

**Three pentraxins C-reactive protein, serum amyloid p component and pentraxin 3 mediate complement activation using Collectin CL-P1**

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Running head: CL-P1 and pentraxins in atypical hemolytic-uremic syndrome

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Keywords: collectin, scavenger receptor, pentraxin, classical pathway, aHUS

## Abstract

*Background:* Pentraxins (PTXs) are a superfamily of multifunctional conserved proteins involved in acute-phase responses. Recently, we have shown that collectin placenta 1 (CL-P1) and C-reactive protein (CRP) mediated complement activation and failed to form terminal complement complex (TCC) in normal serum conditions because of complement factor H (CFH) inhibition. It is still unknown whether other structurally related PTXs have similar effects on CL-P1. PTXs were recently shown to be associated with atypical hemolytic uremic syndrome (aHUS), a renal disease with 40% of the cases being associated with CFH gene mutation. However, the exact role PTXs in aHUS remains to be determined.

*Methods:* We used CL-P1 expressing CHO/IdlA7 cells to study the interaction with PTXs. For a cell free system, soluble type CL-P1 was used in an ELISA assay for the binding, C3 fragment deposition and TCC deposition experiments. In the cell-included condition, we used our previously established CL-P1 expressing HEK293 cells for the C3 fragment and TCC deposition assay.

*Results:* In this study we demonstrated that CL-P1 **also bound** serum amyloid p component (SAP) and pentraxin 3 (PTX3) to activate the classical pathway as well as the alternative pathway. CRP and PTX3 further amplified complement deposition by properdin. We further demonstrated that CRP and PTX3 recruit complement factor H (CFH), whereas SAP recruits C4 binding protein (C4BP) on CL-P1 expressing cell surfaces to prevent the formation of terminal complement complex (TCC) in normal serum conditions, while the depletion of CFH or C4BP failed to prevent TCC formation. **Furthermore**, an aHUS associated CFH mutation showed increased TCC formation on CL-P1 expressing cells and soluble CL-P1 coated ELISA wells.

*Conclusion:* Our current study hypothesizes that the interaction of pentraxins with CL-P1 might have a putative role in worsening complement mediated damage in some aHUS patients.

*General significance:* The interaction of CL-P1 with PTXs might protect host cells from excessive complement attacks in the case of acute PTXs concentration as well as prevent CL-P1 and PTXs mediated complement activation which could be useful in treating some aHUS patients.

## Abbreviations

Pentraxins (PTXs); CRP, C-reactive protein; PTX3, pentraxin 3; SAP, serum amyloid P component; neuronal pentraxin 1 (NPTX1); neuronal pentraxin 2 (NPTX2); neuronal pentraxin receptor (NPTXR); APR, acute phase reaction; CL-P1, collectin placenta 1; CRD, carbohydrate recognition domain; TCC, terminal complement complex; SR, scavenger receptor; CHO, Chinese hamster ovary; HEK, human embryonic kidney; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; PBS,

phosphate-buffered saline; TBS, Tris-buffered saline; ELISA, enzyme linked immunosorbent assay; CFH, complement factor H; aHUS, atypical hemolytic uremic syndrome; oxLDL, oxidized low density lipoprotein; LDL; aHUS, atypical hemolytic uremic syndrome; AMD, age-related macular degeneration; CFH, complement factor H; CFB, complement factor B.

## INTRODUCTION

Pentraxins (PTX) are multimeric proteins forming cyclic structures, distinguished by the presence of a C-terminal 'pentraxin domain' of 200 amino acids and a conserved 'pentraxin signature motif' of an eight amino acid-long sequence (HxCxS/TWxS, where x is any amino acid) (1). This superfamily of proteins can be further classified into short and long PTX. The pentraxins C-reactive protein (CRP) and serum amyloid P-component (SAP) (2, 3) are evolutionarily conserved classic short pentraxins, share a high amino acid sequence homology and similar annular disc-like pentameric structure. The CRP and SAP participate variably in the acute phase reactions (APR) in different species. For example, CRP is a major acute-phase protein in humans (4), while in mice it is a trace plasma component and only a minor acute-phase protein (5–7). In contrast, SAP is highly inducible during the APR in mice but only slightly in humans (8).

Pentraxin 3 (PTX3), the prototypic long pentraxin, which contains an additional N-terminal domain, is an octamer composed of two disulphide linked tetramers (9). In addition to PTX3, other long pentraxins identified in humans are pentraxin 4 (PTX4), neuronal pentraxin 1 (NPTX1), neuronal pentraxin 2 (NPTX2), and a neuronal pentraxin receptor (NPTXR) (10). The long PTXs share the pentraxin signature sequence and high homologies with short PTX in their carboxypentraxin domain. Unlike classic PTX made in the liver, PTX3 is produced locally, primarily by macrophages, neutrophils, endothelial cells, epithelial cells, and vascular smooth muscle cells (11–15). PTX3 could increase significantly during acute phase reactions both in humans and mice (16, 17)

The CRP, SAP and PTX3 bind to C1q and activates the classical complement pathway (18, 19), resulting in the removal of cell debris (20). A recent report suggested that CRP activates the classical pathway on nucleated cells without activating the membrane attack complex (MAC) or causing cytolysis (21). Another report also showed that CRP binds apoptotic cells and protects the cells from assembly of the terminal complement components by recruiting complement factor H (CFH) (22). A similar study showed that PTX3 binds FH without interfering with its complement inhibitory function and may contribute to focusing CFH regulatory action, preventing excessive complement activation, and thus has an important function in the control of inflammation in response to tissue injury (23). Unlike CRP and PTX3, SAP binds C4 binding protein (C4BP) and plays a potential role for the regulation of the classical

complement pathway (24).

Polymorphisms and mutations in CFH are associated with age-related macular degeneration (AMD) and atypical hemolytic uremic syndrome (aHUS), suggesting that impaired control of complement activation in the retina and the kidney endothelium, respectively, is involved in these diseases (25). Recently, it has been shown that the level of CRP is increased in aHUS and AMD (26, 27). Another study raised the possibility that CFH mutations may affect CFH-CRP binding in host renal epithelial cells, thus making these more susceptible to inflammatory attack (28). Kopp et al. has reported that PTX3 recruits functional CFH to the extracellular matrix in kidneys; however this interaction is impaired by certain CFH mutations and autoantibodies affecting CFH which could amplify local complement-mediated inflammation, as well as endothelial cell activation and damage in atypical hemolytic uremic syndrome (aHUS) (29). On the other hand, deficiency of the C4BP was present in a patient with a disease clinically resembling Behçet's disease (30). However, it is not clear whether the disease itself, or only the complicating angioedema, was a consequence of the C4BP deficiency.

We recently identified collectin placenta 1 (CL-P1) (31), also known as a scavenger receptor with C-type lectin (SRCL), collectin-12, SCAR4 is a hybrid protein with the structural traits of both collectins and scavenger receptors (SR). CL-P1 is a type II membrane glycoprotein and is structurally characterized by an N-terminal intracellular domain, transmembrane region, an  $\alpha$ -helical coiled-coil region, a collagen-like region and a C-terminal carbohydrate recognition domain (CRD) (31). The combination of a C-type CRD and a collagen-like region defines it as a collectin, whereas the extra-cellular projection of an  $\alpha$ -helical coiled-coil and a collagen-like region with exposed polycationic residues is a classical trait of a class A SR (32, 33). We found CL-P1 is an endothelial receptor that can endocytose and phagocytose Gram-negative and -positive bacteria and yeast as well as oxidized low density lipoprotein (OxLDL) in vascular endothelial cells (34). A recent study indicates that the fluid phase molecule of CL-P1 might initiate complement activation through the alternative pathway (AP) on *Aspergillus fumigatus* by directly interacting with properdin (35). Very recently, we investigated the ability of CL-P1 to interact with CRP and drive the classical complement pathway (36). Furthermore, CRP recruited CFH to protect the cells from the formation of terminal complement complex (TCC).

Based on existing information, it is logical to speculate that SAP and PTX3, structurally similar to CRP, might also interact with CL-P1. In this study, we examined the interaction of CL-P1 with CRP, SAP and PTX3 as well as their ability in CL-P1 mediated complement activation. We have also discussed the role of CFH and C4BP in CL-P1 and PTX mediated complement regulation in protecting the cells from the formation of terminal complement complex. Moreover, we addressed the role of an aHUS-associated CFH mutation in complement activation mediated by CL-P1 and PTXs.

## **Materials and methods**

### **Cells and reagents**

CHO/IdIA7 cells, which lack functional LDL receptors, were kindly provided by Dr. M. Krieger (MIT). Human embryonic kidney (HEK293) cells were from ATCC. The following reagents were used: Native CRP, C1q depleted serum, and anti-rabbit IgG HRP (Merck Millipore); recombinant human pentraxin 2 (SAP), recombinant human pentraxin 3 (PTX3), recombinant human CD35, biotinylated anti-mouse pentraxin 2 (cross reacted with human pentraxin 2), biotinylated anti-human pentraxin 3 (R&D Systems); purified native C1q, purified human factor H, purified human properdin, purified human factor B, rabbit anti-human C5b-9, goat anti-human factor H, goat anti-human factor B, goat anti-human properdin, properdin depleted serum, factor B depleted serum and factor H depleted serum (Complement Technology); murine anti-human C4BP, purified human C4BP, and MicroVue SC5b-9 plus EIA kit (Quidel); HAM's F-12, Dulbecco's minimal essential medium (DMEM)-high glucose, fetal bovine serum (FBS), and human complement serum (Sigma-Aldrich); Anti-myc monoclonal antibody, Alexa Fluor 555 antibody-labeling kit, EZ-Link Sulfo-NHS-LC-LC-Biotin, and Alexa Fluor conjugated antibodies (Invitrogen); rabbit anti-human C3d complement (Dako).

### **Cell culture and transfection**

CHO/IdIA7 cells were cultured in HAM's F-12 medium supplemented with 5% heat inactivated FBS. HEK293 cells were maintained in DMEM-high glucose supplemented with 10% FBS. Cells were maintained in a humidified incubator at 37°C and 5% CO<sub>2</sub>. 24 h before the transfection, cells were seeded onto poly-L-lysine-coated 35 mm glass based dishes (CHO-IdIA7) or collagen-coated dishes (HEK293) (Iwaki, Japan). Constructs containing *myc*-tag were transiently transfected into cells using Lipofectamine LTX transfection reagent (Invitrogen) in accordance with the manufacturer's instructions. At 24 h after transfection, cells were used for the binding and complement related assay.

### **Construction of expression vectors and recombinant CL-P1 expression and purification**

A cDNA encoded full length human CL-P1 was introduced into pcDNA3.1/myc-HisA expression vectors as previously described (30). The extracellular domain of human CL-P1 (59–742) containing insulin leader peptide and FLAG tag was produced as described earlier. Expi293F<sup>TM</sup> cells (Invitrogen) were used for transient expression of recombinant CL-P1. The cell line had been adapted to grow in a serum free medium (Expi293<sup>TM</sup> Expression Medium) (Invitrogen) and transfected using ExpiFectamine<sup>TM</sup> 293 transfection reagent. After growth of the cells for 7 days, the culture supernatant was harvested after centrifugation at 3,500 rpm for 30 min and stored at 4 °C. Anti-FLAG M2 affinity gel (Sigma) was used to purify the recombinant CL-P1 from the culture supernatant.

### **Binding of CRP, SAP and PTX3 to cell surface CL-P1**

The binding of Alexa 555-CRP and non-labelled SAP and PTX3 with CL-P1 was performed as previously described with little modification (35). Briefly, CHO/IdIA7 cells transfected with the indicated cDNAs were incubated with 10 µg/ml Alexa 546-CRP or SAP or PTX3 in ice-cold HAM's F12 medium /10 mM HEPES at 4 °C for 1 h. The cells were fixed with 4% phosphate-buffered formalin (Wako Pure Chemical Industries) for 30 min at room temperature, washed and incubated with anti-*myc* antibody or anti- *myc* antibody combined with biotinylated anti-mouse pentraxin 2 (cross reacted with human pentraxin 2) or biotinylated anti-human pentraxin 3 for 30 min at room temperature. After being washed, the cells were incubated with Alexa 488-anti-mouse IgG or Alexa 488-anti-mouse IgG with streptavidin Alexa 555 conjugate for 30 min at room temperature. The cells were counterstained with Hoechst 33342 (Invitrogen). The cells were photographed under a fluorescent microscope (BZ-9000, Keyence) at × 40 magnifications.

#### **Binding of pentraxins to CL-P1 in solid phase ELISA**

Assays were conducted in TBSTC (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5 mM CaCl<sub>2</sub>, pH 7.4); using MaxiSorp microtiter plates (Nunc). Proteins (0.1 µg) were immobilized overnight at 4 °C. Remaining binding sites were blocked with BlockAce/PBS (DS Pharma Biomedical) at 37 °C for 1 h. If not stated otherwise, ligands were added for 1 h at 37 °C. Biotin conjugated antibodies were added for 1 h at 37 °C. Elite ABC kits (Vector laboratories) were used to detect the biotin conjugated antibodies. SureBlue TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories) was used for the visualization, and the absorbance was measured at 450 nm with a model 680 microplate reader (Bio-Rad Lab.)

#### **Complement deposition ELISA**

Maxisorp microtiter plates (Maxisorp, Thermo Fisher Scientific) were coated with recombinant human CL-P1 (100 µl of 5 µg/ml) or heat inactivated BSA (100µl of 5 µg/ml) diluted in a coating buffer overnight at 4 °C. The plates were washed 3 times with PBS and blocked for 1 h at room temperature with 2% BSA in PBS. Thereafter, the plates were incubated with 1% human complement serum or C1q-depleted serum or properdin depleted serum or CFB depleted serum, or aHUS patient serum at 37 °C for 1 h with or without 20 µg/ml (full serum) CRP, SAP or PTX3 in a veronal buffer (0.82 mM MgCl<sub>2</sub>, 145.45 mM NaCl and 0.25 mM CaCl<sub>2</sub>, 3.11 mM barbitol, 1.8 mM sodium barbitol) containing 0.1% gelatin. For TCC deposition, 10% human complement serum or CFH depleted serum or C4BP depleted serum or aHUS serum was used. To recover the complement activation we added C1q (200 µg/ml full serum) or native properdin (6 µg/ml full serum), or CFB (200 µg/ml full serum). In contrast, CFH (400 µg/ml full serum) or C4BP (160 µg/ml full serum) was added to the corresponding depleted serum to CFH and C4BP function. We used 10.65 µg/ml soluble complement receptor 1 (sCR1) to inhibit TCC

deposition. C3 and TCC were detected with 3.5 µg/ml rabbit anti-human C3d antibody (Dako) or rabbit polyclonal anti-human C5b-9 antibody followed by horseradish peroxidase (HRP)- conjugated anti-rabbit IgG antibody (1:5000, Merck Millipore) diluted in PBS containing 1% BSA, 0.05% Tween 20. The reaction was developed with SureBlue TMB microwell peroxidase and the absorbance was measured at 450 nm as described above.

### **C3 fragment and TCC deposition on HEK293 cells expressing CL-P1**

HEK293 cells were seeded in collagen-coated dishes and transfected with pcDNA3.1 vector, CL-P1 full length plasmid. Cells were washed with PBS, thereafter incubated with 10% human complement serum or C1q depleted serum or properdin depleted serum or factor B depleted serum for C3 fragment deposition and with 10% CFH depleted serum or C4BP depleted serum or with serum from an aHUS patient (5%) in the presence or absence of 20 µg/ml CRP, SAP or PTX3 (full serum) at 37 °C for 1 h. Depleted serum was replenished with native C1q (200 µg/ml full serum) or native properdin (6 µg/ml full serum), or CFB (200 µg/ml full serum) or CFH (400 µg/ml full serum) or C4BP (160 µg/ml full serum). For the inhibition assay, we added 10.65 µg/ml sCR1 to the CFH depleted serum or C4BP depleted serum or aHUS patient serum. At the end of the incubation step cells were fixed in 4% phosphate-buffered paraformaldehyde and stained with rabbit anti-human C3d antibody (3.5 µg/ml) or rabbit polyclonal anti-human C5b-9 followed by Alexa-conjugated secondary antibody. A fluorescent microscope (BZ-9000, Keyence) was used for acquisition of fluorescent staining on cell surfaces. The signal intensity was calculated by the BZ-HIC program.

### **CFH, C4BP, factor B and properdin recruitment assay**

We incubated transiently transfected HEK293 cells with a final concentration of 10% human complement serum in the presence or absence of 20 µg/ml CRP, SAP or PTX3 (full serum) at 37 °C for 1 h. Cells were then washed, fixed and incubated for 30 minutes at room temperature with goat polyclonal anti-human factor H or goat anti-human properdin or goat anti-human factor B or anti-human C4BP mAb followed by Alexa-conjugated secondary antibodies. Cells were imaged using a fluorescence microscope.

### **Preparation of C4bp-depleted serum**

Approximately 10 mg of sheep, anti-C4bp IgG (Abcam Inc., Cambridge, MA) was coupled to a HisTrap, NHS-activated column (1 ml; GE Healthcare) following the manufacturer's instructions. To prepare C4bp depleted human complement serum, 40% human complement serum (diluted in phosphate-buffered saline (PBS) was passed through the column by syringe injection at room temperature. The C4bp levels in the resulting sera were measured by Western blot analysis with an anti-C4bp antibody (Abcam).

### **Patient serum**

aHUS patient's serum was a kind gift from Professor Yoshihiko Hidaka. The heterozygous G3717A mutation and heterozygous polymorphisms of C-257T, A2089G, G2881T, and G1492A were found in FH1. Anti-factor H antibody was not detected.

### **Quantification of soluble SC5b-9**

Transiently transfected HEK293 cells were exposed to 5% aHUS patient serum or aHUS patient serum supplemented with purified CFH (400 µg/ml full serum) in a culture medium (without FBS) and incubated at 37°C for 1 h. The serum containing medium was then collected and SC5b-9 were quantified using commercially available MicroVue SC5b-9 Plus EIA kits (Quidel).

### **Statistical Analysis**

Statistical analysis was conducted using the unpaired two-tailed Student's *t* test included in the JMP statistics software package (version 7, SAS). Data are mean ±S.E. *p*<0.0001 is considered statistically significant.

## **RESULTS**

### **Interaction of CRP, SAP and PTX3 with CL-P1**

In previous studies, we reported the binding of CL-P1 with CRP using CHO/IdIA7 cell lines with ELISA experiments (36). In this study, we first examined whether other pentraxin family proteins, SAP and PTX3, can bind to cell surfaces expressing CL-P1. A CHO/IdIA7 cell transient transfection system was used in this study. Alexa-555 labelled CRP or purified recombinant human SAP or purified recombinant human PTX3 were allowed to interact with CL-P1 on cell surfaces. As shown in Fig. 1a CRP, SAP and PTX3 clearly bound to CL-P1, whereas pcDNA3.1 control vector transfected cells did not. The positions of receptor CL-P1 (green) and ligands (red) on the cell surfaces confirm the colocalization. The intensity of the signal in the fluorescence images showed that the binding of CRP was comparatively lower than SAP and PTX3 (Fig. 1b).

We next performed the direct interaction of purified recombinant CL-P1 with SAP and PTX3 in the cell free system to eliminate the possibility that the observed SAP and PTX3 binding might be due to the indirect effects of CL-P1 expression. In this system, the recombinant extracellular domain of CL-P1 prepared with a mammalian cell line was used. We were able to observe a dose dependent interaction (0-30 µg/ml) of CL-P1 with SAP and PTX3 (Fig. 1c and d). A control protein, BSA, exhibited only background signals, indicating the binding of CL-P1 with SAP and PTX3 was specific. The SAP and PTX3 which were heat denatured in boiling water lost their ability to bind CL-P1. Taken together, these data demonstrate that not only CRP but other pentraxins, SAP and PTX3, can interact with the extracellular domain of CL-P1.



### **Pentraxins predominantly activate complement through the classical pathway on CL-P1**

A common theme for CRP, SAP, and PTX3 is that they all interact with C1q from the classical complement pathway and may, upon binding to a ligand, mediate complement activation (18, 19). We recently reported on the CRP-induced C1q dependent classical complement pathway on HEK293 cells expressing CL-P1 (36). Hence, we envisaged that the interaction of CL-P1 with PTX3 or SAP might also invoke the C1q dependent classical pathway. To substantiate this hypothesis, we established a sensitive quantitative ELISA using soluble CL-P1. We found C3 fragment deposition on soluble CL-P1 coated wells using C1q depleted serum which was increased only when C1q was supplemented (Fig. 2a). The PTX3 showed higher C3 fragment deposition compared to CRP and SAP in our ELISA setup.

We then assayed C3 fragment deposition on HEK293 cells. C1q depletion causes a significant decrease in CRP, SAP and PTX3 and induced complement activation as with our ELISA experiments, which was recovered when we added C1q to the depleted serum (Fig. 2b-d). We found an increase in C3 fragment deposition on pcDNA3.1 transfected cells in the C1q supplemented condition, which was likely due to the interaction of CL-P1 with unknown cell derived materials, perhaps dead cells. These data suggest that the interaction of CL-P1 with CRP, SAP and PTX 3 predominantly activate the C1q dependent classical pathway.

### **Pentraxins further amplify complement activation through the alternative pathway on CL-P1**

The alternative pathway of complement activation can amplify the classical or the lectin pathway by forming an alternative C3 convertase. Human CFB is a centrally important component of the alternative pathway activation of the complement system (37). The recruitment of CFB was evident on the CL-P1 expressing HEK293 cells when we incubated the cells with human complement serum including excess CRP, SAP and PTX3 (Fig. 3a). To address the role of the alternative pathway in our ELISA system, CFB-depleted human serum was used; the deposition of C3 fragments were abrogated and were restored by the addition of purified CFB (Fig. 3b). We next confirmed our ELISA assay using an HEK293 cell based assay. We observed similar abrogation and restoration in the deposition of C3 fragments on CL-P1 expressing HEK293 cells as in ELISA (Fig. 3c and d).

In a recent paper, we showed that the interaction of CRP with CL-P1 recruits properdin to further amplify the classical pathway (36). In our present study, therefore, we aimed to evaluate the recruitment of properdin on CL-P1 and other pentraxin interactions. We found recruitment of properdin on HEK293 cells for CRP and PTX3 (Fig. 4a). Surprisingly, we did not find such recruitment of properdin for SAP and CL-P1 interaction. Our ELISA, using soluble type CL-P1, showed the involvement of properdin in complement amplification for CRP and PTX3 (Fig. 4b). Furthermore, in agreement with our ELISA assay, we found the decrease of C3 fragment deposition for CRP and PTX3 on CL-P1 expressing HEK293 cells

(Fig. 4c and supplemental figure 1). Repeated freeze-thawing of commercially available properdin may form higher order oligomers, likely to bind with ligands with higher avidity, or perhaps non-specifically, to surfaces that native forms of properdin may not. So, we avoided repeated freeze-thaw of purified properdin. Hence, we came to the conclusion that the interaction of CL-P1 with CRP and PTX3 amplifies the complement through the alternative pathway which is further amplified through the properdin dependent pathway. However, CL-P1 and SAP interaction amplifies complement activation only through the alternative pathway using CFB and could be the reason for the lower C3 fragment deposition for SAP.

#### **CRP and PTX3 recruits CFH while SAP recruits C4BP on CL-P1 to prevent TCC formation**

In our previous study we found that CRP recruits CFH on CL-P1 expressing cells to prevent TCC formation (36). This data prompted us to perform further experiments to evaluate whether SAP and PTX3 induced complement activation which proceeded to TCC formation on CL-P1. Parallel to our previous work, we found that the addition of excess SAP and PTX3 also can not initiate the formation of TCC in normal complement serum condition on HEK293 cells expressing CL-P1 (Fig. 5a). We speculated that these results could be because of the recruitment of CFH or C4BP, known ligands of PTX and SAP respectively (23, 24). Interestingly, we found the recruitment of CFH on the PTX3 and C4BP on SAP (Fig. 5b and c). These data suggest that the inhibition of TCC formation in the normal complement serum condition in Fig. 5A might be the recruitment of CFH and C4BP to PTX3 and SAP respectively, on CL-P1 expressing cell surfaces.

#### **CFH and C4BP depleted serum failed to prevent TCC formation on CL-P1**

CRP has been shown to activate the classical complement pathway on nucleated cells without activating TCC or causing cytolysis (38). Recently, we reported that recruitment of CFH on CRP was responsible for the reduction in TCC formation in CL-P1 coated ELISA and cell experiments (36). In the current study, we first tested the TCC deposition with ELISA on soluble CL-P1 and found that the CFH depleted serum failed to prevent TCC formation in an excess CRP and PTX3 condition, which was inhibited when CFH was added back to the serum (Fig. 6a). sCR1, the inhibitor of all three complement pathways also potentially inhibited the TCC deposition. As expected, we found no deposition of TCC in an excess SAP condition using CFH depleted serum. In the HEK293 cell based assay we found TCC formation due to CRP and PTX3 on the surface of CL-P1 expressing HEK293 cells which was significantly inhibited by addition of CFH to the CFH depleted serum (Fig. 6b-d). Next, we prepared C4BP depleted human complement serum (Fig. 7a) to check the SAP induced TCC formation. Our results showed that depletion of C4BP can not prevent TCC formation in an excess SAP condition both in ELISA and cell experiments (Fig. 7b, c and supplemental figure 2). In addition, C4BP and sCR1 inhibited SAP induced TCC formation. Therefore, we concluded that the recruitment of CFH to CRP and PTX3

and C4BP to SAP suppresses TCC formation on CL-P1 expressing HEK293 cells.

#### **aHUS associated CFH mutation impairs CFH recruitment and leads to TCC formation on CL-P1**

A growing body of evidence is accumulating to show that mutation or polymorphisms in CFH impairs the interaction of CFH with CRP and PTX3 (28-29, 39). We found that aHUS associated CFH mutation G3717A causes no recruitment of CFH to CRP and PTX3 on CL-P1 expressing cell surfaces resulting in TCC formation on the cell surfaces (Fig. 8a and c). The results were reversed when purified CFH was added to the aHUS serum (Fig. 8b and c). We then measured the SC5b-9 in the aHUS serum after 1 h of TCC formation reaction and observed a significant increase of SC5b-9 in the aHUS serum (Fig. 8d). Because the interaction of CRP and PTX3 with CFH prevents TCC formation in a normal complement serum condition, we suggest that the reduction of function due to mutation in CFH (G3717A) might impair the interaction with CRP and PTX3 in aHUS and could lead to enhanced TCC formation and complement mediated damage in aHUS patients.

#### **sCR1 inhibits TCC formation**

Recently, it has been shown that sCR1 inhibits the common complement pathways to prevent the activation of the terminal pathway induced by aHUS serum (40). We observed that sCR1 greatly inhibits TCC formation mediated by aHUS serum and CFH depleted serum on CL-P1 expressing HEK293 cells, indicating that the staining of TCC in our experiments was specifically related to complement activation products (Fig. 9a-c). Finally, we tested the TCC deposition on soluble CL-P1 using aHUS serum and found that TCC deposition was greatly increased in aHUS compared with human complement serum both in CRP and PTX3 included conditions, which was decreased significantly when purified CFH or sCR1 was added back to the aHUS serum (Fig. 9d).

### **Discussion**

The pentraxins, CRP, SAP, and PTX3, are multifunctional soluble pattern-recognition molecules highly upregulated under inflammatory conditions (41). The interaction of PTX with C1q and its role in the activation of the classical complement pathway are well described (18–19). Recent studies have provided compelling evidence that the upregulated PTX may contribute to the etiology of aHUS (26, 28-29). Very recently, we showed the interaction of CRP with CL-P1 mediates complement activation (36). In this study we investigated whether SAP and PTX3, which have structural and functional similarities with CRP, interacts with CL-P1 to mediate complement activation. We also analyzed the interaction of PTX and CL-P1 with complement regulator CFH in the context of aHUS.

In the literature, CL-P1 is often referred to as an endothelial scavenger receptor, and based on the original association of CL-P1 with endothelial cells, subsequent work has focused on CL-P1 functionality

(31, 42-43). Recently, we found CRP is a novel ligand for CL-P1 (36), which inspired us to evaluate whether other pentraxins are likely to bind to CL-P1. When we examined the interaction between the two other pentraxins and CL-P1, and using cells and the ELISA platform as the read-out, we demonstrated a direct interaction of CL-P1 with CRP as well as SAP and PTX3. The somewhat different binding specificity of CRP, SAP and PTX3 might be due to differences in their source.

It is well described that C1q interacts with the pentraxin family members CRP and SAP and PTX 3 and that this complex formation may mediate classical pathway complement activation (44). Our current data also suggested that the binding of two other pentraxins, SAP and PTX3, with CL-P1 which resulted in the activation of the C1q dependent classical pathway and enhanced C3 fragment deposition on CL-P1 expressing cells and ELISA. The deposition of C3 fragments was higher in CRP and PTX3 compared to the SAP including condition which might be due to the difference in the interaction of C1q with CRP, SAP and PTX3 or the differences in the quality of our protein sources. These also could be due to the inability of SAP to use properdin for complement activation in our system. These data demonstrated that the CL-P1 and PTX induced classical complement pathway may play a critical role in tissue damage and inflammation in acute phase PTX concentration.

The alternative pathway can be constitutively activated at a background level or amplify the classical pathway by forming an alternative C3 convertase and depositing more C3b molecules. Fig. 5 demonstrates a profound involvement of the alternative pathway in PTXs and CL-P1 mediated complement activation. In our previous report we found that properdin further amplifies the CRP and CL-P1 mediated complement activation (36). These findings lead us to investigate whether other pentraxins can amplify the classical pathway using the same system. We found PTX3 drives the similar properdin dependent amplification pathway. To our surprise, we observed that SAP could not amplify the complement using properdin. Recently, SAP has been reported to inhibit the capacity of properdin to initiate complement activation (45). SAP interacts with both properdin and properdin-binding surfaces to block and/or displace properdin:surface complexes. All this suggests that SAP might be involved in the inhibition of properdin mediated complement amplification in our assay system by a hitherto unknown mechanism.

Binding of CFH to CRP is a critical interaction through which CRP participates in the regulation of complement activation. A direct binding of CFH to CRP was described, suggesting regulation of CRP-mediated complement activation on cell surfaces (46). However, under infection/inflammatory conditions (e.g., during the acute phase response or at sites of tissue damage and local inflammation), CRP can bind CFH and locally focus its complement inhibitory activity. PTX3 can recruit CFH to the surface of apoptotic cells, which prevents excessive complement activation and cell lysis (23). Another

study proposed that ECM-bound PTX3 increases the recruitment of CFH, resulting in enhanced local complement regulation (29). In this study we identified SAP as well as PTX3 as new ligand of CL-P1 to prevent TCC formation by recruiting C4BP and CFH respectively. Although C4BP is also a known ligand of CRP and PTX3, we could not find any recruitment of C4BP on CL-P1 expressing surfaces (47).

In our previous paper (36) we showed that CFH depleted serum failed to prevent CRP and CL-P1 induced TCC formation. In the present study, we demonstrated that CFH in a similar way can prevent PTX3 and CL-P1 induced TCC formation. Furthermore, we have found that SAP utilizes C4BP to inhibit TCC formation. This could be a very important mechanism in inflammatory situations where certain complement components might have been consumed or in various inherited or acquired complement deficiency states.

aHUS is a severe kidney disease mainly associated with dysregulation of the alternative complement pathway and characterized by endothelial injury. aHUS is sometimes associated with CFH mutations or autoantibodies against CFH in up to 40% of patients (29). It was demonstrated previously that C-terminal CFH mutations and autoantibodies disturb the physiological interaction of CFH with C3b, causing reduced protection of host cells from complement-mediated lysis (48-50). A recent report suggested that PTX may exhibit direct CFH regulatory activity to specific sites to control excessive local complement activation (23). Our data show that elevated levels of CRP and PTX3 recruit functional CFH to CL-P1 expressing cells and this interaction is impaired by mutation affecting CFH. Thus, certain mutations can impair multiple interactions and functions of CFH. This could amplify local complement-mediated inflammation, as well as endothelial cell activation and damage in aHUS. Therefore, the impaired interactions of CFH–PTX3 and CFH–CRP in the CL-P1 expressing cells may exacerbate renal damage in some aHUS patients.

In many clinical conditions associated with massive complement activation, a cytolytically inactive TCC (SC5b-9) accumulates in plasma as an apparently irrelevant byproduct of the complement sequence (51). A recent study has shown that the inactive complex not only binds to endothelial cells (ECs), it stimulates these cells to express adhesion molecules and tissue factor to promote transendothelial migration and proinflammatory activities on EC (52). Our data suggest that the SC5b-9 released in the aHUS serum due to PTX and CL-P1 interaction may cause an unrestricted inflammatory process that is deleterious to tissue.

In summary, the results of the current investigation disclose a yet unrecognized function of CL-P1 and PTXs in complement regulation. To our knowledge this study is the first to demonstrate that CL-P1 interacted not only with CRP but also with SAP and PTX3 and promoted complement activation through the classical and alternative pathway which was further regulated by CFH and C4BP. The novelty of our

observation is that TCC deposition in the ELISA system using soluble CL-P1 and PTX could be used as a helpful tool to differentiate at least some aHUS patients from healthy individuals as well as to evaluate the drug regulating the complement activation. Taken together, the control of CL-P1 on PTXs could represent a novel therapeutic target in some aHUS patients. Further studies are certainly warranted and may contribute to a better understanding of the interaction of CL-P1, PTXs and the complement system and their association with aHUS.

### ***Acknowledgements***

\*This work was supported by grants from JSPS KAKENHI Grant Number (22390113, 26293124) and from the Northern Advancement Center for Science & Technology (Sapporo, Japan). This work was also supported by grants from the Smoking Research Foundation, Takeda Science Foundation, and the Mizutani Foundation for Glycoscience.

### ***Conflict of interest***

The authors declare that they have no competing interests.

### ***Author contributions***

NR, KO, YM, KM, IH, and NW carried out the experiments and/or helped to analyse and interpret the data. NR, KO, and NW wrote the manuscript. NR, KO, YH, YA, and NI devised the experiments. All authors read and approved the final manuscript.

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## FIGURE LEGENDS

Fig. 1. Binding of pentraxin family proteins to CL-P1.

(a, b) Alexa 555-CRP (10  $\mu$ g/ml) (red), recombinant human SAP (10  $\mu$ g/ml) (red) and recombinant human PTX3 (10  $\mu$ g/ml) (red) binds CL-P1 (green) on CHO/IdlA7 cells. The blank vector pcDNA3.1 was used as a negative control. Recombinant human SAP and PTX3 bound to CL-P1, were detected using Biotin-anti SAP and Biotin-anti PTX3. The binding was normalized with the expression level of the CL-P1. The results are expressed as mean  $\pm$ SD derived from three experiments. (c, d) SAP and PTX3 (0-30  $\mu$ g/ml) interacts with the soluble CL-P1 dose dependently (●) but not with BSA, used as a negative control (■). Denaturation of SAP and PTX3 (heated at 95°C, 5 min) showed almost no binding with CL-P1 (▲). Data are expressed as mean  $\pm$  S.D of three independent experiments. The asterisks indicate significant differences vs the BSA control (\* $p$ <0.0001). The double daggers indicate significant differences vs active SAP and PTX3 control (\* $p$ <0.0001). Scale bars, 20  $\mu$ m for all images. OD, optical density; MFI, mean fluorescence intensity.

Fig. 2. Pentraxins activate the classical complement pathway on CL-P1.

(a) Immobilized CL-P1 and BSA, used as a negative control, were incubated with C1q depleted serum in presence or absence of 20  $\mu$ g/ml CRP, SAP or PTX3. C3 fragment deposition was detected by a C3 specific antibody. Data are means  $\pm$ S.D of three independent experiments. (b-d), 20  $\mu$ g/ml CRP, SAP or PTX3 was added to C1q depleted serum and incubated with CL-P1 transfected HEK293 cells as described in Materials and methods. We detected C3 fragments using a C3 specific antibody. C1q depleted serum was supplemented with purified human C1q for recovery experiments. Data were relative to CL-P1 expression represented as mean  $\pm$  S.D of three independent experiments. \* $p$ <0.0001. Scale bars, 20  $\mu$ m for all images. ns - not significant; MFI, mean fluorescence intensity;

Fig. 3. Pentraxins and CL-P1 mediated classical pathway provoke the alternative pathway.

(a) HEK293 cells transfected with pcDNA3.1 and CL-P1 were incubated with human complement serum and CFB (red) were detected using CFB specific antibody. (b) CFB depleted serum with or without 20  $\mu$ g/ml CRP, SAP or PTX3 was added to BSA and CL-P1 coated ELISA wells. C3 fragments were then detected as described above. Data shown are mean  $\pm$  SD derived from three independent experiments. (c, d) HEK293 cells transfected as described above were exposed to CFB depleted serum to detect C3 fragments (red). CFB was added back to the CFB depleted serum in some experiments. A representative experiment of three performed is shown. The data were normalized to receptor expression. \* $p$ <0.0001. ns - not significant;; MFI, mean fluorescence intensity; OD, optical density.

Fig. 4. Properdin further amplifies pentraxin and CL-P1 induced complement activation.

(a) To analyse the properdin recruitment to the CL-P1 expressing HEK293 cells, we incubated the cells with human complement serum and detected properdin with a properdin specific antibody. One representative of three experiments is shown. (b) C3 fragment deposition was measured on the BSA and CL-P1 coated wells using properdin depleted serum. Data represent mean  $\pm$  SD from three experiments. (c) Quantitative analysis of CRP, SAP and PTX3 induced C3 fragment deposition. The expression of CL-P1 was used to normalize the signal intensity. The results shown are representative of three independent experiments.  $*p < 0.0001$ . ns - not significant; MFI, mean fluorescence intensity; OD, optical density.

Fig. 5. CRP and PTX3 recruits CFH while SAP recruits C4BP on CL-P1 surfaces

(a) HEK293 cells transiently transfected with pcDNA3.1 and CL-P1 were incubated with 10% human complement serum with or without CRP, SAP and PTX3 (20  $\mu$ g/ml) and TCC formation was detected on the cell surface using an anti-C5b-9 antibody. Experiments were repeated at least three times with similar results. (b, c) HEK293 cells were transfected and incubated with 10% human complement serum as described above and immunostained with anti-CFH (red) and anti-C4BP antibody (red). Representative images were from three independent experiments. White boxes highlight the magnified images showing the clear recruitment of CFH and C4BP on the cell surfaces. Scale bars, 20  $\mu$ m for all images.

Fig. 6. CFH depleted serum failed to prevent CRP and PTX3 induced TCC formation on CL-P1.

(a) Deposition of TCC after incubation of CFH depleted serum with BSA and CL-P1 coated wells in presence or absence of CRP, SAP and PTX3 (20  $\mu$ g/ml). Reconstitution with purified CFH showed the decrease in TCC deposition. Addition of sCR1 (10.65  $\mu$ g/ml) to the CFH depleted serum inhibits TCC deposition. Data are presented as mean values and range from two independent experiments. (b, c) HEK293 cells showing TCC formation using CFH depleted serum. Like our ELISA assay, TCC formation was inhibited when CFH added to the depleted serum. For CRP and PTX3 lower panels are magnified images of the region in a white box. Typical findings in four experiments are shown. (d) Quantitative analysis of TCC formation of the images from Fig b and c. Data are means  $\pm$  S.D (n=4).  $*p < 0.0001$ . Scale bars, 20  $\mu$ m for all images. OD, optical density; ns - not significant; MFI, mean fluorescence intensity.

Fig. 7. SAP prevent TCC Assembly on CL-P1 expressing cells by the recruitment of C4BP.

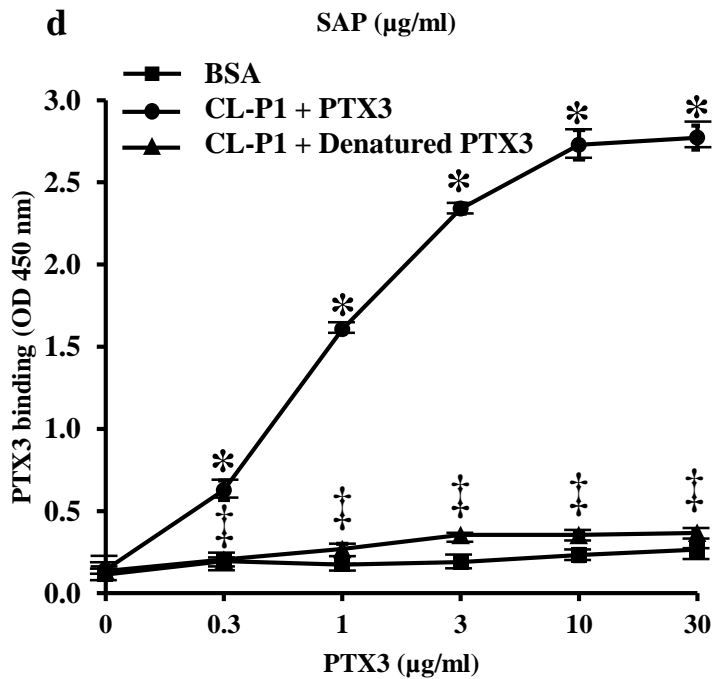
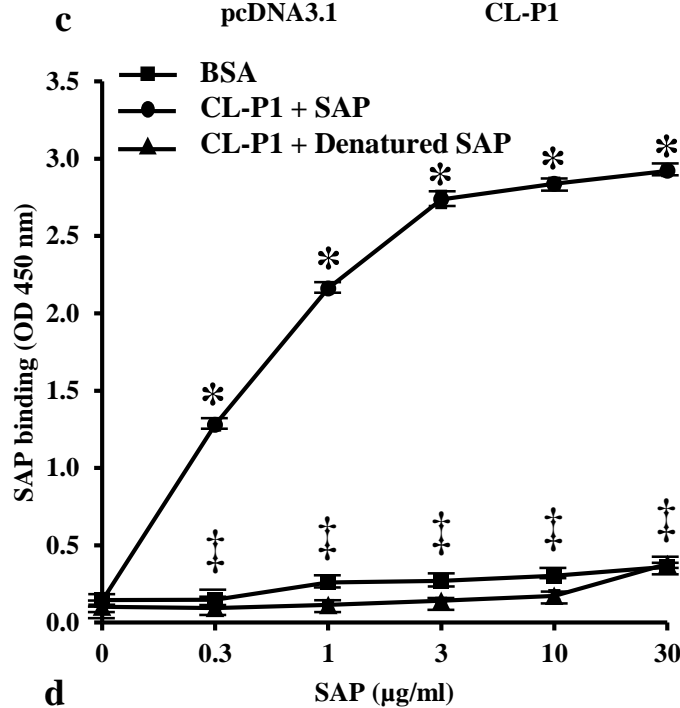
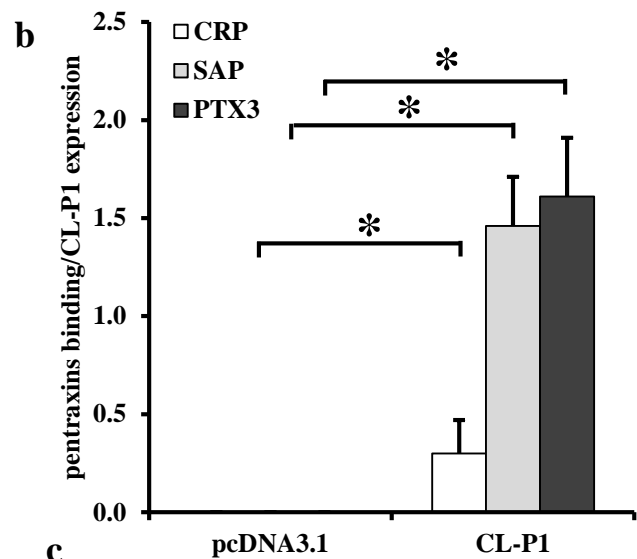
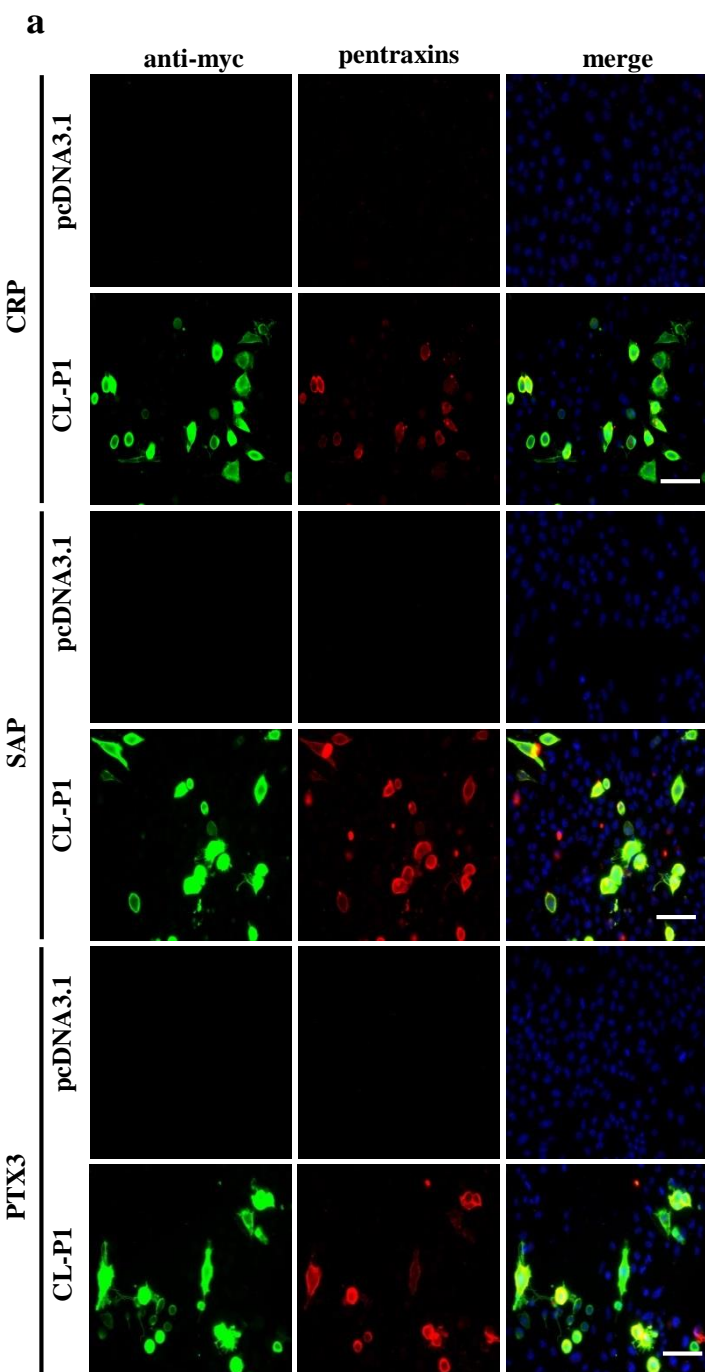
(a) Human complement serum was depleted using an anti-C4BP polyclonal antibody and the depletion was confirmed by western blotting (lane 1, human complement serum, lane 2, C4BP depleted human

complement serum). (b) TCC deposition was measured in a solid phase ELISA using C4BP depleted human complement serum. For the rescue experiment purified human C4BP was added to the depleted serum. sCR1 potentially inhibits SAP induced TCC formation. The results represent mean  $\pm$  S.D (n=3). (c) Mean fluorescence intensity of TCC deposition using C4BP depleted serum. Data are means  $\pm$  S.D of three independent experiments.  $*p<0.0001$ . Scale bars, 20  $\mu$ m for all images. MFI, mean fluorescence intensity.

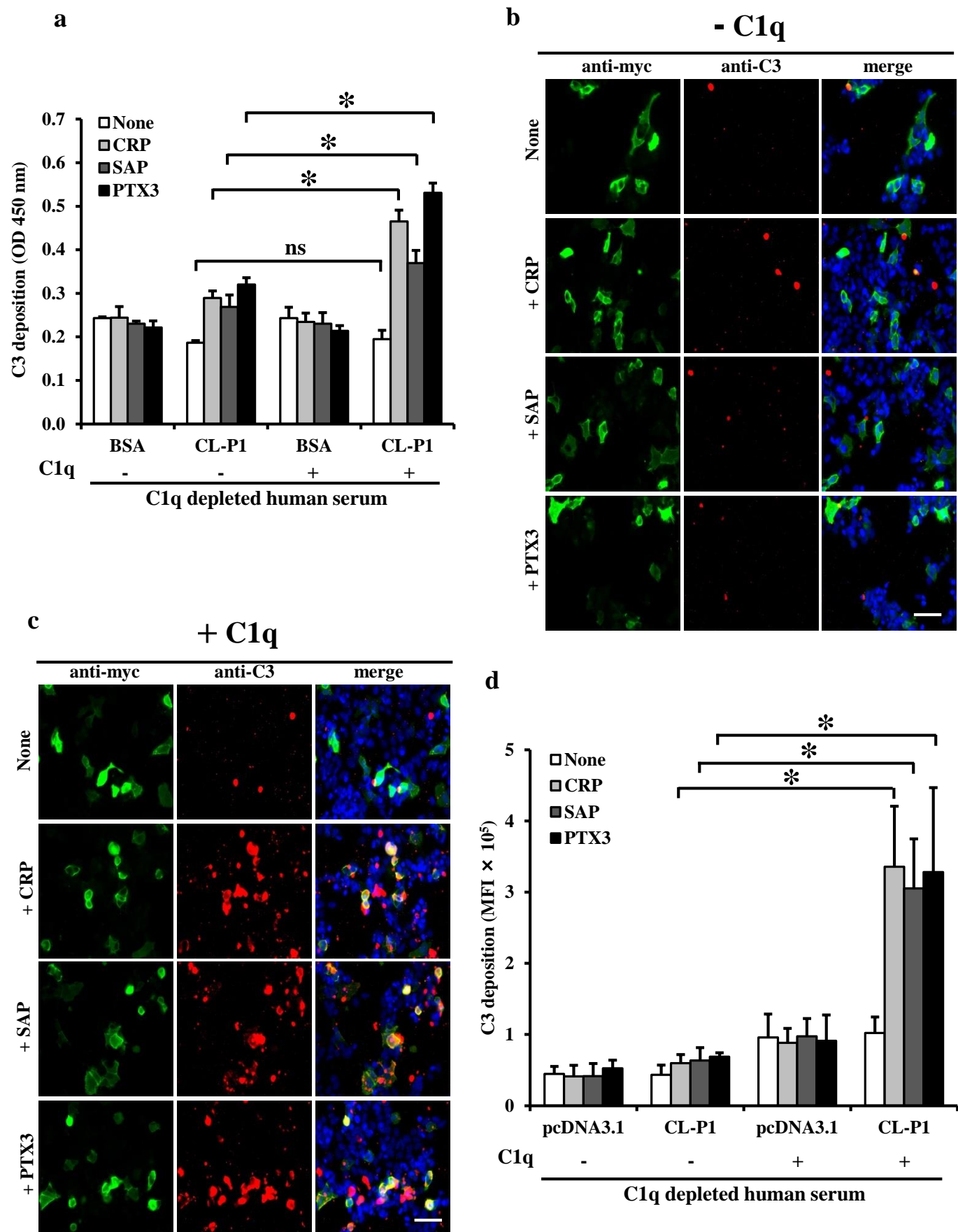
Fig. 8. aHUS associated mutation failed to prevent CRP and PTX3 induced TCC formation on CL-P1. CRP, SAP or PTX3 (20  $\mu$ g/ml) were added to aHUS serum and incubated with transiently transfected HEK293 cells and C5b-9 was detected by polyclonal anti-C5b-9 (red) antibody. All images were acquired at a magnification of  $\times 40$ . For CRP and PTX3 the lower panel shows the magnified images from the white box clearly demonstrating the formation of TCC on the cell surfaces. (b) Purified CFH was added to aHUS serum and C5b-9 was detected as described above. A representative result of three experiments is shown. (c) Quantification of the TCC deposition showed in Fig. a and b. (d) The culture medium containing aHUS serum after 1 h reaction from Fig a and b was used to measure SC5b-9. Data are shown as mean  $\pm$  SD of three experiments.  $*p<0.0001$ . Scale bars, 20  $\mu$ m for all images. MFI, mean fluorescence intensity; ns - not significant; OD, optical density.

Fig. 9. sCR1 potentially inhibits CL-P1 and pentraxins induced TCC formation.

(a-c) Representative fluorescence images ( $\times 40$ ) of TCC (red) staining of CL-P1 expressing HEK293 exposed to serum from an aHUS patient or CFH depleted serum. Serum without sCR1 was used to normalize the data. Data are presented as the mean (n = 3)  $\pm$  SD. C5b-9 deposition were prevented by addition of sCR1 to patient serum, indicating that sCR1 greatly reduced the TCC formation. (d) Human complement serum or aHUS serum was added to BSA and soluble CL-P1 coated wells in presence or absence of CRP, SAP and PTX3. Purified CFH was added to aHUS serum to restore the inhibitory function. sCR1(10.65  $\mu$ g/ml) was used to inhibit the complement activation. Data are means  $\pm$  S.D of three independent experiments.  $*p<0.0001$ . Scale bars, 20  $\mu$ m for all images. MFI, mean fluorescence intensity; OD, optical density.

**Figure 1**

**Figure 2**





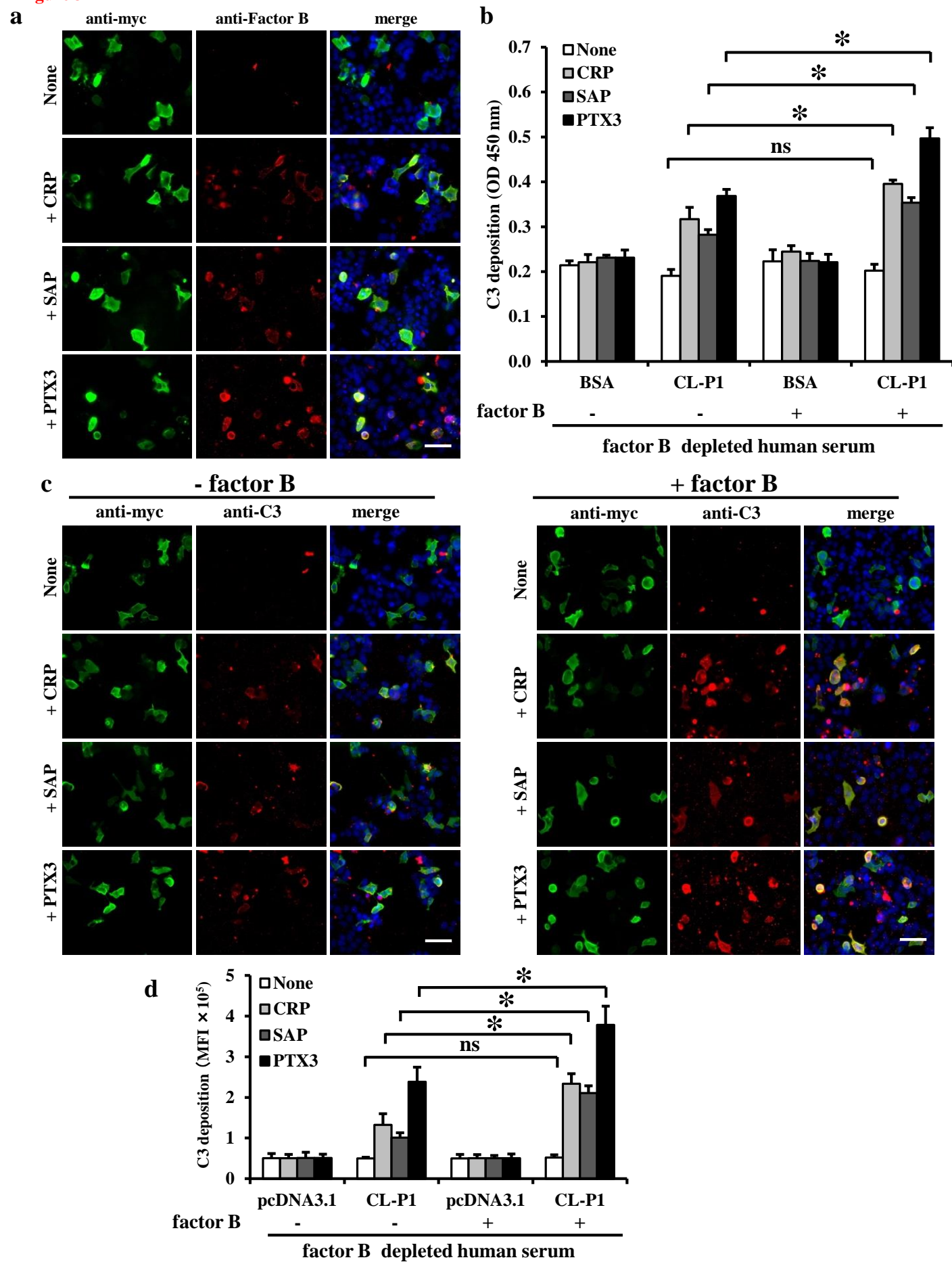
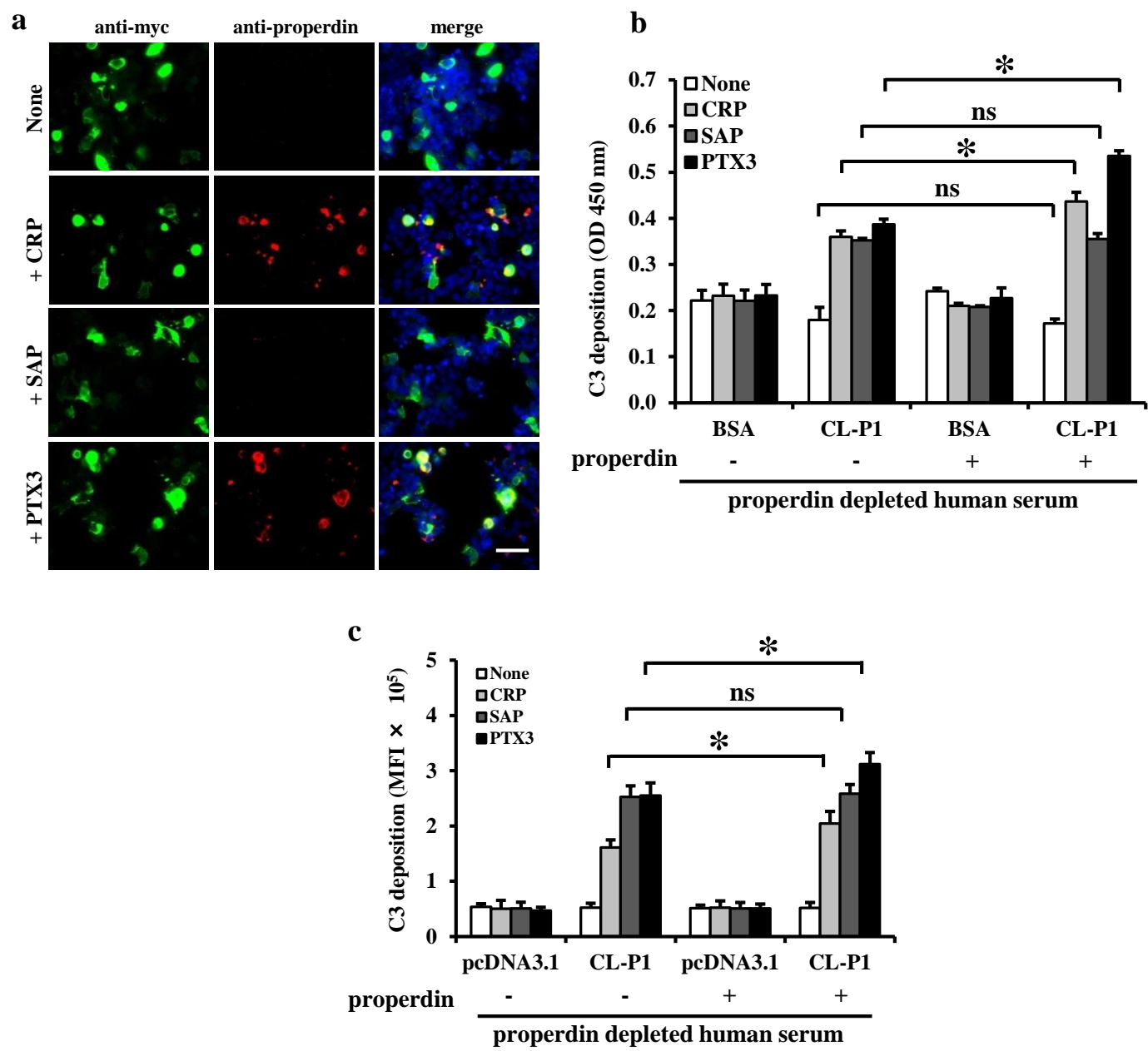
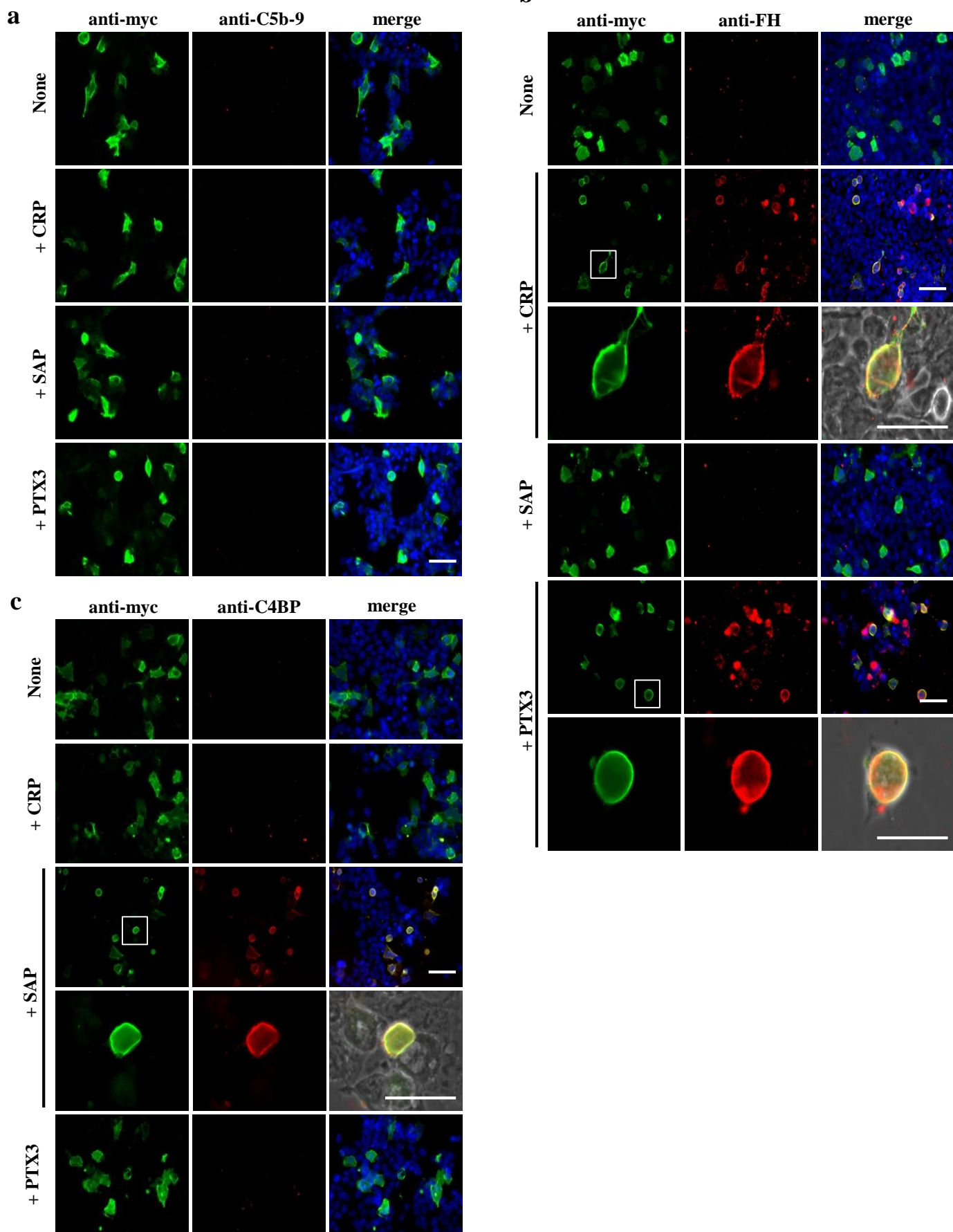
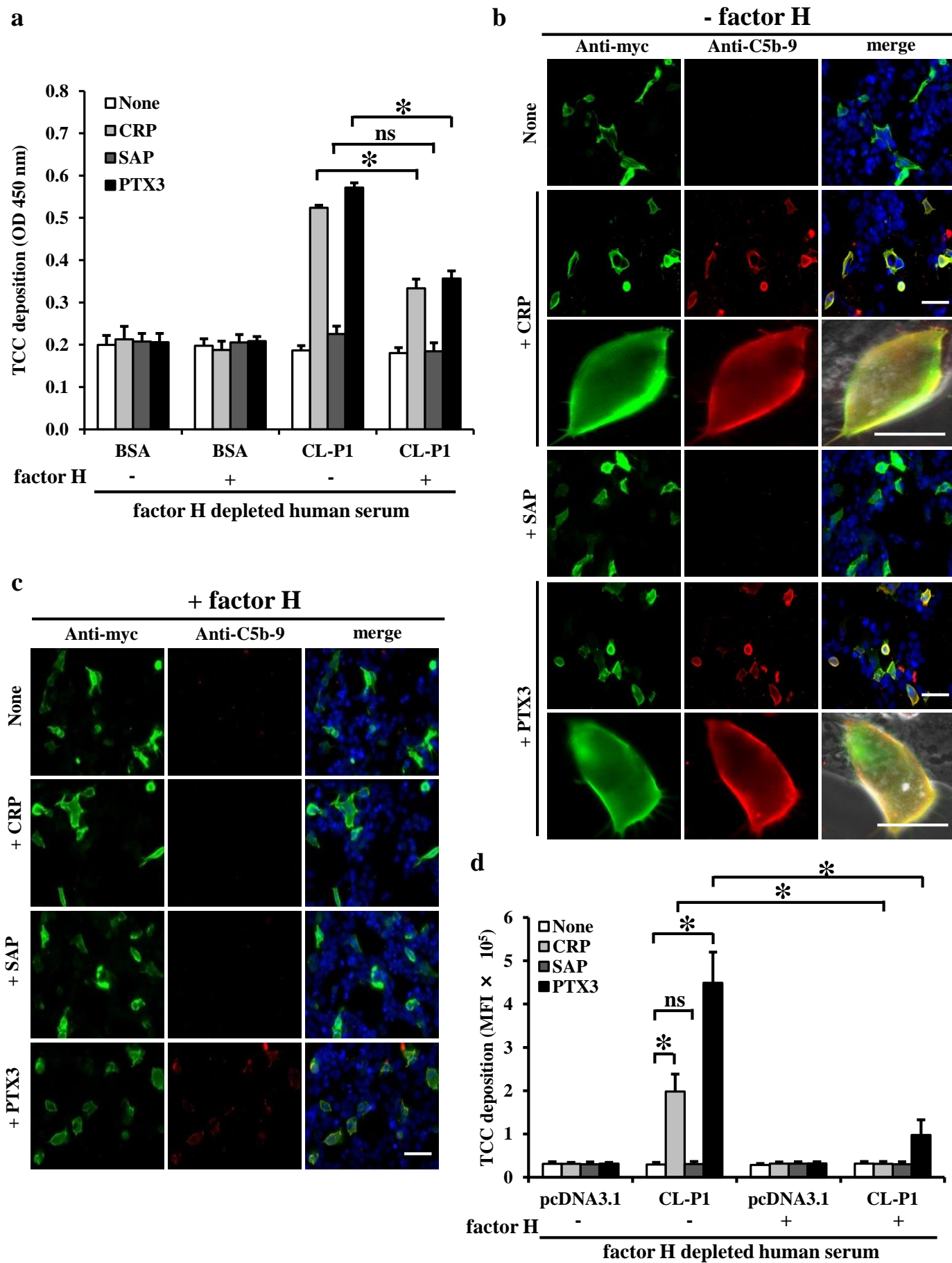
**Figure 3**

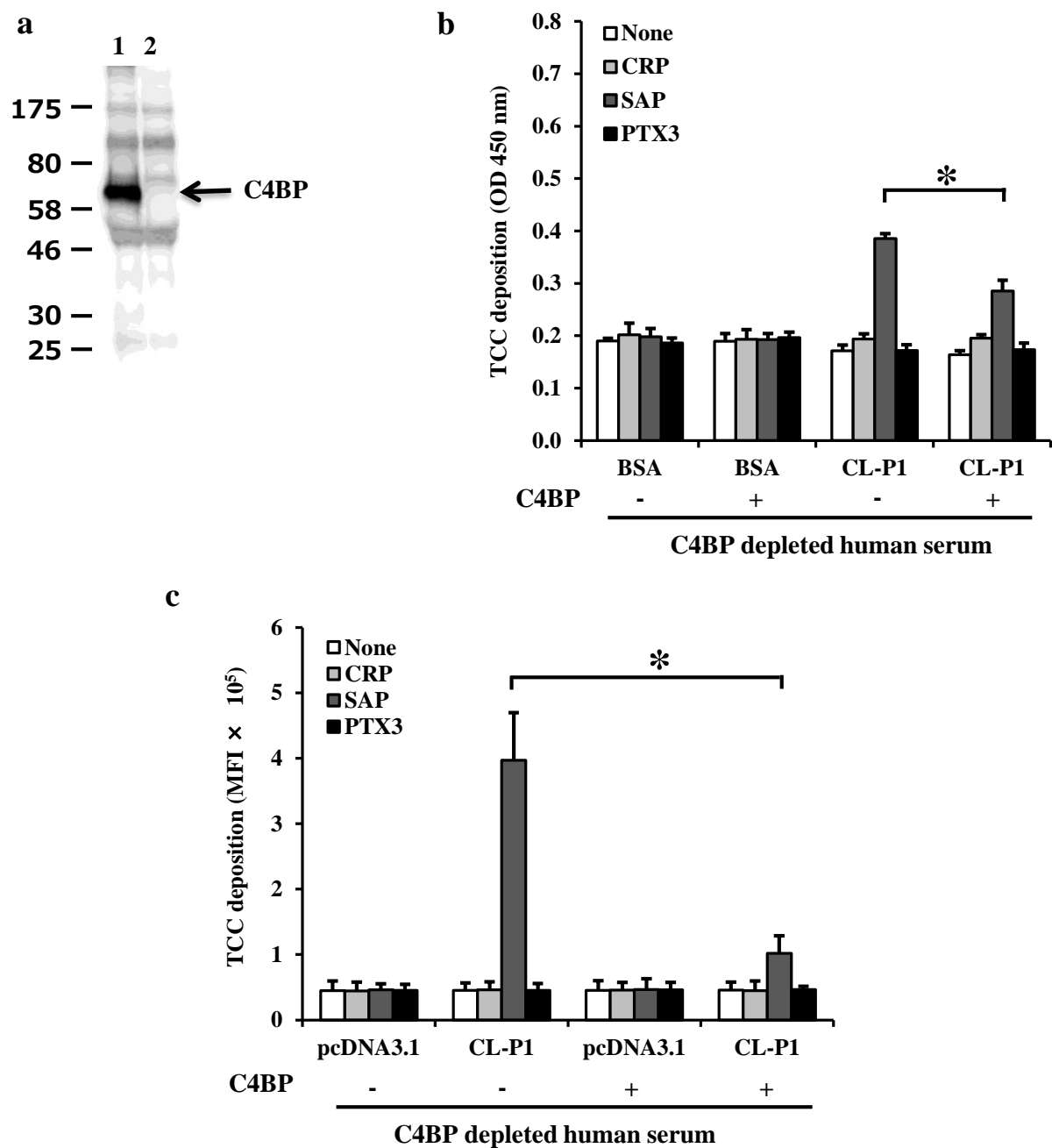
Figure 4

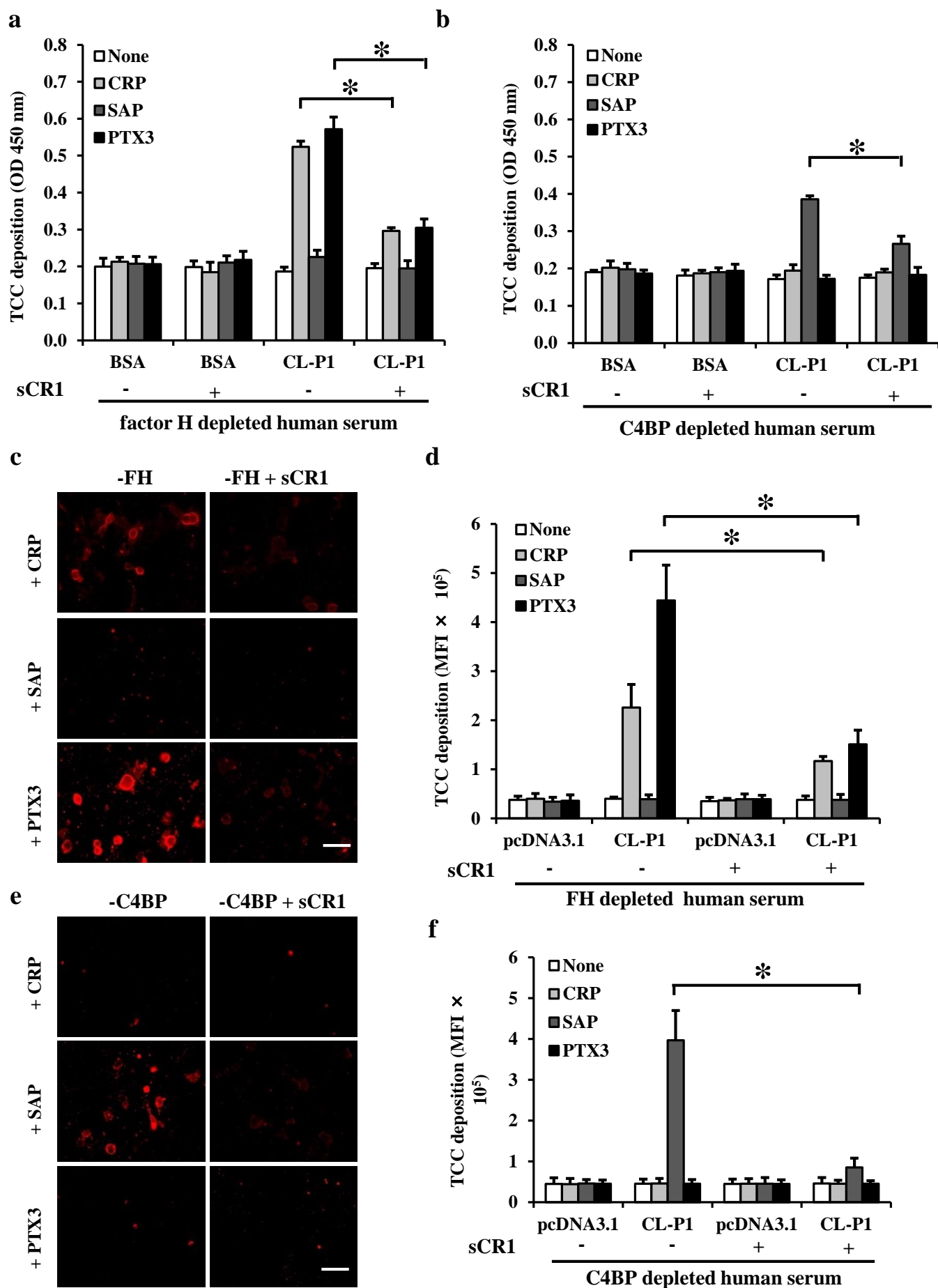


**Figure-5**

**Figure-6**

**Figure-7**



**Figure-8**



**Figure-9**

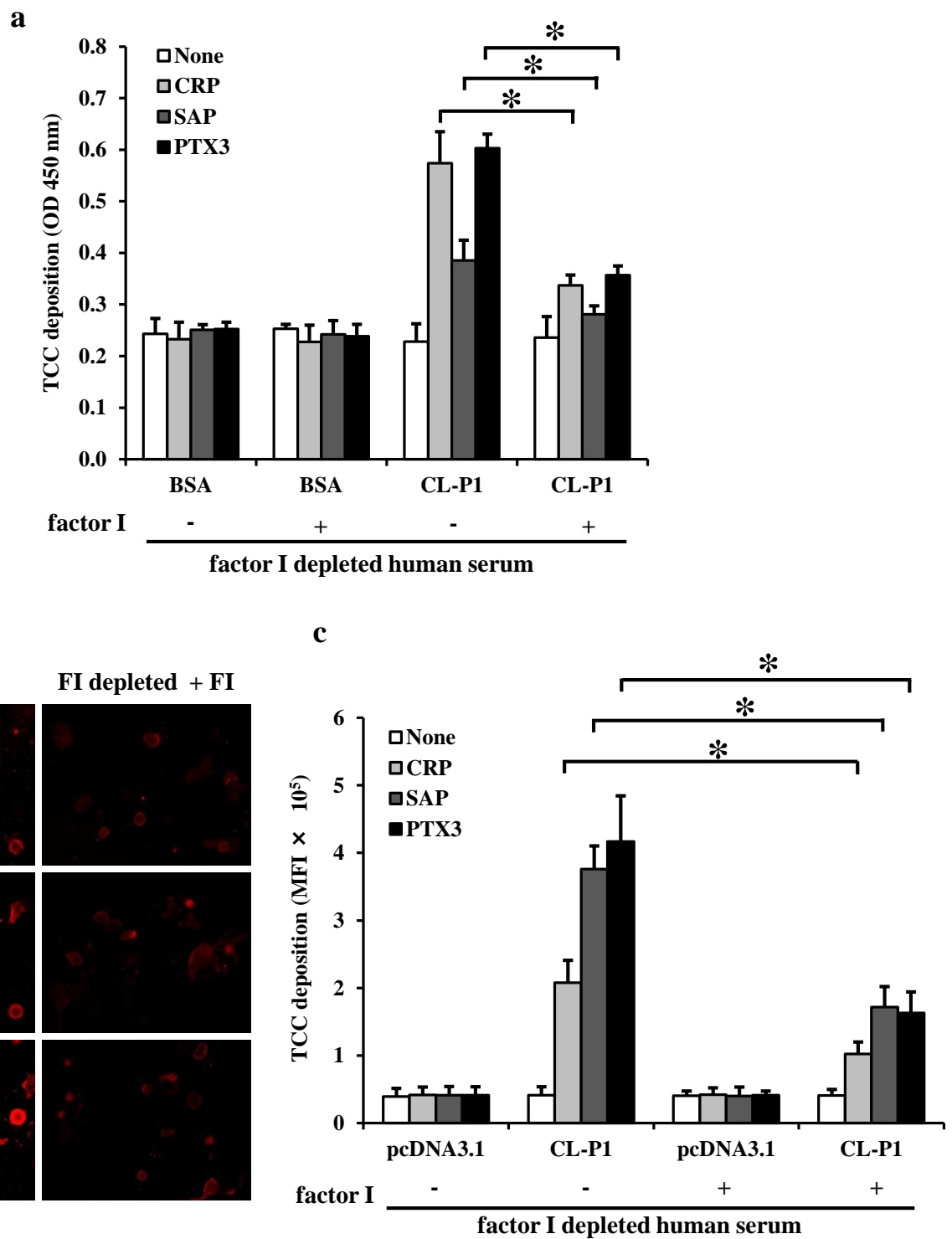


Figure 10

