Properdin binding independent of complement activation in an *in-vivo* model of anti-GBM disease

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Abstract

Properdin is the only known positive regulator of complement activation by stabilizing the alternative pathway (AP) convertase through C3 binding, thus prolonging its half-life. Recent *in vitro* studies suggest that properdin may act as a specific pattern recognition molecule. To better understand the role of properdin *in vivo*, we used an experimental model of acute anti-glomerular basement membrane (anti-GBM) disease with wild-type, C3- and properdin-knockout (KO) mice. The model exhibited severe proteinuria, acute neutrophil infiltration and activation, classical (CP) and AP activation, and progressive glomerular deposition of properdin, C3 and C9. Although the acute renal injury was likely due to acute neutrophil activation, we found properdin deposition in C3-knockout (KO) mice that was not associated with IgG. Together our results show for the first time that properdin may deposit in injured tissues *in vivo* independent of its main ligand C3.

Introduction

The complement system consists of three activation pathways, classical (CP), lectin (LP) and alternative (AP), which converge at the level of C3 activation, proceeding to C5 cleavage and initiating the terminal pathway C5 – C9 activation. Both the CP and LP have pattern recognition molecules (PRM), like C1q and MBL, which in combination with the associated proteases initiate the complement cascade[1]. AP has an important role in augmenting CP and LP, once C3 is activated the AP amplification loop enhances the complement cascade activation[2,3]. In addition, there is a constant low-level auto-activation of C3 (tick-over), which allows C3b formation on surfaces of pathogens and injured host cells[1,4]. The membrane-bound C3b associates with factor B forming a short lived AP C3 convertase C3bBb, which properdin binding stabilises significantly and making properdin the only positive regulator of complement[5–8].

Recent *in vitro* studies have shown that properdin may exhibit pattern recognition molecule activity (PRM)in vitro. Studies have shown targeted complement fixing activity on certain ligands such as lipopolysaccharides (LPS)[9], oxidized-LDL[10] and myeloperoxidase[11]. Similar results have been demonstrated with apoptotic and necrotic cells, and heparan sulfates expressed at the surface of renal epithelial cells[12–16]. In this model properdin attaches to specific ligand and forms stable C3bBbP convertase, possibly overcoming the local threshold for C5 convertase (C3bBbC3b) formation[8,9,12]. However, it is not yet clear how properdin could act as a PRM as it lacks known recognition domains[17].

AP and properdin have been shown to contribute to arthritis, abdominal aortic aneurysm and renal ischemia reperfusion injury (I/RI). However, these studies have not been able to differentiate whether the deposition of properdin in tissues takes place via membrane bound

C3b or through epitopes present in the injured tissues[18–21]. To study the sequence of properdin binding *in vivo* we established an acute model of anti-GBM in wild type (WT), C3- and properdin knockout (KO) mice. Recent studies on anti-GBM have shown that properdin, and other AP components are prominently present in anti-GBM affected glomeruli in humans and that AP contributes to the injury in mice[22–24], making the model attractive for studying properdin.

Results

Characterisation of acute complement activation after anti-GBM initiation

Acute complement activation was performed with comparison of mouse plasma before and 2 hours post-administration of anti-GBM, showing significantly increased C3b/C3c/iC3b in WT mice (3.6 fold), which was not seen following administration of control IgG or in properdin-KO (fP KO) mice (**Fig 1A**). Moreover, basal levels of C3 activation fragments was two-fold lower in fPKO mice compared to the WT mice.

The novel mouse properdin ELISA has linear detection range with recombinant mouse properdin (**Fig 1B**) with no detectable properdin in fP KO mouse serum (**Fig 1C**). No changes in circulating properdin was found 2h after injection of WT mice with anti-GBM or control antibody (**Fig 1C**).

Functional ELISAs established intact serum complement in WT mice, and total AP deficiency in the fP mice. Two hours after anti-GBM injection prominent consumption of CP (**Fig 1D**) and AP (**Fig 1E**) was seen, whereas for LP consumption was inconclusive (**Fig 1E**). No prominent or consistent complement consumption was observed with control antibody injected mice. In properdin-KO mice anti-GBM injection did not result in significant CP or LP pathway

consumption (**Fig 1F**). Together these results show prominent complement activation in this anti-GBM model, with partial dependence on properdin.

Time course histological analysis of complement activation in glomeruli

Kinetics of renal complement activation following anti-GBM injection was evaluated in groups of WT mice sacrificed at different time points. The Anti-GBM Rabbit IgG was clearly present in glomerular vasculature from 2h – 72h (**Fig 2, Rb IgG**). The C1q staining was performed as a marker of CP involvement, showing acute and unchanged staining pattern from 2h – 72h, closely resembling that of Rb IgG (Fig 2, C1q). The C3 deposition was present in glomeruli at 2h and increased until 72h. The C3 deposition closely resembled the pattern of rabbit IgG until 48h, after which a focal staining pattern became more apparent (**Fig 2, C3**). Properdin deposition was present at 2h, with clear increase from 24h onwards. Interestingly, properdin staining did not exhibit linear pattern found with IgG and C3 (**Fig 2, fP**). The staining for both C6 and C9 exhibited late kinetics, emerging only after 24h and becoming most prominent at 48h (**Fig 2, C6 and C9**). In all cases specificity of the staining was confirmed with isotype and biological positive and negative controls (Supplemental Fig 1). We did not observe gender specific differences in staining pattern or intensity in any of the studied complement factors (data not shown).

C3 and properdin deposition show progressive increase and colocalisation

The rapid deposition of C3 in the glomerulus, combined with the systemic consumption of CP and linear deposition of both Rb IgG and C1q, suggested a IgG and CP-mediated activation mechanism. To verify this, we assessed glomerular colocalisation of Rb-IgG and C3 with anti-GBM injected WT mice. The time-course histology showed that the deposition of C3 closely

resembles both rabbit IgG and C1q staining in the glomerular endothelium at 2h – 6h, with clearly defined vascular loop-structure. From 48h onwards, the C3 deposits lining the vascular endothelium become more prominent and significant C3 staining is present in the lumen independent from rabbit IgG deposits (**Fig 3A**). With the same mice, we found clear colocalisation of properdin and C3 throughout the observation period, suggestive of AP-mediated C3 activation (**Fig 3B**). However, the single deposits of both C3 and properdin were also observed, especially at 48 – 72h when properdin stain was most prominent (**Fig 3B**).

Properdin deposition in injured glomeruli independent of C3 involvement

Properdin deposition in C3-KO mice was studied at 2h, 24h and 72 h following anti-GBM treatment (Fig 4A). Colocalisation of Rabbit IgG with properdin was only incidental and properdin exhibited strong single stain in both WT and C3KO mice, with no evident staining in fp KO mice (Fig 4B). Colocalisation of TUNEL staining with Rabbit IgG, C3 and properdin was evaluated and found to be also incidental with prominent TUNEL stain in the glomerular lumen together with occasional double stain with C3 and properdin (Fig 4C). Analysis of Colocalisation of TUNEL and properdin staining in C3 KO mice showed close but non-overlapping colocalisation within glomeruli at 72h (Fig 4D).

Glomerular injury and proteinuria is not prevented in C3- and Properdin-KO mice

Next, we evaluated the impact of properdin-KO on the anti-GBM induced renal injury using U-ACR as a functional read-out (**Fig 5**). The injection of anti-GBM in WT C57bl/6 resulted in severe albuminuria in 24h-urine collected between 72h – 96h. The U-ACR in WT mice was significantly higher in anti-GBM mice compared to control antibody injected mice (60761 ± 30011 and 1205 ± 282 mg/g respectively). Compared to WT mice the properdin-KO and C3-KO mice showed 36 – 40% lower level of albuminuria (U-ACR of 36420 ± 16994 and 40373 ± 18247 mg/g respectively). However, statistical difference was not reached. Female WT and

C3-KO mice exhibited higher degree of renal injury than male mice. With properdin-KO mice no gender differences were observed (Supplemental Table 1).

Neutrophil activation and inflammation after administration of anti GBM

Neutrophils have been shown to contribute to renal injury in experimental models of anti-GBM disease[24]. Time course analysis of neutrophils using a GR1 staining, showed that infiltration within the glomeruli was biphasic, being most prominent at 2h, returning to low levels at 24h – 48h, and increasing at 72h (**Fig 6A**). Acute infiltration and activation of neutrophils was supported by a transient threefold increase in serum MPO at 2 – 6 h (**Fig 6B**) which was followed with SAP peaking at 24h and remaining elevated until 72h (**Fig 6C**). Serum properdin showed a gradual increase from baseline, which reached significance at 48h (**Fig 6D**).

Dependence of neutrophil infiltration on properdin

Complement anaphylatoxins C3a and C5a are potent chemoattractants for inflammatory cells. Additionally neutrophils contribute to systemic properdin pool and can secrete it locally[25,26]. There was no clear colocalisation with properdin and GR1-positive neutrophils in the affected glomeruli at 2h and neither properdin nor neutrophils were present in control antibody treated mice (**Fig 7A**). Furthermore, the degree of neutrophil infiltration was not decreased in properdin-KO mice (**Fig 7B**), suggesting that in this model properdin-regulated activation of complement does not contribute to recruitment of neutrophils.

Discussion

Recent studies have established that properdin has differing roles in disease pathologies, with contributing role in experimental models of renal I/RI and possibly arthritis, and a protective role in C3 glomerulopathy and sepsis [18,20,27,28]. The role of properdin as a positive regulator of AP is clear. However the possible PRM role remains unsolved. Recent *in vitro* studies show that properdin has PRM activity and can act as a focus point of local C3b deposition on several ligands[8,9,13,15,29]. Conversely, in other cases properdin has been shown to act only as a positive regulator, requiring preceding C3b deposition to bind[30,31]. To better understand the mechanism of properdin binding and function *in vivo,* we explored an experimental model of anti-GBM disease in wild type, properdin-KO and C3-KO mice.

Our model exhibited typical characteristics of an experimental anti-GBM disease, which include systemic complement consumption, activation of AP and CP pathways, deposition of complement factors in the glomeruli, neutrophil activation, inflammation and severe renal injury[23,24,32–34]. Acute complement activation was verified with plasma C3b/C3c/iC3b fragments at 2h, which also revealed the impact of properdin as a positive regulator of AP as properdin-KO mice had lower basal level of C3 activation fragments and less prominent generation of C3 fragments. Furthermore, we observed acute consumption of CP and AP , which was not present in properdin-KO mice underlining the role of properdin and AP amplification loop[5]. Interestingly, we did not observe serum properdin consumption in WT mice. Conversely, the levels increased in tandem with SAP suggesting that systemic inflammation may affect the secretion of properdin to circulation from activated inflammatory cells[25,35,36]. It must be noted that the range of mouse properdin seem excessively high compared to human reference range, which may be due to the use of recombinant mouse properdin as a standard that may not have the same distribution of properdin isoforms as is found in vivo [37].

Time course immunohistochemistry revealed an acute C1q, C3 and properdin deposition in line with serum determinations, suggesting CP and AP mediated complement activation. However, C6 and C9 deposition became evident only after 24h and continued to intensify from 48h – 72h, coinciding with increased C3 and properdin deposition. Together, these results suggest that in this model of anti-GBM disease, the complement mediated injury may be attenuated until 48h – 72h. Further analysis of C3 colocalisation confirmed that initial complement activation is most likely CP dependent and is present in the vascular endothelium. However, from 48h the C3 staining intensifies and colocalised more with properdin suggesting AP and properdin involvement in complement activation. We demonstrated that properdin deposition was present in anti-GBM injected C3 KO mice glomeruli, and that properdin did not colocalise prominently with IgG or TUNEL positive cells excluding the possibility that properdin binds prominently to glomerular anti-GBM IgG immunocomplexes or apoptotic cells [12,38].

Properdin-KO and C3-KO resulted in reduced proteinuria compared to the WT mice; however, this protection was not statistically significant. This is in contrast to previous studies with two-step model, which established involvement of both complement and neutrophils in the experimental renal injury in male mice[24] . Although small group size did not allow proper statistical analysis, female mice seemed to exhibit markedly higher proteinuria in WT and C3KO mice this model. However, we did not observe any difference between female and male renal histology stains on any complement factor, including C6 and C9. We have recently shown that female C57bl/6 mice have significantly reduced terminal complement pathway functionality[39]. In light of our results here, we can conclude that even residual complement activity results in prominent complement deposition *in vivo* when the injury is localised such as in the glomeruli. The increased renal injury in female mice is an interesting finding requiring further characterisation in this model. However, the finding is in line with known impact of sex

in mice and autoimmune disease, where oestrogen is a major contributing factor and may be one of the underlying causes [40,41] .

Our results show an acute and transient infiltration of neutrophils, with simultaneous increase of serum MPO and SAP which precede the full complement activation. Furthermore, properdin-KO did not impact neutrophil infiltration and no colocalisation between neutrophils and properdin was observed. Together our results show that in this model the initial CP- and AP-mediated C3 activation and anti-GBM IgG are sufficient to recruit neutrophils to the glomeruli together with the rabbit IgG Fc. Our results show only limited amelioration of renal injury using C3 KO mice, suggesting that the neutrophils are the likely driver of the acute injury, and that the full complement activation dependent on CP, AP and properdin is attenuated and may become a contributing factor later[24,42].

Taken together, our results show that properdin is present in injured glomeruli *in vivo,* independent of C3 involvement. The mechanism and putative ligand facilitating the deposition remains unclear, as only incidental colocalisation was found with C3, TUNEL positive cells, neutrophils and immunoglobulins. Further studies should aim to identify whether this finding is a result of specific epitopes or passive entrapment within the injured glomeruli, and whether deposition of properdin may direct further C3 activation *in vivo.* These findings are interesting for various AP and complement-mediated diseases. The ability of properdin to interact independent of C3 *in vivo* expands its possible roles in diseases and injuries. In particular, the role of properdin in removal of apoptotic material[12,16] and in neutrophil mediated diseases[29,43–45] can be expanded further with future *in vivo* studies using the methods, animals and reagents described here.

Methods

Animals used in this study

C57BL/6 wild type (WT) mice were purchased from Charles River laboratories. C3 knockout (KO) mice were a kind gift from Mike Carroll (Harvard Medical School, Boston, MA, USA), properdin-KO mice were maintained by Cordula Stover (University of Leicester, Leicester, UK)[28]. All experiments were approved by the Leiden University animal ethical committee and performed according to institutional and national guidelines.

Induction of glomerulonephritis

Rabbit anti-rat GBM, cross reacting with mouse GBM was prepared in house as previously[46], preparation and cross reactivity was confirmed in pilot experiments with anti-GBM and control antibodies injected into wild-type (WT) C57BL/6 mice. For the model, both male and female age-matched (ranging from 7 – 10 weeks) mice (C57BL/6 WT, C3-KO mice and properdin-KO mice) were used. At day 0, the mice were injected intravenously with rabbit anti-GBM or control rabbit IgG (0.5mg in 200µl physiological saline).

Acute anti-GBM was evaluated at 2h after injection. WT mice were injected with anti-GBM or control antibody, whereas properdin-KO mice were injected only with anti-GBM. For complement determinations, EDTA-plasma was collected by tail-cut from each mouse before the injection and at 2h after injection without anaesthesia. Serum was collected after CO_2-euthanasia via heart puncture. All blood samples were place directly on ice and prepared as plasma or serum as described previously[47].

The impact of C3 and properdin deficiency was investigated by injecting either anti-GBM or control IgG (0.5 mg) in WT, C3-KO and properdin-KO mice. The severity of renal injury was evaluated by 24h urine collection, and proteinuria analysis from anti-GBM injected WT

(n=10), properdin-KO (n=8), C3-KO (n=9) and and WT mice injected with control antibody (n=5). For urine collection mice were placed in metabolic cages 72 h after anti GBM administration with free access to food and water. The collected urine was centrifuged, aliquoted and stored at -20°C.

Kinetics of complement and neutrophil activation was studied with groups of WT mice sacrificed at 2h – 72h after injection of anti-GBM. Additional kidneys were collected at 2h from properdin-KO mice, and at 72h from C3-KO mice that were injected with either anti-GBM or control IgG. The mice were euthanized with CO_2, kidneys were harvested and snap frozen for later histological analysis.

Measurement of creatinine and proteinuria

Urine albumin was quantified using rocket immune-electrophoresis as previously described[24]. Urine creatinine was quantified with creatinine strips for the Reflotron Plus system (Roche Diagnostics, Germany). Degree of proteinuria was calculated as urinary albumin-creatinine ratio (U-ACR).

Properdin ELISA

A specific ELISA for mouse properdin was developed with mouse anti-mouse properdin monoclonal antibody (mAb) (clone 17-17), and rabbit polyclonal antibody (pAb) raised against recombinant mouse properdin[48]. The capture mAb was coated at 2 µg/ml in 96 well ELISA plates (Nunc Maxisorp; Thermo Fisher Scientific, United States). Following coating, the wells were blocked with 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Normal mouse CD-1 serum, recombinant mouse properdin or properdin-KO serum was serially diluted in PBS, 0.05% Tween 20, 1% BSA (PTB) and added to the blocked wells. Captured properdin was detected using DIG-labelled rabbit anti-human properdin (in house, LUMC),

anti-DIG-POD (11207733910, Roche Diagnostics), followed by colorimetric quantification with a colorimetric substrate step with 3,3',5,5'-tetramethylbenzidine (TMB) for 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 (BioRad, Japan).

Complement activation fragments and functional complement analysis

Measurement of functional mouse complement pathway activities was performed with an ELISA based system as described earlier [39,47]. In brief, human IgM coated plates were used for CP ELISA, Mannan coated plates for LP, and LPS coated plates for AP. For CP and LP assessment plasma samples were diluted in $MgCl_2$ and $CaCl_2$ supplemented Veronal buffered saline, and for AP samples Veronal buffer supplemented with $MgCl_2$ and Ca^{2+}-chelating EGTA. Complement activity at the level of C3 was determined with biotinylated rat anti-mouse C3b/C3c/iC3b mAb clone 2/11[49] (HM1065, Hycult Biotech, the Netherlands) and Streptavidin-HRP conjugate (Hycult Biotech).

Determination of activated mouse C3, MPO and SAP

Commercial assays were used to measure SAP (HK215, Hycult Biotech) and MPO (HK210, Hycult Biotech) according to manufacturer's specifications. The C3b/C3c/iC3b quantification was performed as described previously[47].

Histological analysis

Kidney sections were sectioned into 4 µm slices using a cryostat and fixed by 10 min incubation in acetone, washed with PBS three times 5 min after each step and all antibodies were diluted in 1%BSA/PBS.

Mouse properdin, C6 and C9 were detected using tyramide-fluorescein isothiocyanate (FITC) amplification based staining. The slides were first treated with 45 min RT incubation in PBS

buffer containing 0.6% H_2O_2 (1.07209.0250, Merck KGaA, Germany) and 0.2% NaN_3 (1.06688.0100, Merck) diluted in PBS.

Properdin was detected with 1µg/ml diluted rabbit anti-mouse properdin-DIG described for the properdin ELISA (in house, LUMC), whereas mouse C6 and C9 was detected with 1µg/ml rabbit anti-mouse recombinant C6-DIG and C9-DIG described previously[47]. For both components, the detection was followed with over-night incubation of the slides with 1/750 diluted sheep anti-DIG-POD (Roche Diagnostics). Finally, the slides were incubated 20 min with 1/500 diluted tyramide-FITC (T9034-4, Sigma-Aldrich, United States) in tyramide buffer (NENTM, Life Science Products, United States) with 0.01% H_2O_2 (Merck).

Mouse GR1 was detected with 1/400 diluted rat anti-mouse GR1 (a kind gift from professor Georg Kraal, VUMC, the Netherlands) and 1/750 diluted goat anti-rat-Alexa 568 (A11011, Molecular Probes, United States). Deposition of rabbit IgG to mouse glomeruli was detected with 1/400 diluted goat anti-rabbit IgG-Alexa 488 (A11008, Molecular probes, United States) or 1/400 diluted goat anti-rabbit IgG-Alexa568 (ab175471, Abcam, United Kingdom). Mouse C3 was detected with 1/1000 rat anti-mouse C3 (CL75603AP, Cedarlane, Canada), and 1/750 diluted goat anti-rat Alexa 568 (A11011, Molecular Probes). Where applicable, nuclear stains were performed with 1/10000 diluted Hoechst (H3569, Invitrogen, United States) according to manufacturer's instructions. Fluorescence microscopy was performed with Zeiss Axio Scope A1 with EX-plan Neofluar 40x lens and captured with Axiocam MRC5 (Carl Zeiss, United states).

Specificity of the staining was confirmed with renal tissue harvested 72h after injection of antibody. Rabbit IgG and C9 stain specificity was controlled with mice injected with control rabbit IgG. Properdin and C3 staining specificity was tested with properdin-KO and C3-KO

mice injected with anti-GBM antibody. In all cases the staining was free of non-specific signal within the glomeruli, as assessed by isotype controls.

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Acknowledgements

This work was financially supported in part by the European Union (Marie Curie TranSVIR FP7-PEOPLE-ITN-2008 No. 238756) and by a Consortium grant from the Dutch Kidney Foundation (COMBAT).

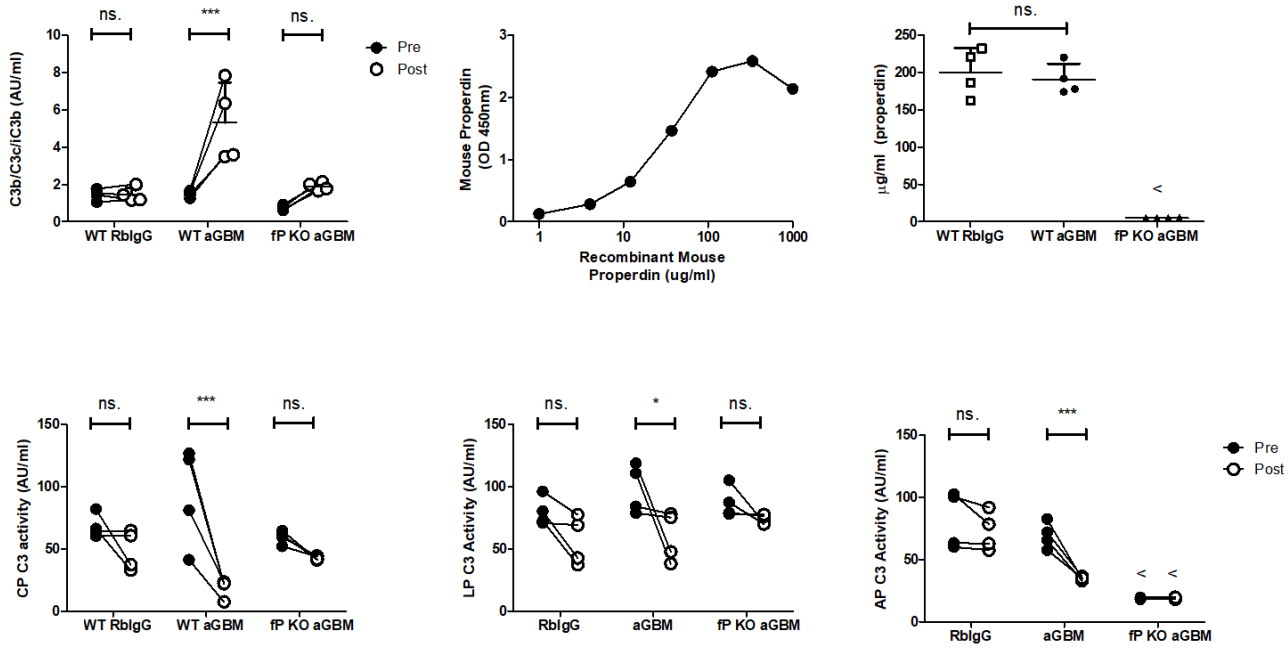


Figure 1. Analysis of systemic complement. **A)** Acute complement activation was determined with plasma C3b/C3c/iC3b at 2h with paired pre – post samples from anti-GBM treated WT and properdin knockout (fP KO) mice and control pAb treated WT mice **B)** properdin sandwich ELISA performance was assessed with reciprocal dilutions of recombinant mouse properdin with fourfold steps from 1000 ng/ml. **C)** Serum properdin levels were assessed 2h after induction of the model with anti-GBM in wild type (WT) and properdin knockout (fP KO) mice, and with control pAb injected WT mice. Serum Classical **(D, CP),** Lectin (**E, LP)** and Alternative (**F, AP)** pathway activities were determined with functional C3 ELISAs. Two way ANOVA was used to determine change specificity in pre – post analysis, one-way ANOVA was used to determine specificity of change between control and anti-GBM antibodies. Error bars represent standard deviation of duplicate measurements.

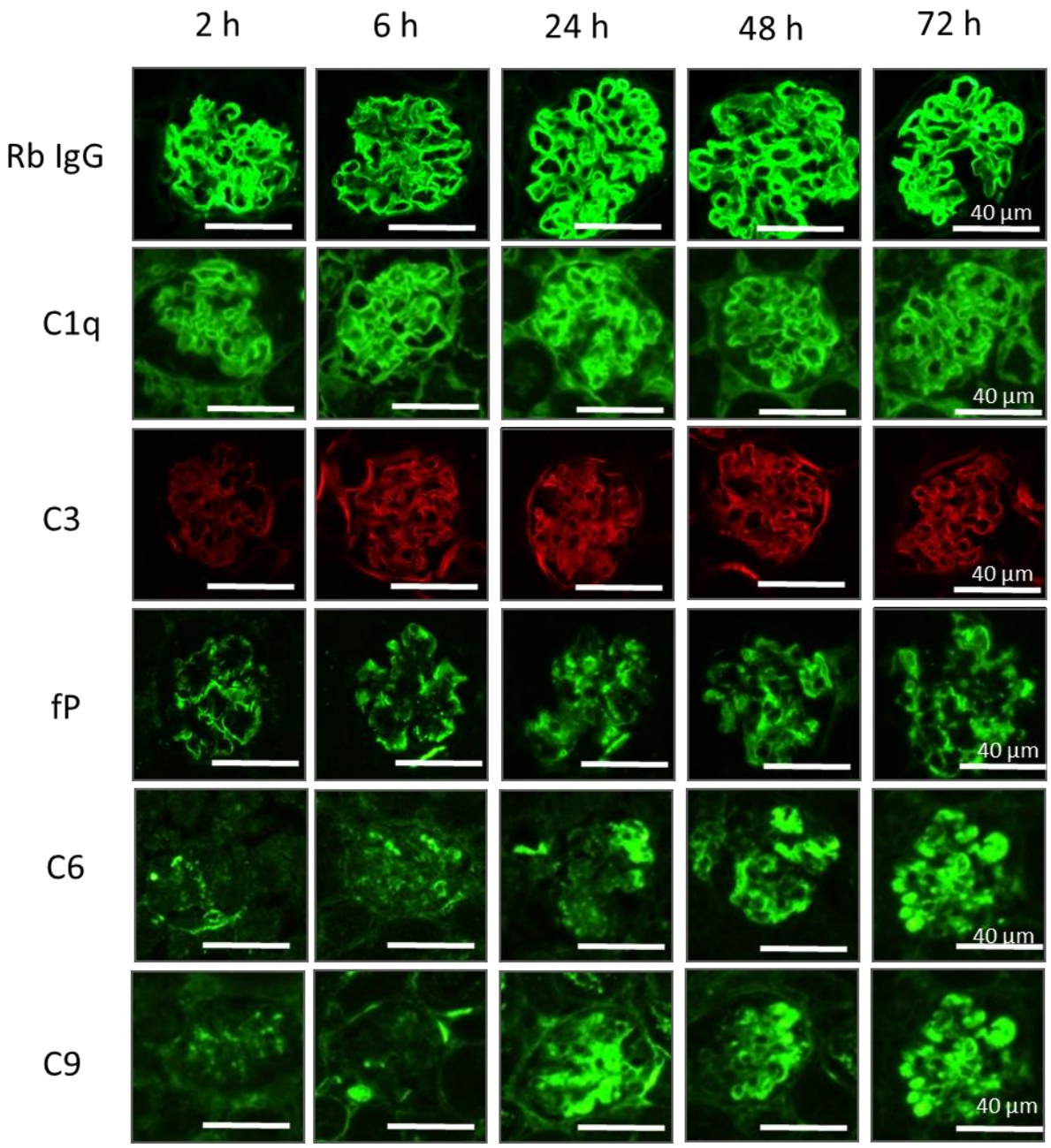


Figure 2. Time course analysis of glomerular activation of complement. Analysis was performed with kidneys harvested from wild type C57bl/6 mice sacrificed after 2h – 72h after administration of anti-GBM.Deposition of rabbit anti-GBM IgG and mouse C1q were detected with Alexa-488 (green) secondary antibody. C3 deposition was detected with Alexa-568 (red) labelled secondary antibody. Mouse properdin, C6 and C9 deposition was detected with HRP-labelled secondary antibody and Tyramide-FITC amplification (green). Images were acquired at 40x magnification, four mice (2 female and 2 male) per group were analysed and representative glomerulus was chosen from images acquired from four different animals.

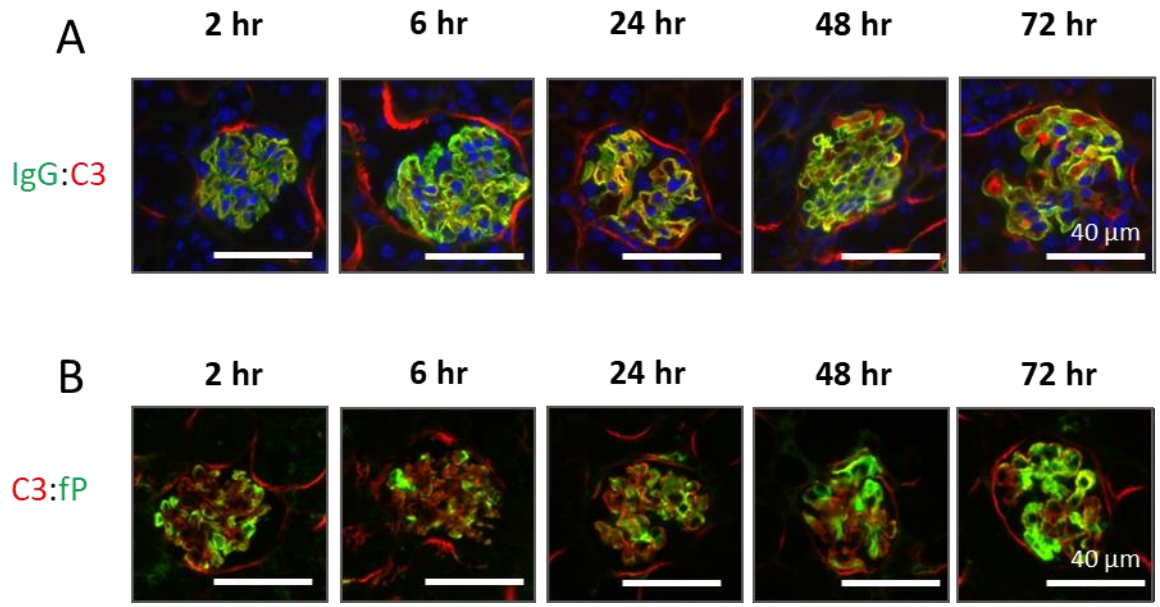


Figure 3. Time course colocalization of Rabbit anti-GBM IgG with mouse C3, and mouse C3 with properdin (fP). Analysis was performed with kidneys harvested from wild type C57bl/6 mice sacrificed after 2h – 72h after administration of anti-GBM. **A)** Anti-GBM Rabbit IgG was detected with Alexa-488 (green) labelled secondary antibody and C3 with Alexa-568 (red) labelled secondary antibody, nuclei were stained with Hoechst (blue) **B)** Mouse Properdin (green) was detected with HRP-labelled secondary antibody and Tyramide FITC amplification and C3 (red) with PE labelled secondary antibody. Images were acquired at 40x magnification, four mice (2 female and 2 male) per group were analysed and representative glomerulus was chosen from images acquired from four different animals.

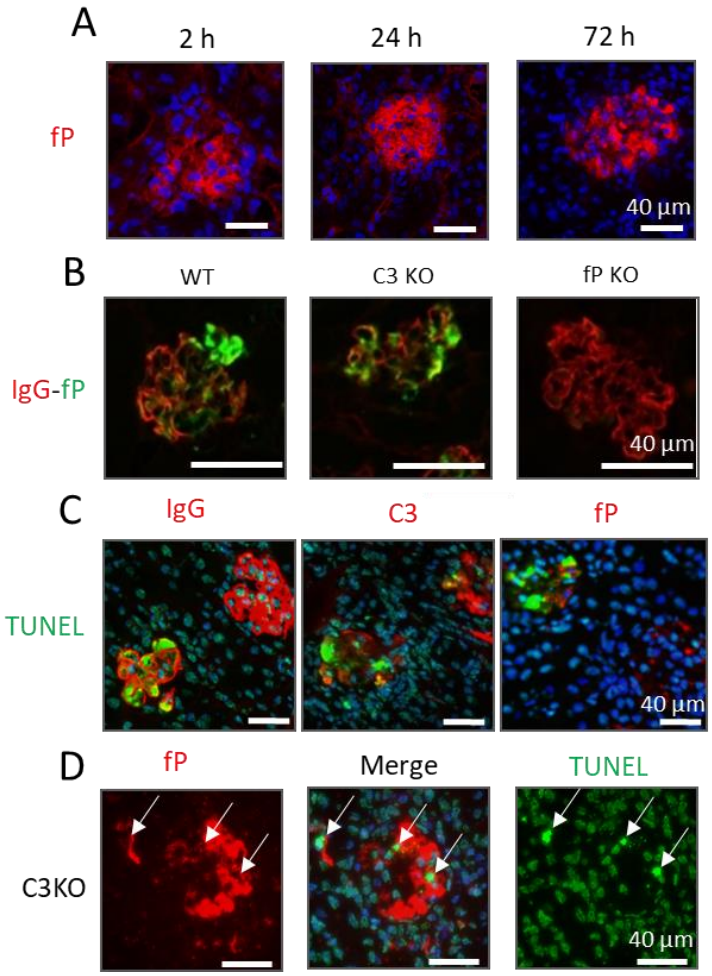


Figure 4. Colocalisation of properdin binding with C3 and rabbit IgG. A) Time course of fP deposition in C3 KO mice treated with rabbit anti-GBM at 2h, 24h and 72h was detected with PE labelled (Red) secondary antibody and counterstained with Hoechst **B)** localisation of fP stained with Tyramide-FITC amplification (green) in relation to disease inducing rabbit IgG stained with PE (red) labelled secondary antibody in WT, C3 KO and fP KO mice at 72h after anti-GBM administration. **C)** Co-localisation of TUNEL (green) stained cells with IgG, C3 and fP deposition detected with PE labelled secondary antibody in WT mice at 72h. Cells counterstained with Hoechts (blue). D) The deposition of properdin and rabbit IgG was analysed 72h after injection of anti-GBM into WT, C3 KO and properdin (fP) KO mice. Properdin was detected with HRP labelled secondary antibody and Tyramide FITC amplification (green), and rabbit IgG with Alexa-568 (red) labelled secondary antibody **(B)** Co-staining of mouse C3 and properdin deposition in wild type mice at 72h after anti-GBM administration, C3 was detected with Alexa-568 labelled secondary antibody and properdin with Tyramide FITC amplification.

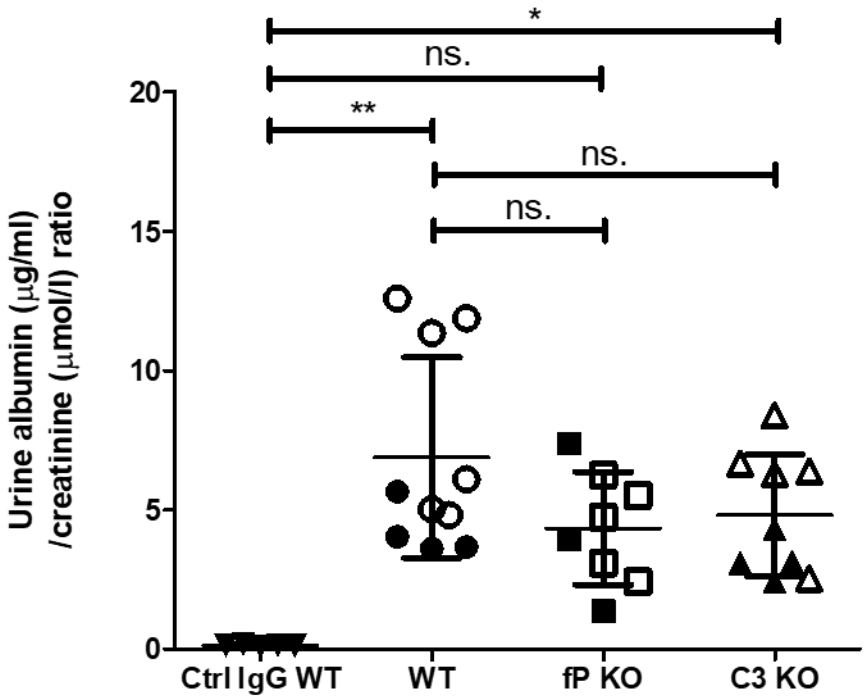


Figure 5. Anti-GBM induced proteinuria. Anti-GBM disease was induced with injection of 0.5 mg of rabbit anti-GBM IgG in wild type C57bl/6 mice (WT, n=10), properdin-KO mice (fP KO, n=8), and C3-KO mice (n=9). The control group, WT C57bl/6 mice were injected with 0.5mg of control rabbit IgG (Ctrl IgG WT, n=5). The gender of each mouse is indicated in the figure as either filled (male) or empty (female) symbols. 24h urine was collected from each mouse in metabolic cages 72-96h for quantification of urinary albumin-creatinine ratio. Non-parametric One-way ANOVA with Dunn's multiple comparison test was used to determine significance. Error bars represent standard deviation.

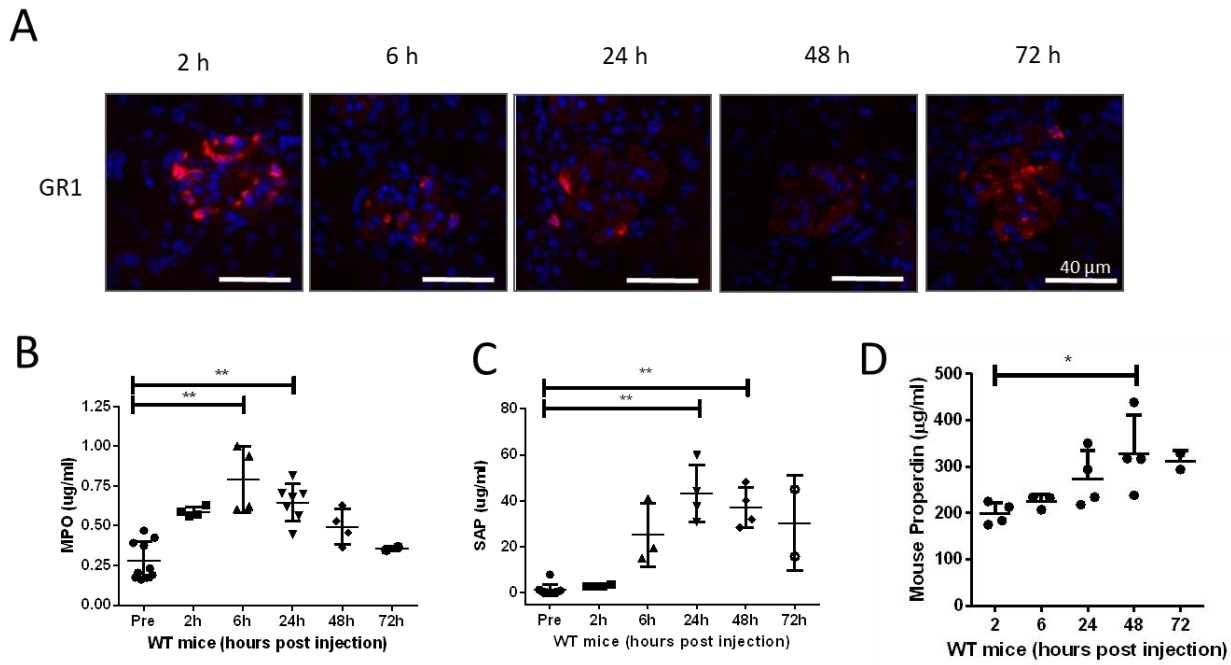


Figure 6. Analysis of neutrophil activation in a one-step model of anti-GBM. A) Wild type mice were injected with anti-GBM and tissues were collected at different time points for staining of infiltrating neutrophils with Alexa-568(red) labelled secondary antibody and rat anti-mouse GR1, and overlaid with Hoechst nuclear stain (blue). Activation of neutrophils was further analysed with **B)** serum myeloperxodase (MPO) measurement, and the impact on general inflammation with **C)** serum amyloid protein (SAP). **D)** Last, the impact of anti-GBM to complement was assessed with time course determination of serum properdin. All measurements were done in at least duplicate and significance was determined with One-Way ANOVA.

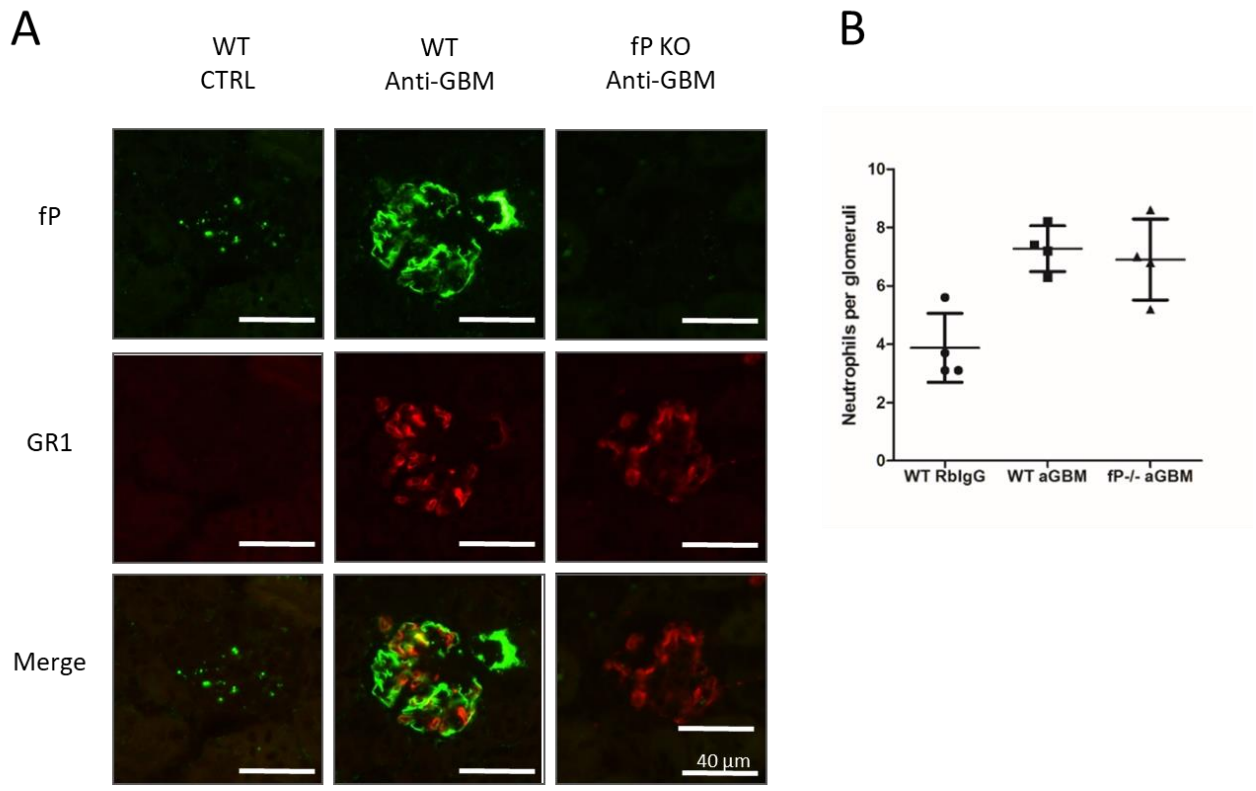
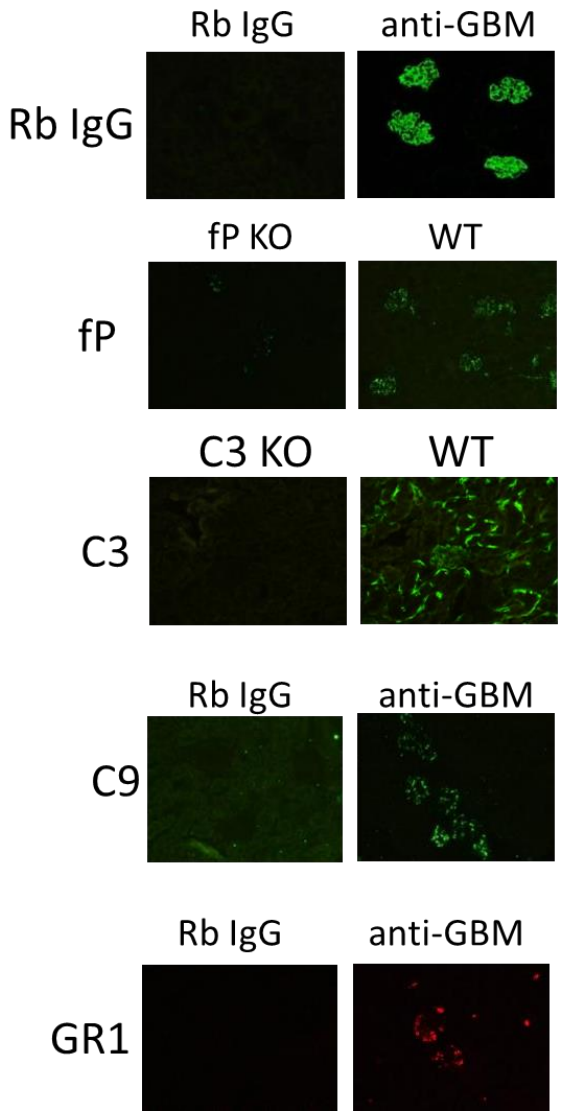


Figure 7. Colocalisation of neutrophil infiltration with properdin deposition in anti-GBM affected glomeruli. A) The double staining of properdin and neutrophils (GR1) was performed 2h after anti-GBM administration. GR1 was detected with Alexa-568 labelled secondary antibody (red) and properdin with tyramide-FITC amplification (green). Deposition was evaluated in WT mice injected with control antibody, WT injected with anti-GBM and properdin-KO mice with injected with anti-GBM. **B)** Based on the GR1 stain the neutrophils were quantified and calculated as infiltrating cells per glomeruli.



Supplemental figure 1. **Figure 6. Stain specificity of renal stainings.** Specificity of RbIgG stain was assessed on WT mouse renal tissue from animals injected with 0.5 mg non-specific Rb IgG and 0.5 mg Rb IgG against GBM, with both tissues harvested 72h post injection. Properdin stain specificity was assessed with **(C)** fPKO mice injected with 0.5 mg RbIgG anti-GBM and **(D)** WT mice injected with 0.5 mg RbIgG anti-GBM, with both tissues harvested at 72h. C3 stain specificity was assessed with **(E)** C3KO mice injected with 0.5 mg RbIgG anti-GBM and **(F)** WT mice injected with 0.5 mg RbIgG anti-GBM, with both tissues harvested at 72h. C9 stain specificity was assessed with **(G)** WT mice injected with 0.5 mg RbIgG control antibody and **(H)** WT mice injected with 0.5 mg RbIgG anti-GBM, with both tissues harvested at 96h. The GR1 stain specificity was assessed with WT mice injected with 0.5 mg RbIgG control antibody harvested 2h post injection and probed with isotype control **(I)**, and with **(J)** specific detection antibody against RbIgG.

	Control	WT	fP KO	C3 KO
n (female)		6	5	5
n (male)		4	3	4
n (all)	5	10	8	9

U-ACR

		WT	fP KO	C3 KO
female		8.63 ± 3.7	4.4 ± 1.61	6.05 ± 2.14
male		4.24 ± 0.96	4.22 ± 3.02	3.25 ± 0.78
All	0.14 ± 0.03	6.87 ± 3.6	4.34 ± 2.02	4.81 ± 2.17

Albumin

		WT	fP KO	C3 KO
female		16346 ± 8805	10273 ± 6695	9370 ± 3229
male		7388 ± 860	5117 ± 2428	4600 ± 1439
All		12763 ± 7491	8340 ± 5867	7250 ± 3509

Creatinine

		WT	fP KO	C3 KO
female		2173 ± 1592	2304 ± 979	1575 ± 304
male		1808 ± 459	1409 ± 414	1408 ± 212
All		2027 ± 1150	1968 ± 901	1501 ± 266

Supplementary Table 1. Analysis of proteinuria and impact of gender to renal injury.