学位論文

Collectin CL-P1 Utilizes C-Reactive Protein for Complement Activation

（コレクチンCL-P1は、CRPを用いて補体経路を活性化する）

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Running head: CL-P1 is involved in complement activation

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ABSTRACT

Background: C-reactive protein (CRP) is a plasma pentraxin family protein that is massively induced as part of the innate immune response to infection and tissue injury. CRP and other pentraxin proteins can activate a complement pathway through C1q, collectins, or on microbe surfaces. It has been found that a lectin-like oxidized LDL receptor 1 (LOX-1), which is an endothelial scavenger receptor (SR) having a C-type lectin-like domain, interacts with CRP to activate the complement pathway using C1q. However it remains elusive whether other lectins or SRs are involved in CRP-mediated complement activation and the downstream effect of the complement activation is also unknown.

Methods: We prepared CHO/ldlA7 cells expressing collectin placenta-1 (CL-P1) and studied the interaction of CRP with cells. We further used ELISA for testing binding between proteins. We tested for C3 fragment deposition and terminal complement complex (TCC) formation on HEK293 cells expressing CL-P1.

Results: Here, we demonstrated that CL-P1 bound CRP in a charge-dependent manner and the interaction of CRP with CL-P1 mediated a classical complement activation pathway through C1q and additionally drove an amplification pathway using properdin. However, CRP also recruits complement factor H (CFH) on CL-P1 expressing cell surfaces, to inhibit the formation of a terminal complement complex in normal complement serum conditions.

General Significance: The interaction of collectin CL-P1 with CFH might be key for preventing attack on "self" as a result of complement activation induced by the CL-P1 and CRP interaction.

Abbreviations

CRP, C-reactive protein; PTX3, pentraxin 3; CRD, carbohydrate recognition domain; Col, collagen; TCC, terminal complement complex; SR, scavenger receptor; CL-P1, collectin placenta 1; SR-AI, scavenger receptor type I; LOX-1, lectin-like oxidized LDL receptor 1; 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; poly(I), polyinosinic acid; poly(C), polycytidylic acid; CFH, complement factor H; oxLDL, oxidized low density lipoprotein; LDL, low density lipoprotein; HCS, human complement serum.

Introduction

C-reactive protein (CRP) is an acute-phase plasma protein produced by hepatocytes in response to inflammation, tissue damage and trauma (1). Like other acute phase proteins, CRP is normally present in trace levels (<10 mg/L) in serum but increases rapidly and dramatically in response to a variety of infectious or inflammatory conditions (2). Mild inflammation and viral infections cause elevation of CRP to 10-40 mg/L while bacterial infections produce levels of 40-200 mg/L. Levels higher than 200 mg/L are found in several bacterial infections and burns (2). CRP is capable of interacting with a variety of ligands such as phosphocholine residues, modified low density lipoprotein (mLDL), and damaged cells as well as activating the complement and opsonizing biological particles (3, 4). The site directed mutagenesis model shows how one globular head group of C1q interacts through the central pore of CRP on the A face of the pentamer (5). The classical pathway is mostly activated in an antibody dependent manner, but it is also initiated by C1q directly through recognition of distinct structural moieties on microbial and apoptotic cells or through various soluble pattern recognition molecules, such as CRP and Pentraxin 3 (PTX3) (6, 7).

Lectin-like oxidized LDL receptor 1 (LOX-1) was discovered as an endothelial scavenger receptor (SR), having a calcium dependent lectin-like protein (8). Recent papers demonstrated that CRP bound to Chinese hamster ovary cells (hLOX-1-CHO) express human LOX-1, and that CRP bind to recombinant

human LOX-1 in a cell-free system (9, 10). These studies also showed that LOX-1 promotes CRP-induced xenogeneic complement activation by interacting with CRP to develop an inflammatory pathogenic response. Moreover, subsequent research has demonstrated that scavenger receptor type I (SR-AI) binds CRP whereas other SRs, CD36 or SR-B1, do not (11).

The ligand specificity of the SR family overlaps considerably (12, 13). We previously identified collectin placenta 1 (CL-P1) (14), a type II membrane protein which contains carbohydrate recognition domain (CRD), a long coiled-coil domain, and a transmembrane domain and showed it to be a SR, in addition to its role as a collectin. It is also referred to as a scavenger receptor C-type lectin (SRCL) and collectin 12 (15). Recent genomic analysis verified CL-P1 as an SR-AI gene of *SCARA4* as well as a collectin gene of *COLEC12* (16, 17). CL-P1 was originally found and identified as 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, and it interacts with OxLDL and microbes through the collagen-like domain whereas it utilizes the CRD to bind sugar ligands (18, 19). Furthermore, very recently Ma et al. have hypothesized the existence of a fluid phase molecule of CL-P1 which may initiate complement activation on *Aspergillus fumigatus* (20).

The aim of this study was to investigate the involvement of CL-P1 in CRP-mediated complement activation, as well as to investigate the downstream effects of this complement activation. In the present

study, we addressed how CL-P1 interacted with CRP and promoted complement activation through the classical and amplification pathway. At the same time, CRP recruits complement factor H (CFH) depending on CL-P1 to protect the cells from the formation of the terminal complement complex.

Materials and methods

Proteins and reagents

Human CRP purified from pleural fluid (AG723) was purchased from Merck Millipore. Sodium azide in the solution was extensively removed by dialyzing 3 times against a 3000-fold volume of PBS. HAM's F-12 and Dulbecco's minimal essential medium (DMEM)-high glucose, fetal bovine serum (FBS), poly(I), poly(C), phosphocholine, polymyxin B and human complement serum were from Sigma. Anti-myc antibody and Alexa Fluor conjugated antibodies were purchased from Invitrogen. We purchased native C1q, purified human factor H, purified human properdin, rabbit anti-human C5b-9, goat anti-human factor H, goat anti-human properdin, properdin depleted serum and factor H depleted serum from Complement Technology. The MicroVue SC5b-9 plus EIA kit were purchased from Quidel.

Cell culture and transfection

Chinese hamster ovary (CHO)/ldlA7 cells lacking functional LDL receptors were cultured and transfected as described previously (19). In brief, cells were grown in HAM's F-12 medium supplemented

with 5% heat inactivated FBS at 37 °C, 5% CO_2. 24 h earlier, transfected cells were seeded onto poly-L-lysine-coated 35 mm glass based dishes (Iwaki, Japan). Transfection was carried out by Lipofectamine LTX transfection reagent (Invitrogen) according to the manufacturer's instructions. Lipofectamine LTX /plasmid DNA complex was replaced with fresh HAM's F12 medium with 5% FBS 6 h after transfection. 24 h post transfection cells were immediately used for a ligand binding assay. HEK293 cells were maintained in DMEM-high glucose supplemented with 10% FBS. HEK293 cells were seeded on collagen-coated dishes (Iwaki, Japan) and transfected as described above.

Construction of expression vectors

Recently, we identified and cloned cDNA sequences of human CL-P1 into pcDNA3.1/myc-HisA expression vectors (18). Constructs for CL-P1 deletion mutants and collagen-like domain positive cluster mutants were prepared as described previously (19). We cloned LOX-1 from human placental cDNA. The plasmid DNA was purified using an EndoFree maxi kit (Qiagen) according to the manufacture's protocol.

Recombinant CL-P1 expression and purification

cDNA encoded extracellular domain of human CL-P1 (59–742) was subcloned into pcDNA3.1 vector with insulin leader peptide followed by FLAG tag in the N terminus. The plasmid was transfected into Expi 293-F cells (Invitrogen). After 7 days, the recombinant protein was purified from culture supernatant with anti-FLAG M2 affinity gel (Sigma) according to the manufacturer's instructions.

Fluorescent labelling and biotinylation of CRP

CRP was fluorescently labeled with an Alexa Fluor 555 antibody-labeling kit (Invitrogen) and dialyzed 3 times (12 h, 4°C) against a 3000-fold volume of PBS. Biotinylation of CRP was performed using EZ-Link Sulfo-NHS-LC-LC-Biotin (Invitrogen) according to the manufacturer's instructions and dialyzed 3 times (12 h, 4°C) against a 1000 fold volume of PBS.

Binding and inhibition assay using the CHO/ldlA7 cell line

The binding of Alexa 555-CRP with full length CL-P1, CL-P1 deletion mutants and LOX-1 was performed as previously described (10). In brief, 24 h after transfection cells were washed twice with ice-cold HAM's F12 medium with 5% FBS. Then, the medium was replaced with ice-cold Ham's F-12/10 mM HEPES containing 10 μg/ml of Alexa 555-CRP and cells were incubated at 4 °C for 1 h. After being washed with ice cold PBS, the cells were fixed with 4% phosphate-buffered paraformaldehyde (Wako Pure Chemical Industries). For the inhibition assay, 10 μg/ml poly(I) or poly(C) were pre-incubated with cells before the addition of Alexa 555-CRP. For the phosphocholine inhibition assay, 1 mM phosphocholine was mixed with Alexa 555-CRP and then incubated with cells for 1 h at 4 °C. The expression of CL-P1 and LOX-1 was visualized using anti-myc antibody followed by Alexa 488 anti-mouse IgG. Nuclear counterstaining was performed with Hoechst 33342 obtained from Invitrogen. The images were taken using a fluorescent microscope (BZ-9000, Keyence). Signal intensity

was calculated by using the BZ-HIC program (Keyence).

CRP and CL-P1 interaction ELISA

Recombinant human CL-P1 (0.1 μg) or BSA (0.1 μg) (Thermo Scientific) was immobilized to 96-well immunoplates plates (Maxisorp, Thermo Fisher Scientific) by incubating overnight at 4 °C in a coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% NaN_3, pH 9.6). After 3 washes with PBS, the plates were blocked with BlockAce/PBS (DS Pharma Biomedical) at 37 °C for 1 h. After washing with PBS, biotin-CRP in TBSTC (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, 5 mM $CaCl_2$, pH 7.4,) was added to each well, and incubated at 37 °C for 1 h. The plates were then washed 3 times with TBSTC and incubated for 1 h with Elite ABC kit (Vector Laboratories) in TBSTC. After washing, 100 μl SureBlue TMB microwell peroxidase substrate (Kirkegaad & Perry Laboratories) were applied to each well and incubated at room temperature for 15 minutes. Finally, we added 100 μl of 1M phosphoric acid to stop the reaction and the absorbance at 450 nm was read with a model 450 microplate reader (Bio-Rad Lab.). For the analyses of the binding of heat-denatured biotin-CRP, CRP solution in PBS was heated in boiling water for 5 min before use. In some experiments a concentration (0-0.1 mM) of phosphocholine or 10 mM EDTA was added to the biotin-CRP in TBSTC and detected as described above.

Complement activation ELISA

An *in vitro* complement activation assay was performed as described previously (21). Recombinant

human CL-P1 (5 μg/ml) or heat inactivated BSA (5 μg/ml) was immobilized to each well of a 96-well immunoplate plate by incubating in a coating buffer overnight at 4 °C. After 3 washes with PBS, the plates were blocked with 2% BSA in PBS at 37 °C for 1 h. After washing, the plates were incubated with 1% human complement serum/PBS (Sigma), C1q-depleted serum (Merck Millipore) or properdin depleted serum at 37 °C for 1 h with or without 20 μg/ml CRP (full serum), inactivated CRP (95 °C, 5 min), or CRP plus polymyxin B (5μg/ml) in a veronal buffer (0.82 mM $MgCl_2$, 145.45 mM NaCl and 0.25 mM $CaCl_2$, 3.11 mM barbitol, 1.8 mM sodium barbitol) containing 0.1% gelatin. In some assays, C1q depleted serum and properdin depleted serum was supplemented with native C1q (200 μg/ml full serum) or purified properdin (6 μg/ml full serum) respectively. We detected C3d using 3.5 μg/ml rabbit anti–human C3d antibody (Dako) in combination with horseradish peroxidase (HRP)-conjugated anti–rabbit IgG antibody (1:5000, Merck Millipore) diluted in PBS containing 1% BSA, 0.05% Tween 20. Finally we determined peroxidase activity with SureBlue TMB microwell peroxidase substrate as described above.

Terminal complement complex (TCC) deposition ELISA

For the TCC deposition assay we used the same procedure as described in the complement activation ELISA except that we used 10% CFH depleted serum. For inhibition of TCC formation, CFH depleted serum was supplemented with purified CFH (400 μg/ml full serum). We detected TCC using rabbit

polyclonal anti-human C5b-9 antibody.

Complement activation on cultured cells

We performed the complement activation assay on cultured cells as previously described (13). In brief, we transfected HEK293 cells seeded in collagen-coated dishes with pcDNA3.1 vector and CL-P1. After washing the cells with PBS, we added 10% human complement serum, C1q depleted serum, and properdin depleted serum in the presence or absence of 20 μg/ml CRP (full serum) in DMEM-high glucose medium. In some assays, C1q depleted serum or properdin depleted serum was replenished with native C1q (200 μg/ml full serum) or native properdin (6 μg/ml full serum). After incubation at 37 °C for 1 h, the cells were washed twice with PBS and fixed with 4% phosphate-buffered paraformaldehyde. We detected the deposition of C3 fragments on the cells by immunostaining with rabbit anti–human C3d antibody (3.5 μg/ml) in combination with Alexa Fluor 594 anti–rabbit IgG antibody. The nuclei of the cells were counterstained with Hoechst 33342. We performed quantitative analysis with the BZ-HIC program.

TCC formation assay

We performed the formation of the TCC on cell surfaces on cultured cells as previously described with few modifications (22). Briefly, HEK293 cells were plated on collagen-coated dishes, in DMEM supplemented with 10% FBS. The next day, cells were transfected with pcDNA3.1 vector and CL-P1. 24

h after transfection cells were washed three times with PBS followed by incubation with a final concentration of 10% human complement serum or CFH depleted serum in the presence or absence of 20 µg/ml of CRP (full serum) at 37 °C for 1 h. In the case of recovery experiments, CFH depleted serum was supplemented with (400 µg/ml full serum) purified human CFH. Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 minutes. After three washes in PBS, cells were incubated for 30 minutes at room temperature with rabbit polyclonal anti-human C5b-9 and anti-myc monoclonal antibody and then for 30 minutes with goat Alexa Fluor 594 anti–rabbit IgG antibody and goat Alexa Fluor 488 anti–mouse IgG antibody. The nuclei were counterstained with Hoechst 33342. Cells were imaged using a fluorescence microscope.

CFH and properdin recruitment assay

HEK293 cells were plated on collagen-coated dishes, in growth medium. Cells were transfected with pcDNA3.1 vector and CL-P1. 24 h after transfection cells were incubated with a final concentration of 10% human complement serum in the presence or absence of 20 µg/ml of CRP (full serum) at 37 °C for 1 h. Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 30 minutes. Cells were then incubated for 30 minutes at room temperature with goat polyclonal anti-human CFH or goat anti-human properdin with anti-myc monoclonal antibody and then for 30 minutes with goat Alexa Fluor 488 anti–mouse IgG antibody or donkey Alexa Fluor 594 anti–goat IgG antibody. We imaged the cells

using a fluorescence microscope.

Quantitative measurement of SC5b-9

HEK293 cells transfected as described above were incubated with CFH depleted serum or CFH depleted serum supplemented with purified CFH (400 μg/ml full serum) in the presence or absence of 20 μg/ml of CRP (full serum). 1 h after incubation the medium was collected and the levels of SC5b-9 in the serum-containing medium were determined using a commercially available ELISA kit (Quidel Corporation).

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

CRP interacts with CL-P1 and LOX-1

It has been reported that LOX-1 binds CRP in cell experiments and ELISA (10). To investigate whether CL-P1 interacts with CRP, we inquired into the binding of Alexa Fluor 555-labeled CRP with CL-P1 and LOX-1 using transiently transfected CHO cells. As shown in Fig. 1a, Alexa 555-CRP binds

CL-P1 and LOX-1 whereas no CRP binding was observed on pcDNA3.1 control vector transfected cells. The CL-P1 and LOX-1 were co-localized with CRP on the cell surface.

We further characterized the CRP and CL-P1 interaction in the ELISA system. The presence of a soluble form of CL-P1 is still under debate. The soluble CL-P1 used in this study was prepared by forcing the extracellular domain to be a secreted protein and attaching an insulin leader peptide. We observed that biotin-CRP bound CL-P1 in a dose dependent manner (0-300 μg/ml) (Fig. 1b) and the heat denatured biotin-CRP lost its ability to bind CL-P1.

Charge dependent interaction of CRP with CL-P1

It is known that CRP interacts with phosphocholine and activates the classical complement pathway (23). The complement activation site in CRP is located in the A-face, whereas the phosphocholine binding site is in the B-face. We found that biotin-CRP and CL-P1 binding in ELISA was inhibited by phosphocholine in a dose dependent manner (0-0.1 mmol/L) (Fig. 2a). We demonstrated that incubation of a phosphocholine and Alexa 555-CRP mixture with CL-P1 transfected cells reduced the CRP binding to CL-P1 (Fig. 2b). Quantitative analysis indicates that 1 mM phosphocholine can completely inhibit the CRP binding (Fig. 2c). These results suggest that CL-P1 might interact with the B-face in CRP.

To check whether this binding of biotin-CRP and CL-P1 was calcium dependent, we performed an EDTA inhibition assay in ELISA which showed no significant decrease in biotin-CRP and CL-P1 binding

(Fig. 2d). These results suggest that the interaction of CL-P1 with CRP might be calcium independent.

We further checked whether the interaction of CL-P1 with CRP is charge dependent. The pre-incubation of Poly(I) with the CL-P1 transfected cells before the addition of Alexa 555-CRP reduced CRP binding to CL-P1, whereas poly(C) did not reduce the binding (Fig. 2e, f). Therefore we concluded that the interaction of CL-P1 with CRP might be mainly charge dependent.

CL-P1 interacts with CRP mainly through the collagen-like and coiled-coil domain

We prepared several CL-P1 deletion mutants to find out the binding domain with different ligands as previously described (19) (Fig. 3a). We confirmed that MIF and western blotting analysis for the expression of full length and deletion mutants of CL-P1 showed almost similar expression levels and patterns on the cell surface except Δcc-col-CRD (19). We found that the collagen-like domain and coiled-coil domain were involved in CL-P1 and CRP interaction (Fig. 3b and supplemental fig. 1)**.** The deletion of CRD could not inhibit the interaction. There are three positively charged clusters in the collagen-like domain of human CL-P1 (19). To verify which positive cluster was involved in the CL-P1 and CRP interaction, we prepared the positively charged cluster mutants (Fig. 3c). We then performed the binding experiments of Alexa 555-CRP with CL-P1 positively charged cluster mutants. Our results showed cluster I and cluster III in the collagen-like domain of CL-P1 were involved in the interaction with CRP (Fig. 3d and supplemental fig. 2). These results also show that the interaction between CRP and

CL-P1 takes place in a charge dependent manner but does not show Ca^{2+} dependent lectin activity, although the detailed mechanism is still unknown.

CRP and CL-P1 interaction activates the classical complement pathway

It is reported that the interaction of LOX-1 with CRP mediates complement activation (9). We tried to detect the interaction of CL-P1 with the CRP activating complement pathway. We examined the complement activation using CRP and recombinant human CL-P1 in an ELISA system. We found that there was complement activation only in the recombinant CL-P1-coated wells but boiled CRP failed to activate the complement system (Fig. 4a). Supplementation of polymyxin B did not affect complement activation (Fig. 4a). These data suggest that the complement activation observed therein is an effect of C-reactive protein in a CRP solution, but not caused by lipopolysaccharide.

Next, we determined the deposition of C3 fragments using CL-P1 expressing HEK293 cells from a human embryonic kidney cell line. The C3 fragments were deposited on CL-P1 in a CRP positive condition (Fig. 4b), although there were some non-specific deposition of C3 fragments. We observed that the degree of specific C3 fragment deposition was 78% on CL-P1 expressing cells and unspecific deposition was 22% in these images. Phase contrast images showed the edges of the cells and that the deposition of C3 fragments occurs all over the surface of CL-P1 expressing cells (Fig. 4b). The signal intensity of the deposition of C3 fragments was increased in CL-P1 expressed cells compared with those

of control pcDNA3.1 when human complement serum was used (Fig. 4c). It is known that CRP can interact with the C1q (19). Next, we tested the possible involvement of C1q in the complement activation in our system. Our ELISA analysis demonstrated that the deposition of C3 fragments occurred in the presence of CRP and C1q (Fig. 5a).

We then analyzed the co-staining of the C3 fragments and C1q. Our results clearly demonstrate the co-staining of C3 fragments and C1q on the cell surface (Fig. 5b). Next, we focused in the deposition of C3 fragments using C1q depleted serum in our HEK293 cell system. In absence of C1q, complement activation was not detected regardless of the presence or absence of CRP (Fig. 5c, d)**.** Supplementation of C1q recovered the deposition of the C3 fragments and it indicates the interaction of C1q and CRP in this system (Fig. 5c, d).

CRP induces the additional activation in the amplification pathway via CL-P1

Activation of the classical pathway inevitably initiates the alternative pathway. To determine whether CRP plays a role in classical pathway-triggered alternative pathway complement amplification, we used ELISA and found a significant decrease in the deposition of C3 fragments which was recovered when purified properdin was added back to the properdin depleted serum (Fig. 6a). We then confirmed the recruitment of properdin on the cell surface in the presence of CRP depending on CL-P1 (Fig. 6b). We next determined, to what degree classical pathway-triggered alternative pathway complement activation

depends on properdin in our assay system. The deposition of C3 fragments was observed even using properdin depleted serum and the supplementation of purified properdin increased the C3 fragments deposition intensity (Fig. 6c, d). Purified properdin apparently has a propensity to form aggregates. So we took extreme care while using purified properdin. Commercially available properdin forms higher oligomers or aggregates upon repeated freezing and thawing were originally called “activated” properdin due to their ability to promote complement activation and consumption when added to serum. So, we avoided repeated freezing and thawing of purified properdin and used in experiments upon receipt to minimize aggregation of the properdin that can occur with prolonged storage. Thus, the interaction of CRP with CL-P1 basically activates the C1q dependent complement pathway and furthermore performs the additive amplification using the alternative pathway.

CRP negatively regulates TCC assembly on CL-P1 expressing cells by recruiting CFH

To determine the effect of CRP on downstream complement components, we examined the assembly of the TCC on CL-P1 expressing cells. As shown in Fig. 7a, we found no TCC formation using human complement serum. It is reported that CRP drives the classical pathway of the complement on nucleated cells without TCC formation or causing cytolysis (24). Other studies have shown that CRP bound to CFH (25, 26), a complement regulatory protein that accelerates the decay of the C3 and C5 convertases and inhibits the assembly of the terminal complement components (27). Our results demonstrated the binding

of CFH with CRP using human complement serum depended on CL-P1 (Fig. 7b). To determine whether CFH was required for the prevention of the TCC formation, we incubated CL-P1 expressing cells with CFH depleted serum with or without CRP. CFH depleted serum failed to prevent the TCC deposition (Fig. 7c). This inhibitory activity could be restored by the addition of CFH (Fig. 7c). Phase contrast images clearly demonstrated the pattern of TCC deposition on the surface of CL-P1 expressing cells (Fig. 7c). We found that a similar phenomenon occurs in the ELISA system (Fig. 7e). Next, we analyzed the soluble TCC formation in the serum-containing culture medium 1 h after TCC formation reaction. The level of SC5b-9 significantly increased in the serum-containing medium after the reaction CL-P1 transfected cells including CRP compared with no CRP addition (Fig. 7d). Therefore, we concluded that the recruitment of CFH by CRP is required for the prevention of the TCC assembly on CL-P1 expressing cells (Fig. 8).

Discussion

CRP is an acute phase protein involved in complement activation through the classical pathway (3). Recently CRP has shown to bind LOX-1 and induce complement activation (9). However the mechanism by which CRP induces complement activation is poorly understood. Our study aimed to identify whether collectin CL-P1 can interact with CRP. Here, we demonstrated that CRP might be a novel ligand for CL-P1 and mediate some biological effects through complement activation.

Fujita et al. reported that LOX-1 interacted with CRP in a calcium dependent manner as EDTA completely ended the interaction (9). In the case of CL-P1, EDTA was unable to inhibit the interaction of recombinant CL-P1 with CRP and this suggests that the interaction of CL-P1 and CRP might be calcium independent. It was proposed that LOX-1 interacted with the B-face of CRP since recombinant LOX-1 and CRP interaction was inhibited by phosphocholine (9). Two inhibition studies using phosphocholine in ELISA and cell experiments indicate that CL-P1 might also interact with the B-face of CRP.

Next, we focused on the inhibition effects by polycations or polyanions on the interaction of CRP with CL-P1. It was shown that polycations bound CRP on the B-face (28, 29). Our microscope data showed the interaction was completely inhibited by the pre-incubation of polyanions but not by polycataions before the addition of CRP in CL-P1 expressed cells. This could be explained by the neutralization of a positive charge on CL-P1 by polyanions that inhibit the interaction with CRP. Lee et al. has found phosphocholine can apparently bind cationic sites on CRP and the binding site does not overlap with the polycationic binding site (30). Therefore we hypothesize that CL-P1 might interact with the B-face of CRP in a charge dependent manner.

The site directed mutagenesis study has shown that the OxLDL and CRP binding site on LOX-1 is different but shares some common features because a carrageenan and anti-LOX-1 antibody were able to act as competitors to both (31). Recently, we have shown that the collagen-like domain of CL-P1 is

responsible for the interaction with OxLDL and microbes using CL-P1 deletion mutants (19). Our results also suggest that the collagen-like domain and coiled-coil domain of CL-P1 is involved in CRP binding. The study using mutants of the positively charged cluster in the collagen-like domain of CL-P1 revealed that the first and third positively charged cluster is involved in interaction with CRP. However, previous mutant experiments demonstrated that the second and third charged cluster was important for the binding of OxLDL and microbes (19). These results suggest that the third positively charged cluster of CL-P1 is an important domain for CRP and OxLDL binding.

CRP activates the complement system in human and mouse serum because CRP is known to bind C1q and activate pathways through its A-face (32). Here, we prepared a complement activation system using human complement serum and human cell line HEK-293 cells. Our Figs. 4 and 5 also demonstrate that the interaction of CRP with CL-P1 mediates complement activation depending on C1q as a classical pathway without antibodies. The deposition of C3 fragments using soluble CL-P1 is low in our ELISA analysis. This is could be due to the randomness of the orientation of the soluble CL-P1 in the ELISA plate.

Activation of the alternative pathway can occur secondary to classical pathway activation or be initiated independently. CRP has been shown to enhance the alternative pathway activation via the C3 convertase generation and function with it as a pre-antibody host defense mechanism (33). Properdin

plays an important role in the activation of the alternative pathway not only by maintaining the stability of C3bBb, but also by providing a platform for the de novo C3 convertase assembly (34). Our results suggest that the interaction of CRP with CL-P1 on the cells first activates the classical pathway through C1q and next drives the alternative pathway as an amplification loop using properdin (Fig. 6). We believe that this auto-activation of the complement on the body's own cells only takes place if a very high concentration of CRP exists in the serum. Furthermore, Ma et. al. have reported that the interaction of soluble CL-P1 and properdin activates the alternative pathway and reacts effectively with *A. fumigatus*. (20). Usually, CL-P1 is expressed in vascular endothelial cells but a natural soluble form of CL-P1 has been found in human umbilical cord plasma (20). Although the soluble CL-P1 was not clearly detected in normal adult venous plasma, CRP might collaborate with soluble CL-P1 for pathogen recognition and complement activation.

Fujita et al. could not demonstrate the final stage of the CRP and LOX-1 mediated complement activation. It has been found that CRP activates the classical pathway on nucleated cells without activating the TCC or causing cytolysis (24). CRP has been shown to bind apoptotic cells and protects the cells from assembly of the TCC by recruiting CFH (35). Our results also demonstrated that the complement activation by CRP and CL-P1 interaction could not form the TCC. Our data show that depletion of CFH allows TCC formation which was prevented when CFH was added back to the CFH

depleted serum (Figs. 7), although the interaction of CL-P1 and CRP initiates complement activation. We suggest that, the complete CFH level in the serum might be able to suppress terminal complement activation even if acute inflammation or injury occurs with up-regulation of CRP. Thus, the interaction of elevated levels of CRP with CL-P1 could be disastrous in a CFH compromised condition. Furthermore, another study showed no enhanced CFH binding or any protective effect of CRP regarding C9 deposition (36). Why these observations differ from some other reports may relate to the quality of proteins and the semantics regarding the stages of cells.

The role of CRP in cardiovascular disease is controversial (37, 38). For example, reports of atherosclerosis resulting in apolipoprotein E deficient mice overexpressing CRP were conflicting, with one study finding a positive relationship between CRP and cardiovascular disease (39), whereas other studies did not (40, 41). Our study suggests that in CFH dysfunctional individuals, CRP might augment endothelial injury by activating the complement via CL-P1 to initiate cardiovascular disease.

Du Clos et al. have found that local production and activation of the complement has an important role in I/R injury and allograft rejection (42). By localizing at sites of tissue damage CRP has the potential to contribute to the complement activation at these sites, but CRP may also regulate this activation by its interaction with CFH depending on CL-P1.

In this paper, we found an important role of collectin CL-P1 in the limited activation of the

complement system induced by the acute phase level of CRP. These results might provide a novel insight into the involvement of CL-P1, CRP and complement factors in complement related diseases. Of course, future studies are needed to characterize more details of the CFH-CRP-CL-P1 interaction and their relationship to diseases.

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FIGURE LEGENDS

Figure 1. CRP binds CL-P1 and LOX-1.

(a) Alexa 555 labelled CRP binds CL-P1 and LOX-1. CHO/ldlA7 cells transiently transfected with pcDNA3.1 vector, CL-P1, and LOX-1 were incubated with Alexa 555-CRP (red) and then anti-myc antibody followed by Alexa 488 conjugated goat anti-mouse IgG (green), and were counterstained with Hoechst 33342 (blue). The analyses were performed in five independent experiments. (b) Soluble CL-P1 binds CRP. Biotin-CRP interacts with recombinant human CL-P1 dose dependently (●) but not with BSA (■). Denatured biotin-CRP showed no binding with CL-P1 (▲). The asterisks indicate significant differences vs BSA control (*p<0.0001). The daggers indicate significant differences vs active CRP

control (*p<0.0001). Data are means ± S.D (n=3). Scale bars, 20 μm for all images. OD, optical density.

Figure 2. Phosphocholine and poly(I) inhibit CRP and CL-P1 interaction on ELISA and cultured cells.

(a) Competition of phosphocholine with biotin-CRP for the binding to recombinant CL-P1. Phosphocholine dose dependently inhibited biotin-CRP binding to CL-P1 in ELISA. Data are means ± S.D (n=3). (b) Phosphocholine inhibition assay using CHO/ldlA7 cells. Phosphocholine almost completely inhibits the interaction of Alexa 555-CRP (red) with CL-P1 (green) (c) Quantification of phosphocholine inhibition efficiency. The analyses were performed in three independent experiments. (d) Calcium independent binding of Biotin-CRP to CL-P1 in ELISA. Data are shown as mean ± S.D (n=3 independent repeat). (e) Poly(I) and poly(C) inhibition assay using CHO/ldlA7 cells. (f) Quantification of poly(I) and poly(C) inhibition efficiency. We imaged the cells using a Keyence microscope (KZ9000). Data are means ±S.D of three independent experiments. *p<0.0001. Scale bars, 20 μm for all images. N.S. - not significant; MFI, mean fluorescence intensity, OD, optical density.

Figure 3. CL-P1 interacts with CRP mainly through a collagen-like and coiled coil domain.

(a) Schematic diagram showing the structure of CL-P1 deletion mutants. (b) Signal intensity for the binding of CRP with CL-P1 deletion mutants. (c) Schematic diagram showing the structure of CL-P1

positively charged cluster mutants. (d) Signal intensity for the binding of CRP with positively charged cluster mutants. The first and third positive cluster mutants show lower affinity to CRP. The results shown are representative of three independent experiments. *p<0.0001. N.S. - not significant; MFI, mean fluorescence intensity.

Figure 4. Interaction of CRP with CL-P1 mediates complement activation on ELISA and cultured cells.

(a) Complement activation by CRP (20 μg/ml) on recombinant human CL-P1 or BSA immobilized on ELISA using human complement serum. Data are means ± S.D (n=3). (b) Complement activation detected by the deposition of C3 fragments (red) on HEK293 cells. The anti-myc staining showed the CL-P1 expression (green) and cell nuclei were counterstained with Hoechst 33342 (blue). Phase contrast images (×40) clearly show the co-localization of CL-P1 and C3 fragments on the surface of the cells. (c) Fluorescence intensity of the deposition of C3 fragments using human complement serum. Values represent the mean of three independent experiments. *p<0.0001. Scale bars, 20 μm for immunofluorescence and phase contrast images. OD, optical density; N.S. - not significant; MFI, mean fluorescence intensity.

Figure 5. Classical pathway activation by CRP via CL-P1.

(a) CRP (20 μg/ml) induced complement activation on recombinant human CL-P1 or BSA immobilized on an ELISA using C1q depleted human serum with/without the complementation of native C1q. Data are means ± S.D (n=3). (b) C3 fragments (red) and C1q (green) co-staining on HEK293 cells. Results are from two independent experiments. (c) Depletion of C1q suppressed the C3 deposition (red) on HEK293 cells depending on CL-P1 (green) and CRP. (d) Mean fluorescence intensity of the deposition of C3 fragments using C1q depleted serum in presence or absence of CRP. Data are means ± S.D (n=3). *p<0.0001. Scale bars, 20 μm for all images. OD, optical density; N.S. - not significant; MFI, mean fluorescence intensity.

Figure 6. CRP additionally activates Classical pathway-triggered alternative pathway amplification on CL-P1.

(a) Properdin depleted serum shows lower deposition of C3 fragments. Properdin depleted serum replenished with native properdin recovered the deposition of C3 fragments on the cells. (b) Properdin (red) was recruited only in CL-P1 (green) and CRP positive conditions on HEK293 cells. Results are representative images from three independent experiments. (c) Mean fluorescence intensity of the deposition of C3 fragments using properdin depleted serum with/without supplementation of properdin. Cell nuclei were counterstained with Hoechst 33342 (blue). Data are means ± S.D of three independent

experiments. *p<0.0001. Scale bars, 20 μm for all images. N.S. - not significant; MFI, mean fluorescence intensity.

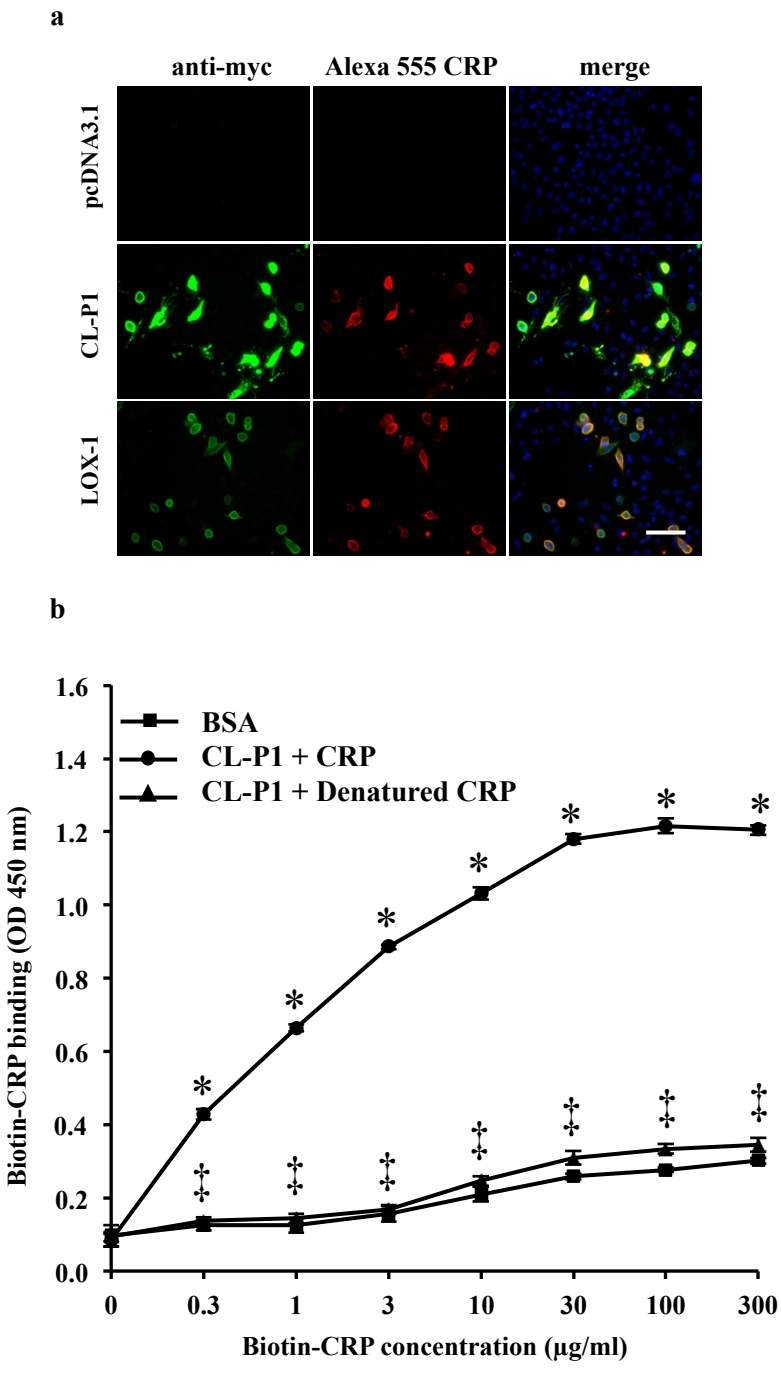
Figure 7. CFH prevent the TCC assembly on CL-P1 expressing cells through CRP.

(a) Complement activation of the TCC assembly was not detected by C5b-9 depositions (red) on HEK293 cells using human complement serum and CRP. The anti-myc staining showed the CL-P1 expression (green) and cell nuclei were counterstained with Hoechst 33342 (blue). Representative images of three independent experiments are shown. (b) CFH (red) was recruited only in CL-P1 (green) and CRP positive conditions on HEK293 cells. The experiment shown is representative of three independent assays. (c) CFH depleted serum failed to prevent TCC formation (red). Phase contrast images (×40) show the TCC formation on the surface of the cells. (d) SC5b-9 formation in medium of Fig. 7c was detected by ELISA. CFH depleted serum induced the SC5b-9 formation which can be inhibited by the addition of CFH. (e) TCC deposition was detected by ELISA. Depletion of CFH failed to prevent CRP dependent TCC formation. The result was reproduced in three independent experiments. Scale bars, 20 μm for immunofluorescence and phase contrast images.

Figure 8. Proposed mechanism for CRP and CL-P1 mediated complement regulation.

CRP is composed of 5 subunits and has an A-face and B-face. First, CL-P1 traps CRP on the B-face. Next, C1q binds to the A-face and activates the complement system through the classical and properdin mediated amplification pathway. The binding of CRP to CL-P1 also recruits CFH, which reduces the downstream activation of the complement and might inhibit the assembly of terminal complement complex (TCC).

Figure 1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anti-myc
Alexa 555 CRP
merge
pcDNA3.1
CL-P1
LOX-1
b
BSA
CL-P1 + CRP
CL-P1 + Denatured CRP
Biotin-CRP binding (OD 450 nm)
Biotin-CRP concentration (μg/ml)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
0.3
1
3
10
30
100
300

Figure 2

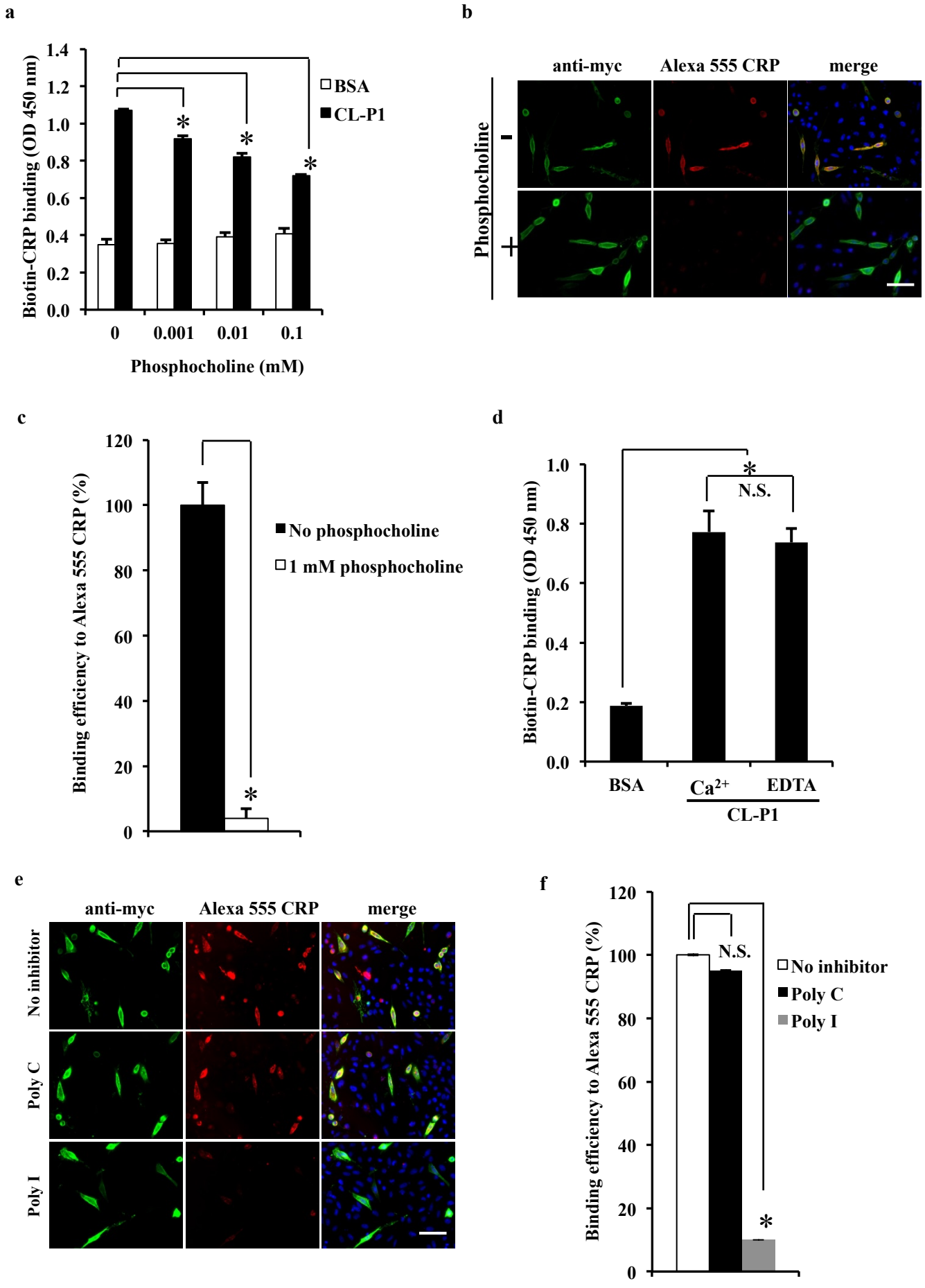

a
Biotin-CRP binding (OD 450 nm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BSA
CL-P1
*
*
*
0
0.001
0.01
0.1
Phosphocholine (mM)
b
anti-myc
Alexa 555 CRP
merge
Phosphocholine
-
+
c
Binding efficiency to Alexa 555 CRP (%)
120
100
80
60
40
20
0
No phosphocholine
1 mM phosphocholine
*
d
Biotin-CRP binding (OD 450 nm)
1.0
0.8
0.6
0.4
0.2
0.0
*
N.S.
BSA
Ca2+
EDTA
CL-P1
e
anti-myc
Alexa 555 CRP
merge
No inhibitor
Poly C
Poly I
f
Binding efficiency to Alexa 555 CRP (%)
120
100
80
60
40
20
0
N.S.
*
No inhibitor
Poly C
Poly I

Figure 3

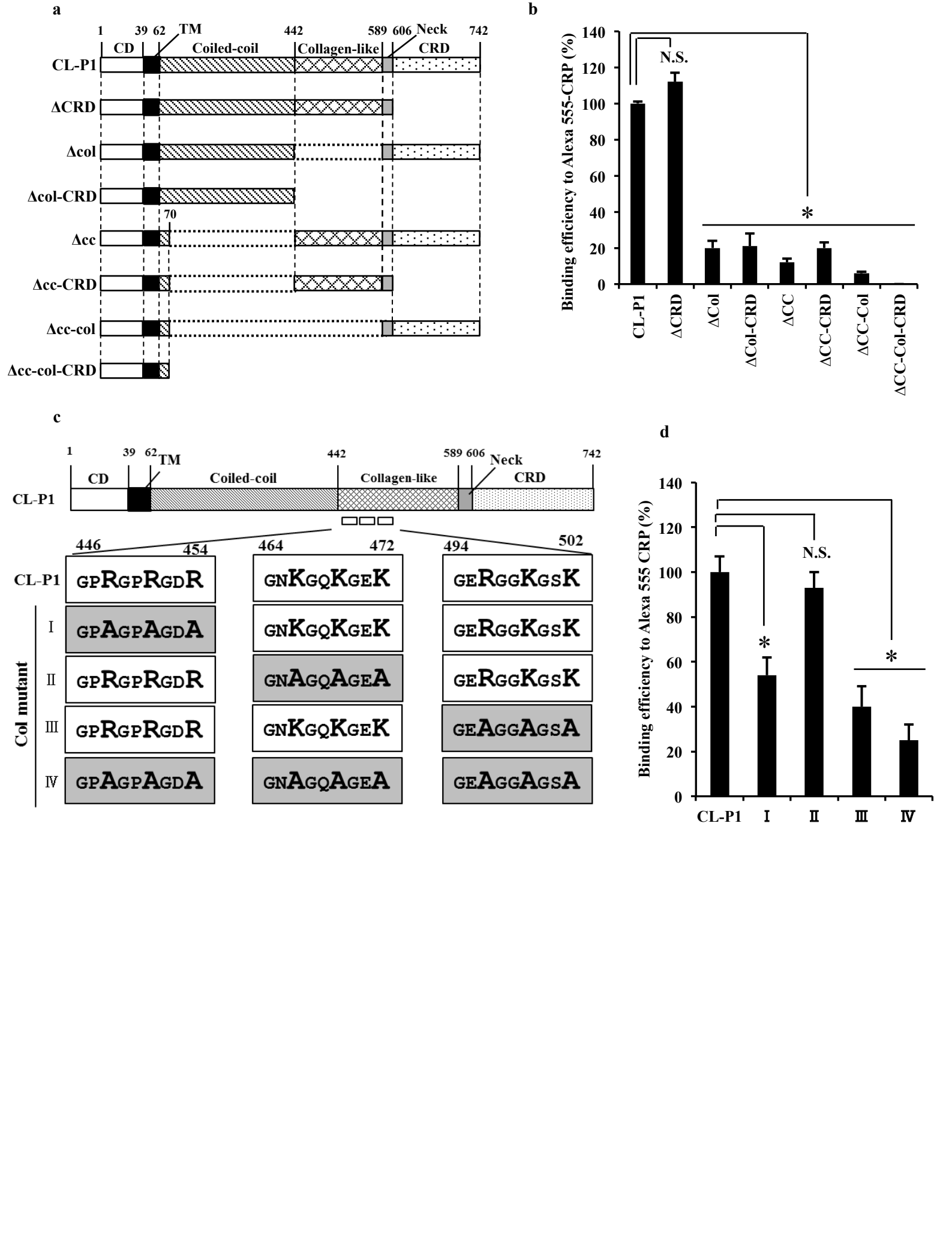

a
1
39
62
TM
442
589
606
Neck
742
CD
Coiled-coil
Collagen-like
CRD
CL-P1
ΔCRD
Δcol
Δcol-CRD
70
Δcc
Δcc-CRD
Δcc-col
Δcc-col-CRD
b
Binding efficiency to Alexa 555-CRP (%)
140
120
100
80
60
40
20
0
N.S.
*
CL-P1
ΔCRD
ΔCol
ΔCol-CRD
ΔCC
ΔCC-CRD
ΔCC-Col
ΔCC-Col-CRD
c
1
39
62
TM
442
589
606
Neck
742
CD
Coiled-coil
Collagen-like
CRD
CL-P1
446
454
464
472
494
502
CL-P1
GPRGPRGDR
GNKGQKGEK
GERGGKGSK
Col mutant
I
GPAGPAGDA
GNKGQKGEK
GERGGKGSK
II
GPRGPRGDR
GNAGQAGEA
GERGGKGSK
III
GPRGPRGDR
GNKGQKGEK
GEAGGAGSA
IV
GPAGPAGDA
GNAGQAGEA
GEAGGAGSA
d
Binding efficiency to Alexa 555 CRP (%)
140
120
100
80
60
40
20
0
N.S.
*
*
CL-P1
I
II
III
IV

Figure 4

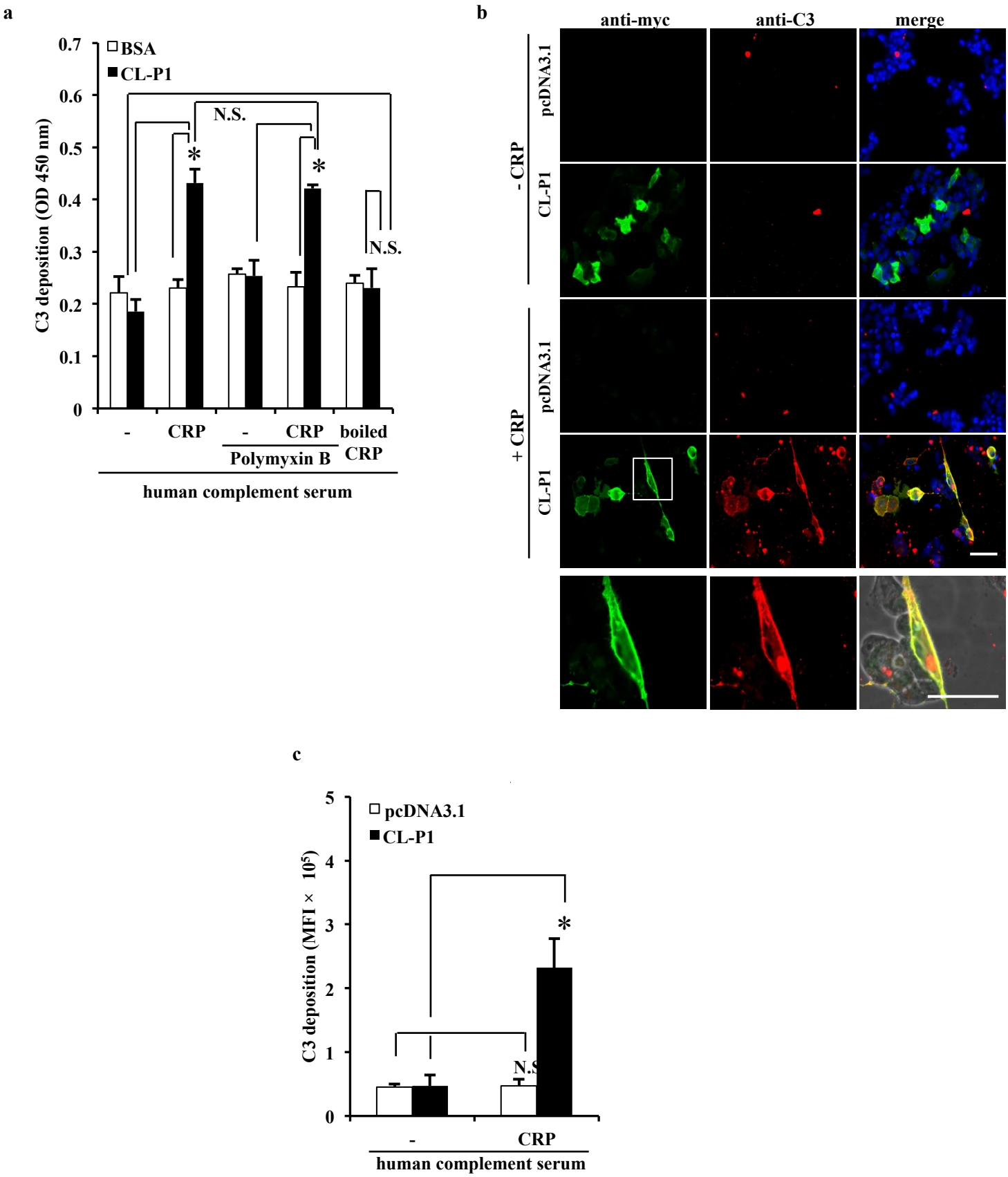

a
BSA
CL-P1
C3 deposition (OD 450 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N.S.
*
*
N.S.
-
CRP
-
CRP
boiled CRP
Polymyxin B
human complement serum
b
anti-myc
anti-C3
merge
- CRP
+ CRP
pcDNA3.1
CL-P1
pcDNA3.1
CL-P1
c
pcDNA3.1
CL-P1
C3 deposition (MFI × 10^5)
5
4
3
2
1
0
*
N.S
-
CRP
human complement serum

Figure 5

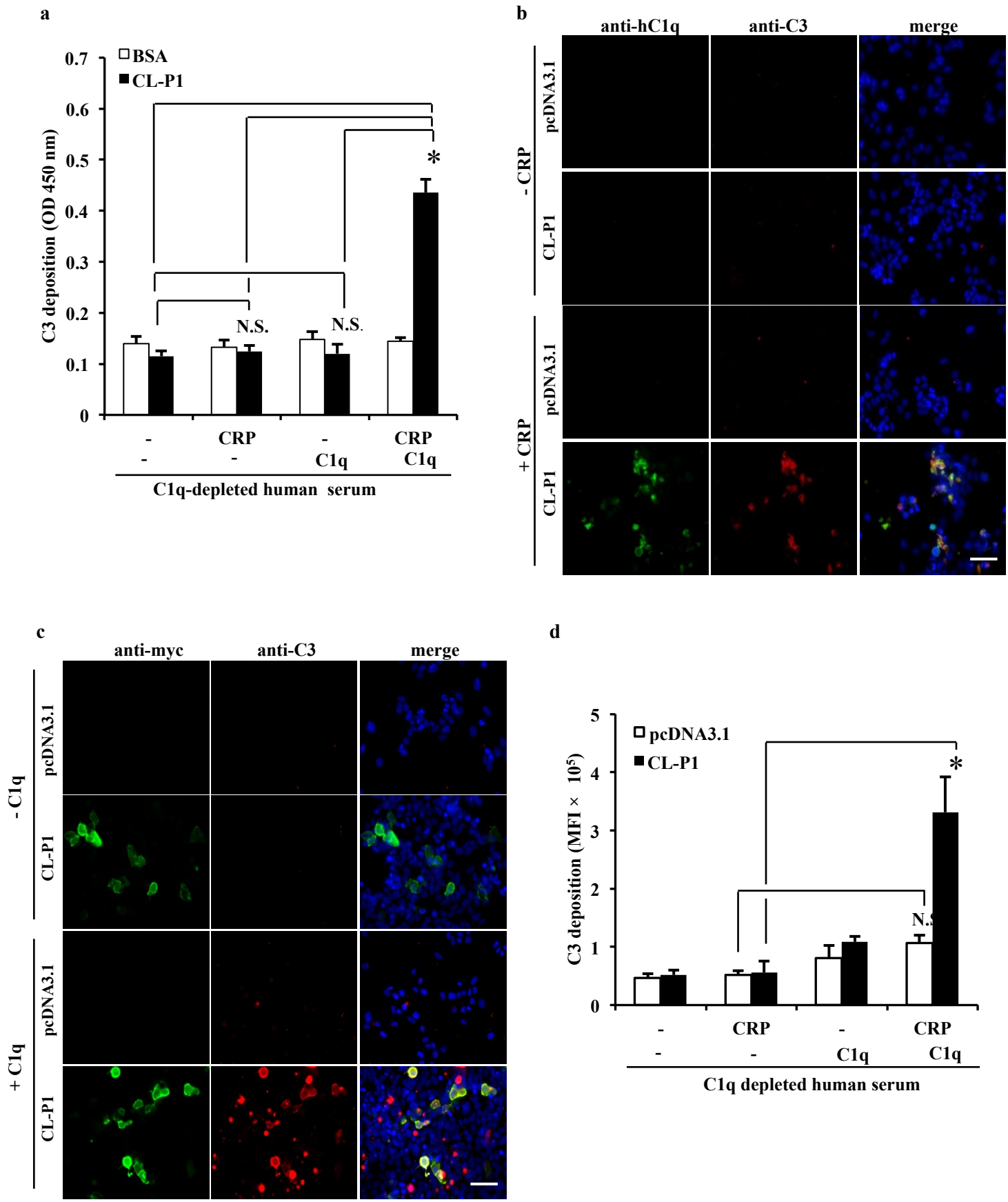

a
BSA
CL-P1
C3 deposition (OD 450 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
N.S.
N.S.
*
-
-
CRP
-
-
C1q
CRP
C1q
C1q-depleted human serum
b
anti-hC1q
anti-C3
merge
- CRP
+ CRP
pcDNA3.1
CL-P1
pcDNA3.1
CL-P1
c
anti-myc
anti-C3
merge
- C1q
+ C1q
pcDNA3.1
CL-P1
pcDNA3.1
CL-P1
d
pcDNA3.1
CL-P1
C3 deposition (MFI × 10^5)
5
4
3
2
1
0
*
N.S.
-
-
CRP
-
-
C1q
CRP
C1q
C1q depleted human serum

Figure 6

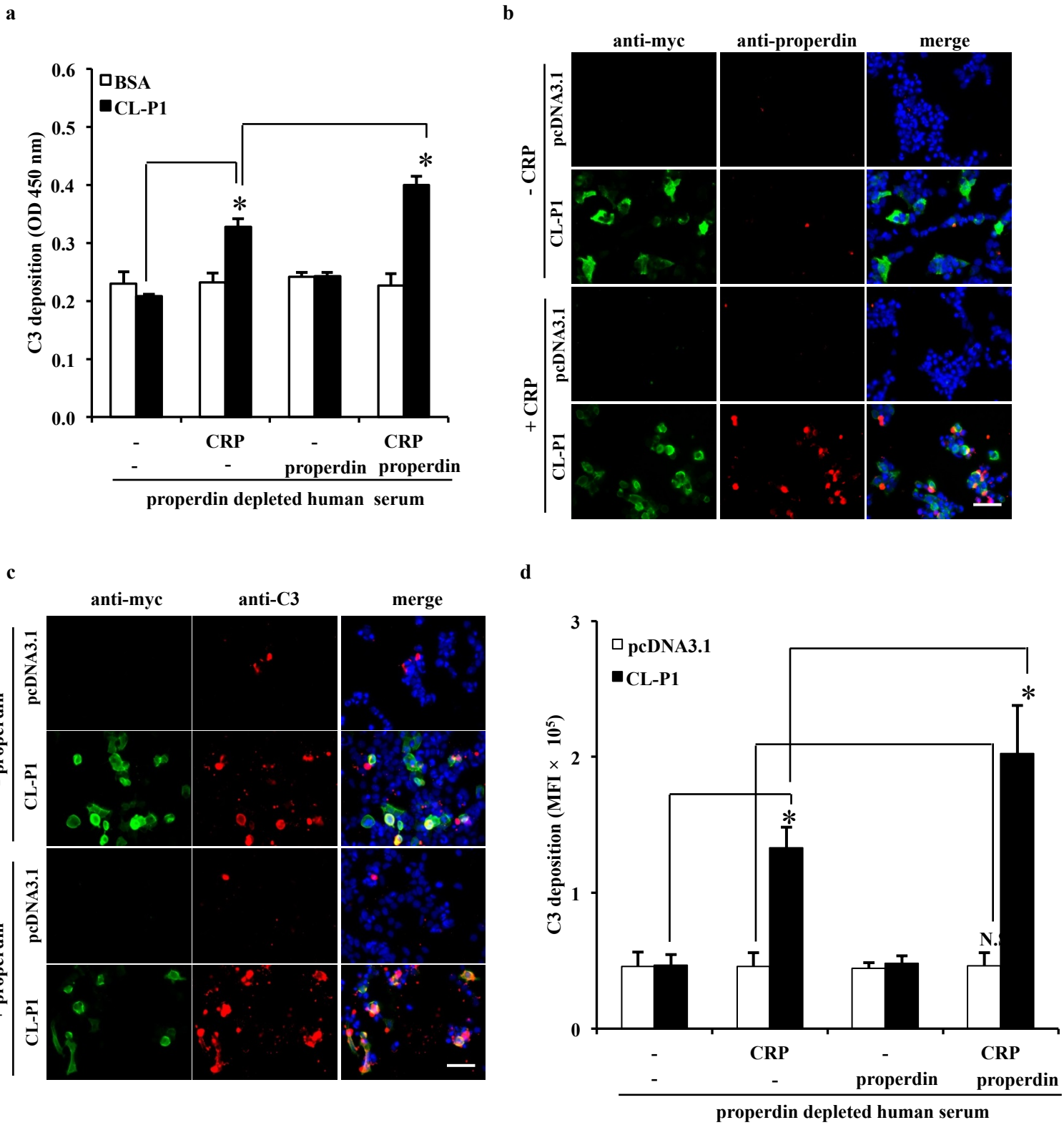

a
BSA
CL-P1
C3 deposition (OD 450 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
-
-
CRP
-
CRP
properdin
properdin
properdin depleted human serum
b
anti-myc
anti-properdin
merge
- CRP
+ CRP
pcDNA3.1
CL-P1
pcDNA3.1
CL-P1
c
anti-myc
anti-C3
merge
- properdin
+ properdin
pcDNA3.1
CL-P1
pcDNA3.1
CL-P1
d
pcDNA3.1
CL-P1
C3 deposition (MFI × 10^5)
3
2
1
0
*
*
N.S
-
-
CRP
-
CRP
properdin
properdin
properdin depleted human serum

Figure 7

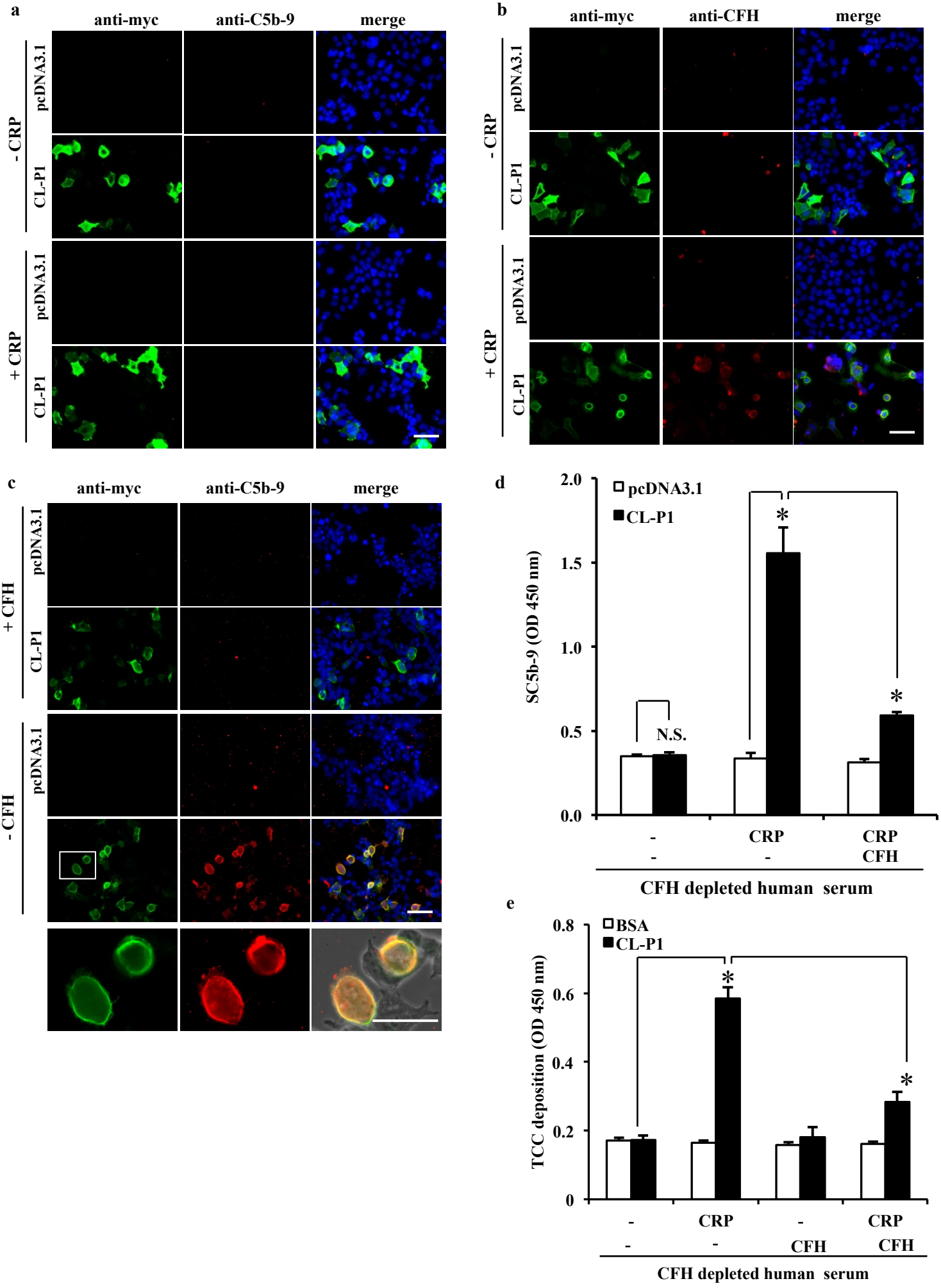
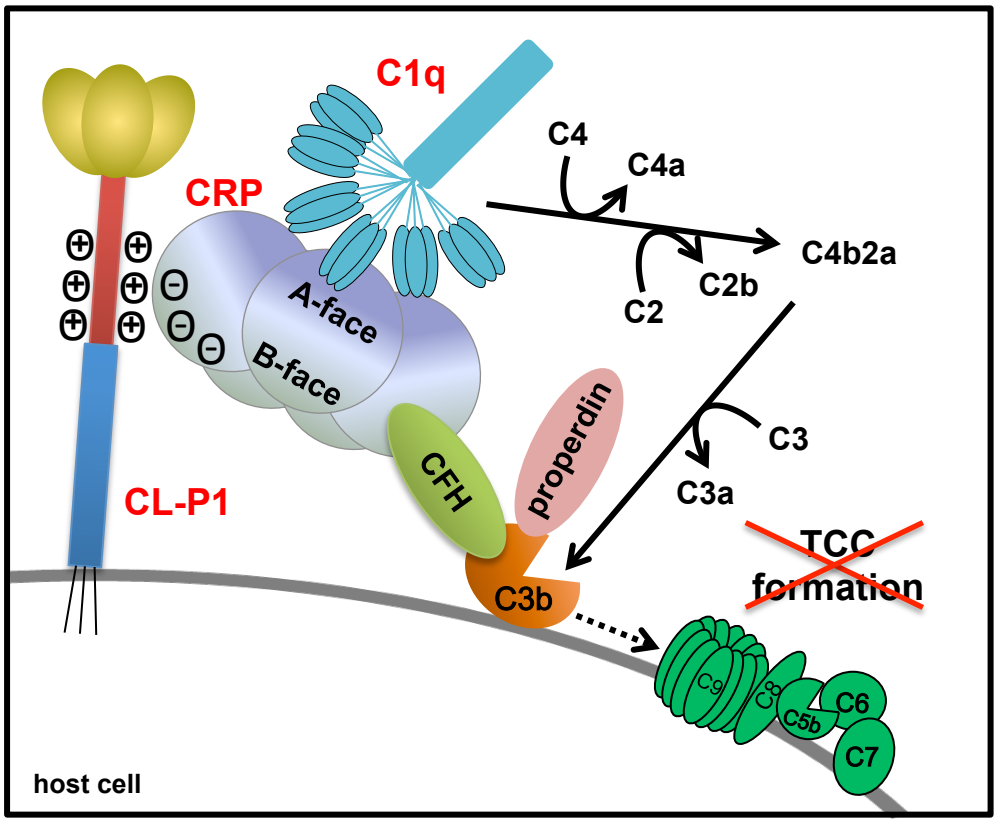

a
anti-myc
anti-C5b-9
merge
- CRP
+ CRP
pcDNA3.1
CL-P1
b
anti-myc
anti-CFH
merge
c
anti-myc
anti-C5b-9
merge
+ CFH
- CFH
d
pcDNA3.1
CL-P1
SC5b-9 (OD 450 nm)
2.0
1.5
1.0
0.5
0.0
N.S.
*
CRP
CFH
CFH depleted human serum
e
BSA
CL-P1
TCC deposition (OD 450 nm)
0.8
0.6
0.4
0.2
0
CFH depleted human serum

Figure 8


C1q
CRP
C4
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C2
C2b
A-face
B-face
C3
C3a
properdin
CFH
CL-P1
TCC
formation
C3b
C9
C8
C5b
C6
C7
host cell