Extracellular Histones Inhibit Complement Activation through Interacting with Complement Component 4

Short article: Histones Inhibit Complement Activation


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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.
**Introduction** The Complement system forms a major part of the host response to infection and cellular injury(1, 2). 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, mannose binding lectin (MBL) and alternative pathways(2). The classical pathway recognises antigen-antibody complexes contained on the surface of pathogenic factors, including gram-negative bacteria, viruses and damaged cells(3). The MBL pathway binds mannose containing pathogenic surfaces(4). Finally, the alternative pathway directly targets surface carbohydrate regions on pathogens such as viruses, bacteria and fungi(5). The terminal pathway of complement activation by different stimuli is the formation of C3 and C5 convertases to lead to assembly of C5b-9 complex, the membrane-attack complex (MAC) to lyse pathogens. Many products generated during complement activation are also able to opsonize damaged cells or pathogens to facilitate phagocytosis(1, 2). In this way, complement activation enhances the ability of antibodies and phagocytic cells to clear invading pathogens and cellular debris from the circulation(6).

Complement activation not only kills pathogens but also damages host cells during an inflammatory reaction and excessive activation contributes to inflammation-driven tissue injury(7). 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 responses(8-10). Histones, the most abundant and important DAMPs, can be detected in blood taken from many critical illnesses, such as severe trauma(11), severe sepsis(12, 13) and necrotising pancreatitis(14). Histones are positively charged proteins and have high affinity for negatively charged phospho-groups in DNA or cell membranes. Histone binding to cell membrane allows ions, particularly Ca^{2+} influx into cells to cause harmful effects to cells contacted(11). 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 (15-17). Histones also interact with coagulation factors in the circulation to promote thrombin generation, fibrin deposition and systemic coagulation activation (18-22). In animal models, extracellular histones have been shown to mediate multiple organ injury and even death in sepsis (13, 23). Clinically, correlation between circulating histone levels and organ injury as well as disease severity has also been demonstrated (12).

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 (24, 25). Upon complement activation, C4 is cleaved by C1S enzyme into C4a (kDa) and C4b, the latter mainly participates in classical and lectin pathways by forming C3 and C5 convertases, whilst C4a as an anaphylatoxin enhances smooth muscle contraction, histamine release and vascular permeability as well as serving as a chemotaxis and inflammatory mediator (26, 27). In many disease conditions, particular in sepsis, complement activation (28) and histone release (12) coexist. The outcome of histones binding to C4 appears important and this study is trying to understand the pathophysiology related to the complement system and extracellular histones.

Materials and Methods

Human plasma and serum

Citrate 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). Human normal serum was purchased from CompTech, USA.
Fractionation of human serum and plasma by ultracentrifugation

Citrate plasma and serum (1 ml) from patients were fractionated by ultracentrifugation at 40,000 rpm (4°C) for one hour and then 6 layers of equal volume fractions (166 ul per fraction) were collected. Histones and Histone-DNA complexes were then measured in each fraction by Western blotting and ELISA (Cell death detection ELISA PLUS, Roche), respectively, as previously described.(11)

Isolation of histone binding protein from plasma and mass spectrometry analysis

Isolated citrated plasma was diluted with 2 x 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 a 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 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.
Detection of Histone-C4 complexes by ELISA

Histone-C4 complexes were detected in normal and critically ill patient plasma using Cell death detection ELISA PLUS kit (Roche) with modification. In brief, normal plasma was pre-incubated with different concentrations of calf thymus histones for 10 minutes. Histones in plasma were first captured by biotinylated-anti-histone antibody immobilized on streptavidin-coated 96 well plates. After extensive washing, rabbit anti-human C4 antibody (Abcam) followed by anti-rabbit-HPR antibody were used to probe histone-C4 complexes. Arbitrary units (AU) were calculated based on the absorbance (450nm) to represent the relative levels of the complexes.

Western blotting using HRP conjugated C4

In order to double confirm the interaction of C4 with individual histones by different assay, 2 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. C4 was purchased from Fitzgerald, USA and conjugated using Lighting-Link HRP conjugation kit (Innova bioscience, Babraham, Cambridge, UK, ref. 701-0000) according to the procedure recommended by the manufacturer.

Surface Plasmon resonance measurements

The binding parameters of C4 to individual histones, including the equilibrium dissociation constant (Kd), affinities on-rates (k_{on}), and off-rates (k_{off}) were measured by surface plasmon resonance analysis on a Proteon XPR36 system (Bio-Rad). Chips coated with 20µg.ml streptavidin (GLH, GE Healthcare), which could directly interact with histones,(29) 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 C4 was injected at a flow rate of 10 µl/min over both captured histone surface and reference surface (blank) at 20°C. Kds were calculated using software provided by the manufacturer.

**Complement activity assay**

The effect of histones on complement activity in the three pathways was measured using COMPL300 Total Complement Functional Screen kit from Wielisab (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. 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, the 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. C3a and C5a were measured using C3a and C5a ELISA kits (e-Bioscience). C5b-9 induced by zymosan (Comp Tech) was measured using an ELISA kit from Quidel Corporation, USA.

**Antibody and heparin blocking assay**

Anti-histone reagent, non-anticoagulant heparin 20µg/ml, 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 Wieslab COMPL CP310 kit. Percentage changes were calculated by comparing to untreated (100%).

**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.

**Cell viability assay**

Viability was assessed using a WST-8 cell proliferation assay kit (Enzo Life Sciences), as described previously(31). 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.

**Statistical analysis**

Intergroup differences were analysed using ANOVA followed by Student-Newman-Keuls test. Two group comparisons with or without treatment used Student’s t test unless otherwise specified.

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

Although it is known that nucleosomes can be released after cell death or NETs formation (32-36), it is not clear whether circulating histones are still exclusively in the form of histone-DNA complexes. Using ultracentrifugation to fractionate plasma or serum with high circulating histone levels into 6 fractions, we found that histones were detectable in all 6 fractions (Figure 1A upper panel). However, DNA-histone complexes (most likely nucleosomes) were in fraction 6 only (bottom fraction) (Figure 1A, lower panel). No difference was found between plasma and serum. This experiment demonstrated that DNA-free histones exist in circulation.

Histone-conjugated Sepharose beads were then 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 (Figure 1B). Following liquid chromatography-mass spectrometry (LC-MS/MS) analysis, complement C4 and C-reactive protein (CRP) were identified (Figure 1C and 1D). CRP has been reported to be a major histone-binding protein that neutralises histone toxicities (37). As to complement C4, we could detect histone-C4 complexes in normal plasma spiked with calf thymus histones (Figure 1E) and also in plasma from critically ill patients with high circulating histone levels (Figure 1F), confirming that histones form complexes with C4 in vivo. In this study, we further investigated the interaction of histones with C4 and its potential biological roles and significance.

Individual histones bind to complement C4 with different affinity

To determine the relative binding extents of individual histones to C4, equal molar concentrations of individual histones were subjected to gel overlay assay (Figure 2A upper) with Coomassie blue stained gel demonstrated equal loading (Figure 2A lower). Figure 2A
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. To determine the comparative binding strengths under physiological conditions, we used surface plasmon resonance (SPR, Biocore X-100) (Figure 2B-F). Table 1 shows that H3 \( (K_D = 0.76 \pm 0.12 \text{ nM}) \) and H4 \( (K_D = 0.91 \pm 0.07 \text{ nM}) \) had much higher binding affinity than equal molar concentrations of H1 \( (K_D = 7.26 \pm 0.80 \text{ nM}) \) and H2B \( (K_D = 9.45 \pm 1.43 \text{ nM}) \), with weak binding to H2A \( (K_D = 12.67 \pm 0.59 \text{ nM}) \).

**Histones dramatically inhibit classical and lectin but not alternative pathways**

To investigate functional consequence, we used a Complement functional screen kit to measure the effects of histones on the activation of classical, MBL and alternative pathways. Pre-incubation of different concentrations of calf thymus histones 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 amount of MAC could be formed in the presence of 50 μg/ml histones (Figure 3A). In contrast, histones showed much less effect on the alternative pathway and 50 μg/ml histones only reduced MAC about 20%. To evaluate the overall effect of histones on complement activation in human serum, zymosan was used to activate complement in the presence or absence of histones. We found that histones at 50 μg/ml could significant inhibit the production of MAC induced by zymosan (Figure 3B). We also assessed the role of individual histones in the classical (C, D), and MBL (E, F) pathway activation and 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.

**Anti-histone reagents can rescue complement activation**
To demonstrate the specificity of histones on complement activation, anti-histone H4 and non-anticoagulant heparin that 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 (Figure 4A, B), whilst anti-histone H4 could significantly rescue the H4-inhibited complement activation of both pathways (Figure 4C, D). Those data demonstrated that the effect of histones on complement inactivation was specific to histones.

**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, a process of C4 activation, was investigated. We found that histones showed no effect on the production of C4a (Figure 6 A-B), indicating histone binding does not affect the ability of C1s to cleave C4 protein. Further investigation showed that histones bind to C4b but not C4a (Figure 6C). However, in the presence of histones, the production of C3a and C5a were significantly reduced in the classical and MBL pathways but not the alternative pathway (Figure 6E-F), 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 6E-F) was significantly reduced by histones due to the suppression of both classical and MBL pathways.

**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 1/3 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 5A, B). However, the zymosan-induced complement activation could be recovered by 300 μg/ml C4 from 25% to 70% of total activity in the presence of 20 μg/ml histone H4 protein (Figure 5C). This observation suggests that histones may also target other components of the complement system rather than C4 alone. On the other hand, C4 protein could significantly reduce the cytotoxicity of histones to cultured endothelial cells (Figure 5D).

**Discussion**

Complement activation generates membrane attack complexes (MAC) to lyse cells and leads to cell death and content release, including histones. The inhibitory effects of histones on complement activation could form a physiological feedback loop to prevent over production of MAC and excessive tissue damage. This finding is novel and with evidence 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 (Figure 7).

Complement C4 is activated by C1s cleavage to produce C4a and C4b. The C4b is the essential component of both C3 and C5 convertase, a common step of both classical and MBL pathways (Figure 7). Histones strongly bind to C4 but do not affect C4 activation because there is no difference in C4a production in the presence or absence of histones. Histones binds to C4b but not C4a, therefore their major effect is to reduce the activity of C3 and C5 convertase, as indicated by reduction of C3a and C5a, the products of C3 and C5 activation. 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. Since the life time of C3 and C5 convertases in solution are very short, it is difficult to distinguish the two potential mechanisms. In contrast, histones have minimal effect on alternative pathways, 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. However, 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 (Figure 7). This needs further
investigation.

It is known that circulating C4 is about 0.4 mg/ml, but no histones could be detected in blood
from heathy donors (39). In critical illness, for example sepsis, histones could surge up to
100-200 mg/ml (12) but C4 was reported to decrease due to consumption (39). Therefore the
high levels of histones are sufficient to inhibit both classical and MBL pathways. Although
high levels of C4 could efficiently detoxify histones in vitro, the low levels of C4 in sepsis
may not be sufficient to neutralise high levels of histones. In non-critical 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.

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**Abbreviations:** AP: alternative pathway; CP: classical pathway; CRP: C-reactive protein;
DAMPs: damage-associate molecular patterns; MAC: membrane- attack complex; MBL:
Mannan binding lectin; LPS: lipopolysaccharides; NETs: neutrophil extracellular traps; SPR:
surface plasmon resonance; TLR: Toll-like receptors.
**Author contributions:** YQ, STA and YA performed experiments, analysed the data and performed statistical analysis. PM and YQ did SPR assay. STA, GW and CHT wrote, edited and reviewed the manuscript and figures. GW, SEC and CHT supervised the work.

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**References**


**Figure legends**

**Figure 1. Identification of Complement component 4 as a histone binding protein.** (A) 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). (B) 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. (C) and (D) The typical spectra of the two major proteins are presented. (E) Histone-C4 complexes were detected by ELISA following the addition of different concentrations of histones to normal plasma. (F) Histone-C4 complexes are elevated in critically ill patient plasma compared to normal (n=3).

**Figure 2. Complement C4 binds to individual histones.** (A) Two μg individual histones and 6 μg S100P as a control were subjected to SDS-PAGE. One gel was transferred onto PVDF membrane and probed with HRP-conjugated C4 protein (upper panel). The other gel
was stained with Coomassie brilliant blue (lower panel). (B-F) SPR analysis. Individual histones were first immobilized on streptavidin surfaces. Different concentrations of C4 were applied onto each surface and typical binding curves are presented.

**Figure 3. 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 antibodies. The complement activity of control wells without histones was set up as 100%. The Means±SD of relative activities were presented. (B) Shows the Means±SD of relative activities activated by zymosan (activating different pathways) in the presence of different concentration of calf thymus histones (0-50 µg/ml). The Means±SD of relative activities of Classical (C) and MBL (D) pathways in the presence of 20µg/ml individual histones. The Means±SD of relative activities of Classical (E) and MBL (F) pathways in the presence of different concentration of individual histones (0-50 µg/ml). Means±SD were calculated from at least 3 independent experiments. ANOVA test, *p<0.05 compared to untreated.

**Figure 4. Anti-histone treatment rescues complement activation.** (A) and (B) non-anticoagulant heparin (20 µg/ml) was used to rescue complement activities of classical (A) and MBL pathways inhibited by individual histones (20 µg/ml). (C) and (D) Anti-histone H4 antibody (12 µg/ml) was used to rescue complement activities of Classical (C) and MBL (D) inhibited by H4 (20 µg/ml). The Means±SD of relative activities from at least 3 independent experiments were presented. ANOVA test, *P<0.05 when compared to untreated. **P<0.05 when compared to that treated with histone alone.

**Figure 5. Histones show no effect on C4 cleavage but significantly reduce C3 and C5 convertase activities.** (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 mins 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 antibody is presented (Upper panel). Fold changes were calculated by setting up C4a intensity without histones as 1.0. The relative fold changes of that with calf thymus histones from 3 independent experiments are presented (Lower panel). Student’s t test, P=0.2. (C) Two µg C4, C4b, C4a and S100P (as a control) were subjected to blotting with HRP-conjugated calf thymus histones. A typical blot is presented. (D-F) Complement in serum was activated by IgM (Classical pathway, CP), Mannan (MBL), LPS (Alternative pathway, AP) or zymosan in the absence or presence of calf thymus histones treated (50 µg/ml) for 1hr at 37°C. Then the C3a (D), C5a (E) or MAC levels (F) were detected by ELISA. Means±SD from at least 3 independent experiments are presented. ANOVA test, *P<0.05 when compared to that without histones.

Figure 6. Effect of C4 protein on histones. (A-B) C4 protein affects histone-inhibited complement activity. Adding C4 protein (0-300 µg/ml) rescued 20 µg/ml histone H4-inhibited activation of Classical pathway (A), MBL pathway (B). (C) 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). (D) Human endothelial cell line, EA.hy926, was treated with 100 µg/ml calf thymus histones in the presence 0-300 µg C4 proteins for one hour. The percentage of cell viability was detected using WST8 cell viability kit. Means±SD from 3 independent experiments are presented. ANOVA test, *P<0.05 when compared to histone alone.

Figure 7. Schematic representation of the effect of histones in the complement pathway.
Figure 2

A

C4 overlay

Coomassie

H1  H2A  H2B  H3  H4  S100P

B

Ligand: H1; Analyte: C4

Response (RU)

Time (s)

0.500 μM
0.250 μM
0.125 μM
0.063 μM
0.031 μM
0.016 μM
0.008 μM
0.004 μM
0.000 μM

C

Ligand: H2A; Analyte: C4

Response (RU)

Time (s)

1.000 μM
0.500 μM
0.250 μM
0.125 μM
0.063 μM
0.031 μM
0.016 μM
0.008 μM
0.004 μM
0.000 μM

D

Ligand: H2B; Analyte: C4

Response (RU)

Time (s)

1.000 μM
0.500 μM
0.250 μM
0.125 μM
0.063 μM
0.031 μM
0.016 μM
0.008 μM
0.004 μM
0.000 μM

E

Ligand: H3; Analyte: C4

Response (RU)

Time (s)

0.063 μM
0.031 μM
0.016 μM
0.008 μM
0.004 μM
0.000 μM

F

Ligand: H4; Analyte: C4

Response (RU)

Time (s)

0.125 μM
0.063 μM
0.031 μM
0.016 μM
0.008 μM
0.004 μM
0.000 μM
Figure 3

A

B

Zymosan

C

D

Classical pathway

Classical pathway

E

F

Lectin pathway

Lectin pathway
Figure 4

A. Classical pathway

B. Lectin pathway

C. Classical pathway

D. Lectin pathway

MAC (%)