Analysis of systemic complement in experimental renal injury and disease

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Chapter 6

Sex matters: Systemic complement activity of female C57BL/6J and BALB/cJ mice is limited by serum terminal pathway components.

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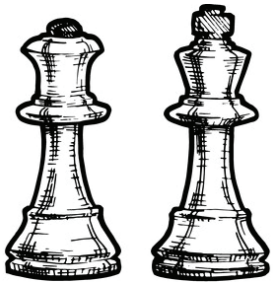

ABSTRACT

Experimental mouse models have been extensively used to elucidate the role of the complement system in different diseases and injuries. Contribution of gender has revealed an intriguing gender specific difference; female mice often show protection against most complement driven injuries such as ischemia/reperfusion injury, graft rejection and sepsis. Interestingly, early studies to the mouse complement system revealed that female mice have very low total complement activity (CH50), which is related to androgen regulation of hepatic complement synthesis. Here, our aim was to understand at which level the female specific differences in mouse complement resides. We have used recently developed complement assays to study the functional activities of female and male mice at the level of C3 and C9 activation, and furthermore assayed key complement factor levels in serum of age-matched female and male C57BL/6 mice. Our results show that the female mice have normal complement cascade functionality at the level of C3 activation, which was supported by determinations of early complement factors. However, all pathways are strongly reduced at the level of C9 activation, suggesting a terminal pathway specific difference. This was in line with C6 and C9 measurements, showing strongly decreased levels in females. Furthermore, similar gender differences were also found in BALB/cJ mice, but not in CD-1 mice. Our results clearly demonstrate that the complement system in females of frequently used mouse strains is restricted by the terminal pathway components and that the perceived female specific protection against experimental disease and injury might be in part explained by the inability promote inflammation through C5b-9.

1. INTRODUCTION

The complement system is an integral, albeit often overlooked, arm of the immune system that consist of more than 30 serum and membrane proteins. Traditionally the complement system has been classified as a serum based system forming the first line of defence against invading pathogens, acting either independently or as the effector arm of antibody specific responses [1, 2]. Recent experiments have shown that the complement system has a versatile role in adaptive and innate immunity, and is central in orchestrating inflammatory responses. Paradoxically, the complement system has also been shown to contribute to tissue injury, for example during ischemia/reperfusion injury (I/RI), sepsis or graft rejection [3].

Interestingly both BALB/c and C57BL/6 female mice have shown marked attenuation of renal I/RI and sepsis, which could be in part explained by the impaired total haemolytic activity in female mice [4–7]. The female-specific low complement activity has been linked to androgen specific control of hepatic expression of serum complement. Several studies have shown that castration of males result in diminished, and androgen supplementation in female mice results in increased total complement activity [8–10].

We have recently developed novel functional and standard complement assays for studying mouse complement, with a possibility to study the functional activities of the three major initiation pathways and the terminal pathway, which is responsible for the generation of complement effectors C5a and C5b-9 [11]. Therefore, to better understand the gender specific complement differences in C57BL/6J mice, we determined the serum concentrations of key complement factors and functional activities of the three major complement pathways at the level of C3 and C9 activation from age-matched female and male C57BL/6J mice. Our results show that female C57BL/6J mice have functionally similar complement systems as the male mice at the level of C3 activation and similar

serum levels of initiation factors and C3. Strikingly, female mice had nearly absent complement activity at the level of C9 activation and were characterized by low C6 and C9 serum concentrations. Furthermore, BALB/cJ mice but not CD-1 mice exhibit the same gender differences. Together these results can in part explain why experimental models reliant on C5b-9 formation show female specific protection.

2. METHODS

2.1 ANIMALS AND SAMPLE ACQUISITION

The Animal Care and Use Committee of the Leiden University Medical Center (LUMC) approved all experiments and material acquisitions from mice performed in Leiden. Material for sex specific difference profiling in C57BL/6J mice was acquired from 8 week old C57BL/6J mice purchased from Charles River Laboratories (Massachusetts, United States). The CD-1 serum (NMS) used as reference standard for functional assays was purchased from Innovative Research (Michigan, United States). Plasma samples were prepared from CO_2 euthanized mice via heart puncture and blood placed directly on ice. EDTA-plasma was collected with syringes pre-treated with EDTA and tubes with final EDTA concentration of 10mM. Blood was kept on ice for 30-120 min, centrifuged twice 3000-5000g for 10 minutes at 4°C. Samples were pooled and aliquoted to single use batches, or collected from individual mice and stored at -80°C. C57BL/6 C1q KO, C3 KO, fP KO and C5 deficient A/J sera were generated for internal control purposes as reported earlier [12, 13].

Strain specific gender differences were analysed in EDTA-Plasma acquired from 8 week old female and male mice housed and bred in Hannover Institute of Laboratory Animal Sciences (Hannover Medical School, Germany). Three strains were studied, including BALB/cJ, CD-1 and C57BL/6J(han-ztm) which

is similar to C57BL/6N in that the strain does not carry the spontaneous nnt-gene mutation (Ronchi et al., 2013).

2.2 DEVELOPMENT OF ANTIBODIES

Mouse anti-mouse mAb against mouse C3 was developed as described previously [15]. In short, mouse C3 was purified from C57BL/6 mouse serum first with DEAE sepharose fractionation, then, C3 containing fractions were pooled and fractionated further with CMC-50, and finally, separated from contaminating mouse IgG with Superdex HR-200 and HPLC. Mouse C3 containing fractions were pooled and used in immunisation of C57BLC57BL/6 $C3^{-/-}$ mice; 30 μg C3 in 100 μl complete Freund's adjuvant (Difco Laboratories Inc., Michigan, United States) was injected subcutaneously, followed by three boosts with 30 μg mouse C3 in 100 μl incomplete Freund's adjuvant (Difco) at 1-week intervals. Reactivity of antisera against C3 was tested on C3 coated plates, and the reactive animals proceeded for hybridoma fusion. Fusions were performed as described previously.

Polyclonal antibodies against mouse C3 and properdin have been described previously (O'Flynn et al., 2015, manuscript in preparation). Polyclonal antibodies against recombinant mouse C6 (rmC6) and C9 (rmC9) were obtained by immunization of male New Zealand White rabbits (Harlan, Indiana, United States) with purified rmC6 and rmC9 (kind gift of Prof Piet Gross, Utrecht, Netherlands). Injection of 30 μg rmC6 and rmC9 in 100 μl complete Freund's adjuvant (Difco Laboratories) subcutaneously was followed by three boosts with 30 μg mouse rC6 or rC9 in 100 μl incomplete Freund's adjuvant (Difco Laboratories) at 2-week intervals. Rabbit pAb was prepared as described previously [15], with minor modifications: fractions were tested for the presence of anti-mouse C6 or C9 reactivity using a direct ELISA. ELISA plate was coated with purified mouse rmC6 or rmC9 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., Pennsylvania, United States). Reactive fractions were pooled, concentrated and dialysed against PBS.

2.3 SDS-PAGE AND WESTERN BLOT ANALYSIS

Analysis of recombinant proteins was performed with 10% SDS-PAGE gel and unreduced samples: mouse IgG (4.2 μg), rmC6 (9.2 μg) and rmC9 (4.8 μg) were loaded and run under standard conditions followed by coomassie staining. Specificity of polyclonal antibodies against rmC6 and rmC9 was analysed with 10% SDS-PAGE run under non-reducing conditions followed by transfer to nitrocellulose membrane: rmC6 (1.2 μg) and rmC9 (0.6 μg) or 0.5μl pooled male C57BL/6 NMS were loaded and detected with either anti-C6-DIG (100ng/ml) or anti-C9-DIG (50ng/ml). Next, replicate westerns were developed for C6 or C9 detection with 0.5μl of NMS from four female and male C57BL/6 mice. Bound pAb was detected with anti-DIG-POD (Prod.no. 11207733910, Roche Diagnostics GmbH, Mannheim, Germany) and chemiluminescence (Supersignal, Pierce, Illinois, United States).

2.4 MEASUREMENT OF FUNCTIONAL PATHWAY ACTIVITIES AT THE LEVEL OF C3 AND C9 ACTIVATION.

Measurement of functional mouse pathway activities was performed as described earlier [13]. In short, purified human IgM (in-house, LUMC, Leiden, the Netherlands; Roos et al., 2003) was coated at 1 μg/ml for CP ELISA, 10 ug/ml mannan for LP (M7504, Sigma-Aldrich, Missouri, United States) and 3 ug/ml LPS from strain Salmonella enteritidis for AP (HK4059, Hycult Biotech, Uden, The Netherlands). IgM and mannan were coated in carbonate buffer pH 9.6 and LPS in PBS / 10 mM $MgCl_2$ over night at room temperature (RT) on Nunc Maxisorp plates (Thermo Fisher Scientific). Each incubation step

was 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 $MgCl_2$). Deposition of mouse C3b/C3c/iC3b on C3 functional ELISAs was detected with biotinylated rat anti-mouse C3b/C3c/iC3b mAb clone 2/11(HM1065, Hycult Biotech) [17] and Streptavidin-HRP conjugate (Hycult Biotech). Deposition of mouse C9 on functional pathway ELISAs was quantified with Digoxigenin conjugated rabbit anti-mouse C9 (in-house, LUMC) and anti-DIG-POD, Fab fragments (Roche Diagnostics GmbH) diluted in PBT (PBS / 1% BSA / 0.05% Tween20). TMB Plus2 was used as substrate for C3-functional ELISAs (Cat.no. 4395 Kem-En-Tek, Taastrup, Denmark), TMB XTRA was used for C9 functional ELISAs (Cat.no. 4800, Kem-En-Tek) and. The Colorimetric substrate step was 15-30 min at room temperature and stopped with 50 µl 1M H_2SO_4 and read at 450 nm with a BioRad 550 instrument (Bio-Rad Laboratories, Tokyo, Japan).

2.5 DETERMINATION OF COMPLEMENT FACTOR SERUM CONCENTRATIONS WITH ELISAS

All ELISAs were coated on Nunc Maxisorp plates (Thermo Fisher Scientific) with CB buffer (100 mM Na_2CO_3 /$NaHCO_3$, pH 9.6) 16 h at room temperature (RT). Assay volume was 50µl/well and each incubation step was 1 h at 37°C. After each step the wells were washed 4 x 5 min with PT (PBS / 0.05% Tween 20). First, plates were blocked with PB (PBS / 1% BSA) and samples diluted in PTB/E (PBS / 1% BSA / 0.05% Tween20 / 10mM EDTA). Mouse C1q was captured with Rabbit anti-mouse C1q pAb coated at 4µg/ml and detected with Rabbit anti-mouse C1q pAb-DIG as described earlier [18]. Mouse C3 was captured with mouse anti-mouse C3 mAb (clone 5F4G6) coated at 0.5µg/ml, and detected with Rabbit anti-mouse C3 pAb-DIG, assay specificity was

controlled with C3 KO NMS. Mouse properdin was captured with mouse anti-mouse properdin mAb (clone 17-17, kind gift of Prof. Wilhelm Schwaeble) coated at 2 μg/ml, and detected with Rabbit anti-mouse properdin pAb-DIG. Properdin ELISA specificity was controlled with properdin KO NMS. Mouse C6 was captured with Rabbit pAb anti-mouse recombinant C6 coated at 10 μg/ml and detected with Rabbit anti-mouse rC6-DIG at 100 ng/ml. Mouse C9 was captured with Rabbit pAb anti-mouse recombinant C9 coated at 10 μg/ml and detected with Rabbit anti-mouse rC9-DIG at 100 ng/ml. Rabbit anti DIG-POD (Roche Diagnostics) and TMB Plus2 (Kem-En-Tek) was used to quantify each ELISA. Specificity of pAbs against rC6 and C9 has been previously described in context of functional C3 and C9 ELISAs [11] and was further expanded with western-blot analysis here. Commercial assays were used to measure MBL-A (HK208, Hycult Biotech), MBL-C (HK209, Hycult Biotech), and mouse C5 (cat.no. abx056439, Abbexa Ltd, Cambridge, United Kingdom) according to manufacturer's specifications.

2.6 STATISTICAL ANALYSIS

Specificity of observed differences between gender groups was determined with nonparametric, two-tailed Mann-Whitney T-test with 95% confidence interval. GraphPad Prism Version 5.0 was used for all statistical analysis.

3. RESULTS

3.1 FUNCTIONAL ANALYSIS REVEALS SPECIFIC REDUCTION IN TERMINAL PATHWAY ACTIVITY IN FEMALE C57BL/6J MICE

To investigate a possible difference in complement activity between male and female C57BL/6J mice, we used the recently described and validated ELISA assays together with standard ELISAs described here and elsewhere [11]. At the level of C9 activation, female sera had a low but detectable dose dependent activity in all three pathways **(Fig 1A)**. At the level of C9, all three pathways exhibited significant, 4–5 fold lower activity: CP C9 (241±39 vs 65±6 AU/ml,

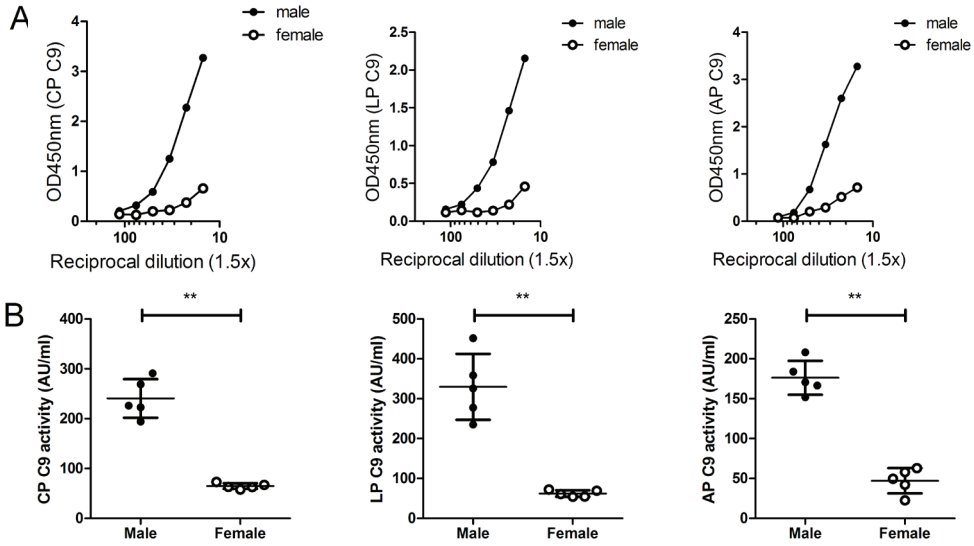

Fig 1. Functional serum complement activities at the level of C9 activation. (A) *Dose dependent activity of pooled male and female C57BL/6J serum on classical(CP), lectin (LP) and alternative (AP) pathway assays at the level of C9 activation was determined with reciprocal 1.5 fold dilutions of pooled serum starting from 1/15 dilution. Error bars represent standard deviation of duplicates.* **(B)** *Quantification of CP, LP and AP activity at the level of C9 activation was determined from individual C57BL/6 male (n=5) and female (n=5) mice. Each sample was tested in duplicate, error bars represent standard deviation. Statistical significance was determined with nonparametric, two-tailed Mann-Whitney test with 95% confidence interval.*

p=0.008), LP C9 (330±83 vs 62±8 AU/ml, p=0.008), and AP C9 (176±21 vs 47±16 AU/ml, p=0.008) **(Fig 1B)**.

At the level of C3 activation, CP, LP and AP had similar dose-dependent activities independent of gender **(Fig 2A)**. Analysis of five age matched female and male C57BL/6 mice showed that the CP C3 activity was slightly reduced in individual females (**Fig 2B**, 190±25 vs 130±36 AU/ml, p=0.02). Similarly also LP C3 was lower in females but did not reach significance (198±19 vs 151±45, p=0.06) AU/ml. The AP C3 activities did not differ between genders, although female serum exhibited higher variation between individual mice

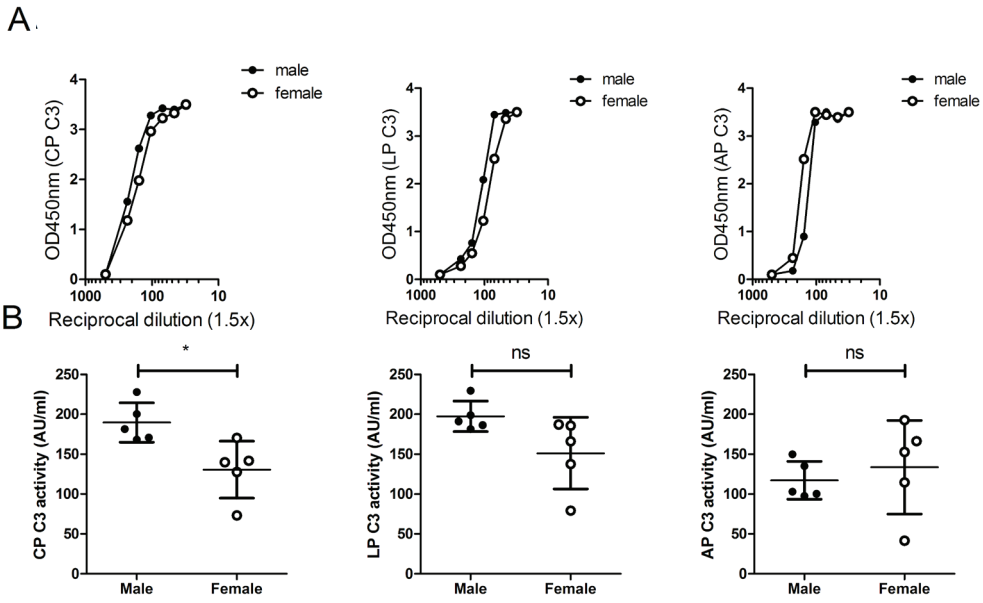

Fig 2. Functional serum complement activities at the level of C3 activation. (A) *Dose dependent activity of male and female serum on the three pathway assays at the level of C3 activation was determined with reciprocal 1.5 fold dilutions of pooled serum starting from 1/30 dilution. Error bars represent standard deviation.* **(B)** *Classical, lectin and alternative pathway activity at the level of C3 activation were determined from individual male (n=5) and female (n=5) C57BL/6J mice. Each sample was tested in duplicate, error bars represent standard deviation. Significance of difference was determined with nonparametric, two-tailed Mann-Whitney test with 95% confidence interval.*

than males (117±24 vs 134±59, p=0.3) **(Fig 2B)**. Together these results suggest that especially the terminal pathway is responsible for the female specific low complement activity in C57BL/6J mice.

3.2 LEVELS OF C6 AND C9 ARE STRONGLY REDUCED IN FEMALE C57BL/6J MICE.

Since the functional ELISA results suggested a gender difference specific for the terminal pathway, we determined the levels of key complement factors. Female mice exhibited higher concentrations of classical pathway initiator C1q (152±22 vs 170±33 μg/ml) **(Fig 3A)**, lectin pathway initiator MBL-C (33±2 vs 38±2 μg/ml) **(Fig 3C)** and alternative pathway pattern recognition and C3-convertase stabilizer, properdin (74±8 vs 114±19 μg/ml) **(Fig 3D)**. The only initiator exhibiting significantly higher concentration in male mice was MBL-A (15±2 vs 12±1 μg/ml) **(Fig 3B)**. Interestingly, although not reaching significance, the female mice had higher C3 serum levels (786±288 vs 938±138 μg/ml) **(Fig 3E)**, but no significant differences were found in the C3 activation fragments (1.1±0.14 vs 0.97±0.29 AU/ml) **(Fig 3F).** Concerning the terminal pathway, levels of C5 were on average higher in male C57BL/6J mice but not reaching significance and male mice exhibited considerable variation (260±165 vs 227±47 ng/ml) **(Fig 3G)**.

In order to be able to analyse the levels of C6 and C9 in serum, we generated polyclonal antibodies against recombinant mouse C6 and C9. C57BLSDS-PAGE analysis of recombinant C6 and C9 showed under non-reducing conditions a molecular size of approximate 70 kDa and 55 kDa respectively **(Supplemental figure 1A)**. The specificity of the pAbs against rC6 and rC9 was shown by western blot, where both pAbs did not cross reacted with other mouse serum proteins **(Supplemental figure 1B, 1C)**.

C57BLWestern blot analysis of four female and male sera showed more intensive staining of both C6 (**Fig 4A)**, and C9 (**Fig 4C**) in male C57BL/6J sera. Furthermore, female C9 seems to migrate at a higher molecular weight than male C9 (**Fig 4C**). Quantification

of serum concentrations of terminal pathway components C6 and C9 using specific sandwich ELISAs confirmed significant, 4-7 times lower concentrations in females: C6 (**Fig 4B**, 9.1±2.0 vs 1.2±0.2 μg/ml) and C9 (**Fig 4D**, 24.5±6.4 vs 6.2±1.0 μg/ml).

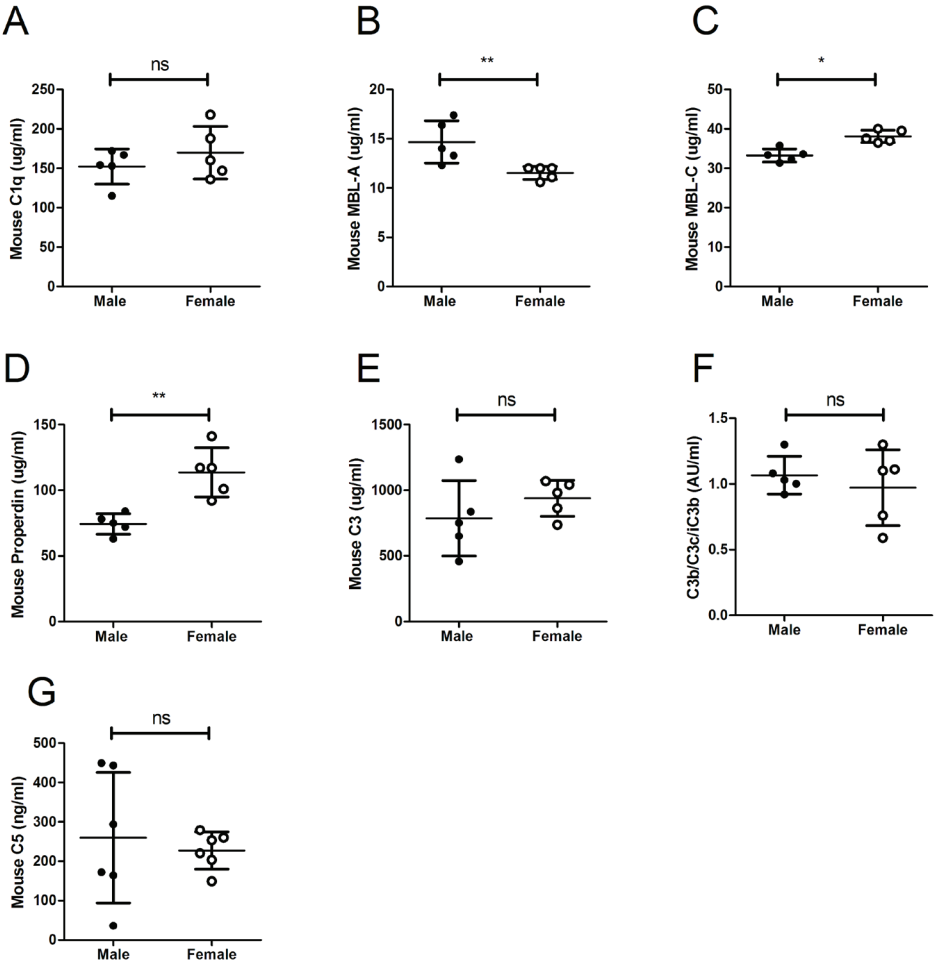

Fig 3. Quantification of individual complement factors. *Commercial and in-house sandwich ELISAs were used to determine serum concentrations of C1q* **(A)**, *MBL-A* **(B)**, *MBL-C* **(C)**, *properdin* **(D)**, *C3* **(E)**, *plasma C3b/C3c/iC3b* **(F)** *and C5* **(G)** *from age matched female and male C57BL/6J mice (n=5). Each sample was tested in duplicate, error bars represent standard deviation. Statistical significance was determined with nonparametric, two-tailed Mann-Whitney test with 95% confidence interval.*

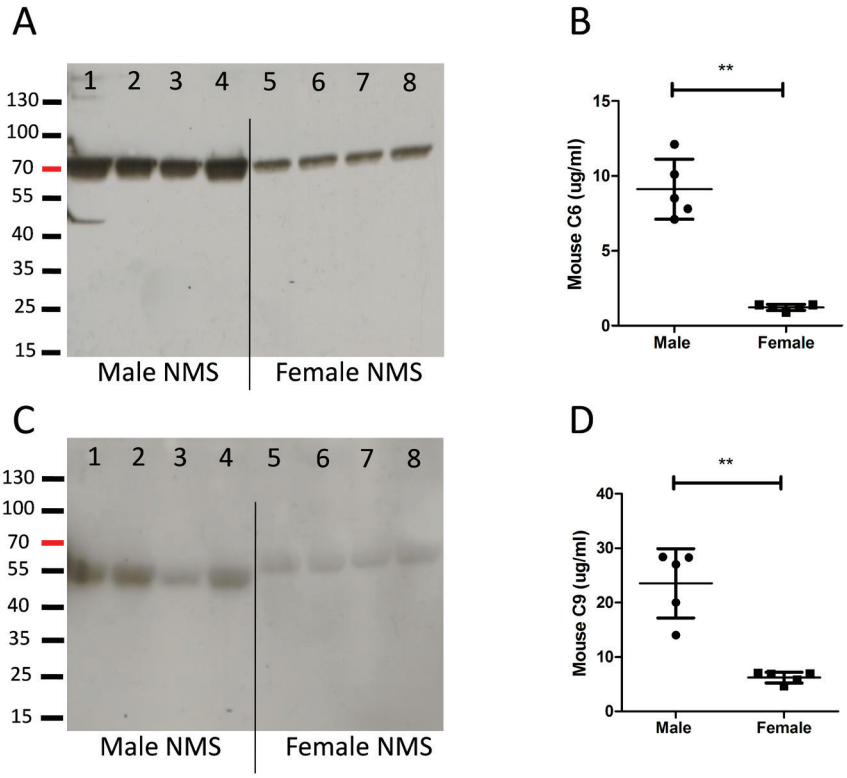

Fig 4. Analysis of mouse C6 and C9 in C57BL/6J mice. *Serum C6* **(Fig. 4A)** *and C9* **(Fig 4C)** *from serum of age matched C57BL/6 male and female mice was analysed by Western blot, with individual male serum in lanes 1 – 4 and female serum in lanes 5 – 8. Immunodetection was performed with chemiluminescence. Quantification of C6* **(Fig. 4B)** *and C9* **(Fig 4D)** *in male and female serum (n = 5) was performed with specific sandwich ELISAs, and values calculated as absolute concentrations. Error bars represent standard deviation. Statistical significance was determined with nonparametric, two-tailed Mann-Whitney test with 95% confidence interval.*

3.3 FUNCTIONAL ACTIVITY AND TERMINAL PATHWAY COMPONENTS ARE ALSO REDUCED IN FEMALE BALB/cJ BUT NOT IN CD-1 FEMALE MICE

To answer the question whether these gender differences in terminal pathway activity are exclusive for C57BL/6J, other commonly used mice strains were

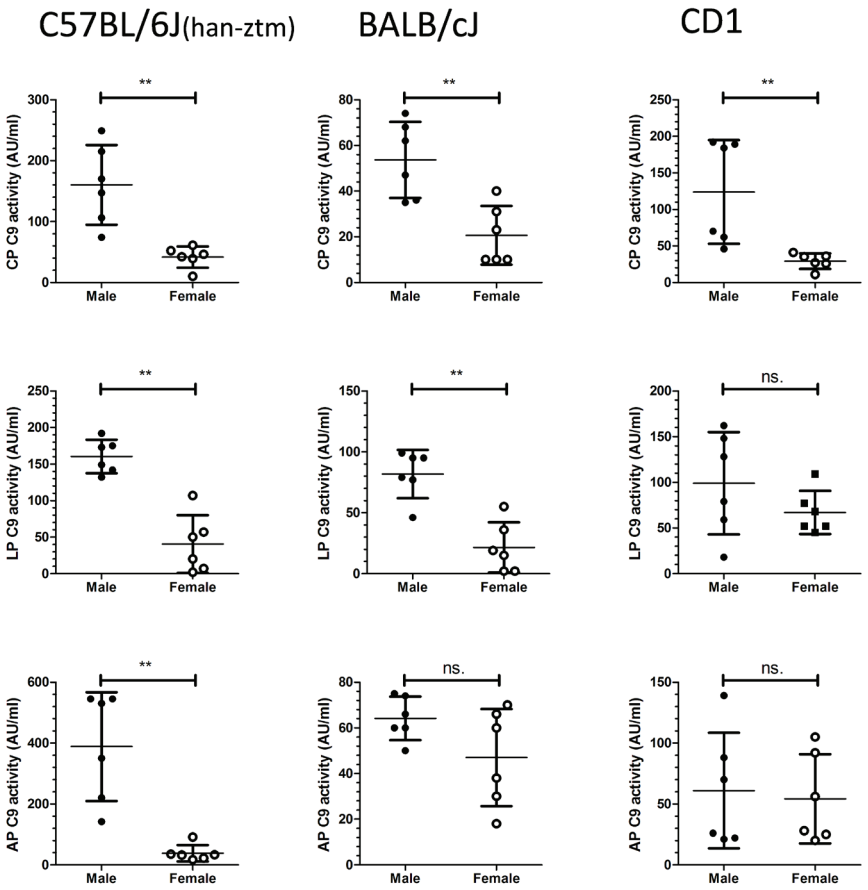

Fig 5. Strain dependence of functional serum complement activities at the level of C9 activation. *Classical (CP), lectin (LP) and alternative (AP) pathway activity at the level of C9 activation were determined from male (n=6) and female (n=6) mice from C57BL/6J(han-ztm), BALB/cJ and CD-1 mice. Each sample was tested in duplicate, error bars represent standard deviation. Statistical significance was determined with nonparametric, two-tailed Mann-Whitney test with 95% confidence interval.*

investigated. Pathway specific functional activities at the level of C9 were reduced for all three pathways in female compared to male BALB/cJ mice, similar to the results with C57BL/6J(han-ztm), the same strain but obtained from a different supplier **(Fig 5)**. In contrast, in CD-1 mice only CP was prominently decreased in female mice and activity of LP and AP were similar **(Fig 5)**.

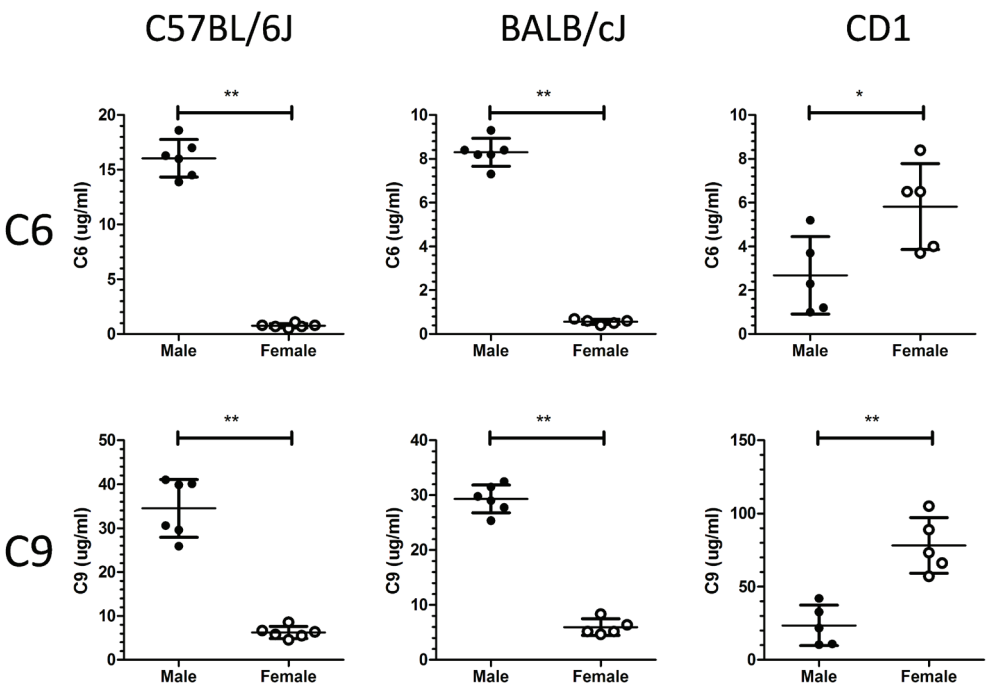

Fig 6. Strain dependence of serum C6 and C9 concentrations in female and male mice. *Serum concentration of C6 and C9 were quantified from C57BL/6J(han-ztm), BALB/cJ and CD-1 mouse strains. Measurements were with age matched male and female mice (n=6) from each strain. Each sample was tested in duplicate, error bars represent standard deviation. Statistical significance was determined with nonparametric, two-tailed Mann-Whitney test with 95% confidence interval.*

Quantification of C6 and C9 in different strains revealed that also male C57BL/6J(han-ztm) mice have higher level of both C6 (16.0±2.0 vs. 8.0±1.0 μg/ml) and C9 (35±7 vs. 6±1 μg/ml), and that the same difference is also present for BALB/cJ C6 (8.0±0.6 vs. 1.0±0.1 μg/ml) and C9 (29±3 vs. 6±1 μg/ml) **(Fig 6)**. In contrast, for the CD-1 strain it is the female mice that have higher levels of C6 (4±2 vs. 6±2 μg/ml) and C9 (24±14 vs. 78±19 μg/ml) **(Fig 6)**.

4. DISCUSSION

The purpose of this study was to characterise the sex-specific difference in the complement system of C57BLcommon laboratory mice in order to understand which pathways of mouse systemic complement are affected and whether our results could in part explain the female specific protection in different experimental mouse disease and injury models.

The literature in general agrees that the functional activities of classical and alternative pathways of mouse complement is much weaker in female mice [7, 19, 20]. However these determinations have been mostly generated with haemolytic assays which cannot fully differentiate the initiation pathways and the terminal pathway activities. Furthermore, there's evidence that haemolytic assays may have compatibility issues with certain mouse strains and possibly with between genders [21]. To fully understand the intricacies of female-male difference in mice, we used recently developed functional and standard ELISA-based assays to investigate the gender differences in C57BL mice.

Our results show that female C57BL/6J mice have fully functioning pathways until the level of C3 activation. The classical and lectin pathways had somewhat lower activity in female serum, which may be due to the sex-limiting protein (C4, slp) expression and impact on formation of CP/LP C3-convertase

C4bC2a [20]. When the complement activation was determined at the level of C9, all three pathways showed uniform 4–7 times lower activity in female C57BL/6J serum, suggesting that terminal pathway is the source of gender specific differences, as described previously with haemolytic complement assays [7, 22]. To verify the role of the terminal pathway, we determined the serum level of key complement factors for each pathway, which accurately reflected the functional complement determinations. There were no major gender-specific differences with serum C1q, MBL-A, MBL-C, properdin, or C3 concentration. These results are in part supported by earlier studies showing that female C57BL/6, BALB/c and C3H/He mice have similar level of serum C3 [23] and CP initiation factors [10]. However these results are in contradiction to previous results generated with BUB mice which show that most serum complement factors would be present in higher concentrations in male mouse sera [7, 19]. One possible explanation was brought forward recently by a study which showed that BUB-mice have higher complement factor concentrations only when determined by haemolytic assays, suggesting that mouse strain, and possibly gender, may influence haemolytic assay compatibility [21].

Previous studies have shown that C5 is androgen inducible, and different methods including haemolytic assays, ELISA and immunoelectrophoresis showed that C5 is lower in females of different mouse strains including C57BL/6 and BALB/c [24–27]. Our results here, using a commercial mouse C5 ELISA kit, only showed slightly higher level of C5 in male mice but the difference was not significant. However, the male C57BL/6J group had a considerable variation that may affect the interpretation of these results. Moreover, mouse C5 has been described to exhibit gender dimorphism with two forms of C5, where one is androgen inducible [25]. Therefore a final answer on possible gender differences in mouse C5 await thoroughly validated assays showing accurate detection of C5 in both male and female mice.

Our results clearly showed that the terminal pathway components C6 and C9 were strongly reduced in female C57BL/6J sera when analysed either by ELISA or by western blot. These results are in line with previous studies on BALB/c mice, which suggested low C6 haemolytic activity with female mouse sera [10]. Our results also show that female C9 migrates at a different molecular weight on SDS-PAGE, which could be due to sex-specific isomorphism in analogy to mouse C5 [25]. Further research is therefore warranted on the gender specific molecular differences of mouse C9.

To answer the question whether the gender difference, as observed in our assays, was exclusive for the C57BL/6J we tested other commonly used laboratory mouse strains. Analysis of the functional activities of the three pathways at the level of C9 activation showed that also female BALB/cJ mice have reduction in all three pathways, suggesting terminal pathway specific difference. This was in agreement with the strongly reduced levels in C6 and C9 in female BALB/cJ mice. In addition we confirmed the reduced terminal pathway activity in C57BL/6J using mice from a different supplier. This means that reduced complement activity in female mice is observed in two commonly used strains. However, these results cannot be extrapolated to all mouse strains.

Importantly we found that in CD-1 mice only the CP C9 activity was reduced in female mice, whereas LP C9 and AP C9 were at the similar levels. This finding suggests that in CD-1 mice the terminal pathway is not responsible for the gender specific differences. Even more, quantification of C6 and C9 showed significantly higher levels in female CD-1 mice, underlining the fundamental difference of complement system in CD-1 mice compared to C57BL/6J and BALB/cJ. Although outside the scope of the current study, the results also suggested that in C57BL/6J mice the overall activity of all three pathways is higher than with BALB/cJ and CD1. Interpretation of the functional assay findings alone should be performed with care as different strains may exhibit

different affinity to the complement activation ligands used in these assays. Our results warrant further research with both functional and standard ELISA determinations to study the potential impact of gender, strain and age to the mouse complement system.

Together our results show that the complement system of female C57BL/6J operates normally until the level of C3 activation, showing no difference in key complement factors or in overall turnover of C3 as measured by C3 activation fragments. Therefore the gender specific protection against experimental I/RI, antibody mediated rejection and sepsis is most likely not due to lower levels of serum C1q, MBL-A, MBL-C, properdin or C3 which *in vivo* studies have shown to be essential for complement mediated injury [28–30]C57BL. This is an important finding as complement activation at the level of C3 is essential for numerous processes, including antigen presentation, clearance of immune complexes through complement receptor 1(Cr1) and the roles of C3a in modulating the allostimulatory activity and recruiting inflammatory cells to the site of complement activation [31–34].

Our results strongly suggest that the terminal pathway activity might be more tightly regulated and shows strain and gender differences, especially at the level of C6 and C9. This will affect the haemolytic activity and the formation of C5b-9. The contribution of C5b-9 in disease and injury has been established through systemic inhibition of key complement factors or with complement deficient rodent strains, clearly showing that inhibition of C5a and C5b-9 generation can markedly reduce damage in sepsis, renal I/RI and ameliorate acute rejection [35–39]. However, in these cases it has not always been possible to distinguish between the effect of C5a and the effect of C5b-9. Unfortunately, based on our experiments the role of C5 remains unclear and warrants further research. This is especially of importance since the availability of C5aR antagonists has introduced a specific therapeutic intervention at the C5a effector functions [40] Recent clinical studies have highlighted the role

of gender, with evidence that women have better tolerance against renal I/RI and kidneys generally have better prognosis [41, 42], however differences in complement activity were not taken into consideration. Studying the gender impact with experimental mouse models should be performed with care as our results show that most common mice strains exhibit very low terminal pathway activity that can directly impact the interpretation of the experimental model in context of human disease and injury.

In conclusion, our results show that the previously reported low complement activity in female C57BL/6J and BALB/cJ can be attributed to low level of terminal pathway complement factors C6 and C9 in serum of female mice. These findings can in part explain the intriguing female specific protection in different experimental disease and injury models, and should be carefully addressed in interpretation of preclinical models.

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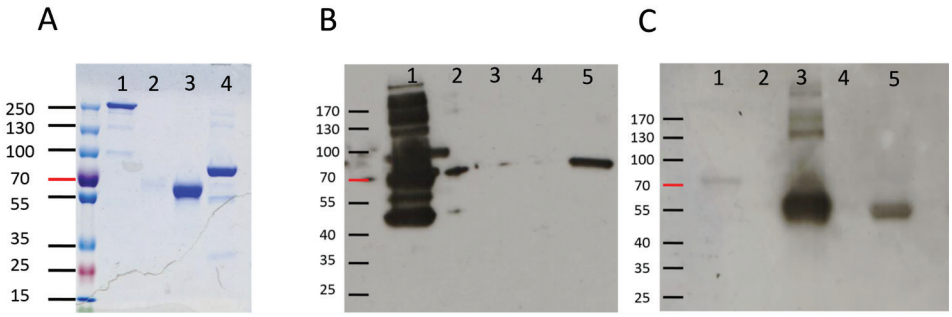

Supplemental figure 1. Analysis of specificity of polyclonal anti-mouse C6 and anti-mouse C9 reagents. (A) *The sizes of mouse rC6 (lane 4) and rC9 (lane 3) were analysed on Coomassie stained non reduced 10% SDS-PAGE together with purified IgG (lane 1). Specificity of anti-rC6* **(B)** *and anti-rC9* **(C)** *was assessed on replicate western blots with rC6 (lane 1), rC9 (lane 3), C57BL76 NMS (lane 5) and loading buffer (empty lanes 2 and 4). Immunodetection was performed with chemiluminescence.*

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