C2 by-pass: cross-talk between the complement classical and alternative pathways

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Abstract

Several disorders associated with the total or partial absence of components of the human complement system are known. Deficiencies of classical pathway (CP) components are generally linked to systemic lupus erythematosus (SLE) or SLE-like syndromes. However, only approximately one-third of patients who lack C2 show mild symptoms of SLE. The relatively high frequency of homozygous C2 deficiency without or with minor disease manifestation suggests that there might be a compensatory mechanism which allows the activation of the CP of complement without the absolute requirement of C2. In this study we show that factor B (FB), the C2 homologue of the alternative pathway (AP) of complement, can substitute for C2. This was confirmed by using C4b as immobilised ligand and FB as analyte in Surface Plasmon Resonance (BIACORE). C2 binding to the immobilised C3b-like molecule $C3(CH_3NH_2)$ was not seen. The estimated binding constant for C4bB complex formation was $2.00 * 10^{-5}$ [M]. We were further able to demonstrate that C4b supports the cleavage of Factor B by Factor D. Finally, cleavage of ^{125}I-C3 by C4bBb was evaluated and gave strong evidence that the "hybrid" convertase C4bBb can cleave and activate C3 *in vitro*. Cleavage activity is very low, but consistent with some of the "C2-bypass" observations of others.

1. Introduction

The complement system is a very important component of the innate (non-adaptive) immune system and can respond to challenges by micro-organisms before an adaptive immune response has developed. Proteins of the complement system detect targets via three means of activation and bind to them. These three routes are named the classical- (CP) (Porter and Reid, 1979), the alternative- (AP) (Pryzdial and Isenman, 1987) and the lectin-pathway (LP) (Collard, et al., 2000, Matsushita and Fujita, 1992) (Fig. 1). Binding of early components of the complement system to the targets results in the formation of unstable protease complexes termed the C3 convertases (Kerr, 1980). The C3 convertases of the LP and the CP (C4b2a) and the AP (C3bBb) are each made up of two components. When one component, C4b or C3b, respectively, becomes covalently attached to the surface of a complement activator, the other component, C2 or factor B, binds in a Mg^{2+}-dependent reaction to form an initial C4bC2 or C3bFB complex. C2 and FB have similar morphology and function (Bentley, 1986) and are both single-chain polypeptides with molecular weights of 100 kDa and 90 kDa respectively. The weight difference is due to glycosylation variants but both components consist of three types of globular domains (Bentley, 1986). The N-terminal domains consist of three complement control protein modules (CCP1, CCP2 and CCP3) and the middle region contains a type A domain, similar to those found in von Willebrand factor. The C-terminal end of C2 and FB is a serine protease (SP) domain (Volanakis and Arlaud, 1998). To become proteolytically active against soluble C3, complexed C2 and FB have to be cleaved and activated by C1s or factor D (FD), respectively. Cleavage of C2 by C1s or FB by FD occurs in the N-terminal region of the vWF-domain, resulting in the generation of C2a (70 kDa) + C2b (30 kDa) or of Bb (60 kDa) + Ba (30 kDa) fragments, respectively. The smaller fragments drift away from the site of activation, leaving behind the active CP-C3 convertase (C4b2a) or AP-C3 convertase (C3bBb) attached to the complement activator. All complement pathways, at one point lead to the turnover of C3, the most abundant complement component

and its breakdown into C3a and C3b. The major fragment C3b binds covalently to complement-activating surfaces (e.g. cells, viruses, immune complexes) and enhances phagocytosis (Merle, et al., 2015). If the complement activator has a lipid bilayer, lysis can also occur through interaction with the membrane and components C5, C6, C7, C8 and C9 which bind together to form the membrane attack complex (MAC). During assembly, the MAC associates with the lipid membranes and may cause lysis and death of the target cell.

It is suggested that the complement system is constantly activated at a low level and hence constant control is required to regulate the system. Absence of such regulatory components (C1-inhibitor, factors I and H, C4-binding protein, decay acceleration factor) is linked to uncontrolled activation of the complement cascade. Various diseases linked to the lack of appropriate regulators, or malfunction and deficiencies of soluble components of complement have been described in recent years (Lachmann, 1974, Tappeiner, 1982, Ross and Densen, 1984, Perlmutter and Colten, 1989, Atkinson, 1989, Walport and Lachmann, 1990, Densen, 1991, Whaley and Schwaeble, 1997, Frank, 2000, Botto, et al., 2009, Schröder-Braunstein and Kirschfink, 2019).

In general, deficiencies of the components which participate in the classical pathway of the complement system are associated with systemic lupus erythematosus (SLE) or SLE-like syndromes (Atkinson, 1989). C2 deficiency is also linked to severe infections caused by encapsulated bacteria (Sjoholm, 1990) and infections with *Streptococcus pneumoniae*, *Neisseria meningitidis* or *Haemophilus influenzae* have been reported from 1 in 3 C2-deficient patients (Figueroa and Densen, 1991). However, SLE is found in only one-third of patients with C2 deficiency (Ruddy, 1986) but almost in all individuals with homozygous C1q or C4 deficiencies (Agnello, 1978). Those C2 deficient patients who are diagnosed with SLE usually show a relatively mild

manifestation of the disease but otherwise have similar clinical features to those of SLE patients without complement deficiency (i.e. photosensitivity, alopecia and arthritis) (Rynes, et al., 1977). The estimated frequency of homozygous C2 deficiency in Caucasian populations is 1:10,000 and may result from either type I or type II defects (Agnello, 1978). In the case of type I deficiency, which is the predominant variation, a 28-base pair deletion at exon/intron junction 6 within the C2 gene in the class III region of the major histocompatibility complex (MHC) results in complete lack of C2 synthesis. In type II, secretion of C2 is blocked due to single amino acid substitutions in the protein (Yu, 1998). The relatively high frequency of homozygous deficiency of C2 without disease manifestation and relatively mild disease symptoms led to the idea that a compensatory mechanism might exist. Considering the similar structures and physiological properties of C2 and FB during the formation of AP and CP C3 convertases, it has long been suggested that both complement components could substitute for each other (Knutzen Steuer, et al., 1989, Farries, et al., 1990). There are several independent reports which contribute towards such a cross-reaction between components of different complement pathways. Two publications (Knutzen Steuer, et al., 1989, May and Frank, 1973) report that sheep erythrocytes sensitised with 20 to 40 times the quantity of IgG or IgM antibody required for lysis via the classical complement pathway undergo lysis in C2-deficient human serum. Even though it was suggested that the AP convertase was facilitated by the provision of a site protected from regulators (rather than a hybrid CP/AP convertase) (Atkinson and Frank, 2006), in a clinical study on immune complex clearance, residual binding of immune complexes to red cells in serum from C2-deficient SLE-patients was observed, and it was suggested that FB may be involved (Traustadottir, et al., 1998). One of the conclusions of the authors was that FB may interact with C4 to provide a low-grade C3 activation thereby promoting immune complex binding to red cells.

As no direct evidence for C2 substitution by binding of FB to C4b has been published yet, the aim of the current study was to elucidate in detail the implied cross interaction between these two components of the alternative (FB) and classical pathways (C4b). To prove the existence of an active "hybrid" C3 convertase (C4bBb), we demonstrate that **i)** FB binds to C4b; **ii)** FB bound to C4b is cleavable by FD to form an active C4bBb-complex and **iii)** that C4bBb is capable of cleaving ^{125}I-C3 to a low but detectable rate.

2. Materials and Methods

2.1 *Complement proteins*

Complement proteins were purified from human serum or plasma. The following complement components were used: C1s, C4, C2, C3, FB, FD, FH and FI. Material used as analyte in BIACORE studies was dialysed against 10 mM HEPES, 140 mM NaCl, pH 7.4 and made 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ prior to the experiment. Material used for cleavage experiments was dialysed against VB^{++} buffer (5 mM sodium veronal, 145 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$).

C1s was purified from human serum and activated as described previously (Sim, 1981).

C4 was purified by ion-exchange or affinity chromatography as outlined elsewhere (Laich and Sim, 2001). C4 for BIACORE experiments was freshly prepared by ion-exchange chromatography and used immediately to avoid hydrolysis of the internal thioester. C4b for cleavage experiments was generated from affinity purified C4 by C1s cleavage and further purified by ion-exchange chromatography (Dodds, 1993). C4b was adsorbed with CNBr-activated Sepharose beads carrying anti-C3 antibodies to remove C3 as possible contaminant.

C2 was purified by antibody affinity chromatography (Laich, et al., 2001). C2 was adsorbed with CNBr-activated Sepharose beads carrying anti-FD antibodies to remove FB as possible contaminant.

C3 was purified from human plasma by ion exchange (Dodds, 1993). For BIACORE experiments, C3 was used immediately after preparation to avoid hydrolysis of the internal thioester. An aliquot of C3 was dialysed against 0.1M K-Phosphate buffer, pH 7.4 and radio-iodinated (160 µg C3; 0.5 mCi) according to (Fraker and Speck, 1978). For cleavage experiments, C3 was used which had been stored at 4 °C for several months to allow conversion into $C3(H_2O)$. $C3(H_2O)$ was adsorbed with CNBr-activated Sepharose beads carrying anti-C4 antibodies to remove C4 as possible contaminant.

FB was purified by dye-ligand affinity chromatography (Williams and Sim, 1993) or purchased from Sigma. FB for cleavage was adsorbed with CNBr-activated Sepharose beads carrying anti-C2 antibodies to remove C2 as a possible contaminant.

FD was a kind gift from J.E. Volanakis. Prior to use in ^{125}I-C3 cleavage experiments, FD was incubated with a 10-fold molar excess of C1-inhibitor to exclude possible activity of trace contaminants of C1s in the preparation.

2.2 *FB-C4b interaction detected by surface plasmon resonance*

To investigate C4b-FB interaction in real time, a BIACORE 2000 system was used. Fresh C4 (80 µg) and C1s (4 µg) both in PBS, pH 7.4 were co-incubated for 1 hour at 37 °C to generate C4b. Fresh C3 (80 µg) in PBS, pH 8.2 was made 25 mM methylamine and incubated at 37 °C to generate the C3b-like molecule $C3(CH_3NH_2)$ (34). C4b and C3b were immobilised onto a 2,2-pyridinyldithio-ethaneamine- (PDEA-) treated CM5 sensor chip, via exposed -SH groups, at a flow rate of 5 µl/min. 7000 RU of either material was immobilised within 2 minutes. As control, inactivated C4b and C3b respectively (after activation both components were made 5 mM iodoacetamide to block all reactive exposed -SH groups and incubated for 1 hour at 37 °C) was passed under the same conditions as reactive C4b and C3b over the chip. Thereafter, the chip-surfaces were blocked and treated in the same way as the test surfaces. FB (serial dilutions 625 – 40 nM) and C2 (serial dilutions 500 – 30 nM) were passed over the sensor chip at 50 µl/min in 10 mM Hepes, 140 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.4 at 25 °C. Stripping buffer between consecutive pulses of increasing analyte concentrations was 10 mM MES, 750 mM NaCl, 10 mM EDTA, pH 5.5.

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2.3 *Cleavage of FB, attached to C4b, by FD*

Purified C4b (2.5 µg) + FB (5 µg) + FD (5 µg) was co-incubated in VB^{++}-buffer at 37 °C for 12, 24, 36, and 42 hours. To avoid unspecific cleavage by trace contamination of other proteases, the mix was made 5 µg/ml soybean trypsin inhibitor (SBTI). In parallel, C4b + FB, FB + FD and FB alone, all in VB^{++}-buffer, were incubated under the same conditions (37 °C for 12, 24, 36, and 42 hours). To test whether the FB-preparation used was functionally active, 2.5 µg of C3(H$_2$O) + 5 µg of FB + 5 µg of FD were incubated for 24 hours at 37 °C. Cleavage of FB was 95%. Complement components were added according to their physiological order of activation. After cleavage, samples were run on 10% acrylamide SDS-PAGE under reducing conditions. The gel was stained with Coomassie Brilliant Blue R250.

2.4 *Cleavage of ^{125}I-C3 by the activated "hybrid" convertase C4bBb*

Using I^{125}-C3 as substrate, the activity of the "hybrid" C3 convertase was monitored directly. In three series of cleavage experiments, 100 ng of ^{125}I-C3 (specific activity was 50.000 cpm) was co-incubated with 5 µg FB + 2.5 µg FD and 10 µg of C4b. Addition of components was performed according to their physiological order of activation. Cold C4b and FB were added to a reaction vial containing the appropriate buffer before I^{125}-C3 was introduced; finally, FD was added to start the reaction. Control incubations with I^{125}-C3 alone, with I^{125}-C3 + FB + FD (but without C4b) were performed in addition. To test the cleavability of the substrate, I^{125}-C3 was incubated with 10 µg of cold C3(H$_2$O) + FB + FD. Incubation time was 1 hour at 37 °C throughout; cleavage was performed in VB^{++}-buffer in the presence of 0.1% (v/v) of TX-100 to prevent radioactivity sticking to the reaction vials used. The reaction volume was 50 µl. Immediately after the incubations were completed, samples were run on 10% acrylamide SDS-PAGE under reducing conditions. The gels were stained with Coomassie to control the amount of applied material, dried down onto filter paper and exposed to a phosphor imager

screen for 12 -24 hours. The extent of I^{125}-C3 cleavage was evaluated by a Storm 860 system; evaluation software was Image-Quant for Windows NT.

2.5 *Protein modelling*

The C4b structure was downloaded from the Protein Data Bank, PDB ID: 6YSQ (Zarantonello, et al., 2020). The C4b was aligned to the C4b model in the C4b2 and C4b2a solution structures (Mortensen, et al., 2016). FB (PDB ID: 7JTQ) or FBb (PDB ID: 2WIN) were aligned to C2 and C2a respectively to generate the figures (Wu, et al., 2009, Xu, et al., 2021).

3. Results

3.1 *Surface Plasmon Resonance reveals low affinity binding between FB and C4b*

Surface Plasmon Resonance experiments showed a direct interaction between FB (625 nM – 20 nM) and C4b (7000 RU immobilised). As a control, C2 (500 nM – 30 nM) was passed over the same chip surface to prove correct immobilisation of C4b. Although 7200 RU of the C3b-like molecule $C3(CH_3NH_2)$ (Pangburn, et al., 1981) were correctly immobilised onto the chip, as proved by a test run using 500 nM – 15 nM FB, C2 did not bind to C3b (Fig. 2d). Although BIACORE experiments were primarily performed to obtain qualitative binding results, for most experiments binding parameters could be calculated. For C4b-C2 interaction (Fig. 2a) a binding constant K_D of 1.78 * 10^{-8}[M] was calculated by steady state analysis, based on the plot "concentration of C2 [M] vs. equilibrium binding" (see insert in Fig. 2a). The K_D for C3b-FB interaction (Fig. 2c) was 3.3 * 10^{-7}[M] and was obtained by direct curve fitting according to a 1:1 Langmuir binding model; Chi^2 was 0.571. In the case of C4bB complex formation (Fig. 2b), quantitative binding data were difficult to evaluate. As equilibrium was not reached, a steady state analysis could not be performed. By direct fitting, an approximate K_D of 2.00 * 10^{-5}[M] was calculated, but the match of the fitted curve gave a Chi^2 value of 56. Chi^2 values provide an approximate indication of how well the fitted curve matches the measured curve and should be <10 (35)). As the calculated K_D for C4b-FB interaction is near the limit of detection of the applied method, it can only give a rough estimation of the binding constant. However, all calculated binding constants are in accordance with previously published results (Laich and Sim, 2001). To confirm that the fB binding on the CM5 chip (Fig. 2b) was specific, the reference cell was checked and deemed clear (not shown).

3.2 *Low FD-mediated cleavage of FB in complex with C4b indicates activation to C4bBb*

In the next series of experiments, we investigated whether FB is cleavable by FD when in complex with C4b. Only minor breakdown was detected during a control incubation of FB on its own (0-42 hours). In the presence of FD also minor, but slightly more FB degradation was seen (Fig. 3a). In the presence of $C3(H_2O)$ + FD (Fig. 3b) however, FB was cleaved to 95% after 24 hours, indicating that the FB preparation used was in good condition (Fig. 3, middle panel of lanes). Increasing cleavage of FB in the presence of C4b and FD was seen over the entire time course. After 24 hours the cleavage was above background and after 42 hours the 60 kDa band of the Bb fragment clearly confirmed that FB was cleaved by FD (Fig. 3c). It should be noted that the Ba fragment (30 kDa) runs at the same position as the C4 γ-chain and is difficult to see. Cleavage was quite low but indicated that the complex C4FB was activated to C4bBb.

Incubation of ^{125}I-C3 with C4bBb showed a very low, but detectable cleavage of C3 (Fig. 4). Incubation of ^{125}I-C3 (100 ng) alone did not lead to any breakdown of the substrate (zero-control); incubation of ^{125}I-C3 (100 ng) with cold $C3(H_2O)$ (10 µg) + FB (5 µg) + FD (2.5 µg) resulted in complete cleavage (100% control). Incubation of ^{125}I-C3 (100 ng) + FB (5 µg) + FD (2.5 µg) showed 63.54%, 72.33% and 66.65% of ^{125}I-C3 cleavage. Under the same conditions but in the presence of 10 µg C4b, ^{125}I-C3 was cleaved to 66.17%, 73.81% and 68.43%. The average increase of cleavage in presence of C4b was 1.96 % (S.D.=0.49). Data were evaluated by a paired t-test (SPSS 9.0) and were significant (n=3; p=0.0294) (Fig. 4).

The structural alignments show the predicted C4b:FB (Fig. 5a) and C4b:FBb (Fig. 5b) conformations. According to this model, which mirrors the homologous interactions in the classical pathway proconvertase C4b2, and convertase C4b2a (46), interactions

would be predominantly between the vWFA domain (green) of FB and the C345c domain of C4b, and the Ba region of FB (yellow) and the α' N-terminal region of C4b. After FB activation, the vWFA domain of FBb would interact with the C345c domain of C4b.

Discussion

This study has presented our indirect evidence for the existence of a cross-reaction between components of the CP and AP of complement activation. From haemolytic assays with C2-deficient human serum it was suggested that a C4bB complex might exist (Selander, et al., 2000). A C3b-C2 interaction has surprisingly never been discussed nor has any evidence for such a complex been noted. In a previous study (Laich and Sim, 2001) it was seen for the first time that FB as analyte cross-reacts with C4b as ligand in Surface Plasmon Resonance. As at that time C4b was immobilised rather unspecifically via amine-coupling to a CM5 sensor chip, we here repeated the experiment with C4b immobilised via an exposed -SH group. Immobilisation by this method was more physiological and we expected better results. Using protocols described earlier (Laich and Sim, 2001), C4bB interaction allowed a quantitative evaluation of the binding constant. With a binding constant K_D of $2.00 * 10^{-5}$ [M] the FB binding was 60 times weaker to C4b than to C3b-like molecule $C3(CH_3NH_2)$. Compared to C4bC2 interaction, the C4bB complex was almost 1,100 times weaker. As such, the detection limit of the system was nearly reached (Chowdhry and Harding, 2001). The finding that C2 did not bind to C3b also corresponds well to previous results (Laich, et al., 2001). It might be possible that properdin (Brade, 1978), a small component of the AP of complement activation, supports or stabilises the binding of C2 to C3b. Therefore, it cannot be totally excluded that such a complex does not exist.

After C4bB interaction was clearly detected, the question as to whether such a complex could be activated to cleave C3 needed to be addressed. It is known that FB cleavage by FD is negligible without the presence of C3b (Volanakis, 1990). A C3-convertase complex containing intact FB is not capable of cleaving its substrate because the serine protease domain in the Bb fragment remains inactive. In control experiments this finding was confirmed. In the presence of $C3(H_2O)$ however, FB was cleaved by FD to 95% indicating that the FB-preparation used was functionally active. As in the

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presence of C4b, FB was minimally cleaved into Bb and Ba, it remained to be demonstrated that the C4bBb complex was capable of C3 cleavage.

First experimental attempts to show the cleavage were performed by incubating ^{125}I-C3 (75000 CPM, 200 ng) + C4b (10 µg) + FB (2 µg) + FD (1 µg) at 37 °C in time courses for up to 48 hours. ^{125}I-C3 cleavage was already seen after 4 hours. After 48 h the cleavage was almost to the same extent as seen in the control experiments using 10 µg of cold C3(H_2O) instead of C4b. Our interpretation of this result was that a C4bBb complex was formed which initially indeed cleaved ^{125}I-C3 to a very low degree. ^{125}I-C3b might then have formed a complex with FB and might have been activated to the AP-C3 convertase ^{125}I-C3bBb. This convertase cleaved the substrate ^{125}I-C3 leading to an exponential increase of more radioactive convertase, finally cleaving the substrate to almost 100%. An initially active C4bBb complex must however have been formed, as ^{125}I-C3 does not bind FB (it was shown by co-incubation with factors H and I (FH and FI) (Sim, et al., 1993) that the ^{125}I-C3 preparation used did not contain ^{125}I-C3(H_2O)).

To avoid the formation of a ^{125}I-C3bBb complex and to get a better cleavage result from the "hybrid" C3 convertase, C4bBb, the experiment was repeated but in the presence of complement regulators FH and FI. The underlying idea was that, as soon as ^{125}I-C3b was generated, FH and FI would break it down to ^{125}I-iC3b which would no longer interfere with the activity of C4bBb (Sim, et al., 1993, Soames and Sim, 1997, Seya, et al., 1995). Autoradiograms showed perfect breakdown of ^{125}I-C3 in the controls (AP-C3 convertase) but no cleavage at all in case of the C4bBb complex. The experiment was performed again and when the radioactive gel was stained with Coomassie, C4b was found to have been broken down to iC4b within 30 minutes. As 30 min was probably not long enough time for the formation of the C4bBb complex, it was decided to return to the initial cleavage protocol but to cut down the incubation

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time to 1 hour at 37 °C. The amount of FB and FD was increased to 5 µg and 2.5 µg respectively and the concentration of ^{125}I-C3 was reduced to 100 ng. The C4b concentration remained 10 µg. At the time the experiment was performed, traces of ^{125}I-C3(H$_2$O) in the ^{125}I-C3 preparation could not be excluded. As it was seen in previous experiments that the formation of ^{125}I-C3bBb could mimic the activity of C4bBb, an additional control experiment was performed. ^{125}I-C3 was incubated with FB and FD to obtain a background cleavage activity. When the autoradiograms were evaluated, a significant difference in ^{125}I-C3 cleavage in the absence and presence of C4b was seen. The very low but significant variation in cleavage activity must therefore be due to the activity of the "hybrid" C3-convertase, C4bBb.

In assembling the AP C3 convertase, the initial interaction between FB and C3b occurs between residues at the Mg^{2+}-dependent Metal Ion-Dependent Adhesion Site (MIDAS), D^{251}GSDS^{255}, and the α1 helix in the vWFA domain which binds C345C at the C-terminus of the α-chain (Hinshelwood, et al., 1999, Hourcade, et al., 1999, Tuckwell, et al., 1997). Initial recognition also involves SCR1-3/CCP domains (Pryzdial and Isenman, 1987, Hourcade, et al., 1995) in particular the third CCP 138-195, which interacts with the C3b α-chain α'NT region, MG2, MG3, MG7 and CUB regions. C3b makes no contact with the SP of FB. In the initial interaction, FB binds C3b in a closed conformation but opens up upon cleavage by FD. After release of Ba, the structure of C3bBb, the AP C3 convertase is maintained by interactions between the vWFA domain of Bb and C345C domain (A^{1492}-N^{1641}) of C3b. The SP domain of Bb is kept away from C3b as Bb projects outwards (Milder, et al., 2007, Rooijakkers, et al., 2009, Torreira, et al., 2009).

In assembling the CP C3 pro-convertase, C4b binds C2 in a Mg^{2+}-dependent manner (Sitomer, et al., 1966). C2 can then be cleaved by C1s, MASP-1 or -2 into C2a (which includes the vWFA and SP domain) leaving C2a bound to C4b (Muller-Eberhard, et

al., 1967)). The C2a-C4b interaction involves the SCR1-3/CCP domains (Nagasawa and Stroud, 1977) and MIDAS (Horiuchi, et al., 1991). Not surprisingly in view of their role in cleaving C3 and C5, the crystal structure of C4b2a and C3bBb are very similar (Mortensen, et al., 2016), in particular the arrangement of the catalytic C2a and Bb domains (Mortensen, et al., 2016). Our molecular modelling of the putative C4bB and C4bBb complexes illustrates interacting regions between the vWFA domain of FBb with C4b. The SP domain within FB protrudes away from C4b. The models indicate a possible mode of binding between the two proteins, based on the homologous complexes of the classical pathway (Mortensen, et al., 2016).

Given that C4bBb is prone to dissociation, and that the rate of activation of C4bB and of C3 cleavage is slow, the physiological relevance of these observations will need further clarification. Nonetheless, a physiological role may be supported by looking at the role AP plays in supporting CP, the dominant pathway in mediating bactericidal activity in mice. In a model of *Streptococcus pneumoniae* pneumonia, CP's functional importance was confirmed, as loss of IgM-induced CP resulted in septicaemia and impaired macrophage-mediated phagocytosis (Brown, et al., 2002). In other work, providing possible insight into the physiological relevance of our findings, AP enhanced MAC deposition and the serum bactericidal activity of CP (Mutti, et al., 2018), although on its own, AP did not demonstrate any bactericidal activity against Gram-negative bacteria. Also important in opsonizing bacteria with C3b/iC3b, and in maintaining a pathogenic inflammatory response, in the absence of C4 or C2 and conventional C4b2a or C3bBb, is the MASP-2 mediated, bypass mechanism for the non-canonical activation of C3 (Yaseen, et al., 2017). For further support of our findings of the "hybrid" convertase, C4bBb, able to cleave and activate C3 *in vitro*, ensuing empirical studies could involve coating red blood cells with C4b and seeing whether formation of a convertase incorporating FB would allow for deposition of C3b.

Declaration of Competing Interest

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Figures

Fig.1 Schematic overview of know pathways of complement activation. The classical pathway (CP) and alternative pathway (AP) C3-convertases. C4b2a and C3bBb are underlined. The possibility of a "hybrid" C3-convertase, C4bBb, as cross-reaction between components of CP and AP is investigated in the current paper.

Fig. 2 Surface Plasmon Resonance experiment using purified FB, C4b, C2 and the C3b-like molecule $C3(CH_3NH_2)$. To prove correct immobilisation of C4b and C3b, control experiments were performed. C4bC2 (Fig. 2a) and C3bFB (Fig. 2c) interaction was seen. FB bound to C4b (Fig. 2b) but C2 did not bind to C3b (Fig. 2d). Binding parameters where evaluated by steady state analysis for the C4bC2 complex formation (see insert in Fig. 2a) or by direct curve fitting according to a Langmuir 1:1 model (Fig 2b and 2c). Measured curves are shown as solid lines, fitted curves as dotted lines. Binding constants (K_D) for C4bC2-interaction were $1.78 * 10^{-8}$[M], for C3bFB-interaction $3.3 * 10^{-7}$[M] and for C4bB-interaction $2.00 * 10^{-5}$[M]. RU, response units (1 RU=1pg of material bound to $1mm^2$ of chip-surface).

Fig. 3 FB (MW 90 kDa) attached to C4b is cleaved by FD into Bb (MW 60 kDa) and Ba (MW 30 kDa) (right). In a time course, FB (5 µg) + C4b (2.5 µg) was incubated without or with FD (5 µg) for up to 42 h at 37 °C. Unspecific FB cleavage was excluded by adding soybean-trypsin inhibitor (SBTI) to the reaction-mix. FB alone (data not shown) or with C4b only was stable (left), no breakdown being observed.

Fig. 4 ^{125}I-C3 MW was incubated for 1 hour at 37 °C under conditions i) – iv). C3-convertases cleave an 8.4 kDa fragment from the C3 α-chain (112 kDa) to convert C3 to C3b. The cleaved C3 α-chain is termed C3α' and is seen in SDS-PAGE under reducing conditions as a distinct band just below C3α. The C3 α-chain (74 kDa) is not cleaved by any C3-convertase. Concentrations used for the cleavage experiment were

100 ng (50.000 CPM) of ^{125}I-C3, 10 µg of cold C4b or C3(H_2O), 5 µg of FB and 2.5 µg of FD. Autoradiograms (left) were evaluated to calculate the percentage of ^{125}I-C3 cleavage (right). In presence of C4b, FB and FD (condition iii) cleavage of ^{125}I-C3 was on average 2% higher then without C4b (background; condition iii). Conditions i) and iv) were performed as controls. A paired t-test * of conditions ii) and iii) was significant (n=3; p=0.0294).

Fig. 5 (a) C4b:FB model shown in cartoon representation in two rotations. C4b is shown in grey, the SP domain of FB in purple, the vWFA domain of FB in green and FBa in yellow. Insets show close-up views of the interfaces between C4b and FB in the complex, involving the α'-Nt region of C4b and Ba (bottom), or the C345c domain of C4b and the vWFA domain of FB (top). (b) C4b:FBb model shown in cartoon representation in two rotations. C4b is shown in grey, the vWFA domain of FBb in green and the SP domain of FBb in red. The inset shows a close-up view of the interacting domains, the C345c domain of C4b and the vWFA domain of FBb. The metal ion is shown in blue in all panels.
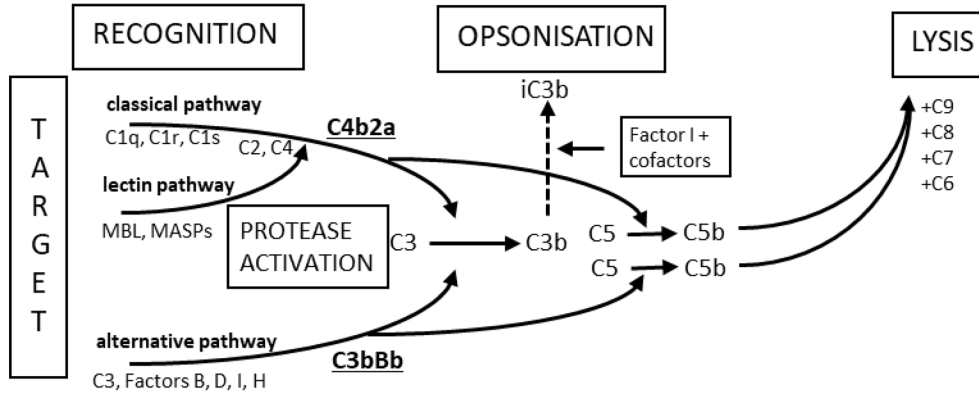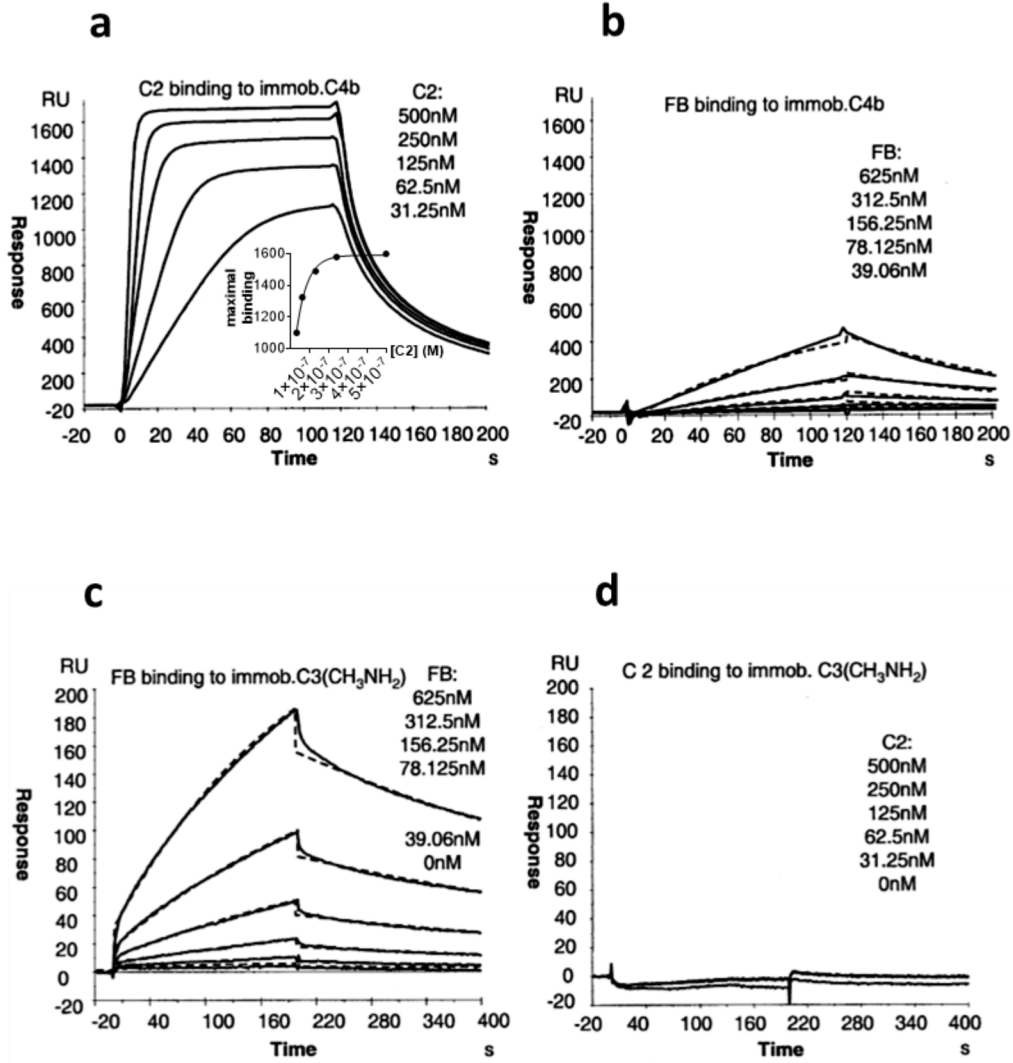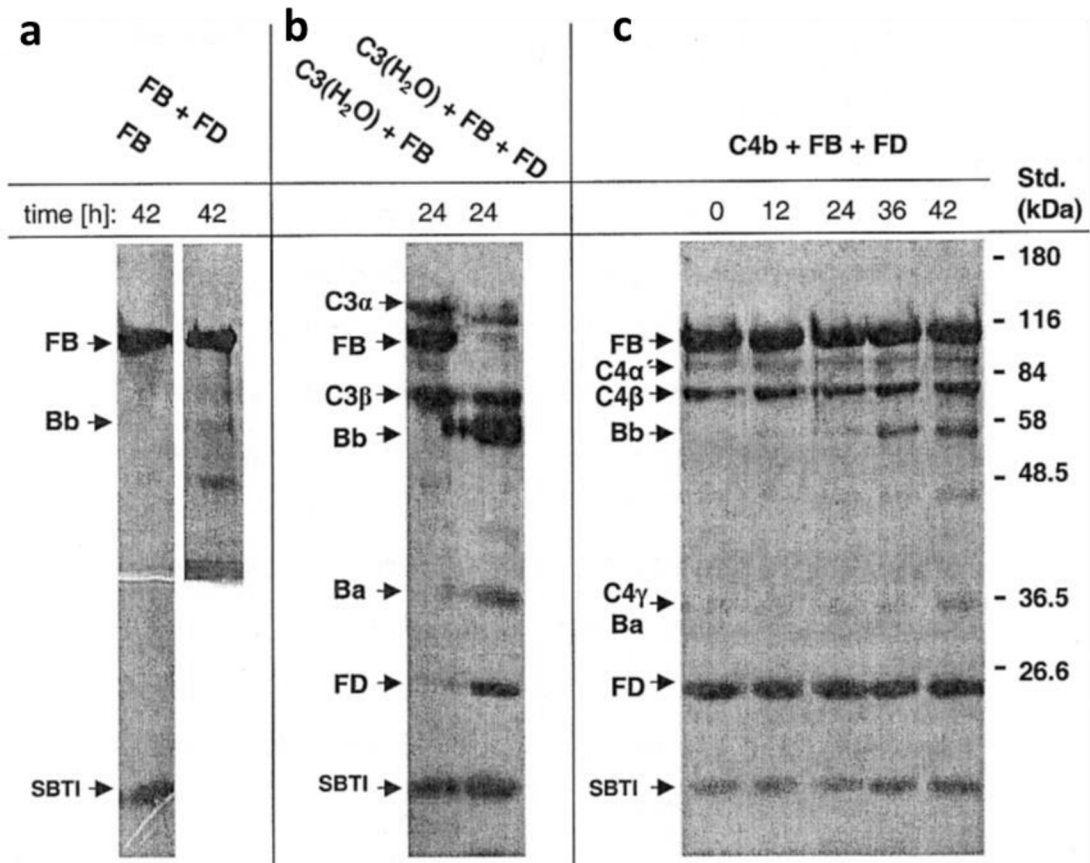
Fig. 1

Fig. 2

a

RU — Response

C2 binding to immob.C4b

C2:
500nM
250nM
125nM
62.5nM
31.25nM

(inset) maximal binding vs [C2] (M): 1×10^{-7}, 2×10^{-7}, 3×10^{-7}, 4×10^{-7}, 5×10^{-7}

Time s

b

RU — Response

FB binding to immob.C4b

FB:
625nM
312.5nM
156.25nM
78.125nM
39.06nM

Time s

c

RU — Response

FB binding to immob.C3(CH_3NH_2) FB:
625nM
312.5nM
156.25nM
78.125nM

39.06nM
0nM

Time s

d

RU — Response

C 2 binding to immob. C3(CH_3NH_2)

C2:
500nM
250nM
125nM
62.5nM
31.25nM
0nM

Time s

Fig. 3

a

FB + FD
FB

time [h]: 42 42

FB →
Bb →

SBTI →

b

C3(H$_2$O) + FB + FD
C3(H$_2$O) + FB

24 24

C3α →
FB →
C3β →
Bb →

Ba →

FD →

SBTI →

c

C4b + FB + FD

0 12 24 36 42

Std. (kDa)

- 180
- 116
- 84
- 58
- 48.5
- 36.5
- 26.6

FB →
C4α' →
C4β →
Bb →

C4γ →
Ba

FD →

SBTI →

23

Fig. 4

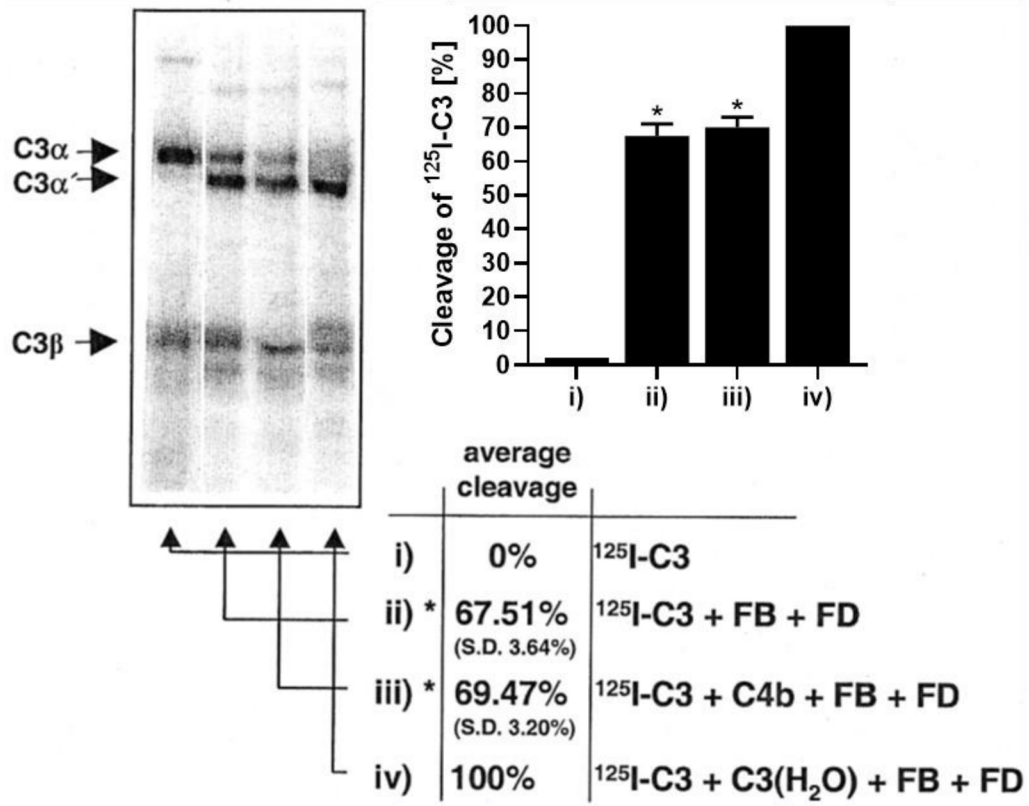|	average cleavage	
i)	0%	^{125}I-C3
ii) *	67.51% (S.D. 3.64%)	^{125}I-C3 + FB + FD
iii) *	69.47% (S.D. 3.20%)	^{125}I-C3 + C4b + FB + FD
iv)	100%	^{125}I-C3 + C3(H$_2$O) + FB + FD

C3α
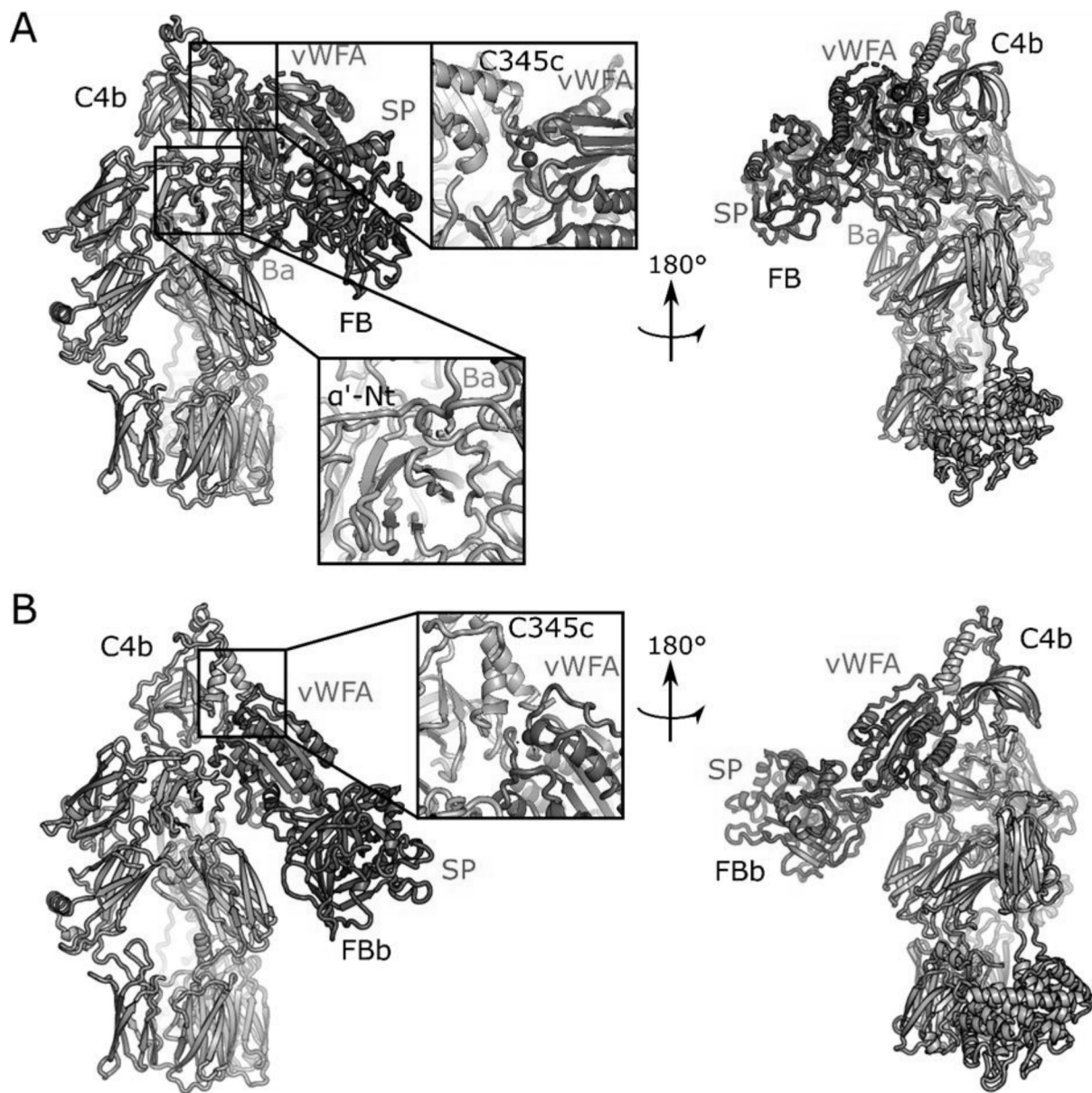C3α´
C3β

Cleavage of ^{125}I-C3 [%]

i) ii) iii) iv)

Fig. 5

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