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#### C4b-binding protein (C4BP) inhibits development of experimental arthritis in mice.

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Keywords: complement, rheumatoid arthritis, C4b-binding protein

**Abbreviations:** C4BP, C4b-binding protein; CVF, cobra venom factor; CII, collagen type II; CAIA, collagen antibody induced arthritis; CIA, collagen induced arthritis; CR1, complement receptor 1; mAb, monoclonal antibody;

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#### Abstract

**Objectives**: To assess the human complement inhibitor C4b-binding protein (C4BP) for treatment of arthritis.

**Methods**: We have used two mouse models of rheumatoid arthritis (RA) to assess the therapeutic effect of C4BP on different phases of arthritis, the collagen antibody induced arthritis (CAIA), an acute antibody induced disease and the collagen induced arthritis (CIA), which carries the full complexity of arthritis.

**Results**: Purified human C4BP injected intraperitoneally alleviated CAIA significantly in a manner similar to cobra venom factor that depletes complement due to massive activation. Furthermore, C4BP was injected before and after the disease development into CIA mice. In the former case, the disease onset was delayed and in the latter, the severity of the disease was reduced in animals treated with C4BP. However, C4BP did not affect the anti-CII antibody synthesis. C4BP present in mouse sera decreased activity of the classical but not the alternative pathway of the complement system when these were assessed in a fluid phase. However, C4BP was efficiently inhibiting the alternative pathway when present on the activating surface. Taken together, the disease ameliorating effect of C4BP appears to be related to inhibition of both pathways of complement.

**Conclusions**: Although human C4BP was cleared relatively fast from the circulation and was only moderately affecting complement activity, its effect on the disease severity was substantial, suggesting that minor alterations in complement activity can have significant therapeutic value in RA.

#### Introduction

Excessive or misguided activation of complement contributes to the pathogenesis of inflammatory diseases such as rheumatoid arthritis (RA). The complement cascade may proceed by the classical, alternative, and lectin pathways depending on the initiating molecule. Central to complement activation is the formation of enzymatic complexes such as C3- and C5-convertases, releasing chemoattractant anaphylatoxins C3a and C5a, which in turn induce production of cytokines and other proinflammatory mediators, and enhance vascular permeability (1). Potentially harmful complement is regulated by inhibitors such as C4b-binding protein (C4BP), a large plasma glycoprotein (570 kDa) that inhibits mainly the classical and lectin pathways (2). It hinders the assembly, as well as accelerates the natural decay, of the C3-convertase (3). In addition, C4BP serves as a cofactor to the plasma protease factor I in the degradation of C4b (4) and C3b so it can also affect the alternative pathway although not as strongly as factor H (5).

The pathogenesis of RA involves immune complexes within the synovial pannus tissue. The bound antibodies may activate complement along with necrotic cells (6) and released cartilage proteins such as fibromodulin (7). Indeed, consumption of complement and elevated levels of complement activation products were detected in synovial fluids of RA patients (8-11). C5a concentrations sufficient to induce neutrophil accumulation and leakage of plasma proteins from microvessels were detected in the synovial fluid of RA patients (8). C3b/iC3b activates complement receptors CR1/CR3 on synovial neutrophils and C3d acts as a molecular adjuvant by binding to collagen type II (CII) (12). The importance of complement for RA in animal models was first implied when rats decomplemented with cobra venom factor (CVF) were resistant to collagen–induced arthritis (CIA) (13). *Cia2* locus encoding C5 was identified to harbor a strong candidate gene for arthritis susceptibility and severity in mice (14-18). Genetic deletion of complement factors prevents arthritis in mice (19-21). Therefore, complement inhibition presents possibilities for developing novel therapeutic agents for arthritis.

CIA is the most commonly used animal model for RA and antibodies play an important role in the inflammatory phase of CIA. CII specific antibodies induce arthritis *in vivo* (22) and

collagen antibody induced arthritis (CAIA) is  $Fc\gamma R$  (23) and complement (21) but not B and T cells (24) dependent; neutrophils and macrophages are the major mediators of this inflammation (25). CAIA provides an opportunity to study the inflammatory phase without involving the priming phase of the immune response. In this report, we show the significant therapeutic potential of moderate complement inhibition by C4BP in both CAIA and CIA.

#### MATERIALS AND METHODS

#### Mice

B10.RIII mice (C5 sufficient; H-2<sup>r</sup>) were bred in the animal facilities of Lund University. The mice were kept in climate-controlled environment with 12-hour light/dark cycles, fed with standard rodent chow and water *ad libitum* (as defined at www.inflam.lu.se). Local animal welfare authorities at Lund University approved the animal experiments. Age-matched 3-4 months old male mice were used in the experiments.

#### **Purification of C4BP**

Human C4BP lacking anticoagulant protein S was purified from 10 liter pooled plasma obtained from local blood bank. Plasma was supplemented with 1 mM phenylmethylsulphonyl fluoride (Sigma Aldrich, Stockholm, Sweden; as other chemicals) and the C4BP-protein S complex along with other y-carboxyglutamic acid domain-containing proteins were removed by precipitation with BaCl<sub>2</sub>. BaCl<sub>2</sub> was added to a final concentration of 80 mM and incubated under stirring for 1h. Plasma was centrifuged for 30 min at 5000 g to remove the precipitate and applied at a flow of 90 ml/h on an affinity column with mAb 104 against C4BP coupled to Affi-Gel 10 at density of 2 mg/ml gel (2.6 cm x 12 cm; BioRad, Hercules, CA, USA) previously equilibrated with TBS (26). The column was washed with TBS, 1 M NaCl and C4BP, subsequently eluted with 3 M guanidinium chloride and dialyzed extensively against PBS. Concentration of C4BP was determined from measurement of absorbance at 280 nm and its purity evaluated to be at least 95% after separation by electrophoresis and silver staining. LPS concentration of C4BP preparation (4 mg/ml) was 2 ng/ml as determined by limulus test (Hycult, Uden, the Netherlands). C4BP was labeled with I<sup>125</sup> using chloramine T method. C4BP is normally present in human serum at approx. 0.2 mg/ml and becomes upregulated by 30-50% in inflammatory diseases (27).

#### Treatment of collagen induced arthritis (CIA)

For induction of arthritis, mice were immunized i.d. with 100  $\mu$ g of CII isolated from bovine nasal cartilage (28) emulsified 1:1 in complete Freund adjuvant (Difco/Becton Dickinson Franklin Lakes, NJ, USA) in a total volume of 100  $\mu$ l. In the preventive experiment C4BP (2 mg/mouse) was injected i.p. every second day from day -1 to +9 after injection of collagen while injections of CVF (12.5 U/mouse; Quidel, San Diego, CA, USA) were done on days -1 and +5 (i.p.). The third group received PBS injections at the same interval as C4BP. There were 12 animals in each group. In the therapeutic experiment, the mice were treated with PBS or C4BP (2 mg/mouse) on days 0 (day of onset), +2, +4 and +6 counting from the day of onset. CVF (12.5 U/mouse) was injected on days 0 and 5. Number of animals: C4BP treated; n=5, CVF treated, n=5 and untreated, n=10. The range of arthritis onset was days 16-26. Mice were observed for arthritis daily starting from day 14 and distributed into the all the treatment groups on any given day.

### Anti-CII antibody response

During CIA experiments, blood samples through retro-orbital plexus were taken using 75 mm capillary tubes (VWR International, Stockholm, Sweden). The amount of total anti-CII IgG in serum were determined through quantitative ELISA as described (29). Sera from non-immunized syngenic mice and pre-immune sera from the experimental animals were used as negative controls.

#### Collagen antibody induced arthritis (CAIA)

The two arthritogenic mAbs combination (M2139 ( $\gamma$ 2b) and CIIC1 ( $\gamma$ 2a)) described earlier (25) was used. Amount of endotoxin in the antibody solutions was 0.02 – 0.08 EU/mg of protein as analyzed by limulus test (30). The cocktail of the mAbs (9 milligrams in total per mouse) was injected i.v. into all the mice in 250-350 µl volume. On day 5, LPS (25 µg/mice; Sigma Aldrich) was injected i.p. into all the mice. Purified human C4BP (2 mg/mouse) was injected i.p. into 10 mice on day -1, +1, +3, +5 and +7 while injections of CVF (12.5 U/mouse) i.p. into 10 mice were done on days -1 and +5. The control group consisted of 20 animals.

#### **Clinical evaluation of arthritis**

Mice were examined daily for the arthritis development for a minimum of 17 days or until the inflammation subsided. Scoring of animals was done blindly using a scoring system based on the number of inflamed joints in each paw, inflammation being defined by swelling and redness as described (31). Scoring was recorded in the phalangeal joints (maximum of 1 point per digit, 5 points per paw), the metacarpus or metatarsus (5 points), and in the wrist and ankle joints (5 points). Thus, the maximum score was 15/paw resulting in a peak of 60 for the total joint count.

#### **Histological Preparations**

Paws were dissected on the indicated days from each group of mice (4 -5 mice per group), fixed in 4% phosphate buffered paraformaldehyde solution (pH 7.0) for 24 hours, decalcified for 3-4 weeks in an solution containing EDTA, polyvinylpyrrolidone and Tris-HCl, pH 6.95 followed by dehydration and embedding in paraffin. Joint sections (6  $\mu$ m) were stained with hematoxylineosin for detection of morphology.

#### **Complement assays**

Activity of the alternative pathway was studied as described with a hemolytic assay using rabbit erythrocytes (18) or with assay measuring deposition of C3b on zymosan (Sigma Aldrich) (32). Activity of the classical pathway was determined from C3 deposition on K562 cells (ATCC, LGC Promochem, Borås, Sweden) opsonized with rabbit polyclonal antibodies. The antibodies, purified by affinity chromatography on protein A Sepharose (GE Healthcare, Uppsala; Sweden), were kind gift of Dr Mike Holers and Dr Damian Kraus. K562 were cultured in suspension in DMEM supplemented with FCS, glutamine and antibiotics (all from Invitrogen, Carlsbad, CA, USA). The cells were washed twice in cold PBS in each condition,  $10^6$  cells were added to reaction tubes containing a PBS with 2 mM MgCl<sub>2</sub>, 0.15 mM CaCl<sub>2</sub>, 5 µg/ml of antibodies and 10% mouse sera in a total volume of 100 µl. After 30 min incubation at 37°C cells were washed with cold FACS buffer (1% FCS in PBS). The amount of deposited C3b was measured using FITC-conjugated goat anti-mouse C3 (ICN/MP Biomedicals, Illkirch, France) diluted 1:100 and allowed to bind for 1 h at 4°C. The particles were washed three times with cold FACS buffer and resuspended in 1% paraformaldehyde and analyzed by flow cytometry (FACS Calibur, Beckton Dickinson).

Effect of C4BP on the alternative pathway was also analyzed when C4BP was immobilized on the activating surface. Microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with 5  $\mu$ g/ml of zymosan, together with either 0-100 nM C4BP or 0-100 nM prothrombin, diluted in 75 mM sodium carbonate, pH 9.6. Each step was followed by extensive washing with 50 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl<sub>2</sub>. 0.1% Tween, pH 7.5.The wells were blocked using 200  $\mu$ l of 1% BSA in PBS. Mouse serum (5%) was diluted in Mg-EGTA buffer (2.5 mM veronal buffer pH 7.3, 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl<sub>2</sub> and 10 mM EGTA) and 50  $\mu$ l added to the plates for 1 h at 37°C, followed by incubation with a goat anti-mouse C3, diluted 1:750 in blocking solution. Horseradish peroxidase-labeled secondary antibodies against goat antibodies (Dako, Glostrup, Denmark; 1:2000 in blocking solution) were then added. Bound enzyme was assayed using 1,2-phenylenediamine dihydrochloride tablets (Dako) and the absorbance was measured at 490 nm.

#### Statistical analyses

The severity of arthritis was analyzed by Mann Whitney U test and the incidence by Chi Square test using the Statview 5.0.1 version. Significance was considered when P < 0.05.

#### RESULTS

#### Effect of C4BP on disease development in CAIA.

CAIA bypasses some of the complexity of the arthritis focusing on the effector phase (24). C4BP was injected starting on day -1 in two days intervals (Fig.1) and compared to CVF injected on days -1 and 5. Injection of C4BP significantly delayed onset of CAIA by several days and yielded significantly milder disease in those mice that were affected (Fig. 1). The effect was transient and subsided upon C4BP withdrawal. Similar effect regarding delay of disease development was observed for CVF but the effect was stronger as compared to C4BP as it leads to full decomplementation (Fig. 1A). On the other hand, the mean arthritic score of the mice that developed disease after cessation of CVF injections was the same as in control mice (Fig. 1B). Staining of joint tissues harvested on day 7 in the three groups of mice showed significant destruction of bone tissue and infiltration of neutrophils in control mice that were injected with mAbs (Fig. 2A). Joints of mice treated with C4BP (Fig. 2B) and CVF (Fig. 2C) were not distinguishable from the healthy control mice (Fig. 2D). Therefore, it appears that complement is an important contributor to the effector phase of arthritis and its inhibition, even partial, can ameliorate the disease.

We have estimated clearance rate of human  $I^{125}$ -C4BP in healthy mice after i.v. and i.p. injections. We found that the maximal concentration of C4BP in the blood after i.p. injection was reached after 4 h and that about 50% of the injected C4BP remained in the bloodstream ten hours after reaching the maximum similarly to i.v. injection (Fig 3A). When sample was taken on the same day C4BP was injected i.p. (day 3) there was 200 µg/ml of human C4BP in the serum as determined by ELISA specific for human C4BP(33) (Fig. 3B). When C4BP concentration was analyzed on day 7, i.e. two days after previous C4BP injection there was little C4BP left in circulation – about 3% (6 µg/ml) of the initial amount detected.

In order to estimate the effect of C4BP on the complement activity in treated mice we used specific assays distinguishing different pathways. The activity of the classical pathway was strongly decreased in mice treated with CVF (Fig. 3C). On day three there was also significant difference in the activity between the mice treated with PBS and C4BP (Fig. 3C). When the activity of the alternative pathway in solution was evaluated by two different assays, the significant difference was detected between the CVF treated mice and PBS treated controls (Fig.

3D and E). C4BP did not affect the alternative pathway in these settings. Interestingly the complement activity in mice treated with PBS was increased at day 7 as compared to day -1. This is probably due to the fact that many complement factors are acute phase reactants. When effect of C4BP on activation of the alternative pathway was evaluated with C4BP captured on activating surface rather than in solution we found that C4BP strongly inhibited the alternative pathway initiated by zymosan (Fig. 3F). We conclude that human C4BP suppress both pathways of mouse complement – the classical one when present is solution and the alternative one when present on activating surface.

#### Effect of C4BP injection on development of CIA (preventive experiment).

Next we wanted to investigate if C4BP would affect the complex CIA model. C4BP was injected into the mice treated with CII from day -1 to day +9 on alternate days. Another group of mice was treated with CVF on days -1 and +5. Control mice were treated with PBS injections at the same intervals as injections of C4BP. A delay in arthritis development was seen in the C4BP group (Fig. 4A and B). The incidence of arthritis in the CII and C4BP treated group was significantly lower on days 18, 20 and 22 when compared to animals treated with CII and PBS (Fig. 4A). The mean arthritis score was significantly lower in the C4BP treated group as compared to PBS animals on days 18, 20 and 26 of the experiment (Fig. 4C). CVF affected significantly the disease score and incidence only at day 26 at concentration and interval used. C4BP may interact with CD40 (34) and CD40L (35) and could affect antibody production. However, this was excluded as the levels of antibodies against CII were the same in all the experimental groups (Fig. 4D). Therefore, observed effect of C4BP on the disease development is most probably due to the effects on complement without operating through the adaptive immune system.

#### Effect of C4BP injection on development of CIA (therapeutic experiment).

In order to investigate if C4BP injection would also affect the already established disease, C4BP, CVF or PBS were injected into mice treated with CII and the injections initiated on a day on which a given animal started developing symptoms of arthritis (day of onset, labeled as day 0). The scheme of injections of C4BP and CVF is presented in Fig. 5A, PBS as a control was injected according to C4BP scheme. We found that C4BP significantly affected the disease score on the two days tested while CVF had a very strong effect that persisted for a week (Fig. 5A). Therefore, these results suggest that inhibition of complement is a viable therapeutic possibility in established disease. When activity of complement in sera taken on day 6 was measured we found that the classical pathway, but not the alternative one, was suppressed in the C4BP treated animals (Fig. 5B and C). CVF at concentrations used strongly inhibited both pathways (Fig. 5B, C and D).

#### DISCUSSION

Complement contributes to pathology of many inflammatory diseases and considerable efforts are invested nowadays in the development of pharmaceutical complement inhibitor. It is important to understand in detail contribution of complement to the development of RA and to evaluate if already a low level of complement inhibition would provide amelioration of the disease. Therefore, we investigated the effect of one of the inhibitors of complement (C4BP) on different phases of arthritis development using two widely used mouse models. We found that C4BP inhibits inflammatory phase of arthritis in CAIA model similar to that of CVF and has preventive and therapeutic value in CIA. CVF leads to massive activation of complement leading to depletion while presence of C4BP leaves complement intact only providing increased level of inhibition of C4b/C3b-mediated effects. It has been suggested that C4BP activates human B cells

through CD40 and thus may affect antibody synthesis (34). However, in the present study, we did not find any effect by human C4BP in anti-CII antibody synthesis in mice. This could be due to the fact that C4BP does not affect antibody-responses to T-cell dependent antigens despite interacting with CD40, that the injected level was not sufficient to observe the effect or that this system does not operate in mice. Although C4BP was cleared relatively fast from the circulation and affected the complement activity of mouse weakly, its effect on the disease severity was substantial suggesting that minor alterations in systemic complement activity can have significant therapeutic value.

It is still a matter of a debate, which complement pathway that is most crucial for the development of RA. We found that C4BP injections caused systemic decrease in activity of the classical pathway but no effect was detected when the alternative pathway was tested in two different assays. However, C4BP was able to efficiently inhibit the alternative pathway when present bound to the activating surface. Since C4BP binds strongly to apoptotic (36) and necrotic cells as well as DNA (6) one can expect that C4BP will be localized to the joint surfaces together with activators of the alternative pathway. Therefore C4BP may be exerting its therapeutic effect by inhibition of both pathways. C4BP has many ligands including C-reactive protein (37) and therefore it can not be excluded that its effect on arthritis is in part related to effects on other systems than complement. One can, however, exclude effect on coagulation as the C4BP used in this study was devoid of  $\beta$ -chain subunit and protein S.

In the current study we injected 2 mg/ml of C4BP per mouse, which doubled C4BP concentration after injection. Within 48 h, the majority of injected C4BP was cleared and next C4BP injection performed. Now we are planning to titrate the amount of injected C4BP in order to find the lowest effective dose. C4BP had more significant effect in the preventive CIA as compared to the therapeutic CIA experiment. This implies that complement is crucial in the initial phases of the disease.

It has been suggested previously that complement inhibitors can be used for treatment of RA. Gene therapy with sCR1 delayed the development and decreased severity in CIA (38). However, C3-convertase inhibitor Crry-Ig did not alter the course of CIA although its activity is essentially identical to that of sCR1. It is possible that the amount of administered Crry-Ig was not sufficient or treatment started too late (39). Complement inhibitors CD55 and CD59 were effective in amelioration of arthritis in a rat model (40). Systemic administration of anti-C5 antibodies ameliorated CIA (41). One important conclusion of our study is that even modest effect on complement activity results in measurable decrease in disease activity in two different models tested. Therefore, our results suggest that developing pharmaceutical complement inhibitors that would not abolish whole complement activity but just provided significant decrease could be tremendously useful by ameliorating the ongoing diseases processes in RA patients. Such inhibitor should carry less risk of unwanted side effects such as increased susceptibility to infections that could follow complete inhibitors in containing the hyperactivity of the complement and innate immune system pathways in human RA.

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Competing interests: the authors of this publications are currently authors on a submitted patent application.

## FIGURE LEGENDS

Fig. 1. Effect of complement inhibition via decomplementation with CVF or injection of C4BP in CAIA. Four months old B10.RIII mice were injected i.v. with a cocktail of two monoclonal antibodies (9 mg in total/mouse) binding to CII on day 0, followed by LPS (25  $\mu$ g/mouse/i.p.) on day 5. Purified human C4BP (2 mg/0.5 ml/mouse) was injected i.p. on days -1, +1, 3, 5 and 7 as indicated by thick arrows while injections of CVF (12.5 U/mouse) on days -1 and 5 are marked with thin arrows. The mice were scored every day and the graphs present A) incidence (maximal possible value is 100) and B) severity of arthritis (maximal possible value is 60). Error bars indicate ± SEM and n-denotes number of mice in each group. The severity of arthritis was analyzed by Mann Whitney U test and the incidence by Chi Square test \* p<0.05; \*\*\* p<0.01; \*\*\* p<0.005; \*\*\*\* p<0.001.

Fig. 2. Effect of complement inhibition in CAIA demonstrated in tissue histology. Another group of four months old B10.RIII mice were injected with antibodies and treated with CVF, C4BP or PBS similar to Fig.1. Seven days after the antibody transfer the mice were sacrificed and the joint morphology was assessed using standard hematoxylin-eosin staining protocol. Joint sections (6  $\mu$ m) from mice injected with mAbs (A), mAbs and C4BP (B), mAbs and CVF (C), PBS (healthy controls, D) were shown. Magnification x 10.

Fig. 3. Effect of injections of C4BP and CVF on complement activity of mouse sera. A) Clearance of <sup>125</sup>I-labelled C4BP injected i.p. or i.v. into mice. Blood samples were taken at indicated intervals and the amount of radioactivity was determined using  $\gamma$ -counter. Results are presented as mean of data obtained for two animals for each mode of injection. B) Sera were collected on indicated days and great care was taken in order to preserve complement activity. Determination of human C4BP level in the sera by ELISA. C) Activity of the classical pathway of complement measured as deposition of C3b on antibody-opsonized K562 cells and incubated with mouse sera for 30 min at 37°C. Deposited C3b was detected with FITC-labelled antibody labeled using flow cytometry. D) Activity of the alternative pathway of complement measured as deposition of C3b on zymosan particles incubated with mouse sera for 20 min at 37°C. Deposited C3b was detected with FITC-labelled antibody labeled using flow cytometry. E) Activity of the alternative pathway of complement measured in hemolytic assay in which rabbit erythrocytes were incubated with mouse sera for 3 h at 37°C. After the incubation the amount of hemoglobin released in supernatants due to lysis was determined spectrophotometrically. Absorbance of serum itself was subtracted and the values are shown as Z, which equals the natural negative logarithm of (1 - % lysis). F) C4BP inhibits efficiently the alternative pathway when bound to activating surface. C4BP and prothrombin were coated on microtiter plates together with zymosan. After incubation with mouse serum C3b deposited as result of activation of the alternative pathway was detected with specific antibodies. Statistical significance of observed differences was estimated with Student's T-test \* p<0.05; \*\* p<0.01; \*\*\* p<0.001.

Fig. 4. Effect of complement inhibition via decomplementation with CVF or C4BP in a preventive model of CIA. B10.RIII mice (3-4 months old) were immunized with 100  $\mu$ g of bovine CII in complete Freund adjuvant. Purified human C4BP (2 mg/mouse) was injected i.p. every second day from day -1 to +9 after injection of collagen while injections of CVF (12.5 U/mouse) were done on days -1 and +5. The third group received PBS injections at the same interval as for C4BP. The mice were scored every second day and the graphs present A) incidence of arthritis B) day of onset C) mean score of arthritis and D) levels of anti-CII antibodies determined in the sera of tested mice. Number of animals: C4BP treated; n=12, CVF treated, n=12 and untreated, n=12. Statistical significance of observed differences observed in B and C was estimated with Mann-Whitney U Rank test, \* p<0.05; \*\* p<0.01.

Fig. 5. Effect of complement inhibition via decomplementation with CVF or C4BP in a therapeutic model of CIA. Groups of B10.RIII mice (n=40) were immunized with 100  $\mu$ g of bovine CII in complete Freund adjuvant i.d. and received treatment starting from the day of onset. A) One group of mice were treated with C4BP, second group with CVF and the third group with PBS according to the scheme, thick arrows represent C4BP and PBS while thin arrows correspond to CVF. B) Activity of the classical pathway measured as deposition of C3b on sensitized K562 cells C) activity of the alternative pathway measured as deposition of C3b on zymosan D) activity of the alternative pathway measured in a hemolytic assay. Number of animals: C4BP treated; n=5, CVF treated, n=5 and PBS treated, n=10. Data in panels B-D were evaluated with Student's T-test: \*\*\* p<0.001.

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Figure 1

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Figure 2

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Figure 3

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Figure 4

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Figure 5