Functional assessment of mouse complement pathway activities and quantification of C3b/C3c/iC3b in an experimental model of mouse renal ischaemia/reperfusion injury.

Juha P. Kotimaa
Maaike B. van Werkhoven
Joseph O’Flynn
Ngaisah Klar-Mohamad
Jan van Groningen
Geurt Schilders
Helma Rutjes
Mohamed R. Daha
Marc A. Seelen
Cees van Kooten.

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ABSTRACT

The complement system is an essential component of our innate immunity, both for the protection against infections and for proper handling of dying cells. However, the complement system can also contribute to tissue injury and inflammatory responses. In view of novel therapeutic possibilities, there is an increasing interest in measurement of the complement system activation in the systemic compartment, both in the clinical setting as well as in experimental models. Here we describe in parallel a sensitive and specific sandwich ELISA detecting mouse C3 activation fragments C3b/C3c/iC3b, as well as functional complement ELISAs detecting specific activities of the three complement pathways at the level of C3 and at the level of C9 activation. In a murine model of renal ischaemia/reperfusion injury (IRI) we found transient complement activation as shown by generation of C3b/C3c/iC3b fragments at 24 h following reperfusion, which returned to baseline at 3 and 7 days post reperfusion. When the pathway specific complement activities were measured at the level of C3 activation, we found no significant reduction in any of the pathways. However, the functional complement activity of all three pathways was significantly reduced when measured at the level of C9, with the strongest reduction being observed in the alternative pathway. For all three pathways there was a strong correlation between the amount of C3 fragments and the reduction in functional complement activity. Moreover, at 24 h both C3 fragments and the functional complement activities showed a correlation with the rise in serum creatinine. Together our results show that determination of the systemic pathway specific complement activity is feasible in experimental mouse models and that they are useful in understanding complement activation and inhibition in vivo.
INTRODUCTION

The complement system is a robust and tightly regulated first line of defence against invading pathogens, and essential in proper clearance of injured host cells (Walport, 2001). However, loss of complement regulation, due to extensive damage or inadvertent activation, is central in several systemic and organ-specific diseases (Lesher and Song, 2010; McCullough et al., 2013). Activation of the complement system is initiated by specific pattern recognition molecules; the classical pathway (CP) is activated for instance via C1q binding to surface deposited immunoglobulins (Kishore and Reid, 2000), the lectin pathway (LP) is activated via mannan binding lectin (MBL) or Ficolins that recognise specific carbohydrate moieties on pathogens (Petersen et al., 2001) and altered self-structures (Collard et al., 2000). The alternative pathway (AP) can be initiated directly through C3 deposition on damaged or unprotected surfaces, or through properdin acting as a specific pattern recognition molecule (Spitzer et al., 2007). Activation for terminal pathway produces C5a, which promotes local inflammation and recruits inflammatory cells, and mC5b-9 (terminal complement complex), which can lyse unprotected cells and promote apoptosis of damaged host cells (Nauta et al., 2002; Walport, 2001).

Renal ischaemia/reperfusion injury (IRI) is a multifactorial condition, where local and systemic factors contribute to the development of acute kidney injury and tubular necrosis. Complement activation has been observed following renal IRI in human renal biopsies, and evidence from experimental animal models suggests prominent contribution to the overall injury (Arumugam et al., 2004; Danobeitia et al., 2014; McCullough et al., 2013). Studies with knockout mice and selective blocking of complement factors such as fB, have shown that AP is the major activation pathway in experimental renal IRI (Miwa et al., 2013; Thurman et al., 2006; Zhou et al., 2000). Furthermore, inhibition of C5a and C5b-9 formation reduces the renal injury, confirming the central role for complement mediated damage in renal IRI (de Vries et al., 2003a; De Vries et al., 2003b).

Although detection of complement deposition in mouse tissues is well established (Mastellos et al., 2004; Trouw et al., 2005), determination of the systemic complement activity in mouse is still challenging. Haemolytic assays for mouse are impaired by their sensitivity (De Vries et al., 2009; Klerx et al., 1983), whereas C3a and C5a are extremely labile ex vivo and are rapidly cleared from circulation by cellular receptors in vivo (Kirschfink and Mollnes, 2003; Oppermann and Götze, 1994). Several groups have recently described the development and use of antibodies specific for neo-epitopes on C3 activation fragments to quantify C3 activation in mouse (Lesher et al., 2013; Møller-Kristensen et al., 2005; Thurman et al., 2013). However, assay sensitivity, analysis of sample type preference and the stability of mouse
C3 activation fragments have not been extensively described. We have recently described methodology for specific ELISAs measuring functional complement pathways in rats, in analogy to described human assays (Seelen et al., 2005). The functional complement ELISAs are analogous to the haemolytic complement assays in that each pathway may be activated independently with a specific ligand. In functional complement ELISAs the resulting formation of intermediate or terminal complement activation products are quantified using specific antibodies, and the degree of product deposition on ELISA plate reflects the activity in biological sample. Together with sensitive measurement of soluble C5b-9 (sC5b-9) we showed that functional complement ELISAs are ideal in validating in vivo inhibition, but that experimental rat renal IRI alone did not lead to significant systemic consumption of complement components, and that generation of sC5b-9 was a relatively late event after IRI (Kotimaa et al., 2014; van der Pol et al., 2012). Until now ELISAs to measure specific pathways of complement activity in mice have been limited to the detection of C3 deposition (Trouw et al., 2005), whereas human rat assays measure the pathway activities to the level of C5b-9 deposition.

To better understand changes in systemic complement following experimental renal IRI in mouse, we developed in parallel an ELISA for C3 activation fragment (C3b/C3c/iC3b) using the neoepitope-specific monoclonal antibody (mAb) clone 2/11 (Mastellos et al., 2004), and six functional complement ELISAs for pathway specific activity measurement at the level of C3 and C9. We provide information on the specificity of these assays and the requirements for sample handling. Together these assays allowed us to profile systemic complement changes after experimental renal IRI in mouse, showing major complement consumption in line with previously published results.

METHODS

2.1 Animal materials
The Animal Care and Use Committee of the Leiden University Medical Center (LUMC) approved all animal experiments. The C57bl/6 mice were purchased from Charles River, the C3−/− and C4−/− mice (both on C57bl/6 background) were provided by Marina Botto (Imperial College, London, U.K) and Mike Carroll (Harvard Medical School, Boston, MA) and bred as described previously (Otten et al., 2009). A/J mice with natural C5-deficiency (Wetsel et al., 1990) were purchased from Jackson laboratories (Bar Harbour, ME). The CD1 serum (NMS) was purchased from Innovative Research (Novi, MI). NMS and plasmas were prepared from CO₂ euthanized mice via heart puncture, stored on ice and prepared as described previously (Kotimaa et al., 2014). Briefly; serum (NMS) was let to clot 1 h at 4°C and supernatant was collected. EDTA- and Lepirudin plasmas were prepared by adding 10 mM EDTA or 50 µg/ml Lepirudin (r-hirudin; Pharmion, Germany) to the collected blood. To remove clot and cells,
the samples were centrifuged twice at 3000-5000 g for 10 min at 4°C, and supernatant was pooled, aliquoted and stored at -80°C.

2.2 Anti-mouse C9 polyclonal antibody
Polyclonal antiserum (pAb) against recombinant mouse C9 (rC9) was obtained by immunization of male New Zealand White rabbits (Harlan) with rC9 (kind gift of Prof. Piet Gross, Utrecht, The Netherlands). Injection of 30 µg rC9 in 100 µl complete Freund’s adjuvant (Difco, Detroit, MI) subcutaneously was followed by three boosts with 30 µg mouse rC9 in 100 µl incomplete Freund’s adjuvant (Difco) at 2-week intervals. Rabbit pAb anti-mouse C9 was prepared as described previously (Trouw et al., 2004), with minor modifications: fractions were tested for the presence of anti-mouse rC9 reactivity with a direct ELISA. ELISA plate was coated with purified mouse rC9 at 2.5 µg/ml, serial dilutions of the fractions in PBS/0.05% Tween/1% BSA were tested and binding of rabbit IgG was demonstrated using goat anti-rabbit IgG conjugated to HRP (Jackson ImmunoResearch Laboratory Inc., PA). Mouse rC9 reactive fractions were pooled, concentrated and dialysed against PBS.

2.3 Mouse C3b/C3c/iC3b ELISA
The mouse C3 fragments are captured with rat anti-mouse monoclonal specific to C3b/C3c/iC3b (clone 2/11, HM1065, Hycult Biotechnology, The Netherlands) (Mastellos et al., 2004), coated at 5 µg/ml on Nunc Maxisorp plates (Thermo Fisher Scientific, NY) with CB buffer (100 mM Na$_2$CO$_3$/NaHCO$_3$, pH 9.6) 16 h at room temperature (RT). Assay volume was 50 µl/well and each incubation step was 1 h at 37°C, except for sample incubation which was performed at 4°C. After each step the wells were washed 4 x 5 min with PT (PBS/0.05% Tween 20). First, the plates were blocked with PB (PBS/1% BSA) and samples were diluted in PTB/E (PBS/1% BSA/0.05% Tween 20/10 mM EDTA). C3b/C3c/iC3b was detected with 8 µg /ml biotinylated rabbit anti-mouse C3 pAb (HP8012, Hycult Biotech), Streptavidin-HRP (Hycult Biotech) and TMB Plus2 (Kem-En-Tek, Denmark). The colorimetric substrate of all ELISAs was 15-30 min at room temperature and stopped with 50 µl 1 M H$_2$SO$_4$ and read at 450 nm with a BioRad 550 instrument (Tokyo, Japan). Standard for the assay was prepared incubating CD1 NMS (IMSCD1-COMPL, Innovative Research) with 4 mg/ml zymosan (Z4250, Sigma-Aldrich, MO) for 2 h at 37°C, centrifuged at 3000 g for 10 min, the supernatant was collected and stored at -20°C. The undiluted standard was set to 100 arbitrary units per ml (AU/ml).

2.4 Mouse C3b/C3c/iC3b ELISA characterisation
The performance of the C3b/C3c/iC3b ELISA was evaluated with reciprocal dilutions of zymosan activated serum (ZAS) and fresh C57bl/6 NMS. Next, C3b/C3c/iC3b was determined from NMS, EDTA- and Lepirudin plasma prepared from C57bl/6 mice, together with NMS from C57bl/6 C3$^+$ and A/J C5$^+$ mice. Generation of C3b/C3c/iC3b during sample preparation was evaluated from matched NMS and EDTA plasma samples from male C57bl/6 mice (n = 5).
Intraplate variation was established with one sample diluted and measured 16 times, intra-assay variation (IAV) was determined with three samples measured on four assays at the same time, and interassay variation (AAV) was established with seven samples measured at four different times. All measurements were done in duplicate.

2.5 C3 and C9 functional complement ELISAs

Functional complement ELISAs were developed and standardized based on published work on human and rat assays (Kotimaa et al., 2014; Seelen et al., 2005). In short, human IgM (in-house, LUMC, Leiden, The Netherlands) (Roos et al., 2003) was coated at 1 µg/ml for CP, 10 µg/ml mannan for LP (M7504, Sigma-Aldrich) and 1 µg/ml LPS from strain S. enteritidis for AP (HK4059, Hycult Biotech). IgM and mannan were coated in CB buffer and LPS in PBS/10 mM MgCl₂ for 16 h RT on Nunc Maxisorp plates (Thermo Fisher Scientific). Each incubation step was 1 h 37°C and plates were washed 3 x 5 min with PT (PBS/0.05% Tween 20). CP and LP plates were blocked with PB and samples diluted into BVB++ buffer (Veralon buffered Saline/0.5 mM MgCl₂/2 mM CaCl₂/0.05% Tween 20/1% BSA, pH 7.5). AP samples were diluted in BVB++/MgEGTA (BVB++/10mM EGTA/5 mM MgCl₂). C3 functional complement ELISAs were detected with 0.5 µg/ml biotinylated rat anti-mouse C3b/C3c/iC3b (HM1065-BIO, Hycult Biotech) and Streptavidin-HRP conjugate (Hycult Biotechnology) in PBT. C9 functional complement ELISAs were detected with 5 µg/ml Digoxigenin conjugated rabbit anti-mouse rC9 (in-house, LUMC) and anti-DIG-HRP (Roche Diagnostics, Germany) in PBT. C3 functional complement ELISA was developed with TMB Plus2 (Kem-En-Tek), and C9 functional complement ELISAs with TMB XTRA (Kem-En-Tek). Assay standard was established with CD1 NMS (Innovative Research), set to 100 arbitrary units per ml (AU/ml).

2.6 Mouse functional complement ELISA performance, specificity and reproducibility

Performance of the functional complement ELISAs was evaluated with fresh (NMS) and heat inactivated (ΔNMS) C57bl/6 sera, with reciprocal 1.5 fold dilutions from 20% NMS in assay buffer. Sample activity was determined as percent activity versus standard sample activity with equation: 

\[
\text{Sample activity} = \frac{(\text{Sample OD450 nm} - \text{Reagent OD450 nm})}{(\text{Reference OD450 nm} - \text{Reagent OD450 nm})} \times \frac{\text{Sample dilution}}{\text{Reference dilution}} \times 100
\]

Specificity of assays was evaluated with complement inhibitory compounds and CD1 NMS: all three pathways are inhibited with 30 mM EDTA (E9884, Sigma-Aldrich), and CP and LP activities were inhibited with 30 mM EGTA (03779, Sigma-Aldrich). CP and AP were inhibited with 200 µg/ml polyanetholesulfonic acid (SPS, P2008, Sigma-Aldrich), as described elsewhere (Palarasah et al., 2010). LP was inhibited with 100 mM D-Mannose and controlled with 100 mM L-Mannose (M3655 and M1134, Sigma-Aldrich). Functional complement activities in complement deficient NMS were determined at concentration ranges 1.5-3.3% (C3) and 3.0-6.6% (C9). Sample type compatibility for functional complement analysis was assessed with NMS, EDTA, and Lepirudin plasmas collected from
age and sex matched C57bl/6 mice. All of the samples were analysed in reciprocal twofold dilutions from 10% (C9) or 2.5% (C3). Intraplate variation was determined measuring single sample 16 times, intra-assay variation (IAV) was determined with three samples analysed in reciprocal dilutions by two operators simultaneously, and AAV was determined with six samples measured four separate times. All measurements were done in duplicate or triplicate.

2.7 Assessment of complement stability in mouse samples
Freeze-thaw stability of C57bl/6 NMS, EDTA and Lepirudin plasmas was assessed with fresh samples subjected to 1–8 cycles of freeze-thaw between -80°C and melting ice (0-2°C), followed by C9 functional complement ELISA and C3b/C3c/iC3b measurements. Temperature stability of mouse NMS pathway activities was tested at 4°C, room temperature (RT, 18-22°C) or at 37°C for 30, 60, 120 or 240 min. C3b/C3c/iC3b temperature stability was tested with both NMS and EDTA plasma. Pathway activities were quantified and calculated as percent change vs fresh sample, and C3b/C3c/iC3b was calculated determined either as arbitrary units or as relative change to fresh sample. All measurements were done in duplicate.

2.8 Mouse model of renal ischaemia/reperfusion injury
The study was approved by the Institutional Animal Care Committee of the University of Groningen. The C57Bl/6 wild type mice were kindly provided by Bao Lu (Harvard Medical School, Boston) and bred at UMCG animal facility. Mice were housed in groups up to the experiments, and individually after surgery until sacrifice. Standard laboratory cages were used for housing with free access to food and water ad libitum.

Animals were anaesthetized with isoflurane/O₂. Body temperature was maintained at 37°C with heating pad during surgery and by a neonatal incubator during ischaemia. A midline abdominal incision was made and bilateral ischaemia was induced by applying two non-traumatic vascular clamps per renal pedicle for 40 min. During ischaemia, the wound was covered with cotton soaked in sterile saline. After removal of the clamps, the kidneys were inspected for restoration of blood flow. The wound was closed in two layers. Buprenorphine was applied subcutaneously for postoperative pain management. The animals were sacrificed 24 h after IRI or sham surgery (n = 8). At time of sacrifice, blood was collected for analysis and prepared as EDTA plasma.

2.9 Statistical analysis
Specificity of change was determined with nonparametric, two-tailed Mann-Whitney test with 95% confidence interval or with Kruskal-Wallis, 1-way ANOVA and Dunn’s Multiple Comparison post-test. GraphPad Prism version 5.00 software package was used for all statistical determinations.
RESULTS

3.1 Mouse C3b/C3c/iC3b ELISA
Zymosan-activated NMS (ZAS) was used to optimize the ELISA for C3b/C3c/iC3b, using mAb 2/11 as capture antibody (Mastellos et al., 2004), and rabbit polyclonal anti-mouse C3 as detecting antibody. The resulting ELISA was sensitive enough to accurately detect basal levels of C3b/C3c/iC3b in normal serum and plasmas, with a linear relation in the dilution range of 1/80 – 1/1280 (Figure 1A). Levels were similar in NMS, Lepirudin plasma and C5-deficient A/J NMS, but lower in EDTA plasma, and completely absent in C57bl/6 C3\(^{-}\) NMS (Figure 1B). Analysis of matched C57bl/6 NMS and EDTA plasma samples shows that levels of C3b/C3c/iC3b significantly increased during serum preparation (Figure 1C). C3b/C3c/iC3b in NMS and EDTA-P was stable at 4°C up to 30 min and rapidly increased after incubation of serum at RT and 37°C, whereas this increase was attenuated and less prominent in EDTA-P. Freeze-thawing did not impact C3b/C3c/iC3b in either NMS or EDTA-P (Supplementary Figure 1). Intraplate variation, IAV and AAV of the assay was 13.2%, 14.3% and 16.5% respectively (Table 1).

Table 1: Intraplate variation, intra-assay (IAV) and Interassay (AAV) variation of the developed ELISAs.

<table>
<thead>
<tr>
<th>C3b/C3c/iC3b</th>
<th>CP C3</th>
<th>LP C3</th>
<th>AP C3</th>
<th>CP C9</th>
<th>LP C9</th>
<th>AP C9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraplate variation</td>
<td>13.2</td>
<td>8.8</td>
<td>12.3</td>
<td>21.3</td>
<td>6.3</td>
<td>13.4</td>
</tr>
<tr>
<td>IAV</td>
<td>14.3</td>
<td>11.5</td>
<td>3.3</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV</td>
<td>16.5</td>
<td>13.0</td>
<td>13.0</td>
<td>24.3</td>
<td>13.3</td>
<td>18.4</td>
</tr>
</tbody>
</table>

Intraplate variation for all assays was determined with single sample which was measured 16 times in duplicate on one occasion. Intra-assay variation (IAV) was determined with three samples measured independently by two operators at the same time. Interassay variation (AAV) of C3b/C3c/iC3b ELISA was determined with measurement of seven samples with different levels of C3b/C3c/iC3b on four different times. AAV of functional C3 and C9 ELISAs was determined with measurement of six samples of different complement pathway activities on four different times.

3.2 Generation of plasma C3b/C3c/iC3b after renal IRI
Because NMS preparation was shown to cause \textit{ex vivo} C3b/C3c/iC3b generation, EDTA-P was used for \textit{in vivo} analysis. The renal IRI resulted in significant increase in plasma C3b/C3c/iC3b at 24 h post reperfusion, returning to baseline at day 7 as compared to sham operated mice (Figure 1D). Plasma creatinine was also significantly elevated at 24 h (Figure 1E), with return towards baseline 72 h and 7 d post reperfusion (data not shown). The observed acute decline of renal function and generation of C3b/C3c/iC3b were both heterogeneous between individual mice and showed a significant association at 24 h post reperfusion (p = 0.0058, \( r^2 = 0.43 \)) (Figure 1F).

3.3 Mouse Functional complement ELISAs
To be able to determine the pathway of complement activation implicated in this transient
response, we developed assays for and assessment of pathway specific complement consumption. We previously described a functional complement ELISA, which measures the complement system activity to the level of C3 activation using a polyclonal antibody (Trouw et al., 2005). To improve this assay we used a C3b/C3c/iC3b specific mAb in this study, replacing the pAb used previously. Furthermore, we developed three novel assays which measure pathway specific complement activity at the level of C9 activation with a novel polyclonal raised against mouse C9. Both for C3 (Figure 2A) and for C9 (Figure 2B), a linear range of detection was established with reciprocal dilutions of fresh C57bl/6 NMS. The assays were specific as demonstrated by the absence of non-specific signal with heat inactivated C57bl/6 serum (ΔNMS).
The specificity of functional complement ELISA at the level of C9 was determined with selective inhibition of complement activation: 30 mM EDTA inhibited C9 deposition in all pathways, whereas 30 mM EGTA left AP activity intact and inhibited all deposition on Ca\(^{2+}\) dependent CP and LP, showing that no AP activity was present on IgM and Mannan coated plates (Figure 3A). Specificity of the LP ELISA was demonstrated by more than 90% inhibition of LP using D-Mannose, but not L-Mannose, whereas D-Mannose did not specifically affect CP or AP. Finally, SPS was shown to inhibit CP and AP but not LP, showing that AP and CP do not contribute to LP (Figure 3A).

The functional complement ELISAs could be used for NMS, EDTA and Lepirudin plasmas, showing similar activity in NMS and EDTA plasma and reduced activity in Lepirudin plasma (Figure 3B). Furthermore, analysis of each sample type with reciprocal dilutions showed similar dose dependent activity with each functional complement assay (Supplementary Figure 2). Further evidence on specificity of the assays was established with complement deficient mouse NMS: C57bl/6 ΔNMS, C57bl/6 C3\(^{-/-}\) and A/J C5-deficient sera showed no signal at the level of C9 (Figure 3B), whereas C5-deficient NMS was active at the level of C3 activity (data not shown). C4\(^{-/-}\) NMS had normal AP activity and 80-90% reduced activity on
CP and LP compared to C57bl/6 NMS (Figure 3B). Intraplate variation of C3 and C9 functional complement ELISAs was 8.8-21.3 and 6.3-13.4 respectively. Intra-assay variation was 1.1-11.5 for the C9 functional complement ELISAs. AAV of C3 and C9 functional complement ELISAs was 13.0-24.3 and 13.3-18.4 respectively (Table 1). Freeze-thawing of serum and plasma did not result in measurable decline on functional complement activities at the level of C9 (Supplementary Figure 3A). However, incubation of serum at 37°C resulted in decline of all three pathways for both C3 and C9 pathway activity after 30 min, with up to 50% of the activity lost in all three pathways by 120 min (Supplementary Figure 3B). Together, the assays are compatible with NMS and plasma, the assays are specific, reproducible and the protocols described for sample preparation and handling do not result in artefacts.

### 3.4 Complement pathway consumption after renal IRI

Since the functional complement ELISAs were compatible with plasma, this allowed us to use the same 24 h EDTA plasma used for C3 fragments also for functional complement...
analysis. Renal injury did not result in significant consumption of the systemic complement when measured at the C3 level (Figure 4A). However, in the same samples a significant reduction in complement activity was measured at the level of C9 (Figure 4B). The observed loss of C9 activity was 39% of CP (p < 0.006), 40% of LP (p < 0.01) and 69% of AP (p < 0.001) when compared to sham operated mice (Figure 4B).

Figure 4: Determination of pathway specific functional complement activities following experimental renal ischaemia/reperfusion.

Plasma functional complement pathway activity was determined at the level of (A) C3 and (B) C9, 24 h post reperfusion from IRI and sham operated animals (n = 8). Significance of change was determined with nonparametric, two-tailed Mann-Whitney test with significance set to p < 0.05; error bars represent standard deviation.

Consumption of all three pathways, measured at the level of C9, showed significant correlation with the C3 activation fragments (p ≤ 0.0002) (Figure 5A). We then analysed the correlation between renal function and complement activity and found the strongest inverse correlation for AP C9 ($r^2 = 0.5402$, $p = 0.002$), followed by LP C9 ($r^2 = 0.392$, $p = 0.009$) and CP C9 ($r^2 = 0.344$, $p = 0.023$) (Figure 5B). Further analysis of IRI samples at 24 h post reperfusion showed a significant correlation between AP C9 and LP C9 but not between AP C9 and CP C9 ($p = 0.033$, $r^2 = 0.415$ and $p = 0.118$, $r^2 = 0.63$ respectively) (data not shown).
DISCUSSION

In this study we show that determination of pathway specific complement activities in mouse is feasible, and that assays described here allowed us to detect and characterise a transient complement activation associated with decline in renal function following experimental renal IRI in mice.

The C3b/C3c/iC3b ELISA is a sensitive tool for assessment of systemic complement activation, detecting basal level of C3b/C3c/iC3b in NMS and plasmas. We found that EDTA plasma has significantly lower basal level of C3 activation fragments compared to serum and Lepirudin plasma, suggesting that sample preparation results in artificial C3 activation, which earlier studies have not reported (Lesher et al., 2013; Mastellos et al., 2004). Coagulation is known to activate C3, which could explain that NMS and Lepirudin plasma, which both have complete or partial activation of coagulation cascades, result in higher levels of C3b/C3c/iC3b (Amara et al., 2008). Furthermore, in contrast to published assays, we perform sample incubation at 4°C with sample buffer containing EDTA to avoid inadvertent ex vivo coagulation and C3 activation. Repeated freeze-thawing did not result in loss or increase

Figure 5: Linear correlation of complement pathway activities, creatinine and C3b/C3c/iC3b.
Linear correlation of complement pathway activities at the level of C9 with (A) plasma C3b/C3c/iC3b and (B) plasma creatinine was determined from IRI and sham operated animals sacrificed at 24 h post reperfusion (n = 16).
in serum or plasma C3b/C3c/iC3b when kept carefully cold. Incubation of both serum and EDTA plasma at RT (18-22°C) or 37°C resulted in generation of C3b/C3c/iC3b fragments. This is in line with previous studies on in vitro lability of C3 (Pfeifer et al., 1999). Our results suggest that addition of futhan or K76COOH to sample and sample buffer, or careful handling as described here are required to avoid measurement artefacts (Hong et al., 1981; Pfeifer et al., 1999). Altogether, our results show that the assay to measure C3b/C3c/iC3b in plasma of mice is reproducible, accurate and sensitive.

The mouse functional complement ELISAs are analogous to the assays described for human (Seelen et al., 2005), for rat (Kotimaa et al., 2014) and in part for mouse (Trouw et al., 2005). The assays described here were shown to be specific through selective inhibition of complement activation and with C-deficient sera, in analogy to rat assays (Kotimaa et al., 2014). Interestingly the C4-deficient NMS showed measurable CP and LP activities present at the level of C3 and C9, which is in line with published literature on AP-mediated C1 bypass (May and Frank, 1973) and MASP-mediated C4 bypass (Schwaebel et al., 2011). Our analysis of sample type applicability shows that both serum and EDTA plasma can be used for mouse functional complement analysis with these protocols, as shown earlier for rat and human (Kotimaa et al., 2014; Seelen et al., 2005).

We found that mouse complement activity is stable for up to 60 min when stored on melting ice and otherwise at -80°C. Loss of functional complement activity in both serum and plasma is observed when serum is stored at ambient (18-22°C) or at 37°C for more than 30 min, which coincides with generation of serum C3b/C3c/iC3b. In analogy to the C3b/C3c/iC3b fragments, we did not observe decline of functional complement activities even after repeated freeze-thaw cycles when samples were handled with care.

We applied the assays to assess and characterise experimental mouse renal IRI. We show that C3b/C3c/iC3b fragments are generated during the first 24 h post reperfusion and that the fragments return to baseline by 72 h-7 d post reperfusion. This is in line with literature describing early complement consumption in experimental mouse renal IRI (De Vries et al., 2003a, 2003b). Although the magnitude of increase was relatively minor, it is in line with previous determinations with C3a desArg biomarker assay (Møller-Kristensen et al., 2005). Next we characterised the nature of complement activation using functional complement ELISAs at 24 h post reperfusion. Our measurements revealed consumption affecting all three pathways, which was detectable only at the level of C9. Consumption was most prominent with AP, in line with the observations that AP is very important for mouse IRI. Loss of pathway activity and generation of C3 activation fragments showed a strong correlation, establishing the usefulness of using C3 activation fragments as sensitive...
biomarkers of systemic consumption. The results suggest that C3 functional complement ELISAs may not be as sensitive in detection of the consumption as C9 assays. However it is possible that systemic consumption of both initiation factors such as properdin, fB and MBL, together with consumption of terminal pathway components C5-C9 is required to find detectable consumption with these functional complement ELISAs. Furthermore, microvascular coagulation has been shown to occur shortly after renal IRI which could result in direct activation of C3 and C5 independent of other initiation factors (Amara et al., 2008; Sutton et al., 2002).

Plasma creatinine had a strong correlation with AP and LP. AP consumption and LP consumption were also associated together, which seems in line with previous work showing prominent roles for both AP and LP but not CP (Miwa et al., 2013; Zhou et al., 2000). However, the current functional complement ELISAs alone may not be sufficient to distinguish heterogeneous complement activation, and further analysis of single factors should be performed. Interestingly, this mouse model showed acute consumption at 24 h post reperfusion, whereas our recent study in an analogous rat model did not show acute systemic complement activation (Kotimaa et al., 2014). This may be in part attributed to lower level of terminal pathway complement factors C5-C9 in mice compared to rats, resulting in more pronounced differences in mice after localised complement activation (Ong and Mattes, 1989). The mouse C3b/C3c/iC3b may also be more sensitive biomarker of complement activation compared to rat sC5b-9. Interestingly, in a rat model of renal IRI we observed a late increase in sC5b-9 (Kotimaa et al., 2014), which within the same time period was not observed with mouse C3b/C3c/iC3b. Together these results warrant careful interpretation of results from experimental models before translating to the human setting.

In conclusion, mouse renal IRI results in acute complement consumption that is associated with decline in renal function, activation via AP and LP and a normalisation 72 h after reperfusion. The assays described here are suitable for comprehensive assessment of complement activation in the course of experimental injury and disease in mice. They are valuable tools in understanding dynamics of complement activation, and useful in assessing the specificity of therapeutic intervention of complement in vivo and vitro.

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REFERENCES


Mouse Complement Pathway Analysis


Supplementary Figure 1: Autoactivation of mouse C3.
Temperature stability of C57bl/6 (A) serum and (B) EDTA plasma was assessed up to 240 min at 4°C, RT (18-22°C) and at 37°C. Sample C3b/C3c/iC3b was determined from duplicate measurements. (C) Freeze-thaw stability of C3b/C3c/iC3b in C57bl/6 serum and EDTA plasma was determined with repeated freeze-thaw cycles between -80°C and 4°C. Change in C3b/C3c/iC3b was calculated as percent change to fresh sample. Error bars represent standard deviation of replicates.

Supplementary Figure 2: Analysis of sample applicability in functional complement ELISAs.
C57bl/6 serum, EDTA and Lepirudin plasmas were analysed in reciprocal twofold dilutions starting from (A) 1/40 for C3 functional complement ELISA or (B) from 1/10 for C9 functional complement ELISAs. All samples were analysed in duplicate.
**Supplementary Figure 3: Stability of mouse complement.**

(A) C57bl/6 serum and EDTA plasma were subjected to repeated freeze-thaw cycles between -80°C and 4°C. Remaining activity was determined at the level of C9 as relative activity versus unthawed sample. (B) Temperature stability of C57bl/6 serum was assessed with incubation up to 240 min at 4°C, RT (18-22°C) and at 37°C. Change in activity was calculated as percent change to fresh sample. Error bars represent standard deviation of replicates.