C4b Binding Protein Acts as an Innate Immune Effector Against Influenza A Virus

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C4b Binding Protein (C4BP) is a major fluid phase inhibitor of the classical and lectin pathways of the complement system. Complement inhibition is achieved by binding to and restricting the role of activated complement component C4b. C4BP functions as a co-factor for factor I in proteolytic inactivation of both soluble and cell surface-bound C4b, thus restricting the formation of the C3-convertase, C4b2a. C4BP also accelerates the natural decay/dissociation of the C3 convertase. This makes C4BP a prime target for exploitation by pathogens to escape complement attack, as seen in Streptococcus pyogenes or Flavivirus. Here, we examined whether C4BP can act on its own in a complement independent manner, against pathogens. C4BP bound H1N1 and H3N2 subtypes of Influenza A Virus (IAV) most likely via multiple sites in Complement Control Protein (CCP) 1-2, 4-5, and 7-8 domains of its α-chain. In addition, C4BP CCP1-2 bound H3N2 better than H1N1. C4BP bound three IAV envelope proteins: Haemagglutinin (~70 kDa), Neuraminidase (~55 kDa), and Matrix protein 1 (~25kDa). C4BP suppressed H1N1 subtype infection into the lung epithelial cell line, A549, while it promoted infection by H3N2 subtype. C4BP restricted viral entry for H1N1 but had the opposite effect on H3N2, as evident from experiments using pseudo-typed viral particles. C4BP downregulated mRNA levels of pro-inflammatory IFN-α, IL-12, and NFκB in the case of H1N1, while it promoted infection by H3N2 subtype. C4BP restricted viral entry for H1N1 but had the opposite effect on H3N2, as evident from experiments using pseudo-typed viral particles. C4BP differentially modulates the efficacy of IAV entry, and hence, replication in a target cell in a strain-dependent manner, and acts as an entry inhibitor for H1N1. Thus, CCP containing complement proteins such as factor H and C4BP may have additional defense roles against IAV that do not rely on the regulation of complement activation.

Keywords: complement, C4BP, influenza A virus, inflammation, pseudo-typed lentiviral particles
INTRODUCTION

The complement system plays a crucial role in our early response against invading pathogens, including viruses. The complement system is activated via three pathways—Classical, Alternative, and Lectin. The classical pathway is activated through the binding of C1q to IgG or IgM-containing immune complexes or other non-immunoglobulin targets. Non-self-carbohydrate targets are recognized by Mannose Binding Lectin (MBL) or ficolins triggering the lectin pathway. The activation of classical or lectin pathways leads to the cleavage of C4 and C2, yielding C3 convertase (C4b2a), which then cleaves C3 to form C3b. For the alternative pathway, C3 is spontaneously hydrolyzed to a C3b-like form \([C3(H_2O)]\) due to the hydrolysis of the internal thioester bond. The C3(\(H_2O\)) binds to Factor B, which enables Factor D to cleave Factor B to Bb. This then forms C3(\(H_2O\))Bb, which is homologous to the C3 convertase, C4b2a. C3(\(H_2O\))Bb then cleaves C3 to C3b, and C3b can bind covalently to target surfaces, on which it forms more convertase, C3bBb. The binding of the C3b to C4b2a or C3bBb converts them into classical or alternative pathway C5 Convertases, respectively. The subsequent cleavage of C5 by the C5 convertases initiates the formation of the Membrane Attack Complex (C5b-C9). This complex binds to the microbial surface and can cause lysis of lipid bilayer membranes (1).

The complement system is kept in check by various regulatory proteins to prevent runaway reactions leading to unnecessary inflammation and death of healthy cells. One such humoral regulator is C4b Binding Protein (C4BP). C4BP, like other complement regulators Factor H, CR1, CD46, CD55, and the Factor H-related family, is encoded by a gene present in the long arm of chromosome 1 and is synthesized and secreted mainly by hepatocytes (2). The normal range of C4BP in human plasma is estimated to be approximately 150–300 µg/ml (3). C4BP regulates complement activation by controlling C4b mediated reactions (4). C4BP, a 570 kDa glycoprotein, functions as a primary fluid phase regulator of the classical and lectin pathways (5). It consists of seven identical 70 kDa \(\alpha\)-chains and a 45 kDa \(\beta\)-chain that are linked at their carboxy-terminal ends by short amphipathic helices further stabilized by disulfide bonds (6). The \(\alpha\)-chains and \(\beta\)-chain contain eight and three Complement Control Protein (CCP) domains, each of ~60 amino acids, respectively. The polymerization of the polypeptide chains of C4BP occurs via a short C-terminal region, downstream of CCP8 (7). Each molecule of C4BP can bind only four molecules of C4b due to steric hindrance, although each of the seven \(\alpha\)-chains has a C4b binding site (8). The binding site for C4b has been localized to CCP1-3, especially within a cluster of positively charged residues at the interface of CCP1 and CCP2 (9, 10). C4BP has been shown to inhibit the formation of C3 and C5 convertases, accelerate the decay of the convertases, and act as a cofactor for Factor I which cleaves and thereby inactivates fluid phase and cell-bound C4b (9–12). The co-factor activity of the protein has been linked to CCP1-4 (13).

Consistent with its role as an immunomodulator, C4BP also engages with pathogens. Flaviviruses limit complement activation by binding C4BP through their NS1 protein: the bound C4BP inactivates soluble or membrane-bound C4b (5). *Streptococcus pyogenes* uses its Protein H to recruit C4BP in order to facilitate the invasion of endothelial cells and reduce the chances of its opsonization by bound C3b, or attack by the MAC (14). C4BP probably is also recruited by other pathogens to limit C3 opsonization, MAC mediated lysis, and immune system activation by C3a/C5a (15).

C4BP has been reported to have properties that are independent of its involvement in complement regulation. For example, C4BP is known to facilitate the uptake of adenoviruses by hepatocytes via its interaction with heparin-sulfate proteoglycans found on cell surfaces (16). C4BP induces proliferation and activation of B lymphocytes by binding with CD40 (17). Interaction between CD40, CD154, and C4BP inhibits apoptosis in epithelial cells. C4BP has also been implicated in inhibiting DNA release from necrotic cells in conjunction with Protein S by binding to phosphatidylserine (18–20). C4BP also have an association with the clotting system as the \(\beta\)-chain binds the vitamin K-dependent anticoagulant protein S (21).

In this study, we investigated potential complement-independent defense functions of C4BP against Influenza A Virus (IAV). Influenza virus causes an upper respiratory tract infection affecting the nose, throat, bronchi, and sometimes the lungs. Of the three genera of Influenza virus that belong to the Orthomyxoviridae family, IAV is known to cause pandemics. IAV causes Influenza in birds and a few species of mammals, including humans and pigs. Its wide host range has led to pandemics such as the 1918 Spanish flu, which caused 20–50 million deaths (22). It has several subtypes based on variants of two proteins on the surface of the viral envelope. These proteins are Hemagglutinin (HA), which causes RBCs to agglutinate, and Neuraminidase (NA), which breaks the glycoside bonds of neuraminic acid (23). Currently, there are 18 known variations of HA, and 11 known variations of NA, theoretically allowing 198 different combinations (24, 25). Some of the well-known combinations are H1N1 that caused the 1918 Spanish flu, 1977 Russian flu, and 2009 flu Pandemic, H3N2 that caused the 1968 Hong Kong flu and H2N2 that caused 1957 Asian flu (26).

Human infection by IAV is achieved through oral or nasal cavities, where the viral HA binds sialic acids in order to attach to lung epithelial cells (27). The HA protein of the human-infecting IAV has increased specificity for \(\alpha\)-2,6-linked sialic acid (28). Sialic acid, a nine-carbon acidic monosaccharide, is commonly found with \(\alpha\)-2,6-linkages and a few \(\alpha\)-2,3-linkages on human tracheal epithelial cells (29). Once bound to the cell surface, the viral particle undergoes receptor-mediated endocytosis. We show here that IAV interacts with the \(\alpha\) chain of C4BP mainly via CCP domains 4–5, CCP domains 7–8, and to a lesser extent CCP domains 1–2. C4BP acts as an entry inhibitor for H1N1 subtype when challenged against A549 (lung epithelial) cells and suppresses the pro-inflammatory cytokine storm induced by IAV.

MATERIALS AND METHODS

Purification of Native C4BP

C4BP was isolated from human plasma as described previously (30). Briefly, C4BP was purified from neutral euglobulin...
precipitate by affinity chromatography using C4c-Sepharose, equilibrated in 5 mM EDTA/25 mM potassium phosphate buffer, pH 7.0. The column was washed in the same buffer and then bound protein was eluted by a linear gradient of NaCl from 0 to 2 M; C4BP was eluted at 0.8 M NaCl. The peak fractions were run on 12% w/v acrylamide SDS-PAGE to assess purity and adjusted to 1 mg/ml concentration. The immunoreactivity of this plasma purified C4BP was assessed via western blotting.

Expression and Purification of Recombinant Complement Control Protein Deletants of C4BP α-Chain

Recombinant α-chain of C4BP (rC4BP) and C4BP α-chain CCP deletants (Table 1) were provided by Prof. Anna Blom, Lund University, Malmo, Sweden. Briefly, CCP deletant constructs were expressed in Human Embryonic Kidney (HEK) 293 cells (ATCC number 1573-CRL). The transfected cells were selected were expressed in Human Embryonic Kidney (HEK) 293 cells (ATCC number 1573-CRL). The transfected cells were subjected to SDS-PAGE with 12% w/v acrylamide/bisacrylamide (10).

The recombinant CCP deletants were subjected to immunoblotting. The medium collected from transfected cells expressing the highest amount of CCP deletants were assessed using neomycin analogue G418 (400 μg/ml), and colonies expressing the highest amount of CCP deletants were selected and incubated for 3 days at 37°C in the infection medium [DMEM + 1% penicillin/streptomycin + 0.3% w/v bovine serum albumin (BSA) + 1 µg/ml of 1-1-Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-Tryptsin (Sigma-Aldrich)]. TPCK treated Tryptsin mediates the cleavage of HA0 into two subunits HA1 and HA2, which are required to make HA fusion competent. After the incubation, the medium was collected and centrifuged in sealed containers at 3,000 × g for 15 min. The supernatant containing the viral particles was harvested, and the titer of the particles was determined via 50% Tissue culture Infective Dose (TCID50) Assay. Purified virus stocks were serially diluted in a 96-well plate up to 10−7-fold with ½ log10 increments. 1 × 105 MDCK cells were added to each well and incubated for 3 days at 37°C under 5% v/v CO2. The titer of the virus was determined to be 1 × 10⁶ PFU/ml by TCID50 using the Reed–Muench method (31).

TABLE 1 | C4BP deletants used in this study.

| Mutants | Deleted regions within the α-chain of C4BP |
|---------|------------------------------------------|
| ΔCCP1  | Asn1-Tyr62 |
| ΔCCP2  | Lys54-Asn124 |
| ΔCCP3  | Val125-Lys188 |
| ΔCCP4  | Lys188-Asn249 |
| ΔCCP5  | Asn249-Ala314 |
| ΔCCP6  | Leu315-Gly375 |
| ΔCCP7  | Asp376-Lys433 |
| ΔCCP8  | Cys433-Trp492 |

TABLE 2 | Plasmids used for pseudo-typed lentiviral particle production.

| Plasmids | H1N1 Envelope Protein-Coding Plasmid | H3N2 Envelope Protein-Coding Plasmid | VSV-G Backbone Plasmid |
|----------|--------------------------------------|--------------------------------------|------------------------|
| pcDNA3.1-swineH1-FLAG-H1 from swine H1N1 A/California/04/09 (Codon optimized H1 (Geneart)) | pcDNA-H3 [H3 from A/Denmark/70/03/H3N22] (Codon optimized H3 (Geneart)) | pCMV-VSV-G (Addgene plasmid # 8454) |
| pcDNA3.1-swineN1-FLAG (N1 from swine H1N1 A/California/04/09) (Codon optimized N1 (Geneart)) | pl.18-N2 (N2 from human H3N2 A/Texas/50/2012) | pHIV-Luciferase (Addgene plasmid # 21375) |
| Packaging Plasmid | psPAX2 (Addgene plasmid # 12260) | | |
The transfected cells in the pellet were lysed using lysis buffer (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 0.1% v/v Triton X-100) and were centrifuged at 1,500 × g for 5 min to remove cell debris. The total lysate and supernatant were filtered with a 22 µm filter. The pseudo-typed lentiviral particles were harvested by centrifuging the filtered cell lysate and the supernatant at 5,000 × g for 10 min at 4°C in a closed container. The lentiviral particles in the supernatant were then concentrated by ultracentrifugation at 25,000 × g for 3 h at 4°C (33). The pseudo-typed lentiviral particles produced were characterized by western blotting (32).

**ELISA to Detect C4BP-Virus Interaction**

Microtiter wells of 96-well plates (Nunc # 4912) were coated (4°C, overnight) with the virus [100 µl of H1N1 or H3N2 subtypes (1,000 PFU/ml)] in carbonate-bicarbonate buffer, pH 9.6 (Sigma # c3041). Other wells were coated with 100 µl/well of C4BP, α-chain CCP mutants, at 5, 2.5, 1.25 µg/ml in the same buffer. In another experiment, 5 µg/ml of rC4BP was similarly coated. Unbound proteins or viruses were removed by washing the wells three times with PBST Buffer (PBS + 0.05% Tween 20) (Fisher Scientific). The wells were then blocked using 2% w/v BSA in PBS (Fisher Scientific) for 2 h at 37°C and washed three times using PBST. Decreasing concentrations of C4BP (5, 2.5, 1.25 µg/ml) were added to the wells in which the virus was coated to study its binding to immobilized viral particles. In parallel experiments, 1,000 PFU/ml of purified H1N1 and H3N2 were added to the protein-coated wells to study the binding of the virus to immobilized C4BP or rC4BP. The wells were incubated for 2 h at 37°C and then washed with PBST to remove any unbound viruses or protein. VSV-G pseudo-typed lentivirus was used as a negative control. The wells were probed using polyclonal rabbit anti-human C4BP (1 mg/ml of purified IgG; MRC Immunofluochemistry Unit, Oxford) to detect the virus-bound C4BP. Monoclonal Anti-Influenza Virus H1 HA, A/California/04/2009 (H1N1)pdm09, Clone 5C12 (produced in vitro) (BEI-Resources, NIAID, NIH, USA), Monoclonal Anti-Influenza Virus N2 Neuraminidase (NA), Clone NA2-1C1 (produced in vitro) (BEI-Resources, NIAID, NIH, USA), Polyclonal Anti-Influenza Virus N2 Neuraminidase (NA), A/shorebird/Delaware/172/1997 (H6N2) (antiserum, Goat) (BEI-Resources, NIAID, NIH, USA), Monoclonal Anti-Influenza Virus N1 Neuraminidase (NA), Clone N1-1A7 (produced in vitro) (BEI-Resources, NIAID, NIH, USA), Monoclonal Anti-Influenza Virus H3 HA, A/Hong Kong/1/1968 (H3N2) (antiserum, Goat) (BEI-Resources, NIAID, NIH, USA), and Protein A-HRP conjugate (1:1,000; Promega #W4011) for 1 h; the blot was developed using 3,3′-diaminobenzidine (DAB) (Sigma) as a substrate.

For direct western blot for viral proteins, the PVDF membrane, containing electro-transferred viral proteins, was incubated with appropriate antibodies: these included monoclonal anti-IAV H1, polyclonal anti-IAV H3, Monoclonal anti-IAV Neuraminidase (NA), Clone NA2-1C1 (produced in vitro) (BEI-Resources, NIAID, NIH, USA), Polyclonal Anti-Influenza Virus N2 Neuraminidase (NA), A/shorebird/Delaware/172/1997 (H6N2) (antiserum, Goat) (BEI-Resources, NIAID, NIH, USA), and monoclonal anti-Matrix protein 1 (M1) antibody (Abcam) as required for 1 h at room temperature on a rotary shaker. Following three washes with PBST, the membrane was probed with either anti-mouse HRP or Protein A-HRP conjugate, as appropriate for 1 h at room temperature on a rotary shaker. Following three washes with PBST, the blot was developed using DAB.

**A549 Cell Infection Assay**

Human alveolar basal epithelial cells derived from an adenocarcinoma (A549) (ATCC® CCL-185™) were cultured for 12 h in each well of a 12-well plate in growth medium. Post-attachment, the cells were serum starved by incubating them in serum-free DMEM for 24 h. H1N1 and H3N2 subtypes (MOI 1) were incubated with C4BP (10, 20, or 40 µg/ml) in PBS for 1 h and added to A549 cells in serum-free DMEM. After 1 h incubation at 37°C, the medium was removed, and cells were washed with PBS to remove any unbound IAV. Infection medium (DMEM + 1% v/v Penicillin-Streptomycin + 0.3% w/v BSA + 1 µg/ml of TPCK-Trypsin) was added to the cells and incubated for 2 or 6 h. The cells were then harvested by scraping with a sterile disposable cell scraper and centrifuging at 1,500 × g for 5 min. Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). Contaminating DNA was removed by treating the RNA samples with DNase I (Sigma) for 15 min. DNase I was inactivated by heating at 70°C for 10 min. The quantity and quality of the extracted RNA were assessed using a Nanodrop 2000 spectrophotometer (Thermo Fisher) via absorbance at 260 nm and 260:280 nm ratio. mRNA was converted to cDNA using TagMan™ High Capacity RNA to cDNA Kit (Applied Biosystems™). The cDNA...
was stored at –20°C for future use. qRT-PCR was performed using a StepOnePlus System (Applied Biosystems™) at 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. The specificity of the assay was established by melting-curve analysis. The relative expression (RQ) was calculated by using C4BP untreated cells as the calibrator. The RQ value was calculated using the formula: RQ = 2−ΔΔCt. Primers were generated for specificity using the Primer-BLAST software (Basic Local Alignment Search Tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 3).

**Luciferase Assay**

MDCK cells were seeded in a 96-well plate (1 × 10⁵ cells per well) and were serum starved. The cells were infected for 24 h in the infection medium with 50 μl of the H1N1 and H3N2 pseudo-typed particles that were pre-incubated with C4BP for 1 h, or pseudo-typed particles that were not treated with C4BP. Post-infection, luciferase activity in the cells was measured using Pierce™ Firefly Luc One-Step Glow Assay Kit (Thermo Fisher) and CLARIOstar Plus Plate Reader (BMG Labtech).

**Statistical Analysis**

GraphPad Prism 8.0 software was used to create all graphs. Two-way ANOVA was used to find the statistical significance of the data generated. Significant values were considered based on *p < 0.1, **p < 0.05, ***p < 0.01, and ****p < 0.001 between treated and untreated conditions. Error bars show the standard deviation among the samples.

**RESULTS**

**C4BP Directly Binds to Influenza A Virus in a Dose-Dependent Manner**

Native C4BP, purified from plasma, was run on a 12% w/v reducing SDS-PAGE, which revealed a band corresponding to α-chain (~70 kDa), and one corresponding to β-chain (~45 kDa) (Figure 1A). The immunoreactivity of the protein was determined by performing a western blot with rabbit polyclonal anti-human C4BP antibodies, which yielded a band corresponding to the α-chain (~70 kDa) of C4BP. However, the β-chain (~45 kDa) was barely visible (Figure 1B). Indirect ELISA was then performed to examine the binding of C4BP to IAV subtypes. Binding of immobilized C4BP to the virus (Figure 2A), and conversely, of immobilized virus to C4BP (Figure 2B) was observed. As evident in Figures 2A, B, C4BP bound to H3N2 and H1N1 in a dose-dependent manner; binding to H3N2 was greater than to H1N1 (Figure 2A) subtype. Recombinant C4BP alpha chain (rC4BP) (Supplementary Figure 1) was also used to confirm its binding to the virus. No significant difference in binding efficiency was observed between the recombinant C4BP and the plasma purified C4BP (Figures 2C, D).

**C4BP Binds HA, NA, and M1 Proteins of Influenza A Virus**

In order to identify the IAV proteins that interact with C4BP, far-western blot analysis was performed. When SDS-PAGE separated IAV proteins were probed with C4BP, it revealed that C4BP interacted with IAV viral proteins Hemagglutinin (HA) (~70 kDa), Neuraminidase (NA) (~55 kDa) and Matrix protein 1 (M1) (~25kDa) (Figure 3A). The identities of C4BP bound IAV glycoproteins were confirmed using separate blots that were directly probed with anti-HA (Figure 3B), anti-NA (Figure 3C), anti-M1 (Figure 3D), or anti-NS1 antibodies (Figure 3E). IAV proteins separated by SDS-PAGE were probed directly with anti-C4BP antibodies to rule out non-specific interaction between the antibody and IAV proteins (Figure 3F). NS1 protein has similar molecular weight to M1, but the blot with antibodies confirmed M1 as the 25kDa species involved in binding.

**Influenza A Virus Binding Sites Within C4BP α-Chain Appear to Be Localized Within CCP4-5 and CCP7-8 Modules**

To identify the likely binding site on C4BP for the IAV, an indirect ELISA was performed using recombinant C4BP α-chain mutants lacking individual CCP domains. Recombinant C4BP lacking CCP4, 5, 7, or 8 showed relatively weaker binding to H1N1 and H3N2 subtypes. The deletion of CCP domain 8 reduced binding by approximately 75% for both subtypes when compared to full-length C4BP. Deletion of CCP7 resulted in reduced binding for H1N1 (~49%) as well as H3N2 (~62%). The CCP4 and 5 deletion mutants lost ~80% binding activity for H3N2 and ~60% for H1N1. Approximately 20% loss of binding for H1N1 was observed in the case of CCP1 and 2 deletants, but the same deletants showed ~80% reduction in binding to H3N2. The loss of CCP domains 3 and 6 caused a small increase in binding by approximately 10% for both IAV subtypes. This indicates that C4BP binds both IAV subtypes lacking CCP4, 5, 7, or 8 showed relatively weaker binding to H1N1 and H3N2 subtypes. The deletion of CCP domain 8 reduced binding by approximately 75% for both subtypes when compared to full-length C4BP. Deletion of CCP7 resulted in reduced binding for H1N1 (~49%) as well as H3N2 (~62%). The CCP4 and 5 deletion mutants lost ~80% binding activity for H3N2 and ~60% for H1N1. Approximately 20% loss of binding for H1N1 was observed in the case of CCP1 and 2 deletants, but the same deletants showed ~80% reduction in binding to H3N2. The loss of CCP domains 3 and 6 caused a small increase in binding by approximately 10% for both IAV subtypes.
most likely involving CCPs 4-5 and 7-8 domains of its α-chain (Figure 4). However, in the case of the H3N2 subtype, and to a lesser extent H1N1 subtype, it appears that there is an additional binding interaction with CCPs 1-2 (Figure 4B). This may be consistent with greater binding to H3N2. VSV-G pseudo-typed lentivirus that was used as negative control showed no significant binding (Figure 4C).

**C4BP Inhibits H1N1 Replication**

HA, NA, and M1 are essential IAV proteins which have many functions from viral entry to virion budding, bringing about IAV infectivity and replication. Therefore, we investigated the effect of C4BP on cell infection. A549 cells were challenged with either H1N1 or H3N2 for 6 h that were previously incubated with C4BP. The efficacy of replication was estimated by comparing the M1 expression levels of the virions treated with C4BP (10, 20, or 40 µg/ml) to the cells infected with IAV but, not treated with C4BP. M1 mRNA levels were downregulated by C4BP in the case of H1N1 (−4 log10), suggesting that the interaction of C4BP with H1N1 reduced the replication efficiency of the virus at an early stage of the replication cycle. However, in the case of A549 cells challenged with H3N2, M1 mRNA levels were found to be upregulated (2 log10), suggesting an increase in IAV replication efficacy. Taken together, these results suggest that C4BP differentially modulates viral entry, and thus, replication efficacy among the IAV subtypes (Figure 5).

**C4BP Is an Entry Inhibitor for H1N1**

As C4BP was found to interact with HA and NA, the IAV proteins that play an integral role in viral host cell recognition and entry, lentiviral pseudo-particles were used to study the entry process independent of other IAV components. The pseudo-particles expressing the respective IAV coat proteins with an HIV-Luciferase backbone were used as a safer alternative to assess the role of C4BP as an entry modulator of H1N1 and H3N2. This system contains a single packaging plasmid (psPAX2) encoding genes including Gag, Pol, and Tat. pHIV-Luciferase was used as a lentiviral transfer plasmid, which is flanked by long terminal repeat (LTR) sequences and designed to express the firefly luciferase reporter. Thus, pHIV-Luciferase is replication-incompetent which contains an additional sequence deletion in the 3’ LTR leading to viral self-inactivation post-integration. Purified H1+N1 and H3+N2 pseudo-typed particles pre-incubated with C4BP (20 µg) were used to infect MDCK cells. The lentiviral particles not treated with C4BP were used as controls. A ~56% reduction in luciferase reporter activity was observed when MDCK cells infected with C4BP-treated H1+N1 pseudo-typed particles were compared to their untreated counterparts. However, an opposite effect, although not so dramatic, was observed following C4BP treatment in the case of H3N2 unmatched pseudo-particles. A ~17% increase in luciferase activity was observed in the treated samples when compared to the control (Figure 6). C4BP protein seems to be acting as an entry inhibitor for H1N1, but a weak facilitator of infection by H3N2.

**C4BP Modulates Pro-inflammatory Cytokine/Chemokine Response in IAV-Challenged A549 Cells**

Pro-inflammatory cytokines and chemokines, such as IL-1, IL-6, IL-12, IFN-α, and NF-κB transcription factor, are a hallmark of
IAV infection (34). In order to understand the effect of C4BP on the pro-inflammatory cytokines produced during IAV infection, A549 cells were challenged with H1N1 and H3N2 viruses (MOI 1) preincubated with C4BP. qRT-PCR was then carried out using total RNA from the cells (Figure 7), using cells infected with IAV that were not treated with C4BP as the control. C4BP treated H1N1 challenged A549 cells showed lower levels of IFN-α (-2 log10) at both 2 and 6 h, whereas cells challenged with C4BP treated H3N2 exhibited higher levels of IFN-α 6 h post-infection (Figure 7A). IL-12 is essential for the generation of cell-mediated immunity via the production of IFN-γ by helper type 1 T (Th1) cells, which leads to the elimination of the virus (35). Levels of IL-12 at 2 h in case of A549 cells challenged with either C4BP treated H1N1 or H3N2 were not detectable (Figure 7B). At 6 h in C4BP treated H1N1 challenged cells, IL-12 levels were downregulated (-2 log10), while in cells challenged with C4BP treated H3N2 the levels were upregulated (1 log10) (Figure 7B). As interferon response is triggered by the detection of viral components within infected cells, and IL-12 induction requires live viruses, these results are consistent with the previously observed levels of M1 transcript.

The transcription factor, NF-κB, is a regulator of antiviral cytokines such as IFN-β, which initiates a strong type I IFN immune response. IAV induces IFN expression via NF-κB as viral titer levels are higher in cells treated with an anti-IFN-α/β receptor antibody and not in dominant-negative IκBα expressing cells (36). NF-κB mRNA levels at 2 h were slightly upregulated in C4BP treated H1N1 challenged cells and no significant change in its levels were observed in cells challenged with C4BP treated H3N2 (Figure 7C). Cells infected with C4BP treated H1N1 at 6 h exhibited lower levels of NF-κB (~1 log10), and no significant changes were observed in C4BP treated H3N2 infected cells (Figure 7C). The lower levels of NF-κB can be attributed to either low viral titer in the case of H1N1 or the IAV non-structural protein NS1 which acts as a major suppressor of NF-κB activation (37).

Increased levels of IL-6, TNF-α, and IFN-γ, have been detected in individuals infected with acquired acute as well as severe seasonal IAV-infected patients (38, 39). Production of TNF-α during IAV infection correlates with morbidity and mortality rates in IAV infected humans and macaques (40, 41). Six hours post-infection, TNF-α mRNA levels were upregulated (2 log10) in A549 cells infected with C4BP treated H1N1 or H3N2, while at the earlier 2-h time point a slight upregulation was observed (Figure 7D).

RANTES is a C-C chemokine and a potent chemoattractant for monocytes, T lymphocytes, basophils, and eosinophils. IAV, via its HA, can induce RANTES expression, probably through the activation of NF-κB, and cause airway hypersensitivity by inducing the continuous infiltration of eosinophils (42). C4BP treated H1N1 challenged A549 cells exhibited lower levels of RANTES expression (~2 log10) at 2 h compared to the control;
no changes were observed in cells challenged with C4BP treated H3N2 (Figure 7E). RANTES expression (2 log10) was upregulated at 6 h in the case of both subtypes (Figure 7E). Enhanced levels of IL-6 have been observed in the serum and lungs of IAV infected patients. It is unknown whether IL-6 in IAV infected patients contributes to lung pathology caused by the viral infection or if increased levels of IL-6 contribute to a protective mechanism against IAV (39, 43). Levels of IL-6 at 2 h in C4BP treated A549 cells challenged with either virus were not detectable (Figure 7F). At 6 h in C4BP treated H3N2 infected cells, IL-6 levels (2 log10) were upregulated; in cells challenged C4BP treated H1N1 the IL-6 levels (0.5 log10) were also upregulated (Figure 7F). Studies also suggest that signals mediated by IL-6 are crucial for survival against a non-lethal dose of H1N1 infection, and IL-6/IL-6R deletion inhibits H1N1 clearance together with reduced neutrophils levels in the lungs of infected mice (44).

DISCUSSION

The Influenza virus, an upper respiratory tract pathogen, is known to have caused some of the deadliest pandemics in history. To develop better IAV treatment strategies and mitigate the risk of the current viral strains evolving to a more pathogenic one by reassorting among themselves, a better understanding of the immune mechanisms against IAV is required. Here, we examined the complement-independent role of C4BP, a complement regulator, against IAV. Many pathogens such as adenoviruses, Yersinia pestis, and Aspergillus spp. are known to bind C4BP to promote survival and proliferation (15). C4BP is a major fluid phase inhibitor of the classical and the lectin pathways, making it a prime target for the exploitation by pathogens to circumvent the host’s potent humoral immune system, such as complement. In this study, we sought to understand the interactions between C4BP and IAV in a serum-free system and explore any complement-independent functions of C4BP with respect to IAV.

We first established protein-protein interactions between C4BP and IAV subtypes. C4BP α chain was found to bind IAV subtypes (Figures 2A, B), and H3N2 was found to bind C4BP with higher affinity than H1N1. This interaction with the α chain of C4BP was further confirmed using rC4BP (Figures 2C, D). The C4BP α chain was found to interact with viral envelope protein HA, NA, and surprisingly, M1 (Figure 3). As the M1 protein lies beneath the lipid layer, the interaction likely occurs by the abundantly present non-packaged M1 that are released from ravaged cells during late stages of infection to help increase the odds of survival of the virus (45).

It was also possible that C4BP interacted with IAV NS1, but the signal was masked by the interaction of C4BP with M1 due to the low concentrations of NS1 when compared to M1, as proteins share similar molecular weights. Avirutnan et al. have shown that Flavivirus NS1 restricted the activation of classical and lectin pathways by binding to C4BP, thereby enabling the virus to evade C4b mediated lysis (5). Since both NS1 proteins share independent immunoregulatory functions, the binding of Influenza NS1 with C4BP was also considered. Far western blot
analysis and direct western blot analysis confirmed that it was M1 that C4BP bound to at ~26 kDa; probing with monoclonal Anti-IAV NS1 did not reveal any bands corresponding to NS1 protein. Furthermore, multiple sequence alignment and identity-similarity analysis on IAV NS1 [(Accession #: P03495; P03496) and Flaviviruses NS1 (Accession #: Q63P1 Residues: 779-1130) (P29991) Residues: 776-1127; P06935 (Residues: 788-1139)] performed using Clustal Omega and Sequence Manipulation Suite (Data not shown) revealed IAV NS1 and Flavivirus NS1 have sequence similarity scores that are lower than 25%.

Use of deletion mutants lacking specific CCP domains of C4BP revealed that H1N1 and H3N2 subtypes interacted via multiple CCP domains, CCP1-2, CCP4-5, and CCP7-8 of the C4BP α-chain. It is known that the Flavivirus NS1-C4BP binding is mediated through CCP2-5 and 8. Since CCP8 is near to the C-terminal oligomerization domain, its loss could affect the conformational structure of C4BP, which can affect the accessibility of NS1 to CCP2-5 (5). Similarly, the deletion of CCP8 reduced IAV-C4BP binding by ~75% (Figure 4). Deletion of CCP4-5 inhibited binding by ~79% to H3N2 and ~67% to H1N1 (Figure 4). C4BP binds C4b through CCP1-3, with domains 2 and 3 being the most critical for the interaction (10). The loss of CCP1-2 reduced binding efficiency of H3N2 and, to a lesser extent, of H1N1 (~80% for H3N2 and ~20% for H1N1) (Figure 4). It is possible that the IAV virus, like other pathogens such as S. pyogenes, Neisseria gonorrhoeae, Bordetella pertussis, Moraxella catarrhalis, Borrelia recurrentis, Candida albicans, and Hemophilus influenzae, is exploiting the natural binding site for C4b (8). This preference for the site can be attributed to its immutability, accessibility and the fact that the region may contain intrinsic properties which favor ligand binding (46). Furthermore, this increased binding affinity between CCP1-2 and H3N2 may explain the difference in the binding efficiency of C4BP to H3N2 when compared to H1N1, as seen in Figure 2. As plasma purified C4BP is a glycoprotein, and therefore, is likely to contain N-glycosides that are sialylated; it is possible for sialic acid residues to bind to HA/NA. C4BP [UniProtKB - P04003 (C4BPA_HUMAN)] has N-linked glycosylation only in CCP3 and CCP8 and so sialic acid is likely to occur only at these sites. Our results do not implicate CCP3 in binding, but it remains a possibility that CCP8 binding could involve sialic acid.

It is likely that C4BP is regulating viral replication levels by modulating viral entry. To establish this, second-generation matched and unmatched lentiviral vectors, pseudo-typed for H1+N1 and H3+N2, respectively, were generated. This system was selected as a safe alternative method to mimic the structure and surfaces of IAV and prove that C4BP acts as an entry inhibitor in cells transduced with pseudo-typed IAV particles that are restricted to only one replicative cycle. A luciferase reporter assay revealed a 56% reduction in H1N1 pseudo-typed viruses treated with C4BP that entered the cells, corresponding with the 4-fold decrease observed in the M1 mRNA levels of H1N1 infected cells (Figures 5 and 6), while a slight increase (~17%) was observed in case of the H3N2 pseudo-particles (Figure 6), consistent with M1 levels. This suggests that C4BP acts as an entry inhibitor for H1N1 while it has limited restrictive potential against H3N2.

Recently, factor H, the principal regulator of the alternative pathway, and vaccinia virus complement control protein (VCP),
a homologue of factor H, were found to interact with IAV in a manner similar to C4BP (32). MDCK cells infected with H1+N1 pseudo-typed lentiviral particles resulted in approximately 25% and 45% reduction in luciferase activity after treatment with factor H or VCP, respectively. However, a 50% increase in luciferase activity in MDCK infected with H3+N2 pseudo-typed lentiviral particles was observed when treated with factor H, and 30% when treated with VCP was observed. Hence, the treatment with these proteins resulted in restriction of IAV entry and replication by 4-fold for the H1N1 subtype, while promoting entry and replication by 2-fold for H3N2. Thus, using lentiviral vectors, pseudo-typed for H1+N1 and H3+N2, Murugaiah et al. demonstrated the ability of Factor H and VCP to modulate IAV entry, in a subtype dependent manner, similar to C4BP (32). As seen in the case of C4BP, Murugaiah et al. also suggested that FH and VCP to accomplished the subtype dependent entry modulation via interaction with HA, NA, and M1. Additionally, FH and VCP were also shown to trigger an anti-inflammatory response in case of H1N1 subtype while leading to pro-inflammatory response in H3N2 subtype. The inflammatory response was also modulated in a subtype-specific manner. Transcriptional levels of IFN-α, TNF-α, IL-12, IL-6, and IFN-α were upregulated, while RANTES was downregulated following factor H treatment for the H1N1 subtype at 6 h post-infection. In the case of the H3N2 subtype, mRNA levels of these pro-inflammatory cytokines were enhanced (32). The findings of this study highlight a possible common mechanism that is used by C4BP, VCP, and Factor H that contain CCP domains in modulating the entry of IAV subtypes. The heparin-binding sites on Factor H (surface of CCP7, CCP13 and CCP20) and VCP (tip of module 4) are also found on C4BP α chain (CCP domains 1-2) (9, 47). These sites may provide a possible explanation for the effect observed on viral entry modulation among the IAV subtypes. In this study, we have shown that IAV subtypes bind C4BP via the heparin-binding site containing CCP domain 1-2 (Figure 4). We suspect the overlapping heparin-binding and IAV binding sites may contribute to the variation observed between the subtypes. C4BP has been shown to enhance adenovirus uptake in hepatocytes by providing a bridge between the adenovirus fiber knob domain and cell surface Heparan Sulfate Proteoglycans (HSP) (16); we speculate that the increased efficiency observed between the binding of C4BP’s CCP1-2 domain and H3N2 subtype compared to H1N1 subtype may contribute to the increased H3N2 virion entry in a similar fashion. The similarity of the results between the interaction of C4BP, Factor H, and VCP to IAV subtypes further support this idea and requires further study by treating cells with heparinase prior to C4BP treatment and infection to confirm whether the variation between the subtypes may be modulated by the interaction with HSP.

Another explanation for differences in outcome in spite of C4BP binding both viruses may arise from the local variations found between H1 and H3 or N1 and N2 or the variation in C4BP binding sites to HA or NA. HA and NA are classified into two groups regardless of their serotype identification. In case of NA, N1 belongs to group 1, which lacks an additional cavity next to the active site that is created by the movement of “150 loop” during conformational changes caused by the binding of substrate to the active site (48). N2 belongs to group 2 which...
lacks the “150 loop”, and hence, the additional cavity (48). NA functions in virus internalization by enabling movement of the virus across the cell surface from an endocytosis inactive site on the cell to an active site, thereby increasing the efficiency of viral uptake (49). NA has also been shown to boost HA-dependent influenza virus fusion and infectivity by employing a cell-cell fusion assay and an HIV-based pseudo-typed infectivity assay (50). It is possible that the structural differences between N1 and N2 after binding to C4BP may affect their role in virus internalization. Similarly, HA can also be classified into two groups based on structural characteristics, with H1 in group 1 and H2 in group 2 (51). Hence, localized variations found on HA could influence potential binding pocket for C4BP on the HA, as seen in the case of Stachylin (52). These structural variations in HA and NA may also explain the increased binding observed between C4BP and H3N2. It will be important to investigate the effect of differential interplay between HA and NA of H1N1 and H3N2 viruses across the cell surface from an endocytosis inactive site on the cell to an active site, thereby increasing the efficiency of viral uptake (49).

We found that IL-12 mRNA levels were too low to be detected 2 h post-infection, but the relative mRNA upregulation (1 log_{10}) was observed 6 h post-infection in cells infected with C4BP treated H3N2 compared to its control (Figure 7B). This clearly indicates the presence of a response that would culminate in the prevention of viral replication, activation of cytotoxic T lymphocytes and early NK cell-dependent gamma interferon production (63). The entry inhibitory effect of C4BP on H1N1 subtype, and the subsequent lower viral replication and load is further demonstrated by the downregulation (−2 log_{10}) of IL-12 mRNA in the C4BP treated H1N1-infected cells compared to their untreated counterparts 6 h post-infection (Figure 7B).

The TNF-α−NF-xB axis leads to the expression of a number of inflammatory genes, induction of apoptosis and production of ROS (64–66). NF-xB mRNA in cells infected with C4BP treated H1N1, 2 h post-infection was found to be relatively upregulated (−0.3 log_{10}), while a downregulation (−0.7 log_{10}) of the mRNA was recorded 6 h post-infection when compared to the untreated
In case of cells infected with C4BP treated H3N2, we found an upregulation of NF-kB mRNA 6 h post-infection, while no significant modulation was observed 2 h post-infection (Figure 7C). This variation in NF-kB mRNA levels 6 h post-infection among the IAV subtype further confirms the subtype dependent entry inhibitory action of C4BP on IAV. When compared to their untreated counterparts, the cells infected with either C4BP treated H1N1 or H3N2 were found to upregulate TNF-α mRNA (Figure 7D), indicating a robust inflammatory response at both 2 h (0.2 log_{10}) and 6 h (−1.7 log_{10}) post-infection (67, 68).

RANTES is an important chemokine that been associated with extensive inflammation of the lung in cases of avian influenza and increased mortality in IAV challenged CCR5−/− mice (69). RANTES mRNA expression levels in treated H1N1 challenged cells 2 h post-infection, was found to be significantly downregulated (−2 log_{10}) while it was not detected in case of H3N2 (Figure 7E). RANTES mRNA levels in cells were upregulated observed in case of both C4BP treated viral subtypes (2 log_{10}) 6 h post-infection, suggesting an active recruitment of leukocytes to the inflammatory sites (Figure 7E).

Another cytokine associated with both pro-inflammatory and anti-inflammatory effects is IL-6 (70). Its synthesis is upregulated during infection to simulate the adaptive immune response by inducing differentiation of activated B cells and CD4+ T cells (71). In the case of IAV infection, IL-6 seemed to have a protective role in murine models, promoting viral clearance and limiting inflammation (72). IL-6 mRNA levels at 6 h was found to be upregulated in case of H1N1 (0.5 log_{10}) and H3N2 (1.5 log_{10}) (Figure 7F) after C4BP treatment. These results suggest that C4BP may entice differential adaptive immune response.

It would be interesting to study if C4BP can also bind avian IAV. Another area to explore further would be investigate in humanized murine models to understand the impact of C4BP-IAV interaction in the microenvironment of the respiratory system, and elucidation of mechanism resulting in the subtype-dependent entry modulation of IAV by C4BP.

In conclusion, we show that C4BP differentially regulates the efficacy of Influenza A virus replication in a subtype-dependent manner by modulating their entry into the cells, and provide an interesting candidate to be developed into a HA and NA-based inhibitor against IAV infections. Recombinant forms of CCP4+5 and/or CCP7+8 are likely to be excellent vehicles for therapeutic development against IAV pandemic H1N1 subtype.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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## AUTHOR CONTRIBUTIONS

PV, VM, and NB carried out most of the experiments. NT, HK, SA, and MA-A provided crucial reagents. FA-M, BN, RS, and UK managed the experimental details and analyzed the data. PV and VM wrote the first draft. UK, RS, and BN edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.585361/full#supplementary-material

**SUPPLEMENTARY FIGURE 1** | (A) SDS-PAGE (12% w/v acrylamide/ bisacrylamide) was loaded with 5 µg of recombinant C4BP α-chain in lane 2 along with a protein ladder with a range of 250 kDa to 10 kDa (ThermoFisher) in lane 1. The denatured and reduced samples were run for 120 minutes at 90V and stained with Coomasie brilliant blue to reveal protein bands corresponding to the α-chain (−70kDa). (B) Immunoreactivity of the recombinant C4BP α-chain was analysed by western blotting using rabbit-anti-human C4BP polyclonal antibodies (1:10000) at room temperature for 1 h, followed by incubation with secondary goat anti-rabbit IgG HRP-conjugate (1:1,000) for 1 h at room temperature. Bands corresponding to α-chain (−70kDa) of the C4BP were observed after developing the colour using 3,3′-diaminobenzidine (DAB) substrate.

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