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Antibodies reactive to cleaved sites in complement proteins enable highly specific measurement of soluble markers of complement activation.

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Abstract

An emerging number of diseases and therapeutic approaches with defined involvement of the complement system justify a need for specific markers reflecting activation of particular effector arms of the complement cascade. Measurement of such soluble markers in circulation is a challenge since the specificity of antibodies must be limited to activated complement fragments but not predominant and ubiquitous parental molecules. Existing assays for the measurement of soluble, activated complement proteins are based on the detection of conformational neoepitopes. We tested an alternative approach based on detection of short linear neoepitopes exposed at the cleavage sites after activation of the actual complement component. Obtained antibodies reactive to C4d and C5b fragments enabled us to set up highly specific sandwich ELISAs, which ensured trustful measurements without false positive readouts characteristic for some of the widely used assays.

Keywords:

antibodies, complement system, leukemia, lymphoma, C4d, C5b

Abbreviations:

aa – amino acid; CAU – complement activation unit; FI - complement factor I; ICS#2 – International Complement Standard #2; HRP – horseradish peroxidase; NHS – normal human serum; HI-NHS – heat inactivated NHS; OPD - *o*-Phenylenediamine dihydrochloride

1. Introduction

The complement system consists of more than 30 proteins organized in a cascade of proteolytic events and conformational changes, which support sequential interactions of activated complement components eventually leading to lysis of target cells (Walport, 2001). Proteolysis takes place during the initiation of the alternative complement pathway (cleavage of C3 and factor B) and classical/lectin pathway (C2 and C4), during generation of C3a and C3b by C3 convertases, during inactivation of C3b and C4b opsonins by factor I (FI) (Nilsson et al., 2011), and finally at the transition from opsonic to lytic stage, i.e. splitting of C5 molecule to C5a and C5b (Walport, 2001). Proteolysis triggers conformational changes resulting in exposure of binding sites for complement components placed downstream in the cascade, which in turn also adopt new conformations allowing interaction with the next component. The best-known soluble complement activation products are anaphylatoxins C3a and C5a (and their desArg forms lacking the C-terminal arginine). However, free anaphylatoxins are rapidly cleared from circulation (Webster et al., 1982) thus making the interpretation of their systemic measurement problematic. Notably, not all of cell-bondable, activated complement components bind target cells but a certain amount remains in their soluble, stable forms, e.g. Bb, C4d, sC5b-9 (Bergseth et al., 2013). Although this gives an opportunity to monitor complement activation in clinical material like plasma and body fluids, measurement of such soluble markers is challenging, as it demands highly specific antibodies not reactive to abundant, non-activated complement components.

Most of existing assays make use of antibodies against conformational neoepitopes but these sites may be mimicked or decayed in the course of sample handling. Manufacturers seem to be aware of the problem as they recommend very strict sampling guidelines and discourage prolonged storage or heat-inactivation of the sample before measurement as well as repetitive freezing and thawing of the samples to be measured; a precaution not always easy to follow, especially with archival clinical material. Therefore, we tested a novel strategy of raising antibodies specific to very short linear neoepitopes exposed in activated complement components.

2. Methods

2.1 Reagents, sera, antibodies and purified complement proteins

Normal human serum (NHS) was prepared as described (Blom et al., 2014) and heat inactivation was performed by heating at 56°C for 30 minutes. C6 and C9-depleted sera were purchased from Complement Technology (Tyler, TX). Function-blocking antibody against FI was bought from Quidel (San Diego, CA). The C5-blocking antibody eculizumab was purchased from Alexion Pharmaceuticals (Lausanne, Switzerland). Zymosan was purchased from Sigma (St Louis, MO). Mouse monoclonal antibody mk54 against protein S was prepared as described (Dahlback et al., 1990).

2.2 Raising of neoepitope antibodies

The whole process of custom antibody production was performed by Agrisera AB (Vännäs, Sweden). Briefly, synthetic peptides embracing five terminal amino acids (aa) residues adjacent to the cleavage site of a given complement component followed by two glycine and one cysteine residue spacer were linked to keyhole limpet hemocyanin and used for rabbit immunization (two rabbits per peptide construct) according to the following schedule: 200 µg of peptide with complete Freund's adjuvant followed by another 200 µg of peptide with incomplete Freund's adjuvant four weeks later and 100 µg of peptide every four weeks. Bleeding was performed two weeks after booster injections starting from round two. Sequences of the peptides used for immunization are given in Table 1.

2.3 Purification of antibodies by affinity chromatography

Synthetic peptides (Genscript, Piscataway, NJ) corresponding to nine aa residues before the cleavage site were immobilized with added Cys residue to Sulpholink beads (Thermo Scientific, Waltham, MA) according to manufacturer's instruction and loaded onto a 10 ml column, further equilibrated with PBS buffer. Antiserum was loaded and bound antibodies were eluted with linear gradient of 0.1 M glycine pH 2.5. After neutralization with 1M Tris-HCl pH 8.0, the eluate was directly applied onto another column containing Sulpholink beads conjugated to the similar peptide but extended 4 aa beyond the cleavage site. At this step we collected flow through and all antibodies with affinity to extended peptide motif, and therefore cross-reactive to the parental complement molecule, were absorbed. Pooled flow through was dialysed against PBS and concentrated. Sequences of the peptides used for antibody purification are given in Table 1.

2.4 Complement activation *in vitro*

Serum at concentration of 2% was diluted in DGVB⁺⁺ buffer (2.5 mM veronal buffer pH 7.3, 72 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂) and 0.1 mg/ml zymosan was added. After 45 min incubation at 37 °C mixture was diluted five times (up to 0.4% final concentration) with AG buffer (0.2% Tween 20, 20 mM EDTA, 0.02% NaN₃ in PBS). Zymosan particles were discarded by centrifugation for 3 min at 5000 x g prior to analysis by ELISA. Alternatively, 8% of serum diluted in DGVB⁺⁺ was activated with 0.8 mg/ml human IgG (Immuno, Vienna, Austria) aggregated at 63 °C for 20 minutes. After 45 min incubation the supernatant was diluted twelve times (up to 0.66% final serum concentration) with AG buffer and prepared for ELISA.

2.5 ELISA detecting soluble C4d

Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated overnight at room temperature with 3.5 µg/ml of affinity-purified C4d neoepitope antibody diluted in PBS + 0.02% NaN₃. Wells were blocked with 3% fish gelatine (Norland Products, Cranbury, NJ) in washing buffer (50 mM Tris-HCl, 150 mM NaCl, 0.2 % Tween 20). Blocking step and subsequent incubation with samples and standards followed by detection with antibodies were performed for 1h at 37 °C. Serial dilutions of ICS#2 (International Complement Standard #2, a pool of sera from healthy volunteers activated with aggregated IgG and zymosan (Bergseth et al., 2013)) were used as a standard. Since ICS#2 is a source of every possible complement activation product, their content was defined as 1000 complement activation units (CAU) per one millilitre of ICS#2. Detection was performed by anti C4d #253 Ab (Quidel) diluted 1:1500 in PBS + 0.2% Tween 20 followed by goat anti-mouse, HRP-conjugated Ab (Dako, Glostrup, Denmark) diluted 1:1000. Assay was developed with OPD tablets (Dako) according to the manufacturer's instruction and absorbance at 490 nm was measured using Cary50 MPR microplate reader (Varian, Palo Alto, CA).

2.6 ELISA detecting sC5-9 and its soluble intermediates.

Quantification of sC5b-9 and its intermediates was performed similarly to C4d ELISA but affinity-purified C5b neoepitope antibody was used for coating and samples/standards were incubated at 4 °C. For detection, goat polyclonal antibodies against C5 or C6 or C8 or C9 (Complement Technology) or C7 (Quidel) were used at dilution 1:2000 with exception of anti C8, which was diluted 1:500. Secondary antibody was rabbit anti-goat, HRP-conjugated (Dako).

2.7 Hemolytic assay

Hemolytic assay was performed as described previously (Blom et al., 2014). Briefly, sheep erythrocytes were sensitized with anti-sheep IgM (amboceptor), washed in veronal buffer

(DGVB⁺⁺) and incubated with 5% NHS in the same buffer in the presence or absence of C5-blocking antibody. Complement-mediated lysis was assessed by measurement of hemoglobin released to supernatant at 405 nm. Readout of the sample of erythrocytes lysed with distilled water was considered as maximal attainable (full) lysis.

2.8 Patients

Thirty-one patients with various B cell malignancies treated with anti-CD20 mAbs between 2012 and 2014 at the Karolinska University Hospital (Stockholm, Sweden) were enrolled in the study upon written informed consent and according to permit from regional ethic committee. We collected EDTA-plasma samples before and at the end of the first infusion of rituximab or ofatumumab and stored them at -80 °C. Detailed description of the patients is given in Table 2.

2.9 Statistical analysis

Statistical calculations were performed with Prism 5.0 (Graph Pad Software, La Jolla, CA) and included two-way analysis of variance (ANOVA) and Spearman rank correlation.

3. Results and discussion.

3.1 Specificity of C4d detection by ELISA based on antibodies reactive to a C-terminal, linear C4d neoepitope.

Given that complement proteases are characterized by very narrow specificity (Sim and Tsiftoglou, 2004), there is likely a chance that antibodies targeted to short aa sequences exposed at the cleavage sites might specifically recognize only activated complement components. A challenge of raising such antibodies is that such short peptides may be of low immunogenicity due to impaired presentation in MHC class II molecules (Nelson et al., 1997) or that raised antibodies would recognize a region spanning both sides of the intact cleavage site. However, we obtained a high titer of antibodies specific to neoepitope exposed at the C-terminus of C4d in both rabbits immunized with the corresponding peptide construct but immunization with the peptide corresponding to the N-terminus of C4d was ineffective (Supplementary Fig.1). To ensure the high quality of antibodies, we purified rabbit antiserum by peptide affinity chromatography using first a positive and then a negative selection strategy. The scheme of physiological events leading to C4d generation from C4b molecule, with indicated peptide sequences crucial for immunization and antibody purification, is given in Fig. 1a.

To test our anti-neo-C4d antibodies, we used them as coating antibodies in a sandwich ELISA setup, which was first validated with NHS and heat-inactivated NHS (HI-NHS, as heat inactivation is known to affect early complement components) activated with aggregated IgG. C4d was not detected when inactivated serum was incubated with IgG (Fig. 2a). FI is a serine protease, which converts C4b to C4c and the C4d fragment. The addition of function-blocking FI antibody but not control antibody of the same isotype resulted in a dose-dependent decrease of the C4d signal (Fig. 2a).

3.2 Impact of temperature alterations on C4d detection by this novel ELISA and commercially available assay.

The most widely used assay for assessment of soluble C4d is manufactured by Quidel and has been used in a number of clinical studies regarding vasculitis (Gou et al., 2013), cardiac arrest (Bisschops et al., 2012) (Jenei et al., 2014), graft rejection (Flechner et al., 2010), mixed cryoglobulinemia (Sansonno et al., 2009), Sjögren's syndrome (Sudzius et al., 2014), lupus (Manzi et al., 1996) and lung cancer (Ajona et al., 2013). According to the manufacturer, this ELISA is based on a neoepitope C4d catching antibody and detection is completed by goat

polyclonal anti-C4d. Also, the manufacturer discourages measuring heat-inactivated or repetitively frozen samples.

Thus, we decided to compare the performance of Quidel's assay with our newly designed ELISA. When the same samples as used in experiment shown in Fig. 2a were analysed with Quidel's assay, we observed a very high readout in heat-inactivated serum (which should be devoid of any complement activity) incubated with aggregated IgG (Fig. 2b). Further, we chose three plasma samples collected from the patients receiving immunotherapy, which contained low, moderate and high levels of C4d as preliminarily assessed by our new C4d ELISA. Then, we froze and thawed them up to twelve times and analysed for C4d content using both C4d assays. Quidel's assay showed increased C4d readouts in a manner depending on the number of freezing cycles whereas our assay showed stable readouts (Fig. 2c,d). Of note, samples A and C showing high C4d content by our ELISA showed very low C4d content in Quidel's assay at the first freezing cycle, suggesting discrepancies in readout between these two assays.

3.3 Testing of antibodies reactive to a linear neoepitope in C5b.

Similarly to the procedure described for production of C4d-specific antibodies, we aimed to obtain antibodies reactive to 5-aa long linear neoepitope in C5b. Figure 1b shows a scheme of C5b generation and indicates amino acid residues important for our strategy. Large production of specific C5b antibodies was found in one of the two immunized rabbits. However, in the second rabbit antibody production was also detectable albeit at low level (Supplementary Fig. 1). Affinity-purified anti-neoC5b antibodies were tested by coating in another sandwich ELISA. After application of serially diluted ICS#2 we aimed at detection of C5, C6, C7, C8 and C9 by polyclonal goat antibodies (Fig. 3a). Since detection of all C5-C9 molecules resulted in a dose-dependent signal, we concluded that neoepitope formed by the C5b cleavage site is accessible in sC5b-9 and all stable sC5-9 intermediates.

Next, we confirmed the specificity of the neoC5b antibody by detection of sC5b-9 intermediates in NHS and HI-NHS as well C6- and C9-depleted sera activated by zymosan. As expected, detection of sC5b-9 components was abolished by heat inactivation of serum whereas depletion from C6 and C9 resulted in detection of sC5b-9 intermediates up to the C5 and C8 level, respectively (Fig. 3b). These results showed, that application of this novel C5b antibody offers an easy setup for screening of deficiencies or functional defects in terminal complement components, which are associated with up to 10,000-fold increased risk of meningococcal infections (Grumach and Kirschfink, 2014). Alternatively, one may use the proposed setup for functional analysis of putative terminal complement inhibitors. Another potential application of the currently described C5b assay is to monitor the systemic concentrations of soluble sC5b-9 and intermediates in patients treated with eculizumab – a therapeutic antibody blocking cleavage of C5 to C5a and C5b (Rother et al., 2007), already approved for treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome (Wong and Kavanagh, 2014). We performed *in vitro* experiments, in which we tested if the C5b ELISA is capable to reflect inhibition of complement by eculizumab. Sensitized sheep erythrocytes were incubated with NHS in the presence of increasing concentrations of eculizumab. We observed that eculizumab dose-dependently inhibited hemolysis and similarly dose-dependently decreased C5b in the supernatants (Fig. 3c).

3.4 Measurement of soluble markers of complement activation in plasma of patients treated with anti-CD20 mAbs.

Next, we wanted to test whether antibodies reactive to linear neoepitopes may be used for monitoring of *in vivo* complement activation. Using sandwich ELISAs, we analysed plasma collected from 31 patients with various B cell malignancies treated with the CD20 mAbs rituximab or ofatumumab. Upon binding to target cell, anti-CD20 mAbs are expected to activate

the classical complement pathway but it is not clear, which effector arm of the complement system contribute to the therapeutic effect (Okroj et al., 2013b). Also, it is not always the case that complement attack on tumor cells will proceed to the lytic stage, as malignant B cells may shed CD20-mAb complexes (Taylor and Lindorfer, 2014) and widely express the complement inhibitors CD46 and CD55 preventing C5 cleavage (Okroj et al., 2013a). Two samples were taken from each patient: one just before and the second immediately after CD20 mAb infusion. We assumed that soluble C4d should be increased after CD20 mAbs application in some of the patients and that some of these patients with increased C4d should have also increased sC5b-9. Therefore, we measured soluble C4d as well as the levels of the first sC5b-9 intermediate, namely sC5b-6 (Hadders et al., 2012) and terminal sC5b-9 complex.

After CD20 mAb infusion 16 of the 31 patients (52%) showed increases in C4d (Fig. 4a), and for 9 of these 16 patients (56%) the response to anti-CD20 treatment was confirmed by clear increases in sC5b-6 and sC5b-9, too (Fig. 4b-c). Only one patient with unaltered C4d after treatment (#20) showed increased C5b-6 and C5b-9, whereas the remaining 14 patients had no or just minor changes according to all three assays. Thus according to our ELISAs, there was not a single case of decrease in C4d, sC5b-6 or sC5b-9 after anti-CD20 treatment. As an explanation for increase of sC5b-9 in only 56% of the patients presenting increase of C4d, we propose the effect of membrane bound complement inhibitors expressed on the surface of tumor cells, which mainly target the level of complement convertases engaged in transition from opsonic to lytic stage (Manches et al., 2003; Okroj et al., 2013a).

Of note, whereas the same standard of *in vitro*-activated complement (ICS#2) was used in all our assays, we observed approx. 100-fold decrease in absolute value of complement activation products in sC5b-6 and sC5b-9 ELISA compared to the C4d assay. This suggests that progression to lytic stage of the cascade may be a real challenge during *in vivo* complement activation on malignant B cells and that such process is much less efficient comparing to complement activation in *in vitro*, cell-free conditions. Nonetheless, we still see a statistically significant correlation between the increase of C4d and sC5b-6 (Spearman $r = 0.57$, $p = 0.0007$) as well as between increase of C4d and sC5b-9 (Spearman $r = 0.58$, $p = 0.0006$) in whole patients' cohort, which confirm that the obtained results remain in relation to one another. Also, correlation between sC5b-6 and sC5b-9 increase in patients was highly significant (Spearman $r = 0.83$, $p < 0.0001$) thus reflecting the fact that C5b-9 is formed on the C5b-6 platform (Hadders et al., 2012). These results support the interpretation that the data describe the same process of complement activation probed at different stages and therefore we conclude that our neoepitope mAbs -based ELISAs offer reliable monitoring of complement activation *in vivo*.

3.5 Advantages of linear neoepitope-based measurement of C4d

We decided to re-evaluate C4d in our clinical samples with Quidel's ELISA. In contrast to the C4d results achieved by our ELISA, there was no tendency in the changes of C4d levels after CD20 mAb infusion in the Quidel's assay, since a clear increase was observed for 8 of the 31 patients (26%), but a clear and unexpected decrease was observed for 6 patients (19%) (Fig. 4d). Our C4d ELISA identified all 8 patients showing an increase by Quidel's ELISA, whereas 4 of the 6 patients showing a decrease according to Quidel's assay had an increase in C4d according to our assay (Fig. 4a). The results of the two C4d ELISAs did not correlate to each other (Spearman $r = 0.27$, $p = 0.14$), and in contrast to our C4d assay, the results of Quidel's assay did not correlate with sC5b-6 and sC5b-9 levels (Spearman $r = 0.24$, $p = 0.18$ and Spearman $r = 0.19$, $p = 0.29$, respectively).

Interestingly, while giving rather unexpected results of C4d measurement in patients' plasma samples, Quidel's assay gave expected results in a model experiment where *in vitro* C4d generation is limited by FI availability (Fig. 2b). We propose that detection in Quidel's assay is dependent on C4d conformation, as evidenced by experiments with heat-inactivated or frozen

serum. Whereas C4b cleavage in *in vitro* experiments probably leads to generation of C4d in its canonical conformation, there may be other factors present *in vivo*, which hinder or mimic conformational C4d neopeptides. Importantly, the C4d fragment contains an internal thioester, which normally mediates covalent binding of C4b/C4d to the cell surface (Cohen et al., 2012) (Fig. 1). Given that cells are able to shed covalently bound, activated complement components, this process may interfere with the conformation of C4d released to fluid phase but not affect a linear neopeptide located at the C terminus of the molecule, detected with our assay.

3.6 Conclusions

Our data show that soluble markers of complement activation with antibodies reactive to short linear neopeptides can be detected and that ELISAs based on such antibodies are less prone to generate artefacts formed in the course of sample handling, which distort measurements in clinical materials. The obtained results of C4d, sC5b-6 and sC5b-9 measurements in clinical samples, which adhere to current knowledge of molecular processes governing complement activation, demonstrate potential usability of linear neopeptide mAb-based assays for monitoring systemic activation of the complement system.

Glossary

Terms "sC5b-9 and "sC5b-9 intermediates" indicate the soluble forms of terminal complement complex (TCC). In contrast, TCC being present on the surface of target cell is termed as membrane attack complex (MAC).

Conflict of interest statement.

Anna M. Blom and Marcin Okroj are named as inventors in a patent application including claims to inventions described in the present article. Anders Österborg has received grants for research from Glaxo-SmithKline and has been principal investigator of clinical trials on ofatumumab. Tom E. Mollnes declares no conflict of interest.

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Figures

Fig.1 Scheme of C4d and C5b generation.

Scheme shows the sequence of events leading to C4d formation from the C4b molecule (A) and C5b generation from C5 molecule (B). Peptide sequences crucial for design and purification of anti-C4d neopeptide and anti-C5b neopeptide antibodies are indicated.

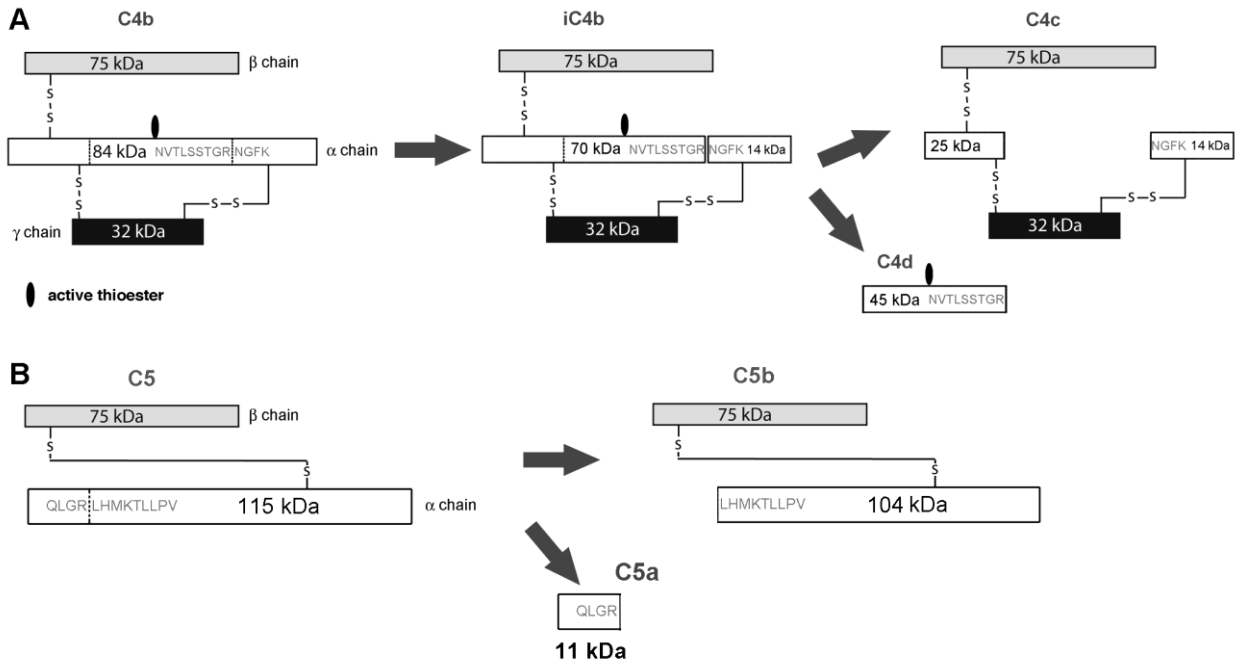


Fig.2 Comparison of C4d detection based on antibodies to short, linear, C-terminal neoepitope and commercially available C4d assay.

A and B) Aggregated IgG was added to NHS or to HI-NHS (negative control) in order to activate the classical complement pathway. In some conditions activation was performed in the presence of FI-blocking antibody (anti-FI#1, Quidel) or control antibody mk54. NHS without IgG added (NHS no IgG) served as another negative control. Samples were loaded onto ELISA plates coated with anti-C4d neoepitope antibodies and bound fragments were detected with anti-C4d monoclonal Ab (#253, Quidel) (A) or measured with Quidel's C4d MicroVue ELISA according to manufacturer's instruction (B). C4d content in panel A was expressed in complement activation units (CAU), according to ICS#2 standard whereas in B the exact concentration was calculated according to the Quidel's standard provided in the kit. C and D) Three clinical EDTA-plasma samples preliminarily tested with our novel C4d ELISA and containing low, moderate and high concentration of C4d were repetitively frozen at -80 °C and thawed for a given number of cycles. Then, freeze-thawed samples were analyzed with either our C4d assay (C) or with Quidel's C4d MicroVue ELISA (D). Original standards of C4d included in Quidel's kit were used as reference in both assays. Every experiment shown in this figure was performed twice and both independent experiments resulted in the same conclusions. Presented data show only one representative experiments of each type. Error bars show standard deviation (panels A and B) calculated from duplicated samples whereas experiments shown in panels C and D were performed as ELISA with single measurements.

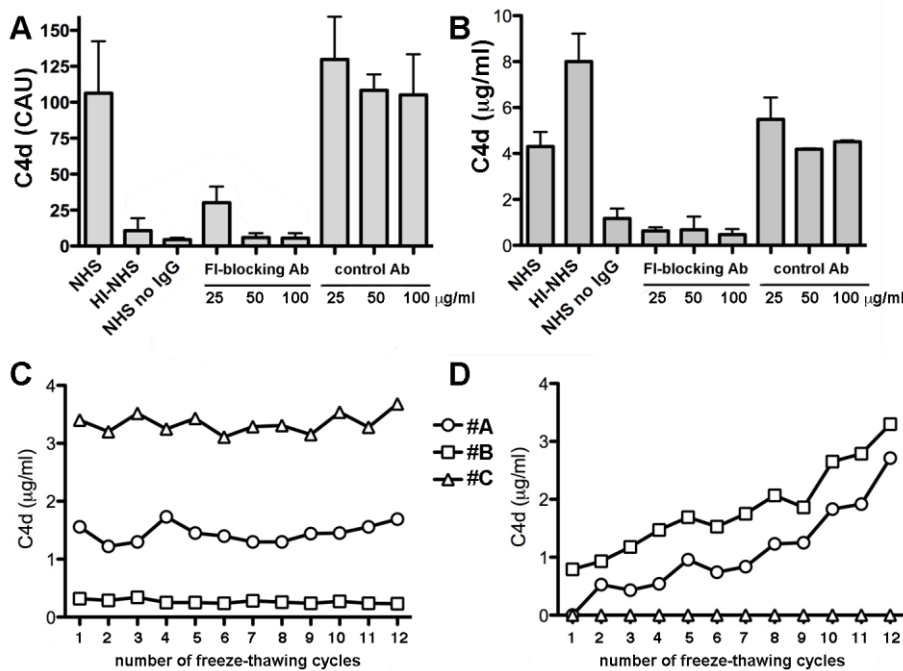


Fig. 3 Validation of anti-C5b neoepitope antibodies in sandwich ELISA assays.

A) Serial dilutions of the ICS#2 complement activation standard were loaded onto ELISA plates coated with anti-C5b neoepitope antibodies. Detection of bound complexes was performed with anti-C5, anti-C6, anti-C7, anti-C8 and anti-C9 polyclonal antibodies. B) NHS, heat inactivated NHS (HI-NHS), C6-depleted serum (Δ C6) and C9-depleted serum (Δ C9) were incubated with zymosan and then MAC intermediates were detected as in panel A. Results were calculated using the standard curve from ICS#2 and presented as percentage of the value obtained for NHS (positive control). All readouts for HI-NHS, readouts for Δ C6 serum from C6 detection onward and readout for Δ C9 at C9 detection were significantly lower than control, $p < 0.001$ according to two-way ANOVA. C) Sensitized sheep erythrocytes were incubated with 5% NHS or HI-NHS (negative control) in the presence of eculizumab. Intensity of complement-mediated hemolysis was measured as 405 nm (left Y axis) and sample of erythrocytes lysed with water was considered as full lysis control. The same supernatants were used for measurement of C5b in sandwich ELISA with the currently developed anti-neoC5b as a catcher antibody and polyclonal goat anti-C5 as detecting antibody. Results are expressed in complement activation units (CAU), according to ICS#2 (right Y axis). All data presented in Fig. 3 are collected from three independent experiments and error bars show standard deviation.

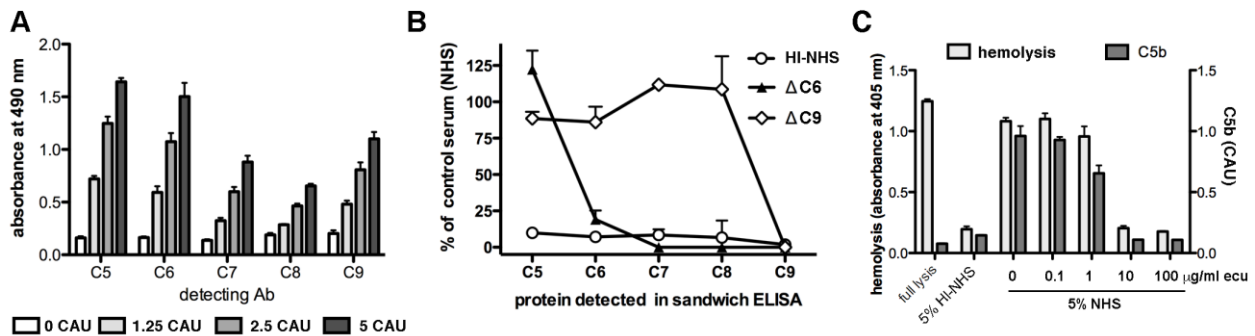
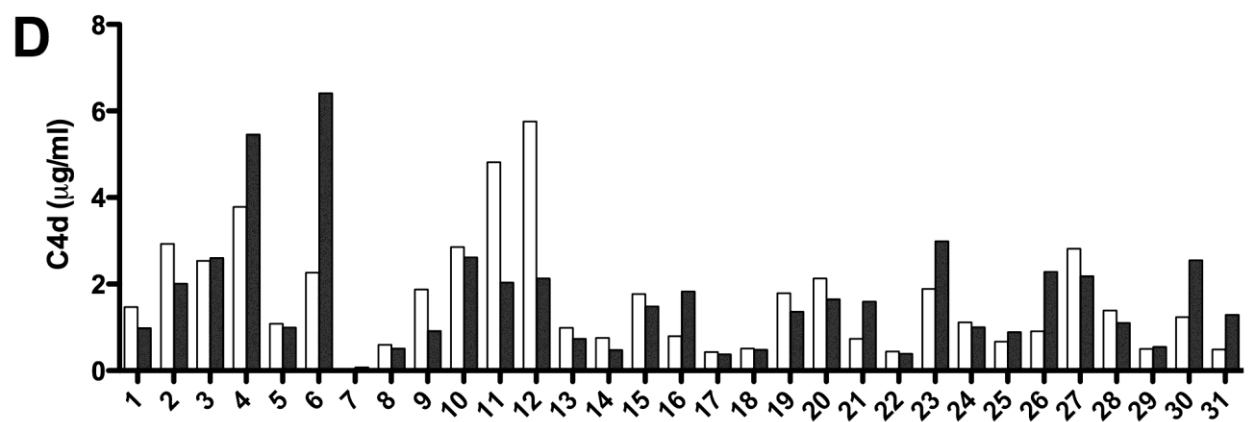
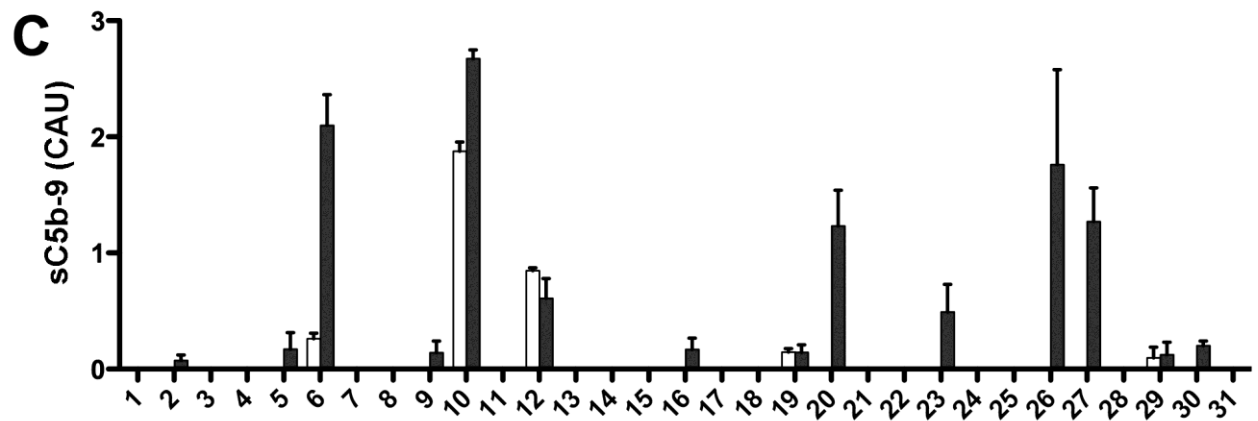
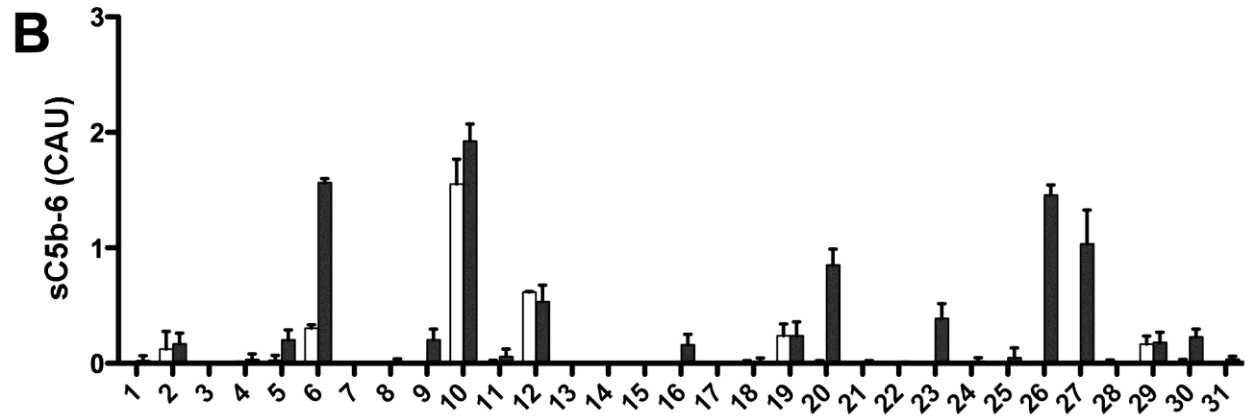
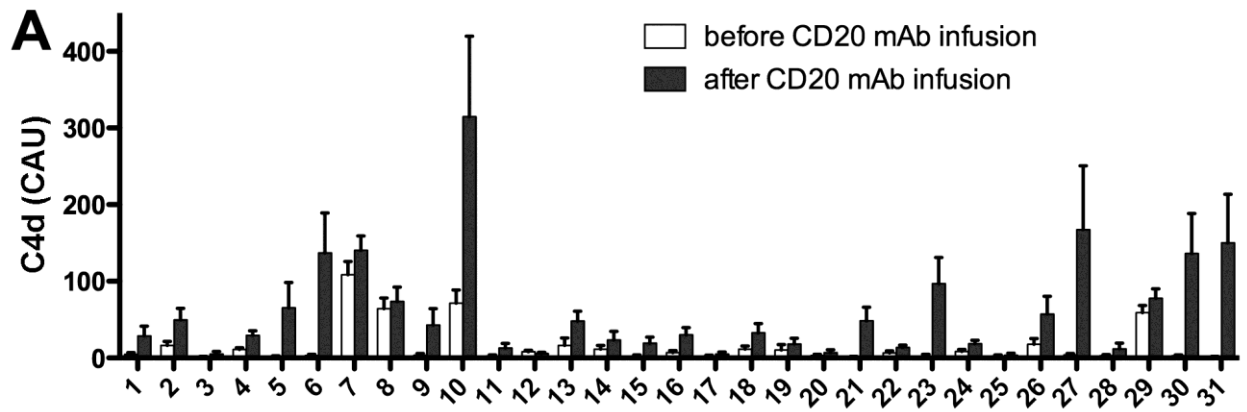
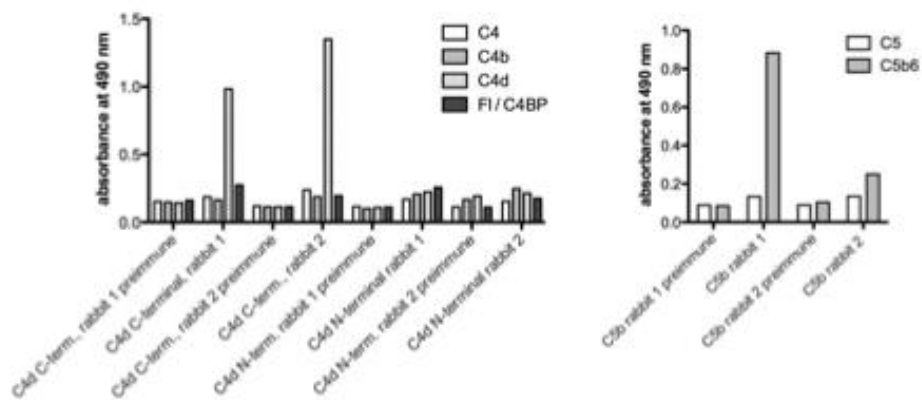


Fig. 4 Increase of soluble markers of complement activation after immunotherapy with CD20 mAbs.

EDTA-plasma samples collected from patients with B cell malignancies before and after the first infusion of therapeutic anti-CD20 mAb were assayed for soluble C4d (A), sC5b-6 (B) and sC5b-9 (C) using sandwich ELISAs based on our new antibodies reactive to short linear neoepitopes. Plasma samples were diluted 1:25 (4 % final concentration) for C4d measurement and 1:2.5 (40 % final concentration) for sC5b-6 and sC5b-9 assay, respectively. Presented data were collected from three independent experiments and error bars show standard deviation. Results are expressed in complement activation units (CAU), according to ICS#2. The same samples were tested for C4d content by Quidel's C4d MicroVue ELISA (D).





Supplementary Figure 1 - screening for specific antibodies in sera from immunized rabbits.

Two rabbits were immunized with peptides corresponding to C-terminal C4d fragment or N-terminal C4d fragment or C5b fragment. Presence of specific antibodies in antisera and pre-immunization sera was checked by direct ELISA. In case of C4d (left panel) plates were coated with 10 µg/ml of C4 or C4b (both from Complement Technologies) or with mixture of C4b and plasma-purified FI and C4BP (10 µg/ml of each, incubated for 30 min at 37°C) or with the same mixture without C4b. In case of C5b, plates were coated with 10 µg/ml of C5 or C5b6 (both from Complement Technologies). Pre-immune serum or antiserum from each rabbit was tested in dilution 1:100, followed by addition of goat-anti rabbit HRP (Dako, 1:1000). ELISA was developed with OPD tablets (Dako) and result was expressed as absorbance at 490 nm.

Table 1. Sequences of peptides used for immunization and purification of antibodies.

antigen	immunization	purification step 1	purification step 2
C-terminal C4d fragment	Cys-GGSSTGR	Cys-NVTLSSSTGR	Cys-NVTLSSSTGRNGFK
N-terminal C4d fragment	TLEIPGGG-Cys	not performed	not performed
C5b	LHMKTGGG-Cys	LHMKTLLPV-Cys	QLGRLHMKTLLPV-Cys

Table 2. Characteristics of the patients.

ID code	Diagnosis	Therapy
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#1	chronic lymphocytic leukemia	rituximab + bendamustine
#2	non-Hodgkin's lymphoma	rituximab + CHOP
#3	non-malignant autoimmune thrombocytopenia	rituximab
#4	diffuse large B cell lymphoma	rituximab + CHOP
#5	chronic lymphocytic leukemia	rituximab
#6	chronic lymphocytic leukemia	rituximab + FC
#7	chronic lymphocytic leukemia	rituximab + bendamustine
#8	mantle cell lymphoma	rituximab + bendamustine
#9	chronic lymphocytic leukemia	rituximab + FC
#10	mantle cell lymphoma	rituximab + CHOP
#11	chronic lymphocytic leukemia	rituximab + FC
#12	non-Hodgkin's lymphoma	rituximab + CHOP
#13	autoimmune haemolytic anemia	rituximab
#14	chronic lymphocytic leukemia	rituximab + bendamustine
#15	follicular lymphoma	rituximab + CHOP
#16	chronic lymphocytic leukemia	rituximab + bendamustine
#17	non-Hodkins lymphoma	rituximab + CHOP
#18	non-Hodgkin's lymphoma	rituximab + CHOP
#19	non-Hodkins lymphoma	rituximab + CHOP
#20	Waldenström's macroglobulinemia	rituximab + bendamustine
#21	follicular lymphoma	rituximab + bendamustine
#22	non-Hodgkin's lymphoma	rituximab + CHOP
#23	chronic lymphocytic leukemia	rituximab + bendamustine
#24	non-Hodgkin's lymphoma	rituximab
#25	follicular lymphoma	rituximab + bendamustine
#26	chronic lymphocytic leukemia	rituximab + bendamustine
#27	Waldenström's macroglobulinemia	rituximab
#28	follicular lymphoma	rituximab
#29	non-Hodgkin's lymphoma	rituximab + bendamustine
#30	chronic lymphocytic leukemia	ofatumumab
#31	chronic lymphocytic leukemia	ofatumumab

Abbreviations:

CHOP: cyclophosphamide, hydroxydaunorubicin, oncovin, prednisone

FC: fludarabine, cyclophosphamide