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Absence of a Neutralizing Antibody Response to Humanized Cobra Venom Factor in Mice.

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Footnote:
Abbreviations: CVF, cobra venom factor; nCVF, natural CVF as it occurs in cobra venom; rCVF, recombinantly produced CVF; hCVF, humanized CVF; huC3, human C3; muC3, murine C3.

Abstract

Cobra venom factor (CVF) is the complement-activating protein in cobra venom. Humanized CVF (hCVF) is a human C3 derivative where the C-terminal 168 amino acid residues were replaced with the homologous sequence from CVF. hCVF has been shown in multiple models of disease with complement pathology to be a promising therapeutic agent, with no observed adverse effects. Here we describe the antibody response to hCVF in two different strains of mice. hCVF was able to repeatedly decomplement the mice after four injections in weekly intervals, demonstrating the absence of a neutralizing antibody response. In contrast, natural CVF caused decomplementation in all mice only after the first administration. After two additional administrations of natural CVF, decomplementation was inconsistent and varied tremendously from mouse to mouse. After the fourth administration, natural CVF was essentially unable to deplete complement, consistent with the known generation of a neutralizing antibody response. We also analyzed the IgG antibody response to hCVF. There was great variation, with approximately one quarter of the mice exhibiting non-detectable levels of anti-hCVF IgG, and another quarter very low levels. The levels of anti-hCVF IgG did not correlate with the levels of remaining C3. The anti-hCVF antibodies cross-reacted with natural CVF, recombinant CVF, and human C3. Whereas overall the level of anti-hCVF IgG cross-reacting with human C3 was lower compared to rCVF or nCVF, mice with higher levels of anti-hCVF IgG exhibited higher binding to CVF and human C3, excluding the possibility that higher antibody levels reflect preferential immunogenicity of CVF-specific or human C3-specific epitopes.

Keywords

Cobra venom factor; humanized cobra venom factor; complement; complement depletion; antibody response

Introduction

Cobra venom factor (CVF) is the complement-activating protein in cobra venom. It is a structural and functional analog of complement component C3. When CVF is added to serum, it binds Factor B and leads to the formation of an alternative pathway C3/C5 convertase. The convertase (CVF,Bb) is physico-chemically stable, and resistant to inactivation by complement regulatory proteins Factors H and I, causing rapid cleavage of C3 and C5, and resulting in the depletion of the serum complement (reviews: Vogel and Fritzinger, 2010; Vogel and Fritzinger, 2017). The functional similarity of CVF to C3 is corroborated by its structure. Both proteins are synthesized as single-chain pro-proteins which are subsequently processed into the mature proteins with corresponding chain structures (Fig. 1). The sequence identity between CVF and mammalian C3 is about 50%, with a similarity of about 70% (Fritzinger et al., 1994). Both proteins exhibit a highly similar crystal structure with identical domains (Janssen et al., 2006, 2009; Krishnan et al., 2009).

Ever since it had been demonstrated that CVF can be safely administered to laboratory animals (Cochrane et al., 1970), the ability of CVF to exhaustively activate, and thereby deplete, complement has been exploited in innumerable studies as a tool to study the role of complement in host defense as well as pathogenesis of disease. As a matter of fact, our knowledge of the role of complement in the pathogenesis of many diseases was established by comparing normal animals with complement-depleted animals. As complement depletion by CVF eliminates the pathogenic contribution of complement to the disease process, complement depletion has more recently been recognized as a potentially valuable therapeutic approach in diseases with complement pathogenesis (Vogel and Fritzinger, 2007, Fritzinger et al., 2008; Vogel et al., 2014). However, CVF is immunogenic, generating an antibody response that limits its usefulness for complement depletion to a single injection (Cochrane et al., 1970, Pryjma and Humphrey, 1975). After identifying that the very C-terminal part of CVF harbors the important structures for forming a stable convertase (Grunwald et al., 1993; Hew et al., 2012), we attempted to overcome the limitation of immunogenicity by creating chimeric proteins in which C-terminal amino acid residues of human C3 were replaced by the homologous sequence from CVF. These chimeric proteins are human C3 derivatives, termed "humanized CVF" (hCVF), which exhibit rapid complement depletion *in vitro* and *in vivo* just like natural CVF (Fritzinger et al., 2009; Vogel and Fritzinger, 2010; Vogel et al., 2014). Our lead hCVF protein (termed HC3-1496) consists of human C3 in which the 168 C-terminal amino acid residues are homologous to CVF. HC3-1496 exhibits an overall protein sequence identity with human C3 of 94.3%; and even within the 168 C-terminal residues from CVF, 44.05% are identical to human C3 (Fig. 1).

hCVF has been shown in multiple preclinical disease models with complement pathogenesis to be a promising therapeutic agent. These include age-related macular degeneration (AMD) (Fritzinger et al, 2010), myocardial and gastrointestinal ischemia reperfusion injury (Gorsuch et al., 2009; Vogel et al., 2014), ventilator-induced lung injury (Takahashi et al., 2011), collagen-induced arthritis (Fritzinger et al., 2008), paroxysmal nocturnal hemoglobinuria (PNH) (Vogel and Fritzinger, 2010), myasthenia gravis (Vogel and Fritzinger, 2014), and monoclonal antibody therapy of lymphoma (Wang et al., 2009). In three disease models (AMD, arthritis, and myasthenia gravis), multiple injections of hCVF resulted in depletion for up to 30 days, suggesting the absence of a neutralizing antibody response to hCVF. This observation was recently extended in a mouse model of hemophilia A, aimed at reducing the anti-Factor VIII immune response to treatment with recombinant Factor VIII in Factor VIII knock-out mice. A significant reduction in the levels of induced anti-Factor VIII antibodies was achieved by four cycles of complement depletion with hCVF in weekly intervals (Rayes et al., 2018). Here we report that repeated injections of hCVF into Factor VIII knock-out mice, as well as wild-type mice, lead to repeated complement depletion; and although antibodies are generated against hCVF, cross-reacting with natural CVF, recombinant CVF, and human C3, they do not neutralize the complement-depleting activity of hCVF.

Material and methods

Materials. Restriction enzymes and calf intestinal phosphatase were from New England Biolabs (Beverly, MA). T4 DNA ligase was either from New England Biolabs, or from Invitrogen (Carlsbad, CA). Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Fastart TAQ DNA polymerase and Fastart High Fidelity DNA polymerase were obtained from Roche Applied Sciences, Inc (Indianapolis, IN). The *D. melanogaster* S2 expression plasmid, pMT/Bip-V5-HisA , all *Drosophila* S2 media, and fetal calf serum were from Invitrogen. Human complement protein C3 was purchased from Merck Millipore (Merck Chemicals Ltd, Nottingham, United Kingdom). Natural CVF was purified from lyophilized *Naja kaouthia* venom (Miami Venom Laboratories, Punta Gorda, Florida, USA) as described (Vogel and Müller-Eberhard, 1984). Recombinant CVF (rCVF) was produced in *D. melanogaster* S2 cells as described (Kock et al., 2004). rCVF is a mixture of C3-like and C3b-like proteins (Kock et al., 2004) (Fig.1). Polyclonal goat anti-mouse C3 antibodies were purchased from MP Biomedicals (Illkirch, France). Biotinylated polyclonal goat anti-mouse C3 antibodies and streptavidin-conjugated horseradish peroxidase (HRP) were from

R&D systems (Lille, France). HRP-coupled polyclonal goat anti-mouse IgG antibody was from Southern Biotech (Anaheim, CA).

Preparation of the plasmid expressing hCVF protein HC3-1496. This plasmid was prepared in a manner very similar to the method described earlier for the preparation of the plasmid expressing hCVF protein HC3-1348 (pMB-HC3-1348) (Fritzinger et al., 2009). Initially, two PCR reactions were performed to obtain the human C3 and CVF portions of the coding sequence, the first using pBS-HuC3(2) as a template and HuC3H5-3-F1 (TCTGTGTGGCAGACCCCTTCGAGG) and HuC3H5-4-R1(2) (GAGAAGGCCTGTTCCTTTATCCGGATGGTAGAACCGGGTAC) as primers, and the second using pCVF-FL3Δ as a template and HuC3H5-4-F2(2) (CCGGTTCTACCATCCGGATAAAGGAACAGGCCTTC) and HuC3H5-3-R2 (CATCCATGACATAGATATCATTACCATCTTG) as primers. Following the PCR reaction, the products were purified using the Qiagen PCR purification kit, and combined in an overlap extension PCR reaction, using the two PCR fragments as templates and HuC3H5-3-F1 and HuC3H5-3-R2 as primers. This PCR product was purified, cut with BstBI and gel purified as described above. It was then ligated into pHC3-1550(-sig) (Fritzinger et al. 2009) that had been BstBI cut and dephosphorylated with calf intestinal phosphatase. Orientation of the inserts was determined by EcoRI digestion, and clones with the inserts in the correct orientation were sequenced to ascertain the correct sequence with a lack of PCR-induced mutations. The resulting plasmid was called pHC3-1496. The HC3-1496 coding sequence was amplified by PCR. There were two amplifications, one to produce a fragment coding for HC3-1496 with the native human C3 signal sequence, and one coding for HC3-1496 with a mouse IgG signal sequence. For producing the coding sequence with the native C3 signal, the following primers were used:
PNAtf: 5'-gcaagcttGCCGCCACCATGGGACCCACCTCAGGTC-3' and Pnatr: 5'-ccgcggccgcTTAAGTAGGGCAGCCAAACTCAGTCAAT-3'. The primers used for producing the HC3-1496 with the mouse IgG signal sequence were Pmsp: 5'-gcaagcttGCCGCCACCATGGAGACCGACACACTGCTGCTGTGGGTGCT GCTGCTGTGGGTCCCCGGCTCCACTGGAAGTCCCATGTACTCTATCATCACC CCCAAC-3' and Pnatr: 5'-ccgcggccgcTTAAGTAGGGCAGCCAAACTCAGTCAAT-3'. The resulting PCR products were gel purified and isolated from the gel using the Qiagen QIAquick Gel Extraction Kit, and cut with HindIII and NotI. The fragments were then cloned into pOptiVEC-TOPO that had been cut with the same enzymes. The expression vectors were called pOptiVEC-1496(sig)-3# and pOptiVEC-1496-3# respectively.

Expression and purification of HC3-1496. pOptiVEC-1496(sig)-3# and pOptiVEC-1496-3# were transfected into CHO cells by electroporation. Following transfection, stably transfected cells were selected using either 200 nM or 500 nM methotrexate in liquid media. High-producing clones were then selected by plating between 2,500 and 10,000 cells onto semisolid media containing a fluorescent anti-CVF antibody in petri dishes. Colonies were allowed to grow for 10 days, and colonies producing HC3-1496 were detected, either by detecting fluorescent colonies, or colonies with a ring of immunoprecipitates. Colonies producing high yields of HC3-1496 were selected, and plaque purified. The HC3-1496 protein was purified by a combination of Capto-Q, Butyl FF HiTrap, and Q-HP HiTrap chromatography. Purified HC3-1496 is a mixture of C3-like and C3b-like proteins (Fritzinger et al., 2009).

Animals and *in vivo* complement depletion. Wild-type C57Bl/6 mice (Janvier, Saint-Berthevin, France) and Factor VIII exon 16-invalidated C57Bl/6 mice (gift from Prof. H.H. Kazazian, University of Pennsylvania School of Medicine, Philadelphia, PA, USA) (Bi et al., 1995) were handled at 8-12 weeks-old, in agreement with French ethical authorities (authorization #23BA53). Complement depletion was performed by intraperitoneal injections of nCVF or hCVF in mice (1 mg/kg, diluted in PBS), once a week for 4 consecutive weeks. Retro-orbital sampling was performed to collect blood once a week for four weeks three hours after each hCVF injection, and EDTA (50 mM) was added to the blood samples to prevent *ex vivo* complement activation. Plasma was isolated from the blood and kept at -80°C until use.

Measurement of circulating C3 in mouse plasma. Ninety-six-well flat-bottomed ELISA plates (Nunc Maxisorp®, Roskilde, Denmark) were coated with a polyclonal goat anti-mouse C3 antibody at 3.3 µg/ml in bicarbonate buffer, pH 9.5, overnight at 4°C. Wells were then blocked in PBS, 0.1% Tween-20 and 3% bovine serum albumin (BSA), pH 7.4. Plasma were serially diluted in blocking buffer and added to anti-C3 coated wells for 1 hour at 37°C. Bound C3 was revealed using a biotinylated polyclonal goat anti-mouse C3 antibody (1.6 µg/ml), followed by streptavidin conjugated to horseradish peroxidase using o-phenylenediamine dihydrochloride (OPD) as substrate. Absorbance was measured at 492 nm. Serially diluted normal plasma was used as standard and considered at 100% C3 level. The level of residual C3 in mice was expressed as a percentage of the value in normal plasma. Although cross-reactivity between CVF and antisera to mammalian C3 is very weak (Vogel et al., 1984), we ensured that the measurements of plasma C3 levels after injection with nCVF or hCVF were not altered by any potential cross-reaction (see Supplementary Fig.1).

Titration of anti-human C3, anti-rCVF, anti-hCVF, and anti-nCVF IgG. ELISA plates were coated with human C3 (hC3), hCVF, nCVF, and rCVF (10 nM) in bicarbonate buffer, pH 9.5, overnight at 4°C. After blocking with PBS, 0.1% Tween-20 and 3% BSA, pH 7.4, serially diluted plasma was incubated for 1 hour at 37°C. Bound IgG were revealed using a HRP-coupled polyclonal goat anti-mouse IgG antibody and the OPD substrate. Absorbance was then measured at 492 nm.

Statistical analysis. Statistical significance was assessed using the double-sided nonparametric Mann-Whitney test.

Results

Repeated administration of hCVF causes repeated depletion of complement *in vivo*. In order to assess the efficiency of hCVF for repeated complement depletion in mice, we compared complement C3 depletion following weekly injections of humanized (hCVF) or natural CVF (nCVF) in mice. This experiment was performed in Factor VIII knock-out mice, a preclinical model of hemophilia A, a pathology in which we have observed an important role of complement in the elicitation of anti-Factor VIII antibodies (Rayes et al., 2018). The first injection of either hCVF or nCVF resulted in rapid and drastic complement depletion in all animals as measured by the residual mouse C3 (muC3) levels in plasma. Indeed, circulating muC3 levels decreased from 100% to 6.4% [4.5-11.4%] (median with [interquartile range]) for nCVF (Fig. 2A) and to 19.5% [8.3-27.8%] for hCVF (Fig. 2B), with significant mouse-to-mouse variation. Figure 3 shows a time course of C3 depletion after a single injection of hCVF in Factor VIII knock-out mice, demonstrating that the absence of Factor VIII does neither affect the rapid depletion of C3 nor its return to normal levels after one to two days as shown previously for wild-type mice and other species (Vogel and Fritzinger, 2010). Subsequent weekly injections of hCVF consistently caused efficient complement depletion to residual levels of C3 of about 20% [14-27%], no different from what was seen after the first injection, even at week four (Fig. 2B). In contrast, second and third weekly administrations of nCVF failed to deplete complement in a consistent manner, with resulting C3 levels varying greatly between normal and essentially fully depleted (Fig. 2A); and only a fourth weekly administration rendered nCVF essentially inactive (Fig. 2A). These results strongly suggest that repeated administration of hCVF, in contrast to nCVF, does not elicit a neutralizing antibody response.

Repeated administration of hCVF induces a non-neutralizing humoral immune response in mice. To exclude the remote possibility that the absence of Factor VIII affected the antibody response to hCVF, we repeated the four weekly injection schedule with hCVF in C57Bl/6 wild-type mice. As previously observed in Factor VIII-deficient mice, injections of hCVF in wild-type mice resulted in a significant reduction of circulating C3 level: 43.9% [30.0-53.4] (Fig. 4A), again with significant mouse-to-mouse variation. After four weeks, we also determined the presence of anti-hCVF IgG antibodies. Mice receiving hCVF treatment developed anti-hCVF antibodies, as compared to the PBS control group (0.236 [0.115-0.507] AU vs 0.052 [0.049-0.056] AU, respectively) (Fig. 4B). However, there was significant variation from mouse to mouse, with approximately one quarter of the animals exhibiting essentially non-detectable antibody levels, and another quarter very low levels (Fig. 4B).

Moreover, the anti-hCVF IgG levels in individual mice did not correlate with the levels of remaining muC3 (r^2=0.0440, p=0.3750) (Fig. 4C). These results suggest that hCVF exhibits an overall low degree of immunogenicity in mice, and that anti-hCVF IgG produced in mice lack neutralizing activity toward hCVF.

Anti-hCVF antibodies recognize antigenic epitopes on both CVF and human C3. hCVF protein HC3-1496 is a human C3 derivative with nCVF-like functions, obtained by replacing a short region of the C-terminus of the human C3 α-chain with the homologous sequence from nCVF (Fig.1). The protein sequence of the chimeric single-chain pro-protein HC3-1496 is 94.3% identical to human C3; this includes the 168 residues long CVF portion of the protein where 44.05% of the residues are identical to human C3. hCVF protein HC3-1496, like other hCVF proteins (Fritzinger et al., 2009) and recombinant CVF (rCVF) (Kock et al., 2004), is usually expressed as a mixture of a C3-like and C3b-like form (Fig.1). In order to identify the immunogenic regions of hCVF in mice, we assessed the cross-reactivity of the anti-hCVF antibodies with nCVF, rCVF, and human C3 (huC3). The anti-hCVF antibodies recognized rCVF and nCVF similarly to hCVF (Fig. 5A, B). Anti-hCVF antibodies also bound to huC3, although many mice had essentially non-detectable levels of anti-hCVF antibodies cross-reacting with huC3 (Fig. 5C). The mean level of anti-hCVF antibodies cross-reacting with huC3 was significantly lower compared to hCVF, albeit only at a *p*-value of 0.02.

There was a highly significant correlation when the levels of anti-hCVF were plotted against the levels of anti-nCVF, anti-rCVF, and anti-huC3 antibodies ($p<0.0001$ in each case; Fig. 5D, E, F), indicating that mice with higher overall levels of anti-hCVF antibodies also showed increased binding to both CVF and huC3 epitopes, excluding the possibility that the higher antibody levels reflect preferential immunogenicity of CVF-specific or huC3-specific epitopes.

As shown above for anti-hCVF (Fig 4C), the levels of anti-nCVF, anti-rCVF, and anti-huC3 did not display any significant correlation with the levels of remaining circulating C3 in individual mice after the fourth injection with hCVF (Fig. 5G, H, I). This observation is consistent with the fact that the antibodies elicited against hCVF do not interfere with its C3-depleting activity, and are devoid of a neutralizing function toward hCVF.

Discussion

Humanized CVF (hCVF) is a term coined for human C3 derivatives where the C-terminal portion of the C3 α-chain has been exchanged with the homologous sequence from CVF, thereby introducing the CVF-specific functions of forming a physico-chemically stable C3 convertase, and exhibiting resistance to inactivation by the regulatory proteins Factors H and I. These two properties of hCVF, like natural CVF from cobra venom, are a prerequisite for continuous C3 activation, leading to complement depletion both *in vitro* and in *vivo* (Fritzinger et al., 2009; Vogel et a., 2014; Vogel and Fritzinger, 2017). hCVF was developed as a biopharmaceutic for therapeutic complement depletion in diseases where the complement system is involved in the disease pathogenicity. The efficacy of complement depletion with hCVF was subsequently demonstrated in multiple animal models of disease (Vogel et al., 2014; Rayes et al., 2018).

CVF has been used for well over four and a half decades for safe complement depletion in laboratory animals, from mice to primates. The sole adverse effect known is an acute but fleeting pulmonitis (Till et al., 1987; Mulligan et al., 1996), observed only after massive complement activation in plasma by nCVF. The nCVF-dependent convertase not only cleaves C3 but also C5. The resulting massive generation of C5a overwhelms the body's protective mechanisms, leading to C5a-mediated neutrophil activation with subsequent sequestration to the lungs and causing pulmonitis. In contrast to nCVF, the convertase formed with hCVF does not cleave C5 (Vogel and Fritzinger, 2010; Vogel et al., 2014). Accordingly, complement depletion with hCVF does not generate C5a; and even intra-arterial injection into the pulmonary artery of cynomolgus monkeys had no effect on pulmonary function. Consistent with the decades-long experience of safe complement depletion with nCVF, and the

absence of C5a generation by hCVF, no adverse effects of complement depletion with hCVF were seen in any of the animal models of disease (Vogel et al., 2014).

In addition to efficacy and safety, immunogenicity is an important property of any biotherapeutic. We found that hCVF efficiently depletes complement in mice after each of four weekly administrations. In contrast, nCVF resulted in consistent and efficient complement depletion only after the first administration. Subsequent injections over two weeks resulted in inconsistent complement depletion, with significant variation from mouse to mouse. A fourth injection rendered nCVF essentially inactive. These results confirm earlier studies that demonstrated that CVF is a potent immunogen, eliciting neutralizing antibodies (Cochrane et al., 1970; Pryjma and Humphrey, 1974), and that nCVF is a reliable reagent for complement depletion in nCVF naïve animals only. Our finding that hCVF exhibits effective complement depletion after repeated administration indicates that hCVF does not induce a neutralizing antibody response, at least after multiple injections over a four-week period.

There is always a significant variation from animal to animal, both with regard to the initial complement levels (Fig. 4A) and the extent of complement depletion (Figs. 2B, 3, 4A). We have no experimental evidence, or any explanation, why complement depletion with nCVF would differ from hCVF, and that the absence of Factor VIII would affect the complement depletion by nCVF or hCVF. Although means and interquartile ranges were somewhat different, 25% of the remaining C3 levels in wild-type mice after depletion with hCVF (Fig. 4A) were at the same level of Factor VIII knock-out mice (Fig. 2B), and that many Factor VIII knock-out mice after depletion with hCVF exhibited similarly low remaining C3 levels as seen after depletion with nCVF (compare Figs. 2B and 3 with Fig. 2A).

We analyzed the antibody response to hCVF after four weekly administrations. We detected anti-hCVF IgG antibodies in the murine plasma. However, the overall antigenicity of hCVF appears to be low. There was significant variation in the antibody levels of individual mice, with approximately one quarter of the animals exhibiting essentially non-detectable antibody levels, and another quarter very low levels. The anti-hCVF antibodies cross-reacted with nCVF and rCVF as well as huC3, indicating that both CVF-specific epitopes and huC3-specific epitopes of hCVF contribute to the immunogenicity of hCVF in mice. Our data do not allow to locate antigenic epitopes on the hCVF protein. Based on primary protein sequence differences to murine C3, there are CVF-specific residues in the human C3 portion of hCVF, and huC3-specific residues in the nCVF-portion of hCVF. However, the number of mouse plasma with very low to non-detectable antibodies cross-reacting with human C3 was much lower compared to plasma cross-reacting with rCVF and nCVF, suggesting that CVF-specific sequence differences are more immunogenic despite the fact that there are significantly more huC3-specific sequence differences. On the other hand, we found that mice with higher overall levels of anti-hCVF antibodies also showed increased binding to both CVF and huC3 epitopes, thereby excluding the possibility that the higher antibody levels reflect preferential immunogenicity of CVF-specific or huC3-specific epitopes.

Although mice mount an antibody response to hCVF, our data demonstrate that these antibodies are not neutralizing the complement-depleting activity of hCVF. This is further supported by the observation that there was no correlation between anti-hCVF antibody levels and remaining C3 levels in complement-depleted mice. There are likely several explanations for the low immunogenicity of hCVF and the lack of neutralizing antibodies. For one, the protein sequence of HC3-1496 is highly homologous to murine C3 (approximately 78% identity in the large human C3 portion, and approximately 43% identity within the C-terminal 168 residues). Moreover, hCVF, just like nCVF and the C3 proteins of all mammals, must contain the identical protein structures required for binding to Factor B and forming a convertase. These include the C345C domain at the C-terminus of C3 which binds the Bb portion of Factor B, and other domains of C3, in particular the the CUB^{g} domain, which is required for the initial contact with the Ba portion of Factor B, first described by us (O'Keefe et al., 1988), and subsequently confirmed by crystallography of the CVF,B complex (Janssen et al., 2009). These structures of C3 are therefore highly conserved throughout the mammals (and likely all

vertebrates), and consequently not immunogenic. Lastly, just like complement depletion with hCVF reduces the antibody response to Factor VIII (Rayes et al., 2018), the hCVF-induced complement depletion may aid in lowering its own immunogenicity.

Our results were obtained by injecting hCVF into mice. It may therefore not be unreasonable to speculate that hCVF might be even less immunogenic in humans as the total number of different amino acids between HC3-1496 and murine C3 will be reduced from approximately 400 to less than 100 residues between HC3-1496 and human C3, all within the 168 amino acid long C-terminal sequence only. Moreover, the three-dimensional structure of the C-terminal sequence has been predicted by *in silico* analysis to be essentially identical between human C3 and hCVF (Vogel et al., 2008; Vogel et al., 2014). However, immunogenicity is not only determined by primary structure differences, and complement depletion by hCVF in itself may affect an antibody response differently in humans than in mice. Ultimately, the degree of immunogenicity of hCVF in humans is unpredictable, and will have to await clinical trials.

In conclusion, hCVF is a promising therapeutic agent for the many diseases with complement pathogenesis. It has been shown to be efficacious in multiple animal disease models, it has no known adverse effects, and as shown here, it does not induce neutralizing antibodies in mice, at least not after multiple injections over a one-month period. The effect of longer-term complement depletion with hCVF on the risk of developing infections is unknown. However, complement depletion with hCVF in never complete. There is always a significant amount of C3 remaining, along with normal levels of C5 and the other terminal complement components, ensuring some protection against infections. This is convincingly demonstrated by CVF-transgenic mice which exhibit a normal life span and, in contrast to C3 knock-out mice, no tendency to develop infections (Andrä et al., 2002; Fritzinger et al., 2010).

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Authors' contributions

Designed research: MI, SLD, CWV, JR
Performed experiments: MI, BEH, DCF, SD, JR
Analyzed data: MI, SLD, CWV, JR
Wrote the paper: MI, SLD, CWV, JR

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Figure Legends

Figure 1: Schematic representation of the chain structures of human C3, hCVF (HC3-1496), and CVF. Shown are the single-chain structures of pro-C3, pro-HC3-1496, and pro-CVF. The N-termini are to the left. The chain homologies are indicated. Please note that the nomenclature of the three chains of nCVF (α-chain, β-chain, γ-chain) was coined before their homology to C3 chains was established by N-terminal sequencing (Eggertsen et al., 1981), immunologic cross-reaction (Eggertsen et al., 1983), and molecular cloning (Fritzinger et al., 1994). Both hCVF and rCVF are usually expressed as a mixture of C3-like and C3b-like proteins (Fritzinger et al., 2009; Kock et al., 2004).

Please note that rCVF is fully active without processing into the three-chain structure of nCVF (Kock et al., 2004).

Figure 2. Repeated injection of hCVF, but not natural CVF, causes repeated complement depletion in mice. Factor VIII-deficient mice were injected intraperitoneally with (*Panel A*) nCVF (n=8) or (*Panel B*) hCVF (HC3-1496, n=24) at a dose of 1 mg/kg, once a week for 4 consecutive weeks. Blood was collected before (Week 0) and weekly 3 hours post-CVF injection (Weeks 1-4). Residual circulating mouse C3 (muC3) level was measured by sandwich ELISA. Results are expressed as a percentage of circulating C3 in normal plasma, measured in mice before depletion and considered at 100%. Horizontal bars represent the median of each time point.

Figure 3. Time course of C3 depletion with hCVF in Factor VIII knock-out mice. Factor VIII knock-out mice were injected intraperitoneally with 20 μg hCVF. Blood was collected at different time points as indicated, and C3 levels were measured in plasma by sandwich ELISA.

Figure 4. Repeated administration of hCVF is associated with a non-neutralizing humoral immune response. Wild-type mice were injected intraperitoneally with PBS (n=20) or HC3-1496 (hCVF, n=20) at a dose of 1 mg/kg, once a week for 4 consecutive weeks. Four weeks following the first injection, blood was collected and residual mouse C3 (muC3) and anti-hCVF antibody levels were measured in the plasma. **Panel A**. Residual muC3 levels in plasma was measured by ELISA. Results are expressed as a percentage compared to a normal C3 plasma level, considered at 100%. Horizontal bars represent the median for each group. **Panel B**. Anti-hCVF IgG levels in plasma were assessed by ELISA. Results are expressed in optical densities measured at 492 nm (arbitrary units, AU). Horizontal bars represent the median for each group. **Panel C**. Correlation between the level of anti-hCVF IgG and the residual percentage of circulating muC3 in mice treated with hCVF. The linear regression curve is represented as a full line curve, and the *p* and r^2 values are indicated.

Figure 5. Antibodies against hCVF recognize both CVF- and human C3-specific epitopes. Wild-type mice were injected intraperitoneally with PBS (n=20) or HC3-1496 (hCVF, n=20) once a week for four consecutive weeks, and blood was collected four weeks following the first injection. **Panels A, B, C**. Levels of anti-natural CVF (nCVF, *Panel A*), anti-recombinant CVF (rCVF, *Panel B*), and anti-human C3 (huC3, *Panel C*) IgG in the plasma were assessed by ELISA. Results are expressed as optical densities measured at 492 nm (arbitrary units, AU). Horizontal bars represent the median for each group. **Panels D, E, F**. Correlations between the levels of anti-nCVF (*Panel D*), anti-rCVF (*Panel E*), and anti-huC3 (*Panel F*) IgG compared to the level of anti-hCVF IgG are shown. The linear regression curves are represented as full line curves, and the corresponding *p* and r^2 values are indicated. **Panels G, H, I**. Correlations between the levels of anti-nCVF (*Panel G*), anti-rCVF (*Panel H*), and anti-huC3 (*Panel I*) IgG and the residual circulating C3 levels in hCVF-treated mice are shown. The linear regression curves are represented as full line curves, and the corresponding *p* and r^2 values are indicated.

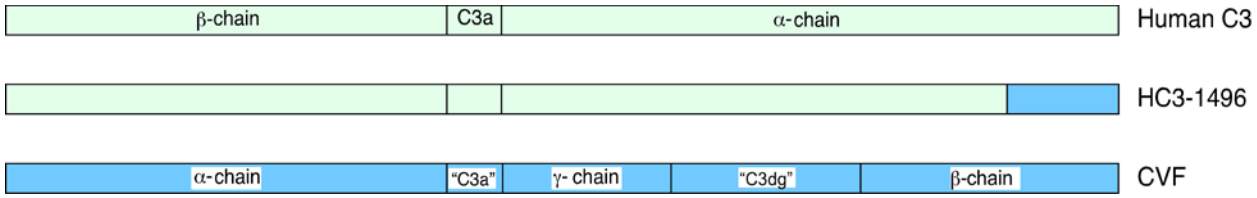
Supplementary Material

To exclude the possibility that the measurements of C3 levels in mice after injection with nCVF or hCVF by the sandwich ELISA assay were falsely overestimated by any cross-reaction of nCVF or hCVF with the anti-muC3antibodies used in the assay, we assessed the possible cross-reaction. Supplemental Fig. 1 shows that neither nCVF, rCVF, nor hCVF of up to 150 μg/ml cross-reacts with the anti-muC3 antibodies employed in the assay. Given that mice (of approximately 20 g body weight) were injected with 20 μg hCVF or nCVF, 150 μg/ml is at least one order of magnitude above the potential peak plasma concentration. We had previously shown that the peak plasma concentration in mice after injection of 10 μg hCVF was 1.5 μg/ml, two orders of magnitude below 150 μg/ml.

Figure Legend

Supplemental Figure 1: Lack of cross-reactivity of nCVF, rCVF, and hCVF with anti-muC3. The sandwich ELISA assay for murine C3 was performed with serial 1:10 dilutions of nCVF (*), rCVF (▼), and hCVF (Δ), starting at 150 μg/ml. Mouse plasma from Factor VIII knock-out mice at a 1:10 dilution (with an estimated concentration of C3 of 150 μg/ml) and serial 1:10 dilutions served as positive control (●). Results are expressed in optical densities measured (arbitrary units, AU).

Figure 1


β-chain
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α-chain
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γ- chain
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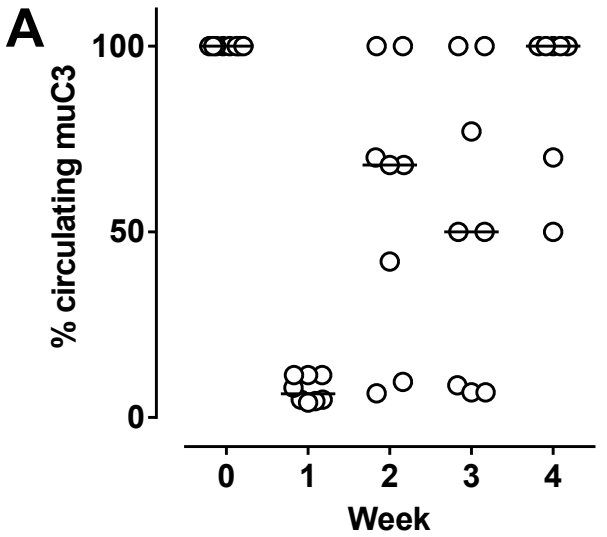
β-chain
CVF

Figure 2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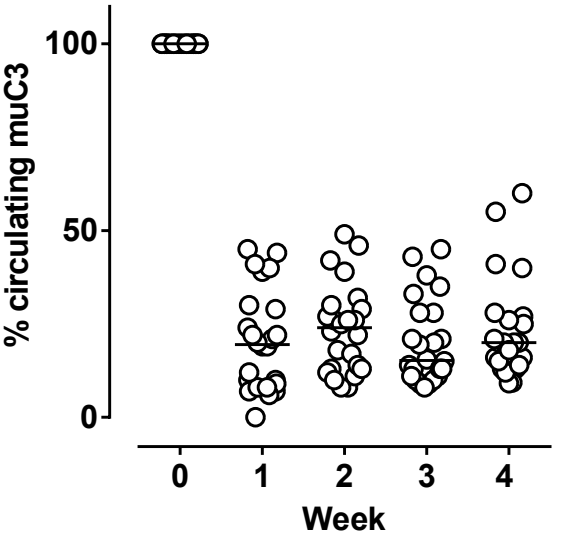
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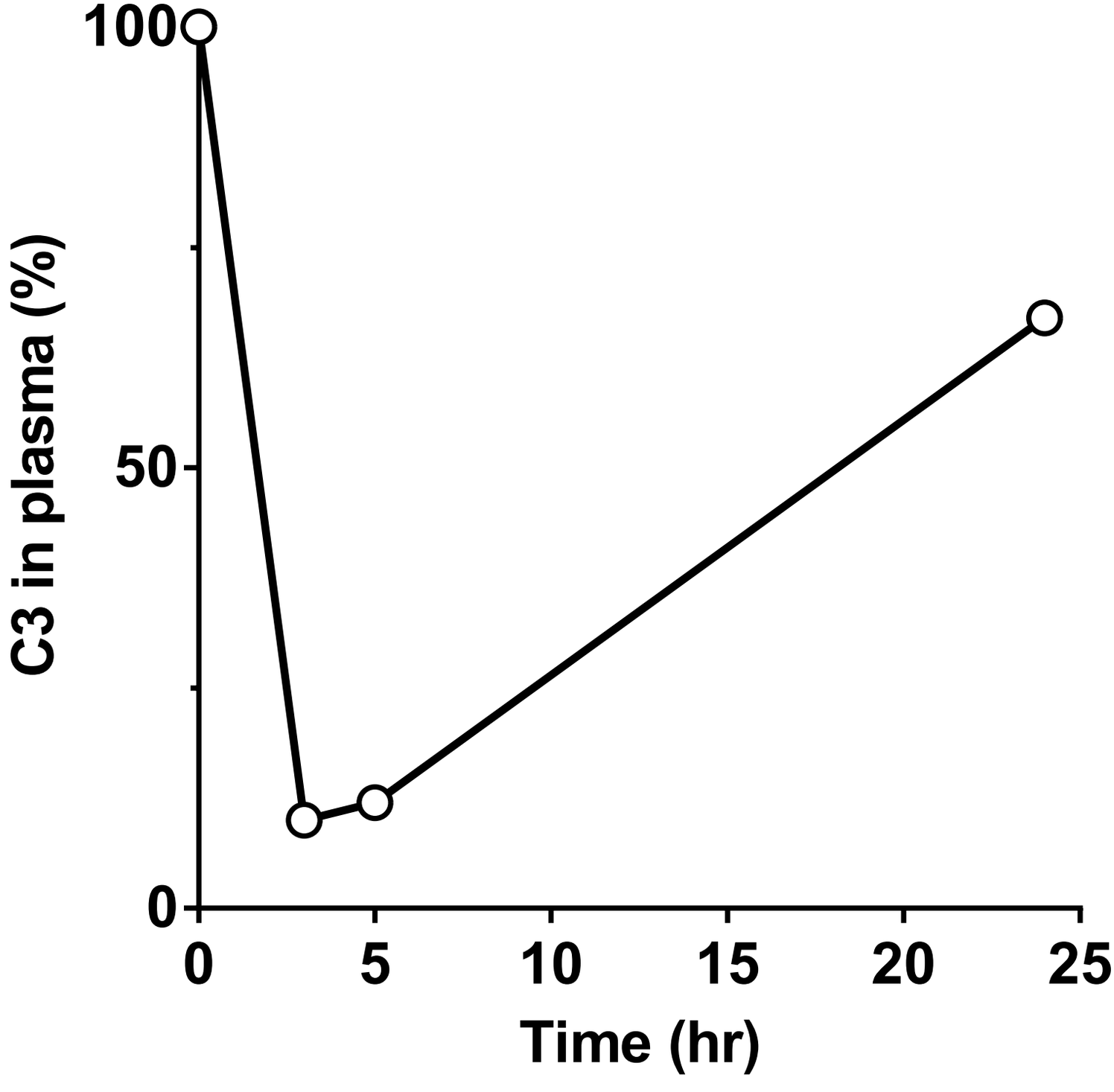
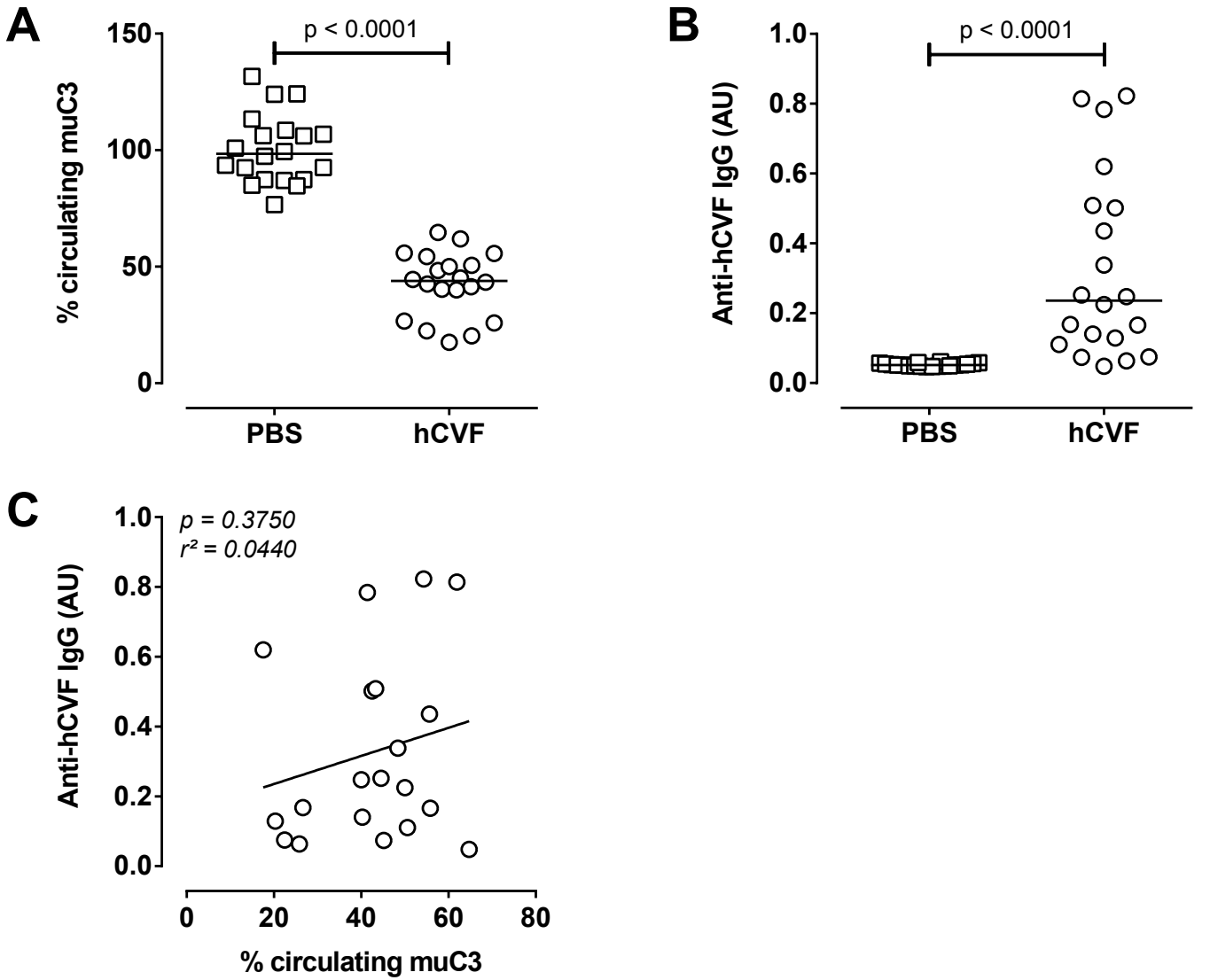
Figure

Figure 3



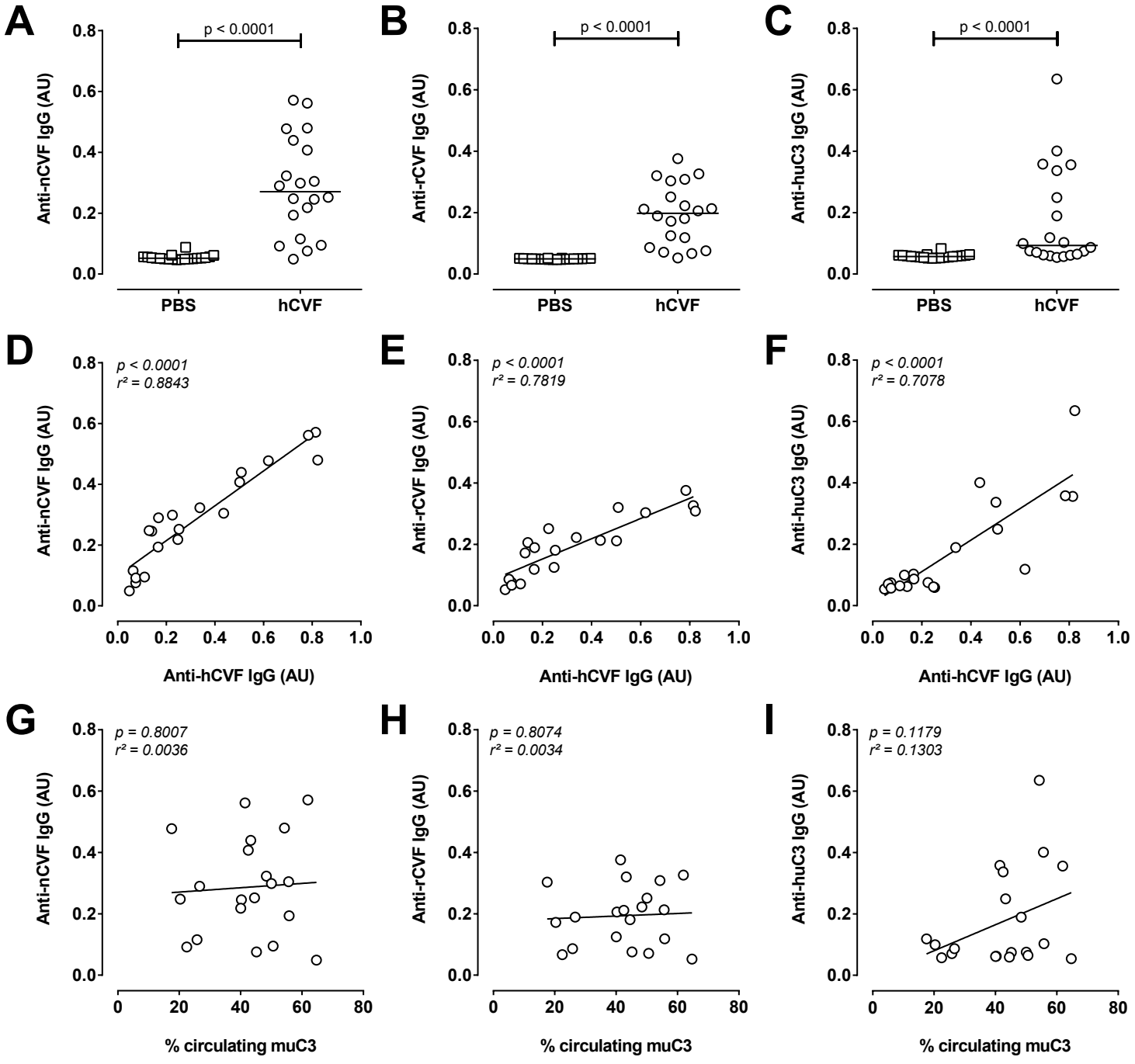
Figure

Figure 4



Figure

Figure 5



Figure

Supplementary Figure 1