizumab, which inhibits complement at the C5 activation level is in clinical use (135, 162, 163).

1.5.3 The complement proteins: Array and dynamics

In addition to the main proteins of the complement cascade (C1-C9), the whole complement network consist of fluid-phase, cell-surface-associated and intracellular proteins that elaborate as pattern-recognition molecules (PRMs), proteases and convertases, regulators and signalling receptors that collectively mediate immune surveillance and tissue homeostasis (130, 158, 164), (Table 1.1). This array of
complement proteins is arranged in a hierarchical structure of the proteolytic cascade which is initiated with danger molecule surface identification, thereafter the subsequent event which is the release of potent proinflammatory mediators (Anaphylatoxins), opsonization and ultimately the analysis of the target by MAC formation (125).

Several complement proteins are zymogens which are disseminated safely through the body and activated at the site of inflammation in a triggered enzyme cascade pattern, whereby a vigorous complement response is induced from the amplification of the start-up proteins by regulated sequential enzymatic reactions (119, 165).

Differently than many other enzymatic cascades, the complement system represents a more sophisticated pattern as it is based on the composition of consecutive activated protein fragments, which subsequently turn into convertases that cleave other complement proteins to form the terminal enzymatic complex (119, 165), these complexes maintain their stability and activity for a very short time as another form of regulatory mechanism operates that the complement system adopts to control activation (119, 166).

Generally, complement proteins are represented by a capital letter followed by a lowercase suffix that should be “a” or “b” in case of low and high molecular weight fragment respectively e.g. C5a & C5b (167), this nomenclature principle has one exception with respect to C2, where C2a refers to the larger fragment while C2b indicates the small one (167). All the nine proteins C1-C9 are numbered chronologically to the cascade events except C4 which is cleaved with C2 and before C3(167).

On the other hand, some MBL and AP pathway unique proteins have variant logical nomenclature like MASPs and factor ”B” (167). To indicate enzymatically active components and protein complexes a bar is used above the name usually e.g.C3Bb, while inactivated complement proteins due to complements’ inhibitors function is indicated with a lower case prefix “i” like iC3b (167, 168).
Table 1.1: Summary of the Complement protein array, adapted from Janeway et al (165); Salvador-Morales et al (169); Leendert (167); Carroll et al (135) Ricklin et al (120); Bajic et al (166).

<table>
<thead>
<tr>
<th>Pattern Recognition</th>
<th>Ligand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Pathway</td>
</tr>
<tr>
<td>C1q</td>
<td>Classical</td>
</tr>
<tr>
<td>e.g. IgG, IgM complexes</td>
<td></td>
</tr>
<tr>
<td>MBL</td>
<td>Lectin</td>
</tr>
<tr>
<td>Carbohydrate moieties</td>
<td></td>
</tr>
<tr>
<td>Ficolin-1,2,3</td>
<td>Lectin</td>
</tr>
<tr>
<td>Carbohydrate moieties</td>
<td></td>
</tr>
<tr>
<td>Properdin</td>
<td>Alternative</td>
</tr>
<tr>
<td>DAMPs, PAMPs, LPS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serine Proteases</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Pathway</td>
</tr>
<tr>
<td>C1r</td>
<td>Classical</td>
</tr>
<tr>
<td>Cleaves C1s</td>
<td></td>
</tr>
<tr>
<td>C1s</td>
<td>Classical</td>
</tr>
<tr>
<td>Cleaves C4,C2</td>
<td></td>
</tr>
<tr>
<td>MASP-1</td>
<td>Lectin</td>
</tr>
<tr>
<td>Cleaves MASP-2, C3, Pro-f-D</td>
<td></td>
</tr>
<tr>
<td>MASP-2</td>
<td>Lectin</td>
</tr>
<tr>
<td>Cleaves C2, C4</td>
<td></td>
</tr>
<tr>
<td>C2</td>
<td>Classical, Lectin</td>
</tr>
<tr>
<td>Part of C3/C5 convertases</td>
<td></td>
</tr>
<tr>
<td>C4bC2a/C4b2C2aC3b</td>
<td></td>
</tr>
<tr>
<td>fB</td>
<td>Alternative</td>
</tr>
<tr>
<td>Part of C3/C5 convertases</td>
<td></td>
</tr>
<tr>
<td>C3bBb/C3b2Bb</td>
<td></td>
</tr>
<tr>
<td>fD</td>
<td>Alternative</td>
</tr>
<tr>
<td>Cleaves fB in C3bB complex</td>
<td></td>
</tr>
<tr>
<td>fI</td>
<td>Alternative</td>
</tr>
<tr>
<td>Inactivates C3b, C4b</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regulators</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Pathway</td>
</tr>
<tr>
<td>C1INH</td>
<td>Classical, Lectin</td>
</tr>
<tr>
<td>Serine protease inhibitor</td>
<td></td>
</tr>
<tr>
<td>C4bp</td>
<td>Classical</td>
</tr>
<tr>
<td>C3 convertase decay</td>
<td></td>
</tr>
<tr>
<td>CR1</td>
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</tr>
<tr>
<td>C3 convertase decay</td>
<td></td>
</tr>
<tr>
<td>FHL-1</td>
<td>Alternative</td>
</tr>
<tr>
<td>C3 convertase decay</td>
<td></td>
</tr>
<tr>
<td>DAF</td>
<td>Classical, Alternative</td>
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<tr>
<td>C3 convertase decay</td>
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<table>
<thead>
<tr>
<th>Signal molecules</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>Name</td>
<td>Pathway</td>
</tr>
<tr>
<td>C4a</td>
<td>Classical, Lectin</td>
</tr>
<tr>
<td>Anaphylatoxin</td>
<td></td>
</tr>
<tr>
<td>C3a</td>
<td>Classical, Lectin, Alternative</td>
</tr>
<tr>
<td>Anaphylatoxin</td>
<td></td>
</tr>
<tr>
<td>C5a</td>
<td>Classical, Lectin, Alternative</td>
</tr>
<tr>
<td>Anaphylatoxin</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Membrane binding proteins and opsonins</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Pathway</td>
</tr>
<tr>
<td>C4b</td>
<td>Classical, Lectin</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td></td>
</tr>
<tr>
<td>C3b</td>
<td>Classical, Lectin, Alternative</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td></td>
</tr>
<tr>
<td>iC3b</td>
<td>Classical, Lectin, Alternative</td>
</tr>
<tr>
<td>Phagocytosis</td>
<td></td>
</tr>
</tbody>
</table>
Functionally, this array of complement proteins in blood and interstitial fluids together supply the immune system with multi-defence strategies upon activation (169), (Fig. 1.8). These include direct lysis of targeted surfaces through MAC assembly (C5b-C9), elicitation of an inflammatory response by potent pro-inflammatory anaphylatoxins and chemoattractants generation (e.g., C3a, C5a), opsonisation and removal of targeted surfaces via the opsonic complement fragments C4b, C3b, iC3b which bind to their corresponding complement receptors (CRs) on phagocytes like macrophages and neutrophils (170-172). The complement system has anti-inflammatory functions too, as it binds to immune complexes and apoptotic cells and helps in their clearance from the circulation and damaged tissues (119).

![Figure 1.8 Consequent functional implications of complement activation, C. Salvador-Morales et al (169).](image)

**1.5.4 The “C4” component of the complement system**

Complement component C4 is a 203 kDa protein which serves as an essential part of the humoral immune response (173, 174). It can be
activated and cleaved by C1s and MASP2 serine proteases in the classical and lectin pathway respectively (174), such activation results in the generation of C4b (195kDa) and C4a (9kDa) (175). In addition to its role in opsonisation (175), the C4b fragment constitutes a subunit of the convertases C3 (C4b2a) and C5 (C4b2a.C3b), which are the enzymatic complexes that activate C3 and C5 of the classical and lectin pathways (130, 176), (Fig. 1.9).

Whilst the anaphylatoxin role of C4 is still controversial (178), the dissociation of this fragment from C4 leads to significant conformational alteration in the C4b molecule which is supportive in both structural and functional aspects (176).

C4 is produced as a single chain precursor protein that is cleaved enzymatically when secreted to form a triple chain molecule of around 210 kDa molecular weight (176, 177). This ternary structure is composed of three different chains including α (~95 kDa), β (~75 kDa) and γ (~33 kDa) which are linked by disulphide bonds (178). Despite sharing up to 30% sequence identity with C3 and C5, C4 is considered a much more sophisticated and developed protein because of the formation of the three chains and the display of posttranslational modifications including four N- and one O-linked glycosylations and sulphation of three tyrosine residues (175, 179), (Fig. 1.10).

![Figure 1.9 The function of C4 in the complement system: Proteolytic processing and function of C4 and C4b in the CP](image-url)
(Including C1s) and the LP (including MASP-1 and MASP-2). Active proteases are in bold font. C4b2a and C4b2a3b are the C3 and C5 convertases respectively, adapted from Mortensen et al (175).

Figure 1.10 A scheme that displays the multi-chain structure of C4 and C4b. Four N-linked (N226, N862, N1328, N1391) and one O-linked (T1244) glycosylation sites are labelled, Mortensen et al (175).

C4 concentration in body fluid is about 600µg/ml in serum (177, 180) and 3.3 µg/ml in cerebrospinal fluid (181), while it is around 5.6 µg/ml in tears (182).

During complement activation C4a (anaphylotoxin, ~9 kDa) is cleaved off the N-terminal of the α chain producing C4b (192 kDa). Cleavage of surface-bound C4b by factor I forms the soluble C4c fragment (~146 kDa) and the cell-bound C4d fragment (~45 kDa) (176, 183, 184). Complement C4c is composed of the C4 β chain (~75kDa), the C4 γ chain (33 kDa) and two of the α chain fragments; an N-terminal 27 kDa fragment (C4cα27) and a 16 kDa C-terminal fragment (C4cα16). These are linked by disulphide bonds as follows: C4β-S-S-C4α3-S-S-C4γ-S-S-C4α4(178). The structures of intracellular C4 precursor and the mature plasma form and its successive degradation molecules are represented in figure 1.11.
Figure 1.11 Polypeptide structure of intracellular prepro human C4 (above dashed line) and of mature plasma C4. For the latter, the polypeptide chain composition of its major activation and secondary degradation products are shown. Indicated are the N- and C-termini of chains, the sites of N-linked glycosylation (closed circle), the site of O-glycosylation (open circle), the thioester bond formed by the side chains of residues C1010 and Q1013, before and after transacylation to a hydroxyl nucleophile (ROH), the location of the isotypic cluster of residues and the sites of tyrosine sulphation. Only inter-chain disulphides are shown. The molecular masses indicated are for the polypeptide segments only; approximately 2 kDa should be added for each carbohydrate chain that is present, adapted from Isenman et al (178).
Complement C4 exists in C4A and C4B variants which share 99% of sequence and vary in six residues only, the plurality of individuals expresses both isoforms (174, 185). C4A and C4B differ in their haemolytic activities and covalent affinity to antigens and immune complexes, in addition to variation in serological reactivity (176). The C4b fragment from C4B tends to create a covalent ester bond with substrates that contain hydroxyl groups; this occurs in a very rapid and active reaction (177, 186, 187). Contrariwise, C4A derived C4b efficiently forms a covalent amide bond with amino groups from antigens (177, 187). C4A is much less efficient than C4B in binding with hydroxyl group containing antigens (176, 188). This difference may lead to many functional variations between C4 isoforms, C4A is more likely to be beneficial for assuring antibody-antigen aggregate solubilisation, or prevention of immune precipitation by binding to antigens in immune complexes or to immunoglobulin G, while C4B propagates the activation pathways that result in the MAC formation to destroy invading antigens (176). This variant binding affinity between C4 isoforms is attributed to the variation in the C4d region of the alpha chain, a region that also specifies Rodgers (Rg) versus Chido (Ch) blood group antigenic determinants (176, 184, 189).

Regardless of C4 isotype, formation of amide or ester bonds protects C4 against hydrolysis and stabilizes C4b on the surface of the pathogen, which in turn restricts the complement activity to the targeted site (187).

Interestingly, this variability of C4 may make this protein more qualified to interact with some other molecules where the establishment of ester or amide bonds is possible. In the same vein, Pietrocola et al. have found that the domain C4b binds to what they called complement interfering protein (CIP) which is produced by the group B Streptococcus (GBS) (190). Furthermore, this interaction between CIP and C4b hindered the latter association with C2 and supposedly suppressed the formation of C3 convertase (C4b2C2a) which consequently decreased the CP and MBL complement activity,
where C4 is the main participant in these pathways (190). In that sense, it seems a matter of importance to study the binding affinity of some molecules that show an inhibitory role to complement activation. From this point, it was interesting to investigate the binding affinity of histones to the complement proteins after I discovered their effects as complement activity suppressors; this can help to understand how extracellular histones affect immune responses in many histone-related medical conditions including sepsis which may contribute to developing a proper strategy of therapy in such clinical cases.

1.5.5 Complement pathways: Different starts, one terminal

In general, complement system activation events occur via three common pathways including CP, LP and AP (191). However, recent researchers have found some other bypass pathways that cause non-traditional complement activation (192-195).

With regard to the classical pathway, the C1 protein multiplex prompts this pathway by binding to variant ligands including antibody-antigen complexes, pathogen surfaces, apoptotic cells and their derivatives, and poly-anionic structures (166, 196). This complex is composed from the pattern recognition subunit C1q which associates with tetrameric proteases C1r2C1s2 calcium dependent complex, in which C1s has high structural pliability that gives it many functional advantages (196, 197). When C1q binds to a ligand surface, detachment of C1r-C1s dimer pairs from the C1 complex leads to C1r auto-activation which in turn activates C1s (166, 197), the latter cleaves C4 and the subcomponent C4b thereafter binds with C2 to form the C3 convertase C4b2a after C2a is being generated via C1s activation (198). As a result of C4b2a enzymatic activity, C3 is cleaved to generate the anaphylatoxin C3a and the C3b molecule which also has opsonic activity binds to C4b2a to produce the C5 convertase C4b2aC3b (199), the latter splits C5 into the powerful
anaphylatoxin C5a. Anaphylatoxins have different controlled functions such as provoking inflammatory mediator release, vascular permeability increase and smooth muscle contraction (200). However, unregulated anaphylatoxin release may lead to serious consequences (200, 201). The crucial role of the other fragment C5b is the initiation of MAC complex formation (C5b-9) to lyse cells via insertion and functional pore construction on the targeted cellular membranes (136), (Fig 1.12).

**Figure 1.12 Complement system overview:** Pathway specific PRMs, interactions and subsequent activation cascade. Garred *et al* (202).

Despite sharing most of the cascade events with the CP pathway upon C4 cleavage, the MBL activation pathway has a different initiation trigger with regard to the PRMs and antibody independent activation (166, 203).
According to the recent complement research, the lectin pathway PRMs are widely expanded to include MBL, collectin-10 (CL-10 or CL-L1), collectin-11 (CL-11 or CL-K1), ficolin-1 (M-ficolin), ficolin-2 (L-ficolin) and ficolin-3 (H-ficolin or Hakata antigen) (202, 204, 205).

In general, these lectin pathway initiators are composed of two structures, one is the collagen-like triple helical that is linked to a second one which is the pattern recognition as well as the descriptive structure, whereby MBL, CL-10, and CL-11 refer to C-type lectins (206, 207) and ficolin 1-3 indicate fibrinogen like proteins (207, 208). The serine proteases MASP1-3 mediate the lectin PRMs function (152, 163, 209).

Lectin PRMs are circulating in complex with zymogen MASPs (203, 210), binding of this complex leads to activation of MASP1 which subsequently activates MASP2, and the latter is able to cleave C4 and C2 to form the C3 convertase and go through a similar cascade to the classical pathway (203, 204, 211). Albeit at a slow rate, MASP-1 can directly cleave C3 and stimulate the AP pathway (119, 136), while MASP3 has an antagonist effect versus MASP2 as a part of activity regulation (198). Both MASP1 and 2 can be autoactivated but MASP1 can cleave C2 only (210, 212), hence MASP2 is a very important enzyme of the lectin pathway like the analogue C1s of the CP pathway (136).

In comparison with the classical pathway, the lectin PRMs circulate in lower concentrations as compared to C1q (135), but they overwhelm the latter by their ability to bind sugar moieties and acetyl groups belong to a wider scope of invading microorganisms and carbohydrates on apoptotic/necrotic cells (135, 166). Despite the structural and functional similarity between MASPs and the serine proteases C1r and C1s, the recognition molecule C1q can associate with tetramer of proteases (2 C1r, 2 C1s), while MBL and the ficolins seem to bind only one homodimer of each MASP (135, 213).
The alternative pathway has both unique initiation and activation events, despite converging with CP and MBL pathways in terminal MAC formation. Furthermore, it is distinctive in the sense of being active constantly at low level, thus ready to tackle any infection quickly by the slow hydrolysis of C3 (Tick over) (203, 214). In the same distinctive way, this pathway is primed without the conventional PRMs in the CP and lectin pathways, while C3 itself plays the role of recognition molecule (136).

However, some molecules can initiate the AP pathway like properdin and P-selectin by recruiting C3 (H₂O) and C3b to the cell surface, whereby they bind directly to the targeted cell surface and act as platforms for local AP pathway C3 convertase formation (135, 153, 212). (Fig. 1.13). Nevertheless, a recent paper has challenged the idea of considering properdin as a PRM, suggesting that binding of this molecule happens only upon C3 activation for the known purpose of stabilising the AP C3 convertase (215).

Figure 1.13 Platforms for AP pathway activation. (A) Properdin is released from activated neutrophils and is bound to the cell membrane where it recruits C3b to compose the alternative pathway C3 convertases. C5a then trigger additional
neutrophils which secrete more properdin. This stimulates a vicious cycle of neutrophil and complement activation. (B) Properdin released from neutrophils or in the plasma binds to activated platelets enhancing C3 (H$_2$O) recruitment and complement activation. (C) Stimulation of endothelial cells with C3a, heme, or other agonists induces expression of P-selectin. P-selectin contains CCP domains and binds C3b, promoting formation of C3 convertases that generate more C3a to stimulate cells, adapted from Merle et al (212).

The view of properdin as a pattern recognition molecule is therefore challenged, and it is argued that properdin typically binds a complement-activating surface subsequent to C3b in order to stabilize the alternative pathway C3 convertase.

On the other hand, many types of carbohydrate structures which exist on the surfaces of bacteria, yeast and multicellular parasites are considered activating surfaces for many C3b binding sites, whereby they initiate the AP cascade via direct binding with C3b (119, 198, 203, 214).

With respect to the tick over process, native plasma C3 goes through reversible structural alterations and displays the thioester-containing domain (TED) (135, 214). This domain is hydrolysed thereafter to form a C3b structural and functional like molecule C3 (H$_2$O), the latter then undergoes conformational configuration and exposes a binding locus for Factor B (fB) (216, 217).

Factor D (fD) cleaves fB to release Ba, resulting in the fluid phase C3 convertase C3 (H$_2$O) Bb (136, 218), this complex is unstable and can be dissociated by the regulatory fH which allow fI to split C3b to iC3b (136). Yet, properdin has been shown to increase the stability of this fluid phase complex and form C3(H$_2$O)BbP, which leads to a tenfold increase its half-life (153). In the absence of pathogen or other
activating surface, the C3b that is generated upon C3 convertase activity will be degraded (214).

In case of activating surface availability, C3b binds covalently to the surface and is able to form new C3 convertase in the presence of factors B and D (119, 214).

Thus, C3b plays an important role in the ‘amplification loop’ mechanism which supports all of the pathways including the AP itself (198). For this reason, the AP pathway should be regulated firmly due to its potent role in rapid amplification and targeting host tissues for C3b deposition and subsequent complement-mediated destruction (214).

Binding of the AP pathway C3 convertase C3bBbP to another C3b domain covalently will create C3bBbP3b, which is the C5 convertase of the alternative pathway (135). In general, these convertases are more potent when assembled on a solid phase such as the cell membrane as compared to the fluid phase (136). Upon C5 cleavage, the AP pathway shares the same terminal events with CP and Lectin pathways (167, 219).

1.5.6 Bypass pathways

Interestingly, it has been reported that complement and coagulation pathways overlap with each other (220). The research work on interconnection between these two systems has developed a new term in this field that is called the “Extrinsic protease pathway” (220, 221).

Accordingly, it was demonstrated that central coagulation factors such as thrombin, plasmin, FXa and FXIa can directly cleave C3 and C5 (214), stimulating an AP pathway mediated complement activation, and generation of C5a which triggers a local inflammation (118, 222).
Moreover, it was shown that complement activation can up-regulate the coagulation system and this process is mostly localized at complement deposition sites (214).

On the other hand, some coagulation system components like thrombomodulin can act as a down-regulator factor for complement activation via FI mediated C3b inactivation (126).

One of the novel bypass mechanisms is intracellular complement activation; in which locally produced C3a and C5a activate T cells via binding to their particular receptors on these lymphocytes (223-225).

Other bypass scenarios include direct cleavage of C3 by MASP-1, bypassing C4b2a (119, 226-228). It has been proposed that C1q or MBL binding with C3b protect against FI and fH down regulating role (193). However, these mechanisms are still under research.

In the same vein, other C2 bypass pathways have been reported, in which C4 can form a hybrid classical/AP C3 convertase (C4bC3bBb) (229) or even enhance C3bBb fixation on tissues (230).

Regarding C3 independent mechanisms, Huber-Lang et al have revealed that macrophage and neutrophil serine proteases can directly split C5 to its subcomponents bypassing the need of C3 (231). However, it is unclear whether such kind of interaction is sufficient to go through the terminal cascade and form C5b-9 or not.

1.5.7 Complement system regulation

The complement system cascade is orchestrated via many soluble as well as membrane bound regulators (120), where this occurs in a complex type of physiological process (232). Fundamentally, complement regulators protect the surfaces of entire host cells from complement targeting and activation (233).

Since eliminating apoptotic particles and necrotic cells from the body is one of the complement system functions, in such cases complement regulatory proteins would permit complement activation to the extent
that it carries out this precise mission, in the sense of blocking any further cascade activation other than C3b surface deposition (131). Furthermore, even in case of the invasion of pathogens and their related derivatives, the complement cascade works in a tightly regulated manner (131).

Soluble complement regulators are present in plasma and other body fluids, and include factor H, factor H-like protein 1 (FHL1), properdin, carboxypeptidase N, C1 inhibitor, C4BP, complement factor H-related protein 1 (CFHR1), clusterin, and vitronectin1 (233). Membrane-bound regulators include CR1, complement receptors, membrane cofactor protein (MCP; CD46), DAF (CD55), and CD59 (131, 166, 232, 234, 235), (Table 1.2).

As compared to membrane-bound regulators, the soluble ones exert pathway specific regulatory effects and inactivate either C3 or C4, while the membrane-bounded regulators can suppress both C3 and C4 and act on all pathways generally (233, 236). These regulators act as cofactors and/or by accelerating the decay of the convertases (232, 235).

Dysregulation of the complement system can cause different pathological consequences, such as types of autoimmune diseases and infection onset (232, 237, 238). The aberrant complement activity that leads to such pathological consequences may due to genetic deficiency of complement proteins and subsequent activity reduction, or hyper-activation results from decrease in expression of complement down-regulators (135).

Thus, it is vitally important that this system holds a proper balance between activation on pathogens and modified self-cells versus inhibition on intact host cells (232, 233).
Table 1.2 Complement regulators, Bajic et al (166).

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Ligand</th>
<th>Function</th>
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<tbody>
<tr>
<td>Soluble regulators</td>
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<td></td>
</tr>
<tr>
<td>Factor H (FH)</td>
<td>C3b, C3d</td>
<td>AP C3 convertase decay and cofactor</td>
</tr>
<tr>
<td>FHL-1</td>
<td>C3b</td>
<td>AP C3 convertase decay and cofactor</td>
</tr>
<tr>
<td>CFHR1</td>
<td>C3b, C3d, C5 convertase</td>
<td>Inhibits C5 convertase, competes with FH, acts as decay for certain pathogens</td>
</tr>
<tr>
<td>CFHR2</td>
<td>C3b, C3d, AP C3 convertase</td>
<td>AP C3 convertase decay, competes with FH</td>
</tr>
<tr>
<td>CFHR3</td>
<td>C3b, C3d</td>
<td>Competes with FH</td>
</tr>
<tr>
<td>CFHR4</td>
<td>C3b</td>
<td>AP C3 convertase decay</td>
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<tr>
<td>CFHR5</td>
<td>C3b, C3/C5 convertase?</td>
<td>Competes with FH</td>
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<td>Factor I (FI)</td>
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<td>Serine protease, degrades C3b, iC3b, C4b, and iC4b</td>
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<td>Properdin (FP)</td>
<td>C3b, C3bB, C3bBb, GAGs</td>
<td>Stabilizes AP convertases</td>
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<td>C4b, S protein</td>
<td>CP C3 convertase decay and cofactor</td>
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<tr>
<td>C1-INH</td>
<td>C1r, C1s, MASP-2s</td>
<td>Serine protease inhibitor</td>
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<td>MAP19</td>
<td>MBL and ficolins</td>
<td>Competes with MASP-2 in vitro</td>
</tr>
<tr>
<td>MAp44</td>
<td>MBL and ficolins</td>
<td>Blocks MASP transactivation</td>
</tr>
<tr>
<td>Clusterin</td>
<td>C7, C8, C9, MAC</td>
<td>Inhibits MAC</td>
</tr>
<tr>
<td>Vitronectin</td>
<td>C5b-C7, MAC, GAGs</td>
<td>Inhibits MAC</td>
</tr>
<tr>
<td>Membrane-associated and transmembrane regulators</td>
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<tr>
<td>MCP (CD46)</td>
<td>C3b, C4b</td>
<td>Cofactor activity, T-cell differentiation and activation</td>
</tr>
<tr>
<td>DAF (CD55)</td>
<td>C3b, C4b, AP and CP C3 convertase</td>
<td>AP and CP C3 convertase decay</td>
</tr>
<tr>
<td>CD59</td>
<td>C8, MAC</td>
<td>Inhibits MAC</td>
</tr>
<tr>
<td>CR1</td>
<td>C3b, C4b</td>
<td>AP and CP C3 convertase decay and cofactor</td>
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1.5.8 Complement function in health and disease

1.5.8.1 Complement in health

There is increasing evidence showing the novel roles that complement proteins have in the body, a matter that paved the way for interesting fields of translational research (122, 123). Complement proteins function in developmental, proliferative, and regenerative pathways in both injured and uninjured tissue (123, 239, 240), (Fig. 1.14).

C3a and C5a contribute to hepatic regeneration by their function of signalling on cytokine and transcription factor expression (241, 242). In addition, complement proteins are also associated with the regeneration of bone, cardiac muscle and skeletal muscle, as well as neurons (243, 244). Thus, it became unambiguous that the complement system undertakes unconventional roles, as well as the traditional inflammatory functions (122, 243).

Figure 1.14 Complement proteins take part in proliferative and regenerative processes in organs including bone, marrow, liver, and connective tissues. Rutkowski et al (123).
1.5.8.2 Complement in disease

The protective role of the complement system to the host as well as its several defensive and innate/adaptive immunological functions is now unambiguous (120, 125, 167, 245, 246). However, any disturbance in this system balance may lead to serious harmful effects, (Fig. 1.15). Disorganised complement activity may be due to several factors including excessive activation, insufficient regulation or/and complement component deficiency (135, 238, 247).

![Figure 1.15 Complement mechanisms of dysregulation, adapted from Harris (247).](image)

Complement hyper-activation with respect to duration or intensity is due to prolonged existence or overwhelming amounts of complement activators such as bacteria, yeasts and damaged host tissue, otherwise excessive complement activity may result from reduction of complement down-regulator expression (130, 135). Such over-activation associates with many pathological and clinical conditions like multiple sclerosis, rheumatoid arthritis, IR, Alzheimer’s, asthma, chronic obstructive pulmonary disease (COPD), sepsis and hyper-acute organ rejection (131, 135), where the intensive complement
response boosts the hyper-inflammatory events in a way that exceeds the initial trigger of destructive effects (130).

In sepsis, complement seems to have a devastating influence in this disease despite being beneficial in the early stages (248). Complement and particularly C5a may lead to organ damage in late stages of sepsis by consumptive coagulopathy in cooperation with a burst of cytokine flow (135, 249). Consequently, C5a affects the immune cells and stimulates an uncontrolled coagulation by C5a mediated TF expression that results in the harmful impact which is seen in sepsis (131, 248, 250).

Many approaches have been made regarding complement therapeutics, including C5 inhibition, pathway initiation prevention, attenuating the amplification loop and complement effector function blocking, (Fig. 1.16). Administrating the C3 inhibitor compstatin to a baboon model of late-stage sepsis significantly saved organs and improved other clinical biomarkers, advocating the use of complement inhibitors as interventional drugs in the second stage of severe sepsis (251, 252). Moreover, Colley et al suggested that C5aR1 and C5aR2 receptors blocking with monoclonal abs has therapeutics effects where C5a mediates the inflammation in sepsis, ischemia-reperfusion injury and similar inflammatory conditions (253). However, many factors still represent a challenge for anti-complement therapy including thorough comprehension of disease mechanisms, disturbing the delicate system balance, adverse effects and complement proteins concentrations (131, 247, 254).

On the other hand, less complement activity can result primarily from genetic deficiency of complement proteins or may occur temporarily due to complement consumption via certain conditions like infection, injury or surgery (135, 167, 238). Deficiency of the early component of the CP in addition to C2 and C4 is often connected with SLE (131, 255), whereas insufficient C3 causes bacterial infection susceptibility (256). The lack of the factors fI or fH leads to C3 deficiency-like
consequences, due to subsequent impaired iC3b generation and hence phagocytosis (135, 256). Lack of MBL is linked to respiratory tract infections, while MASP2 deficiency results in recurrent infections (131). Deficiency of fH and disorder of C3 tick-over may lead to renal diseases including typeII membranoproliferative glomerulonephritis and atypical haemolytic-uremic syndrome (aHUS) (257, 258), whereas properdin deficiency was found to increase *Neisseria meningitides* mediated mortality (259). C5-C9 shortage correlates with predisposition to neisserial infections and susceptibility to dextran sulphate sodium-induced murine colitis (135, 260-262). Figure 1.17 shows several pathogenic conditions due to local or systemic complement disorders.

**Figure 1.16 The complement drug development scheme.**

Complement-targeted drugs and drug candidates that are in preclinical or clinical development as of September 2017 are shown. This schematic was found depending on publications, conference abstracts and publicly available information on company websites. The main target is listed beside each drug name. In the preclinical part, drug candidates are ordered according to target, and their order does not reflect the stage of development. In the phase I through phase III parts, drugs are
ordered chronologically according to the start date of the clinical trial; only the ultimate advanced trial is reported for each candidate drug, Ricklin et al (254).

Figure 1.17 Major mechanisms of the pathogenic involvement of complement in systemic and local disorders. Even when the complement system is operating ordinarily, adverse activation can occur upon exposure to enormous amounts of pathogen, DAMP stimuli or foreign surfaces such as transplanted organs or biomaterials. In several chronic disorders, genetic alterations result in a systemic or local imbalance of complement that can lead to inflammation, thrombosis and tissue damage. Insufficient removal of apoptotic cells, debris or immune complexes due to clearing capacity of the complement system being overwhelmed or lack in complement components can prompt or aggravate autoimmune and neurodegenerative diseases. aHUS, atypical haemolytic uraemic syndrome; AMD, age-related macular degeneration; C3G, C3 glomerulopathy; CARPA, complement activation-related pseudo allergy; PNH, paroxysmal nocturnal haemoglobinuria; SIRS, systemic inflammatory response syndrome; SLE, systemic lupus.
Substitution with fresh frozen plasma or either purified complement proteins like MBL or C1-INH is a reconstitutive strategy that been used to tackle the primary complement deficiency problem (167, 263, 264).

All in all, both too much and not enough complement lead to pathological consequences as this double edge sword like system is meant to be tightly regulated to keep the body safe (135, 265).

1.5.9 Complement detection assays.

In order to thoroughly evaluate the potency of the complement system, researchers have developed different methodologies that are based on three major parts, which are: Functional assays, complement analysis and the detection of activation products (Neoepitopes) (129).

Haemolytic assays were used usually to estimate complement activation in the CP and AP pathways (169, 199, 266, 267). In the classical CH50 test, this method depends on the use of IgG antibody coated sheep erythrocytes (EA) which are lysed in serum diluted in a Ca\(^{++}\) and Mg\(^{++}\) containing buffer, as these ions are important for these pathways’ convertase stability (169, 268), the results are commonly represented as the reciprocal dilution of serum needed to lyse 50% of a fixed amount of EA (269, 270).

AH50 is the corresponding test to detect the AP activity, it requires rabbit (RbE) or guinea pig (GpE) erythrocytes for AP activation in serum that is diluted in a buffer containing only Mg\(^{++}\) ions, while Ca\(^{++}\) ions should be chelated with EGTA to block the classical and lectin pathways which are dependent on both ions rather than Mg\(^{++}\) only like the AP (129).
To be mentioned, the haemolysis assay has many limitations despite being readily carried out, these includes the short shelf-life of SRBCs, the inconsistent inter-lot performance of SRBCs, and the low sensitivity of the assay with regard to measuring the activation product such as C5a and SC5b-9 (169).

Enzyme immunoassay (EIA) techniques are widely used in complement work. Initially, this method was first described by Zwirmer et al who found that many complement proteins could be deposited on an appropriate solid phase during complement activation including the terminal products (271).

Thereafter, developing EIAs for inspecting the deficiencies of the classical and the alternative pathways was ongoing (272), to specifically activate an individual pathway, plates were coated with IgM, mannan and LPS to stimulate the CP, MBL and the AP respectively (129, 273, 274).

EIAs tests that include the three pathways were developed and they are commercially available nowadays (Wieslab Complement System Screen, Wieslab AB, Malmö, Sweden) (129, 275, 276). This assay has many advantageous features over the hemolytic assays, since it is easily implemented, does not rely on sensitized erythrocytes, comprises the three pathways and easy fP deficiency detection in the AP (129).

Serum samples are used in this method to detect activation via detecting the complement cascade terminal product (MAC), by targeting the neoepitope of C9 with anti-C5b-9 abs (277), (Fig. 1.18). Other techniques to quantify complement activity via MAC detection or other earlier components and their activating products may include ELISA, cell-ELISA, flow cytometry, immunofluorescence assay (IFA) and Western blotting (275, 278-283).

Zymosan is a polysaccharide prepared from the cell wall of actively growing yeast *Saccharomyces cerevisiae* (284, 285). This molecule
has served as a mimic model for microbial recognition for many decades, and was incorporated in many innate immunity studies including stimulation of inflammatory mediator production, mechanisms of phagocytosis, and activation of complement deposition (132, 286, 287).

![Figure 1.18 EIA principle for assay of activation of the three distinct complement pathways (Wieslab Complement System Screen®). Adapted from Harboe et al (129).](image)

In respect of complement activation, zymosan has been used both in vitro and in vivo to activate CP, MBL and AP in several complement research studies (279, 288-293). Thus it represents a good choice to stimulate the complement system cascade and study the difference in the complement proteins levels or the total terminal activity versus different assigned conditions (279, 294).

There are three types of epitope on a complement protein and its subsequent activation fragments, therefore the specificity of antibodies is a very important consideration while studying complement activation in the laboratory (129, 276, 295), (Fig. 1.19).
Selecting the proper type of anticoagulant is important while studying the complement activity in whole blood; the recombinant hirudin analogue lepirudin has been shown to be a suitable anticoagulant without adverse effects on complement as compared to other anticoagulants like EDTA (129, 296).

**Figure 1.19 Activation-dependent epitope specificity.** Specificity of epitope is pivotal in studies of complement activation. Three distinct types of epitope on a complement component and its activation products detectable by monoclonal antibodies are indicated: “native restricted epitopes” which exist only in the native form of complement component; “activation independent epitopes” are present both in the native component and in the activation product, while “neoepitopes” are existent on activation products only. Adapted from Harboe et al (129).

The patient’s complement profile which reflects the disease severity and the pathophysiological role of complement used to be variable (235, 277, 283). Consequently, complement tests have an important role in the disease follow up, monitoring complement-targeted therapy
as well as the initial disease diagnosis (277). Figure 1.20 displays the recent complement diagnostic approaches.

Figure 1.20 Overview of major classes of complement diagnostic approaches, In addition, to the methods shown here, genetic analyses become increasingly prevalent in the complement diagnostics field. Ricklin et al (277).

1.5.10 Complement role in innate immunity and phagocytosis

The complement system represents an old arm of innate immunity that bridges the adaptive immune response and has expanded to cross talk with other systems such as coagulation (119, 220, 297). Consequently, this sophisticated network of interactive proteins has association with many events of the innate immune system including direct killing of pathogen by MAC, inflammatory response stimulation via its active anaphylatoxins and phagocytosis, whereas
the latter is the indirect strategy that the complement mediates to clear pathogens (114, 298).

Phagocytosis is a receptor-mediated, actin and ATP-dependent mechanism that is stimulated via binding between the targeted surface antigens to the membranous receptors of the phagocytes like macrophages, dendritic cells and polymorphonuclear leucocytes (299, 300).

There are two types of phagocytes receptors, non-opsonic receptors that can straightway recognize the targeted surface like PAMPs, the other type is TLRs that help with other receptors in the uptake of a target and the phagosome modulation (301, 302). The Opsonic receptors are the other main type of phagocytosis associated receptor; they include the heterogeneous binders of the Fc region of immunoglobulins as well as the complement receptors like CR3 which are able to distinguish complement coated particles (301-303). Opsonisation is a vital mechanism to enhance phagocytosis and helps in efficient pathogen clearance (304), where an opsonin like C4b, C3b, iC3b act as adaptors that bind and activate potent phagocytic receptors (305), (Fig. 1.21.A).

The process of phagocytosis is basically performed to internalise and engulf large particles (≥0.5µm), microorganisms, and cell debris then to degrade them and eventually present peptides derived from particulate antigens (306-308). Binding of a targeted particle-opsonin complex to one of the phagocytes’ opsonic receptors stimulates the rearrangements in the actin cytoskeleton which results in the internalization of the opsonised particle (132, 307).

The term “Efferocytosis” refers to the process where apoptotic cells are scavenged by phagocytes (309). Some structural rearrangements accompany phagocytosis including membrane deformation, phagocyte pseudopod expansion, and their subsequent contraction to ingest particles and form an endocytic vesicle known as a phagosome (300, 310, 311). Microbes and apoptotic cells submit to variant oxygen
dependent and independent processing upon being ingested inside the phagosome (302, 305).

Pathogen breakdown results in further ligand release which is detected by receptors on phagolysosomal membrane or in the cytosol, a matter that stimulate the inflammatory response, while the expression of digested bacterial peptides on the active phagocytes’ major histocompatibility complex class II enhances acquired immunity (305, 312).

In addition to the effect of complement activating products (C4b, C3b and iC3b) on phagocytosis via opsonisation (172, 313), the release of anaphylotoxins C3a and C5a contributes to phagocytosis triggering by recruiting more phagocytic cells to the inflammation site which promotes the uptake and destruction of pathogens by phagocytic cells (165, 172, 199), (Fig 1.21.B).

Several assays have been developed in order to evaluate opsonophagocytic potency and are used in vaccine production by testing whether the vaccine-induced antibodies drive efficient complement deposition and subsequent opsonophagocytic killing (314-316).

Thus, any disorder in complement activation kinetics or interference with complement opsonic molecules may lead to impacts on complement mediated phagocytic function and host defence (317, 318), which is a vital mechanism of rapid immune defence since opsonisation via complement molecules can occur even before the CP that relies on antibodies and mounting of an adaptive immune response (172).
Figure 1.21 Effects of complement activation on phagocytosis. A: Phagocytosis enhancement via opsonisation. B: Activation products like C3a and C5a recruit more phagocytes and trigger the phagocytosis process by binding to the N-terminal regions of granulocytes including the phagocytic cells. Dunkelberge et al (125).
1.5.11 Hypothesis of the proposed work and aims

The role of extra-cellular histones in inflammation and tissue injury was established well in several diseases, whether they are part of neutrophil extracellular traps NETs or necrotic cell debris (31, 35, 37, 39). Little research has demonstrated the relationship between histones and the complement system which is the part of innate immunity that tackles both PAMPs and DAMPs, Leffler et al. reported NETs as potent complement activators and clarified that this interaction may have a main role in SLE, targeting complement with inhibitors or by elimination of complement activators like NETs could be advantageous for SLE patients (319). Bosman et al. found that complement activation via C5aR and C5L2 triggers the release of nucleosome components such as histones, resulting in a cycle of tissue injury and inflammation (55).

On the other hand, other studies linked complement activity alteration to some other microbial protein secretion interference (190), they revealed that some microbial products are able to reduce complement activation and cause further innate immunity impairment (320).

Recently, the group used a pull-down assay and found that histones interact with complement C4. We hypothesize that histones may affect the complement activation and subsequently affect phagocytosis. Therefore, the aim of my study was to 1: Demonstrating the binding affinity pattern between histones and C4 through different tests and under variant conditions (denatured and physiological) 2: Investigate the potential effect of histones bound C4 on complement activation and related phagocytosis.
Chapter 2: Extracellular Histones Show High Binding Affinity to Component C4

2.1 Introduction

The Complement system forms a major part of the host response to infection and cellular injury (130, 131). This system is intricately involved in these processes and consists of a cascade of more than 50 proteins, participating in three activation pathways; namely the classical (CP), mannose binding lectin (MBL) and alternative pathways (AP) (131). The classical pathway recognises antigen-antibody complexes contained on the surface of pathogenic factors, including gram-negative bacteria, viruses and damaged cells (321). The MBL pathway binds mannose containing pathogenic surfaces (322). Finally, the alternative pathway directly targets surface carbohydrate regions on pathogens such as viruses, bacteria and fungi (323).

These pathways are linked into a common termination pathway, leading to the formation of the membrane attack complex (MAC), this complex is amphipathic and composed of C5b, C6, C7, C8 and several C9 molecules and causes lysis to the pathogen (123, 128, 129).

Although the complement factors in circulation are generally produced in liver, it is now understood that most tissues and inflammatory cells like neutrophils and macrophages, are effective origins of complement factors (154-158). On the other hand, recent studies have shown the essential impact of complement deposition and anaphylatoxin-mediated signalling on active surface, which leads to adaptive immune response propagation (159-161)

Many products generated during complement activation are also able to opsonize damaged cells or pathogens to facilitate phagocytosis (130, 131). In this way, complement activation enhances the ability of antibodies and phagocytic cells to clear invading pathogens and cellular debris from the circulation (132).
Complement activation not only kills pathogens but also damages host cells during an inflammatory reaction and excessive activation contributes to inflammation-driven tissue injury (324). Host cell lysis will release cell breakdown products, including DNA and histones, and those damage-associate molecular patterns (DAMPs) have been demonstrated to play important roles in disease progression and host immune response (14, 325, 326). Histones, the most abundant and important DAMPs, can be detected in blood taken from many critical illnesses, such as severe trauma (35), severe sepsis (34, 37) and necrotising pancreatitis (327).

Histones are positively charged proteins and have high affinity for negatively charged phospho-groups in DNA or cell membranes. Normally, they are highly alkaline intra-nuclear proteins that are present in eukaryotic cells (20). The basic function of these nuclear proteins is to pack and sort the DNA into functional units (Nucleosomes) as well as gene regulation (24). Misplaced histones in the extracellular space can display a toxic and pro-inflammatory distressing side both in vitro and in vivo (34), this happens when they are released passively or actively by dead or triggered cells (20, 35). Furthermore, extracellular histones represent a cell death alarm that stimulates the immunological response as well as the body repair programme (36). Histones are also released through neutrophil extracellular traps (NETs), these traps have a significant protective role against exogenous pathogens (25, 26).

Histone binding to cell membranes allows ions, particularly Ca\(^{2+}\) influx into cells to cause harmful effects to cells contacted (35). In addition, histones are also the ligands of Toll-like receptors (TLR)-2, 4 and 9 receptors to trigger immune response; including inflammasome activation and cytokine release (54, 62, 66).

Histones also interact with coagulation factors in the circulation to promote thrombin generation, fibrin precipitation and systemic coagulation activation (40, 61, 63, 71, 328). In animal models,
extracellular histones have been shown to mediate multiple organ injury and even death in sepsis (34, 329). Clinically, correlation between circulating histone levels and organ injury as well as disease severity has also been demonstrated (37).

Recently, we found that extracellular histones interact with complement component 4 (C4) protein. Complement C4 coded by both C4A and C4B genes is synthesised into a single peptide (Precursor) and then cleaved into α (98kDa), β (73kDa) and γ (33kDa) chains (176, 330). Upon complement activation, C4 is cleaved by the C1s enzyme into C4a and C4b, the latter mainly participates in classical and lectin pathways by forming C3 and C5 convertases, whilst C4a as an anaphylatoxins enhances smooth muscle contraction, histamine release and vascular permeability as well as serving as a chemotaxis and inflammatory mediator (331, 332).

In many disease conditions, particularly in sepsis, complement activation (333) and histone release (37) coexist. The outcome of histones binding to C4 appears important and this study is aiming to understand the pathophysiology related to the complement system and extracellular histones.

2.2 Materials and Methods

2.2.1 Human plasma and serum

Citrated plasma and serum were isolated from whole blood drawn from critically ill patients according to the protocol granted by Liverpool Adult Ethical Committee (Ref: 13/NW/0089). Normal human serum was purchased from CompTech, USA.
2.2.2 Fractionation of human serum and plasma by ultracentrifugation

Citrated plasma and serum (1 ml) from patients were fractionated by ultracentrifugation at 274,400 x g (4°C) for 1 h and then six layers of equal volumes fractions (166 µl/fraction) were collected. Histones and Histone-DNA complexes were then measured in each fraction by Western blot and ELISA (Cell Death Detection ELISA PLUS; Roche, West Sussex, U.K.), respectively, as previously described (35).

2.2.3 Isolation of histone binding protein from plasma and mass spectrometry analysis

Isolated citrated plasma was diluted 1:2 with phosphate buffered saline (PBS) (v/v) and centrifuged to eliminate insoluble contents. The harvested supernatant was then pre-cleared using blank Sepharose resin and then loaded on CNBr-activated Sepharose 4B (GE Healthcare, Little Chalfont, UK) column conjugated with calf thymus histones (Roche, West Sussex, UK). After a high stringency wash with PBS+0.5% (v/v) Tween-20 (Sigma-Aldrich, Dorset, UK) followed by PBS, histone-binding proteins were eluted and separated by gel electrophoresis. Gel slices from SDS-PAGE were washed (2 x 30 min) with 50% acetonitrile, 0.2M ammonium bicarbonate pH 8.9 and then dried in a rotary evaporator. The slices were re-swollen in rehydration buffer RHB [2 M urea, 0.2 M ammonium bicarbonate pH 7.8] containing 0.2 µg trypsin and incubated at 37°C overnight. Excess RHB was then removed and peptides were extracted from the gel slices with 60% acetonitrile, 0.1% TFA. The total peptide extract was concentrated in a rotary evaporator and then desalted using C18 ZipTips according to the manufacturer’s instructions. MS analysis was performed using a MALDI-Tof instrument (Waters-Micromass) using a saturated solution of alpha-cyano-4 hydroxycinnaminic acid in 50% acetonitrile/0.1% trifluoroacetic acid.
2.2.4 Detection of Histone-C4 complexes by ELISA

Histone-C4 complexes were detected in normal and critically ill patient plasma by modified Cell death detection ELISA PLUS (Roche) with modification. In brief, normal plasma was pre-incubated with different concentrations of histones for 10 minutes prior to ELISA, allowing for histones to bind to C4 within the plasma.

For the ELISA procedure, diluted plasma was incubated for 2 hours at room temperature with a biotinylated-histone capture antibody, in streptavidin coated 96 well plates (Cell death detection ELISA PLUS, Roche). Samples were washed and subject to sequential 1 hour incubations with anti-human C4 abs (Abcam) and anti-rabbit-HRP antibodies. Histone-C4 complexes were calculated in normal and patient samples (arbitrary units; AU) based sample absorbance (450nm), compared to those of the standard curve.

2.2.5 Gel overlay assay with C4 and histones individually

Equal molar concentration of H1, H2A, H2B, H3 and H4 besides 4µg of S100P (as a control) were loaded into a 15% SDS-PAGE gel then subjected to Western blotting with 0.5µg of C4 and 4µg of S100P which is the negative control. Upon the transfer, each histone’s membrane was overlayed overnight at 4˚ C with overlay buffer containing the corresponding concentration of the recombinant histone (H1, H2A≈1.6µg/ml, H2B, H3 and H4 ≈1.26µg/ml). Later, particular anti-histone abs were used to detect the band of each overlay histone that might bind to the loaded C4. Recombinant histones (Produced in an E. coli strain) were purchased from New England Biolabs (Massachusetts, USA), while the anti-histone abs and anti-C4 abs were made by Abcam (Cambridge, UK). C4 was purchased from Fitzgerald, USA.

Another experiment was designed to verify the binding affinity of each type of histone to C4. Four microgram of S100P and equal molar
concentration of H1, H2A, H2B, H3 and H4 in addition to 0.25µg of C4 were used in a 15% twin SDS-PAGE gels for the overlay assay and Coomassie staining. The membrane was overlayed with 10µg/ml of C4 at 4˚C overnight then incubated with anti-C4 abs.

2.2.6 Western blotting using HRP conjugated C4

To avoid the possible effect of antibodies, HRP-conjugated C4 was used to confirm the interaction between complement C4 and individual histones. Two µg of H2A, H2B, H3 and H4, 4 µg of H1 and 6 µg of S100P (as control) were subjected to Western blotting with HRP-conjugated C4 protein. Another identical gel was stained with Coomassie blue to demonstrate the equal loading. C4 protein was purchased from Fitzgerald, USA and conjugated to HRP using a Lighting-Link HRP conjugation kit (Innova Bioscience, Babraham, Cambridge, UK, ref. 701-0000) according to the procedure recommended by the manufacturer. The HRP-conjugated C4 was used to incubate the membrane at 4˚ C overnight and the bands were visualized using enhanced chemiluminescent (ECL) substrates (Thermo Fisher Scientific, USA).

2.2.7 Production of complement C4 Beta chain domains for gel overlay assay

2.2.7.1 Plasmid design and Production

In order to have a greater chance to get the desired protein, C4 beta chain production involved generating two domains which altogether form that protein, to eliminate a higher molecular weight protein production problem. Thus, Beta chain production work included the synthesis of recombinant proteins C4 1-3 and 4-5 by *E coli* (334), (Fig.2.1).
Figure 2.1 Peptide sequence of Complement C4 beta chain.

Domain 1-3 starts from no.20 and ends at the amino acid no.365 (Highlighted with light blue), while the rest of C4 β fragment (Domain 4-5) starts and ends at 416 and 725 amino acids respectively (Highlighted with yellow). Adapted from UniProt.
In summary, the peptide sequence was obtained from UniProt. The coding DNA sequence was then translated from peptide sequence and synthesised (335). A pET expression system was used for plasmid construction and proliferation (336). C4 domains 1-3/4-5 were subcloned individually into a pET-16b vector in an expression system that contains a T7 promoter and ampicillin resistant marker, as well as a His\textsubscript{10}-tag. The pET-16b-C4 domains expression constructs were sequenced to confirm the right coding sequences subcloned. The maps of these constructs were generated (Fig. 2.2.A and B) using SnapGene®.

The detailed procedure is as following: the coding DNA for human C4 beta chain 1-3 and 4-5 domains were synthesised by life technology containing BamHI and NdeI restriction sites. The DNA was amplified using polymerase chain reaction (PCR), extracted by QIAquick Gel Extraction Kit (QIAGEN, Netherlands) and digested with NdeI and BamHI restriction enzymes as inserts. Ten µl of pET16 (b+) DNA was also digested using the same enzymes as vector. The digested pET-16b vector (Novagen, Germany) was ligated with inserts using T4 DNA ligase. After transformation into DH5α competent cells, resultant single clones were cultured and plasmid DNAs were isolated using QIAprap Spin Miniprep Kit and checked using NdeI and BamHI digestion. Plasmids containing the correct sizes of DNA inserts were subsequently sequenced for confirmation. The correct plasmids were transformed into Shuffle® T7 and BL21 (DE3) \textit{E. coli}, to express domain 1-3 and domain 4-5 peptides respectively.
C4 β chain domain 1-3
pET-16b
6747 bp

C4 β chain domain 4-5
pET-16b
6639 bp
**Figure 2.2 Plasmids for C4 domains expression.** DNA of C4 domain 1-3 (A) as well as C4 domain 4-5 (B) were digested with BamHI and NdeI and ligated to pET-16b vector to form a novel plasmid for each C4 fragment production. The digested domains were subjected to an 1 % (w/v) agarose gel and showed identical base pair bands.

### 2.2.7.2 Protein induction

2 µL of each domain plasmid was transformed into Shuffle® T7 express *E. coli* and BL21 (DE3), for the production of C4 domains 1-3 and 4-5 respectively. The following day, single colonies were selected and each incubated in 3 mL LB broth containing 100 µg/mL (w/v) ampicillin for 1 h at 37°C. Bacteria were then induced at 18°C overnight with 1 mM IPTG (Melford, UK), with a non-induced colony acting as negative control. After incubation, a sample of 30 µL was analysed by 15% SDS-PAGE. Once the small scale induction was successful, a large scale induction was conducted. Bacteria were continually incubated in 500 mL LB broth containing 100 µg/mL ampicillin at 37°C and the bacteria optical density (OD$_{600}$) measured at 600 nm until reaching an OD reading of between 0.5 and 0.6. The bacteria were subsequently induced at 18°C overnight. Proteins from the non-induced and induced bacteria were then run on a 15% SDS-PAGE gel, (Fig. 2.3).
**Figure 2.3 β chains domains induction.** Domain 1-3 (Upper panel) and domain 4-5 (Lower panel) expression plasmids were transformed to T7 and BL21 *E. coli* respectively, then induced at 18°C with 1 mM IPTG overnight. The non-induced *E. coli* was used as negative control.
2.2.7.3 Protein purification using a Nickel-Nitrilotriacetic acid (Ni-NTA) resin (QIAGEN, USA) column

After large scale induction, bacteria were harvested at 6,000 × g for 10 min at 4 °C. The pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 500 mM NaCl, 10 mM imidazole and 5% glycerol). Upon a 30 min of centrifugation at 25000 g, the remaining pellet was resuspended with denaturation buffer (8 M urea, 40 mM Tris-HCl, pH 7.4) and sonicated eight times for 30 seconds with a rest period of 1 min through each sonication. Next, the sonicated yield was spun down again and the supernatant was diluted with ddH2O that time. In order to identify the desired proteins, each supernatant was run on a 15% SDS-PAGE gel.

For C4 domains denaturation purification, the denatured inclusion bodies were applied to a Ni-NTA resin (QIAGEN, USA) column, pre-equilibrated with the denaturation buffer (4 M urea, 20 mM Tris-HCl, pH 8). 20 ml of the supernatant was passed through the column, then many buffers were used to wash the column including (Denaturation buffer+30 mM imidazole), (Denaturation buffer+10 mM β-mercaptoethanol), (20 Mm Tris-HCl pH 7.4, 100 mM NaCl with then without 5 mM β-Cyclodextrin). Finally, the C4 domain was eluted and refolded by 20 Mm Tris-HCl pH 7.4, 100 mM NaCl+300 mM imidazole). A sample of 30 µl of each domain elution was loaded into a 15% SDS-PAGE gel. Thereafter, the proteins were buffer exchanged and concentrated using Thermo Scientific™ Pierce™ Protein Concentrator tubes.

2.2.7.4 SDS-PAGE and Coomassie brilliant blue staining

Proteins were loaded into 10 – 15% SDS-PAGE gels and subjected to electrophoresis at 30 mAmp/gel for 1 h. Following electrophoresis, the gels were stained using Coomassie brilliant blue staining buffer containing 0.25% (w/v) Coomassie brilliant blue, 10% (v/v) acetic
acid and 30% (v/v) methanol for 30 min at room temperature, as described (337). After staining, the gel was destained with 10% (v/v) acetic acid and 30% (v/v) methanol for 6 hours, changing the destaining buffer four times, (Fig.2.4).

**Figure 2.4 C4β chains domains purification.** Domain 1-3 as well as 4-5 were insoluble, the inclusion bodies were denatured in urea and refolded on Ni-NTA resin column. 15% SDS-PAGE was loaded with serial concentrations of C4β 1-3 (Left panel) and 4.5 (Right panel).
2.2.7.5 Determination of produced protein concentrations

The concentration of purified proteins was tested using a DC™ Protein Assay (Bio-Rad, USA) and the Quick Start™ BSA standard set (Bio-Rad, USA) referring to the instruction manual. In addition, the Nanodrop ND-1000 Spectrophotometer (USA) was also used for calculation of the C4 domain concentrations using OD readings at 280 nm (338).

2.2.8 Gel overlay assay for C4 beta chain domains

This method was used to detect the binding affinity of C4 domains to H3. In brief, a 15% SDS-PAGE gel was loaded with 2µg of recombinant H3 (New England Biolabs, Massachusetts, USA), 4µg of S100P that was produced by me as a negative control, 0.5µg of C4 β chain domains 1-3, 4-5 and the same amount of full length C4 (Fitzgerald, USA) was loaded in the last well. Upon electrophoresis and transfer, the procedure was resumed as previously described (75) and the membrane was overlayed with around 1.26µg/ml of the recombinant H3 at 4°C overnight. Thereafter, bound H3 was detected by anti-H3 antibodies (Abcam, Cambridge, UK).

2.2.9 Surface Plasmon resonance measurements

The binding parameters of C4 to individual histones, including the equilibrium dissociation constant (KD), affinities on-rates (kon), and off-rates (koff) were measured by surface plasmon resonance analysis on a Proteon XPR36 system (Bio-Rad) in collaboration with the University of Manchester. Chips coated with 20µg/ml streptavidin (GLH, GE Healthcare), which could directly interact with histones (339), were used for immobilizing individual histones and measuring binding affinities to C4. Running buffer (10 mM HEPES pH7.4, 150 mM NaCl, 0.05% Tween 20) and regeneration buffer (0.1 M glycine pH2.2) were used throughout the assay. Five µg/mL of each
recombinant histone (H1, H2A, H2B, H3 or H4) in running buffer was captured only on the surface of flow cells Fc (2-6) with Fc1 set as blank. For kinetics, a concentration series of C4 was injected at a flow rate of 10µl/min over both captured histone surface and reference surface (blank) at 20°C. KDs were calculated using software provided by the manufacturer.

2.2.10 Statistical analysis

Intergroup differences were analyzed using ANOVA followed by Student–Newman–Keuls test. Two group comparisons with or without treatment used Student t test unless otherwise specified. P<0.05 was considered statistically significant.
2.3 Results

2.3.1 Free histones exist in circulation and can form complexes with complement C4

It is known that nucleosomes could be released actively from dying cells or passively through neutrophil extracellular trap formation and degraded into histones and DNA (27, 340-343). Nevertheless, it is not obvious if circulating histones are still in the form of histone-DNA complexes exclusively. Using ultracentrifugation to fractionate plasma or serum with high levels of circulating histones into six fractions, we found that histones were detectable in all the six fractions (Fig.2.5, upper panel), but DNA-histone complexes (most likely nucleosomes) were precipitated in fraction 6 only (bottom fraction) (Fig.2.5, lower panel). No difference was found between plasma and serum. This experiment demonstrated that DNA free histones exist in circulation and they themselves can play the role as damage associate molecular patterns (DAMPs) upon release as demonstrated by many previous publications (35, 37-40).

Figure 2.5 Histones can exist in the circulation in a free form.

Critically ill patient plasma was separated into 6 fractions (1
= upper fraction; 6 = lower fraction) based on density using ultracentrifugation. Circulating histones (top panel) were measured by Western blot and histone-DNA complexes (bottom panel) quantified by ELISA (n=4).

Histone-conjugated Sepharose beads were used to pull down human plasma proteins. Following extensive washing, proteins bound to histone-beads were eluted. Multiple proteins were visualised on Coomassie blue stained gels with two major protein bands at approximately 70 kDa and 25 kDa (Fig. 2.6.A). Following liquid chromatography-mass spectrometry (LC-MS/MS) analysis, complement C4 and C-reactive protein (CRP) were identified (Fig. 2.6.B and C).
Figure 2.6 Identification of histones bound C4 as well as CRP in pulled down human plasma proteins. (A) Using histone-conjugated Sepharose, a few proteins were pulled down. Among them, there were 2 major bands on Coomassie blue stained gel, one was complement C4 and the other was CRP, as identified by mass spectrometry. (B and C) The typical spectra of the two major proteins are presented.

CRP was reported to be a major histone-binding protein to neutralising histone toxicities (75). Using ELISA kit, we then found that histone-complement C4 complexes were detectable in normal plasma spiked with calf thymus histones, and were proportional to the increase of histones concentration (Fig. 2.7.A). Histone-complement C4 complexes were also existent in critically ill patients’ plasma that have high levels of circulating histones (Fig.2.7.B), with a significant difference as compared with normal plasma. This data confirmed that histones form complexes with C4 in vivo.

The previous outcomes were encouraging enough to further study the interaction pattern between histones and C4 to highlight the structural
engagement between these two types of proteins and verify it using different assays as will be explained later.

**Figure 2.7 Identification of complement component 4 as a histone binding protein.** (A) Histone-C4 complexes were detected by ELISA following the addition of different concentrations of histones to normal plasma. (B) Histone-C4 complexes are elevated in critically ill patient plasma compared with normal plasma. (n=3), *p < 0.001 compared with normal subjects.
2.3.2 Individual histones show binding affinity to complement C4 through different assays

To determine the relative binding extents of individual histones to C4, equal molar concentrations of individual histones were subjected to gel overlay assay accompanied with Coomassie blue stained gel to demonstrate equal loading.

In this experiment, each membrane was overlayed with one type of histones and then C4-histone band was targeted with anti-histone abs for that type of histone. It was shown that there was an engagement trend between histones and C4 which seems mainly with the β chain (~75 kDa) of C4 (Fig. 2.8. A, B, C, D and E),
Figure 2.8 Individual histone shows binding affinity to C4 mainly through its β chain. (A, B, C, D and E) Five pairs of 15% SDS-PAGE gels were loaded with 0.25μg of C4, 2μg of
certain histone type and 6µg of the negative control S100P. Following the electrophoresis step, one gel of each pair was stained with Coomassie while the other copy was transferred onto polyvinylidene difluoride membrane, then overlayed with one of the histone proteins before probing with the abs which are specific for that type of overlayed histone.

To determine the comparative binding strengths under physiological conditions, I used surface plasmon resonance (SPR, Biocore X-100) in collaboration with the University of Manchester. Calf thymus histones immobilized on the surface of a streptavidin coated chip was incubated with soluble human C4 concentrations ranging from 0.04 to 0.5µM. The histones’ proteins bound to C4 in a dose-dependent manner, (figure 2.9.A-E).
Figure 2.9 The response by time (-60 - 120S) to different concentration ranges of native complement component C4 to each histone in RU. (A) Ligand, 5µg/ml H1, analyte C4 0.5- 0.03125µM two folds serial dilutions. (B) Ligand, 5µg/ml H2A, analyte C4 1.000- 0.063µM two folds serial dilutions. (C) Ligand, 5µg/ml H2A, analyte C4 1.000- 0.063µM two folds serial dilutions. (D) Ligand, 5µg/ml H3, analyte C4 0.063- 0.004 µM two folds serial dilutions. (E) Ligand, 5µg/ml H4, analyte C4 0.125- 0.008 µM two folds serial dilutions.

Further experiments were done to reconfirm the binding between histones and C4. Individual histones shown a comparable binding pattern to C4 in a different design of gel overlay assay experiment, when the membrane was incubated with C4 and then probed with anti C4 abs, the results clarified that C4 did bind to histones in a various pattern of affinity, (Fig. 2.10. upper). Coomassie blue stained gel indicates the equal loading (Figure 2.10 lower).
Figure 2.10 C4 binds to individual histones. 0.25 µg of C4 besides two microgram of each type of histones were loaded into a couple of 15%SDS-PAGE. Upon the transfer of one gel, 10µg/ml C4 was used to overlay the membrane based then probed with anti-C4 abs (Upper panel), while the other gel was stained with Coomassie Brilliant Blue (lower panel).

In order to eliminate any primary abs interfering role in demonstrating histones-C4 binding, another gel overlay method was designed for this purpose, where HRP-conjugated C4 was used to overlay a membrane of transferred equal molar concentration of histones, in a direct protein-protein interaction experiment. Figure 2.11 shows that H3 and H4 predominantly bound to C4 and to a lesser extent H1 and H2B, with H2A-C4 binding undetectable using this method.

Figure 2.11 C4 binds to individual histones. Two micrograms of H2A, H2B, H3 and H4, 4 µg of H1, and 6 µg of S100P as a control were subjected to SDS-PAGE. One gel was transferred onto polyvinylidene difluoride membrane and probed with HRP-conjugated C4 protein (upper panel). The
other gel was stained with Coomassie Brilliant Blue (lower panel).

Interestingly, the Surface Plasmon resonance measurements have shown the same pattern of affinity between histones and C4, with regard to H3 and H4. Table 2.1 shows that H3 (KD = 0.76±0.12 nM) and H4 (KD = 0.91±0.07 nM) had much higher binding affinity than H1 (KD = 7.26±0.80 nM) and H2B (KD = 9.45±1.43 nM), with weak binding to H2A (KD = 12.67±0.59 nM). Such a link between native C4 and histones in this assay has suggested the possibility of its occurrence in vivo which may lead to various consequences in the complement network.

Table 2.1 Kinetics of complement C4 binding to individual histones

<table>
<thead>
<tr>
<th>Interaction</th>
<th>$K_a$ (M^{-1} s^{-1})^a</th>
<th>$K_d$ (s^{-1})^b</th>
<th>$K_D$ (nM)^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>4.19 ±4.72X 10^4</td>
<td>7.28 ±9.52 X 10^{-1}</td>
<td>7.26±0.80</td>
</tr>
<tr>
<td>H2A</td>
<td>5.04±3.37 X 10^4</td>
<td>6.39 ±5.77X 10^{-3}</td>
<td>12.67±0.59</td>
</tr>
<tr>
<td>H2B</td>
<td>8.31 ±6.76 X 10^4</td>
<td>8.31±2.69 X 10^{-4}</td>
<td>9.45±1.43</td>
</tr>
<tr>
<td>H3</td>
<td>4.68±5.29X 10^{-7}</td>
<td>7.63±4.33 X 10^{-7}</td>
<td>0.76±0.12</td>
</tr>
<tr>
<td>H4</td>
<td>3.38±5.64 X 10^{-7}</td>
<td>1.03±8.9 X 10^{-4}</td>
<td>0.91±0.07</td>
</tr>
</tbody>
</table>

In most of the previous overlay experiments, the results demonstrated that C4 beta chain was the part that showed strongest binding to histones. In order to verify this outcome, the C4 beta chain was produced. The synthesis of C4 β chain included two domains production (Fragment 1-3, Fragment 4-5) to avoid technical production problems that associate with larger molecular weight proteins. Thereafter, these two domains that together compose C4 β
were submitted to another gel overlay experiment versus H3, which showed a remarkable binding affinity to the beta chain of purchased C4 in another experiment. (Please review fig 2.8.D). The results revealed and confirmed the same binding affinity between the complement component C4 and histones, where both C4 β chain constituents (Domain 1-3 and 3-5) bound to H3 in the overlay buffer and notably detected by anti-H3 abs, (Fig. 2.12).

Figure 2.12 C4 Beta chain domains show binding affinity to H3.

0.25µg of purchased C4, 0.5µg of the produced beta chain fragments 1-3 and 4-5 were subjected to 15% SDS-PAGE gel besides 6µg of S100P and 2µg of H3 as negative and positive controls respectively. One gel stained with Coomassie (Left panel) while the other one was transferred and then overlayed with H3 before probing with anti-H3 abs.
As my results demonstrated that the complement component C4 and especially the β chain have an obvious binding affinity to different types of histones through various tests and experimental designs, and since the beta chain of C4 is the part of the potent molecule C4b (178) that has different roles in the complement system(130, 175, 176), so it was a matter of concern to investigate whether such binding will generally affect the complement system activity or any other functions that are related to C4 and subsequently the domain C4b, that is covered in the following results chapters.

2.4 Discussion

Our group demonstrated previously that histones bind to CRP protein and form complexes in serum from patients with both elevated CRP and histones, and such binding decreased the cytotoxicity of histones and rescued mice infused with lethal doses of histones (75). My study found out another protein, complement C4, could bind to histones when a pull down technique was utilized using histone-conjugated beads, to identify histone-binding proteins in critical ill patients’ sera. CRP as well as complement component 4 (C4) were identified by mass spectrometry. This has been confirmed to naturally exist by ELISA, histone-complement C4 complexes in patient sera. This data suggests circulating histones could directly interact with complement C4.

Using gel overlay assay, I demonstrated that all histone (H1-H4) interacted with complement C4, mainly through C4 beta chain binding. Further mapping of the histone binding sites on C4 beta-chain was performed by producing two recombinant proteins with domain 1-3, or domain 4-5 of beta-chain. However, both proteins were shown to interact with histone H3 and this indicates that it is difficult to precisely map the binding site using current technique.
Since IgG has a weak binding to histones, I used HRP-conjugated C4 protein in gel overlay assay to avoid the possible cross reaction. I found that all histones except H2A bind to C4 protein (Fig. 2.11) although the single overlay was positive for H2A (Fig. 2.8 B). This may be due to the relatively weak interaction between H2A and C4, which has been confirmed by Biosensor assay.

Chapter 3: Effects of Histones on Complement Activation

3.1 Introduction

The complement system represents a unique and complex pattern since it is based on the composition of consecutive activated protein fragments, which subsequently form the convertases that cleave other complement proteins to compose the next enzymatic complex (119, 165). These complexes are stable and active for a very short time as another form of regulatory mechanism that the complement system adopts to control activation (119, 166).

The C4 concentration is about 600µg/ml in serum (177, 180). C4 full length protein is about 203 kDa, which is an essential part of the humoral immune response (173, 174). C4 is cleaved by C1s and MASP2 serine proteases in the classical and lectin pathway respectively (174), such activation results in the generation of C4b (195kDa) and C4a (9kDa) (175). The C4b fragment constitutes a subunit of the convertases C3 (C4b2a) and C5 (C4b2a.C3b), which are the enzymatic complexes that activate C3 and C5 of the classical and lectin pathways (130, 176).

Despite sharing up to 30% sequence identity with C3 and C5, C4 appears to be a much more sophisticated and developed protein, due to the formation of the three chains and the display of posttranslational modifications including four N- and one O-linked glycosylations and sulphation of three tyrosine residues (175, 179). C4 variants (C4A and C4B) share 99% of their sequence and vary in six residues only; most individuals express both isoforms (174). C4A and C4B differ in their haemolytic activities and covalent affinity to antigens and immune complexes, in addition to variation of the serological reactivity (176).

This variability of C4 could make this protein susceptible to interact with other molecules, where ester or amide bonds can be formed. In the same vein, it was found that the domain C4b binds to the
complement interfering protein (CIP), which is produced by the group B Streptococcus (GBS); this binding had suppressive effects on CP and MBL pathways where C4 is an important participant in their convertase enzymes (190).

Although it converges with CP and MBL in terminal MAC formation, the alternative pathway has both unique initiation and activation events. Furthermore, it is distinctive in the sense of being active constantly at low level, thus it is primed to cope with any infection quickly by the slow hydrolysis of C3 (Tick over) (203, 214). In case of activating surface availability, C3b binds covalently to the surface and is able to form new C3 convertase C3bBbP in the presence of factors B and D (119, 214). Binding of the AP pathway C3 convertase to another C3b domain covalently, will create C3bBbP3b, which is the C5 convertase of the alternative pathway (135).

Different methodologies have been developed to measure complement system activity. Mainly they are based on three major parts, which are: functional assays, complement analysis and the detection of activation products (Neoepitopes) (129). Enzyme immunoassay (EIA) techniques that include the three pathways are widely used in complement work and are commercially available nowadays (Wieslab Complement System Screen, Wieslab AB, Malmö, Sweden) (129, 275, 276). This assay has many advantages over the haemolytic assays, since it is easy to carry out, does not require sensitized erythrocytes and can include the three pathways together (129).

Other techniques to quantify complement activity via MAC detection or other earlier components and their activating products may include ELISA, cell-ELISA, flow cytometry, immunofluorescence assay (IFA) and Western blotting (275, 278-283).

Zymosan has been used both in vitro and in vivo to activate CP, MBL and AP in several complement studies (279, 288-293). Thus it represents a good choice to stimulate the complement system cascade
and study the difference in the complement proteins levels or the total terminal activity versus different assigned conditions (279, 294).

In order to block histone toxicity, heparin (17, 63, 74) and anti-histone abs (38, 54, 107) have been used and found to neutralize histone toxicity and possess several advantageous effects both in vitro and in vivo.

In this chapter, the functional effects that histones may have on individual and total complement activity through their binding with C4 which was well demonstrated in the previous chapter, will be studied as this may affect the related roles that are played by the CP, MBL and AP pathways. Thus it was interesting to understand the complement system response variation and the possible scenario during the related physiological conditions that is characterized by histone release and inflammatory response induction, such as sepsis (10) or even while complement clears apoptotic particles and necrotic cells (36, 131), which can be another source of extracellular histones and in other words DAMPs (35).

3.2 Materials and Methods

3.2.1 Complement activity assay

The effect of histones on complement activity in the three pathways was measured using a COMPL300 Total Complement Functional Screen kit from Wieslab (Sweden). Briefly, mixtures of the reaction were added to strips of wells for classical pathway (CP) estimation, that were precoated with IgM, strips for alternative pathway (AP) determination were coated with LPS, while Mannan binding lectin pathway (MBL) strips were coated with mannan. Normal human sera were diluted 1/101 (CP and MBL) and 1/18 (AP) assay in specific kit buffers, to ensure that only the pathway in question was activated (275). After one hour of incubation at 37 °C then washing the strips, alkaline phosphatase-conjugated antihuman C5b-9 was added before
incubation at room temperature for 30 min. Additional washing was performed, substrate was added, and the wells were incubated for 30 min. Finally, absorbance values were read at 405 nm. In each assay, standard positive and negative control sera provided in the kit were used. The complement activity for each pathway was expressed as a percentage of the activity of the calibrating serum.

3.2.2 C3a and C5a assays

Serum C3a and C5a levels were measured using C3a and C5a ELISA kits (e-Bioscience). Samples were pre-incubated for one hour at 37°C in 100 µl assay buffer in the absence or presence of calf thymus histones (50 µg/ml) added to wells coated with IgM, mannan or LPS to activate CP, MBL and AP pathways respectively (190). In order to estimate the C3a and C5a levels upon total complement activation, zymosan (Comp Tech) activated serum samples (279, 289, 294) were pre-incubated in the same conditions without and with the corresponding concentration of calf thymus histones that was used to treat the serum samples in each pathway. Thereafter, complement levels of C3a and C5a were detected by the ELISA assay kit.

3.2.3 Quantification of the Terminal Complement Complex (TCC, SC5b-9) yielded from different pathways

The final reaction of the complement system that includes C5b-9 formation was induced by zymosan (Comp Tech), and then measured using an ELISA kit from Quidel Corporation, USA. In brief, upon pre-incubation with zymosan (279, 289, 294) in the absence or presence of different concentrations of calf thymus histones (0–50 µg/ml), the specimens were loaded into wells coated with a mouse monoclonal antibody that binds specifically to the C9 component of SC5b-9 and incubated for one hour at room temperature. Afterwards, the kit
instructions were applied. The control was set up as 100% and the effects of calf thymus histones or nucleosomes were calculated.

### 3.2.4 Effect of nucleosomes on complement activation

Human mono-nucleosomes purified from HeLa cells were used in this experiment (EpiCypher, USA). In brief, zymosan activated serum on its own (control), or with calf thymus histones 50µg/ml, or human mononucleosomes, 50µg/ml were incubated and the levels of C5b-9 were detected by the ELISA kit from Quidel Corporation, USA. The samples were loaded into wells coated with a mouse monoclonal antibody that binds specifically to the C9 component of SC5b-9 and incubated for one hour at room temperature. Afterwards, the kit instructions were applied. The control was set up as 100% and the effects of calf thymus histones or nucleosomes were calculated.

### 3.2.5 Antibody and heparin blocking assay

An anti-histone reagent, non-anticoagulant heparin (20µg/ml, Sigma-Aldrich, Dorset, UK) was incubated with 20µg/ml H1, H2A, H2B, H3 or H4 proteins, or 20 µg/ml anti-histone H4 antibody incubated with H4, prior to complement activation using a Wieslab COMPL CP310 kit. The manufacturer’s procedure was followed and percentage changes were calculated by comparing to untreated (100%).

### 3.2.6 C4 cleavage assay

C1s (50µg/ml, Comp Tech, USA) was incubated with C4 (250µg/ml) in the presence or absence of histones (100µg/ml) at 37°C for 30 mins, then 2X SDS loading buffer was added and boiled for 10 mins prior to SDS-PAGE. The gel was stained with Coomassie brilliant blue or subjected to Western blotting with anti-C4a antibody (Comp Tech). The C4a band intensities were measured using software 7.05 GeneSnap from Syngene and fold changes were calculated.
3.2.7 Cell viability assay

Viability was assessed using a WST-8 cell proliferation assay kit (Enzo Life Sciences), as described previously (38). Briefly, 5x10⁴ cells were seeded into each well of a 96-well plate and grown until fully confluent (24 hours). Cells were treated with histones at 100 µg/ml with and without different concentrations of C4 (10-300 µg/ml) for 1 hour. After treatment, the medium was changed to a fresh 100 µl growth media and 10 µl of WST-8 dye was added to each well, followed by further incubation for 2 hrs. Viability was assessed by measuring the absorbance at 450 nm against a reference 650 nm using a microplate reader (Multiskan Spectrum, Thermoelectron Corporation). Viability of untreated cells was set as 100% for comparison.

3.2.8 Statistical analysis

Intergroup differences were analyzed using ANOVA followed by Student–Newman–Keuls test. Two group comparisons with or without treatment used Student t test unless otherwise specified. P<0.05 was considered statistically significant.
3.3 Results

3.3.1 Histones dramatically inhibit the classical and MBL pathways but have much less effect on the AP pathway

To investigate the functional consequences of histones binding to C4 that was demonstrated in chapter two, I used a complement functional screen kit to measure the effects of histones on the activation of the CP, MBL, and AP pathways. Pre-incubation of different concentrations of calf thymus histones (0-50µg/ml) with human serum significantly reduced the production of MAC by activated classical and MBL pathways. Significant reduction could be detected at 10 µg/ml histones, and only trace amounts of MAC could be formed in the presence of 50 µg/ml histones (Fig. 3.1.A). In contrast, the same figure displays that histones showed much less effect on the AP, and 50 µg/ml histones only reduced MAC formation (C5b-9) by around 20%.

To evaluate the overall effect of histones on complement activation in human serum, zymosan was used to activate complement in the absence or presence of histones. I found that histones at 50µg/ml could significantly inhibit the production of MAC induced by zymosan (Fig. 3.1.B).
Figure 3.1 Histones inhibit complement activation. (A) Classical, MBL, and alternative complement pathways were activated by IgM, mannan, and LPS respectively, in the absence or presence of different concentrations of calf thymus histones (0–50 µg/ml). MAC was detected by anti-human C5b-9 abs. The complement activity of control wells without histones was set up as 100%. The mean ± SD of relative activities were presented. (B) Shows the mean ± SD of relative activities activated by zymosan (activating different pathways) in the presence of different concentrations of calf thymus histones (0–50 µg/ml). *p <0.05 compared with untreated (n=3).

I also assessed the role of individual histones in the classical (Figure 3.2.A and B) and MBL (Figure 3.2.C and D) pathway activation using a concentration range of H1, H2A, H2B, H3 and H4 (0-50µg/ml), I found that 20 µg/ml individual histones started to significantly reduce activation of both classical and MBL pathways, with H4 and H2B showing the most significant effects.
These data highlighted that histones bound C4 could have functional influence on the terminal step of certain activation pathways where C4 is a vital constituent of the convertases, taking into consideration the variation of individual histone impact which may due to structural differences among histones.
Figure 3.2 Individual histones inhibit complement activation. (A and C) The mean ± SD of relative activities of classical and MBL pathways respectively, in the presence of different concentrations of individual histones (0–50 µg/ml). (B and D) The mean ± SD of relative activities of classical and MBL pathways respectively, in the presence of 20 µg/ml individual histones. Mean ± SD were calculated from at least three
independent experiments. ANOVA test, *p < 0.05 compared with untreated.

3.3.2 Anti-histone reagents can rescue complement activation

To demonstrate the specificity of histones on complement activation and in particular on the CP and MBL suppression, anti-histone H4 and non-anticoagulant heparin, which have been shown to specifically inhibit histone toxicity both in vitro and in vivo (11, 38), were used. Heparin could reverse the inhibition of both classical and MBL pathways by all individual histones significantly and the activity was excessively close to the untreated samples in many histones types (Fig. 3.3.A and B).

As clarified before, H4 had the strongest binding affinity to histones as well as being the robust CP and MBL inhibitor among the other types of histones, thus serum samples which are infused with 20µg/ml of H4 were used with and without anti-histone H4 abs. The outcomes demonstrated that neutralizing H4 by its corresponding abs could significantly rescue the H4-inhibited complement activation of both CP and MBL pathways (Fig. 3.4.A and B).
Figure 3.3 Anti-histone treatment (Heparin) rescues complement activation. Non-anticoagulant heparin (20µg/ml) was used to rescue complement activities of classical (A) and MBL (B) pathways inhibited by individual histones (20µg/ml). *p < 0.05 when compared with untreated, †p < 0.05 when compared with that treated with histone alone (n=3).
Figure 3.4 Anti-H4 abs treatment rescues complement activation.

Anti-histone H4 Ab (12 mg/ml) was used to rescue complement activities of classical (A) and MBL (B) inhibited by H4 (20 µg/ml). The mean ± SD of relative activities from at least three independent experiments were presented. ANOVA test, *p < 0.05 when compared with untreated, †p < 0.05 when compared with that treated with histone alone.

3.3.3 Excess C4 protein only partially rescues histone-inhibited complement activation but significantly reduces cytotoxicity of histones

Using C4 protein up to 300 µg/ml, only one third of the maximal complement activity of classical and MBL pathways could be recovered in the presence of calf thymus histones up to 20 µg/ml (Figure 3.5 A and B).
Figure 3.5 Effect of C4 protein on histones-mediated CP/MBL inhibition. (A and B) C4 protein affects histone-inhibited complement activity. Adding C4 protein (0–300 µg/ml) rescued 20 µg/ml histone H4-inhibited activation of CP (A) and MBL (B) pathway, (n=3).
However, the zymosan-induced complement activation could be recovered by 300 µg/ml C4 from 25 to 70% of total activity and hit the significance level in the presence of 20 µg/ml histone H4 protein (Fig. 3.6.A).

This observation suggests that histones may also target other components of the complement system rather than C4 alone. In contrast, C4 protein could significantly reduce the cytotoxicity of histones to cultured endothelial cells (Figure 3.6.B). This result may reveal that C4 binding sites to histones could include some loci that are responsible for histones binding to cell membrane phospholipids, which is one of the histones mechanisms to cause cell toxicity and calcium ion influx (34, 35, 74).
Figure 3.6 Effect of C4 protein on histones-mediated total complement activity inhibition and cytotoxicity (A) Zymosan activated complement activity in the absence or presence of histone H4 20 µg/ml and C4 protein 300 µg/ml (histone H4/C4 molar ratio = 1:1). ANOVA test, *p <0.05 compared to control, †P<0.05 compared to H4 alone, (n=3). (B) Human endothelial cell line, EA.hy926, was treated with 100 µg/ml calf thymus histones in the presence of 0–300 µg C4 proteins for 1 h. The percentage of viable cells was detected using WST-8 cell viability kit. Mean ± SD from three independent experiments are presented. ANOVA test, *p < 0.05 when compared with histone alone, (n=3).
3.3.4 Histones do not affect C4 cleavage but significantly reduce C3 and C5 convertase activity

To clarify the molecular mechanism of histone-inhibited complement activation through interaction with C4, the effect of histones on the cleavage of C4 to C4b and C4a by C1s as well as the process of C4 activation were investigated. I found that histones showed no effect on the level of production of C4a (Fig. 3.7 A and B), indicating that histone binding does not affect the ability of C1s to cleave C4 protein.

Further investigation was carried out using gel overlay assay with HRP-conjugated calf thymus histones, the outcomes revealed that histones bind to C4b but not C4a, represented by two bands in the corresponding molecular weight of β and γ chains that compose C4b domain (Figure 3.7. C).
Figure 3.7 Histones show no effect on C4 cleavage and bind to C4b. (A) *In vitro* cleavage of C4 by C1s in the presence or absence of histones. C4 (250 µg/ml) was incubated with C1s (50 µg/ml, active enzyme to cleave C4 into C4a and C4b) ± calf thymus histones (100 µg/ml) at 37°C for 30 min and subjected to 8–18% gradient SDS-PAGE along with calf thymus histones, C4a, C4b, C4, and C1s proteins. A typical Coomassie Brilliant Blue–stained gel is presented. (B) A typical Western blot with anti-C4a Ab is presented (upper panel). Fold changes were calculated by setting up
C4a intensity without histones as 1. The relative fold changes of cells treated with calf thymus histones from three independent experiments are presented (lower panel). Student t test, $p = 0.2$. (C) Two micrograms of C4, C4b, C4a, and S100P (as a control) were subjected to blotting with HRP-conjugated calf thymus histones. A typical blot is presented.

However, in the presence of histones, the production of C3a and C5a were significantly reduced in the classical and MBL pathways but not the AP and were correlated with their terminal reaction suppression trend (Figure 3.8.A, B and C), suggesting that histone bound C4b is not as efficient as C4b alone in forming active C3 and C5 convertases.

The overall C3a, C5a, and C5b-9 production induced by zymosan (Figure 3.8.A, B and C) were significantly reduced by histones because of the suppression of both classical and MBL pathways.

These outcomes illustrate that the role of histones on complement activation not only affect the terminal step but also involves different upstream levels in the cascade, and significantly in the CP and MBL pathways where C4 participates.
Figure 3.8 Histones significantly reduce C3 and C5 convertase activities. (A–C) Complement in serum was activated by IgM (CP), mannan (MBL), LPS (AP), or zymosan in the absence or presence of calf thymus histones treated (50 µg/ml) for 1 h at 37°C. Then, the C3a (A), C5a (B), or MAC levels (C) were detected by ELISA. Mean ±SD from at least three independent experiments are presented.
ANOVA test, *$p< 0.05$ when compared with that without histones.

### 3.3.5 Intact nucleosomes could not inhibit complement activation

In relation to how long histones are present in the circulation, we have reported that in critical illness when the buffering or clearance capacity is overwhelmed, histones are detectable for many days longer than nucleosomes (35, 37, 39, 344). Moreover, in Chapter two (2.3.1) it was shown that DNA-free histones can be detected in plasma and serum fractions of high level circulating histones upon ultracentrifugation, whilst DNA-histone complexes were in fraction 6 only.

However, in order to eliminate the possibility of nucleosomes impacting on complement activation, I further investigated whether the nucleosomes themselves have any effect on complement activity rather than the free histones. Intriguingly, the data in Figure 3.9 shows that while histones could significantly suppress complement activation in zymosan induced serum, nucleosomes had no influence on such complement potency which supports the hypothesis about the effects of the free histones specifically on complement activation levels.
Figure 3.9 Effect of nucleosomes on complement activation.

Complement activity was measured using the MicroVue SC5b-9 Plus Enzyme Immunoassay. Zymosan activated serum on its own (control), or with calf thymus histones 50µg/ml (CT histones), or human mononucleosomes (50µg/ml) was incubated and the levels of C5b-9 was detected. The control was set up as 100% and the effect of calf thymus histones or nucleosomes were calculated. Means±SD from 5 experiments are presented, Student t test, *P<0.01.

As is known, the complement system is involved in several parts of the immune system including the innate and adaptive response, and even beyond that by crosstalk with the coagulation system (119, 220, 297). Thus, any unusual interference with this cascade may lead to dysregulation of any other tasks that the complement system performs.

In this context, I was keen to study the impact of the demonstrated histone-mediated inhibition of complement activation on another vital function that is related to complement.

The phagocytosis process interacts with the complement system through several parts, whether through opsonisation by molecules like C4b, C3b, iC3b (304, 305), or the release of anaphylotoxins C3a and C5a which subsequently promote the uptake and destruction of pathogens by phagocytic cells (165, 172, 199). Thus, I investigated this further in chapter four.
3.4 Discussion

The Complement fixation process takes place via three activation pathways including CP, MBL and AP, despite being different in their recognition strategy and effector molecules, they all share the main steps through the cascade which involve the C3/C5 enzymatic cleavage then MAC assembly (345). Complement activation generates MAC to lyse nucleated cells and leads to cell death and content release, including histones (346, 347). C4 is activated by C1s cleavage to produce C4a and C4b (348). The C4b is the essential component of both C3 and C5 convertases, a common step of both classical and MBL pathways. In line with C4, my data revealed that histones strongly bind to C4 but do not affect C4 activation as there was no difference in C4a production in the presence or absence of histones. Furthermore, the overlay outcomes demonstrated that histones bind to C4b in particular.

Taking these result into consideration with major effect of histones in reducing the activity of C3 and C5 convertases, as indicated by reduction of C3a and C5a generation (products of C3 and C5 activation), we assume that one mechanism could be the interruption of the convertase formation and the other could just affect the catalytic activity, even though the complexes are formed. Because the lifetime of C3 and C5 convertases in solution are very short (349), it is difficult to distinguish the two potential mechanisms. To be mentioned, the concentrations of histones used in this work were mainly 20-50µg/ml. For the C1s cleavage assays, we used 100µg/ml to demonstrate that even very high levels of histones could not affect C1s cleavage. From our previous publications, we have demonstrated that circulating histones can reach up to several hundred µg/ml in critically ill patients or mouse models (35, 37). However, we do not know if local histone levels after cell death or NET formation are much higher or lower.
In support with my data regarding C4b-mediated complement inhibition, Pietrocola et al clarified that C4b binds to what they called complement interfering protein (CIP) which is produced by the group B Streptococcus (GBS) (190). This interaction between CIP and C4b handicapped the latter association with C2 and supposedly suppressed the formation of C3 convertase (C4b2C2a) which consequently decreased the CP and MBL complement activity, where C4 is an essential component in these pathways, this finding comes in parallel with my results with regard to histones-C4b binding effects on CP and MBL activity.

In the other hand, there is one study suggests that histones activate MBL pathway (350). However, I have used their methods and repeated the experiments but could not find activation of complement by histones (Fig. 3.10). The trace amounts of signals were similar to that of buffer control. Besides, their work only compared the difference between MBL-deficient (B/B) human serum and normal serum without proper negative or positive controls. Therefore, their conclusion is not fully supported.

![Figure 3.10 Complement activation](image)

**Figure 3.10 Complement activation.** In accordance with the publication of Nakamura et al, 2014, I immobilised calf thymus histones, recombinant human histone H1, H2A, H2B, H3, and H4 as well as S100P protein (for negative control)
on a 96-well plate using the Takara Peptide Coating Kit (Cat. MK100, Takara Bio, Shiga). Activation of the MBL-dependent lectin pathway was examined using the Complement System Screen Wieslab kit (Euro Diagnostica, Malmo) with some modifications. Positive control well was coated with mannan, (n=3).

The inhibitory effects of histones on CP and MBL pathways activity that I demonstrated in this study could be eliminated significantly upon treating the samples with non-anticoagulant heparin, which have been shown to specifically inhibit histone toxicity both in vitro and in vivo (11, 38). Furthermore, Neutralizing H4 which has the strongest binding affinity with C4 by anti-H4 abs could significantly recover the complement activation of both CP and MBL pathways, which demonstrates the specificity of histones on complement activation and in particular on the CP and MBL suppression and expand the roles of both histones and their blocking molecules beyond the toxicity side to be engaged with complement activity inhibition/recovery.

Thus, the mechanism by which histones-mediated complement inhibition occurs may form a physiological feedback loop to prevent overproduction of MAC and excessive tissue damage. This finding is novel, and with evidences that histone–C4 complexes exist in the circulation of critically ill patients, adds relevance to filling the unknown gap on communication between targeted cells and complement.

In contrast, histones have minimal effect on AP, in which C4b is not required. However, the overall effect is the significant reduction of C3 and C5 activation as well as the MAC formation. This finding suggests that C4 is a major target of the complement system. Nevertheless, excess of C4 could not fully restore the complement activation in the presence of histones. This finding suggests that
histones may, have more targets on those pathways, such as C1 or C2 (Fig 3.11). This needs further investigation.

It is known that circulating C4 is ~ 0.4 mg/ml, but no histones could be detected in blood from healthy donors (39). In critical illness (for example, sepsis), histones could surge up to 100–200 µg/ml (12), but C4 was reported to decrease because of consumption (39). Therefore, the high levels of histones are sufficient to inhibit both classical and MBL pathways.

High levels of C4 could efficiently detoxify histones in vitro, as we found in this study via the cell viability assay when human endothelial cells treated with histones, this protective effect of histones binding proteins phenomenon that we displayed was demonstrated in other publications but with other proteins rather than C4. In association with our groups work, Histone-CRP complexes have been recognised in ICU patients’ serum with high levels of both CRP and histones (75). When this serum incubated with endothelial cells, CRP has shown a cytoprotective effect due to preventing histones from integration to plasma membranes and causes the consequent calcium influx and cytolysis, this prophylactic role due to competing for binding with histones to membranous phospholipids loci (75, 101).

Furthermore, Bustos et al clarified the advantageous side when histones engaged with several serum proteins, as these histones binding serum proteins (HBSC) like CRP compete with anti-histones abs and can enhance the degradation of chromatin by complement and nucleases, suggesting that CRP may be important in the clearance of nuclear material, and illustrating that HBSC like CRP, could contribute to inhibit interaction of antibodies to histones and forming the pathological histones-immune complex, as well as preventing histones from engaging with glomerular basement membranes, cell surfaces or joints during SLE (351).
On the other hand, the low levels of C4 in sepsis (352, 353) may not be sufficient to neutralize high levels of histones. In noncritical illness, such as chronic inflammatory diseases with complement activation, the circulating histones could be very low, but the local concentration of histones released from lysed cells may be high and sufficient to suppress further complement activation and prevent excessive injury of host tissues. However, further laboratory experiments and clinical investigation are required to clarify those points.

Figure 3.11 Schematic representation of the effect of histones in the complement pathway.
4.1 Introduction

Phagocytosis is the process by which particles greater than 0.5µm in size are ingested (354). Immunologically, this mechanism is essential for pathogen destruction and infection elimination, as well as host defence stimulation (284). This form of endocytosis is a receptor-mediated, actin- and ATP-dependent phenomenon which is initiated by the binding of particles or organisms to specific plasma membrane receptors (299, 354).

There are four major steps that summarize this process, these include recognition of the target particle, signalling to trigger the internalization system, phagosome formation, and phagolysosome maturation (355). The mature phagolysosome represents the eventual microbicidal vesicle, where pathogens are destroyed (356). This can be accomplished via several anti-microbial mechanisms, such as acidic (357), enzymatic (358) and scavenging by dedicated molecules like lactoferrin (359), that remove the iron required by some bacteria and the NADPH oxidase that generates oxygen radicals (355).

The internalization of damaged or senescent cells is termed as efferocytosis and represents the non-inflammatory form of phagocytosis, where ‘find-me’-signal release including nucleotides (ATP, UTP and the lipids lysophosphatidylcholine) recruit the phagocytes towards the dying cells (360). Subsequently, dedicated engulfment receptors identify the target ‘Eat-me’-signals such as phosphatidylserine and calreticulin and engulf the apoptotic cells. This process is followed by the release of several mainly pro-inflammatory cytokines to adjust the local immune response and keep the neighbouring cells safe (360, 361).

Professional phagocytes include macrophages, neutrophils and dendritic cells which interact with the non-professional phagocytic
cell types such as epithelial cells, in order to mediate their activities (362).

Monocyte–macrophage lineage cells exist in blood (~10% of leucocytes) primarily as monocytes, which are large (10 to 18 μm) cells with peanut-shaped, pale purple nuclei as determined by Wright staining (363). Mature monocytes exit the bone marrow and circulate in the bloodstream until they enter tissues, where they become tissue macrophages (363-365).

Several assays have been developed in order to evaluate opsonophagocytic potency, which is implicated in vaccine production by testing whether the vaccine-induced antibodies drive efficient complement deposition and subsequent opsonophagocytic killing (314-316).

Toll-like receptors (TLRs) and Nod-like receptors (NLRs) represent two salient members of innate immune pattern recognition receptors, which can elicit immediate responses against pathogenic invasion or tissue injury (366). DAMPs and PAMPs can both be recognized by TLRs and NOD-like receptors (367). TLRs are widely expressed on or within cells of the immune system such as macrophages and the epithelia and can detect a wide variety of pathogens (368). TLR2 and TLR4 were found to mediate phagocytosis (369), West et al demonstrated that the macrophage anti-microbial potency was due to TLR2 and TLR4 mediated stimulation (370). Yet, the innate immune system seems more complex than was proposed upon the discovery of non-TLRs, such as the NOD-like and C-type lectin receptors (371), e.g. macrophages isolated from NOD1-deficient mice are hyporesponsive to certain bacterial amino acids, mice lacking NOD2 were more susceptible to Toxoplasma gondii infection (372).

The complement system cross-talks with the phagocytic process in different ways (345). For example, the generation of C3a and C5a upon activation results in enhanced phagocytosis, since these phlogistic peptides will recruit more phagocytic cells, together with
their anaphylatoxin role will promote pathogen degradation (165, 172, 199). In addition, complement activation also produces other essential molecules like C4b and C3b, which facilitate further phagocytosis via their opsonisation function (172, 313). In this context, microbes are susceptible to phagocytosis via complement receptors on phagocytes. Complement receptor type 1 (CR1) is a high-affinity receptor for C3b and C4b fragments of complement and mediates the uptake of C3b- and C4b-engaged particles (368).

Interference of the complement cascade can in turn affect complement fragment generation; such overlap may subsequently impact other activities that communicate with complement like phagocytosis, and represents one of the strategies that some pathogens adopt to suppress phagocytosis (190, 373). Based on this approach of complement-mediated control of phagocytosis, I studied the variation of phagocytosis rate in phagocytes in relation to histone exposure, since I demonstrated that histones lead to significant reduction in C3 and C5 cleavage, so it was a matter of concern to investigate the suppressive effects on phagocytosis.

### 4.2 Materials and methods

#### 4.2.1 Effect of histones on phagocytosis rate and oxidative burst in whole blood specimens by flow cytometry.

In order to thoroughly understand the histone-phagocytosis relationship, it was important to study this phenomenon while phagocytes are within whole blood, to mimic the in vivo physiological conditions and cell interactions through the haematopoietic network (374, 375). This novel assay measures both phagocytic uptake and the superoxide burst in the phagocyte populations in whole blood (376) and was applied according to the setting and principle displayed in figure 4.1 which was devised by a Liverpool School of Tropical Medicine research group (377). First, silica beads (Kisker Biotech,
Germany) were prepared from the stock by several washes and re-suspension steps using RPMI-1640 medium. Citrated blood was drawn from healthy donors according to University of Liverpool Interventional Ethics Committee (RETH000658.089)

The whole blood specimens (90µl) were then transferred into the FACS tubes that contained 10µl of the silica beads solution in duplicates, then 0-100µg/ml of calf thymus histones were added to each pair. After that, the samples were incubated with shaking at 37˚C for 60min. Upon incubation and terminating the phagocytosis by 10 min in an ice bath, 2ml of 1x Facs lysing solution was added to each tube and mixed gently before 20 min of incubation at 4˚C. Subsequently, specimens were centrifuged for 5min at 300 g, and washed twice with 2ml 1xPBS before being re-suspended in 250µl PBS. Samples were kept on ice and phagocytosis examined by Flow Cytometry (BD LSR II (BD Bioscience)).

Figure 4.1 Phagocytosis and oxidative burst in whole blood samples by flow cytometry. Each bead is coated with IgG (to enhance uptake), reporter (activated green fluorescent emission by oxidation after entering neutrophil phagosomes) and calibrator (constant red fluorescent signal for calibration the number of beads). Therefore, the two fluorescent signals can be read at the same time and their ratios can be used to differentiate the associated beads from the beads that are oxidized by H₂O₂ in phagosomes.
4.2.2 Effect of histones on *E coli* GFP phagocytosis by U937 macrophage like cells

A cell line of U937 (ATCC® CRL1593.2™) was grown in RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin-streptomycin solution until an optimum growth density was achieved. Thereafter, cell numbers were adjusted to 1.3 ×10^5 cells in each specimen using a haemocytometer (viability >95% as assessed by trypan blue staining). Phorbol-12-myristate-13-acetate (PMA) was used to trigger cell differentiation into adherent macrophages. 100nM PMA and the adjusted number of U937 cells in a total volume of 400µl of RPMI-1640 only were used to activate cells in eight well BD Falcon chambered cell culture slides, these slides were incubated for 48hrs at 37°C and 5% CO2 for macrophage maturation. Meanwhile, 10µl of Green Fluorescent Protein (GFP) labelled *E. coli* (ATCC® 25922GFP™) stock was incubated overnight in 5ml LB Broth, including 100µg/ml AMP in an orbital incubator at 37°C. The next day, 0.5ml of the bacterial growth was transferred into 10ml fresh LB broth and incubated for 30mins under the same conditions. Subsequently, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added (final concentration of 1mM) and the bacteria were incubated for a further 2hrs under the same conditions. Activated bacterial growth was then centrifuged at 2800 g for 10mins, the pellet was washed two times and re-suspended in 5ml PBS.

Upon standardizing the bacterial growth density, the phagocytosis experiment was performed. In brief, different concentrations of calf thymus histones (0-100µg/ml) were loaded into the chamber slide wells containing the adherent PMA stimulated U937 cells, then *E coli* GFP bacteria were added (total volume of 200µl RPMI-1640). Slides were incubated for 1hr at 37°C and 5% CO2. Slides were then washed with PBS, then fixed and stained with 4% paraformaldehyde and Propidium iodide (PI) stain for 5min. Finally, slides were mounted and left to dry before examination with a Nikon Eclipse E600 Fluorescence Microscope.
4.2.3 Western blotting and TLR blocking

The phagocytosis experiment was carried out using U937 cells as mentioned in 4.2.2 with some modifications. Adherent PMA stimulated U937 cells were treated with 0-20 μg/ml histones. To investigate the role of TLR inhibition, histone (20μg/ml) treated wells were also incubated without or with anti-TLR2 or/and anti-TLR4 abs (5μg/ml final) (InvivoGen, USA). Following incubation, the slides were washed with PBS, but instead of being fixed as in above (4.2.2), 60μl of clear lysis buffer was added to each well for 3min.

Thereafter, a cell scraper was used to remove cellular extracts which were transferred to Eppendorf tubes for Western blotting. Samples were separated using 15%SDS-PAGE. Following transfer on to PVDF membranes, mouse monoclonal anti-GFP ab (Abcam, Cambridge, UK) was used to detect GFP in the cellular extracts. This experiment was repeated three times. Equal sample loading was determined by probing the same membrane with an anti-beta actin monoclonal ab (Sigma-Aldrich, Dorset, UK).

4.2.4 Image processing software

The Java-based image processing program (ImageJ 1.51) was used to count the GFP+ phagocytic cells and for Western blotting band quantification; data was then normalized in order to make it suitable for statistical analysis.

4.2.5 Statistical analysis

Intergroup differences were analyzed using ANOVA followed by Student–Newman–Keuls test. Two group comparisons with or without treatment used Student t test unless otherwise specified. P<0.05 was considered statistically significant.
4.3 Results

4.3.1 Histones increase bead uptake by neutrophils in whole human blood

Histones were found to interact with C4 and subsequently cause complement inhibition as demonstrated in Chapters 2 and 3. Nevertheless, it is unclear whether this inhibition in complement activity has other impacts on the vital early defense of innate immunity, such as phagocytosis, as certain complement activation products are opsonins for phagocytosis.

To clarify the effects of histones on phagocytosis, we used a novel flow cytometry procedure to measure the phagocytic activity of neutrophils of specific beads within whole blood samples by gating the neutrophil population and counting 10,000 cells (Fig.4.2. A). Each bead has both reporter (activated/oxidized after entering neutrophil phagosomes) and calibrator (which is demonstrated neutrophil association but not oxidization), these two fluorescents readouts separate the neutrophil population into two: associated/internalized with or without oxidization (Figure 4.2B). The blue dots represent the bead associated with neutrophils but not entered phagosomes. In this way, we can clearly detect the increase in true phagocytosis and not the beads becoming adhered to the cells.

Surprisingly, treating blood samples with 20 µg/ml of histones significantly increased the mean fluorescent intensity (MFI) (green) emitted by oxidation of beads inside phagosomes of neutrophils (Fig. 4.2.C), which indicates a significant increase in phagocytosis. A dose response curve was then obtained using different concentration of histones (0-100µg/ml), showing a plateau of uptake reached in the presence of 20µg/ml histones (Figure 4.2 .D).
20 μg/ml Histones

Calibrator Pacific Blue-A

Reporter FITC-A

D

Reporter FITC (MFI)

Histones (μg/ml)
**Figure 4.2 Whole blood neutrophil phagocytosis assay.** Normal whole blood was incubated without and with different concentrations of histones for 60 mins at 37°C. Phagocytosis was assessed by quantifying the uptake by neutrophils of intraphagosomal silica beads, with calibrator (Pacific Blue) and reporter (OxyBURST Green) fluorochromes. (A) Typical scatter plot demonstrating scatter properties of different cellular populations (neutrophils gated red). Uptake of intraphagosomal beads in the gated neutrophil population, following treatment without (B) and with (C) 20 µg/ml histones (Calibrator beads = blue; Reporter beads = green). (D) Concentration-dependant effect of histones on phagocytosis. Means ±SD of fluorescent intensity of oxidised beads from 3 independent experiments are presented. *ANOVA test P<0.05.

### 4.3.2 Effect of histones on *Escherichia coli* GFP phagocytosis by U-937 macrophage like cells

To demonstrate that extracellular histones could enhance phagocytosis of live bacteria, I used PMA-activated U937 cells as a model of phagocytes uptake of GFP-expressing *E.coli*. Histones were found to enhance phagocytosis of live bacteria, by PMA-activated U937 cells in a dose dependent manner (0-20µg/ml) (Figure. 4.3 A- D), confirming the data demonstrated above.
Figure 4.3 Histones increase phagocytosis in U937 cells. U937 cells (1.3×10^5) were activated by 100nM PMA for two days and adhered to eight wells chamber slide, 10:1 (Bacteria: Cells) *E. coli* GFP were added to each well in a total volume of 200µl RPMI-1640 media. The green dots represents the GFP within the phagocyte, the faint rounded shapes are the phagocytic U937 macrophage like cells. (A) Without histones. (B) 5µg/ml of histones. (C) 10µg/ml of histones. (D) 20µg/ml of histones, slides were examined by fluorescence microscope (20X).

My data revealed that the percentage of phagocytes that engulfs bacteria to the total number of phagocytic cells has increased
significantly and dependently on increasing doses of histones, where 20µg/ml of histones causes a large rise in this percent (Fig.4.4.A.)

Moreover, the rate of GFP bacterial uptake was proportional to increase in histone dose and there was a significant difference between the GFP bacteria/phagocytes ratio of histone-treated U937 cells, as compared with untreated cells (Fig. 4.4.B).
Figure 4.4 GFP bacteria/Phagocyte ratio difference. Images of untreated slides from three experiments as well as three of 5, 10 and 20µg/ml histone treated chambers were processed using Image J analysis software. (A) The percentage of phagocytes that contain GFP *E. coli* to all phagocytes count. (B) The fine particles of engulfed GFP *E. coli* and number of phagocytes were quantified, the ratio of GFP bacteria to phagocytes’ number then was calculated. ANOVA, *p* < 0.01 when compared with untreated.

4.3.3 Histones may interact with phagocytes through their PRRs

Western blotting was used to further investigate the influence of histones on phagocytosis with *E. coli* GFP, using anti-GFP abs. We investigated the possibility of histones interacting with phagocytes directly via PRRs, e.g. TLRs, since such sensors are widely expressed on phagocytes (368) and can interact with both DAMPs and PAMPs (367) and enhance the phagocytosis process (369, 370).

As demonstrated in figure 4.5 (Lower panel), Western blotting indicated that histones trigger phagocytosis at several concentrations (0-20µg/ml) and there was a very strong GFP band in the 20µg/ml lane, which paralleled my previous findings regarding the significant increase in GFP particles between 20µg/ml histones and non-treated samples. Furthermore, use of TLR abs slightly weakened the GFP bands, with anti-TLR4 seeming to have the greatest effect. The upper panel of the same figure shows the total loaded cells indicated by beta actin proteins.
**Figure 4.5 Determination of phagocytosis potency via GFP.** U937 macrophage-like cells were stimulated with 100nm PMA then *E coli* GFP bacteria were incubated with these cells, different concentrations of histones (5, 10, 20µg/ml) were used in this phagocytosis experiment along with a control well without histones. Anti-TLR2/4 blocking antibodies (5µg/ml) were also used in wells containing 20µg/ml histone (Lower panel). Following phagocytosis, experiments were terminated by the addition of clear lysis buffer, then subjected to Western blotting using anti-GFP abs (n=3). Equal loading was determined using a mouse monoclonal anti-beta actin abs for each membrane (Upper panel).

Using Image J software to quantify the density of each band following phagocytosis of GFP *E. coli* (for both GFP and beta actin), figure 4.6 shows that the increase of GFP protein was significant among histone
as compared with no histone controls. In addition, anti-TLR2 and TLR4 abs decreased phagocytosis, demonstrated by less GFP protein. However, this was not statistically significant when compared to non-TLRs abs samples with same dose of histones (Fig. 4.6). Further experiments need to be performed to clarify the role of TLRs on histone-induced phagocytosis, where by different doses of the antibody could be used.

Figure 4.6 Estimation of phagocytosis via GFP bands quantification. The membrane images of three Western blotting experiments were processed using ImageJ software to evaluate the density of both GFP and beta actin bands. The ratios of GFP/actin (mean±S.D) for each treatment are presented. ANOVA test, $p < 0.01$ when compared with untreated; $P<0.05$ when TLRs abs samples compared with non-histones alone.
4.4 Discussion

The complement system and phagocytosis are two interactive processes of innate immune response, that are closely linked (345). Increases in C3a and C5a generation upon activation augment phagocytosis through recruiting more phagocytes to the site of inflammation (165, 172, 199).

Complement activation cross-talks with phagocytosis via opsonins fragments, such as C4b and C3b, which facilitates enhanced phagocytosis (172, 313) and mediate the uptake of C3b- and C4b-engaged particles, through binding to CR1 receptors on phagocytes (368). Consequently, disturbing complement activation and these fragments levels (C3a, C5a, C3b and C4b) are associated with altered phagocytosis (190, 373).

In this study, I investigated the effect of histone-induced complement suppression on phagocytosis, since their presence could significantly reduce the production of opsonins from complement activation, such as C5a, C3a and C3b. Interestingly, I found that histones enhance phagocytosis of bacteria by both human peripheral neutrophils and activated U937 cells, in a dose dependent manner. Within the range of 0-20µg/ml histones, phagocytosis increases in a concentration dependant manner. In the range of 20-50µg/ml, the phagocytosis activity reached a plateau. I also used anti-GFP antibody to quantify the amount of GFP that was taken up by activated U937 cells. The results are consistent with bacteria counting.

As to the mechanism of histone-enhanced phagocytosis, it is not clear. In this study, I used anti-TLR2 and anti-TL4 antibody to block the known histone receptors. I found that blocking TLR4, not TLR2, could reduce histone-enhanced phagocytosis although these data did not reach significance, suggesting histone-TLR4-myoD88 pathway may be one of the mechanisms. Further investigation is required.
Nevertheless, the roles of other receptors need to be also explored. Histones acting as DAMPs have been found to share several types of PRRs with PAMPs such as TLR4, TLR3, TLR7-9 as well as NLRs and RLRs (21, 378). It was found RLRs deficient mice show reduced phagocytic activity upon TLRs stimulation by LPS, and were also more susceptible to infections (379), suggesting a synergetic role between other receptors types in this process. In addition, macrophages isolated from NOD1-deficient mice are hypo-responsive to certain bacterial amino acid, mice lack NOD2 were more susceptible to *Toxoplasma gondii* infection (372). NOD1 and NOD2 were also able to induce the auto-phagosomes formation, leading to uptake of invading bacteria (380). Experiments exploring the role of these additional receptors/pathways could be further investigated in near future.
Chapter 5: General Discussion, conclusions and future work

5.1 Discussion

Within cells histones are intra-nuclear proteins that pack and sort the DNA into functional units (Nucleosomes) and contribute in gene regulation (24, 381). Histones are released extracellularly via neutrophil extracellular traps through a process of NETosis, whereas a bundle of both intra-cellular and intra-nuclear components including histones perform a protective role against exogenous pathogens (382). In many critical illnesses, during cellular damage, nuclear chromatin is cleaved into nucleosomes, which are released extracellularly (383) and further degraded into individual histones (340). Histones are rapidly cleared by the liver (384) and are rarely detected in blood. Since extracellular histones were reported as a major mediator of death in sepsis models (34), clinical studies have found increased circulating histones in many critical illnesses, including severe sepsis, acute pancreatitis and trauma (35, 385, 386). We found that in patient samples, histone concentrations >50 µg/ml express direct toxic effects on endothelial cells (35, 75), with levels below 50µg/ml, are still able to trigger pro-inflammatory cytokine release (75). In human sepsis, median histone levels are 60 µg/ml (Quartiles 20 to 80 µg/ml), indicating the possible contribution of histone toxicity to the pathological process in at least half of septic patients (386). Importantly, circulating histone levels strongly correlate with disease severity and organ failure scores (35, 386). Circulating histones are also directly cardiotoxic causing elevated circulating cardiac troponin, but their dose-differential effect on left and right ventricular (RV) failure suggests the added contribution of impaired lung perfusion at high histone levels (387).

In addition to histone binding to phospholipid on cell membranes to cause calcium influx and cell damage (35), TLR-2, -4 and -9 are also involved (62, 66). Extracellular histones are the most important DAMPs in activating immune cells, inflammasomes and releasing
pro-inflammatory cytokines (20, 388). Clinically, high circulating histone levels correlate significantly with cytokine levels and translate into disease severity and organ-specific injury scores, e.g. pancreatic necrosis (35). Histones have been shown to enhance thrombin generation through platelet activation and aggregation (63). High histone levels (>50 µg/ml) directly cause endothelial damage and low levels activate endothelium to release von Willebrand factor (VWF), recruit leucocytes/platelets and reduce thrombomodulin-dependent protein C anticoagulant effects (20). Whilst these are indirect mechanisms that link histones to thrombin generation, a more direct effect has been proposed, whereby histone H4 can promote prothrombin auto-activation into thrombin (73). However, its clinical relevance is unclear, since this process takes over 60 hrs to occur. Physiologically, prothrombin is activated very quickly by the prothrombinase complex, comprising the enzyme Factor Xa (FXa), co-factor FVa, calcium and phospholipid (PL) surfaces to localise clot formation (389, 390). Thus, direct molecular mechanisms of histone pro-thrombotic effects remain unclear. Besides their deleterious role (391), released to the extracellular spaces passively upon cell apoptosis and/or necrosis (392, 393), or actively through uncontrolled NETosis (27, 43, 382), histones serve as an alarmin to inform the body that damaging processes are occurring (66). Extracellular histones cause immediate increase in IL-6 release (within 1 hour) and lead to acute phase response. The increase in acute phase proteins, such as C-reactive protein (CRP), could neutralise histones to reduce its toxic effects (75). Histones are also able to directly kill bacteria through their bactericidal properties. Therefore, elevated circulating histones are not solely harmful, but also trigger protective mechanisms within the host. My findings provide additional evidence for the protective mechanisms. Histone-attenuated complement activation and enhanced phagocytosis may be a major mechanism to facilitate pathogen clearance and reduce host injury.
Complement cascade activation has a leading role in defending the host against both DAMPs and PAMPs (120, 133) and the possible relationship between histones and the complement system has previously been proposed. Some studies covered the relationship between histones and complement system, but were mainly based on complement-mediated-histone release through NETosis (55, 115, 394-396). My new approach investigating complement-histone interactions further aids in understand the pathology of several diseases, that are associated with histone release, expanding the scope of the effect of histone on the innate immunity and signalling (397).

In order to directly address the confusion as to whether histones themselves interact with complement or histone-DNA complexes, my study has clarified whether free histones circulate in blood during inflammatory diseases (24, 55, 66, 72, 111, 398, 399). I have clearly demonstrated that free histones were identified in six fractions of critically ill patients’ plasma following ultracentrifugation, whilst histones-DNA complexes were in the bottom fraction only.

My study also highlighted that histones may interact with complement system in several diseases, as C4-histones complexes were increased significantly in critically ill patients’ plasma. C4-histones binding affinity was also confirmed using different assays and under different physiologically relevant condition (H4 > H3 > H1 >H2B > H2A). C4 β chain displays the main binding region of C4 to histones. These data further extend from data presented in a previous study, where a shotgun proteomics study has suggested that exogenous histones precipitate numerous plasma proteins including complement. These authors were unsure as to whether these binding was a simply due to protein deposition or an actual binding. Data presented in my study clarified that histones-C4 binding occurs under physiological conditions, which was out of the scope of the previous publication (109).
Besides using HRP-conjugated histones in gel overlay assays against C4, biosensors data demonstrated the binding affinities, which further confirms the pattern of affinity that each histone has against C4 and provided detailed analysis of binding between histones and C4. On a molecular basis, I also confirmed the C4 domains, produced in our lab were binding to histones via overlay assay. My study was the first to conclusively demonstrate that histones-complement binding using several assays, and further demonstrated that C4b is the specific region of the C4β chain to be the most part of C4 that interacts with histones.

Complement activation generates MAC to lyse nucleated cells and leads to cell death and content release, including histones (346, 347). Interestingly, my study illustrates that histones bound to C4 affects complement activation in a selective manner; whereas CP and MBL pathways activity were significantly reduced, while this binding has less impact on AP pathway activation. In addition to the suppressive effect that histones have on CP and MBL activation, the upstream activity of these pathways were also decreased, as there was significant inhibition of both C3a/C5a generation. These data demonstrate inhibited convertases activity as a result of histones-C4 binding. My data revealed that histones-C4 binding does not affect C4 activation, since there was no difference in C4a production in the presence or absence of histones. Furthermore, I demonstrated that histone binds to C4b, an essential component of both C3 and C5 convertases activity in classical and MBL and was able to reduce the activation of these pathways through inhibiting convertase activity. Histones had little effect on the activity of the AP pathway since C4b is absent in this pathway.

Thus, it is proposed that this reduction in CP and MBL activity may due to histone interrupting the convertase formation through binding to C4. This hypothesis of C4b-mediated complement inhibition agrees with another study. These authors indicated that a bacterial protein bound to C4b and hinders its association with C2. Subsequently, the
formations of C3 convertase (C4b2C2a) as well as CP and MBL pathways activity were reduced (190). Another reason why histones may inhibit CP and MBL activity is the catalytic alteration of C3a/C5a convertases upon histone binding. However, this hypothesis is difficult to verify experimentally due to the short half-life of C3a/C5a (349).

When both suppressive effect of nucleosomes and free histones were tested on complement activity, my study demonstrated that nucleosomes have no effect on complement activation, while free histones significantly reduce complement activation. These data clarifies that free histones but not nucleosomes affect complement activation during inflammatory conditions.

Histones-mediated reduction of complement activity could significantly reversed when histones blocking reagents such as non-anticoagulant heparin and anti-H4 abs (11, 38), indicating that the reduction in CP and MBL pathways activity is histone specific. Yet, other complement components rather than C4 may take part in this phenomenon, as C4 rescue experiments could partially recover the CP and MBL activity.

Histones-mediated inhibition of complement activation may form a protective physiological strategy to prevent hyper tissue damage via excessive MAC formation (400). With evidence that histone–C4 complexes exist in the circulation of critically ill patients, this novel finding exposes another aspect of cross-talk between complement and targeted cells, may occur in relevant medical conditions (i.e. sepsis), where histones levels are dramatically high and can cause CP and MBL activity inhibition via binding to C4, where the latter deposits are low due to consumption.

In addition, the association of C4 with histones could be cytoprotective since my study has indicated that C4 was able to neutralize histones toxicity and protect endothelial viability. However, it is still unclear whether histones levels following cell death or NETs
formation are much higher or lower in local tissues, and whether the low deposits of C4 in such pathological conditions are adequate to neutralize the toxicity of circulating histones. Further laboratory and clinical work is necessary to clarify these issues.

The complement system activity affects phagocytosis in different scenarios via its opsonins (C3b and C4b) and anaphylatoxins (C5a, C3a, C4a) (165, 172, 199, 313). So, the alteration of complement activity by histones demonstrated in this study, may have an impact on the rate of phagocytosis.

Despite the data generated in this study demonstrating the reduction in anaphylatoxins C3a/C5 generation as well as C4b the opsonin being engaged with histones, the effect of histones on phagocytosis was enhanced. This phenomenon of increased phagocytosis was confirmed and quantified using various methodologies, such as whole blood phagocytosis and GFP E. coli uptake (quantified microscopically and by Western blot).

Since the inhibitory effect of histones on complement activity did not interfere with phagocytosis process, we assume that histones may interact directly through direct cellular binding via their receptors or by aggravating the inflammatory events and related mediators release which in turn propagate the process of phagocytosis (54, 378, 401). This theory would suggest a role of histones that act directly on phagocytosis independently of the suppression impact they caused to complement and its subsequent effects on phagocytosis via opsonins generation impairment.

In this context, this study has found that blocking the macrophage like U937 cells TLRs2 and 4 receptors could reduce the phagocytosis, although not significantly. In support with my data, it was found that histones may act as DAMPs and interact with TLR2, 4 and TLR9 (54, 61, 62, 66). Yet, other receptors role also needs to be investigated in this aspect, since histones as DAMPs were found to share several types of PRRs with PAMPs such as TLR4, TLR3, TLR7-9 and both
NLRs and RLRs (21, 378). Since histones may act through various receptors, this information may illustrate why the histones-stimulated phagocytosis could not be completely inhibited upon blocking TLR2/4 and as such other types of PRRs may be involved in this mechanism.

The limitation of this work is that the principle has not been fully demonstrated in vivo, including animal models and patients. This will be my future work to keep this research direction and try to benefit patients in the long run.
5.2 Conclusions

- In critical illness, histones can exist in circulation freely not only within nucleosomes.
- Histones bind to complement component C4 in different affinities. This binding decreased total complement activity as well as the classical and MBL pathways, as C4 is the main protein in the C3 and C5 convertases of these pathways. While the AP activation is slightly influenced by C4-histones interaction, due to the different composition of C3, C5 convertases.
- The mechanism by which histones-mediated complement inhibition occurs may form a physiological feedback loop to prevent overproduction of MAC and excessive tissue damage.
- Other complement components may interact with histones, since rescue C4 experiments could only partially restore the activity.
- Anti-histone antibodies may have promising therapeutic purposes in both histones toxicity neutralization and complement function recovery in certain cases where such immune defense is required.
- Histones may enhance phagocytosis in a complement independent manner that may act through TLRs and other PRRs.
- Comprehensive understanding of the crosstalk inside this vital triangle of innate immunity (Complement-phagocytosis-TLRs) and histones impacts on this interaction may help in developing effective therapeutic strategies in related medical conditions such as sepsis. Thus further studies are requisite to achieve this target.
5.3 Future work

- Investigating other possible interactions of complement proteins like C2 with histones.
- Applying complement-histone approach locally, by studying the effect of this binding in body fluids of damaged tissue.
- Studying the effect of histones on phagocytosis in association with other signaling molecules like NOD-like receptors. In parallel with complement activity evaluation under the same conditions.
- Using animal models to demonstrate the principles established \textit{in vitro}.
- Clinical studies to explore how to benefit patients via my discovery.
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