Analysis of systemic complement in experimental renal injury and disease

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Chapter 2

Functional assessment of rat complement pathway activities and quantification of soluble C5b-9 in an experimental model of renal ischemia/reperfusion injury.

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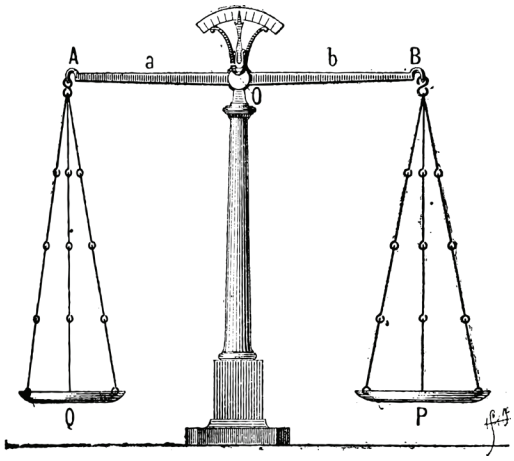

ABSTRACT

There is a growing interest in the monitoring of complement activation, not only in clinical settings but also in experimental models. However, for rodents only a limited number of tools are available to assess complement activity and activation. Here we describe three ELISAs for measurement of rat classical (CP), MB-lectin (LP) and alternative (AP) pathway activities in serum and plasma. Moreover, we optimised a soluble C5b-9 (sC5b-9) ELISA for detection of low level complement activation in rat. We determined the conditions for correct sample handling and showed that the assays had low inter- and intra-assay variation. We applied these assays to monitor complement activation in an experimental rat model of renal ischemia/reperfusion injury. We did not observe major complement consumption following reperfusion in CP or LP, and only minor AP consumption at 24h post reperfusion. However, MBL depletion prior to ischemia/reperfusion using a monoclonal antibody, transiently and specifically inhibited 75% of LP activity and ameliorated the AP consumption at 24h. To further assess complement activation during renal IRI, we monitored serum sC5b-9 and found that it was only significantly increased 72h post-reperfusion, but not when rats were pre-treated with anti-MBL or after sham surgery. In conclusion the described assays enable sensitive, reproducible and comprehensive assessment of complement activation in experimental rat models.

1. INTRODUCTION

The complement system is an essential component of the host innate immune system, not only with an important role in protection against pathogens, but also in the clearance of apoptotic or necrotic cells [1]. The complement system consists of more than 30 abundant serum and membrane-bound proteins, and three major activation pathways. The classical pathway (CP) is initiated via C1q binding to immunoglobulins, the lectin pathway (LP) is initiated via mannan binding lectin (MBL) or ficolins 1-3, which recognise carbohydrate moieties on pathogens and altered self-structures. The alternative pathway (AP) can be initiated directly through C3 deposition on unprotected surfaces or through the specific pattern recognition molecule properdin. The three pathways converge at the level of C3 resulting in activation of terminal sequence of complement activation, and formation of soluble effectors (C3a, C5a), which recruit inflammatory cells to site of activation, membrane bound opsonins (C3b, C4b) which promote phagocytosis, and C5b-9 which induces lysis of pathogens or damaged host cells [1].

Deficiencies in complement can result in susceptibility to recurrent infections [2] whereas deficiencies in complement regulators can lead to uncontrolled complement activation and destruction of unprotected tissues, like in atypical haemolytic uremic syndrome (aHUS) [3]. The CP is essential for antibody mediated host protection. CP can also be activated through allo- or autoantibodies, resulting in damage to transplants and destruction of host tissues in autoimmune diseases such as in systemic lupus erythematous (SLE), rheumatoid arthritis (RA) and anti-glomerular basement membrane disease [5]. Organ damage associated with ischemia/reperfusion injury can also lead to complement activation, which further aggravates existing injury and promotes local inflammation [6].

Assessment of complement activation is an important tool to monitor complement-mediated diseases and may be studied at different levels; 1) the functional assessment of serum complement activity, 2) the quantification of complement factors and/or their activation products in sera or other body fluids, or 3) histological determination of complement factors in affected tissues. Traditionally haemolytic assays have been used to determine classical (CH50) and alternative (AP50) pathway activities in serum, both for *in vivo* and *in vitro* applications. Recently, standardised ELISAs based assays have been described for measurement of human functional complement activities of the three major pathways [7]. ELISA-based assays can be used for the determination of major complement abnormalities, for the monitoring of complement pathway activities following therapeutic intervention and to enable the *in vitro* efficacy and function of inhibitory compounds. Finally, soluble products of complement activation such as soluble C3d, C5a and sC5b-9 have been used to assess human complement activation in different diseases *in vivo* and *vitro* [8–10].

Experimental rat and mouse models are essential to understand the role of complement activation in disease and to determine the efficacy of novel therapeutic intervention strategies. Recent developments in complement therapeutics, such as the approval of a novel C5 targeting antibody (Eculizumab) for treatment of aHUS and paroxysmal nocturnal hemoglobinuria (PNH), has opened possibilities for treatment of other disease conditions [11, 12]. In addition, a number of novel complement therapeutics have been developed and are being evaluated both in human trials and animal models [13]. However, investigating the complement system and complement therapeutics in rodents remains challenging due to the limited number of species specific assays.

In the current study we describe three ELISAs for the measurement of the three functional complement pathways in rat serum and plasma. Furthermore we optimised a previously published rat sC5b-9 ELISA, to measure rat sC5b-9

in serum and plasma with high sensitivity [14]. We established that our assays were sensitive, specific and reproducible and determined the conditions of sample handling to prevent artefacts of complement measurements. These assays were used to monitor complement activation in an experimental model of renal ischemia/reperfusion injury (IRI) in rats, where we recently have shown that *in vivo* depletion of MBL prior to renal IRI ameliorates renal damage and results in improved renal function [15].

2. METHODS

2.1 ANIMAL SAMPLE MATERIAL ACQUISITION

The Animal Care and Use Committee of the Leiden University Medical Center approved all experiments and material acquisitions from rats. Standard material for assay setup and sample stability studies were obtained from 12 week old Female Wistar rats, 7 weeks old male PVG rats and 12 weeks old female PVG/c C6-deficient rats. Serum and plasma samples were prepared from blood drawn from CO_2 euthanized rats through heart puncture and blood was placed directly on ice. EDTA-plasma was collected with syringes pre-treated with EDTA and tubes with a final EDTA concentration of 10 mM. Lepirudin-plasma was collected with syringes pre-treated with Lepirudin (r-hirudin; Refludan, Pharmion Germany GmbH, Hamburg, Germany) and tubes with a final concentration of 50 μg/ml. Blood was kept on ice for 30-120 min, centrifuged twice 3000g for 10 min at 4°C. All plasma and serum samples were aliquoted to single use batches and stored at -80°C. Heat inactivated serum was prepared by incubating fresh rat serum at 56°C for 2h before aliquoting and storing at -80°C.

2.2 FUNCTIONAL ASSESSMENT OF RAT COMPLEMENT ACTIVITY WITH PATHWAY ELISAs

The pathway activity of rat serum was assessed with functional pathway ELISAs, which were developed and standardized based on published work on human assays [7]. In short, purified human IgM (in-house, LUMC, Leiden, the Netherlands; Roos et al., 2003) was coated at 3 μg/ml for the CP ELISA, 10 μg/ml mannan for the LP ELISA (M7504, Sigma-Aldrich, St. Louis, MO) and 3 μg/ml LPS from strain Salmonella enteritidis for the AP ELISA (HK4059, Hycult Biotech, Uden, the Netherlands). IgM and mannan were coated in carbonate buffer pH 9.6 (100 mM Na_2CO_3 / $NaHCO_3$) and LPS in PBS with 10 mM $MgCl_2$ overnight at room temperature (RT) on Nunc Maxisorp plates (Thermo Fisher Scientific, Rochester, NY). Following steps were incubated 60 minutes at 37°C and after each step the plates were washed three times with PT (PBS / 0.05% Tween 20). CP and LP plates were blocked with PB (PBS / 1% BSA) and samples diluted into BVB++ buffer (Veronal buffered Saline / 0.5 mM $MgCl_2$ / 2 mM $CaCl_2$ / 0.05% Tween 20 / 1% BSA, pH 7.5). AP plates were not blocked and samples were diluted in BVB++/MgEGTA buffer (BVB++ / 10mM EGTA / 5 mM $MgCl_2$). C5b-9 deposition on functional pathway ELISAs was quantified with biotinylated mouse anti-rat C5b-9 neoepitope detecting mAb 2A1 (HM3033-IA, Hycult Biotech) and Streptavidin-HRP conjugate (Hycult Biotech) diluted in PBT (PBS / 1% BSA / 0.05% Tween20). TMB (3,3`,5,5`-Tetramethylbenzidine) K-Blue was used as substrate (Neogen Europe, Auchincruive, Scotland, United Kingdom). The colorimetric substrate step was 30 min at room temperature and was stopped with 50 μl 1 M H_2SO_4 and read at 450 nm with a BioRad 550 instrument (Tokyo, Japan).

2.3. RAT FUNCTIONAL PATHWAY ELISA PERFORMANCE, SPECIFICITY AND REPRODUCIBILITY

The performance of the functional pathway ELISAs was evaluated with Wistar serum (NRS) and heat inactivated NRS (ΔNRS) with reciprocal dilutions from 10% (CP, twofold), 5% (LP, twofold) and 7.5% (AP, 1.5 fold). A standard for these assays was established with lyophilized Fischer F334 rat serum (Hycult Biotech). Undiluted standard was set to 100% pathway activity (AU/ml). Next, pathway activities were assayed from 5% Wistar serum (NRS), EDTA- and Lepirudin plasma and from PVG/c C6-deficient serum. Specificity of functional pathway ELISAs was assessed with selective inhibition of the different pathways. All inhibitions were done in 5% Wistar serum and the inhibitors or chelators were incubated with the serum for 30 minutes at 4°C before transfer to the ELISA plates and analysis in triplicate. CP was inhibited with 100 μg/ml rabbit anti rat C1q (HP8021, Hycult biotech) and controlled with 100 μg/ml rabbit IgG (X0936, Dako, Glostrup, Denmark), LP was inhibited with 30 mM D-mannose (M3655, Sigma- Aldrich). CP and LP activity was inhibited with 10 mM EGTA (Ethylene glycol-bis(2-aminoethylether)-N,N,N´,N´-tetraacetic acid; 03779, Sigma-Aldrich). Inhibition of all three pathways was achieved with 30 mM EDTA (Ethylenediaminetetraacetic acid; E9884, Sigma-Aldrich). The intra-assay variation (IAV) of functional pathway ELISAs was assessed with measurement of single sample in 96 replicates and calculating the variation in the obtained OD450nm values. The interassay variation (AAV) was evaluated with six serum samples measured by two different operators. IAV and AAV measurements were done in duplicate from single dilution and values calculated as pathway specific activity (AU/ml).

2.4. RAT sC5b-9 ELISA

Sensitive rat sC5b-9 ELISA was developed based on published work [14], with optimization of coat and detection antibody concentrations, and improved detection reagents, resulting in standardised assay (HK106, Hycult Biotech). In short, the rat sC5b-9 complex is captured with mouse anti-rat C5b-9 neoepitope recognizing mAb clone 2A1 (HM3033-IA, Hycult Biotech, Uden, the Netherlands), detected with biotinylated mouse anti-rat C6 mAb 3G11 (HM3034, Hycult Biotech) and Streptavidin-poly-HRP (Hycult Biotech) with TMB K-Blue as substrate (Neogen Europe). A standard for the rat sC5b-9 ELISA was prepared by incubation of rat serum with 2 mg/ml zymosan (Z4250, Sigma-Aldrich) for 2h at 37°C, centrifuged at 3000g for 10 min, aliquoted, lyophilized and stored at 4°C or -20°C. The undiluted standard was set to 100 sC5b-9 units (AU/ml). Antibodies and enzymes were provided in lyophilized or prediluted stocks that were reconstituted into dilution buffer (Hycult Biotech). Samples were prepared in PTE (PBS / 0.05% Tween / 10mM EDTA), analysed in duplicate and incubated on plate for 60 minutes at 4°C, detection steps were incubated for 60 minutes at room temperature. After each step the plate was washed three times with wash buffer (PBS, 0.05% Tween20). The Colorimetric substrate step was 30 min at room temperature and stopped with 50 µl 1M H_2SO_4 and read at 450 nm with a BioRad 550 instrument (Tokyo, Japan).

2.5. RAT sC5b-9 ASSAY PERFORMANCE, SPECIFICITY AND REPRODUCIBILITY

The performance of the rat sC5b-9 ELISA was evaluated with reciprocal dilutions of zymosan activated serum (ZAS), normal Wistar rat serum (NRS) and C6-deficient PVG/c serum. Linearity of the detection was established by plotting ZAS standard OD450nm values against known sC5b-9 (AU/ml) values. SC5b-9 was determined in Wistar serum, EDTA- and Lepirudin-plasma. The

intra-assay variation of the sC5b-9 ELISA was established with six samples, each diluted separately for four times and measured in duplicate in one assay. The interassay variation was established by measuring six samples in duplicate on four different days.

2.6. ASSESSMENT OF SERUM AUTOACTIVATION AND FREEZE-THAW STABILITY

The stability of functional complement activities and sC5b-9 levels were evaluated at different temperatures and followed over time. Aliquots of serum were incubated at 4°C (either with or without dilution in ELISA buffer), at room temperature (RT), or at 37°C, and incubated for periods up to 120 minutes. Pathway activity and sC5b-9 levels were quantified and compared to fresh sample. Change in pathway activity was determined as percent change compared to a fresh sample. Change in sC5b-9 concentration was determined as fold increase compared to a fresh sample. All measurements were done in duplicate.

Stability upon repeated freeze-thawing of functional complement activity and sC5b-9 levels was investigated for Wistar serum, EDTA- and Lepirudin-plasma by using individual aliquots of samples with one to five freeze-thaw cycles between -80°C and melting ice. Complement pathway activities and sC5b-9 were determined from single dilutions of these sample measured in duplicate. Change in pathway activities and sC5b-9 were determined as percent activity left in the sample compared to fresh sample.

2.7. RAT MODEL OF RENAL ISCHEMIA/REPERFUSION INJURY

A rat model of renal kidney ischemia/reperfusion injury (IRI) was performed as described previously [15] on eight-week-old male Lewis rats (200–250 g) purchased from Harlan (Horst, The Netherlands). The animals were housed in standard laboratory cages and were allowed free access to food and water ad libitum. Unilateral ischemia was induced by clamping of the left renal pedicle for 45 min using a bulldog clamp (Fine Science Tools, Heidelberg, Germany). After clamping the contralateral kidney was removed. Anti-MBL treated animals received intravenously anti-rat MBL-A mAb (IgG1;P7E4; 1 mg/kg) 5 min before reperfusion. In sham-treated animals the contralateral kidney was removed but the remaining kidney was not clamped.

Two groups were established of IRI (n=11) and anti-MBL treatment (n=5) in which serum was collected before clamping (Pre), 1h, 24h, 48h, and 72h post reperfusion. Serum creatinine was measured from all samples using standard autoanalyser methods by LUMC hospital research services. In a second experiment, IRI (n=18) was compared with sham operated rats (n=6) and animals were sampled for serum before clamping (Pre) and 72h post reperfusion.

2.8. STATISTICAL ANALYSIS

Kurskall-Wallis one-way analysis of variance was used to analyse the results of the first study with anti-MBL, followed by Dunn's multiple comparison test with significance set to $P<0.05$ to determine significance of change compared to the pre-ischemia/reperfusion samples in each group. In the follow-up study for determination of sC5b-9 in renal IRI, two way ANOVA and bonferroni post-test was used to compare IRI, Sham and MBL-treated animals with significance set to $p<0.05$.

3. RESULTS

3.1 DEVELOPMENT AND CHARACTERISATION OF FUNCTIONAL COMPLEMENT ELISAS FOR RAT

Functional complement ELISAs were developed in analogy to the human assays which measure C5b-9 deposition resulting from classical (CP), MB-lectin (LP) and alternative pathway activation on specific immobilised ligands on ELISA-plates [7]. A monoclonal antibody specific for a rat C5b-9 neoepitope [14] was used to quantify *in vitro* formed C5b-9 complex on the ELISA plates as a readout of serum activity of the respective pathways. Normal rat serum showed a dose-dependent activity for all three pathways, reaching a plateau with a 1/10 dilution, whereas heat-inactivation completely abolished C5b-9 deposition (Fig 1A). The assay was specific for detection of the terminal pathway, as shown by absence of C5b-9 deposition in C6-deficient PVG/c sera (Fig 1B). Importantly, complement activity could be determined with similar efficiency in serum, EDTA-plasma and lepirudin plasma (Fig 1B).

Specificity of the assays was further assessed with selective inhibition of the activation pathways. In comparison to fresh rat serum, 30 mM EDTA completely inhibited all three pathways, whereas 30 mM Mg-EGTA did inhibit CP and LP, but left AP activity intact (Fig 1C). Inhibition of mannan binding lectin (MBL) with D-mannose resulted in absence of LP activity on mannan coated plates, but did not affect CP or AP activity. C1q-neutralization with a polyclonal antibody resulted in a specific inhibition of the CP (Fig 1C). Therefore we conclude that the assays described can specifically determine the three pathways of complement activation in rat serum or plasma samples.

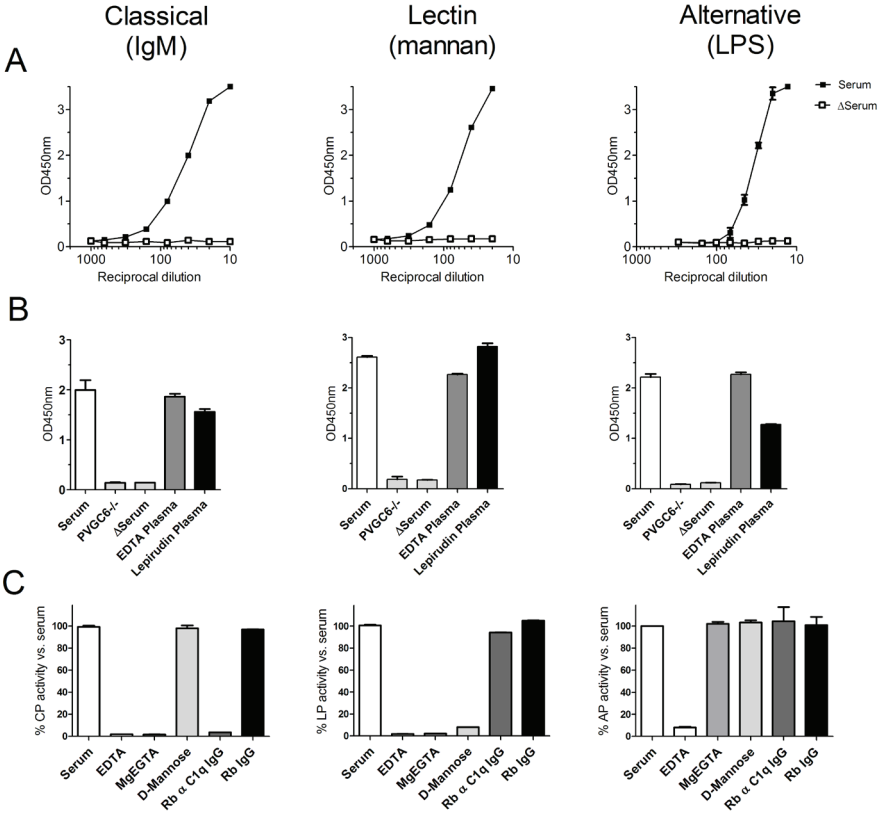

Fig 1. Development and specificity of rat functional pathway ELISAs. (A) *The activities of three complement pathways were assessed in Wistar serum in comparison with heat-inactivated serum (⊥serum) to determine aspecific signals in these assays. Samples were analysed in duplicate starting from 10% sera in twofold reciprocal dilutions.* **(B)** *Complement pathway activities in Wistar serum, EDTA- and Lepirudin plasma were assessed together with ⊥serum and PVG/c (C6-deficient) serum as controls. Activity was measured in duplicate; CP from 1/40, LP from 1/20 and AP from 1/40 diluted sample.* **(C)** *The specificity of each pathway ELISA was assessed using selective inhibition of complement activation with 30 mM EDTA which blocks all activation or 10 mM MgEGTA, which blocks only CP and LP. LP was specifically inhibited with 30mM D-Mannose which blocks LP activation via MBL. CP was specifically inhibited with 100 μg/ml polyclonal anti-C1q, equal amount of isotype polyclonal antibody was used as control. Activity was determined from two or more replicates and calculated as relative to fresh rat serum.*

3.2. STABILITY OF RAT COMPLEMENT ACTIVITY AND ASSAY REPRODUCIBILITY

To investigate how sample handling might affect complement activity, we tested the stability of serum at different temperatures (Fig 2A). CP and LP were found to remain stable for 120 min at 4°C and RT, with only minor loss of activity after 120 min at 37°C. In contrast, we found that 20-25% of AP activity was lost after 30 min at 4°C. However this could be avoided when samples were stored on melting ice. More than 50% of AP activity was lost within first 30 min at RT and 37°C. Diluted samples incubated at 4°C followed the same pattern as undiluted serum at 4°C (data not shown).

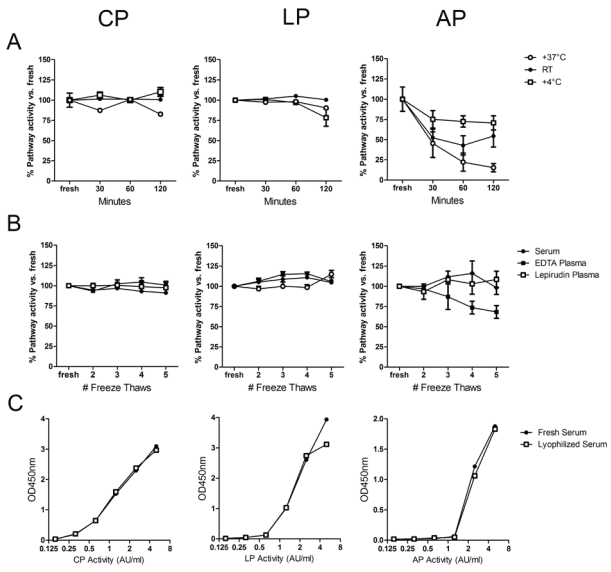

Fig 2. Stability of rat complement activity. (A) *The stability of complement activity was assessed by incubation of fresh serum at 4°C, Room Temperature (RT) and 37°C up to 120 minutes, as indicated, after which the pathway specific activity was measured. All samples were analysed in duplicate and change in activity was calculated as percent change relative to fresh sample* **(B)** *The effect of freeze thawing on serum, EDTA- and Lepirudin plasma complement activity was assessed by subjecting samples to several rounds of freeze-thaws between -80°C and melting ice as indicated followed by measurements. Change in activity was assessed as percent change to fresh sample.* **(C)** *Activity of lyophilized and fresh F334 rat serum standards (set to 100 AU/ml) were analysed in reciprocal dilutions to assess impact of serum lyophilisation to complement activity.*

INTERASSAY VARIATION OF THE FUNCTIONAL PATHWAY ACTIVITY ELISAS

	CLASSICAL PATHWAY			*LECTIN PATHWAY*			*ALTERNATIVE PATHWAY*		
Sample	**CP Activity (AU/ml)**	**SD**	**CV**	**LP Activity (AU/ml)**	**SD**	**CV**	**AP Activity (AU/ml)**	**SD**	**CV**
Serum 1	*98.2*	*4.8*	**4.9**	*199.5*	*6.7*	**3.3**	*98.6*	*0.5*	**0.5**
Serum 2	*46.7*	*0.5*	**1.0**	*105.9*	*1.4*	**1.3**	*37.8*	*1.0*	**2.8**
Serum 3	*45.9*	*0.6*	**1.4**	*116.9*	*2.4*	**2.1**	*68.3*	*1.0*	**1.5**
Serum 4	*31.0*	*3.8*	**12.3**	*85.3*	*5.0*	**5.8**	*69.9*	*6.6*	**9.4**
Serum 5	*30.4*	*4.3*	**14.0**	*85.0*	*3.3*	**3.9**	*68.5*	*5.2*	**7.6**
Serum 6	*29.6*	*4.5*	**15.3**	*87.5*	*4.0*	**4.6**	*71.3*	*5.9*	**8.2**
	Average CV		**8.1**	*Average CV*		**3.5**	*Average CV*		**5.0**

Table 1. Interassay variation of functional pathway activity ELISAs. *Six serum samples of different activities were assessed for pathway activities. Samples were analysed in duplicate on four assays performed on different days.*

Next we determined whether repeated freeze-thawing had an impact on complement activities measured in serum, EDTA- or lepirudin plasma, provided that samples were kept on melting ice and handling time was minimised (Fig 2B). Only AP measured from EDTA-plasma was observed to lose 25% of activity after five cycles of freeze-thawing. Lyophilisation of serum did not change the functional activity of any of the three pathways, thereby enabling long term storage and generation of a standard (Fig 2C). This standard was set at 100 AU/ml, thereby allowing quantification and comparison between different experiments. The performance of the assay was further validated by determination of intra- and interassay variation, which were found to be 7.7 – 10.9% and 3.5 – 8.1% (Table 1) respectively.

INTRA- AND INTERASSAY VARIATION OF THE sC5b-9 ELISA

	INTRA-ASSAY VARIATION			*INTERASSAY VARIATION*		
Sample	**sC5b-9 (AU/ml)**	**SD**	**CV**	**sC5b-9 (AU/ml)**	**SD**	**CV**
Serum 1	*0.362*	*0.037*	*10.3*	*0.396*	*0.036*	**9.2**
Serum 2	*0.182*	*0.018*	**9.9**	*0.19*	*0.018*	**9.3**
Serum 3	*0.355*	*0.06*	**16.9**	*0.389*	*0.061*	**15.6**
Serum 4	*0.217*	*0.008*	**3.9**	*0.233*	*0.013*	**5.4**
Serum 5	*0.211*	*0.018*	**8.6**	*0.218*	*0.019*	**8.7**
EDTA-Plasma	*0.187*	*0.02*	**10.5**	*0.188*	*0.018*	**9.5**
	Average CV		**10.0**	*Average CV*		**9.6**

Table 2. Reproducibility of the sC5b-9 ELISA. *Variation of the sC5b-9 ELISA was assessed with six samples; five sera and one EDTA-plasma sample. Intra-assay variation was established by diluting each sample four times and analysis in duplicate. Interassay variation was established by measuring the six samples in duplicate on four different days.*

3.3. FUNCTIONAL COMPLEMENT ACTIVITIES IN AN EXPERIMENTAL RAT MODEL OF RENAL IRI

The functional complement ELISAs were used to monitor complement activity following experimental renal ischemia/reperfusion injury (IRI). This model is characterized by a rapid decline in renal function and a significant increase of serum creatinine from 24h onwards (Fig 3A). Apart from a 30% reduction of AP at 24h, significant complement consumption was not observed following I/R. In contrast we observed a significant increase of the CP 48 – 72h post reperfusion ($p<0.0001$) (Fig 3B). As shown before [15], we observed that treatment with anti-MBL, prior to reperfusion, ameliorated the loss of renal function as measured by serum creatinine (Fig 3A). Anti-MBL treatment resulted in transient and significant inhibition of the LP observed at 1h ($P<0.05$) (Fig 3E), without affecting CP or AP (Fig 3C, 3G). LP activity returned to

baseline at 24h. Despite the protective effect of anti-MBL on renal function, the observed increase of CP was also present in the anti-MBL treated rats, although not reaching statistical significance. In conclusion, we have shown that the functional complement pathway activity assays are suitable tools to assess *in vivo* complement activity.

3.4. RAT SOLUBLE sC5b-9 ELISA SETUP AND SPECIFICITY

We hypothesized that the detection of soluble products of complement activation may be more sensitive for the detection in circulation of local renal complement activation. We optimised a previously described rat sC5b-9 ELISA [14], resulting in a commercially available standardised assay for detection of rat sC5b-9 in fresh serum (Fig 4A). In a direct comparison we found that the amount of circulating sC5b-9 in normal serum represents approximately 1% of the maximum, as observed in zymosan- activated serum (Fig 4A). No reactivity was observed in PVG/c (C6-deficient) serum, further confirming the specificity of this assay (Fig 4A).

Standard for the assay was generated by activating serum with Zymosan at 37°C. Lyophilisation of this standard did not result in loss of sC5b-9 activity. Therefore lyophilised zymosan activated serum could be used as a stable standard. Linearity of sC5b-9 detection was good (R^2=0.985) and detection limit of sC5b-9 in normal serum was found to be 0.02 AU/ml, which is at least 10 fold lower than typical serum basal level (Fig4B). Furthermore, sC5b-9 can be measured in serum, EDTA- and Lepirudin plasma (Fig 4C).

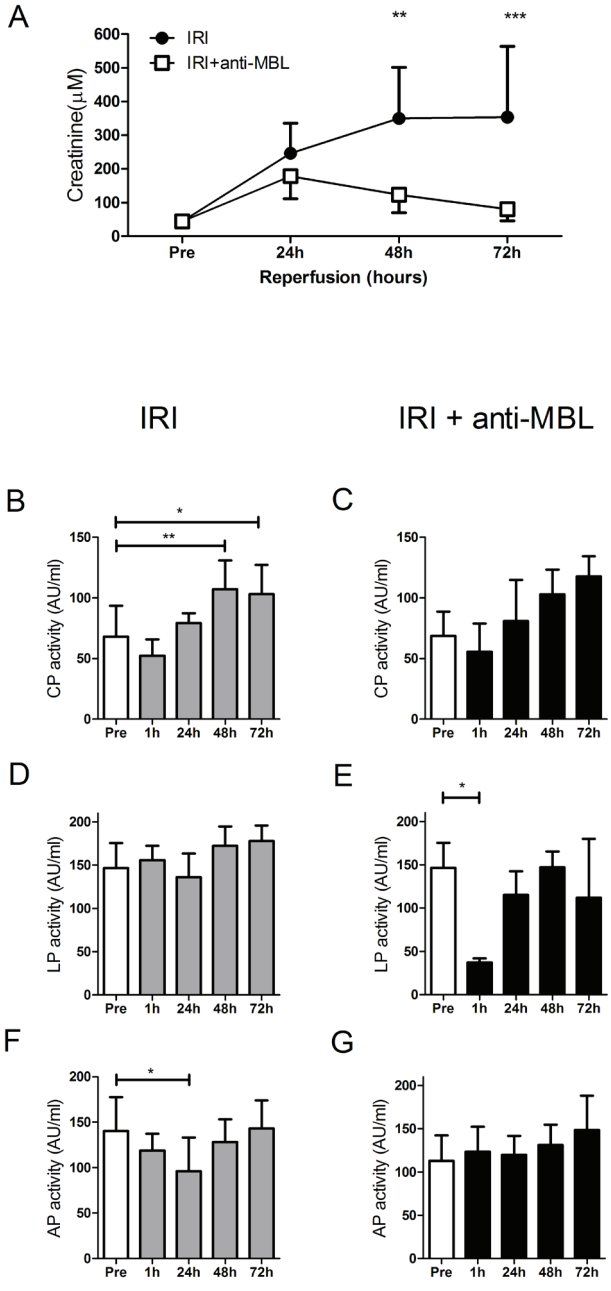

Fig 3. Serum creatinine and functional complement pathway activities in a rat model of renal ischemia/reperfusion injury. *IRI group (n=11) and anti-MBL treated IRI group (n=5) were analysed by measuring serum creatinine* **(A)**, *classical pathway* **(B and C)**, *lectin pathway* **(D and E)** *and alternative pathway activity* **(F and G)** *in pre-IRI samples and at 24h, 48h and 72h after reperfusion. Statistical analysis was performed with two-way ANOVA to compare the effect of treatment to creatinine levels and Kurskall-Wallis one-way ANOVA followed by Dunn's multiple comparison test with significance set at P<0.05 to assess changes in pathway activities following reperfusion. Data are plotted as the mean + SD.*

3.5. RAT sC5b-9 STABILITY AND ASSAY VALIDATION

To avoid inadvertent *ex vivo* generation of rat sC5b-9, we determined whether serum sC5b-9 remains stable up to 120 min when samples are stored at 4°C (Fig 4D) or when diluted in assay buffer at 4°C (data not shown). However, storage of serum at ambient temperature (18-20°C) or at 37°C resulted in rapid increase in serum sC5b-9 already after 15 min (Fig 4D).

The SC5b-9 present in serum or plasma is relatively stable when samples are subjected to repeated freeze-thaw, showing only 25% loss of sC5b-9 after five consecutive thawing cycles (Fig 4E). The sC5b-9 ELISA intra- and interassay variations were determined, and did not exceed 11.0% (Table 2).

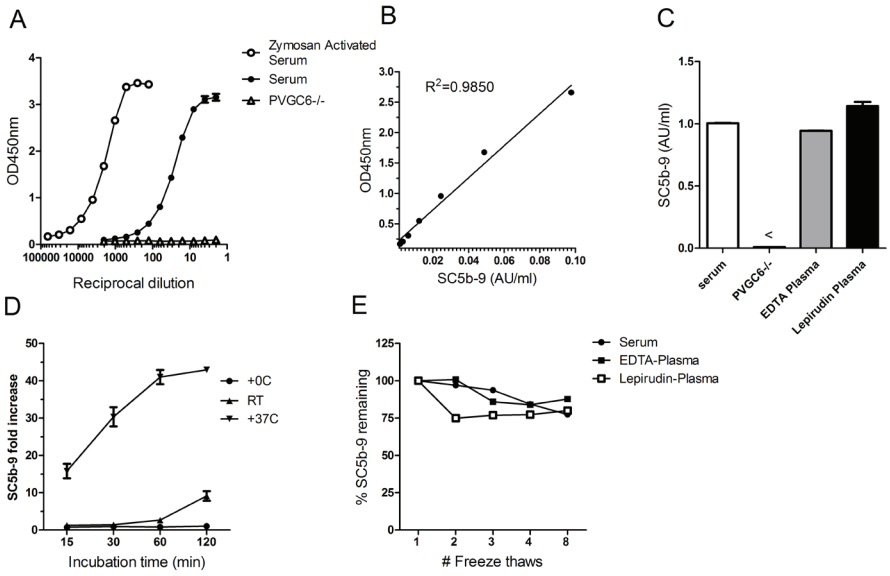

Fig 4. Development of a rat sC5b-9 ELISA and determination of sample stability. (A) *Soluble C5b-9 was measured in Wistar Zymosan-activated serum (ZAS), Wistar serum and C6-deficient (PVGC6-/-) serum, with reciprocal dilutions starting from 2% (ZAS) or 50% (Serum).* **(B)** *Linear regression of standard curve was determined by plotting OD450nm values of sC5b-9 standard against known sC5b-9 (AU/ml).* **(C)** *sC5b-9 was determined in fixed dilution of serum, EDTA- and Lepirudin plasma prepared from age and sex matched Wistar rats. PVG/c C6-deficient serum was used to determine non-specific background* **(D)** *Serum autoactivation to the level of sC5b-9 was assessed by incubating fresh serum at 4°C, Room Temperature (RT) and 37°C up to 120 minutes as indicated and then sC5b-9 was measured.* **(E)** *Freeze-thaw stability of sC5b-9 in serum, EDTA- and Lepirudin-plasma was assessed by subjecting samples to repeated freeze-thaw between -80°C and melting ice and then assayed for sC5b-9 in duplicate. Freeze-thaw loss was determined as percentage loss compared to fresh sample. For all measurements error bars represent standard deviation.*

3.6. SERUM SC5B-9 IN RAT ISCHEMIA/ REPERFUSION INJURY

The optimised sC5b-9 ELISA was used to assess complement activation following experimental renal ischemia/reperfusion injury. Serum sC5b-9 shows only minor variation in pre-IRI samples, and did not change early after IRI (Fig 5A). However, from 48h onwards there was an increase in sC5b-9, reaching significance at 72h (p=0.02) (Fig 5A). It should be noted that individual rats showed a large heterogeneity in the amount of sC5b-9. This increase of sC5b-9 at 72h was specific for the IRI group, and not observed in sham-operated rats (p=0.0005) (Fig 5B), or when rats were treated with anti-MBL (p=0.05) (Fig 5C).

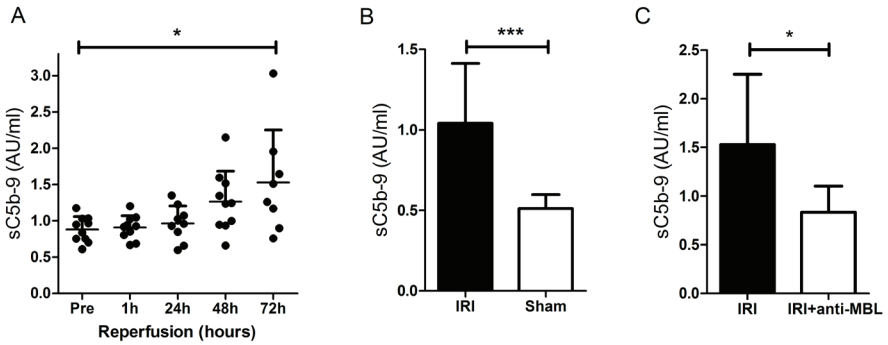

Fig 5. Serum sC5b-9 in a rat model of renal ischemia/reperfusion injury. (A) *sC5b-9 was determined in serum of rats with IRI (n=11), before IRI (Pre) and at 1h, 24h, 48h and 72h after reperfusion.* **(B)** *Specificity of serum sC5b-9 increase to renal IRI was assessed with determination of SC5b-9 from IRI (n=18) and Sham (n=6) treated animals sacrificed at 72h post reperfusion.* **(C)** *Effect of systemic anti-MBL treatment to sC5b-9 following IRI was assessed at 72h after reperfusion with renal IRI (n=8) and anti-MBL treated IRI group (n=4). One-way ANOVA with Dunn's post-test (A) and Two-way ANOVA with bonferroni post-test (B and C) with significance level set to 0.05 were used in analysis of statistical significance.*

4. DISCUSSION

Studies on the contribution of complement activation in experimental animal models have predominantly relied on histological assessment of complement deposition at the damaged site. To broaden the possibilities to study complement in experimental models we have established three ELISAs to measure functional complement pathways in rat serum or plasma and which can be applied both for *in vivo* and *in vitro* studies. Furthermore we have established a rat sC5b-9 ELISA for monitoring of complement activation *in vivo*. We show that this sensitive ELISA is able to detect signs of complement activation in an experimental model of rat renal IRI.

The assays were shown to be broadly applicable to different types of sample, including fresh serum, EDTA- or Lepirudin plasma, which is in line previous studies on functional assays for human complement activity (Seelen et al., 2005; Mollnes et al., 2007). Although these assays are not directly analogous to traditional haemolytic assays, which use primed red blood cells for complement activation, the use of C5b-9 deposition as a read-out guarantees that the complete complement cascade will be monitored. Still since ELISAs use immobilised ligands for initiation of a specific pathway, this might lack the natural cell surface conditions for activation, as recently illustrated to be essential for CP-mediated activation via IgG hexamers [17]. Nevertheless, measurements of CP and AP in human serum using both types of assays has been shown to be strongly correlated [7]. The ELISA based system has the additional advantage that lower amounts of material are required and information can also be obtained for LP. In our experiments we have therefore fully concentrated on the ELISA and have not made direct comparisons with haemolytic assays.

Concerning the different sample types, rat lepirudin plasma was shown to have less AP activity in comparison to serum or EDTA plasma. Therefore, for proper comparison within an experimental model, only one type of sample should be used. Our results show that in contrast to the human assays, the rat assays do not require additional inhibitors to ensure specificity [7]. The rat SC5b-9 ELISA optimisation resulted in sensitive and reproducible assay. The improved sensitivity of the SC5b-9 assay, compared to the original format [14], allowed detection of basal levels of rat sC5b-9 from serum or plasma, and thereby the small increases observed during IRI. This is in line with the analogous human assay [18].

The complement system can autoactivate rapidly if samples are handled or stored improperly [19]. Here we show that exposure of rat serum to elevated temperatures results in rapid generation of sC5b-9, gradual loss of AP activity, but not in prominent loss of CP and LP activity. AP linked autoactivation is therefore the likely source for sC5b-9 that is observed to increase during incubation. However, rat complement activity can be preserved and *ex vivo* activation avoided if serum or plasma is kept on melting ice while handling and when samples are stored at -80°C. In addition, repeated freeze-thawing of fresh serum or Lepirudin plasma does not result in major degradation of complement activity or sC5b-9. However our results showed that EDTA-plasma seems to be more prone to AP specific degradation of activity compared to serum or Lepirudin plasma. In conclusion correctly handled samples can be analysed repeatedly for functional activities, sC5b-9 or other complement factors of interest, which is also true for human sC5b-9 [20].

Functional complement assays were used to validate the efficacy of therapeutic inhibition of MBL prior to experimental renal IRI, showing a transient and near complete inhibition of lectin pathway 1h post reperfusion. No other changes in lectin pathway activity were observed, suggesting that renal MBL deposition occurring after renal IRI [21] is not extensive enough to be

reflected in systemic LP consumption in our model. Intriguingly we observed a progressive and specific increase in CP activity, irrespective of treatment. This would suggest a non-specific inflammatory response to anaesthesia or surgery, possibly through upregulation of C1q as LP and AP were not affected. Although C1q has not been reported to be an acute phase protein, there is evidence of C1q upregulation in response to IL-6 and tissue damage [22–24], which are prominently present in acute kidney injury such as I/R [25].

As a next step we evaluated whether sC5b-9 would be more sensitive in monitoring complement activation following localised complement activation. Our results show that sC5b-9 increases relatively late after reperfusion, reaching a maximum at 72h post reperfusion. Furthermore, the results suggest that the increase in sC5b-9 is related to the IRI damage, as sham operated animals and anti-MBL treated animals had significantly less sC5b-9 at 72h. Reports on sC5b-9 from human and animal studies have shown that the sC5b-9 does not increase prominently following localized complement activation but can be useful in situations where significant complement consumption is not present [9, 26].

The current results suggest that generation of sC5b-9 is a relatively late process in renal IRI. However, this seems in contradiction with the prevailing information that C5b-9 might be pivotal for the induction of renal IRI injury [27]. Still the results are in line with our clinical study showing that early after renal transplantation there is only minimal and transient release of sC5b-9 [28]. Furthermore, in our rat model of renal IRI, C5b-9 deposition in the kidney is detected only after 24h [15]. Future studies are required to disseminate the role of sC5b-9 in renal IRI and how measurement in circulation reflects complement activation at the tissue level.

Together these results show that sC5b-9 and functional pathway ELISAs are specific, sensitive and relatively simple to perform especially for large

scale studies. More importantly we show that the standardised assays can be implemented into experimental rat models and are valuable tools to evaluate complement inhibition *in vivo* and *in vitro*. In conclusion, the functional pathway and sC5b-9 ELISAs enable comprehensive monitoring of complement activity to better understand the contribution of complement to disease and to evaluate novel therapeutic strategies.

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