The Production and Use of Transgenic Mouse Models to Study the Role of Complement Pathway Activation in Progressive Kidney Disease

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Statement of originality

This accompanying thesis submitted for the degree of PhD entitled “The Production and Use of Transgenic Mouse Models to Study the Role of Complement Pathway Activation in Progressive Kidney Disease” is based on work conducted by the author at the University of Leicester mainly during the period between May 2012 and July 2016.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

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Dedication,

This thesis is dedicated to the memory of my late mother who never left my heart and my mind.
The production and use of transgenic mouse models to study the role of complement pathway activation in progressive kidney disease

Samy Taha AlGhadban

Abstract

The complement system mediates inflammatory diseases, including kidney disease, through one or more of three pathways named the classical, the alternative and the lectin pathway. The existing experimental data do not allow comprehensive understanding of the individual complement pathway or pathways that mediate the renal injury. This study therefore aimed to determine the complement pathway(s) that are involved in mediating the injury in three models of kidney disease: protein overload proteinuria, unilateral ureteric obstruction nephropathy and adriamycin nephropathy.

In protein overload proteinuria, a highly significant increase in the expression levels of TGF-β, TNF-α and IL-6 was observed in WT mice compared to the lectin pathway deficient mice. Macrophage infiltration, apoptosis and the expression of the gene for Col4α1 were also significantly increased. These data suggested a significant role for the lectin pathway in mediating the injury. Most interestingly, the results indicated that antibody mediated inhibition of the lectin pathway led to less injury in some of the examined parameters. These data might suggest a novel therapeutic approach to relief the renal injury in proteinuric nephropathies by targeting the activation of the lectin pathway.

In unilateral ureteric obstruction nephropathy, results indicated that deficiency in either the lectin or the classical pathways led to reduced macrophage infiltration and renal fibrosis. The expression levels of Il6, Ifn-γ, TGFβ-1 and Col4α1 were reduced in the lectin pathway deficient mice but not in C1qKO mice when compared to WT. However, lower expression levels of these genes were not up to statistical significance. These data suggested that both the lectin and the classical pathways mediate the injury. Similarly, adriamycin nephropathy mice deficient in the lectin pathway showed significant reduction in renal inflammation and fibrosis when compared to WT mice.

Additionally, two gene targeting approaches were utilized in order to establish a novel mouse line with specific deficiencies of both the lectin and the classical pathways of the complement. This mouse model should provide a powerful tool to investigate the possible synergetic cooperation between these two pathways in mediating inflammatory renal pathology.
Table of Contents

1 INTRODUCTION ................................................................................................................. 1

1.1 THE IMMUNE SYSTEM ................................................................................................. 1
  1.1.1 Innate and adaptive immunity ................................................................. 1
  1.1.2 Inflammation ..................................................................................................... 3

1.2 THE COMPLEMENT SYSTEM ......................................................................................... 4
  1.2.1 Activation of the complement system .......................................................... 4
    1.2.1.1 Classical pathway of complement ...................................................... 5
    1.2.1.2 Alternative pathway of complement .................................................. 6
    1.2.1.3 The lectin pathway of complement .................................................... 7
    1.2.1.4 Membrane attack complex ................................................................. 7
  1.2.2 Complement system regulators ............................................................................ 8
  1.2.3 Receptors of complement system ....................................................................... 9
  1.2.4 Renal disease and the complement deficiency .............................................. 9
  1.2.5 Synthesis of complement proteins .................................................................... 11
  1.2.6 Local synthesis of complement in renal tissue ............................................. 11

1.3 CHRONIC KIDNEY DISEASE ....................................................................................... 12
  1.3.1 Complement activation in renal disease ....................................................... 13
    1.3.1.1 Complement activation in clinical proteinuric disease ...................... 14
    1.3.1.2 Complement activation in experimental models of proteinuria .......... 15
    1.3.1.3 Complement activation in non-proteinuric kidney disease ................ 17
    1.3.1.4 Complement activation in other phenotypes of kidney disease .......... 17
  1.3.2 Effects of complement activation on renal cell function ................................ 18

1.4 THREE MODELS OF KIDNEY DISEASE ARE INVESTIGATED IN THE CURRENT STUDY ........................................................................................................ 20
  1.4.1 Protein overload proteinuria ............................................................................ 20
  1.4.2 Unilateral ureteric obstruction ........................................................................ 22
  1.4.3 Adriamycin nephropathy ................................................................................. 24

1.5 TRANSGENIC MOUSE MODELS OF COMPLEMENT DEFICIENCY; A USEFUL TOOL TO UNDERSTAND KIDNEY DISEASE .............................................. 25
  1.5.1 Production of transgenic mice .......................................................................... 25
  1.5.2 Current models of complement deficiencies .................................................. 28
    1.5.2.1 MASP-2<sup>−/−</sup> mice ........................................................................ 28
    1.5.2.2 Clq<sup>−/−</sup> mice ............................................................................... 29
  1.5.3 Generation of a mouse lines deficient in both classical and lectin pathways of complement ........................................................................................................ 31
    1.5.3.1 Previous attempts to generate classical/lectin pathways double deficient mouse model................. 31
    1.5.3.2 Current approaches ............................................................................... 31

1.6 AIMS OF THE WORK ................................................................................................. 36
2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Experimental animals

2.1.2 Reagents used and ready-made kits

2.1.3 Chemicals, media and solutions

2.1.4 Gene targeting vectors

2.1.4.1 PL451 gene targeting vector

2.1.4.2 pGEM®-T Easy vector

2.1.5 Oligonucleotides

2.2 METHODS

2.2.1 Establishment of the protein overload proteinuria mouse model and model specific techniques

2.2.1.1 Experimental animals

2.2.1.2 Unilateral nephrectomy surgery

2.2.1.3 BSA administration to induce protein overload proteinuria

2.2.1.4 Antibody mediated inhibition of the lectin pathway in wild type mice with protein overload proteinuria

2.2.2 Establishment of the unilateral ureteric obstruction mouse model and model specific techniques

2.2.2.1 Unilateral ureteric obstruction surgery

2.2.2.2 Antibody-mediated lectin pathway inhibition in unilateral ureteric obstruction mice

2.2.2.3 Lectin pathway inhibition assay

2.2.3 Adriamycin nephropathy mouse model

2.2.3.1 Experimental animals

2.2.3.2 Establishment of the model by administration of adriamycin

2.2.4 Renal injury models: general analysis techniques

2.2.4.1 Immunohistochemistry

2.2.4.2 Image analysis

2.2.4.3 Statistical analysis

2.2.4.4 Detection of apoptosis

2.2.4.5 Sirius Red staining

2.2.4.6 Haematoxylin/eosin staining

2.2.4.7 Determination of total protein concentrations in urine and serum

2.2.4.8 Creatinine estimation

2.2.4.9 Active C3c titration method using enzyme linked immunosorbent assay (ELISA)

2.2.4.10 Gene expression in injured kidneys

2.2.4.11 Total RNA extraction

2.2.4.12 Genomic DNA extraction

2.2.4.13 Agarose gel electrophoresis

2.2.5 Gene targeting procedures of murine C1rA and MASP-2 genes
2.2.5.1 Recombineering approach for conditional targeting of C1rA gene ........................................ 63
2.2.5.2 Polymerase chain reaction (PCR) .......................................................................................... 72
2.2.5.3 CRISPR/Cas based approach to target MASP-2 gene in C1qKO mice ..................................... 76

3 THE ROLE OF THE COMPLEMENT SYSTEM ACTIVATION IN PROTEIN OVERLOAD PROTEINURIA. 79

3.1 OPTIMIZATION OF THE PROTEIN OVERLOAD PROTEINURIA MODEL IN MICE ........................................... 79
3.1.1 Dosing regimen, strain and unilateral nephrectomy ............................................................... 79
3.1.2 Proteinuria time-course profile in BALB/c mice over 24 hours post-BSA dose administration ................................................................................................................................. 83
3.1.3 C3 expression in renal tissues after POP .................................................................................. 85

3.2 THE USE OF TRANSGENIC MASP-2KO MICE TO STUDY THE ROLE OF THE LECTIN PATHWAY OF COMPLEMENT

ACTIVATION IN PROTEIN OVERLOAD PROTEINURIA ......................................................................................... 86
3.2.1 Experimental design .............................................................................................................. 87
3.2.2 MASP-2KO mice genotyping .................................................................................................. 87
3.2.3 Determination of serum total proteins in WT and MASP-2KO mice ....................................... 88
3.2.4 Determination of proteinuria following BSA overload ............................................................. 89
3.2.5 Macroscopic appearance of dissected mice ........................................................................... 90
3.2.6 Histopathological examination of WT and MASP-2KO kidneys after BSA challenge .............. 91
3.2.7 Kidney ultrastructure by electron microscopy ........................................................................ 92
3.2.8 Macrophage infiltration ........................................................................................................ 93
3.2.9 Expression of transforming growth factor-β (TGF-β) in the mouse kidney .............................. 95
3.2.10 Determination of tumour necrosis factor α (TNF-α) expression levels in MASP-2KO mouse kidneys after BSA challenge .............................................................................................................. 97
3.2.11 Expression levels of interleukin 6 in WT and MASP-2KO mouse kidneys after BSA challenge 99
3.2.12 Determination of apoptosis in WT and MASP-2KO mice kidneys ........................................ 101
3.2.13 Determination of selected gene expression profiles in WT and MASP-2KO mouse kidneys 103

3.3 ANTIBODY MEDIATED INHIBITION OF THE LECTIN PATHWAY IN A MOUSE MODEL OF PROTEIN OVERLOAD

PROTEINURIA ............................................................................................................................................. 106
3.3.1 Experimental design .............................................................................................................. 106
3.3.2 Determination of proteinuria induced in protein-overload mice treated with anti-MASP-2 antibody 106
3.3.3 Measurement of lectin pathway inhibition after antibody treatment ........................................ 107
3.3.3.1 Flow cytometry based determination of the inhibition of the lectin pathway in serum of antibody treated mice ......................................................................................................................... 107
3.3.3.2 Determination of the lectin pathway inhibition using ELISA ................................................ 109
3.3.4 Histological examination of proteinuric mouse kidney sections after inhibition of the lectin pathway ........................................................................................................................................... 111
3.3.5 Macrophage infiltration in the lectin pathway inhibited mouse kidneys .................. 112
3.3.6 TGF-β expression profile in mouse kidneys after inhibition of the lectin pathway .... 114
3.3.7 Assessment of the degree of expression of TNF-α in MASP-2 inhibited mouse kidneys. 115
3.3.8 Assessment of IL-6 expression after administration of anti-MASP-2 antibody .......... 117
3.3.9 Determination of apoptosis in lectin pathway inhibited mouse kidneys ................. 118
3.3.10 Expression profiles of inflammation marker genes in the mouse kidney after inhibition of the lectin pathway ........................................................................................................ 120
3.4 DISCUSSION .................................................................................................................. 123
3.5 CONCLUSIONS ............................................................................................................ 133

4 THE ROLE OF THE COMPLEMENT SYSTEM ACTIVATION IN UNILATERAL URETERIC OBSTRUCTION 134
4.1 UUO MODEL OPTIMISATION ..................................................................................... 134
4.1.1 Experimental design ............................................................................................... 134
4.1.2 Gross histological changes in mouse kidneys after one, three and seven days of UUO. 134
4.1.3 Collagen deposition in mouse kidney sections after one, three and seven days of UUO 135
4.1.4 Animal welfare assessment and pain scoring after one, three and seven days of UUO 137
4.2 UTILISATION OF COMPLEMENT COMPROMISED TRANSGENIC MOUSE LINES TO STUDY THE ROLE OF THE CLASSICAL AND THE LECTIN PATHWAYS OF COMPLEMENT IN UNILATERAL URETERIC OBSTRUCTION ........................................ 138
4.2.1 Experimental design ............................................................................................... 138
4.2.2 Genotyping of mice ................................................................................................. 138
4.2.3 Histopathological examination of WT, C1qKO and MASP-2KO mice kidney sections after UUO 139
4.2.4 Transmission EM examination of kidney ultrastructure ......................................... 140
4.2.5 Visualisation and quantification of fibrosis in mouse kidneys from WT, C1qKO and MASP-2KO groups after UUO ........................................................................................................ 141
4.2.6 Visualisation and quantification of macrophage infiltration in kidney tissues ......... 143
4.2.7 Determination of the expression profiles of selected genes in UUO mouse kidneys ..... 145
4.3 ANTIBODY MEDIATED INHIBITION OF THE LECTIN PATHWAY IN UNILATERAL URETERIC OBSTRUCTION MICE ...... 149
4.3.1 Experimental design ............................................................................................... 149
4.3.2 Determination of the lectin pathway activity in antibody treated mice ................. 149
4.3.3 Estimation of fibrosis in UUO kidney sections after administration of anti-MASP-2 antibody 151
4.3.4 Macrophage infiltration after antibody-mediated inhibition of the lectin pathway ..... 154
4.3.5 Serum creatinine levels in UUO mice after inhibition of the lectin pathway .......... 156
4.3.6 Serum urea in UUO mice after inhibition of the lectin pathway ............................. 156
4.4 DISCUSSION .................................................................................................................. 158
4.5 CONCLUSIONS ............................................................................................................ 163
5 THE ROLE OF THE LECTIN PATHWAY OF COMPLEMENT IN ADRIAMYCIN NEPHROPATHY .......................... 164

5.1 MODEL OPTIMISATION ............................................................................................................. 164
  5.1.1 Experimental design .......................................................................................................... 164
  5.1.2 Changes in the mouse body weight after 1, 2 and 4 weeks of AN ........................................ 164
  5.1.3 Proteinuria induced after 1, 2 and 4 weeks of AN .................................................................. 165
  5.1.4 Changes in total serum protein in AN mice .......................................................................... 166
  5.1.5 Renal histopathological changes after 1, 2 and 4 weeks of AN ............................................ 167
  5.1.6 Renal inflammation in AN mice after 1, 2 and 4 weeks of injury ......................................... 168

5.2 THE USE OF MASP-2\textsuperscript{KO} MICE TO DETERMINE THE ROLE OF THE LECTIN PATHWAY ACTIVATION IN AN .......... 170
  5.2.1 Experimental design .......................................................................................................... 170
  5.2.2 Changes in the body weight of WT and MASP-2\textsuperscript{KO} mice after AN ............................. 170
  5.2.3 Proteinuria induced in WT and MASP-2\textsuperscript{KO} mice after AN ........................................ 171
  5.2.4 Changes in the total serum protein of WT and MASP-2\textsuperscript{KO} mice after two weeks of AN 172
  5.2.5 Renal histopathological changes in WT and MASP-2\textsuperscript{KO} mice after AN .................... 173
  5.2.6 Renal inflammation in WT and MASP-2\textsuperscript{KO} mice after two weeks of AN .................. 174
  5.2.7 Renal fibrosis in WT and MASP-2\textsuperscript{KO} mice after two weeks of AN ............................ 176

5.3 DISCUSSION .................................................................................................................................. 178

5.4 CONCLUSIONS ................................................................................................................................. 180

6 ESTABLISHMENT OF A NOVEL MOUSE MODEL OF COMPLEMENT DEFICIENCY WITH TARGETED DEFECTIVE GENES FOR KEY COMPONENTS OF BOTH THE CLASSICAL AND THE LECTIN PATHWAYS OF COMPLEMENT ................................................................. 181

6.1 INTRODUCTION ............................................................................................................................ 181

6.2 GENERATION OF A CONDITIONAL TARGETING CONSTRUCT TO TARGET THE MURINE C1rA GENE USING A RECOMBINEERING-BASED TECHNOLOGY .............................................................................................................. 182

  6.2.1 Distribution of the expression levels of C1rA and C1rB genes in male and female mouse tissues 182
  6.2.2 Design of the C1rA targeting construct .................................................................................. 184
  6.2.3 Restriction fragment length polymorphism gene targeting screening ................................ 186
  6.2.4 Assembly of the construct homology arms in bacterial cloning vector .................................. 187
    6.2.4.1 PCR amplification of the construct cassettes ...................................................................... 187
    6.2.4.2 Bacterial cloning of the construct homology arms ............................................................ 191
    6.2.4.3 Assembly of the two arms of cassette B ........................................................................... 192
    6.2.4.4 Insertion of a LoxP recombination site into cassette B .................................................... 196
  6.2.5 Assembly of the C1rA targeting construct in the vector PL451 ............................................ 199
  6.2.6 Confirmation of proper assembly of the construct by DNA sequencing ................................ 201
  6.2.7 Electroporation of the C1rA construct into 129/Sv ES cells ................................................. 202
6.3 Establishment of a novel mouse model of complement deficiency through combined disruption of the C1q and the MASP-2 genes ................................................................. 203
  6.3.1 Design of the CRISPR based targeting of the MASP-2 gene ........................................ 203
  6.3.2 Validation of the C1qKO mouse line sequence preservation ........................................ 206
  6.3.3 Synthesis of the CRISPR gRNAs .................................................................................. 208
  6.3.4 Microinjection of the CRISPR reagents into C1qKO embryos and embryo transfer .......... 208
  6.3.5 Genotyping of the CRISPR targeted pups ..................................................................... 211
  6.3.6 Confirmation of CRISPR pups genotyping by DNA sequencing .................................... 213
  6.4 Discussion ......................................................................................................................... 216
  6.5 Conclusions ....................................................................................................................... 219

7 SUMMARY AND FUTURE WORK ......................................................................................... 220
  7.1 Summary ........................................................................................................................... 220
  7.2 Future work ....................................................................................................................... 226

8 APPENDIX I ........................................................................................................................ 228
  Automated Image Analysis Macro Code ............................................................................. 228

9 REFERENCES ......................................................................................................................... 229
Table of Tables

Table 1: The annotation map of the PL451 vector.................................................................42

Table 2: Optimisation of protein overload proteinuria mouse model........................................80

Table 3: Sizes of the restriction fragment length polymorphism of the murine C1rA and C1rB genes and their predicted targeted recombinants.................................................187

Table 4: PCR primer pairs used to amplify the homologous cassettes of the C1rA gene targeting construct........................................................................................................189

Table 5: CRISPR gRNAs used to target the murine MASP-2 gene.............................................205

Table 6: Outcomes of 6 microinjection sessions performed to inject the CRISPR reagents into C1qKO embryos.................................................................................................210
# Table of Figures

Figure 1: Activation pathways of the complement system ........................................5

Figure 2: Renal injury mediated by the complement system ................................. 19

Figure 3: General protocol for the production of gene targeted mice ..................... 27

Figure 4: Targeting event for the establishment of the MASP-2 knockout mouse line. 29

Figure 5: Schematic diagram for targeting the mouse C1q gene .............................. 30

Figure 6: Schematic representation of the CRISPR mediated DNA double strand break 33

Figure 7: Schematic structure of the murine C1rA gene ........................................ 34

Figure 8: Schematic structure of the murine MASP-2 gene and its transcript variants .... 35

Figure 9: PL451 vector map showing the restriction enzymes available in the two polylinker regions ...................................................................................................................................................... 43

Figure 10: pGEM®-T Easy vector map ........................................................................ 44

Figure 11: Representative images for negative control slides .................................. 53

Figure 12: Representation of the amplification blot of the GAPDH gene in all tested samples. 60

Figure 13: A schematic diagram for the CRISPR mediated targeted deletion of the MASP-2\(^{\text{KO}}\) gene in C1q\(^{\text{KO}}\) mice ...................................................................................................................................................... 77

Figure 14: Fold increase in proteinuria from seven experiments conducted to optimise the protein overload proteinuria mouse model ...................................................................................................................................................... 82

Figure 15: Urine total protein concentration of BALB/c mice over 24 hours post-BSA injection. ................................................................................................................................................................................................ 84

Figure 16: Proteinuria profile of BALB/c mice over 24 hours measured as urine PCR. ....... 85

Figure 17: Light microscopy images for mouse kidney sections stained with C3 after POP ...... 86

Figure 18: Agarose gel image of a multiplex PCR for genotyping of mouse MASP-2 gene. ..... 88
Figure 33: Numbers of apoptotic cells counted manually in 20 adjacent high power fields (HPFs) of the kidney cortex. ................................................................. 103

Figure 34: qPCR analysis of expression levels of seven complement and renal inflammation related genes in non-proteinuric controls and protein overloaded kidneys of WT and MASP-2\(^{\text{KO}}\) mice........................................................................................................ 105

Figure 35: 24 hours urinary protein excretion in anti-MASP-2 and non-anti-MASP-2 treated mice.............................................................................................................. 107

Figure 36: Flow cytometry examination of the deposition of lectin pathway based active C3 in minimally diluted serum samples. ................................................................. 108

Figure 37: Flow cytometry examination of deposition of lectin pathway based active C4....... 109

Figure 38: Determination of inhibition of the lectin pathway after anti-MASP-2 antibody treatment using non-BSA based blocking buffer (skimmed milk). ................................. 110

Figure 39: ELISA determination of lectin pathway dependant C3 deposition using BSA based blocking buffer. ........................................................................................................ 110

Figure 40: H&E stained kidney sections from proteinuric mice with and without inhibition of the lectin pathway. .............................................................................................. 111

Figure 41: Macrophage infiltration in mouse kidney sections from all experimental groups ... 112

Figure 42: Computer based image analysis quantitation of F4/80 stained macrophages in mouse kidney sections. ................................................................. 113

Figure 43: Quantitation of the macrophage infiltration in the juxtamedullary and capsular zones of the mouse kidney cortex................................................................. 113

Figure 44: Representative images of kidney sections stained with transforming growth factor β. .............................................................................................................. 114

Figure 45: Quantification of TGF-β stained areas ................................................................. 115

Figure 46: Representative images of kidney sections stained with TNF-α antibody......... 116

Figure 47: Quantitative analysis of mouse kidney sections stained with anti- TNF-α antibody. ..................................................................................................................... 116
Figure 48: Representative images of kidney sections stained with interleukin-6. 117

Figure 49: Quantitation of IL-6 stained sections using one-way ANOVA. 118

Figure 50: Representative images of apoptotic cells, red arrows, stained in kidney sections from all treated groups. 119

Figure 51: Statistical evaluation of apoptotic cells stained by TUNEL with and without antibody-mediated inhibition of the lectin pathway. 119

Figure 52: qPCR analysis of expression levels of seven genes in WT mouse kidneys with and without inhibition of the lectin pathway. 121

Figure 53: H&E stained mouse kidney sections after one day (B), three days (C) and seven days (D) of unilateral ureteric obstruction. 135

Figure 54: Representative micrographs of mouse kidney sections stained with Sirius Red after one day (B), three days (C) and seven days (D) of unilateral ureteric obstruction. 136

Figure 55: Computer based image analysis of mouse kidney sections stained with Sirius Red after one day, three days and seven days of UUO. 137

Figure 56: Agarose gel image of a multiplex PCR for genotyping of mouse C1q gene. 139

Figure 57: Representative micrographs of mouse kidney sections stained with H&E after seven days of UUO. 140

Figure 58: Transmission electron micrographs of wild type, C1q^KO and MASP-2^KO mouse kidney sections after seven days of UUO. 141

Figure 59: Representative micrographs of Sirius Red stained mouse kidney sections from wild type, C1q^KO and MASP-2^KO after seven days of UUO. 142

Figure 60: Analysis of Sirius Red stained kidney sections from WT, C1q^KO and MASP-2^KO mice after seven days of UUO. 143

Figure 61: Representative micrographs of mouse kidney sections stained with macrophage specific antibody F4/80 after seven days of UUO. 144

Figure 62: Analysis of F4/80 macrophage stained areas in kidney sections from WT, C1q^KO and MASP-2^KO mice after seven days of UUO. 145
Figure 63: Relative mRNA expression levels of a group of selected inflammation and fibrosis related genes in WT, C1qKO and MASP-2KO mouse kidneys after seven days of UUO. ..........147

Figure 64: Relative mRNA expression levels of a group of selected inflammation and fibrosis related genes in WT, C1qKO and MASP-2KO mouse kidneys after seven days of UUO. ..........148

Figure 65: Flow cytometry estimation of the activity of endogenous C3 in mice serum after seven days of UUO. .................................................................150

Figure 66: Flow cytometry based activation assay of endogenous C4 in mice serum after seven days of UUO. .......................................................................151

Figure 67: Representative micrographs of mouse kidney sections stained with Sirius Red after seven days of UUO .................................................................152

Figure 68: Estimation of the collagen area stained with Sirius Red in mouse kidney sections from all groups after seven days of UUO ...........................................153

Figure 69: Estimation of the collagen area stained with Sirius Red in UUO mouse kidney sections .................................................................153

Figure 70: Representative micrographs of infiltration of macrophage cells stained with F4/80 antibody in mouse kidney section after UUO .................................154

Figure 71: Analysis of F4/80 macrophage stained area in mouse kidney sections from all treated groups after seven days of UUO ..............................................155

Figure 72: Estimation of the macrophage area stained with F4/80 antibody in UUO mouse kidney sections .................................................................155

Figure 73: Estimation of the serum creatinine levels of UUO mice treated with anti-MASP-2 antibodies .................................................................156

Figure 74: Estimation of the serum urea levels of UUO mice treated with anti-MASP-2 antibodies .................................................................157

Figure 75: Changes in the body weight of BALB/c mice after adriamycin nephropathy (AN). ..............................................................................................165

Figure 76: Proteinuria induced in BALB/c mice after 1, 2 and 4 weeks of AN ...............166

Figure 77: Total serum protein of AN mice after 1, 2 and 4 weeks of the injury ..........167
Figure 78: Histopathological examination of mouse kidney sections after 1, 2 and 4 weeks of AN.................................................................168

Figure 79: Macrophage infiltration in mouse kidney sections stained with F4/80 antibody after 1 week (B), 2 weeks (C) and 4 weeks (D) of AN.................................................................169

Figure 80: Changes in the body weight of WT and MASP-2KO mice after two weeks of AN...171

Figure 81: Urine total proteins excreted in 24 hours from WT and MASP-2KO mice after two weeks of AN.................................................................................172

Figure 82: Total proteins in the serum of WT and MASP-2KO mice after two weeks of AN ...173

Figure 83: Representative images of mouse kidney sections stained with H&E after two weeks of AN .................................................................................174

Figure 84: Mouse kidney sections stained with F4/80 macrophage specific antibody after two weeks of AN.................................................................................175

Figure 85: Estimation of the F4/80 macrophage infiltration in WT and MASP-2KO mouse kidney sections after two weeks of AN .............................................................................176

Figure 86: Collagen depositions stained with Sirius Red in WT and MASP-2KO mouse kidney sections after two weeks of AN.................................................................................177

Figure 87: Analysis of the collagen deposition in WT and MASP-2KO mouse kidney sections stained with Sirius Red after two weeks of AN ...........................................................................177

Figure 88: Tissue distribution of C1rA and C1rB mRNA expression levels measured by qPCR in male (A) and female (B) mice ..........................................................................................184

Figure 89: Schematic diagram represents the murine C1rA gene and showing the locations of the construct homologous cassettes A and B..............................................................................185

Figure 90: Schematic diagram for the design of a conditional knockout construct for a targeted deletion of exon 3 from the murine C1rA gene ..............................................................................186

Figure 91: Estimation of the truncated protein product from the targeted C1rA gene after deletion of exon3 ............................................................................................................................................186

Figure 92: Agarose gel image showing a thermal gradient PCR to optimize the annealing temperature of the CasB1 primers pair ..................................................................................................190

XVIII
Figure 93: Agarose gel images showing the PCR amplicons of CasA (A), CasB1 (B) and CasB2 (C)...............................................................................................................................190

Figure 94: Sequence of the leading strand of cassette A and the modified primers that were used to amplify it ........................................................................................................................................191

Figure 95: Schematic representation of the construction of Cassette B elements in pGEM®-T Easy vector ........................................................................................................................................193

Figure 96: The leading strand sequences of CasB1, LoxP and CasB2 ligated to each other in the right order........................................................................................................................................194

Figure 97: Agarose gel image showing the DNA bands of the digestion of CasB2 out of the pGEM®-T Easy vector (lanes 1 and 2) and CasB1 ligated into the vector (lanes 3 and 4) .....195

Figure 98: Agarose gel image showing the restriction digestion analysis of CasB1 and CasB2 ligated into the pGEM®-T Easy vector. ........................................................................................................................................195

Figure 99: Agarose gel images showing PCR products of positive (A) and negative (B) insertion of LoxP site in the sense direction........................................................................................................................................198

Figure 100: Sequencing data of a cassette B ligate with a single LoxP recombination site (blue box) inserted in the desired place and in the sense direction.................................................199

Figure 101: Agarose gel image showing the ligation of cassette B to the mammalian vector PL451 (lanes 1, 2, 3, 5 and 6). Lane 4 is showing the vector only. ................................................200

Figure 102: Agarose gel image showing the ligation of cassettes A and B into the PL451 vector ...................................................................................................................................................201

Figure 103: Sequencing data confirming the assembly of the designed construct ...............202

Figure 104: 129/Sv ES cells transfected with the C1rA conditional knockout construct and growing on MEF cells in a neomycin-supplemented selection medium.................................203

Figure 105: MASP-2 gene showing the gRNAs predicted by the web tool ChopChop®........206

Figure 106: Sequencing of the targeted loci in C1qKO mice.................................................207

Figure 107: In vitro transcribed gRNAs ..................................................................................208

Figure 108: Optimization of the CRISPR genotyping PCR primer pairs...............................212
Figure 109: Thermal gradient PCR to determine the optimum annealing temperature for the CRISPR genotyping PCR primer pairs...........................................................................................................................................212

Figure 110: Genotyping of the CRISPR targeted pups .........................................................................................................................213

Figure 111: DNA sequencing of the mouse ID 141 (A) compared to a WT mouse (B)........214

Figure 112: DNA sequencing of the mouse ID 141 (A) compared to a WT mouse (B)........215
Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Eukaryotic 18S rRNA</td>
</tr>
<tr>
<td>Actn1</td>
<td>Actinin alpha 1</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AN</td>
<td>Adriamycin nephropathy</td>
</tr>
<tr>
<td>AP</td>
<td>Alternative pathway</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial artificial chromosome</td>
</tr>
<tr>
<td>BBS</td>
<td>Barbital buffered saline</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C1qKO</td>
<td>C1q knockout mouse line</td>
</tr>
<tr>
<td>C3</td>
<td>Complement component 3</td>
</tr>
<tr>
<td>C3aR</td>
<td>C3a receptor</td>
</tr>
<tr>
<td>C4bp</td>
<td>C4 binding protein</td>
</tr>
<tr>
<td>C5aR</td>
<td>C5a receptor</td>
</tr>
<tr>
<td>Cas9</td>
<td>CRISPR associated protein 9</td>
</tr>
<tr>
<td>CCP</td>
<td>Complement control protein</td>
</tr>
<tr>
<td>Cdh1</td>
<td>Cadherin 1</td>
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XXI
cDNA  Complementary DNA
Cfp  Complement factor properdin
CFU  Colony forming unit
CHO  Chinese hamster ovary
CKD  Chronic kidney disease
CKO  Conditional knockout
Col4α1  Collagen type IV alpha 1
CP  Classical pathway
CR  Complement receptor
CRISPR  Clustered regularly-interspaced short palindromic repeats
DAB  Diaminobenzidine
dATP  Deoxyadenosine triphosphate
dCTP  Deoxycytidine triphosphate
DEPC  Diethyl pyrocarbonate
DMF  Dimethyl formamide
DMSO  Dimethyl sulfoxide
DNase  Deoxyribonuclease
dNTP  Deoxyribonucleoside triphosphate mixture
DTT  Dithiotheritol
EDTA  Ethylene diamine tetra-acetic acid
EGF  Epidermal growth factor
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>EGTA</td>
<td>Ethylene glycol tetra-acetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal trans-differentiation</td>
</tr>
<tr>
<td>ES cells</td>
<td>Embryonic stem cells</td>
</tr>
<tr>
<td>ESRD</td>
<td>End stage renal disease</td>
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<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorter</td>
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<tr>
<td>fH</td>
<td>Factor H</td>
</tr>
<tr>
<td>fI</td>
<td>Factor I</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>Fn1</td>
<td>Fibronectin 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>gRNA</td>
<td>CRISPR guide RNA</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Gusβ</td>
<td>Glucuronidase beta</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin stain</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>HLH</td>
<td>Hemophagocytic lymphohistocytosis</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HUS</td>
<td>Haemolytic uremic syndrome</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>Ifn-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Il-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>Il-12a</td>
<td>Interleukin-12a</td>
</tr>
<tr>
<td>Il-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base pair</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
</tr>
<tr>
<td>LoxP</td>
<td>Locus of X-over P1 bacteriophage</td>
</tr>
<tr>
<td>LP</td>
<td>Lectin pathway</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MAp19</td>
<td>MBL-associated protein of 19 kDa</td>
</tr>
<tr>
<td>MASP</td>
<td>Mannan associated serine protease</td>
</tr>
<tr>
<td>MASP-2KO</td>
<td>MASP-2 knockout mouse line</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannan binding lectin</td>
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<td>Abbreviation</td>
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<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescent intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MPGN</td>
<td>Membrane proliferative glomerulonephritis</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for biotechnology information</td>
</tr>
<tr>
<td>NHS</td>
<td>National health services</td>
</tr>
<tr>
<td>NP</td>
<td>Non-proteinuric</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PAMPS</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PMSG</td>
<td>Pregnant mare’s serum gonadotropin</td>
</tr>
<tr>
<td>POP</td>
<td>Protein overload proteinuria</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PTEC</td>
<td>Proximal tubular epithelial cells</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RCA</td>
<td>Regulators of the complement activation</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RQ</td>
<td>Relative quantitation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>rt-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of means</td>
</tr>
<tr>
<td>SGPI</td>
<td><em>Schistocerca gregaria</em> protease inhibitor</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SP</td>
<td>Serine protease</td>
</tr>
<tr>
<td>β-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TCCs</td>
<td>Terminal complement components</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptors</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N′, N′-tetramethylenediamine</td>
</tr>
<tr>
<td>TGFβ-1</td>
<td>Transforming growth factor beta-1</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureteric obstruction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>Vim</td>
<td>Vimentin</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
1 Introduction

1.1 The immune system

The immune system provides the body with defence mechanisms against infectious disease and clears debris and injured cells and toxins as a scavenger system. This system is composed of highly sophisticated and specialized networks of cellular and non-cellular components that protect the body from invading pathogens as well as aberrant or infected cells. As such, immunity maintains the body’s integrity and maintains homeostasis (Harvey & Morgan, 2014). If challenged, the immune system initiates a vast group of activation cascades that lead to the destruction and elimination of the source of harm. These activation processes are carried through a variety of interactive humoral and cell-mediated responses that orchestrate a combined response. The cell-mediated responses involve the recruitment and activation of white blood cells that can attack and destroy pathogens (Litman et al., 2005). The humoral immune response involves the release of fluid phase proteins that can initiate the clearance of pathogens. Among these are cytokines, chemokines, anaphylatoxins, complement proteins and other peptides and growth factors (Medzhitov, 2007). Uncontrolled activation of the immune system can lead to secondary immune-deficiencies due to depletion of effector components and/or inflammatory disease that can shift from an acute phase state to chronic inflammatory conditions collectively known as autoimmune diseases. Inadequate immune responses are harmful to the body and lead to a large variety of health complications.

The immune system is composed of two major branches: i) innate immunity and ii) the adaptive (or acquired) immunity.

1.1.1 Innate and adaptive immunity

The innate immune response delivers the initial response to pathogens that enter the body. An innate immune response is characterized by its instantaneous action, fighting invading microorganism instantly and does not require complex
adaption processes. It is equipped with a group of specific and non-specific mechanisms that can respond to all kinds of challenges. Innate immune defence mechanisms extend from the external barriers of the body, like for example the skin or the mucous membranes and the stomach’s acidic environment, to groups of innate immune cells and fluid phase components like for example antimicrobial peptides.

Cells of the innate immune system are mainly of haematopoietic origin and include cells living inside tissues e.g. dendritic cells, and cells that circulate in blood and lymph (O'Byrne & Dalgleish, 2001). Circulating cells comprise a group of phagocytes that recognise and kill pathogens through universally encoded pattern on microbes known as pathogen associated molecular patterns (PAMPs). Once a pathogen enters the body, phagocytic cells scan the invading pathogen for PAMPs using a group of various PAMPs receptors, the pattern recognition receptors PRRs, found on their surface to identify danger signals (Gallucci & Matzinger, 2001; Pradeu & Cooper, 2012). If not recognised as a self-antigen, the protein will be opsonised, engulfed and destroyed by macrophage lytic enzymes. The innate immune response depends on pre-existing pattern recognition molecules and lacks memory function (Medzhitov, 2007).

In contrast to the rapid activation of innate immune defence, the adaptive immune response takes longer to initiate a custom-made response. However, it retains memory of the invading microbes thus facilitating a rapid defence and effective response to subsequent attack.

Thus, the acquired immune response represents a highly sophisticated defence mechanism able to recognise and eliminate foreign bodies, even microorganisms that are highly mutational (Borghesi & Milcarek, 2007). Its core components are T and B lymphocytes that utilise a variety of antigen specific cellular receptors (T cell receptors, TCR, and B cell receptors, BCR) which recognise and release several humoral immunoglobulin antibodies specific to the pathogen. Antibodies recognise and bind specifically to the pathogen to facilitate its phagocytosis and
clearance. Adaptive immunity requires the pre-recognition of the innate immune response to the antigen in order to build up its specific antibodies as well as storing a memory about it for future possible interactions (Playfair & Chain, 2009).

1.1.2 Inflammation

Inflammation is part of the complex immune system response considered as one of the innate immunity mechanisms. Essentially inflammation is a protective response that involves activation of immune cells and other tissue factors to eliminate the initial cause of injury and initiate tissue repair processes. Inflamed tissue usually is characterized by heat, redness, swelling, and loss of function due to increased blood flow in the site, activation of the complement system and subsequent fighting of pathogens (Ferrero-Miliani et al., 2007).

Inflammation can be classified as acute and chronic. Acute inflammation is the initial stage that is triggered by the harmful stimulus. It is mediated by increased permeability of blood vessels and capillaries at the site of injury and facilitates migration of leucocytes into the tissue. A series of biochemical processes are triggered involving the immune system cells and the cells of the injured tissue. Chronic inflammation is a kind of injury that exerts stress on the tissue elements and lead to a progressive shift in the type of cells present at the site of inflammation (Harvey & Morgan, 2014). Chronic inflammation though is characterised by simultaneous destruction and healing of the tissue from the inflammatory process.

In general, inflammation is a fundamental process that is needed for the destruction and clearance of pathogens; however it exerts stress on cells and tissues. Deregulated chronic inflammation leads to progressive tissue destruction in affected organs and tissues and a variety of diseases and health complications, including chronic kidney disease (CKD) (Coussens & Werb, 2002; Rather, 1971).
1.2 The complement system

The complement system is a biochemical cascade of proteins that represents essential part of the innate immunity. It responds rapidly to defend the host against a variety of invading microorganisms. It also orchestrates many homeostatic processes as a connection between innate and adaptive immune responses. Complement system also participates in the inductive phase of the acquired immune response by contribution to the recognition and presentation of non-self antigens, enhancing the immunologic memory and helping in the augmentation of the antibody responses (Walport, 2001). Complement was first identified in the late 19th century by the German scientist Paul Ehrlich as a heat-labile blood serum component with non-specific antimicrobial activity that “complements” other immune functions; hence the name “the complement system” was coined. The system is composed of a group of approximately forty serum and membrane-bound proteins. The cascade activation and assembly of those proteins lead to the formation of functional units that play three essential physiological activities to maintain the body homeostasis:

a) The release of a group of polypeptides in order to initiate inflammation
b) Attachment of opsonins to the surface of the antigen to facilitate phagocytosis
c) Formation of the membrane attack complex that may lead to the cell lyses

1.2.1 Activation of the complement system

Three major pathways are known to activate the complement system namely classical, alternative and lectin pathways. Figure 1 is a diagrammatic representation for the complement components and its cascade activation.
1.2.1.1 Classical pathway of complement

The Classical pathway is the first described complement pathway. The activation of it is initiated by the activation of the first recognized complement complex C1. It starts by the binding of the tulip shaped hexamer complement subcomponent C1q to the Fc region of IgG or IgM of an antibody bound to the surface of bacteria. Upon the C1q binding, C1r cleaves and activates the serine protease...
C1s. Activated C1s then catalyses the cleavage of C4 into two subcomponents: the smaller C4a and the larger C4b. C4b then attaches to the cell surface with one C2 unit attached to it. Cleavage of the C2 by the C1s leads to the formation of C4b2a, an enzyme that cleaves the key component C3 into C3a and C3b. the C3b then attaches to the C3 convertase enzyme (C4b2a) in order to form the C5 convertase enzyme (C4b2a3b). Cleavage of C5 into C5a and C5b lead to the aggregation of C5b with one unit of C6, C7 and C8 and initiates polymerization of C9 on the cell membrane of the attacked cell. This forms a membrane attack complex that integrates into the cell membrane and lead to the leakage of the cell components and cell death (Playfair& Chain, 2009).

1.2.1.2 Alternative pathway of complement

Activation of the alternative pathway of complement is dependent on the spontaneous low level hydrolysis of the internal thio-ester bond of C3 to C3 (H$_2$O). C3 (H$_2$O) acts as C3b and binds to a hydroxyl group on cell-surface carbohydrates and proteins of a pathogen. This might happen in the absence of pathway specific recognition molecules and it does not require specific antigen antibody interaction. The activation initiates by low-grade cleavage of C3 in plasma. The second step of the activation comprises the attachment of Factor B molecule to the hydrolysed C3b to form C3bB that is converted into active enzymatic form by Factor D and Properdin to become C3bBb, the alternative pathway C3 convertase enzyme. The binding of the properdin gives stability to this process. The continuous deposition of cleaved C3b on the surface of the pathogen around C3bBb then shifts the specificity of the C3bBb towards C5. This occurs by the attachment of one more C3b molecule to form C3bBbC3b which is one form of the C5 convertases. Cleavage of the C5 leads to the formation of the membrane attack complex the same way like in the classical pathway (Inchley, 1981). The alternative pathway also amplifies the classical and lectin pathways and therefore is critical for the full activity of the complement.
1.2.1.3 The lectin pathway of complement

The mannose-binding lectin pathway is initiated by binding of the complex of the hexamer tulip shaped mannose-binding lectin and the two mannose binding lectin- associated serine proteases 1 and 2 (abbreviated MASP-1 and MASP-2 respectively) to specific recognition patterns of carbohydrate group (mannose or ficolin for example) on the surface of bacterial cell. This complex acts the same way like the classical pathway initial activation component C1 complex cleaving C4 into C4a and C4b and cleaving the C4bC2 into the C3 convertase active enzyme C4b2a. Activation of the C3 lead to the formation of active C5 convertase which is in turn leads to the formation of active C5 and the assembly of the membrane attack complex the same way of the classical pathway. It is also believed that MASP-1 enzyme is able to cleave C3 directly (Playfair & Chain, 2009; Inchley, 1981) forming active C5 convertase. MASP-2 is the effector component of the lectin pathway that acts the same way as the serine protease C1s in the classical pathway (Schwaeble et al., 2002).

1.2.1.4 Membrane attack complex

The membrane attack complex formation is facilitated by cleaving the complement protein C3 into a small fragment C3a and a large fragment C3b, by any of the C3 convertase produced by one of the complement pathways (either the classical and the lectin pathways C3 convertase (C4b2a) or the alternative pathway C3 convertase C3bBb). Accumulations of C3b to the C3 convertase leads to forming C5 convertase, C4b2a(C3b)n for classical and lectin pathways or C3bBb(C3b)n for the alternative pathway. The C5 convertase then cleaves C5 into a large portion C5b and a small portion C5a. The C5b binds to the cell membrane of a pathogen, leading to the formation of C5b-8 complex. This allows the polymerisation of C9 that forms pores in the lipid bilayer of the pathogen cell membrane leading to disturbing the balance inside the pathogens and leak of the cell contents. The attachment of complement proteins on the cell surface also facilitates opsonisation and cell phagocytosis.
1.2.2 Complement system regulators

Activation of the complement is controlled by a group of regulatory proteins collectively named Regulators of the Complement Activation (RCA). These proteins are either membrane bound or fluid phase that keep the complement activation to the required level to prevent damage to host cells. They mediate the regulation by promoting the decay of the complement convertases C3bBb and C4b2a or by preventing the assembly of the MAC (Norman et al., 1991). The complement regulatory proteins also act as cofactor to facilitate the degradation of complement active proteins. Members of the RCA family include Complement receptor 1 (CR1 or CD35), C4 binding protein, Decay-accelerating factor (CD55), membrane cofactor protein (CD46) and Complement factor H (Hageman et al., 2005; Fearn & Sheerin, 2015). Also, complement factor I and C1-inhibitor protein are two of the widely studied complement regulatory proteins, yet they do not belong to the RCA in terms of proteins classification (Zipfel & Skerka, 2009). The importance of regulatory proteins is seen when they do not function properly leading to excessive complement activation and tissue injury. Improper regulation of the complement system has been recognized as a central concern in many renal disorders.

Inherited and acquired deregulation of the complement plays an important role in the development of many renal diseases. The recent recognition of disease-causing mutations and genetic variants in complement regulatory proteins has provided a basic understanding to the pathogenesis and aetiology of complement associated renal injuries. One example of these injuries occurs by the dysfunctional deposition of activated complement component C3 on the glomerular basement membrane. This leads to a group of renal injuries collectively named “C3 glomerulopathies” where inflammation is predominant (Boterashvili et al., 2012). One effective strategy for the treatment of this injury is to re-establish the complement normal regulation by the complement blockade anti-C5 monoclonal antibody (Heeringa & Cohen, 2012). The attention to the negative consequences of mal-adaptive complement response or deregulated
complement activation opens a promising avenue in the treatment of many kidney disorders like kidney transplantation rejection and haemolytic uremic syndrome (Stegall et al., 2012).

1.2.3 Receptors of complement system
Complement receptors are a group of membrane bound proteins which bind to a wide range of protein fragments generated during the course of complement activation. They are mainly expressed on the cell surface of immune cells involved in the immune response in the event of tissue injury (Leslie, 2001). Complement receptors also bridges the link between complement and adaptive immunity (Fischer et al., 1998). The receptors can be grouped into three categories: (1) those recognizing the anaphylotoxic polypeptides, C3a, C5a and C5a des-arginine, (2) those binding the active C3 fragment, C3b, and its degradation products, iC3b and C3dg, and (3) receptors for C1q and related collagenous lectins.

Degradation products of the activated C3 and C4 remaining on the surface of pathogens and injured cells are detectable by the complement receptors CR1 and CR3 expressed on neutrophils and macrophages leading to phagocytosis of the opsonized cell (Leslie, 2001; Wetsel, 1995). This draws the attention to the impact of sub lytic levels of complement activation on the proper functioning of self-cells that encountered some sort of injury (Jacquier-Sarlin et al., 1995).

1.2.4 Renal disease and the complement deficiency
Renal disease is a common feature of a number of complement deficiency conditions. Despite it is not necessary that the complement deregulation causes renal disorder, it is well supported that the susceptibility is high. For instance, autoimmune diseases are commonly associated with the classical pathway deficiency and often involving the kidney. Some of these defects produce the most severe diseases of any complement disorders (Welch & Blystone, 2009).
Deficiencies were described for all the three components of the C1 complex; the most common of them is that of C1q. Most of the cases with C1q deficiency have systemic lupus erythematosus (SLE), prominent cutaneous disease and glomerulonephritis of variable severity (Kallel-Sellami et al., 2007; Bowness et al., 1994). Despite therapy, some cases have progressed to an end stage renal failure (Topaloglu et al., 1996). Deficiency of either C1r or C1s is much less common than that of C1q. deficiency of the two genes together is the typical case because these two genes are contiguous harbouring the same locus on chromosome. The available data suggests that approximately two thirds of patients with C1s / C1r deficiency have SLE with glomerulonephritis. There is also a suggestion that this deficiency might be a risk factor for hemophagocytic lymphohistocytosis (HLH) (Endo et al., 1999).

Moreover, deficiency of C2 is the most common deficiency of non-regulatory human complement components. Membrane proliferative glomerulonephritis (MPGN) (Loirat et al., 1980), SLE (Roberts et al., 1978) and Henoch–Schönlein purpura have all been reported in C2-deficient individuals (Gelfand et al., 1975).

On the other hand, deficiency of C3 is rare in human and patients have no consistent renal phenotype associated (Botto & Walport, 1993). A mutation in the C3 molecule that affects the binding site for the regulatory protein factor H has been described. The consequence of this is unregulated C3 consumption by the circulating alternative pathway C3 convertase, C3bBb, which may result in MPGN (Marder et al., 1983).

Furthermore, C4 deficiency is extremely rare, but all the cases documented with C4 deficiency have had SLE, severe cutaneous disease with anti-Ro antibodies. Some patients occasionally develop IgA nephropathy, Sepsis and end stage renal disease (ESRD) (Welch et al., 1989).

Reported cases for deficiency of mannose-binding lectin (MBL) indicate that it is one of the most common complement deficiencies. There are case reports of its role in infectious complications in kidney transplant patients (Manuel et al.,
On the other hand, it was recently reported that the deficiency of MASP-2 confers protection from myocardial and gastrointestinal ischemia/reperfusion injury (Schwaeble et al., 2002). There are no well-documented examples of complete factor B deficiency in humans. Although some association between factor B polymorphism and renal disease have been described, these may be attributable to other linked genes in the MHC, rather than factor B itself (Welch & Blystone, 2009; Welch et al., 1989). A recent study showed an association between haemolytic uremic syndrome (HUS) and a gain-in-function mutation in factor B in two families (Goicoechea de Jorge et al., 2007). There is no typical renal phenotype associated with deficiency of factor D, however, individuals are at risk of meningococcal infection (Sprong et al., 2006).

1.2.5 Synthesis of complement proteins

The primary location for the complement proteins biosynthesis is the liver. Despite this was described by Erlich and Morgenroth since 1900, this was only confirmed on 1976 by Alper et al. who described the contribution of allotype C3 in a host received liver transplantation (Alper & Rosen, 1976). This is also supported by in vitro and in vivo hepatocyte cell function studies (Morris et al., 1982). In addition to the hepatocytes, other tissues in the body synthesis complement protein as well. It’s now supported that approximately 10% of circulating C3 is sourced from organs other than the liver (Naughton et al., 1996). Among these other tissues are fibroblasts, epithelial cells, lymphocytes and macrophages derived from different organs, including the kidney (Sheerin et al., 1997a).

1.2.6 Local synthesis of complement in renal tissue

Despite the liver is the main source of complement proteins in the body, kidney also locally synthesis a major part. Decades ago, studies showed that the human renal proximal tubular cells synthesized and secreted the complement main effector enzyme C3 (Brooimans et al., 1991). Later on, several studies showed that most of the complement components are produced from various renal cells
including glomerular and tubular epithelia and mesangial and endothelial cells (Tang et al., 2001; Gerritsma et al., 1996). Several studies showed that the complement local synthesis was stimulated by the presence of infiltrating immune cells and pro-inflammatory cytokine synthesis in several injuries including chronic kidney disease, acute graft rejection and inflammatory glomerulonephritis (Sacks et al., 1993; Andrews et al., 1994; Wilkinson et al., 2014). In some cases of renal acute transplant rejection, local synthesis of C3 contributed by 10% of the circulating C3 (Tang et al., 1999).

Renal synthesis of complement was shown to mediate the inflammation in many cases of renal disease (Welch et al., 2000). Transplantation of complement deficient kidney tissue into complement competent recipients is one approach to elucidate the contribution of local synthesis in inflammation. Some studies showed that the local synthesis of complement mediated inflammation in transplant rejection, ischemia reperfusion injury and tubulointerstitial injury in proteinuric kidney injuries (Farrar et al., 2006; Sheerin et al., 2008).

1.3 Chronic kidney disease

Chronic kidney disease (CKD) affects 6-8% of the adult population in the UK and exhibits a similar prevalence in other developed countries (Stevens et al., 2007). In 1990, the disease was ranked 27th in the list of causes of total number of deaths worldwide, this rank was raised to 18th in 2010 (Jha et al., 2013). CKD is common in individuals with other long-term conditions such as hypertension and diabetes, and is associated with a risk of progressive renal impairment and cardiovascular morbidity and mortality.

In the United Kingdom around 60,000 patients are in receipt of renal replacement therapy by dialysis or transplantation (Rao et al., 2015). Dialysis costs circa £30,000 per annum for one patient, and according to a UK NHS study in 2014, the cost of the treatments of the disease is more than that of the treatment of breast, lung, colon and skin cancer combined (Kerr et al., 2014); similar figures are estimated elsewhere the developed world. Although not all patients with
CKD will develop into end stage renal failure, all stages of CKD are associated with elevated levels of morbidity and mortality and lower quality of life.

Proteinuria, the presence of excess serum proteins in the urine, indicates either excess filtration of protein due disruption of glomerular permselectivity, or a defect in the re-absorption of filtered proteins by the proximal tubular epithelial cells (PTEC) (Oberbauer et al., 1995; Oliver et al., 1994). Importantly, proteinuria is a significant risk factor for progressive renal failure and the development of CKD (Sano et al., 2002; Metcalfe, 2007). Proteinuric nephropathy describes the development of a pro-inflammatory scarring environment in the kidney as a direct result of excess tubular proteins interacting with PTEC. The consequence is progressive destruction of nephrons and the eventual development of end stage chronic renal failure (Clark et al., 2008; Abbate et al., 2006).

### 1.3.1 Complement activation in renal disease

It is well supported that the complement system is activated in the immune mediated glomerular nephropathies like post-infectious glomerular nephritis, lupus nephritis and membranous nephropathy (Bao & Quigg, 2007; Wyatt et al., 1988; Ma et al., 2013). The complement also is known to be activated in other renal haemolytic conditions like thrombotic microangiopathies, atypical haemolytic uremic syndrome and during antibody mediated transplant rejection (Fearn & Sheerin, 2015; Davin & van de Kar, 2015; Wong et al., 2013). What is less understood is the degree the complement contributes in the development of tissue injury and fibrosis and non-disease specific inflammation. Contribution in these symptoms could be as harmful to nephrons functionality as the disease itself. This suggests strong therapeutic avenues through manipulation of the activation levels of the complement during the injury.

The processes underlying tubulointerstitial fibrosis are complex involving multiple pathways and mediators due to the number of mechanisms that interact to promote the injury. Cellular stress promotes a pro-inflammatory and pro-
fibrogenic status that involves the expression of a number of growth factors and cytokines as well as the activation of the renin-angiotensin system (Bottinger & Bitzer, 2002; Yang & Liu, 2002). Chemotaxis leads to infiltration of inflammatory cells and proliferation and activation of myofibroblasts as well as excess deposition of the matrix (Wetsel, 1995). This promotes the tissue inflammation which in turn exerts more stress on the cells. There is a growing body of evidence that the immune system is leading this process with the complement system extensively involved, it can directly affect the function of the cell or escalate several responses of the innate and adaptive immunity (Tapmeier et al., 2010; Eis et al., 2004).

1.3.1.1 Complement activation in clinical proteinuric disease
In many clinical renal diseases, the link between proteinuria, Tubulointerstitial fibrosis and impaired renal function is well established. However, the mechanism of developing interstitial scarring after proteinuric glomerular disease is not clear. Impaired glomerular filtration and loss of the selective permeability causes the complement proteins to go through the glomerular barriers and enter the tubular part of the nephron. Complement activation by-products can be found in the urine of patients with several proteinuric kidney diseases including focal segmental glomerulosclerosis, IgA nephropathy and diabetic nephropathy (Morita et al., 2000). This could be the result of activation of complement either in the glomerular or the tubular part of the nephron. In the diseases where the glomerular complement activation is not a main feature like diabetic nephropathy and focal segmental glomerulosclerosis, the presence of complement activation components in the urine indicates the complement activation occurs in the tubules (Ogrodowski et al., 1991). Gaarkeuken and others in 2008 reported that the complement activation in the tubules occur on the tubular epithelium apical surface via the alternative pathway (Gaarkeuken et al., 2008). Several reports suggest different explanations to this (Camussi et al., 1982; Camussi et al., 1985). The alteration in the glomerular filtrate pH or ammonia release from affected epithelial cells could activate C3. The brush border of proximal tubules
is known to be relatively deficient in complement regulatory proteins allowing the attachment of complement proteins (Ichida et al., 1994). Attachment of properdin to the epithelial cells glycosaminoglycans stabilises the convertases of the alternative pathway and might help promote the activation (Gaarkeuken et al., 2008). Also the presence of albumin on the apical surface might compete with factor H binding sites reducing the chance of complement inhibition. By all means, the presence of albumin on a cell surface that lacks the proper complement regulation leads to the activation of complement and formation of MAC (Zaferani et al., 2012; Zaferani et al., 2011). Deposition of the MAC on the surface of proximal cells is quantitatively related to interstitial infiltration of inflammatory cells and fibrosis and increased MAC presence in the urine (Brenchley et al., 1992; Buelli et al., 2009). However, it’s difficult to prove the mechanism by which this causes tubulointerstitial scarring and fibrosis in clinical cases.

1.3.1.2 Complement activation in experimental models of proteinuria

Experimental models of proteinuria have significantly contributed to the understanding of the role the complement plays in the development of proteinuric disease. Protein overload proteinuria is one of the well-established models of renal tubulointerstitial injury in rats and mouse (Ishola et al., 2006). Overload of the albumin in the circulation causes the development of tubulointerstitial scarring leading to renal inflammation and chronic failure (Lawrence & Brewer, 1981). The model represents an outstanding platform to study the disease mechanisms of non-glomerular origin tubulointerstitial injury in a non-haemodynamic and non-immunologic condition (Ruggenenti et al., 2001). Also, establishment of this model in mice, after rats, gives a valuable opportunity to utilize the wide variety of available mouse transgenic lines to help investigate the underlying mechanisms of the development of the disease (Ishola et al., 2006).

The mechanism connecting between proteinuria, tubulointerstitial injury and chronic renal failure was extensively investigated by many authors (Hodgkins & Schnaper, 2012; Eddy, 2004; Gorriz & Martinez-Castelao, 2012). Some suggests
that the albumin load has a toxic effect on proximal tubular epithelial cells causing damage to it, thus starting a condition of renal interstitial fibrosis and deregulated renal filtration (Thomas et al., 2002). Many in vitro studies showed that the albumin has an important effect on the proximal tubular epithelial cells not only on the growth and proliferation of the proximal tubular epithelial cells but also to induce the secretion of a group of tubular chemokine expression that lead to inflammatory cell infiltration in the renal interstitium like macrophages and constant fibrogenesis (Chana et al., 2008; Remuzzi, 1999). This explains the kidney inflammatory response to albumin overload. Some authors reported that albumin overload was the cause of proximal tubular epithelial cells apoptotic death (Tejera et al., 2004).

Adriamycin and puromycin are aminoglycoside antibiotics that are well-known to disrupt the glomerular epithelial function leading to glomerulosclerosis, proteinuria and tubulointerstitial fibrosis in rodents. Administration of one of these agents into the circulation of the mouse gives a valuable model for studying the developmental mechanisms of renal disease of glomerular origin. Several reports showed that induction of the disease in complement deficient mice has shown that the complement activation contributes to glomerulosclerosis and tubulointerstitial inflammation and fibrosis (Lenderink et al., 2007; Turnberg et al., 2006a). Some studies highlighted the importance of complement regulatory proteins in controlling the activation of complement and the degree of injury (Nomura et al., 1997). This suggests that the formation of MAC and the release of the anaphylatoxins in the interstitium may be the cause of tissue scarring. There is a growing body of evidence that complete depletion or inhibition of the complement activation reduces the severity of the symptoms related to tubulointerstitial injury (Tang et al., 2009; Morita et al., 1997). The renal injury proteinuria models also give the potential of targeting therapeutic approaches by manipulating the complement activation on the tubular cells through selective recombinant proteins (Morita et al., 1997; Couser et al., 1991).
1.3.1.3 Complement activation in non-proteinuric kidney disease

Information available about the role of complement activation in non-proteinuric kidney nephropathy is less compared to proteinuric disease. Unilateral ureteral obstruction, induced by the ligation of the ureter, is the most commonly used model to study progressive non-proteinuric kidney disease. The model is characterized by the gradual development of interstitial inflammation and fibrosis, macrophage and T-cells infiltration, fibroblast proliferation in the interstitium and loss of functioning nephrons (Rangan et al., 2005). The model is closely similar to the development of the disease in patients.

Deficiency of complement in this model showed a significant reduction in fibrosis and interstitial inflammation in C5$^{-/-}$ mice compared to complement competent mice after five days of ligation (Boor et al., 2007). This suggests the complement system activation contributes to the inflammation of the tissue. Similarly, fibronectin, vimentin and platelet derived growth factor B and D showed significant reductions in complement depleted mice. Also in the same report, authors highlighted a protective effect when wild type mice were treated with C5a receptor antagonist with UUO compared to non-treated mice. Similar data were observed with C3 deficient mice deficient of all complement pathways (Zhou et al., 2013). From these studies, evidence is showing that the activation of complement in this model contributes significantly to the development of tissue inflammation and fibrosis. However, what is less clear is the particular complement pathway responsible for this tissue injury and the exact mechanisms of the injury development.

1.3.1.4 Complement activation in other phenotypes of kidney disease

Diabetic nephropathy is another phenotype of kidney disease that the complement system might be involved in the progression of the disease in the glomerular and tubulointerstitial compartments. Serum levels of the mannose-binding lectin are higher in diabetic patients with albuminuria and nephropathy. In a mouse study, MBL deficient mice with diabetic nephropathy developed less glomerulosclerosis and albuminuria compared to their wild type littermates.
(Hovind et al., 2005; Ostergaard et al., 2007). This suggests the role of the lectin pathway of complement in the progression of the glomerular and tubular injury, yet the data showed that the effect is strain dependent (Ostergaard et al., 2012).

In diabetic patients with nephropathy, the deposition of MAC in the glomeruli and tubules is a characteristic (Qin et al., 2004). Also, complement components genes are differentially regulated in the kidney compared to healthy individuals suggesting a role of the local synthesis of the complement in the disease (Woroniecka et al., 2011). In addition, some reports elucidate a role of the complement system, particularly the lectin pathway, in the coronary and renal vascular disease associated with diabetic nephropathy (Ostergaard et al., 2005).

Complement activation is evident also in the development of polycystic kidney disease. In C3 deficient mice, fewer cysts were developed and reduced renal volume and inflammatory cells infiltration in the interstitium compared to complement competent mice (Zhou et al., 2012). In addition, inhibition of complement in complement sufficient mice reproduced similar protection (Su et al., 2014). This suggests a strong avenue of therapeutic approach via the administration of complement blockades.

1.3.2 Effects of complement activation on renal cell function

Activation of the complement through secretion of anaphylatoxins and formation of sub-lytic concentrations of MAC could alter the renal cell functions. This can provide explanations on the contribution of the complement system in the development of renal disease. Deposition of the MAC on the apical surface of the tubular cells promotes the synthesis and release of a group of pro-inflammatory cytokines including interleukin 6 and tumour necrosis factor α (David et al., 1997). It also increases reactive oxygen species (ROS) and the interstitial matrix protein synthesis (Abe et al., 2004; Biancone et al., 1994). Complement activation can also induce the expression of antigens to some histocompatibility on the tubular cells, allowing these cells to derive T-cell proliferative response. This could potentially promote isogenic and autoimmune responses against the
tubular cells (Li et al., 2004). Figure 2 shows a schematic representation of the tubular injury mediated by the complement activation.

Anaphylatoxins also could affect the renal epithelia to express C3a and C5a receptors (Braun et al., 2004; Zahedi et al., 2000). When cells are exposed to C3a or C5a, they might increase the synthesis of collagen and adopt a more mesenchymal like state (Tang et al., 2009). The effect of anaphylatoxins could also be mediated partly by the increased activation of transforming growth factor beta and other growth factors (Liu et al., 2011). The escalation of these conditions in the tissue promotes tissue inflammation and could develop fibrosis.

Figure 2: Renal injury mediated by the complement system, adapted from (Fearn & Sheerin, 2015).
1.4 Three models of kidney disease are investigated in the current study

Three models of renal disease are investigated in the current study: i) protein overload proteinuria, a model for proteinuric nephropathies that originate in the tubular compartment of the kidney, ii) unilateral ureteric obstruction, a model for obstructive non-proteinuric nephropathies and iii) adriamycin nephropathy that is related to focal segmental glomerulosclerosis.

1.4.1 Protein overload proteinuria

Progressive renal diseases end at the same point; fibrosis of the renal tubulointerstitium (TI). This common end is the aim of many therapeutic research works that try to provide new treatment strategies that can save lives. The deterioration of renal excretory function in glomerular diseases is linked directly to the level of renal TI injury (Risdon et al., 1968; Schainuck et al., 1970). The toxic effect of protein passes into the proximal tubules due to loss of glomerular permselectivity is referred as proteinuric nephropathy (Brunskill, 2004; Baines & Brunskill, 2011). This condition is related to many glomerular diseases, and it leads to pro-inflammatory status that injure the renal tissue leading to successive scarring in the kidney tissues in addition to changes in the proximal tubular cells reproduction with apoptosis. It also leads to increase expression of inflammatory cytokines as a result of disturbances in the signalling systems due to stimulation by the proteinuric tubular fluid (Baines & Brunskill, 2011). Proteinuric nephropathy is regarded as a key factor that contributes to the progressive damage of renal tissues following different primary renal diseases.

In addition to its role in immune-complex mediated and other types of glomerulonephritides, complement system has been found to play a role in TI fibrosis in non-glomerular diseases (Quigg, 2003; Naik et al., 2013; Mathern & Heeger, 2015). Complement activation May happen due to abnormal production of complement factors by the tubular cells or other filtrating cells or may be due to dysregulation of protein activation as a result of changes in complement
regulator proteins production or it may occur due to the filtered protein factors (Sheerin et al., 2008).

A study shows that deficiency of CR1-related gene/protein (Crry), which is a complement regulating protein can result in activation of the complement system in TI complicated by inflammation and fibrosis as similar to what happens in human TI pathologies (Bao et al., 2007).

Complement anaphylatoxins C3a has the ability to activate mesenchymal transition upon exposure of epithelial cells to this factor (Tang et al., 2009). Blocking of C3a receptors can stop this changes and decrease TI fibrosis in both non-proteinuric and proteinuric animals. Thus, targeting complement activation can give a promising therapeutic utility in treatment or prevention of inflammatory pro-fibrotic activation in different renal pathologies, opening new ways for research in prevention of renal fibrosis and renal failure (Bao et al., 2011).

Proteinuria is defined as the presence of excess amount of the serum proteins in the urine, and indicates a dysfunction either in the filtration of proteins by the glomeruli or in the reabsorption of proteins by the proximal tubular epithelial cells. One consequence is an inflammatory insult to the kidney resulting in tubulointerstitial injury that is a common precursor for a progressive destruction of nephrons and the development of end stage chronic kidney disease (Clark et al., 2008).

Unilateral nephrectomy is an experimental procedure frequently used in rodent models of renal disease which exerts a compensatory overload on the remaining kidney and usually causes mild renal dysfunction (Schnaper et al., 2003). Many rodent models of renal disease, including protein overload nephropathy, were originally developed in the rat. When transferred across species, mice were often found to be more resistant to renal injury than rats, and therefore unilateral nephrectomy is used to amplify renal injury in the remaining kidney. The model
Introduction

of protein overload nephropathy in unilaterally nephrectomised mice is relevant to the mechanisms occurring in human kidney disease (Ishola et al., 2006).

During proteinuria, complement activation provides a compounding factor underlying tubular and interstitial injury via cytotoxic, pro-inflammatory, and fibrogenic effects (Abbate et al., 2006). Tubular cells may be activated by excess glomerular filtration of serum complement proteins. Many studies indicate that tubular epithelial cells are susceptible to complement mediated injury because of the relative lack of membrane-bound complement regulators on the apical surface (Zoja et al., 2014). Targeting complement biosynthesis and/or activation processes in the proximal tubule might offer new avenues for therapeutic interventions. In this experiment, we employed a novel transgenic mouse model with a deficiency in one of the three different activation routes of complement, i.e. the lectin pathway, to elucidate the role of the activation of this pathway in protein overload proteinuria mediated renal injury.

1.4.2 Unilateral ureteric obstruction

The main goal of all therapeutic trials is to decrease level of fibrosis affecting kidney tissue and complicating renal pathologies. The rarity of the treatments, which can target the pro-fibrotic pathways in kidney diseases, and the increasing need for such successful therapies form a real pressure for more research studies.

The complement activation system which is a major part in the innate immune response with its interaction with the adaptive immune response has been encountered in many renal pathologies as haemolytic uremic syndrome (subtype of thrombotic micro angiopathies), ischemia reperfusion injury, glomerulonephritis and kidney transplant rejection disease (Quigg, 2003; Naik et al., 2013). Inflammation of the renal tubulointerstitial tissue accompanies the renal proteinuric renal pathological conditions (Brunskill, 2004). Complement system has been found to be participating in the pathophysiology of proteinuric nephropathy (Nangaku et al., 2002; He et al., 2005). Kidneys of mice that had unilateral ureteric obstruction (UUO) show evidences of complement activation
suggesting a crucial role of complement system in the pathogenesis of non-proteinuric fibrosis of renal tissue (Boor et al., 2007).

Inflammation and fibrosis of the tubulointerstitium is commonly found in patients with progressive renal diseases and is usually proportionate with the functional decline of the renal excretory system, therefore treatment that can deter or prevent fibrosis will be of great value to big number of patients in the clinical wards (Risdon et al., 1968; Schainuck et al., 1970).

The lectin pathway of the complement activation system depends in its activation on the binding of pattern recognition molecules as mannose binding lectin (MBL), collectin-11 and ficolins to their carbohydrate targets that are expressed by pathogens or abnormal cells (Wallis et al., 2010). The activated bound Recognition molecules then bound to the MBL-associated serine protease-2 (MASP-2) forming an activation complex that cleaves C2 and C4 complement factors generating C4b and C2a that gives the back bone of C4b2a which is the C3 convertase of the lectin pathway that can cleave C3 complement factor (Endo et al., 1998). Activation of the lectin pathway has been approved by many studies to have a role in renal diseases as diabetic nephropathy, transplant rejection, IgA nephropathy, and ischemia reperfusion injury. Lectin pathway activation has also been found in fibrosis outside the kidneys as in case of severe fibrosis due to hepatitis C virus infection (Asgari et al., 2014; Brown et al., 2007; Hisano et al., 2005; Roos et al., 2006; Liu et al., 2013).

Unilateral ureteric obstruction (UOO) is a well-established experimental model of renal injury leading to tubulointerstitial inflammation and fibrosis which are common histopathological features of progressive renal disease (Chevalier et al., 2009). It is characterized by mechanical disturbance resulting from ureteric ligation, hypoxia induced by a marked decline in renal plasma flow, upregulation of monocyte chemo attractant peptides, macrophage influx into the interstitium and production of macrophage derived cytokines (Kawada et al., 1999). The
Introduction

model of unilateral ureteric obstruction is of clinical relevance to obstructive non-proteinuric kidney injuries.

1.4.3 Adriamycin nephropathy

Adriamycin is an anthracycline cytotoxic antitumor chemotherapeutic agent used in the treatment of a wide range of cancers including haematological malignancies, soft tissue sarcomas and many types of carcinoma (Karch et al., 2016; Kanno et al., 2014). Adriamycin nephropathy (AN) is a rodent model of chronic kidney disease that has been studied extensively and has enabled a better understanding of the progression of chronic proteinuric kidney disease (Wang et al., 2000). AN is a model of focal segmental glomerulosclerosis with proteinuria and is characterised by podocyte injury followed by glomerulosclerosis, tubulointerstitial inflammation and fibrosis. It has been shown also in numerous studies to be modulated by both non-immune and immune factors (Lee & Harris, 2011; Yasuda et al., 2010).

AN provides an excellent experimental model of kidney disease. Firstly, it is a highly reproducible and predictable model of renal injury. This is because it is characterised by the induction of renal injury within few days of drug administration, which facilitates the design experiments as the timing of injury is relatively short and consistent (Pippin et al., 2009). It is also a model in which the degree of tissue injury is severe but with an acceptable low risk of mortality (<5%) and morbidity and weight loss (Zheng et al., 2005). The severity and timing of renal injury means that it is a model suitable for testing interventions that either worsen or protect against renal injury. The type of structural and functional injury is very similar to that of chronic proteinuric renal disease in humans (Jeansson et al., 2009).

A disadvantage of this model is that adriamycin has a narrow window of affectivity where doses as little as 0.5 mg/kg lower or higher than the optimum dose may lead to either insufficient renal injury or excess toxicity leading to death (Wang et al., 2000).
1.5 Transgenic mouse models of complement deficiency; a useful tool to understand kidney disease

To dissect the complexity of the physiology and physiopathology of the immune system, immunologists have used numerous mutant animals in their research. Until about two decades ago, the number of available mutant mouse models to study specific immune responses was limited. Nude, beige and aly mice are examples for old valuable immune system experimental models that existed naturally. Such mutations occurred spontaneously and hence the understanding of the genetic background of the mutation was limited (Koller & Smithies, 1992). The introduction of knockout technology resolved these limitations by providing a widely diverse group of models of well-designed immune system related genes mutations, with a full understanding of the genetic basis of the alterations.

The first problem in immunology resolved by the production of a knockout mouse was the function of major histocompatibility complex (MHC) in lymphocytes (Mak et al., 2001). In 1990, Zijlstra et al. and Koller et al. separately produced a mouse model in which the β2 microglobulin gene was knocked out suppressing the expression of the MHC class I protein. Despite the mutant animals developing normally, they had severely reduced numbers of CD4–CD8+ cytotoxic T lymphocytes (CTLs). This showed that MHC class I molecules are not essential for the overall mouse development. However it is needed for the selection of MHC-class-I-restricted T cells and for antigen recognition by these cells (Koller et al., 1990; Zijlstra et al., 1990). Subsequently, in 1991 Kitamura et al. produced an immunoglobulin u chain gene targeted mouse model and hence they produced a B lymphocyte deficient mouse (Kitamura et al., 1991). This model was a breakthrough establishment in the genetic alteration trend of studying the immune system.

1.5.1 Production of transgenic mice

Gene targeting in mouse has become the most powerful tool of the twenty first century for determining gene functions in mammals. The revolutionary outcome of the technology was impactful enough to award doctors Mario R. Capecchi,
Martin J. Evans and Oliver Smithies the 2007 Nobel Prize in physiology or medicine (NoblePrize.org, 2007) for their work in developing the technology. The introduction of specific gene modifications in mice by the use of embryonic stem cells is not only revolutionary in the determination of the role of specific genes in development and physiology, but also plays a key role in the development of medical therapy.

Creating designed genomic modifications takes all advantage of the resources provided by the known sequences of the mouse and human genomes. This, in conjunction with the vast available genetic engineering techniques, enables the investigator to choose precisely which genetic locus to manipulate and give a complete control of how to modulate the chosen genetic locus. Examples of the utilization of the technology comprises the creation of null mutations in what is called gene knockout modifications and the introduction of reporter genes like green fluorescent protein gene (GFP) to study particular gene expression or to track one particular cell lineage (Mak et al., 2001). Homologous recombination based strategies for the production of transgenic mouse models incorporate two independent yet integrated technologies. First, the insertion of the mutation into cultured multi-potent mouse embryonic stem cells by homologous recombination. Second, the introduction of mutated embryonic stem cells into normal mouse embryo in order to produce a mouse model built partly from the mutated cells called a chimera. Cross mating mouse chimeras leads to the production of homozygous mutated strains. Homologous recombination is a natural phenomenon occurs in the cell to repair the DNA faults and during meiosis in order to produce new combinations of DNA sequences lead to the genetic variation of the offspring. It is utilized in the knockout technology to integrate an extrinsic DNA segment, generally a vector carrying the designed mutation (Figure 3). Thousands of mouse genes have been knocked out with ongoing international efforts to make a knockout mouse for every gene available in the near future (the Knockout Mouse Project website, www.komp.org).
A. Gene targeting of embryonic stem cells

Figure 3: General protocol for the production of gene targeted mice ("The 2007 Nobel Prize in Physiology or Medicine - Advanced Information" Nobelprize.org. Nobel Media AB 2014. Web. 1 July 2016)
1.5.2 Current models of complement deficiencies

C1q and MASP-2 are two pro-inflammatory proteins of the complement system involved in the activation of the classical and lectin pathways respectively. Deficiency of either of these two components leads to the lack of the activation of the corresponding pathway while the system remains functioning through the remaining pathway(s).

1.5.2.1 MASP-2\textsuperscript{KO} mice

MASP-2 is the enzyme that translates recognition of pathogen by lectin pathway recognition molecules (MBL or ficolin) into downstream complement activation. The construct used to generate the MASP-2 deficient mouse disrupted the exons encoding the C-terminal part of the CCP-2 domain and the serine protease domain. This recombination event inserted a frame shift mutation and replaced the coding sequence for key residues for serine protease activity with a neomycin resistance gene cassette (Schwaeble \textit{et al.}, 2011).

For the generation of MASP-2 deficient mice, a homologous recombination targeting construct, designed to disrupt the murine MASP-2 gene, was used to interrupt the gene in 129/Sv embryonic stem cells. This construct contained two arms of homology to exons 10 and 12 and a neomycin resistant gene as a positive selection marker between them and was cloned in topKO-NTKV1901 vector expressing ampicillin resistant gene for negative selection. Homologous recombination of this construct with the murine MASP-2 gene of the ES cells resulted in the replacement of exon 11 and part of exon 12 with the neomycin cassette (Figure 4). These exons encode for part of the CCP-2 domain and the serine protease domain and lacking of them leads to the synthesis of truncated non-functional form of the MASP-2 enzyme. Targeted cells were positively selected by means of neomycin resistance and positive clones carrying the disrupted gene, confirmed by specific southern blot assay, were microinjected into blastocysts of C57BL/6J mice. Integration of these cells into blastocysts generated mouse chimeras that were backcrossed to C57BL/6J mice to allow
germ line transmission of the disrupted gene and establishment of the new mouse line (Schwaeble et al., 2011).

Figure 4: Targeting event for the establishment of the MASP-2 knockout mouse line. The construct used to generate the MASP-2 deficient mouse disrupts the exons encoding the C-terminal part of the CCP-2 domain and the serine protease domain, inserting frame shift mutation and replacing the coding sequence for key residues for serine protease activity with a neomycin resistance gene cassette. The figure is adapted after Schwaeble et al., 2011.

1.5.2.2 C1qKO mice

C1q is the recognition molecule of the classical pathway of complement. Its primary role is to bind immune complexes (complexes of immunoglobulin and antigen) and activate complement via the serine proteases C1r and C1s. C1q deficient mice have been established for almost two decades and are well characterised and show no gross abnormalities (Botto et al., 1998). They are somewhat more susceptible to infection than their wild-type litter mates and suffer from an age-related immune-complex disease, similar to SLE, due to an inability to clear apoptotic cell debris.

To establish the C1q deficient mouse colony, a homologous recombination vector was used to target the murine C1q gene in 129/Sv embryonic stem cells. The construct was designed to interrupt the C1q gene by inserting a neomycin resistant gene (neo) into exon 1 as represented in Figure 5. The Neo gene also
played as a positive selection marker in the vector. The insertion occurred by means of homologous recombination through two homology arms of 7 kb and 5 kb before and after exon 1 respectively. The construct also contained a herpes simplex virus thymidine kinase cassette (HSV-tk) for negative selection. The vector was delivered into the embryonic stem cells by electroporation and targeted cells were positively selected by means of neomycin resistance. Positive clones carrying the disrupted gene, confirmed by specific southern blot assay, were microinjected into blastocysts of C57BL/6J mice. Integration of these cells into blastocysts generated mouse chimeras that were backcrossed to C57BL/6J mice to allow germ line transmission of the disrupted gene and establishment of the new mouse line (Botto et al., 1998).

Figure 5: Schematic diagram for targeting the mouse C1q gene to establish a transgenic mouse line deficient in the classical pathway of the complement system, the figure is adapted from Botto et al., 1998. Exons 1 and 2 of the C1q gene are represented as filled boxes and the neo and HSV-tk selection cassettes are represented as empty boxes. Straight lines represent the two homology arms and the construct backbone is represented as zigzag line.
1.5.3 Generation of a mouse lines deficient in both classical and lectin pathways of complement

Several publications, involving the use of either MASP-2 or C1q deficient mice highlighted the significant roles of both the lectin and the classical pathways in mediating the injury in inflammatory disease phenotypes (Schwaeble et al., 2011; Cervera et al., 2010). To establish a mouse line deficient in both the lectin pathway (MASP-2\(^{-/-}\)) and the classical pathway (C1q\(^{-/-}\)) would generate a unique animal model to assess functional activity of the alternative pathway on its own.

1.5.3.1 Previous attempts to generate classical/lectin pathways double deficient mouse model

Separate mouse models of classical and lectin pathways deficiencies are available, mice deficient in C1q (the classical pathway recognition complex) and MASP-2 (a serine protease essential for LP activation), respectively. However, a model of combined CP and LP deficiency is not available. It has not been possible to backcross these mouse lines deficient of both C1q and MASP-2 because the genes are located on the same chromosome in close proximity.

A previous attempt has been made by to establish a mouse strain deficient of both pathways. The gene for murine C1s, one essential serine protease of the CP, was targeted with the aim of producing a C1s/MASP-2 double deficient mouse (Jassal, 2010).

C1sA was targeted and the germ line transmission of the disrupted allele was confirmed. However, backcrossing of heterozygote mice did not result in the expected generation of homozygous C1sA deficient mice. Analysis of the offspring indicated that homozygous deficiency for C1sA appears to be lethal.

1.5.3.2 Current approaches

To achieve the same target of producing a mouse model deficient in both lectin and classical pathways of complement, two transgenic strategies have been approached:
1.5.3.2.1 Recombineering

Recombination-mediated genetic engineering (Recombineering) is a genetic engineering technique based on homologous recombination. The system is considered an improvement to the older method of using restriction enzymes and ligases to combine DNA sequences in a specified order (Ellis et al., 2001). Recombineering is widely used for bacterial genetics, in the generation of target vectors for making a conditional mouse knockout and for modifying DNA of any source often contained on a bacterial artificial chromosome (BAC), among other applications (Lee et al., 2001; Yu et al., 2000).

LoxP (locus of X-over P1) is a site on the bacteriophage P1 consisting of 34 bp. This short target sequence includes an asymmetric 8 bp sequence, variable except for the middle two bases, in between two sets of palindromic 13 bp sequences (Turan et al., 2011). Cre/LoxP recombination is a site-specific recombinase technology and is widely used to carry out deletions, insertions, translocations or inversions at specific sites in the DNA of cells (Sauer & Henderson, 1988; Orban et al., 1992). It allows the DNA modification to be targeted to a specific cell type or be triggered by a specific external stimulus. It is implemented both in eukaryotic and prokaryotic systems.

The system consists of a single enzyme, Cre recombinase, which recombines a pair of LoxP sequences. This system can be implemented without inserting any extra supporting proteins or sequences (Tsien, 2016). The Cre enzyme and the original LoxP site are derived from bacteriophage P1, placing LoxP sequences appropriately allows genes to be activated, repressed, or exchanged for other genes (Missirlis et al., 2006).

1.5.3.2.2 CRISPR/Cas9 system

Clustered regularly-interspaced short palindromic repeats (CRISPR) are short repeats of base sequences found on the DNA of prokaryotes followed by short segments of spacer DNA from previous exposure to a bacterial virus or plasmid (Lander, 2016). The CRISPR/Cas9 system is an immune adaptation of acquired
immunity of prokaryotes that confers protection from invading bacteriophages and plasmids (Horvath & Barrangou, 2010; Kaminski et al., 2016). It recognizes the foreign DNA and initiates an enzymatic mediated double strand break of the exogenous DNA elements (Wang et al., 2016). Elements of CRISPR are found in approximately 40% of bacteria sequenced to date and in 90% of sequenced archaea (Ledford, 2016). The system has been discovered decades ago in prokaryotes and characterized by several authors. However, adoption of the system in mammalian cells was a recent revolutionary discovery that allowed endless opportunities of programmed genome editing (van Diemen et al., 2016). Figure 6 shows a schematic representation of the CRISPR mediated DNA double strand break and its application in various research models.

Figure 6: Schematic representation of the CRISPR mediated DNA double strand break. The Cas9 endonuclease driven by the gRNA catalyses a DNA double strand break. This can be repaired either by NHEJ or HDR pathways. The system can be utilized in a wide range of research models. Image source: origene.com.
1.5.3.2.3 C1rA gene

Complement component 1r subcomponent A (Also mC1rA for murine C1rA) is fluid phase enzyme that plays an essential role in the activation of the classical pathway of complement. Upon the binding of C1q molecule to specific antibody region, the C1r cleaves and activates the serine protease C1s which in turn activates and catalyses the cleavage of C4. The lack of functional activity of C1r is predicted to lead to complement deficiency of the classical pathway.

The gene comprises 11 exons located in the *Mus musculus* mouse genome on the forward strand of chromosome 6 in the range 124,512,405 - 124,523,443. The gene was given the ID number 1355313 on the MGI mouse genome database and ENSMUSG00000055172 on Ensembl. The gene has 1 splice transcripts of 707 amino acids. Figure 7 shows the gene schematic diagram.

![Figure 7: Schematic structure of the murine C1rA gene](image)

1.5.3.2.4 MASP-2 gene

Mannan-binding lectin serine peptidase 2 (MASP-2) is a serum protease that plays an essential role in the activation of the complement system via the mannose-binding lectin activation pathway. In fact, MASP-2 is the effector enzyme of the lectin pathway of complement that the lack of its functional activity leads to complete depletion of the activation of the lectin pathway. After activation, it cleaves the complement components C2 and C4, leading to their activation and to the formation of C3 convertase.
The gene comprises 11 exons located in the *Mus musculus* mouse genome on the forward strand of chromosome 4 in the range 148,602,554–148,602,499, as diagrammatically represented in Figure 8. The gene got the ID number 1330832 on the MGI mouse genome database and ENSMUSG00000028979 on Ensembl. The gene has 4 splice transcripts, two of them not coding for any proteins and the other two are coding for proteins that are 185 and 685 amino acids (MAp19 and MASP-2, respectively).

Figure 8: Schematic structure of the murine MASP-2 gene and its transcript variants
1.6 Aims of the work

The existing experimental data regarding the activation of the complement system in renal disease do not allow comprehensive understanding on the complement pathways(s) that particularly mediate the injury. Current availability of complement deficient, pathway specific, transgenic mouse models provides a useful tool to dissect of the complexity of the activation of the complement system in renal disease. This study was therefore designed to define the complement pathway(s) that mediate the injury in three phenotypes of kidney disease using targeted mouse models of complement deficiency of either the lectin or the classical pathways. The study also aimed to establish a novel mouse line of complete deficiency of both the lectin and the classical pathways of complement in order to establish a possible synergistic co-operation between these two pathways in mediating inflammatory renal pathology.

The specific aims of this project are:

- To study the role of the lectin and the classical pathways of complement in protein overload proteinuria
- To study the role of the lectin and the classical pathways of complement in unilateral ureteric obstruction nephropathy
- To study the role of the lectin pathway of complement in adriamycin nephropathy
- To establish a novel mouse line of complete deficiency of both the lectin and the classical pathways of complement
2 Materials and Methods

2.1 Materials

2.1.1 Experimental animals
Animals used in the current study were wild type male C57BL/6 or BALB/c mice aged between 7-10 weeks at the beginning of experiments (Charles River, UK), MASP-2\textsuperscript{KO} male mice on a C57BL/6 or BALB/c background and male or female C57BL/6 C1q\textsuperscript{KO} mice (provided by Prof. W. Schwaeble University of Leicester). All animals used in this study are from inbred mouse lines. This means they have been backcrossed for at least 20 consecutive generations of sister x brother matings. This insures that the mice from the same mouse line are almost identical in their genotypes. All experimental animal procedures applied in this study were authorised and conducted under the Home Office License number (PPL 60/4438) held by Professor Nigel Brunskill, University of Leicester in accordance with the Animals Scientific Procedures Act and approved by the University of Leicester ethics committee.

While all WT mice used in this study were supplied from Charles River, UK, transgenic mice were supplied from the Central Research Facility (CRF) at the University of Leicester. These genotypes used in the current study could not be sourced from the same supplier due to the fact that neither Charles River supplies the needed transgenic mice nor the CRF supplies WT mice from the same colonies of the transgenic mice. This is why I had to source the animals used in this study from these two suppliers. Establishing new inbred WT mouse lines from the same colonies of all the transgenic mice used in this study was proven out of the feasibility of this project. This is due to the amount of the time needed to establish 20 consecutive generations of sister x brother matings in all colonies that might take years and also due to the cost this work incurs. Nevertheless, this highlights one of the method limitations in this project, however all the mice used in this study are inbred mouse lines based on the same mouse strain. This
gives enough evidence on the intimate similarity of their genetic backgrounds and justifies using them in comparable groups in the study.

All mice were kept under standardised conditions of 12hr/12hr day and night light cycle. Animals were caged 5 per cage in a pathogen free environment in the University of Leicester Central Research Facility. Standardised food pellets and water were provided *ad libitum*. For the purpose of acclimatisation, newly delivered animals were given at least one week before any research work was carried out on them.

### 2.1.2 Reagents and ready-made kits

- **ABC mouse antibody Staining system**
  - Santa Cruz (SC2017)
- **ABC rabbit antibody Staining system**
  - Santa Cruz (SC2018)
- **ABC rat antibody Staining system**
  - Santa Cruz (SC2019)
- **AMV Reverse Transcription System**
  - Promega (A3500)
- **ApopTag® Fluorescein In Situ Apoptosis Detection Kit**
  - Millipore (S7110)
- **Coomassie Plus™ Assay Kit**
  - Fisher Scientific (23236)
- **Diaminobenzidine (DAB) substrate kit**
  - Vector labs (SK-4100)
- **GeneArt™ Precision gRNA Synthesis Kit**
  - Thermo Fisher (A29377)
- **Nucleobond PC 500 Kit**
  - Macherey-Nagel (740574.50)
- **NucleoSpin RNA II**
  - Macherey Nagel (740955.50)
- **QIA Quick Gel Extraction Kit**
  - Qiagen (28704)
Materials & Methods

QuantiChrom creatinine assay kit  BioAssay Systems (DICT-500)

Superscript II TM Reverse Transcriptase kit  Invitrogen (18064014)

Turbo DNA-free kit  Thermo Fisher (AM1907)

Wizard® Genomic DNA Purification Kit  Promega (A1120)

Wizard® Plus SV Minipreps DNA Purification System  Promega (A1330)

2.1.3 Chemicals, media and solutions

5X first strand buffer  Invitrogen (Y02321)

AccuMate differentiation solution  Sigma (A3179)

Acetic acid, minimum 99%  Sigma (A6283)

Albumin from bovine serum  Sigma (A9647)

Albumin, Fluorescein iso thio-cyanate conjugate from bovine serum  Sigma (A9771)

Avidin / Biotin blocking system  Abcam (AB64212)

C1q rat anti mouse monoclonal antibody  HyCult Biotechnology (HM 1044)

C3 rabbit anti human polyclonal antibody  DAKO (A-0062)

Cell culture water  Sigma (W3500)

Collagen solution type 1 from bovine calf skin (1 mg / ml)  Sigma (C8919)
<table>
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<th>Material/Reagent</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Collagenase Type II (1 mg / ml)</td>
<td>Sigma (C1764)</td>
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<tr>
<td>DBX mounting medium</td>
<td>Fisher Scientific (D/5319/08)</td>
</tr>
<tr>
<td>DMED F12 Growth medium 1X</td>
<td>Invitrogen (21331-020)</td>
</tr>
<tr>
<td>dNTP Mix</td>
<td>Promega (U151A)</td>
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<tr>
<td>DTT</td>
<td>Invitrogen (P/N Y00147)</td>
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<tr>
<td>Eosin Y aqueous solution</td>
<td>Sigma (HT 110232)</td>
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<tr>
<td>Folin ciocalteu’s phenol reagent 2N</td>
<td>Sigma (F 9252)</td>
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<tr>
<td>Haematoxylin solution Gill No. 3</td>
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<tr>
<td>Hank’s Balanced Salt Solution (HBSS) 1X</td>
<td>GIBCO (14175)</td>
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<tr>
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<td>Sigma (H0135)</td>
</tr>
<tr>
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<td>Sigma (I1884)</td>
</tr>
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</tr>
<tr>
<td>L-Glutamine 200 mM</td>
<td>Invitrogen (25030-024)</td>
</tr>
<tr>
<td>Low endotoxin bovine serum albumin</td>
<td>Sigma (A4919)</td>
</tr>
<tr>
<td>Oligo (dT)</td>
<td>Sigma (O4387)</td>
</tr>
<tr>
<td>Penicillin (100 IU / ml) / streptomycin (100 µg / ml)</td>
<td>Invitrogen (15070-063)</td>
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<tr>
<td>Polysine 72 slides</td>
<td>VWR (631-0107)</td>
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<tr>
<td>Recombinant human epidermal growth factor</td>
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<tr>
<td>RNase out recombinant ribonuclease inhibitor</td>
<td>Invitrogen (100000840)</td>
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Scott’s tap water bluing agent  
Sigma (S5134)

SuperScript II reverse transcriptase  
Invitrogen (100004925)

Tri-iodothyronine (4 pg / ml)  
Sigma (T5516)

Triton X100  
Sigma (T8532)

Trypsin / EDTA  
Invitrogen (25300-054)

Xylene  
Fisher Chemical (X/0250/17)

2.1.4 Gene targeting vectors

Two vectors were used to build up the targeting construct for the murine C1rA gene.

2.1.4.1 PL451 gene targeting vector

PL451 is a 4,834 Kb gene targeting vector. It is designed to contain restriction sites in two polylinker regions with a neomycin gene positive selective marker and an Ampicillin gene negative selection marker. The vector was provided by the University of Leicester Gene Targeting and Transgenic Facility (GENETA). The annotation map of the vector is shown in Table 1 and the vector map demonstrating the two polylinker restriction regions is shown in Figure 9.

The vector contains two FRT and one LoxP recombination sites providing the possibility to use it as conditional knockout vector, a characteristic that provides the needed flexibility to knockout genes that might be required for mouse development.
Table 1: The annotation map of the PL451 vector

<table>
<thead>
<tr>
<th>No.</th>
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<th>Qualifiers</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gene</td>
<td>705..738</td>
<td>FRT</td>
</tr>
<tr>
<td>2</td>
<td>Gene</td>
<td>796..1310</td>
<td>PGK promoter</td>
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<tr>
<td>3</td>
<td>Gene</td>
<td>1313..1378</td>
<td>EM7 promoter</td>
</tr>
<tr>
<td>4</td>
<td>CDS</td>
<td>1379..2179</td>
<td>Neo</td>
</tr>
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<td>5</td>
<td>Gene</td>
<td>2196..2498</td>
<td>bGHpA</td>
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<tr>
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<td>FRT</td>
</tr>
<tr>
<td>7</td>
<td>Gene</td>
<td>2551..2584</td>
<td>LoxP</td>
</tr>
<tr>
<td>8</td>
<td>CDS</td>
<td>3847..4704</td>
<td>Amp</td>
</tr>
</tbody>
</table>
Figure 9: PL451 vector map showing the restriction enzymes available in the two polylinker regions

2.1.4.2 pGEM®-T Easy vector

The pGEM®-T Easy Vector (Promega cat number A1360) was used for the cloning of the PCR products of the C1rA gene homology arms (Figure 10). The vector contains single 3'-T overhangs at both sides of the insertion site to permit the ligation of A-tailed PCR products into the plasmid by preventing re-
circularization of the vector and providing compatible T-overhangs. Successful cloning of DNA inserts into pGEM®-T Easy vector is confirmed by blue/white screening.

![pGEM®-T Easy vector map](image)

**Figure 10: pGEM®-T Easy vector map**

### 2.1.5 Oligonucleotides

All primers were designed using CLC Sequence Viewer genomic workstation version 6.8.1 from CLC Bio bioinformatics software developers (www.clcbio.com). Primers design was also checked on the National Centre for biotechnology information (NCBI) (http://www.ncbi.nlm.nih.gov) and the web based primer design software Primer3Plus® (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). All oligos used in the study were purchased from MGW Biotech, Germany and Thermo Fisher Scientific, UK. The following is a list of all the nucleotides used in the study and their 5’ → 3’ sequence:

- **C1rA F_cDNA** (CAATGCCAACCCAAATGC)
- **C1rA R_cDNA** (CTTCTGTATCCGGAATCCGC)
- **C1rB F_cDNA** (CTATCTATGGGCCAGGGG)


- C1rB_R_cDNA (AGGTATCCTATTTTCTGTGTCCTCC)
- C1rA_AF_PL451 (CAAGGAGAAGTCGCTTGCT)
- C1rA_AR_PL451 (ACAATGGAGAGAAGCCCTGC)
- C1rA_BF_PL451 (TGCCCTCGACATAGACAGA)
- C1rA_BR_PL451 (AGAGCTGCCCAGTCTGAGTA)
- C1rA_AF_ (CAACAAGATCTAAGTTCACTGGG)
- C1rA_AR (AGGAGACCTCTGGGTACCATT)
- C1rA_BF (GGAGAATTTCATGGCCAGCTT)
- C1rA_BR (TGGAGACCGCTGGATCCTCA)
- C1rA_CasA_F1 (CAGGAGACTAAAGAAAATAACGAGA)
- SalI_F_CasA (gtcgACGAGAAACTCCACACTTCC)
- HindIII_R_CasA (aagcTTCTGCTTCGTCCTCAATTCT)
- C1rA_CasA_R1 (GGATTGAGCCTGTAGTTCTTCT)
- C1rA_CasB1_F1 (GCCTCCAAGTCTTCAACCTA)
- NotI_F_CasB1 (GCTCCCCATGTATTCCCTCCTGTAA)
- Xbal_R_CasB1 (tctagAACAGGGAGGTAGATGTAAGAGC)
- C1rA_CasB1_R1 (GGTTGTTAACAGGGAGGTAGAT)
- C1rA_CasB2_F1 (TGCTCTACACCTACCTCCCT)
- Xbal_F_CasB2 (aatctagACATCTACCTCCCTGTTAACAACC)
- SacII_R_CasB2 (accgctGTCCAAGGTCCAGGAAGGA)
- C1rA_CasB2_R1 (TGTCCTACTTCAGTCCAAGGT)
- LoxP_Xbal_Sense
  (ctagATAACTTCGTATAATGTATGCTATACGAAGTTAT)
- LoxP_Xbal_Antisense
  (ctagATAACTTCGTATAGCATACATTATACGAAGTTAT)
- MASP2_L1_gRNA_pL1 (CTCAGAGGCCACTATCTCCACT)
- MASP2_L1_gRNA_pR1 (TGAGGGCTCAAGTTCCAAATAC)
- MASP2_L1_gRNA_pL2 (CACTATCTCCACTGCACAGACC)
- MASP2_L1_gRNA_pR2 (CAAGTTCCAAATACCACCCATC)
- MASP2_L2_gRNA_pL1 (TAGTTATTGATTGTGGCCCTCC)
- MASP2_L2_gRNA_pR1 (TTCACCTTAGCACATGGGAGAAA)
- MASP2_L2_gRNA_pL2 (ATGTGGACTATATCACAGGCCC)
- MASP2_L2_gRNA_pR2 (TCAGATTCCTGCAAATTGACAT)
- MASP2_R1_gRNA_pL1 (AGACTTCACTGGAACTGTGGCT)
- MASP2_R1_gRNA_pR1 (ATTAATGGAACCCCAGGAAACT)
- MASP2_R1_gRNA_pR2 (AAACTATTCCTCCCACAACCAA)
- MASP2_R2_gRNA_pL1 (GTAAGCGCTAACATGCTCTGTG)
- MASP2_R2_gRNA_pR1 (AGGTACTTGGCGATATGAGGAA)
- MASP2_R2_gRNA_pR2 (TATGAGGAACAATGATTGGACG)
Materials & Methods

- IVT-gi-37209910-gRNA-T4-fwd
  (TAATACGACTCACTATAGTGACCTACCCAATGGGC)

- IVT-gi-37209910-gRNA-T4-rev
  (TTCTAGCTCTAAAAACCATGGCCATTTGGGTAGGTC)

- IVT-gi-37209910-gRNA-T2-fwd
  (TAATACGACTCACTATAGGTACCTTCTTC)

- IVT-gi-37209910-gRNA-T2-rev
  (TTCTAGCTCTAAAAACCATGAAGAAGGTACTTAC)

- IVT-gi-37209910-gRNA-T3-fwd
  (TAATACGACTCACTATAGGTACCTTCTTC)

- IVT-gi-37209910-gRNA-T3-rev
  (TTCTAGCTCTAAAAACCAGTCTCTAAGCCAGCAG)

- IVT-gi-37209910-gRNA-T6-fwd
  (TAATACGACTCACTATAGGGTTCCATTAATTGT)

- IVT-gi-37209910-gRNA-T6-rev
  (TTCTAGCTCTAAAAACCACAATAATGGAAA"

2.2 Methods

2.2.1 Establishment of the protein overload proteinuria mouse model and model specific techniques

2.2.1.1 Experimental animals
Male BALB/c mice (WT and MASP-2KO), 7 weeks of age, were kept in standardised conditions of 12hr/12hr day and night light cycle, fed on
standardised food pellets and given free access to food and water. Animals were caged 5 per cage in a specific pathogen free environment in the University of Leicester central research facility.

### 2.2.1.2 Unilateral nephrectomy surgery

Prior to the commencement of protein overload, mice were subjected to unilateral nephrectomy. Eight week old mice were anaesthetised with 2.5 % isoflurane in 1.5 L/min oxygen and the left kidney was exposed through a 1 cm incision in the flank area. The kidney blood vessels and the ureter were all ligated completely using 6/0 polyglactin suture and the kidney was excised. The wound then closed using the same suture type and mice allowed to recover. Buprenorphine (Vetergesic) analgesic (0.1 mg/kg) was provided preoperative and meloxicam (Metcam) (5 mg/kg) was given pre and post-operative for four doses at 24 hour interval. Also local anaesthetic bupivacaine (Marcain) 5 mg/kg was given once during the surgery. Mice were allowed seven days for complete recovery before starting albumin overloading.

### 2.2.1.3 BSA administration to induce protein overload proteinuria

To induce protein overload nephropathy, mice were given i.p. doses of low endotoxin bovine serum albumin (BSA) (Sigma cat number A4919), diluted in saline, on daily basis. BSA concentrations were increased incrementally from 2 mg/gm body weight for the first dose to 15 mg/gm body weight by the seventh dose, continuing at this concentration for 15 doses in total.

BSA was dissolved in saline at a concentration of 40% w/v and then filter-sterilised. The final concentration then was determined after filtration, and different doses were prepared from the stock solution to be injected i.p. in 1 ml/per 25g mouse and kept at 4°C until use. After the administration of the last dose, animals were caged separately in metabolic cages for 24 hours to collect urine.
Mice were sacrificed 24 hours after the last BSA administration and various tissues collected for analysis. Blood was collected by cardiac puncture under anaesthesia, and mice were culled by exsanguination. Blood was allowed to clot on ice for 2 hours and serum was separated by centrifugation and kept as aliquots at −80°C.

2.2.1.4 Antibody mediated inhibition of the lectin pathway in wild type mice with protein overload proteinuria

Inhibition of the lectin pathway in wild type mice was mediated by the 721-SGMI-H2 antibody (Omeros Inc., Seattle, USA). This is a recombinant human monoclonal antibody targeting the murine MASP-2 serine protease. 721-SGMI-H2 antibody is the second generation of the OMS721 antibody with an SGMI-2 polypeptide chain fused to the C-terminus of the OMS721 heavy chain. The SGMI-2 polypeptide is a highly specific and highly potent MASP-2 inhibitor that is based on the *Schistocerca gregaria* protease inhibitor SGPI and is fully described in (Heja et al., 2012). Targeting the MASP-2 serine protease with these antibodies selectively inhibits the lectin pathway of the complement system while leaving intact the rest of the complement pathways functional activities.

721-SGMI-H2 was administered i.p. at a dose of 10 mg/kg into groups of eight mice alongside two control groups; a saline control and an isotype control group given human IgG4 isotype antibody, 10 mg/kg. Mice were dosed twice weekly starting 1 week before proteinuria induction, and then continuing throughout the study. Blood samples were taken prior to dose administration and at the end of the experiment to assess LP-functional activity.

2.2.2 Establishment of the unilateral ureteric obstruction mouse model and model specific techniques

2.2.2.1 Unilateral ureteric obstruction surgery

Ten week old mice, 6 per group, were anaesthetized with 2.5 % isoflurane in 1.5 L/min oxygen and the right kidney was exposed through a 1cm flank incision. The right ureter was completely obstructed at two points using a 6/0 polyglactin
suture. Buprenorphine analgesia was provided peri-operatively every 12 hours for up to 5 doses depending on pain scoring. Local bupivacaine anaesthetic was given once during the surgery.

Mice were sacrificed 7 days after the surgery and various tissues collected for analysis. Blood was collected from MASP-2−/− and WT mice by cardiac puncture under anaesthesia, and mice were culled by exsanguination after nephrectomy. Blood was allowed to clot on ice for 2 hours and serum was separated by centrifugation and kept as aliquots at −80°C.

2.2.2.2 Antibody-mediated lectin pathway inhibition in unilateral ureteric obstruction mice

Murine MASP-2 activity in this experiment was depleted using two versions of a recombinant human monoclonal antibody selectively targets MASP-2. These antibodies, named OMS721 and 721-SGMI-H2, are known to inhibit the LP functional activity in mice (Schwaebel et al., 2011) and were established and supplied by Omeros Inc., Seattle, USA. Targeting the MASP-2 serine protease with these antibodies selectively inhibits the lectin pathway of the complement system while leaving intact the rest of the complement pathways functional activities.

Male WT C57BL/6 mice were injected with 10mg/kg of either OMS721 or 721-SGMI-H2 administered to groups of 6 mice by i.p. injection on day -1 prior to UUO surgery and again on day +3 post-surgery. Blood samples were taken prior to antibody administration and at the end of the experiment to assess lectin pathway functional activity. Control groups were UUO operated mice and given either normal saline or a non-specific human IgG4 antibody as an isotype control.

2.2.2.3 Lectin pathway inhibition assay

To assess the pharmacodynamics effect of anti-MASP-2 mAb administration in mice, systemic lectin pathway activity was evaluated by quantifying lectin-induced C3 activation in minimally diluted serum samples collected at the indicated times after MASP-2 mAb or control mAb administration to mice. This
analysis has been performed and the data has been generated by a team of collaborative researchers at OMEROS Inc. (Seattle, WA, USA). Briefly, 7µm diameter polystyrene microspheres (Bangs Laboratories, Fisher, IN, USA) were coated with mannan by overnight incubation with 30µg/mL mannan (Sigma) in of sodium carbonate-bicarbonate buffer (pH 9.6), then washed, blocked with 1% foetal bovine serum in PBS and re-suspended in PBS at a final concentration of 1x10^8 beads/ml. Complement deposition reactions were initiated by the addition of 2.5 µl of mannan-coated beads (~250,000 beads) to 50 µl of minimally diluted mouse serum sample (90% final serum concentration), followed by incubation for 40 min at 4°C. Following termination of the deposition reaction by the addition of 250 µl of ice-cold flow cytometry buffer (FB: PBS containing 0.1% FBS), beads were collected by centrifugation (3000 g for 3 min at 4°C) and washed two more times with 300 µl of ice-cold FB.

To quantify lectin-induced C3 activation, beads were incubated for 1 hour at 4°C with 50 µl of rabbit anti-human C3c antibody (DAKO A-0062) diluted in FB. Following two washes with FB to remove unbound material, the beads were incubated for 30 min at 4°C with 50 µl of goat anti-rabbit antibody conjugated to PE-Cy5 (Southern Biotech, Birmingham, AL, USA) diluted in FB. Following two washes with FB to remove unbound material, the beads were re-suspended in FB and analysed by a FACS Calibur cytometer. The beads were gated as a uniform population using forward and side scatter, and C3b deposition in each samples was quantified as mean fluorescent intensity (MFI).

### 2.2.3 Adriamycin nephropathy mouse model

#### 2.2.3.1 Experimental animals

Animals used in this model were BALB/c wild type and MASP-2KO male mice aged between 8 and 10 weeks. Mice were kept in standardised conditions of 12hr/12hr day and night light cycle. Animals were caged 5 per cage in a pathogen free environment in the University of Leicester CRF.
2.2.3.2 Establishment of the model by administration of adriamycin

A single dose of 10.5 mg/kg adriamycin hydrochloride (Sigma cat number D1515), dissolved in 100 µl normal saline was administered intravenously. To define the time point at which the mice develop least injury yet satisfactory renal inflammation, three groups of mice were injected with adriamycin and culled at three time points: one week, two weeks and four weeks after adriamycin administration, and various tissues collected for further analysis. Control mice were injected with saline only.

2.2.4 Renal injury models: general analysis techniques

2.2.4.1 Immunohistochemistry

Formalin fixed, paraffin embedded, 5 µm kidney sections from each mouse were deparaffinised and rehydrated. Antigen retrieval was performed in citrate buffer at 95°C for 20 mins followed by quenching the endogenous peroxidase activity by incubating tissues in 3% H₂O₂ for 10 min. Tissues then were incubated in blocking buffer (10% serum from the species the secondary antibody raised in and 1% BSA in PBS) with 10% avidin solution for 1 hour at room temperature to block the possible antibody non-specific binding. Sections were washed in PBS three times, 5 mins each, after each step.

Primary antibody then applied in blocking buffer with 10% biotin solution for 1 hour at a concentration of 1:100 for the antibodies F4/80 (Santa Cruz cat number sc-25830), TGFβ (Santa Cruz cat number sc-7892), IL-6 (Santa Cruz cat number sc-1265) and C3 (DAKO A-0062) and at 1:50 for the TNFα antibody (Santa Cruz cat number sc-1348). A biotinylated secondary antibody then applied for 30 min at a concentration of 1:200 for the F4/80, TGFβ, IL-6 and C3 sections and 1:100 for TNFα, followed by HRP conjugate enzyme for another 30 min. The colour was developed using diaminobenzidine (DAB) substrate kit (Vector labs, cat number SK-4100) for 10 min and slides were washed in water, dehydrated and mounted without counter staining to facilitate the computer based image analysis.
Negative control slides have been processed along with the rest of the stained slides to insure the specificity of the used antibodies. In this negative control staining, only the primary antibodies have been excluded from the staining process while the rest of the steps have been performed. Figure 11 shows representative images of negative control slides from batches of staining with each primary antibody used. All negative control slides showed only the background basic tissues appearance. However, this negative control slides have been used to threshold the image analysis software to exclude this background baseline staining.

![Figure 11](image)

**Figure 11**: Representative images for negative control slides from staining batches using A) F4/80, B) TGFβ, C) TNFα and D) IL-6 antibodies. No primary antibodies have been used to stain these slides.

### 2.2.4.2 Image analysis

The percentage of the antibody stained area in the cortex in each kidney section was estimated by a modification of the method described in more detail in (Furness *et al.*, 1997). In brief, 24 bit colour images were captured from sequential non-overlapping fields of renal cortex just beneath the renal capsule
around the entire periphery of the section of kidney. After each image capture, the freeware program NIH ImageJ (Schneider et al., 2012) was used to extract the red channel as an 8 bit monochrome image. Unevenness in the background illumination was subtracted using a pre-recorded image of the illuminated microscope field with no section in place. The image was subjected to a fixed threshold to identify areas of the image corresponding to the staining positivity. The percentage of black pixels was then calculated, and after all the images around the kidney had been measured in this way the average percentage was recorded, providing a figure corresponding to the percentage of antibody binding area in the kidney section. A set of macros was written to automate this process (see appendix I for details).

2.2.4.3 Statistical analysis

The statistical analyses of this study were performed using the GraphPad prism software (v7.0). One-way analysis of variance (ANOVA) and unpaired student’s t-test were used at 95% confidence levels to estimate the significance of differences between compared groups in all experiments. When multiple comparisons were made simultaneously on a single experimental data set, the post hoc Bonferroni’s correction method was applied. Group means and standard error of means values (Mean±SEM) are also calculated and presented in the results.

2.2.4.4 Detection of apoptosis

Apoptotic cells in the kidney sections were stained using ApopTag® Peroxidase Kits (Millipore, cat number S7100). Paraffin embedded, formalin fixed, kidney sections from each mouse were de-waxed and then rehydrated then protein permeabilised using proteinase K (20 µg/mL) applied to the specimens for 15 min at room temp. Specimens were washed in PBS between steps. Endogenous peroxidase activity was quenched by incubating tissues in 3% H₂O₂ for 10 min then tissues were incubated in equilibration buffer until TdT enzyme was applied for 1 hour at 37°C. After washing in stop/wash buffer for 10 min, anti-
Materials & Methods

digoxignenin conjugate was applied for 30 min at room temp followed by washing. Colour was developed in DAB substrate kit for 4 min followed by washing in water. Tissues were counter stained in haematoxylin and mounted in DBX. Brown coloured apoptotic cells were counted manually using a Leica DBXM light microscope in 20 adjacent non-overlapping cortical high power fields.

2.2.4.5 Sirius Red staining

In order to determine renal fibrosis, 5 µm paraffin embedded kidney sections were stained with picro-Sirius Red staining specific for collagen as previously described (Whittaker et al., 1994). Briefly, sections were de-waxed and rehydrated and nuclei were stained with Weigert's haematoxylin for 8 min. Slides were washed for 10 min in running tap water then stained with picro-Sirius Red 0.5 gm (Sirius Red Sigma cat number 365548) in 500 ml saturated aqueous solution of picric acid for 1 hr. Slides then washed in two changes of acidified water (5 ml glacial acetic acid to 1 litre of distilled water). Excess water was physically removed from the slides by vigorous shaking and sections were dehydrated in three changes of 100% ethanol then cleared in xylene and mounted in a resinous medium.

2.2.4.6 Haematoxylin/eosin staining

Paraffin embedded kidney sections were de-waxed and rehydrated in serial dilutions of ethanol (100 %, 90% and 70%) for 2 min each. Slides were stained in haematoxylin solution Gill No. 3 for two minutes then washed in tap water. Differentiation was carried out using Accumate solution for 20-60 seconds followed by a wash in tap water. Blueing agent, Scott’s tap water substitute, was applied for 5-60 sec and washed again in tap water. Acidified Eosin Y aqueous solution was then applied for 30-60 seconds then washed three times in tap water. Slides were dehydrated with increasing concentration of serial solution of ethanol and cleared by merging the slides in xylene for 10 seconds. Tissues were mounted in a drop of DBX mounting medium and a cover-slip applied.
2.2.4.7 Determination of total protein concentrations in urine and serum

Total protein in urine and serum samples was measured based on the Bradford method (Bradford M.M., 1976) using Coomassie Plus™ Assay Kit (Fisher Scientific, cat number 23236). In brief, samples were diluted in distilled water (1:20 for urine and 1:200 for serum) and 10 μl from standard or unknown samples were pipetted in duplicate to 96 well plate wells. 300 μl of the Coomassie reagent were added and plate placed on orbital shaker for 30 sec followed by 10 min incubation at room temperature. Colour development was read using plate reader at wavelength 595 nm. A standard curve was prepared using the Thermo Scientific standard albumin ampoules (Catalogue number 23209) and sample readings were plotted and calculated against it.

2.2.4.8 Creatinine estimation

Creatinine concentration in serum and urine samples was estimated using the QuantiChrom creatinine assay kit (BioAssay Systems, cat number DICT-500). For urine samples, the test was performed by adding 200 μl working solution to 5 μl urine sample or 50 mg/dl standard in a well of 96-well plate in duplicates. The plate was tapped briefly to mix and then immediately read at 520 nm wavelength (OD₀) and after 5 minutes (OD₅) for both sample and standard duplicates. For serum samples procedure is the same but the sample or standard volume is 30 μl and the control concentration is 2 mg/dl. Concentration was calculated from the following equation:

Sample concentration = \[
\frac{[(OD₅ \text{ sample} - OD₀ \text{ sample}) / (OD₅ \text{ standard} - OD₀ \text{ standard})] \times \text{standard concentration (mg/dl)}}
\]

2.2.4.9 Active C3c titration method using enzyme linked immunosorbent assay (ELISA)

The assay was primarily designed to assess lectin pathway dependent C3c deposition on mannan. Nunc Maxisorb microtiter plates were coated with 1μg/well mannan diluted in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and incubated overnight at 4°C. Residual protein binding sites were
saturated by incubating the plate with 1% BSA or 5% skimmed milk, separate plates, in TBS blocking buffer (10 mM Tris-CL, 140 mM NaCl, 1.5 mM NaN₃, pH 7.4) for 2 hours then washed in TTBS/Ca²⁺ buffer (TBS with 0.05% Tween 20 and 5 mM CaCl₂). Serum samples that were stored at -80°C were slowly left to thaw on ice and 2.5% serum dilution from each sample was prepared in barbital buffered saline (BBS; 4 mM barbital, 145 mM NaCl, 2 mM CaCl₂, 1mM MgCl₂, pH 7.4), 100 μl diluted serum was added to each well. Wells receiving only BBS buffer were used as negative controls. Samples were loaded on ice and placed at 37°C for 1 hour. Following incubation the plates were washed in TTBS/Ca²⁺ buffer (x4) and bound C3c was detected with a polyclonal anti-human-C3c Antibody (DAKO, A0062) diluted in TTBS/Ca²⁺ buffer at 1:5000. The antibody was incubated at 4°C overnight followed by 30 min at room temperature. Another washing step followed antibody incubation (x4) and administration of the secondary antibody took place using goat anti-rabbit IgG (whole molecule) conjugated to alkaline-phosphatase (Sigma A-3812) diluted 1:5000 in TTBS/Ca²⁺. This was incubated for 1 hour at room temperature. The extent of C3c deposition and subsequently lectin pathway activation was determined by adding 100 μl of the substrate solution (Sigma Fast p-Nitrophenyl Phosphate tablets) and incubation at room temperature. The absorption (OD) at 405 nm was then measured 8 mins after the addition of the substrate using a BioRad micro-titre ELISA plate reader.

2.2.4.10 Gene expression in injured kidneys

Expression of genes relevant to renal inflammation and fibrosis in mouse kidney were measured by quantitative PCR (qPCR). Total RNA was isolated from kidney cortex using Trizol (Thermo Fisher Scientific, Paisley, UK) according to the manufacturer’s instructions. Extracted RNA was treated with the Turbo DNA-free kit (Thermo Fisher Scientific) to eliminate DNA contamination and then first strand cDNA was synthesised using AMV Reverse Transcription System (Promega, Madison, WI, USA).
The cDNA integrity was confirmed by a single qPCR reaction using TaqMan GAPDH Assay (Applied Biosystems, Paisley UK, cat number 4485712) followed by qPCR amplification using Custom TaqMan Array 96-well Plates (Life Technologies, Paisley, UK). The following genes were studied:

- Collagen type IV alpha 1 (Col4α1) assay ID: Mm01210125_m1
- Transforming growth factor beta-1 (TGFβ-1) ID: Mm01178820_m1
- Cadherin 1 (Cdh1) ID: Mm01247357_m1
- Fibronectin 1 (Fn1) ID: Mm01256744_m1
- Interleukin-6 (Il-6) ID: Mm00446191_m1
- Interleukin-10 (Il-10) ID: Mm00439614_m1
- Interleukin-12a (Il-12a) ID: Mm00434165_m1
- Vimentin (Vim) ID: Mm01333430_m1
- Actinin alpha 1 (Actn1) ID: Mm01304398_m1
- Tumour necrosis factor-α (TNF-α) ID: Mm00443260_g1
- Complement component 3 (C3) ID: Mm00437838_m1
- Interferon gamma (Ifn-γ) ID: Mm01168134_m1
- Complement factor properdin (Cfp) ID: Mm01341415_m1

In addition, the following housekeeping control genes were used:

- Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ID: Mm99999915_g1
- Glucuronidase beta (Gusβ) ID: Mm00446953_m1
Materials & Methods

- Eukaryotic 18S rRNA (18S) ID: Hs99999901_s1
- Hypoxanthine guanine phosphoribosyl transferase (HPRT) ID: Mm00446968_m1
- TATA-box binding protein (TBP) ID: Mm01277042_m1

20 µl reactions were amplified using TaqMan Fast Universal Master Mix (Applied Biosystems, cat number 4352042) for 40 cycles. Real time PCR amplification data were analysed using Applied Biosystems 7000 SDS v1.4 software.

For analysis, the murine house-keeping gene GAPDH has been used as an endogenous control. Amplification blots of the gene showed a consistent expression pattern in all tested samples as represented in Figure 12. Amplification started at around cycle 20 in all samples and continued homogenously and exponentially till the end of the experiment. The GAPDH gene is commonly used in gene expression studies as an internal reference for its expression stability (Silver et al., 2006).
2.2.4.11 Total RNA extraction

Snap frozen tissues, up to 30 mg, were homogenized in 350 µl of buffer RA1 and 3.5 µl β-mercaptoethanol (β-ME). Viscosity of the homogenate was reduced and lysates were cleared by filtering through a membrane filter. Lysates were applied to the filter and placed in a collection tube (2 ml) and centrifuged for 1 min at 11,000 g. Filtrates were discarded and 350 µl of ethanol (70%) was added to the homogenised lysates and mixed by pipetting up and down to adjust RNA binding conditions. To bind the RNA to the silica membrane, homogenised lysates were applied to Nucleospin® RNA II Column placed in a collection tube and centrifuged for 30 sec at 11,000 g, the column was then placed in a new collection tube (2 ml). Silica membranes were desalted by adding 350 µl MDB (Membrane Desalting Buffer) and centrifuged at 11,000 g for 1 min to dry the
membrane. DNA was digested by adding 95 μl DNase reaction mixture directly applied to the centre of the silica membrane of the column. The DNase reaction mixture was prepared by adding 10 μl reconstituted rDNase to 90 μl rDNase reaction buffer supplied from the kit and mixed by flicking. Reactions were then incubated at room temperature for 15 min and then silica membranes were washed and dried as the following:

1st wash: 200 μl Buffer RA2 were added to the column then centrifuged for 30 sec at 11,000 g and the column was placed into a new collection tube (2 ml), buffer RA2 inactivates the rDNase.

2nd wash: 600 μl RA3 buffer were added to the column and centrifuged for 30 sec at 11,000 g. Flow through discarded and columns were placed back into the collection tube.

3rd wash: 250 μl Buffer RA3 were added to the column and centrifuged for 2 min at 11,000 g to dry the membrane completely. Columns were placed into a nuclease free collection tube (1.5 ml).

RNA was eluted in 60 μl RNAse-free water and centrifuged at 11,000 g for 1 min. To ensure the RNA stability, RNA extracts were kept at -20°C for short term storage and at -80°C for long-term storage.

2.2.4.12 Genomic DNA extraction
Genomic DNA from mouse tissues was isolated using Wizard Genomic DNA Purification Kit (Promega, cat number A1120). 20 mg of the tissue was homogenized in 600 μl of pre-chilled nuclei lysis solution provided with the kit and then incubated at 65°C for 30 min. 3 μl of RNase solution then added to the cell lysate mixture to digest the RNA and mixture incubated at 37°C for another 30 minutes then cooled to room temperature. Protein content was precipitated by adding 200 μl of the kit protein precipitation solution and vortex and chill on ice for 5 minutes. Samples were centrifuged at 16000 g for 4 minutes to pellet the proteins and supernatant was transferred to another fresh tube containing 600 μl
of isopropanol to precipitate the DNA. After a brief mix, the mixture was centrifuged at 16000 g for 1 minute and supernatant removed. DNA was then washed in 600 µl 70% ethanol at room temperature then ethanol aspirated and DNA was air dried. The extracted and purified DNA pellet was rehydrated in 100 µl of the kit DNA rehydration solution for 1 hour at 65ºC and stored at -20ºC.

2.2.4.13 Agarose gel electrophoresis

Agarose gel electrophoresis is used to separate and analyse DNA depending on size. DNA is negatively charged and therefore migrates towards the anode in an electrical field. When DNA samples are loaded into an agarose gel and electrical field is applied the DNA fragments will travel through the gel and they will be separated based on their size. The bigger the size of the DNA fragments the smaller the distance it travels through the gel and vice versa. By increasing the concentration of the agarose gel smaller sizes of DNA fragments can be visualised better however, this applies more resistance to the bigger fragments. The DNA can be visualised by the addition of ethidium bromide which binds to the DNA by intercalating between the bases and allows the DNA to be visualized upon the exposure to UV light.

1% agarose gel was prepared by dissolving 1g of agarose in 1x TAE buffer containing ethidium bromide. The agarose gel is the poured in the gel plate and the comb was placed for the formation of the wells. Once the gel was solidified the comb was removed carefully and the gel tray was placed in the electrophoresis tank with 1X TAE buffer. DNA samples to be analysed were mixed with a required volume of 6X loading dye and were loaded into the wells against 10µl of 1 Kb ladder (100 ng/µl). The gel was run at 120V till the dye front reached the end of the gel and was later visualized and analysed under UV light.
2.2.5 Gene targeting procedures of murine C1rA and MASP-2 genes

2.2.5.1 Recombineering approach for conditional targeting of C1rA gene

2.2.5.1.1 Gene targeting construct and primer design

The software used to design the C1rA knockout construct and the primers is the CLC Sequence Viewer genomic workstation version 6.8.1 from CLC Bio bioinformatics software developers “website: www.clcbio.com”. Genomic databases used are those of the National Centre for Biotechnology Information (NCBI) database “http://www.ncbi.nlm.nih.gov“ and the Ensembl mouse genome project “http://www.ensembl.org/index.html”. The percentage of homology of chosen cassettes was checked against other loci in the mouse genome using the web based search tool of the National Library of Medicine BLAST accessible through the website of the National Centre of Biotechnology Information “http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome”.

Based on the vector design, two homology segments of the target gene were selected to be the homology arms. They guide the vector to the recombination event so the gene targeting occurs selectively to the selected gene; these cassettes were given the names Cassette A and Cassette B. These pieces fulfil the criteria of having restriction sites suitable to one of the vector poly linker sites, having appropriate primers and not intervening restriction sites for any enzyme that may be used in any of the other restriction digestions in the course of manipulation.

2.2.5.1.2 Setting up LB agar plates

LB agar plates are used to grow bacteria of cloning or transformation. For 400ml of LB agar medium add 4g of tryptone, 2g of yeast extract, 2g of NaCl and 6g of agar in double distilled water and autoclave. While the medium is cooling down to room temperature, prepare 20 x 90mm petri plates in a laminar airflow cabinet cleaned with 70% IMS. Once the medium cools down, add 800μl of ampicillin (stock concentration 50mg/ml) to LB medium to make a final concentration of 50μg/ml and mix. Pour the agar quickly into petri dishes, filling up to about a
quarter of an inch of the plate and close lid immediately. Once they solidify, store at 4°C.

**2.2.5.1.3 Preparation of chemically competent *E. coli* strains**

The protocol used for harvesting the competent *E. coli* strain TOP10F is a modification of the methodology described by (Hanahan, 1983) as the following:

Streak the host strains on LB agar plates containing 10mM Mg2+ and incubate at 37°C overnight. On the following day pick a single colony from the plate and inoculate 5ml of LB medium with that colony in a 50ml sterile falcon tube. Incubate the cultured tube at 30°C in a shaking incubator overnight. On the next day inoculate 100ml of fresh LB medium with 1ml of the bacterial culture, incubate overnight and let it grow at 37°C in a shaking incubator until it reaches an OD550 of 0.7 to 0.8. Collect the cells by centrifugation at 2000g for 10 min and decant the supernatant. Resuspend the bacterial cells in 30 ml sterile ice cold TfbI buffer and incubate on ice for approximately 10mins then centrifuge 2000g at 4°C for 10 min and cells are resuspended in 4ml ice cold TfbII buffer. Aliquot 100 µl fractions into Eppendorf tubes and store them at -80°C.

**2.2.5.1.4 Ligation of insert into vector**

A necessary part of cloning is the ligation of DNA fragments. Covalently joining together two linear DNA fragments is known as ligation and it is proficiently done by using the T4 DNA ligase enzyme from New England Biolabs which is encoded by bacteriophage T4. It catalyses a joining reaction between DNA molecules involving the 3'-hydroxy and the 5'-phosphate termini. T4 ligase works best at 37°C. If the DNA of interest has to be cloned into an uncut plasmid by ligation then the plasmid should be pre-digested with specific restriction enzyme producing a linear DNA molecule with overhanging sticky ends for efficient ligation.
2.2.5.1.5 Ligation of PCR product into pGEM®-T Easy vector

*Taq* polymerase has a non-template-dependent terminal transferase activity which adds a single deoxyadenosine to the 3’ ends of the PCR product. The vector has a single 3’ thymidine overhang that allows PCR inserts to ligate easily with the plasmid. Briefly centrifuge the tube containing the pGEM®-T Easy vector and prepare the ligation reaction in following order, mix the reagents and incubate overnight at 4°C.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Ligation Reaction</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 DNA ligase buffer</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>T4 DNA ligase</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>PCR Product (~60 to 150 ng/ μl)</td>
<td>2μl</td>
<td>------</td>
</tr>
<tr>
<td>pGEM®-T Easy vector (50ng/ μl)</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>Double distilled H₂O</td>
<td>5μl</td>
<td>6μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>10μl</td>
<td>10μl</td>
</tr>
</tbody>
</table>

2.2.5.1.6 Optimizing insert: vector molar ratios

The concentration of PCR product or DNA fragment and vector which are restriction digested are estimated by comparison to DNA standards on agarose gel. The amount of insert DNA to be added was calculated using the following formula for insert- vector optimization. Insert: vector molar ratio was 3:1.

\[ \text{ng of vector} \times \text{kb size of insert} \times \text{insert: vector molar ratio} = \text{ng of insert kb size of vector} \]

2.2.5.1.7 Chemical transformation of plasmid into competent *E. coli* cells

The technique is to introduce a foreign plasmid (a carrier of the DNA of interest) into bacteria to be amplified. This technique is based on the natural function of
the plasmid where the transfer of genetic information through the plasmid is vital for the survival of the bacteria.

Thaw 50 μl of Top 10 F’ competent cells on ice and add to a pre cooled Eppendorf tube. Add 2μl of the ligation reaction and mix it and incubate on ice for 20min. To allow the transformation, heat-shock the cells at 37ºC for 5 mins and incubate back on ice for 1min immediately. Add 450μl of LB medium and incubate the reaction in a 37ºCshaking incubator for 1hour. After incubation the bacterial culture is plated out on plates containing LB agar and ampicillin (50μg/ml), X-gal (25 μg/ml) and IPTG (100 μg/ml) for the selection of recombinant bacterial colonies by blue/white screening.

2.2.5.1.8 Blue/white screening for the selection of recombinant clones
Successful cloning of DNA inserts into pGEM®-T Easy vector is confirmed by blue/white screening. pGEM®-T Easy contains a Lac-Z gene that codes for β-galactosidase, an enzyme that digests and converts X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactside) into a blue coloured product. For the production of the enzyme by the active transcription of the DNA, IPTG (Isopropyl β-D-thiogalactopyranoside) is delivered to the bacteria through the medium. Within the Lac Z gene is a multiple cloning site (MCS) where the gene of interest can be inserted. Hence, if the insert DNA is present in the MCS of the LacZ gene, the expression of LacZ becomes disrupted and no functional β-galactosidase will be expressed. This disruption in enzyme activity is observed as white colonies as no functional Lac Z is available to digest X-gal and produce blue colour. Therefore, if the insert is present in the vector the colonies appear white in colour and their absence leads to blue colour colonies on the LB agar plate.

2.2.5.1.9 Culture colonies for isolation of DNA
Select about 8-10 colonies from the transformed plate using a sterile pipette tip and add individual clones into 15ml falcon tube containing 2.5ml of LB medium with 50 μg/mL ampicillin. Incubate the falcon tube at 37ºCin an orbital shaker overnight.
2.2.5.1.10 Small scale isolation and purification of plasmid DNA (Minipreps)

Minipreps are used to extract plasmid DNA from bacterial cell suspensions. The plasmids are a relatively small supercoiled molecule whereas bacterial chromosomal DNA is large in size and less supercoiled. This difference in topology helps in selective precipitation of chromosomal DNA and protein from plasmid DNA.

Small scale isolation and purification of plasmid DNA was carried out using the Wizard® Plus SV Minipreps DNA Purification System from Promega. The kit combines two techniques; alkaline lysis and silica resin based DNA purification. Cells are partially lysed using an alkaline solution of the detergent SDS. This allows small plasmid DNA molecules to escape from the cells, while genomic DNA remains within the cells. When a concentrated potassium acetate solution is added to the cell lysate, cell debris is precipitated, while plasmids and soluble proteins remain in solution. If the cell membrane is dissolved completely, plasmid and soluble proteins remain in solution. If the cell membranes are dissolved completely, sheared genomic DNA may be released, contaminating the plasmid prep. To avoid this, the lysis step is carried out for just enough time for the solution to clear. The adhesion of DNA to a silica matrix is based on the fact that nucleic acids adhere to silica in high but not in low salt conditions. DNA binds to silica in the lysis solution and is eluted from the silica matrix by TE buffer.

Centrifuge 5 ml of overnight bacterial culture for 5min at a speed of 10,000g in a centrifuge, discard the supernatant and collect the pellet. Resuspend the pellet in 250μl of cell suspension solution by repeatedly pipetting then add 250μl of cell lysis solution and mix by inverting the tube 3-4 times. Add 10μl of alkaline protease solution and mix by inverting the tube and incubate for 5mins at room temperature, the timing is critical. The cell lysis reaction was stopped by adding 350μl of neutralization solution and mixing by inverting the tube and the lysate was then centrifuged at 14,000g for 10 mins. Transfer the cleared lysate to the spin column by decanting; avoid disturbing or transferring any of the white
precipitate with the supernatant. Centrifuge the supernatant at the highest speed in a micro centrifuge for 1 min at room temperature and remove the spin column from the tube and discard the flow through from the collection tube. Add 750μl of column wash solution and centrifuge for another 1 min at the highest speed then remove the spin column from the tube and discard the flow through. Repeat the wash step by using 250μl of column wash solution. Spin the column for another 2 mins at room temperature and transfer the spin column into another fresh sterile 1.5μl Eppendorf tube. Elute the plasmid DNA by adding 100μl of double distilled water to the spin column and centrifuge the tube at highest speed for 2 mins. The plasmid DNA was stored at -20ºC for further analysis. The plasmid identity can then be checked by using restriction digestion.

2.2.5.1.11 Large scale isolation and purification of plasmid DNA (MaxiPreps)

In order to obtain a working quality of large amounts of purified plasmid DNA the Nucleobond PC 500 Kit were used. The principle on which this kit works is that bacteria are harvested from an overnight culture and lysed using the alkaline lysis procedure. The bacterial lysate is cleared and loaded onto the equilibrated column and the plasmid DNA binds to the anion-exchange resin. The column is first washed using equilibration buffer to wash out residual lysate from the filter and obtain maximum recovery of DNA. After subsequent washing, the purified plasmid DNA is eluted in a high-salt buffer and precipitated with isopropanol. The method is described below:

100ml of LB medium containing ampicillin was inoculated with the original culture in a ratio of 1/1000 and incubated in an orbital shaker overnight at 37ºC. The bacterial growth in 50ml of overnight culture was pelleted by centrifugation for 15min at 10,000g and the supernatant was discarded. Re-suspend the pellet in buffer S1, from the kit, containing RNase A, mix well and then add buffer S2 to lyse the cells and incubate for 2 mins. Add chilled buffer S3 for neutralization and incubate for 5 mins on ice. In the meantime, neutralize the NucleoBond Xtra column with buffer N1 and pour the bacterial lysate onto it and let the solution flow through the column drop by drop. Wash the column with buffer N2 and
elute by using buffer N5. Add isopropanol to the eluted plasmid DNA and centrifuge for 30 min at high speed. Decant the supernatant and collect the pellet and wash with 70% ethanol. Re-suspend the purified plasmid pellet in double distilled H\textsubscript{2}O and store at -20°C.

2.2.5.1.12 Glycerol stocks

For long term storage of positive transformants, bacterial cultures were mixed 1:1 with sterile 50% glycerol in cryotubes and frozen down at -80°C.

2.2.5.1.13 Spectrophotometric determination of nucleic acids concentration

The device used to determine the concentration of DNA and RNA samples was Thermo Fisher Scientific NanoDrop 1000 spectrophotometer. It uses only 1 µl of the sample to measure the sample at 230, 260 and 280 nm wavelengths and calculate the concentration.

The ratio of readings at 260nm and 280nm [A\textsubscript{260} / A\textsubscript{280}] provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have ratios in the range of 1.8–2.2.

2.2.5.1.14 Dephosphorylation of a linearized plasmid

During ligation, DNA ligase will catalyse the formation of a phosphodiester bond between adjacent nucleotides only if one nucleotide contains a 5'-phosphate group and the other a 3'-hydroxyl group. Recircularization of plasmid DNA can therefore be minimized by removing the 5' phosphates from both ends of the linear DNA with calf intestinal alkaline phosphatase (CIAP) (Seeburg et al., 1977; Ullrich et al., 1977). As a result, neither strand of the duplex can form a phosphodiester bond. For dephosphorylation, the following protocol was used as provided by Roche:
Materials & Methods

### Reagents

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>The vector DNA digest (50ng/μl)</td>
<td>X μl</td>
</tr>
<tr>
<td>10X CIAP buffer</td>
<td>10μl</td>
</tr>
<tr>
<td>CIAP enzyme (0.05Units)</td>
<td>5μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Y μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>100μl</td>
</tr>
</tbody>
</table>

The reaction is then incubated for 30 mins at 37ºC. The dephosphorylation reaction is followed by phenol extraction and ethanol precipitation.

### 2.2.5.1.15 Restriction digestion

Restriction enzymes cleave DNA at specific sites. Individual restriction enzymes have their unique DNA recognition sequence on a given DNA. To perform restriction digest with a particular restriction enzyme, the DNA is incubated with the enzyme and a buffer that is appropriate for its optimal performance. To set up a restriction digest with two different enzymes, one common buffer is used. The amount of enzyme required and the procedure varies depending on the DNA samples subjected to digestion. The amount of DNA used is a matter of judgement depending on the concentration of the DNA samples. Set up the restriction digest as described below:
### Materials & Methods

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Reaction Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (200ng/μl)</td>
<td>X μl</td>
</tr>
<tr>
<td>Restriction enzyme 1 (20,000 units/μl)</td>
<td>1μl</td>
</tr>
<tr>
<td>Restriction enzyme 2 (20,000 units/μl)</td>
<td>1μl</td>
</tr>
<tr>
<td>10X BSA</td>
<td>2μl</td>
</tr>
<tr>
<td>Buffer (Compatible to both enzymes)</td>
<td>2μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Y μl</td>
</tr>
<tr>
<td>Final volume</td>
<td>20μl</td>
</tr>
</tbody>
</table>

All the reagents were added in an Eppendorf tube to make up to a final volume of 20μl. The samples were kept for digestion for about 2 hours at the optimal reaction temperature of the used enzyme which is usually 37ºC. Later on, samples were analysed using 1% agarose gel electrophoresis in *TAE buffer* at 120 volts.

#### 2.2.5.1.16 Extraction and purification of DNA from agarose gel

The QIA Quick Gel Extraction Kit was used for purification of the DNA fragments obtained from the agarose gel. QIAquick Kit contains a silica membrane assembly for binding of DNA in high-salt buffer and elution with low-salt buffer or water. The purification procedure removes primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide, and other impurities from the DNA samples, the manufacturer protocol was followed.
2.2.5.2 Polymerase chain reaction (PCR)

PCR is a method of amplifying a specific nucleic acid target sequence present in a complex template to produce a large amount of specific DNA fragment of defined length and sequence in vitro (Saiki et al., 1988).

2.2.5.2.1 Basic PCR

The basic PCR reaction takes place in vitro where the primer strand is added in the form of deoxyoligonucleotide and Taq polymerase enzyme is added to helping polymerization. Unlimited supply of amplified DNA is obtained by repeating the reaction, which is made possible by regular denaturation of freshly synthesized double stranded DNA molecules by heating it to 90-98°C. At this high temperature the two strands separate. Taq polymerase is the DNA polymerase which is isolated from Thermus aquaticus growing in hot springs. The enzyme acts best at 72°C and the denaturation temperature of 90°C does not destroy its enzymatic activity.

In vitro amplification using polymerase chain reaction (PCR) was performed in a programmable thermal cycler. The PCR mix for reaction of 25μl contained the following reagents and make sure to prepare the master mix on ice.

<table>
<thead>
<tr>
<th>Reaction Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Template</td>
<td>1.0μl</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5μl</td>
</tr>
<tr>
<td>10X PCR Buffer</td>
<td>1.5μl</td>
</tr>
<tr>
<td>dNTPs (2.5 mM each)</td>
<td>0.3μl</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>1.5μl</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1.5μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>7.58μl</td>
</tr>
<tr>
<td>Taq Polymerase (5 units/μl)</td>
<td>0.12μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>15μl</td>
</tr>
<tr>
<td>Step</td>
<td>Temperature</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>denaturation</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing</td>
<td>55-68°C</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
</tr>
<tr>
<td>Cooling</td>
<td>4</td>
</tr>
</tbody>
</table>

2.2.5.2.2 Touchdown PCR

Touchdown Polymerase Chain Reaction is variation of standard PCR which avoids amplification of non-specific sequences by forward and reverse primers. The annealing temperature of the first amplification is adjusted higher than the calculated melting temperature (Don et al., 1991). The annealing temperature is lowered by 1-2°C for each succeeding cycle. At some point temperature accurate for specific priming will be reached and amplification of the sequence will begin (Hecker & Roux, 1996).

In touchdown PCR Extensor Hi Fidelity enzyme is used from AB gene instead of Taq DNA polymerase. The particular enzyme contains thermoprime plus DNA polymerase with a proofreading activity. Therefore, extensors can amplify DNA with four times higher fidelity and double yield than Taq polymerase enzyme.
2.2.5.2.3 Gradient PCR

Gradient PCR is mostly done to determine the optimum annealing temperature of the primer to the DNA. This type of PCR involves setting up a temperature gradient across the thermo block, starting at 5°C below the primer melting point (Tm) and continues increasing till it reaches 5°C above the Tm. With the help of this technique, in a single PCR reaction, the particular annealing temperature at which the primer amplifies the specific gene sequence can be found.

2.2.5.2.4 Reverse transcription PCR

Reverse transcription polymerase chain reaction (rt-PCR) is designed for the synthesis of cDNA from total RNA using the enzyme Superscript II TM Reverse Transcriptase (Invitrogen). This enzyme works by eliminating RNase H activity that degrades mRNA during first strand reaction and helps in obtaining full length cDNA. Conventionally RT-PCR involves two steps: the RT reaction and PCR amplification. Use of Oligo (dT) primers is a more specific method for
Materials & Methods

Priming first strand cDNA synthesis. In order to synthesize first strand cDNA for the experimental purposes the Invitrogen Superscript II TM Reverse Transcriptase kit was used.

All the reagents were centrifuged briefly before starting the reaction and all steps were performed on ice. RNA and the primers were mixed in sterile 0.5ml PCR tubes as the following:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1μg RNA</td>
<td>10.5μl (e.g. 5μl RNA + 5.5μl H₂O)</td>
</tr>
<tr>
<td>Oligo(dT) (0.5μg/μl)</td>
<td>1.0μl</td>
</tr>
<tr>
<td>Total</td>
<td>11.5μl</td>
</tr>
</tbody>
</table>

Reactions were then incubated at 70°C for 10 mins and the PCR master mix was prepared by adding the following components in the same order:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Buffer (10X)</td>
<td>2μl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>2μl</td>
</tr>
<tr>
<td>DTT (0.1M)</td>
<td>2μl</td>
</tr>
<tr>
<td>dNTP mix (10nM)</td>
<td>1μl</td>
</tr>
<tr>
<td>Superscript II TM Reverse Transcriptase enzyme</td>
<td>1μl</td>
</tr>
<tr>
<td>RNase OUT (RNase inhibitor)</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Total</td>
<td>8.5μl</td>
</tr>
</tbody>
</table>

The master mix was pre-warmed then added to the reaction tube and the reaction was incubated for 60 mins at 45°C. Reaction was then incubated at 70 °C for 10 mins and then put on ice. RNA was then digested out by adding 1 μl of RNase H
Materials & Methods

and incubation at 37°C for 30 min. The reverse transcription PCR product was stored at -20°C for short term and -80°C for long term storage.

2.2.5.2.5 Quantitative real time-PCR (qPCR)

qPCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production at each PCR cycle (in real time) as opposed to the endpoint detection. Real-time PCR was conducted by amplifying 1µl of cDNA with the SYBR Green master mix. SYBR Green I fluorescence is increased when it binds to double-stranded DNA. During the extension step, more and more SYBR Green I bind to the PCR product, resulting in large increase in the fluorescence. GAPDH (Glucose-6-phosphate dehydrogenase) is used as an endogenous control to normalize the amount of starting material in the tube. A cycling program usually consists of 35-45 cycles.

2.2.5.3 CRISPR/Cas based approach to target MASP-2 gene in C1qKO mice

2.2.5.3.1 Design of CRISPR gRNAs

All gRNA guides were designed using the Harvard University web tool ChopChop (Montague et al., 2014) and all guides were having no predicted off-target activity.

The deletion event was designed to excise a portion of exon 9, exon 10 and most of exon 11 of the MASP-2 gene, approximately 2.3 Kb in total, by Non-Homologous End Joining approach NHEJ. Schematic representation of the deletion event is shown in Figure 13. This resembles the deletion event used previously in the establishment of MASP2KO mice. The locations of used gRNAs are explained in the following schematic diagram:
Materials & Methods

Figure 13: A schematic diagram for the CRISPR mediated targeted deletion of the MASP-2\textsuperscript{KO} gene in C1q\textsuperscript{KO} mice. A: MASP-2 gene, exons 8 to 11. B: Targeted gene after deletion of part of exon 9, exon 10 and most of exon 11. Red arrows indicate the CRISPR/Cas9 guide RNA sites.

2.2.5.3.2 Synthesis of gRNAs by in vitro transcription
Synthesis of the gRNAs was performed using the GeneArt\textsuperscript{TM} Precision gRNA Synthesis Kit (Thermo Fisher, cat number A29377). In brief, guide RNA specific DNA templates were designed and supplied by the kit manufacturers. These oligonucleotides were used in a PCR reaction to be assembled with the CRISPR tracrRNA fragment and T7 promoter according to the kit protocol. The PCR product then used as the template for an in vitro transcription using the TranscriptAid\textsuperscript{TM} RNA polymerase enzyme to synthesis the gRNA. The final step is the purification of the gRNA which was performed using a silica membrane spin column RNA purification system provided with the kit.

2.2.5.3.3 Superovulation, mating and embryo harvesting
C1q\textsuperscript{KO} female mice were injected i.p. with 5IU of pregnant mare’s serum gonadotropin PMSG (MSD, cat number VM 01708/4309) followed by 5IU of human chorionic gonadotropin hCG (Intervet, cat number VM 01708/4301) 48 hours later. After hCG administration, females were introduced to C1q\textsuperscript{KO} stud male cage and the next morning copulatory plugs were checked and embryos harvested. Cumulous cell batches surrounding the embryos were digested with hyaluronidase (Sigma, cat number H3506-100MG) in M2 medium (Sigma, cat
numberM7167-50ML) and embryos were washed and stored in CO₂ equilibrated M16 medium (Sigma, cat numberM7292-50ML) until microinjected.

2.2.5.3.4 Microinjection
Cas9 mRNA (TriLink Biotechnologies, cat numberL-6125) (100 ng/µl) and four gene specific gRNAs (50 ng/µl each) in RNAse free PBS were injected into the pronuclear / cytoplasm of harvested embryos using a Eppendorf TransferMan® NK 2 micromanipulator fitted on a Nikon® eclipse microscope.

2.2.5.3.5 Embryo transfer to surrogate mother
Up to 15 injected embryos were surgically transferred to CD1 foster mothers mated with vasectomized males the same night of embryo transfer. For surgery, mice were anaesthetized with 2.5 % isoflurane in 1.5 L/min oxygen and the ovary and oviduct were exposed through a half centimetre incision in the mouse flank area. Using a stereo microscope, the ovarian bursa was torn and the oviductal infundibulum exposed. Embryos, loaded in a mouth controlled embryo transfer pipette, were transferred into the oviduct. The wound was then closed using 6/0 polyglactin absorbable sutures and mice allowed to recover. Buprenorphine (Vetergesic) (0.1 mg/kg) and Carprofen (Vetprofen) (5 mg/kg) analgesics were provided preoperative and mice kept warm in quiet room for full recovery and checked for pregnancy two weeks later.

2.2.5.3.6 Vasectomy
8 weeks old male CD1 mice were vasectomized to be used to generate embryo recipient foster mothers. The surgery comprises the exposure and cauterization of vasa deferentia of both testes through an incision in the midline of the mouse scrotal sac under general anaesthesia. Wound then closed using 6/0 polyglactin suture and mice allowed to recover. Buprenorphine (Vetergesic) (0.1 mg/kg) and Carprofen (Vetprofen) (5 mg/kg) analgesics were provided preoperative. Mice were kept warm in quiet room for full recovery and checked for sterility two weeks later.
3 The Role of the Complement System Activation in Protein Overload Proteinuria

3.1 Optimization of the protein overload proteinuria model in mice
Protein overload proteinuria is a well-established model of tubulointerstitial injury in rats, however the employment of this model in mice is less well characterised. This could be due to the variable response of different inbred mouse strains to the protein overload challenges (Ishola et al., 2006). Therefore prior optimisation of the experimental conditions that cause necessary levels of proteinuria and inflammation in the mouse kidney were required to study complement activation.

3.1.1 Dosing regimen, strain and unilateral nephrectomy
In order to determine the optimal model setup to study the complement activation during this injury, three major factors were optimised:

- Mouse strain
- Dosing regimen
- Necessity for unilateral nephrectomy

To validate these parameters, seven optimisation experiments were conducted as shown in Table 2 and Figure 14. In the first five experiments, the mice used were on C57BL/6 genetic background, while in the sixth and seventh experiments mice used were on BALB/c genetic background. C57BL/6 mice were chosen for the study because it is the most commonly used mouse strain for gene-targeted mice and hence most of available complement deficient knockout mouse models are on this background; however it was not known as a strain with a high degree of susceptibility to proteinuria induced inflammation. Both the lectin pathway deficient mice and the classical pathway deficient mice, i.e. MASP-2\(^{\text{KO}}\) and C1q\(^{\text{KO}}\) mice, were available on C57BL/6 background.
The first three experiments were conducted beginning with 8 doses of albumin given five days a week, increasing to 12 doses, with and without unilateral nephrectomy. The number of albumin doses given in the fourth experiment was increased to 20 administered five days a week without performing unilateral nephrectomy.

Table 2: Optimisation of protein overload proteinuria mouse model. Proteinuria is expressed as fold increases in protein to creatinine ratio (PCR) compared to controls. PCR was calculated after determination of urine total protein and creatinine both expressed in mg/ml. n=3 in all experiments.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Mouse strain</th>
<th>Unilateral nephrectomy</th>
<th>BSA doses</th>
<th>Exp. Duration (Days)</th>
<th>Mean proteinuria ±SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>C57BL/6</td>
<td>No</td>
<td>8</td>
<td>11</td>
<td>1.9±0.31</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>C57BL/6</td>
<td>No</td>
<td>12</td>
<td>17</td>
<td>2.2±0.44</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>C57BL/6</td>
<td>Yes</td>
<td>12</td>
<td>17</td>
<td>2.2±0.9</td>
</tr>
<tr>
<td>Exp. 4</td>
<td>C57BL/6</td>
<td>No</td>
<td>20</td>
<td>25</td>
<td>3.6±0.19</td>
</tr>
<tr>
<td>Exp. 5</td>
<td>C57BL/6</td>
<td>Yes</td>
<td>15</td>
<td>16</td>
<td>1±0.03</td>
</tr>
<tr>
<td>Exp. 6</td>
<td>BALB/c</td>
<td>Yes</td>
<td>15</td>
<td>16</td>
<td>6.2±0.37</td>
</tr>
<tr>
<td>Exp. 7</td>
<td>BALB/c</td>
<td>No</td>
<td>15</td>
<td>16</td>
<td>4.4±0.27</td>
</tr>
</tbody>
</table>

In the fifth experiment, 15 doses of albumin were given continuously seven days a week into C57BL/6 mice unilaterally nephrectomised. The total amount of proteins excreted in the urine in these five experiments did not go beyond a 3.6 fold increase compared to the relevant controls. Proteinuria was calculated as fold increases in protein to creatinine ratio (PCR) compared to controls. PCR was calculated after determination of urine total protein and creatinine both expressed in mg/ml. Table 2 shows proteinuria fold increases in all experiments.
Resistance of C57BL/6 mice to develop proteinuria suggested the use of other mouse strains. BALB/c mice were used in experiments 6 and 7 as one of the known mouse strains reported to be more susceptible to proteinuria (Wang et al., 2000; Chen et al., 1995). Also, the BALB/c strain was chosen because it is one of the few genetic backgrounds other than C57BL/6 on which the MASP-2\(^{\text{KO}}\) mouse line is available. However, the C1q\(^{\text{KO}}\) mouse model was available to me only on C57BL/6 genetic background but not BALB/c. Due to the strict regulations of the Specific Pathogen Free animal unite at the University of Leicester, I was not able to import the C1q\(^{\text{KO}}\) BALB/c mice, yet this model is already established and available at the laboratory of Professor Marina Botto, Imperial College London (Botto, 1998).

BALB/c mice in experiment 6 were unilaterally nephrectomised and given 15 doses of BSA while mice in experiment 7 were injected with 15 doses of BSA without nephrectomy. The dosing regime in both experiments required daily BSA injections. The mean values of the total amount of proteins excreted in the urine at the end of the experiments 6 and 7 revealed proteinuria fold increase of 6.2 and 4.4 respectively compared to the mean value of the corresponding control groups.
Figure 14: Fold increase in proteinuria from seven experiments conducted to optimise the protein overload proteinuria mouse model. The fold increase was calculated against the amount of proteins excreted in the sham control mice of each experiment. Experiments 1 to 5 were conducted in C57BL/6 mice while experiments 6 and 7 were in BALB/c mice.

From these pilot experiments, it was concluded that the use of the BALB/c mouse line overloaded continuously with 15 doses of BSA after unilateral nephrectomy produced the highest level of proteinuria and hence represents the best mouse model of protein overload proteinuria to study complement activation. However, due to the unavailability of C1q\textsuperscript{KO} mice on BALB/c genetic background, I was not able to study the role of the classical pathway of complement in this model of protein overload proteinuria. Further experimentation was conducted using MASP-2\textsuperscript{KO} mice to determine the role of the lectin pathway of complement in this model.
3.1.2 Proteinuria time-course profile in BALB/c mice over 24 hours post-BSA dose administration

Proteinuria 24 hours profile was studied in BALB/c mice after BSA injections. Urine samples were collected at seven time points from all mice in experiments 6 and 7 (Table 2). The time points were: 0, 1, 3, 6, 12, 18 and 24 hours after the administration of BSA dose 13.

Urine total protein in experiment 6 samples increased after the administration of the dose for three hours. The analysis of the six hours samples showed reduction in proteinuria followed by a further peak at 12 hours (Figure 15A). However, this pattern could not be recognized with non-nephrectomised mice in experiment 7 (Figure 15 B).

Protein/creatinine ratio (PCR) was calculated for all samples (Figure 16) from both experiments. The data showed a clear pattern of a peak increase in PCR after 12 hours from the dose administration in overloaded mice in both experiments. The mean value of urine total proteins fold increases measured at this time point in experiments 6 and 7 were 9.6 and 3.3 respectively. This highlights the unilateral nephrectomy to increase the protein creatinine ratio three folds at this time point more than non-nephrectomised mice.
Figure 15: Urine total protein concentration of BALB/c mice over 24 hours post-BSA injection. Urine samples were collected from mice of (A) experiment 6, nephrectomised, and (B) experiment 7, non-nephrectomised BALB/c mice at seven time points: 0, 1, 3, 6, 12, 18 and 24 hours after dose 13.
3.1.3 C3 expression in renal tissues after POP

To determine the activation of the complement system in the kidney tissues after POP, kidney sections from experiments 1 and 2 were stained using anti-C3 antibody (DAKO A-0062). Staining showed no deposition of C3 on the brush border of saline control mice, despite the C3 is generally expressed in the kidney cells (yellow asterisks). On the other hand, POP mice showed remarkable
deposition of C3 on the brush borders of distal and proximal tubules (red arrows) indicating complement activation in the tubular compartments of the kidney (Figure 17).

![Figure 17](image)

Figure 17: Light microscopy images for mouse kidney sections stained with C3 after POP. A) Negative control staining to confirm antibody specificity, B) Non-proteinuric control mouse, C) POP section from experiment 1 and D) POP treated mouse section from experiment 2. C3 depositions were stained on the inner lining of the tubules in C and D plates.

3.2 The use of transgenic MASP-2\(^{KO}\) mice to study the role of the lectin pathway of complement activation in protein overload proteinuria

Deficiency of the MASP-2 gene leads to the lack of functional activity of the lectin pathway of complement (Schwaebel et al., 2011). Utilization of this valuable transgenic mouse line allows the assessment of the complement
activation consequences in the absence of lectin pathway functional activity. In other words, it allows the determination of the role the LP plays in the development of the injury.

### 3.2.1 Experimental design

Proteinuria was induced in groups of 7 WT and MASP-2\(^{\text{KO}}\) mice on pure BALB/c background after unilateral nephrectomy as described before (Section 2.2.1.3). In addition, two groups of 4 mice, WT and MASP-2\(^{\text{KO}}\), were nephrectomised and given saline doses as controls.

#### 3.2.2 MASP-2\(^{\text{KO}}\) mice genotyping

Transgenic mice used in the study were genotyped to confirm homozygosity of the MASP-2 targeted defective allele prior to inclusion of every individual mouse in the experiment. This was performed by multiplex PCR using a set of primers specific for the mouse MASP-2 gene and designed to indicate the genotype through one of three outcomes:

a. Single 500 bp band indicates homozygous MASP-2\(^{\text{KO}}\) genotype

b. Single band at 750 bp indicates WT

c. Two bands at 500 and 750 bp indicate heterozygosity

Examples are shown in Figure 18. The genotype of all animals used in the study was confirmed prior to use.
3.2.3 Determination of serum total proteins in WT and MASP-2\(^{\text{KO}}\) mice

Overloading mice with BSA increased the serum total protein of both wild type and MASP-2\(^{\text{KO}}\) groups to more than double the levels of the sham control groups. Both WT and MASP-2\(^{\text{KO}}\) mice showed similar level of hyperproteinaemia with no significant differences between the groups (WT 60.7 ± 4.1 mg/ml vs. MASP-2\(^{\text{KO}}\) 64.7 ± 4.7 mg/ml) (Figure 19). Also, non-proteinuric (NP) sham control mice in both genotypes showed baseline total serum protein levels with no significant difference between groups (NP WT 26.5 ± 0.8 mg/ml vs. NP MASP-2\(^{\text{KO}}\) 26.3 ± 0.6 mg/ml).
Figure 19: Serum total protein of proteinuric and non-proteinuric WT and MASP-2KO animals. Proteinuric mice from WT and MASP-2KO groups did not show significant difference when compared to each other. Control groups also showed no significant difference when compared to each other.

3.2.4 Determination of proteinuria following BSA overload

To evaluate the extent of proteinuria, urine samples were collected over 24 hours at the end of the experiment from all groups using metabolic cages. Samples showed approximately seven-fold increase of total excreted proteins in the treated groups compared to the sham NP mice. No statistically significant differences were observed between treated groups (WT 126.4 ± 13.9 mg vs. MASP-2KO 130.3 ± 20.7 mg) or between control groups (WT 16.2 ± 5.3 mg vs. MASP-2KO 16.5 ± 2.4 mg) as represented in Figure 20.
3.2.5 Macroscopic appearance of dissected mice

Dissection of mice at the end of the experiment showed some distinct features. Overall, the abdominal cavity in BSA injected animals revealed some yellowish colouration of the connective tissues compared to sham groups (Figure 21B). The macroscopic appearance of treated kidneys showed pale cortex (Figure 21D) and overall pale colouration compared to sham control kidneys (Figure 21C).
3.2.6 Histopathological examination of WT and MASP-2KO kidneys after BSA challenge

Kidney sections from all groups were stained with haematoxylin and eosin and examined to assess the impact of protein overload on renal histology. While control kidneys had a normal healthy appearance, treated kidneys showed changes of acute tubular injury. These changes included patchy tubular dilation, blebbing of proximal tubular cell cytoplasm, vacuolation of tubular epithelial cells, patchy interstitial inflammation and/or peritubular capillary congestion with leukocytes. Also, accumulation of albumin casts in the lumen of several tubules as well as in glomerular space was observed. These features were observed in all BSA injected mice but subjectively appeared less in the MASP-2KO mice (Figure 22).
3.2.7 **Kidney ultrastructure by electron microscopy**

To further investigate the renal injury observed by light microscopy, sections were examined by transmission electron microscopy (Figure 23). In all treated mice, vacuoles of proteinaceous materials are observed within the endothelial space of capillaries as well as reabsorption vacuoles in the cytoplasm of several tubular cells. However, tubular structures of wild type specimens (Figure 23A), showed more prominent degradation of tubular cells brush border cilia and abundant cells bursting into the tubular lumen compared to MASP-2\(^{\text{KO}}\) specimens, Plate B. In the glomeruli, both WT and MASP-2\(^{\text{KO}}\) showed similar appearance of podocyte foot processes (red arrows in Plates C and D).
3.2.8 Macrophage infiltration

F4/80 is an antigen expressed on the surface of macrophages, including those infiltrating the kidney. Computer based analysis of kidney sections from all groups stained with F4/80 antibody showed a notable increase in macrophage infiltration in BSA treated groups compared to saline control groups (Figure 24). This finding is indicative of tubulointerstitial inflammation as a result of protein overload and proteinuria. Statistical analysis using one-way ANOVA showed a highly significant difference between groups (****P<0.0001). Interestingly,
comparison using Bonferroni’s post hoc analysis between protein overloaded WT and MASP-2\(^{\text{KO}}\) kidneys showed a significant reduction in macrophage infiltration in MASP-2\(^{\text{KO}}\) mice (MASP-2\(^{\text{KO}}\) 2.29 ± 0.23 % vs. WT 3.4 ± 0.44 %), (Figure 25).

Figure 24: Representative images of WT and MASP-2\(^{\text{KO}}\) kidney sections stained with F4/80 macrophage antibody. Sections were not counter-stained in order to permit computer based image analysis. (A) Non-proteinuric wild type control; (B) Non-proteinuric MASP-2\(^{\text{KO}}\) control; (C) Proteinuric wild type; and (D) Proteinuric MASP-2\(^{\text{KO}}\).
Figure 25: Image analysis quantitation of macrophage stained area in WT and MASP-2\(^{\text{KO}}\) mouse kidneys. Proteinuric MASP-2\(^{\text{KO}}\) mice showed a significant reduction in the stained area compared to proteinuric wild type mice (*P=0.013).

3.2.9 Expression of transforming growth factor-\(\beta\) (TGF-\(\beta\)) in the mouse kidney

TGF-\(\beta\) is a member of a group of cell signalling proteins, cytokines, released in the tissue to moderate many cellular functions including the control of the cell growth and differentiation as well as elimination of aberrant cells by apoptosis. TGF-\(\beta\) also plays a role in glomerular and tubulointerstitial injuries in chronic kidney diseases contributing to fibrogenesis and epithelial-to-mesenchymal transdifferentiation (EMT) (Bottinger & Bitzer, 2002).

Staining of protein overloaded kidney sections with anti-TGF-\(\beta\) antibody showed an increase in the stained area compared to sham control groups (Figure 26). Computer based image analysis of the sections revealed a significant increase in TGF-\(\beta\) staining in wild type mice compared to MASP-2\(^{\text{KO}}\) mice (WT 28.2 ± 2.6 % vs. MASP-2\(^{\text{KO}}\) 20.8 ± 2.1 %) while sham control groups showed a baseline staining pattern with no significant difference between groups (WT 5.6 ± 0.9 % vs. MASP-2\(^{\text{KO}}\) 7.5 ± 1.3 %). Analysis data are represented in Figure 27, ANOVA
analysis showed a highly significant difference between treated groups (****P=0.0001).

Figure 26: Representative images of WT and MASP-2<sup>KO</sup> kidney sections stained with transforming growth factor beta antibody. Sections were not counter-stained to facilitate the computer based image analysis. (A) Non-proteinuric wild type control, (B) Non-proteinuric MASP-2<sup>KO</sup> control, (C) Proteinuric wild type and (D) Proteinuric MASP-2<sup>KO</sup>.
3.2.10 Determination of tumour necrosis factor α (TNF-α) expression levels in MASP-2KO mouse kidneys after BSA challenge

TNF-α is a potent pro-inflammatory cytokine produced mainly from activated macrophages to augment the acute phase inflammatory reaction. TNF-α is also produced by many other cell types in inflamed tissues and hence is considered an important mediator of inflammatory tissue damage. It regulates other immune cells functions to induce inflammation, inhibit tumour genesis and initiate apoptotic cell death (Locksley et al., 2001). The role the TNF-α plays in mediating renal inflammation is highlighted in many experimental studies, and some suggest a therapeutic effect of TNF-α blockades (Vielhauer & Mayadas, 2007).

To determine the amount of renal TNF-α in proteinuric mice, paraffin embedded kidney sections were stained with anti-TNF-α antibody (Figure 28). Analysis of the sections from BSA overloaded mice showed elevated levels of TNF-α
expression in both WT and MASP-2KO mice with significant increase in WT compared to MASP-2KO mice (WT 14 ± 1.5 % vs. MASP-2KO 9.6 ± 1.3 %) (Figure 29). Control mice showed matching baseline staining patterns with no significant difference between groups (WT 3.3 ± 0.4 % vs. MASP-2KO 3.5 ± 0.2 %). Analysis with one-way ANOVA showed a highly significant difference between treated groups (****P<0.0001). Bonferroni’s post hoc correction analysis showed a significant difference between proteinuric WT and proteinuric MASP-2KO groups.

Figure 28: Representative images of WT and MASP-2KO mouse kidney sections stained with anti-TNF-α antibody. (A) Non-proteinuric wild type control; (B) Non-proteinuric MASP-2KO control; (C) Proteinuric wild type; and (D) Proteinuric MASP-2KO.
Figure 29: Image analysis quantitation of WT and MASP-2\textsuperscript{KO} kidney sections stained for TNF-\(\alpha\). Proteinuric MASP-2\textsuperscript{KO} mice showed significantly reduced staining area compared to proteinuric WT mice (*P=0.014).

3.2.11 Expression levels of interleukin 6 in WT and MASP-2\textsuperscript{KO} mouse kidneys after BSA challenge

Similar to TGF-\(\beta\) and TNF-\(\alpha\), interleukin 6 (IL-6) is a cytokine involved in acute phase inflammatory response and mediates the activation and proliferation of lymphocytes, differentiation of B cells and leukocyte recruitment. IL-6 is secreted from activated monocytes, macrophages, fibroblasts, adipocytes and endothelial cells in response to various stimuli, such as TNF-\(\alpha\), bacterial endotoxins, and oxidative stress. IL-6 is demonstrated at high concentrations to promote the progression of renal injury; while at lower levels may be involved in regulating repair mechanisms (Kayama \textit{et al}., 1997). Also, it has been reported that IL-6 is released in the kidney as a consequence of the activation of the complement system (David \textit{et al}., 1997).

Kidney sections stained with anti-IL-6 specific antibody showed an increased and intense IL-6 staining in BSA overloaded WT kidneys but not MASP-2\textsuperscript{KO}
Role of Complement in POP
kidneys (Figure 30). Quantitative analysis showed an increase in proteinuric WT mice compared to MASP-2\textsuperscript{KO} mice (WT 31.2 ± 2.1 % vs. MASP-2\textsuperscript{KO} 18.1 ± 2.5 %) and no significant difference between sham control groups (WT 18.5 ± 1.6 % vs. MASP-2\textsuperscript{KO} 17.3 ± 1.7 %) (Figure 31). Analysis using one-way ANOVA showed a highly significant difference between groups (***P=0.0004). Bonferroni’s correction test showed a highly significant difference between proteinuric WT and proteinuric MASP-2\textsuperscript{KO} groups (***P=0.0002).

Figure 30: IL-6 stained mouse kidney sections from WT and MASP-2\textsuperscript{KO} mice. (A) Non-proteinuric wild type control, (B) Non-proteinuric MASP-2\textsuperscript{KO} control, (C) Proteinuric wild type and (D) Proteinuric MASP-2\textsuperscript{KO}.
Figure 31: Image analysis quantitation of WT and MASP-2\textsuperscript{KO} kidney sections stained for IL-6. Proteinuric wild type mice showed a significantly increased staining area compared to sham control groups and proteinuric MASP-2\textsuperscript{KO} mice. A highly significant statistical difference was observed between proteinuric WT and MASP-2\textsuperscript{KO} mice in this analysis (**P=0.0002).

3.2.12 Determination of apoptosis in WT and MASP-2\textsuperscript{KO} mice kidneys

Apoptosis is a process of programmed cell death. Apoptotic cells are characterised by cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. Apoptosis of renal tubular epithelial cells is a prominent contributor to renal injury (Thomas et al., 1999). Moreover, presence of apoptotic cells in the tissue during the development of renal injury promotes inflammation to recruit phagocytosis of cell debris, which in turn can exacerbate renal injury and scarring (Savill, 1994).

TUNEL assay is a common method in detecting DNA fragmentation in apoptotic cells by labelling the terminal end of the broken DNA. TUNEL stained kidney sections (Figure 32) were examined by light microscopy and apoptotic cells in 20 adjacent cortical high power fields were counted. While sham control slides showed similar minimal presence of apoptotic cells (WT 7.5 ± 1 vs. MASP-2\textsuperscript{KO}...
8.3 ± 1.1), the degree of apoptosis in WT overloaded kidneys is greatly increased compared to that observed in MASP-2\textsuperscript{KO} mice (WT 45.9 ± 3.5 vs. MASP-2\textsuperscript{KO} 22.1 ± 2.5), (Figure 33). ANOVA analysis showed a highly significant difference between all groups (****P<0.0001). When Bonferroni’s correction test was applied between proteinuric WT and MASP-2KO mice, it showed a highly significant difference.

Figure 32: TUNEL stained mouse kidney sections for the detection of apoptosis in WT and MASP-2\textsuperscript{KO} mice. Populations of apoptotic cells were observed in both proteinuric mouse groups; however MASP-2\textsuperscript{KO} mice exhibited distinct lower numbers of cells compared to WT mice. (A) and (B) represent proteinuric WT mice and (C) and (D) represent proteinuric MASP-2\textsuperscript{KO} mice.
Figure 33: Numbers of apoptotic cells counted manually in 20 adjacent high power fields (HPFs) of the kidney cortex. Highly significant decrease in apoptotic cells was observed in MASP-2<sup>-KO</sup> mice compared to WT mice (****P<0.0001).

### 3.2.13 Determination of selected gene expression profiles in WT and MASP-2<sup>-KO</sup> mouse kidneys

To examine the extent of renal injury in different groups at the gene level, the expression profiles of seven genes relevant to complement activation, renal inflammation and fibrosis were examined in a qPCR study. The genes are: complement component C3, complement factor properdin, collagen type IV, interferon γ, interleukin 6, transforming growth factor β and tumour necrosis factor α. Along with these genes, the expression levels of three housekeeping genes were also studied for quantification of the examined marker genes. These control genes were glyceraldehyde 3-phosphate dehydrogenase (GAPDH), TATA-binding protein (TBP) and 18s ribosomal RNA (18s).

Compared to sham control groups in WT and MASP-2<sup>-KO</sup> mice, BSA overloaded kidneys from WT and MASP-2<sup>-KO</sup> groups demonstrated significantly increased expression of all studied genes (Figure 34). In contrast to BSA overloaded
kidneys from WT mice, overloaded kidneys from the MASP-2^{KO} group showed reduced expression of the renal fibrosis linked gene, collagen type IV (WT 0.35 ± 0.06 Log10 RQ vs. MASP-2^{KO} 0.14 ± 0.06 Log10 RQ), ANOVA analysis with Bonferroni’s correction showed significant difference between these two groups. Quantitation showed that the mean values of the expression levels of the rest of studied genes in MASP-2^{KO} group were lower than that of the wild type group; however there were no significant differences between the control non-proteinuric groups. When all treated groups were analysed with ANOVA, it showed highly significant differences between groups in all studied genes.
Figure 34: qPCR analysis of expression levels of seven complement and renal inflammation related genes in non-proteinuric controls and protein overloaded kidneys of WT and MASP-2\textsuperscript{KO} mice. Proteinuric groups demonstrated upregulated expression
levels of all studied genes compared to control groups. MASP-2\textsubscript{KO} group overloaded with BSA exhibited lower mean values of expression of all genes when compared to WT mice at the same level of BSA challenge. The renal fibrosis related gene collagen type IV showed a statistically significant down regulation in proteinuric MASP-2\textsubscript{KO} mice compared to WT proteinuric group (*P=0.013).

### 3.3 Antibody mediated inhibition of the lectin pathway in a mouse model of protein overload proteinuria

The degree of protection observed in proteinuric MASP-2 knockout mice compared to wild type mice supported the need to investigate the effects of potential therapeutic MASP-2 inhibition using anti-MASP-2 antibodies in wild type mice.

#### 3.3.1 Experimental design

In this experiment, three groups of WT BALB/c mice, n=8, have been set with the protein overload model as described before (Section 2.2.1.3). One group of these mice was treated with 721-SGMI-H2 anti-MASP-2 antibody while the other two groups were controls. The antibody was administered by i.p. injections twice a week at a dose of 10 mg/kg starting one week prior to protein overload and was continued until the end of the experiment. Control groups were injected the same way with either balanced saline solution or the antibody isotype control.

#### 3.3.2 Determination of proteinuria induced in protein-overload mice treated with anti-MASP-2 antibody

Estimation of total urinary excreted protein of albumin injected mice at the end of the experiment showed an almost seven fold increase in proteinuria compared to wild type control mice not challenged by albumin injections. This observation is in line with the data observed when comparing albumin treated and non-treated MASP-2 deficient mice. All proteinuric groups showed similar proteinuria and no statistically significant differences were observed between groups using one-way ANOVA statistical analysis. Importantly, anti-MASP-2 treated animals had similar proteinuria to non-antibody treated proteinuric groups (Figure 35).
3.3.3 Measurement of lectin pathway inhibition after antibody treatment

To validate the 721-SGM1-H2 antibody mediated inhibition of the lectin pathway, serum samples from all mice were examined using either a flow cytometry based assay or enzyme linked immunosorbent assay (ELISA) for titration of lectin pathway -mediated C3c deposition.

3.3.3.1 Flow cytometry based determination of the inhibition of the lectin pathway in serum of antibody treated mice

To assess the pharmacodynamics effects of 721-SGM1-H2 mAb administration on the inhibition of the lectin pathway in mice, systemic LP activity was evaluated by quantifying lectin-induced C3and C4 deposition in minimally diluted serum samples. Samples from all mice were analysed by immunosorbent assay on beads sorted by flow cytometry in two formats, with and without the addition of the antibody to the assay, i.e. unspiked and spiked samples respectively. Mean fluorescent intensity (MFI) measurements of samples from all
BSA overloaded groups showed anomalous data pattern either for C3 deposition assay (Figure 36) or C4 deposition (Figure 37). The data were considered anomalous for two reasons. Firstly, deposition levels of either C3 or C4 did not show homogeneity in any of the groups; and secondly none of the spiked samples showed any distinguishable effect on the amount of deposition as expected in antibody spiked samples. The distortion in the test could occur due to interference between the high protein content of the sera and the test reagents. This inconclusive data suggested the use of diluted serum samples in enzyme linked immunosorbent assay (ELISA) to assess the extent of the lectin pathway inhibition.

Figure 36: Flow cytometry examination of the deposition of lectin pathway based active C3 in minimally diluted serum samples. Samples from all experimental groups were examined unspiked and spiked with 600 nM of 721-SGMI-H2 antibody. Similar mean fluorescent intensity (MFI) readings were collected from unspiked and spiked samples of the same mouse in any group, suggesting invalid test conditions.
3.3.3.2 Determination of the lectin pathway inhibition using ELISA

ELISA was performed using serum samples diluted to 2.5%, thus reducing the possibility of reagent interference with the high protein content in the serum. The assay was performed twice using skimmed milk and BSA separately as blocking media to examine the possibility of the formation of anti-BSA antibody in the serum of overloaded mice.

Whereas both saline and isotype control groups showed robust C3c deposition levels in both assays, strong inhibition of C3c deposition in 721-SGMI-H2 group was observed (Figure 38 and 39), suggesting a powerful inhibition of lectin pathway functional activity following treatment with the 721-SGMI-H2 antibody. The reproducibility of this data in BSA and skimmed milk based blocking buffers suggests C3 activation in all groups is not due to the presence of anti-BSA-antibodies in the sera of BSA overloaded mice.
Figure 38: Determination of inhibition of the lectin pathway after anti-MASP-2 antibody treatment using non-BSA based blocking buffer (skimmed milk). Whereas similar activation levels were observed in both control groups, the 721-SGMI-H2 treated group showed robust inhibition of the lectin pathway dependant C3.

Figure 39: ELISA determination of lectin pathway dependant C3 deposition using BSA based blocking buffer. The LP inhibition levels of the 721-SGMI-H2 treated sera were similar to that observed in non-BSA blocking conditions, suggesting no antigen-antibody C3 activation occurred due to the presence of large amounts of albumin in the serum.
3.3.4 Histological examination of proteinuric mouse kidney sections after inhibition of the lectin pathway

Similar to the data presented before in the knockout experiment, H&E stained sections from all groups showed several features of acute tubular injury. The changes included tubular dilation, blebbing of proximal tubular cell cytoplasm, vacuolization of tubular epithelial cells and patchy interstitial inflammation and peritubular capillary congestion with leukocytes. Although only assessed qualitatively, these tissue injury features are similar in the saline and isotype control groups and to a lesser extent in the antibody treated group (Figure 40). Slides from all treated kidneys were also assessed in a blinded study by a third party pathologist (Prof. Chester Alper, Washington University, Seattle USA) who reached the same conclusion.

![Figure 40: H&E stained kidney sections from proteinuric mice with and without inhibition of the lectin pathway. (A) Non-proteinuric wild type control; (B) proteinuric saline control; (C) proteinuric isotype control; (D) 721-SGMI-H2 antibody group.](image-url)
3.3.5 Macrophage infiltration in the lectin pathway inhibited mouse kidneys

Kidney sections from all groups were stained with F4/80 antibody to evaluate the degree of tissue inflammation as indicated by macrophage infiltration. Representative images are shown in Figure 41. Assessment of these tissue sections by image analysis did not show significant differences between groups using one-way ANOVA test, Figure 42. This evaluation has been repeated to examine two other levels of the cortex, capsular and juxtamedullary zones, yet no significant differences have been observed between all groups (Figure 43).

Figure 41: Macrophage infiltration in mouse kidney sections from all experimental groups, (A) Proteinuric saline control, (B) Proteinuric isotype control and (C) 721-SGMI-H2 antibody group.
Figure 42: Computer based image analysis quantitation of F4/80 stained macrophages in mouse kidney sections. No significant differences between groups were observed after the evaluation of the middle zone of the cortex.

Figure 43: Quantitation of the macrophage infiltration in the juxtamedullary and capsular zones of the mouse kidney cortex. Despite the analysis showed variation in the infiltration rates between different zones, no significant differences were observed between treated groups.
3.3.6 TGF-β expression profile in mouse kidneys after inhibition of the lectin pathway

TGF-β stained sections showed a measured reduction in the stained area in the antibody treated group compared to isotype control group (721-SGMI-H2 20.6 ± 1.6 % vs. isotype control 25.6 ± 1.3 %). Figure 44 shows representative images and Figure 45 shows the formal quantification. One-way ANOVA test showed significant difference between treated groups (*P=0.0311). Bonferroni’s multiple comparison correction method showed significant difference between Isotype control group and the 721-SGMI-H2 treated groups (*P=0.022).

![Representative images of kidney sections stained with transforming growth factor β. (A) Proteinuric saline control, (B) Proteinuric isotype control and (C) 721-SGMI-H2 antibody group.](image-url)
Figure 45: Quantification of TGF-β stained areas showed a significant reduction in the expression levels in the 721-SGMI-H2 antibody group compared to the isotype control group. Evaluation of the data using one-way ANOVA test showed significant difference between groups (*P=0.0311). Bonferroni’s correction showed a significant difference between isotype control group and the antibody treated group (*P=0.022).

3.3.7 Assessment of the degree of expression of TNF-α in MASP-2 inhibited mouse kidneys

Unlike other examined inflammation markers, quantification of TNF-α in stained kidney sections (Figure 46) did not show significant difference between treated groups. Despite 721-SGMI-H2 group showed a noticeably reduced percentage of stained area in comparison to the isotype control groups (721-SGMI-H2 14.4 ± 0.6 % vs. isotype control 16.1 ± 0.9 %) no statistical significant difference has been determined using one-way ANOVA test (Figure 47).
Figure 46: Representative images of kidney sections stained with TNF-α antibody, (A) Proteinuric saline control, (B) Proteinuric isotype control and (C) 721-SGMI-H2 antibody group.

Figure 47: Quantitative analysis of mouse kidney sections stained with anti-TNF-α antibody. Statistical analysis using one-way ANOVA showed no significant difference between groups.
3.3.8 Assessment of IL-6 expression after administration of anti-MASP-2 antibody

Kidney sections from all mice were stained with anti-IL-6 antibody and the percentage of the stained area was determined using image analysis software. 721-SGMI-H2 treated group exhibited reduced staining areas compared to saline and isotype control groups as could be seen in the representative images in Figure 48 and in the image analysis values represented in Figure 49. However, image analysis quantitation using one-way ANOVA showed no significant difference between the groups.

Figure 48: Representative images of kidney sections stained with interleukin-6. (A) Proteinuric saline control; (B) Proteinuric isotype control and (C) 721-SGMI-H2 antibody group.
Figure 49: Quantitation of IL-6 stained sections using one-way ANOVA showed no significant difference between groups.

### 3.3.9 Determination of apoptosis in lectin pathway inhibited mouse kidneys

Examination of frequency of apoptotic cells in kidney sections stained with TUNEL showed a highly significant difference between groups. Treatment with the 721-SGMI-H2 antibody reduced the apoptotic cells per 20 microscopic high power fields (721-SGMI-H2 41.6 ± 4.4 vs. isotype control 71.8 ± 3.8). Figure 50 shows representative images of the stained sections. Apoptotic cells in the sections are marked with red arrows. Analysis of the data is demonstrated in Figure 51. Bonferroni’s post hoc analysis also showed a highly significant difference between 721-SGMI-H2 and isotype control groups with corrected P value = 0.001**.
Role of Complement in POP

Figure 50: Representative images of apoptotic cells, red arrows, stained in kidney sections from all treated groups. The frequency of the apoptotic cells in the antibody treated group (C) was significantly lower than the saline (A) and isotype (B) control groups.

Figure 51: Statistical evaluation of apoptotic cells stained by TUNEL with and without antibody-mediated inhibition of the lectin pathway. Determination of the apoptotic cells numbers showed a highly significant decrease in the antibody treated mice compared to
the isotype control group (\(**P=0.001\)) using Bonferroni’s multiple comparison correction method. ANOVA analysis showed a highly significant difference between groups (\(**P=0.0013\)).

3.3.10 Expression profiles of inflammation marker genes in the mouse kidney after inhibition of the lectin pathway

The expression profiles of the group of genes described before in the knockout experiment have been studied in the kidneys of 721-SGMI-H2 treated mice in comparison to saline and isotype control groups. These genes are: complement component C3, complement factor properdin, collagen type IV, interferon γ, interleukin 6, transforming growth factor β and tumour necrosis factor α as well as GAPDH, TBP and 18s housekeeping genes.

Similar to what have been observed in the knockout experiment, BSA overloaded kidneys of all groups demonstrated upregulated expression levels of all studied genes when compared to non-proteinuric control mice (Figure 52). In comparison to proteinuric saline control mouse, the 721-SGMI-H2 antibody treated kidneys showed a tendency of downregulated gene expression in all studied genes as indicated by lower mean values of groups. However, statistical analysis showed no significant difference between the 721-SGMI-H2 treated mice and the isotype control groups in any of the examined genes. A highly significant difference between groups in the renal inflammation related gene TNF-α has been determined (saline control 2.51 ± 0.18 Log10 RQ vs. isotype control 1.86 ± 0.11 Log10 vs. 721-SGMI-H2 1.98 ± 0.1 Log10 RQ), however application of Bonferroni’s multiple comparisons test showed no significant difference between 721-SGMI-H2 vs. isotype control. Unexpectedly, the isotype saline control group demonstrated no significant difference with the 721-SGMI-H2 antibody treated mice in any of the studied genes.
Figure 52: qPCR analysis of expression levels of seven genes in WT mouse kidneys with and without inhibition of the lectin pathway. All proteinuric mouse groups showed
upregulated expression levels of all genes compared to non-proteinuric WT control animals. ANOVA statistical analysis showed no significant differences between groups in all studied genes except for TNF-\(\alpha\) (\(\text{**P}=0.005\)). However, in TNF-\(\alpha\) Bonferroni’s multiple comparisons test showed no significant difference between 721-SGMI-H2 vs. isotype control.
3.4 Discussion

The complement system plays a fundamental role in the development of inflammatory diseases including chronic kidney disease. Activation of complement is mediated via three distinctive and interdependent pathways; the classical pathway (CP), the lectin pathway (LP) and the alternative pathway (AP). Each of these activation pathways initiates upon encountering certain pathway-specific activation conditions and all lead to the formation of C3 and C5 convertase complexes, that catalyse the enzymatic cleavage of the most abundant serum component of complement, i.e. C3 and the terminal cleavage substrate C5. The enzymatic cascades of activation of each pathway reactions involve the conversion of different pathway specific complement zymogens into their active form, assembly of proteolytic complexes. After the cleavage of C5, all enzymatic activation steps are completed and all three pathways channel into the terminal activation cascade which is initiated by the major C5 cleavage product C5b that forms complexes through intermolecular binding reactions with the terminal cascade components C6, C7, C8 and C9 in the order of their given numbers, i.e. C5b binds to C6, C5bC6 binds C7, the C5bC6C7 complex binds C8 and C8 fixes this C5b,C6,C7,C8 complex to cellular membranes and initiates the formation of poly C9 that can integrate into the membrane forming a membrane penetrating cylindrical pore called the membrane attack complex since it can induce osmolysis of non-nucleated cells, such as erythrocytes. Complement activation is strictly regulated through a number of fluid and solid phase regulators to prevent auto-reactive damage of host cells.

In the kidney, the involvement of local complement activation in the pathophysiology of chronic kidney disease is well established (Morita et al., 2000; Gaarkeuken et al., 2008; Zaferani et al., 2012; Turnberg et al., 2006a). Several authors highlighted the role of complement activation via the AP (David et al., 1997; Abe et al., 2004) and the CP (Meszaros et al., 2010; Valenzuela et al., 2014) in the augmentation of the renal injury in experimental models of disease. However, the contribution of the LP in the development of renal disease
is presently less understood. This is likely to be due to the relatively recent discovery of this activation mechanisms. This also could be due to the only most recent availability of models of LP deficiency that allow to study of the LP role in the development of renal disease.

This study aimed to evaluate the role of the LP of complement activation in an experimental model of POP in mice using the only available murine model with complete lectin pathway deficiency; a mouse strain with a targeted deletion of the lectin pathway key serine protease MASP-2 (Schwaeble et al., 2011). In addition, this study also aimed to investigate the role of the CP activation in POP using a mouse line with complete deficiency of the CP key enzyme C1q (Botto et al., 1998).

Optimization experiments conducted to establish the model of POP in mice showed that C57BL/6 mice were resistant to develop proteinuria. In comparison, BALB/c mice developed approximately six fold increase in proteinuria more than C57BL/6 mice at the same level of treatment. This suggested the use of BALB/c mice in this study. However, due to the resistance of C57BL/6 mice to develop proteinuria and due to the unavailability of C1qKO mice on BALB/c genetic background, investigation of the role of the CP pathway in this model of POP could not be performed.

The results of the present work indicate that the LP contributes significantly to the development of renal tubulointerstitial injury in POP in mice. Importantly, these results suggest that antibody mediated inhibition of the LP showed some evidence of reduced severity of tubulointerstitial injury in this model. The murine POP model used is known to produce kidney injury reminiscent of the early stages of human proteinuric kidney disease (Abbate et al., 2006; Weening et al., 1987), thus my findings provide insight into the therapeutic utility of MASP-2 inhibition to achieve reduction of renal injury and halt disease progression in human proteinuric chronic kidney disease.
The current study showed that BALB/c mice are more susceptible to proteinuria than C57BL/6 mice at the same levels of protein overloading. BALB/c mice overloaded with 15 doses of BSA produced a mean of approximately six fold increase the proteinuria produced from C57BL/6 mice given the same dosing regimen. This is in agreement with the findings of Ishola et al., 2006 who concluded that C57BL/6 mice are more resistant to proteinuria after POP injury.

The results also show that unilateral nephrectomy increased the levels of proteinuria by around 30% compared to non-nephrectomised mice. Nephrectomised mice also showed higher urinary protein excretion at midday checkpoint in 24 hrs protein excretion profile compared to non-nephrectomised mice. Similar observations in this model have been previously reported (Abbate et al., 2008; Benigni et al., 2004; Eddy et al., 2000; Ma & Fogo, 2003).

Unlike previous authors who injected the BSA doses at 10 mg/gm body weight (Benigni et al., 2004; Eddy et al., 2000), I found that the mice tolerated the doses at 15 mg/gm body weight with no apparent complications like pulmonary oedema that was reported by Eddy et al., 2000 after giving daily BSA injections for up to 24 days. This could be due to the relative short duration of my experiment, 15 days, compared to other models extending up to six weeks. However, some authors, such as Abbate et al., 2008 also reported that mice were injected with doses as high as 15 mg/gm body weight BSA in their studies.

In MASP-2\textsuperscript{KO} mice, establishment of the POP injury showed similar degrees of proteinuria and hyper-proteinaemia compared to WT mice with no significant differences between the groups. Proteinuric mice showed almost two fold increases in the serum protein levels compared to their control littermates, and an almost seven fold increase in proteinuria levels. This suggests that differences in renal injury are not explained by reduced proteinuria in complement deficient animals.

H&E stained kidney sections from both WT and MASP-2\textsuperscript{KO} experimental mouse groups revealed distinct histopathological changes after POP injury. Despite
MASP-2\textsuperscript{KO} mice showing some evidence of renal tissue injury a qualitative evaluation suggested that the extent of the injury in MASP-2\textsuperscript{KO} mice was to slightly less than in WT mice. However, evaluation of H&E section by light microscopy is not necessarily the best method to quantify the degree of injury. Examination of the kidney ultrastructure by transmission EM revealed features of renal injury in both WT and MASP-2\textsuperscript{KO} mice and suggested that the injury is more prominent in WT mice compared to MASP-2\textsuperscript{KO}. These observations were validated further by other quantifiable markers described hereunder.

Renal inflammation is a key factor of developing kidney disease and remains a key target for renal therapeutics. To estimate the degree of the kidney inflammation in the lectin pathway deficient MASP-2\textsuperscript{KO} mice and to compare this phenotype to that of WT control mice, sections from each experimental group were stained with the mouse macrophage specific marker F4/80. Several studies used this specific marker to validate the degree of renal tissue inflammation in POP studies (Abbate \textit{et al.}, 2008; Donadelli \textit{et al.}, 2003; Sanchez-Nino \textit{et al.}, 2013). While both POP groups showed a significant increase in macrophage net influx compared to their relevant saline control groups, analysis of the kidney sections from the proteinuric lectin pathway deficient mouse group MASP-2\textsuperscript{KO} showed significantly lower infiltration of F4/80 positive cells compared to similarly proteinuric WT mice. This suggests that the activation cascade of the LP in the kidney could mediate some pro-inflammatory mechanisms and hence, lack of the LP functional activity results in reduced renal inflammation.

These findings are similar to the results obtained by Abbate \textit{et al.}, 2008 and Tang \textit{et al.}, 2009 who reported that lack of complement activation significantly reduced the infiltration of the F4/80 specific macrophages in renal tissues in proteinuric nephropathies. In these two studies, authors investigated the lack of total complement functional activity, not just the lectin pathway. These studies were carried out using a mouse model of C3 deficiency which results in the loss of all the complement activation pathways. The availability of a MASP-2\textsuperscript{KO}
mouse model allowed to dissect the complexity of the complement activation pathways since my study specifically targets the LP only while the other two activation pathways remain operational.

Expression of TGF-β was also compared between MASP-2\(^{K0}\) and WT mice. The expression levels estimated in the MASP-2\(^{K0}\) mice were significantly lower than that of the WT mice. The reduced TGF-β expression levels in MASP-2\(^{K0}\) mice kidneys suggests a potent role for LP in the development of the tissue scarring and fibrosis since TGF-β plays an essential role in renal fibrogenesis, epithelial-to-mesenchymal trans-differentiation and in apoptosis clearance (Bottinger & Bitzer, 2002). Several \textit{in vivo} and \textit{in vitro} studies showed that kidney cells overloaded with protein upregulated the expression of TGF-β (Gekle \textit{et al}., 2003; Eddy & Giachelli, 1995; Wolf \textit{et al}., 2004). Fearn & Sheerin, 2015 highlighted that the activation of complement in renal injury mediates the expression of TGF-β and facilitates renal fibrosis.

Similar to the TGF-β data, expression levels of TNF-α in MASP-2\(^{K0}\) mice were significantly lower than the expression levels in WT mice. As a pro-inflammatory cytokine, TNF-α lower expression levels in MASP-2\(^{K0}\) mice might suggest that the absence of the LP functional activity leads to less inflammation in POP renal injury. Activation of the complement system in kidney cells was reported to induce the expression levels of TNF-α among other inflammatory cytokines (David \textit{et al}., 1997; Zoja \textit{et al}., 2003). On the other hand, Sheerin \textit{et al}., 1997b reported that stimulation of rat glomerular cells with TNF-α in culture upregulated the basic complement component C3. This sheds some light on the nature of the feedback-mechanism in developing renal inflammation influenced by complement activation, and suggests that reducing complement activation helps to mitigate release of pro-inflammatory cytokines which in turn helps to reduce the activation levels of the complement.

IL-6 is also a macrophage-derived inflammatory cytokine involved in acute phase inflammation response and mediates the activation and proliferation of
lymphocytes, differentiation of B cells and leukocyte recruitment. In the current study, IL-6 expression levels in POP MASP-2\textsuperscript{KO} kidneys were significantly less than the levels expressed in WT mice. Computer based image analysis showed that the IL-6 expression in some MASP-2\textsuperscript{KO} mice was comparable to the expression in non-proteinuric control mice. Several authors highlight the link between downregulation of IL-6 and protection from renal disease and several related clinical complications. These included diabetic nephropathy (Dalla Vestra \textit{et al.}, 2005; Fornoni \textit{et al.}, 2008), hypertension (Harrison \textit{et al.}, 2011), obesity (Knight & Imig, 2007; Bastard \textit{et al.}, 2000) and in IgA nephropathy (Suzuki \textit{et al.}, 2011). Downregulation of the IL-6 in MASP-2\textsuperscript{KO} mice observed in this study strongly suggests a protective effect of the absence of the LP from developing renal inflammation in POP mice.

Interestingly, the frequency of apoptotic tubular cells counted across kidney sections from MASP-2\textsuperscript{KO} mice was much lower than in WT mice. Augmented apoptosis in WT kidneys indicate a degree of tissue degeneration, tubular cells turnover and repair mechanisms that are higher than the extent in the LP deficient mice. When compared to control groups, POP mouse groups exhibited elevated levels of apoptotic cells due to the injury. These data are in agreement with many studies showing that proteinuria induced higher levels of apoptotic cells (Thomas \textit{et al.}, 1999; Eddy, 1989). In a recent study, a mouse line deficient in the complement component C6 was used in a POP study to investigate the relation between the MAC formation and the development of tubulointerstitial injury (Rangan, 2016). The author concluded that the formation of the C5-9 complex does not mediate the renal injury in this model. One of the criteria they evaluated was the count of apoptotic cells which did not change in kidney sections from C6\textsuperscript{-/-} and C6\textsuperscript{+/+}. In contrast to these findings, as shown from the results of this study and reported by other authors (Abbate \textit{et al.}, 2006; Turnberg \textit{et al.}, 2006b), deficiency of complement activating components like MASP-2 or C3 reduced renal inflammation and apoptosis. This suggests that the renal tissue injury could be mediated by the pro-inflammatory effects of complement activation products and not by cell lysis through MAC formation.
The expression levels of a group of seven genes related to renal inflammation, fibrosis and complement activation were studied. Compared to control groups, POP mice from WT and MASP-2\textsuperscript{KO} showed a significantly upregulated gene expression levels in all studied genes. Similar results were reported by Nagasawa et al., 2001 who showed that the expression levels of a large group of inflammation-related genes were upregulated shortly after POP in mice. No significant differences were observed between control mice from WT and MASP-2\textsuperscript{KO} groups. Similar baseline expression levels in the control groups suggest that differentiation in gene expression after POP results from the deficiency in MASP-2 gene and not due to other strain variation factors. The renal fibrosis-related gene collagen type IV showed a significant increase in POP WT mice compared to POP MASP-2\textsuperscript{KO} mice, suggesting that WT mice are more susceptible to initiate a fibrosis cascade at this level of treatment but not MASP-2\textsuperscript{KO} mice. The rest of studied genes did not show significant differences between POP WT and MASP-2\textsuperscript{KO} groups. However, there was a non-statistically significant trend towards lower expression of all studies genes in the MASP-2\textsuperscript{KO} group compared to WT mice.

The degree of protection observed in MASP-2 knockout mice compared to wild type mice suggested the need to investigate the effects of MASP-2 inhibition on the scar development in wild type mice. LP functional activity was inhibited in a group of WT mice after using a specific monoclonal anti-MASP-2 antibody. The degree of renal injury in this group was compared to the scar development in two control groups; saline and Isotype.

The proteinuria levels expressed from these three experimental groups were similar after challenging with BSA. All groups expressed approximately seven fold the proteinuria of non-proteinuric sham controls. Importantly, the antibody treated group showed similar proteinuria compared to the other two control groups, this confirms that the amounts of stress exerted on the three groups were similar.
To confirm the inhibition of the LP functional activity after antibody administration, systemic LP activity was evaluated by quantifying lectin-induced C3 and C4 deposition in minimally diluted serum samples. The data obtained from these assays were not conclusive and showed unconventional patterns of C3 and C4 depositions in all groups. Samples showed similar mean fluorescent intensities after spiking with 600 nm of the antibody. The distortion in the test could occur due to interference between the high protein content of the sera and the test reagents. This suggested the use of ELISA to investigate diluted serum samples.

ELISA showed a strong inhibition of the LP-induced C3 deposition in the antibody treated group and robust activation levels in the saline and isotype control groups. The same strong inhibition was observed by investigating the deposition of the C3 on BSA-based and non-BSA-based blocking buffers. These low binding levels observed in the antibody treated group only confirmed the efficacy of the 721-SGMI-H2 antibody in inhibiting the LP functional activity and allowed the study of its protective role in the development of the renal injury.

In addition, the comparable binding affinity of C3 observed in the two assays conducted, using BSA and non-BSA based blocking media, suggests that the mouse sera of all groups did not generate an anti-BSA antibody complexes due to overloading with BSA during the experiment. Determination of the lack of anti-BSA antibodies formation in the sera of this experiment importantly suggest that the C3 activation levels observed in the control groups are due to activation of the LP and not the CP. This sheds some more lights on the optimisation of this model of POP to study the activation of the LP independently with no interference from the other two complement pathways.

Inhibition of the LP functional activity in the antibody treated group showed a significant reduction in the expression levels of some of the studied inflammatory markers. TGF-β expression levels were significantly reduced in the kidneys of the antibody treated group compared to controls. These observations are in
agreement with the results obtained using MASP-2\textsuperscript{KO} mice in this study. Several authors highlighted the roles the TGF-β plays to mediate renal injury (Fearn & Sheerin, 2015; Wolf \textit{et al.}, 2004; Zoja \textit{et al.}, 2003; Harrison \textit{et al.}, 2011; Suzuki \textit{et al.}, 2011). The reduced expression levels of this cytokine in the antibody treated mouse group strongly suggest a protective effect of the inhibition of the LP. This protective effect was also noticed subjectively by examining H&E section by light microscopy. While the control sections showed several features of acute tubular injury, the antibody treated mice appeared to show these features to lesser extent. This qualitative unquantifiable observation is supported by the quantitative measurements of some of the other studied markers.

Moreover, the frequency of apoptotic tubular cells observed in the antibody treated group was significantly lower than the cells observed in the control groups. Reduced apoptosis after inhibition of the LP is in agreement with the data obtained using MASP-2\textsuperscript{KO}. In line with the findings of Thomas \textit{et al.}, 1999; Eddy, 1989 who showed that the occurrence of apoptotic cells is highly increased in proteinuric nephropathy, reduced apoptosis in the LP inhibited kidneys strongly suggests the protective effect of the inhibition of LP functional activity.

Unexpectedly, the antibody treated group showed macrophage infiltration similar to the infiltration in the control groups with no significant differences. Even when studied in three different zones of the cortex, the amount of the macrophage infiltration remained similar in all groups. Also, quantitative analyses of the expression levels of TNF-α and IL-6 did not show significant differences between the antibody treated groups compared to the isotype control groups. These data are unlike what was observed after using MASP-2\textsuperscript{KO} mice. This could highlight one of the main differences between the use of MASP-2\textsuperscript{KO} mice and the phenotype of inhibition of MASP-2 functional activity in WT mice. In contrast to the MASP-2\textsuperscript{KO} mice (where no MASP-2 is produced), the anti-MASP-2 treated mice are able to synthesise MASP-2 and release MASP-2 from the liver (its unique location of biosynthesis) which then is inhibited by anti-MASP-2 antibodies in serum and other body fluids. This inhibition depends on
the bioavailability of the antibody which may not be as readily available in the renal tubules as it is in the blood stream. The synthesis and then the on-site inhibition of the MASP-2 protease in the antibody treated mice could lead to the initiation of some of the early events of the inflammation cascade and one of the earliest events is the macrophages recruitment and attraction to the injured site. However, the lack of the functional activity of one of the main pro-inflammatory effectors, the LP, may not lead to complete macrophage activation as suggested by reduced inflammatory markers examined in this study. To investigate this hypothesis further, two future work directions are suggested; first the need to investigate the pharmacodynamics of the 721-SGMI-H2 antibody inside the renal tissue. Second, the need to further investigate the activation and cell type characterisation of the infiltrated macrophages in this model.

The results also showed a noticeable reduction in the mean values of the expression levels of all studied genes in the antibody treated kidneys compared to saline controls. However, this lower expression levels are not up to statistical significance. These genes are the renal fibrosis related gene TGF-β, the inflammation related gene TNF-α, the basic complement activation component C3 and the complement factor properdin. The sub statistical difference might also suggest the need to refine the model more probably by increasing the number of individuals in studied groups. These results also are in line with the expression levels of TGF-β and TNF-α examined by immunohistochemistry and described earlier in this thesis. Lower expression levels of the properdin, which is a key element in the activation of the alternative pathway, emphasises the interdependent activation of the alternative and the lectin pathways of the complement system. This suggests that inhibition of the LP functional activity may lead to inhibition of the AP activation as well. Previous authors described that the activation of the alternative pathway mediated glomerulosclerosis and tubulointerstitial fibrosis in proteinuric nephropathies in mice (Lenderink et al., 2007; Turnberg et al., 2006a). This suggests that inhibition of the LP functional activity might involve the inhibition of the AP activation as well and hence, block the mechanisms the AP contribute to in the renal injury.
3.5 Conclusions

- The results obtained from this mouse model of POP suggest that the LP plays a role to mediate the progression of renal inflammation and fibrosis and the deficiency of the LP functional activity reduced the renal injury.
- Most importantly, the results showed that antibody-mediated inhibition of the lectin pathway showed similar protection in some of the studied parameters.
- Protective effects of the absence of the MASP-2 observed in this study might suggest a novel therapeutic approach to relief renal injury in proteinuric nephropathies.
4 The Role of the Complement System Activation in Unilateral Ureteric Obstruction

4.1 UUO model optimisation

Unilateral ureteric obstruction is a well-established mouse model of renal injury. The injury is characterised by progressive inflammation and renal fibrosis which escalate progressively from the time of ligation. To achieve sufficient injury levels to see activation of the complement in the kidney, and at the same time to consider animal welfare, optimisation of the time points of the model was undertaken.

4.1.1 Experimental design

Three groups of 8 week old C57BL/6 mice were subjected to surgical UUO and culled at three different time points to determine the course of renal fibrosis development. The observation time points chosen were one day, three days and seven days post-UUO surgery. The degree of tissue injury and inflammation were compared in these three groups of mice to define the most informative time point to monitor the pathophysiological changes induced in this model of kidney disease.

4.1.2 Gross histological changes in mouse kidneys after one, three and seven days of UUO

Compared to sham operated mouse kidneys, no histological changes were observed in UUO mice after one day of the injury. However, after three days, focal dilatation of proximal and distal tubules of the kidney was observed along with effacement of some tubular villous brush borders. Tubular cells were attenuated with reduced cytoplasm. At seven days post-UUO these changes were observed globally in most kidney regions and to greater severity (Figure 53).
4.1.3 Collagen deposition in mouse kidney sections after one, three and seven days of UUO

Collagen deposition and fibrosis are two major characteristics of the UUO kidney injury in mice and other species. To estimate the amount of collagen deposition at these three time points, sections were stained with Sirius Red and examined under bright field light microscope (Figure 54).

Analysis of the sections using a computer based image analysis software showed a linear relation between the time and the collagen stained area (Figure 55). One-way ANOVA analysis showed a highly significant difference between groups (****P<0.0001). After one day of the injury and compared to sham operated mice, no difference could be detected in collagen deposition (sham 5.7 ± 1.4 % vs. one day UUO 5.4 ± 2 %). However, the amount of collagen detected after
three days of the injury was increased to almost double the amount of the sham operated mice and to almost four-fold increase at seven days time point (sham 5.7 ± 0.8 % vs. three days UUO 11.1 ± 0.8 % vs. seven days UUO 21.8 ± 1.7 %). These figures represent the mean±SEM of the percentage of collagen stained area.

Figure 54: Representative micrographs of mouse kidney sections stained with Sirius Red after one day (B), three days (C) and seven days (D) of unilateral ureteric obstruction. (A) is taken from mice operated in a sham operation without ureteric ligation. While no difference in collagen deposition has been observed between sham and one day groups, three days mouse kidneys exhibited increased staining of collagen around most of the kidney tubules and glomeruli, n=3 in all groups.
4.1.4 Animal welfare assessment and pain scoring after one, three and seven days of UUO

To ensure that the UUO surgery was within acceptable severity limits, all animals in this experiment were kept under close observation throughout the experiment with regular pain scoring checks points. Apart from pain assessments during the post-surgical recovery period, all mice in all experimental groups showed normal behaviour and zero pain scoring assessments at all check points and up to the end of the seven day experiment. These observations, alongside the amount of inflammation, fibrosis and tissue changes observed in this pilot experiment, suggested the use of seven days UUO model to further investigate the activation of the complement system.
4.2 Utilisation of complement compromised transgenic mouse lines to study the role of the classical and the lectin pathways of complement in unilateral ureteric obstruction

The complement system is activated by a pattern of cascading pathway specific activation steps that lead ultimately to the formation of the membrane attack complex. This pattern of activation allows for manipulation of specific pathways by targeting certain key elements of each pathway.

As described previously, deficiency in the C1q enzyme leads to absence of the classical pathway functional activity. Similarly, lack of the MASP-2 serine protease functional activity leads to complete deficiency of the lectin pathway of complement. Two transgenic mouse models deficient in each gene, given the symbols $\text{C1q}^{\text{KO}}$ and $\text{MASP-2}^{\text{KO}}$, were used to investigate the role of the lectin and the classical pathway mediated activation of complement in the development of renal fibrosis in UUO mice.

4.2.1 Experimental design

In this experiment, a group of six 8 week old from WT, $\text{C1q}^{\text{KO}}$ and $\text{MASP-2}^{\text{KO}}$ mice on C57BL/6 genetic background were subjected to right UUO. Another group of three mice from each genetic background were sham operated as controls.

4.2.2 Genotyping of mice

All transgenic mice used in the study were genotyped to confirm the homozygous deficiency of either the MASP-2 or the C1q gene prior to the inclusion in the UUO study. The genotyping of the $\text{MASP-2}^{\text{KO}}$ mice was described previously (Section 3.2.2). Genotyping of the $\text{C1q}^{\text{KO}}$ colony was performed by a multiplex PCR reaction using a set of primers specific for the mouse C1q gene interrupted with a neomycin cassette (Figure 56). The multiplex PCR was designed to indicate the genotype of the animal through one of three outcomes:
a. single band at the size 160 bp indicates homozygous C1q\textsuperscript{KO} genotype

b. single band at about 360 bp indicates WT mouse

c. two bands at 160 bp and 360 bp indicate heterozygosity

Figure 56: Agarose gel image of a multiplex PCR for genotyping of mouse C1q gene. C1q\textsuperscript{-/-} showed a single 160 bp band (C1q\textsuperscript{KO}) while C1q\textsuperscript{+/-} showed a single band at 360 bp (WT). C1q\textsuperscript{-/+} heterozygous mice shows both bands at 160bp and 360bp (heterozygous)

4.2.3 Histopathological examination of WT, C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mice kidney sections after UUO

H&E stained kidney sections from each mouse in all groups were examined under light microscope. While sham operated mice showed normal kidney structure, UUO kidney sections across all groups showed typical changes related to ureteric obstruction. Global dilatation of proximal and distal tubules was observed across the sections with a prominent loss of the brush border structures. These changes were observed equally in all UUO groups to similar extents (Figure 57).
4.2.4 Transmission EM examination of kidney ultrastructure

Sections from each experimental group were examined by TEM to estimate the degree of cellular damage. In all UUO mice, the tubular ciliary brush borders were completely effaced (Figure 58B, C and D) whilst the sham operated mice showed normal structures (Figure 58A). The tubular cells of UUO mice displayed a reduced number of mitochondria. However, these changes were observed in all UUO mice and appeared similar between UUO groups (Figure 58).
4.2.5 Visualisation and quantification of fibrosis in mouse kidneys from WT, C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} groups after UUO

Kidney sections from all experimental mice were stained with Sirius Red and examined under bright field light microscope to evaluate the degree of renal fibrosis.

While all sham operated mice showed minimal baseline collagen deposition, UUO mice showed an increased area of collagen in all groups (Figure 59). The amount of collagen deposited in WT mice was greater than the amounts measured in C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mice. Analysis of the stained area in all groups using one-way ANOVA test followed by Bonferroni’s post hoc analysis showed a highly significant difference between groups (**P=0.003). Analysis
showed an increase in WT UUO group compared to both the C1q\(^{\text{KO}}\) group and the MASP-2\(^{\text{KO}}\) group (WT 24.8 ± 4.7 % vs. C1q\(^{\text{KO}}\) 14.2 ± 5.7 % vs. MASP-2\(^{\text{KO}}\) 16.6 ± 3.2 %), (Figure 60). Sham operated mice showed similar baseline staining patterns across groups. These observations suggest that activation of the complement system via the classical and the lectin pathways exacerbates renal fibrosis in UUO kidneys.

Figure 59: Representative micrographs of Sirius Red stained mouse kidney sections from wild type, C1q\(^{\text{KO}}\) and MASP-2\(^{\text{KO}}\) after seven days of UUO. (A) Sham control wild type mice; (B) Wild type UUO mice; (C) Sham control C1q\(^{\text{KO}}\) mice; (D) C1q\(^{\text{KO}}\) UUO mice; (E) Sham control MASP-2\(^{\text{KO}}\); mice and (F) MASP-2\(^{\text{KO}}\) UUO mice. n=3 in sham control groups and 6 in UUO groups.
Role of Complement in UUO

4.2.6 Visualisation and quantification of macrophage infiltration in kidney tissues

Kidney sections from all experimental groups were stained with the macrophage antibody F4/80 to evaluate inflammation as indicated by macrophage infiltration.

In agreement with the Sirius Red studies, examination of these slides by light microscopy showed a noticeable reduction in macrophage infiltration in C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mice when compared to wild type mice (Figure 61). Computer based analysis of these slides showed a highly significant increase in the F4/80 stained area in the WT mice compared to C1q\textsuperscript{KO} mice and to MASP-2\textsuperscript{KO} mice (WT 2.49 ± 0.44 % vs. C1q\textsuperscript{KO} 0.63 ± 0.14 % vs. MASP-2\textsuperscript{KO} 0.58 ± 0.06 %), (Figure 62). One-way ANOVA test showed a highly significant difference between UUO groups (**P=0.0026). No significant differences were observed
between the C1q\textsuperscript{KO} and the MASP-2\textsuperscript{KO} groups or between any of the sham control groups.

Figure 61: Representative micrographs of mouse kidney sections stained with macrophage specific antibody F4/80 after seven days of UUO. (A) Sham control wild type mice, (B) Wild type UUO mice; (C) Sham control C1q\textsuperscript{KO} mice; (D) C1q\textsuperscript{KO} UUO mice; (E) Sham control MASP-2\textsuperscript{KO} mice; and (D) MASP-2\textsuperscript{KO} UUO mice. \( n=3 \) in sham control groups and 6 in UUO groups.
Figure 62: Analysis of F4/80 macrophage stained areas in kidney sections from WT, C1q\(^{\text{KO}}\) and MASP-2\(^{\text{KO}}\) mice after seven days of UUO. WT mice showed a highly significant increase in the stained area compared to C1q\(^{\text{KO}}\) mice (**\(P=0.0004\)) and MASP-2\(^{\text{KO}}\) mice (**\(P=0.0003\)) using Bonferroni’s post hoc analysis. Plots depict mean ± SEM.

4.2.7 Determination of the expression profiles of selected genes in UUO mouse kidneys

The expression of a variety of genes linked to renal fibrosis, complement activation and kidney inflammation in UUO kidneys was compared in WT, C1q\(^{\text{KO}}\) and MASP-2\(^{\text{KO}}\) mouse groups. Twelve genes were studied:

- collagen type IV alpha 1 (Col4α1)
- transforming growth factor beta-1 (TGFβ-1)
- cadherin 1 (Cdh1)
- fibronectin 1 (Fn1)
- interleukin 6 (Il6)
- interleukin 10 (Il10)
- interleukin 12a (Il12a)
vimentin (Vim)  
actinin alpha 1 (Actn1)  
tumour necrosis factor-α (TNF-α)  
complement component 3 (C3)  
interferon gamma (Ifn-γ)

In addition to that, four housekeeping genes were used as a control in this study:

- glyceraldehyde-3-phosphate dehydrogenase (GAPDH)  
- glucuronidase beta (Gusβ)  
- eukaryotic 18S rRNA (18S)  
- hypoxanthine guanine phosphoribosyl transferase (HPRT).

Compared to sham operated mice, the expression levels of all studied genes were upregulated after seven days of UUO in all groups. Comparison between WT and MASP-2\textsuperscript{KO} mice demonstrated a biologically significant upregulation of four genes in WT mice compared to the expression levels of these genes in the MASP-2\textsuperscript{KO} animals. These genes are the inflammation related genes IL-6 (WT 2.34 ± 0.1 vs. MASP-2\textsuperscript{KO} 1.99 ± 0.09 log10RQ) and IFN-γ (WT 1.55 ± 0.1 vs. MASP-2\textsuperscript{KO} 1.4 ± 0.08 log10RQ) and the fibrosis related genes TGFβ-1 (WT 1.16 ± 0.04 vs. MASP-2\textsuperscript{KO} 1.01 ± 0.05 log10RQ) and Col4α1 (WT 1.76 ± 0.06 vs. MASP-2\textsuperscript{KO} 1.57 ± 0.11 log10RQ). However, using ANOVA test to compare between treated groups did not show significant differences between groups in any of the studied genes (Figure 63 and Figure 64). This might suggest the need to increase the group size in this study in order to highlight the differences between groups.
Figure 63: Relative mRNA expression levels of a group of selected inflammation and fibrosis related genes in WT, C1q^{KO} and MASP-2^{KO} mouse kidneys after seven days of UUO. Despite the lower expression levels of the studied genes in MASP-2^{KO} mice compared to WT mice, no statistically significant differences were observed between any of the treated groups. Plots depict mean ± SEM. (RQ) stands for relative quantitation.
Figure 64: Relative mRNA expression levels of a group of selected inflammation and fibrosis related genes in WT, C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mouse kidneys after seven days of UUO. No significant differences were observed between any of the treated groups.
4.3 Antibody mediated inhibition of the lectin pathway in unilateral ureteric obstruction mice

The data obtained from the MASP-2KO mice suggested that absence of the lectin pathway functional activity reduced kidney inflammation and fibrosis in UUO mice. To further investigate this, WT mice pre-treated with a MASP-2 specific inhibitory antibody and its respective isotype control were examined after UUO.

4.3.1 Experimental design

Four groups of WT mice, n=6, were used in this experiment:

a. saline control group

b. Isotype control group was given an unspecific human IgG4 mAb

c. Group treated with the OMS721 antibody

d. Group treated with the 721-SGMI-H2 antibody

Antibodies were administered 10mg/kg by i.p. injections on day -1 prior to UUO surgery and on day +3 post-surgery. All groups were culled seven days after the UUO surgery and relevant samples collected.

4.3.2 Determination of the lectin pathway activity in antibody treated mice

Terminal serum samples collected at the end of this experiment were analysed to evaluate inhibition of the lectin pathway. This analysis has been performed and the data has been generated by a team of collaborative researchers at OMEROS Inc. (Seattle, WA, USA). Inhibition of the lectin pathway was estimated by measuring the activity of endogenous murine C3 (Figure 65) and C4 (Figure 66) in spiked and unspiked conditions. Samples from the saline control group, the isotype control group and the 721-SGMI-H2 antibody group in this experiment were spiked with 600 nM of 721-SGMI-H2 antibody while the serum samples from the OMS721 group were spiked with 600 nM of OMS721 antibody.
Evaluation of lectin pathway functional activity using this assay revealed robust activation levels of both C3 and C4 in the control groups treated with saline and the isotype control antibody and this activation was significantly reduced by spiking the reaction with antibody.

OMS721 treated mice showed variable levels of C3 and C4 activation and spiking the reaction with OMS721 antibody did not block the activation of both C3 and C4 in the tested samples. Treatment with 721-SGMI-H2 showed a robust inhibition of C3 and C4 in all treated mice. This suggests that systemic administration of 721-SGMI-H2 is highly efficient in blocking lectin pathway functional activity.

Figure 65: Flow cytometry estimation of the activity of endogenous C3 in mice serum after seven days of UUO. Four groups of UUO mice (n=6) were treated with either normal saline solution as a control, isotype antibody as a control, OMS721 anti-MASP-2 antibody or 721-SGMI-H2 anti-MASP-2 antibody. MFI: mean fluorescence intensity.
Figure 66: Flow cytometry based activation assay of endogenous C4 in mice serum after seven days of UUO. Four groups of UUO mice (n=6) were treated with either normal saline solution as a control, isotype antibody as a control, OMS721 anti-MASP-2 antibody or 721-SGMI-H2 anti-MASP-2 antibody. MFI stands for Mean Fluorescence Intensity.

4.3.3 Estimation of fibrosis in UUO kidney sections after administration of anti-MASP-2 antibody

Kidneys from all treated groups were collected at the end of the experiment after seven days of UUO. Sections from all kidneys were stained with Sirius Red to evaluate the collagen deposition after inhibition of the lectin pathway. Examination of these slides under light microscope did not show significant differences between any of the treated groups, representative images are shown in Figure 67. Computer analysis of images taken from these sections and analysed using ANOVA test showed no significant differences between any of the treated groups (Figure 68).

These observations were validated by taking three kidney sections from each mouse in the saline and 721-SGMI-H2 groups, 100 µm apart, to study the distribution of collagen deposition in the Z axis of the tissue. Analysis of these sections showed a lower mean value of the stained area in the antibody treated
group compared to the saline control group (WT 27.8 ± 3.02 % vs. saline 32.9 ± 1.8 %), However, statistically the difference was not significant (Figure 69).

Figure 67: Representative micrographs of mouse kidney sections stained with Sirius Red after seven days of UUO and treatment with (A) saline control, (B) Isotype control, (C) OMS721 anti-MASP-2 antibody or (D) 721-SGMI-H2 anti-MASP-2 antibody, n=6 in all groups.
Figure 68: Estimation of the collagen area stained with Sirius Red in mouse kidney sections from all groups after seven days of UUO. No significant differences between any of the treated groups were observed. n=6 and bars represent mean±SEM.

Figure 69: Estimation of the collagen area stained with Sirius Red in UUO mouse kidney sections at three levels of the Z axis of the transverse section of the tissue, 100 µm apart. Despite the mean value of the 721-SGMI-H2 antibody treated group was lower than the mean value of the saline control group in this analysis, the difference between groups was not statistically significant (p value = 0.17). n=6 and bars represent mean±SEM.
4.3.4 Macrophage infiltration after antibody-mediated inhibition of the lectin pathway

Similar to Sirius Red staining, sections from the middle zone of the longitudinal section of the kidney and from two other levels 100 µm apart were stained with F4/80 antibody. Examination of these slides by light microscopy did not show major differences between groups (Figure 70). Analysis of the first level sections is represented in Figure 71. Analysis with one-way ANOVA showed no significant differences between any of the treated groups. Examination of two other levels from the kidneys of the saline control and 721-SGMI-H2 mice showed comparable macrophage infiltration with no significant differences between these groups (Figure 72).

Figure 70: Representative micrographs of infiltration of macrophage cells stained with F4/80 antibody in mouse kidney section after UUO. (A) Saline control group; (B) Isotype control group; (C) OMS721 anti-MASP-2 antibody group; and (D) 721-SGMI-H2 anti-MASP-2 antibody group. N=6 in all groups.
Figure 71: Analysis of F4/80 macrophage stained area in mouse kidney sections from all treated groups after seven days of UUO. No significant difference between any of the treated groups was observed. n=6 and bars represent mean±SEM.

Figure 72: Estimation of the macrophage area stained with F4/80 antibody in UUO mouse kidney sections at three levels of the Z axis of the transverse section of the tissue, 100 µm apart. No significant difference between the saline control group and the 721-SGMI-H2 antibody treated group was observed (P value = 0.81). n=6 and bars represent mean±SEM.
4.3.5 Serum creatinine levels in UUO mice after inhibition of the lectin pathway

Serum creatinine levels in treated mice were measured to examine any possible effects of the anti-MASP-2 treatment on the kidney function. All groups showed creatinine levels within mouse normal values with no significant differences between groups (Saline 0.26 ± 0.03 mg/dL vs. isotype 0.32 ± 0.13 mg/dL vs. OMS721 0.22 ± 0.05 mg/dL vs. 721-SGMI-H2 0.23 ± 0.05 mg/dL), (Figure 73).

![Graph showing serum creatinine levels](image)

Figure 73: Estimation of the serum creatinine levels of UUO mice treated with anti-MASP-2 antibodies. No significant differences between any of the treated groups were observed. n=6 and bars represent mean±SEM.

4.3.6 Serum urea in UUO mice after inhibition of the lectin pathway

Serum urea was also measured in all experimental groups to examine kidney functions. The values from individual mice in all groups were within the normal mouse serum urea levels and no significant differences were observed between
any of the treated groups (Saline 152 ± 25 mg/dL vs. isotype 160 ± 39 mg/dL vs. OMS721 155 ± 21 mg/dL vs. 721-SGMI-H2 148 ± 19 mg/dL), (Figure 74).

Figure 74: Estimation of the serum urea levels of UUO mice treated with anti-MASP-2 antibodies. No significant differences between any of the treated groups were observed. n=6 and bars represent mean±SEM.
4.4 Discussion

Unilateral ureteric obstruction in mice is a well characterised model for renal tubulointerstitial inflammation and fibrosis (Chevalier et al., 2009). Several authors highlighted the role of complement activation in the development of various renal disease models including UUO (Boor et al., 2007; Naik et al., 2013; Nangaku et al., 2002; He et al., 2005; Thurman, 2015). Some studies highlighted the importance of the AP (David et al., 1997; Abe et al., 2004) and the CP (Meszaros et al., 2010; Valenzuela et al., 2014) in mediating renal injury. However, the contribution of the LP in the development of renal disease is less investigated.

This study aimed to establish the role of the lectin and the classical pathways in renal tubulointerstitial inflammation and fibrosis in UUO mouse model. To achieve this, I used unique transgenic mouse models completely deficient in either the LP (Schwaeble et al., 2011) or the CP (Botto et al., 1998). UUO was established in these transgenic mice and compared to WT mice to identify the role of LP and CP independently in the development of renal injury. In addition, UUO was studied in WT mice depleted in LP functional activity.

The results demonstrate a significant beneficial effect of deficiency of the LP on renal tubulointerstitial inflammation, tubular cell injury, pro-fibrotic cytokine release and scarring. However, inhibition of the LP functional activity using specific antibodies did not show similar reduced renal injury. Lacking of CP activation showed significant reductions in renal inflammation and fibrosis but no significant differences were observed in cytokines gene expression profiles compared to WT. These findings suggest that the tissue injury in this model of non-proteinuric kidney disease is mediated by the activation of the classical and the lectin pathways of the complement.

Genetic background is one of the factors contribute to the extent of renal injury in murine UUO model. Many authors reported that C57BL/6 mice are more susceptible to renal inflammation and fibrosis in UUO and initiates the injury
earlier compared to some other strains including BALB/c (Puri et al., 2010; Bersani-Amado et al., 2016), therefore C57BL/6 was used in the current study. In addition to that, C57BL/6 was chosen due to the availability of the C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} transgenic mice on this genetic background.

Optimization of the model at three time points, which are one, three and seven days after UUO, showed that UUO produced a progressive time-dependent renal injury. While H&E stained kidney sections after one day of UUO showed no observable gross histological changes, three days group demonstrated a set of mild to moderate tissue injury features. These were worsened further in the seven days group. Furthermore, determination of tubulointerstitial collagen deposition in these three groups showed similar results. While the kidney sections from the one day group showed collagen accumulation comparable to sham operated control mice, three days group showed approximately two fold the amount of the collagen accumulated in the one day group. Moreover, the seven days group showed approximately two fold the amount of collagen accumulated in the three days group. These data are similar to the findings of Hiatt et al., 2013 and Khalil et al., 2012 who showed that UUO caused gradual renal injury escalated by time.

Despite the moderate to severe renal injury developed in the seven days UUO mice, all animals showed normal behaviour and all pain assessments showed zero pain score. These data, alongside the amount of renal fibrosis and tissue changes observed in this optimisation experiment, suggested the use of seven days UUO model to further investigate the activation of the complement system. Likewise, several authors previously reported the use of the murine seven days UUO model in C57BL/6 mice in their studies (Moriyama et al., 1998; Forbes et al., 2013; Forbes et al., 2012).

Induction of UUO in WT, C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mice showed similar kidney histopathological changes in all groups. These were observed in H&E stained kidney section examined by light microscopy and in sections examined by transmitting EM. The histopathological changes observed in these three
Role of Complement in UUO
genotypes due to UUO were similar and no significant differences were observed between the groups. However, determination of collagen accumulation in these kidneys showed significant differences. First, all UUO mice showed significant increase in collagen deposition when compared to sham controls. Second, interestingly WT mice showed a highly significant increase in collagen accumulation compared to both C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mice. These data are similar to the results previously reported by Boor et al., 2007 who studied the renal fibrosis in C5\textsuperscript{−/−} UUO mice and by Zhou et al., 2013 who conducted similar studies on C3\textsuperscript{−/−} mice. Unlike the data presented in the current study, C5\textsuperscript{−/−} and C3\textsuperscript{−/−} mouse models are deficient in later components of the complement activation cascade shared by all three complement activation pathways, hence these studies investigate the role of the complement system as a whole in the injury and are not specific to any particular pathway.

In support to these observations, renal inflammation was also estimated in the three experimental groups by measuring the infiltration of F4/80 macrophages. Quantitative analysis of the kidney sections showed that all UUO kidneys are significantly infiltrated with macrophages compared to controls. Yet, WT mice showed a highly significant increase in macrophage infiltration compared to both C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mice. These findings are similar to the results obtained by Boor et al., 2007 who reported that lack of complement activation significantly reduced the infiltration of the F4/80 specific macrophages in obstructive nephropathies.

The expression of a variety of twelve genes linked to renal inflammation and fibrosis in obstructed kidneys was compared between WT, C1q\textsuperscript{KO} and MASP-2\textsuperscript{KO} mice. Compared to sham controls, UUO kidneys from all groups showed upregulated gene expression profiles in all studied genes. These data are similar to the findings of Zhou et al., 2013 who reported that UUO in mice led to upregulation of gene expression of some pro-inflammatory and fibrogenic cytokines including TGF-β and TNF-α. Rohatgi & Flores, 2010 also reported that intra-tubular biomechanical pressure in obstructive kidney disease generated
changes in intracellular signalling and cytokine gene expression in mice. Silverstein et al., 2003 and Sommer et al., 2000 reported similar upregulation levels of several cytokine genes in neonatal and adult rats after UUO.

Interestingly, WT mice showed a noticeable biologically significant increase in the gene expression levels of the inflammation related genes Il6 and Ifn-γ, and the fibrosis related genes TGFβ-1 and Col4α1 compared to MASP-2KO mice. However, these differences between treated groups were not up to statistical significance. Showing such limited differences between small groups in this model (n=6) strongly suggest the need to expand these experiments further in larger group sizes in order to achieve better understanding of the differences between groups. These data support the data from the current study showing reduced renal fibrosis and inflammation in MASP-2KO mice compared to WT mice. Similar to these findings, Boor et al., 2007 reported that the gene expression of some renal fibrogenic factors including TGF-β were significantly down-regulated in C5Δc mice after UUO. However, while C1qKO mice also showed lower levels of collagen deposition and macrophage infiltration in their kidneys, no significant differences were observed between C1qKO mice and WT mice in the expression profiles of any of the studied genes. This might suggest the need to further investigate the C1qKO UUO model by assessing other tissue injury markers in larger groups of animals to develop better understanding of the model.

These findings demonstrate a significant contribution of the LP activation in the development of renal inflammation and fibrosis in obstructive kidney disease. The degree of protection observed in the LP deficient mice compared to WT suggested the need to investigate the inhibition of LP functional activity in WT mice using specific antibodies.

To achieve that, two versions of a recombinant monoclonal anti-MASP-2 antibody, named OMS721 and 721-SGMI-H2, known to inhibit the LP functional activity in mice (Schwaebel et al., 2011) were used. 721-SGMI-H2
antibody is a genetically modified form of OMS721 with a SGMI-2 polypeptide coding sequence added to the coding sequence for C-terminus of the OMS721 heavy chain. The SGMI-2 polypeptide is a potent MASP-2 inhibitory peptide as described in Heja et al., 2012. Each of these antibodies was injected into a group of WT UUO mice and the data were compared to saline and isotype controls.

The data obtained suggested that 721-SGMI-H2 antibody is a better inhibitor of mouse MASP-2 that the human specific MASP-2 inhibitor OMS721 achieving a complete inhibition of the murine lectin pathway as shown in C3 and C4 deposition assays in serum of all 721-SGMI-H2 treated mice. In contrast, OMS721 treated mice showed only partial inhibition of the C3 and C4 depositions in some mice. These data indicate that the 721-SGMI-H2 antibody is more effective than the OMS721 antibody in inhibiting the LP functional activity in the serum of mice.

Unlike the data obtained from MASP-2\(^{\text{KO}}\) mice, antibody-mediated inhibition of the LP in this experiment did not show reduced renal fibrosis or renal inflammation compared to controls. Estimation of collagen deposition and macrophage infiltration in the OMS721 and 721-SGMI-H2 kidneys showed injury levels comparable to controls even when studied at three levels from the Z axis of the kidney. However, in this examination the mean stained area of collagen in the 721-SGMI-H2 kidneys were lower than that of the saline control group, yet statistically the difference was not significant. This difference suggests the need to investigate larger groups of mice to confirm or disprove the phenomenon.

Differences between the MASP-2\(^{\text{KO}}\) data and the antibody-mediated inhibition of the LP in WT mice highlight some of the limitations of the UUO model. Unlike renal injury in spontaneous CKD, UUO is a potent model of accelerated renal fibrosis, and interventions that reduce fibrosis in this model are likely to be transferable to other more chronic models of renal disease. Furthermore, it is unlikely that administered antibodies will enter the kidney tubules, either before
the induction of UUO when the glomerular barrier is intact or after UUO when glomerular filtration has ceased. Therefore anti-MASP-2 antibodies may penetrate poorly into an extravascular tubulointerstitial site of injury in this model. These data suggest the need to further investigate the antibody bioavailability in the kidneys of the UUO mice.

Other factors could be optimised to shed more light on these findings like for example the time course of administration of the antibodies. In the current experiment, two doses of the antibodies were administered; on day -1, i.e. one day prior to the UUO surgery, and one at day +3 after surgery. Different dosing regimens could be more effective in inhibiting the LP functional activity in the renal tissue. For example, administration of the antibody a week before the surgery could lead to better depletion of the LP on the day of the surgery and afterward.

4.5 Conclusions
- Genetic-based deficiencies of the CP and the LP of complement showed significant reductions in renal fibrosis and inflammation in UUO mice. These data suggest that the tissue injury in this model is mediated by the activation of these two pathways.
- The degree of tissue protection observed in MASP-2\(^{\text{KO}}\) mice was not reproduced after antibody-mediated inhibition of the LP.
- The data suggest the need to further investigate the model in larger group size of mice to allow better understanding of the differences between the groups.
- Further investigations of the bioavailability of the used antibodies in the UUO kidneys is recommended to determine its permeability into obstructive nephropathies.
5 The Role of the Lectin Pathway of Complement in Adriamycin Nephropathy

5.1 Model optimisation
In BALB/c mice, administration of adriamycin triggers progressive kidney inflammation that initiates within few days after the injury. The model in BALB/c mice is reproducible and relatively predictable. However, a pilot experiment was conducted in order to define the optimal time point that produces sufficient tubulointerstitial inflammation to study activation of the complement system.

5.1.1 Experimental design
In this optimization experiment, three groups of male BALB/c mice (n=3 in each group) were injected with a single i.v. dose of 10.5 mg/kg adriamycin hydrochloride in 100 µl of balanced saline solution. These three groups were culled at three different time points, 1 week, 2 weeks and 4 weeks after the onset of the injury. The data collected were compared to a control group of mice (n=3) injected with saline solution only.

5.1.2 Changes in the mouse body weight after 1, 2 and 4 weeks of AN
The body weight of AN mice was significantly reduced after administration of adriamycin. While control mice displayed normal body weight gain over the course of the experiment, all AN mice showed body weight loss that started as soon as the next day after administration of the adriamycin (Figure 75). This rate of body weight loss was maintained during the first week of the injury in all mice and by the end of the first week, mice lost approximately one third of their body weight. During the second week of the experiment, mice started to gain weight slightly and the gain was notable and increasing during the third and fourth week of the experiment. By the end of the fourth week, mice approximately regained half of the body weight loss they encountered.
Figure 75: Changes in the body weight of BALB/c mice after adriamycin nephropathy (AN). Control mice were injected with a single dose of 100 µl saline solution IV. AN mice were intravenously injected with a single dose of 10.5 mg/kg adriamycin hydrochloride dissolved in 100 µl of saline solution. N=3 for control mice while for AN mice n=9 in week 1, n=6 in week 2 and n=3 in weeks 3 and 4, as 3 mice were culled at the end of weeks 1, 2 and 4.

5.1.3 Proteinuria induced after 1, 2 and 4 weeks of AN
To estimate the extent of proteinuria produced after adriamycin nephropathy, urine samples were collected over 24 hours by means of metabolic cages from all mice at the end of each time point during the experiment. AN mice showed an approximately 4 fold increased urinary excretion of protein by the end of week 1, compared to the control mice (Figure 76). At the end of week 2, the mean value of proteinuria was slightly decreased compared to week 1 and further decreased at the end of week 4 (Week1 20.1±0.77 mg/24hrs vs. week2 18.98±1.34 mg/24hrs vs. week4 16.15±0.71 mg/24hrs).
Figure 76: Proteinuria induced in BALB/c mice after 1, 2 and 4 weeks of AN. AN mice showed an onset increase in proteinuria after one week of administration of 10.5 mg/kg adriamycin hydrochloride. Bars represent mean±SEM.

5.1.4 Changes in total serum protein in AN mice
The amount of total proteins measured in terminal serum samples showed lower values in all AN mice compared to the control group (Figure 77). While in week 1 AN mice showed serum protein levels approximately 25% lower than that of the saline treated control mice, the amount of total serum protein concentrations in AN mice dropped even lower in week 2. At the end of week 4 (Control 24.8±0.67 mg/ml vs. week1 19.6±0.66 mg/ml vs. week2 17.29±0.48 mg/ml vs. wee4 20.3±0.68 mg/ml), mice recovered to some extent from the loss of serum proteins reaching serum protein levels comparable to those seen in the week 1 group.
5.1.5 Renal histopathological changes after 1, 2 and 4 weeks of AN

Kidney sections from all experimental groups were stained with H&E and examined by light microscopy. Examination revealed evident kidney injury at 1 week after adriamycin injection that progressively worsened in subsequent weeks (Figure 78). At weeks 1 and 2, focal changes were observed in several tubules with an increase in resorption droplets in tubular cells and intratubular cast formations. Some glomeruli were increased in size with intraglomerular deposition and vacuoles. These histopathological changes were mild in week 1 and moderate to severe in week 2 sections and widely observed across the tissue. At time point 4 weeks, the changes were more widespread and severe. In addition to protein cast formation that could be observed in some tubules of the diseased kidney at week 2 after AN, several tubules also displayed degenerated brush borders, with flattened and vacuolated tubular epithelium. Glomeruli were reduced in size with several vacuoles and some of them were collapsed.

Figure 77: Total serum protein of AN mice after 1, 2 and 4 weeks of the injury. n=3, bars represent mean±SEM.
Figure 78: Histopathological examination of mouse kidney sections after 1, 2 and 4 weeks of AN. Compared to control mice (A), all changes were merely noticeable after one week (B), obvious after two weeks (C) and predominantly global and severe after four weeks (D).

5.1.6 Renal inflammation in AN mice after 1, 2 and 4 weeks of injury

Kidney sections from all groups stained with F4/80 macrophage antibody were studied to estimate the degree in renal inflammation. Examination revealed that the degree of kidney inflammation in this model is time dependent. It was noticeable from the macrophage net influx into the tissues that the inflammation started in week 1 (Figure 79B), progressed further in week 2 (Figure 79C) and tissues became heavily infiltrated with macrophages in week 4 (Figure 79D). Plate A in the figure represents a control mouse.
Figure 79: Macrophage infiltration in mouse kidney sections stained with F4/80 antibody after 1 week (B), 2 weeks (C) and 4 weeks (D) of AN, (A) represents control mice. A mild and moderate increase was observed in intertubular macrophage infiltration in 1 and 2 weeks mice respectively. However, after four weeks the macrophage infiltration became more predominant and severe. No background staining was applied to these sections to facilitate the computer based image analysis of them.

The data obtained from this optimization experiment suggested that the two weeks injury produced a moderate renal injury and inflammation. Based on these observations and to comply with the home office licence of this project that allows the study of moderate injuries, the two weeks time point was chosen to further study the role of the lectin pathway activation in mouse adriamycin nephropathy.
5.2 The use of MASP-2\(^{\text{KO}}\) mice to determine the role of the lectin pathway activation in AN

5.2.1 Experimental design

AN was induced in groups of eight WT or MASP-2\(^{\text{KO}}\) mice by administration of a single i.v. dose of 10.5 mg/kg adriamycin hydrochloride dissolved in 100 µl balanced saline solution. Control mice, n=3 from each strain, were injected with saline solution only. All mice were culled two weeks after the initiation of the injury and various tissues collected for analysis.

5.2.2 Changes in the body weight of WT and MASP-2\(^{\text{KO}}\) mice after AN

One of the main characteristics of adriamycin nephropathy in mice is loss of body weight. Initiation of the AN in this experiment caused a significant loss in body weight in all AN mice compared to control mice. The loss of the body weight was progressive during the first week of the injury and thereafter animals stabilised and some of them started to gain weight in the second week (Figure 80). During the first week adriamycin injected mice lost approximately 25% of their body weight. Mean values of groups’ body weight ± SEM were 24.14 ± 0.43 gm and 24.45 ± 0.43 gm in Day 0 for WT and MASP-2\(^{\text{KO}}\) mice respectively and decreased to 18.1 ± 0.24 gm and 18.4 ± 0.25 gm after one week in WT and MASP-2\(^{\text{KO}}\) mice respectively. At the end of the experiment, the mean values of groups’ body weight ± SEM were 18.4 ± 0.31 gm for WT and 18.2 ± 0.22 gm for MASP-2\(^{\text{KO}}\) mice. No significant differences were observed between WT and MASP-2\(^{\text{KO}}\) mice in this experiment.
5.2.3 Proteinuria induced in WT and MASP-2KO mice after AN

Initiation of AN in WT and MASP-2KO mice produced approximately a 4 fold increase in total urine protein in treated mice compared to control mice (Figure 81). Urine samples collected over 24 hours at the end of the experiment by means of metabolic cages showed approximately four fold increase in proteinuria in AN mice compared to controls (WT AN 18.2 ± 0.76 mg/24hrs vs. WT control 4.7 ± 0.54 mg/24hrs) and (MASP-2KO AN 17.7 ± 0.89 mg/24hrs vs. MASP-2KO control 4.9 ± 0.21 mg/24hrs). Both WT and MASP-2KO mice produced similar proteinuria in this experiment with no significant differences between treated groups.
Figure 81: Urine total proteins excreted in 24 hours from WT and MASP-2\(^{KO}\) mice after two weeks of AN. No statistically significant differences were observed between groups at the same level of treatment. \(n=3\) for WT and MASP-2\(^{KO}\) control groups and \(n=8\) for WT and MASP-2\(^{KO}\) AN mice. Bars represent mean±SEM.

5.2.4 Changes in the total serum protein of WT and MASP-2\(^{KO}\) mice after two weeks of AN

Serum samples from all experimental animals were collected at the end of the experiment and analysed using Bradford protein estimation assay as explained in the material and methods to investigate the changes in the serum total proteins. While control mice from both strains showed normal levels of serum proteins, AN mice showed significantly reduced serum protein concentrations (Figure 82). WT mice reduced from \((24.7 \pm 0.53 \text{ mg/dL})\) in the control group to \((17.4 \pm 0.39 \text{ mg/dL})\) in the AN group and MASP-2\(^{KO}\) mice reduced from \((25.1 \pm 0.18 \text{ mg/dL})\) to \((17.7 \pm 0.47 \text{ mg/dL})\) at the end of the experiment. Despite the change, no significant difference was observed between the groups.
Figure 82: Total proteins in the serum of WT and MASP-2KO mice after two weeks of AN. Both AN groups showed similar loss in the serum total protein concentration compared to control mice. The loss in both groups was approximately one third of the serum protein content. N=3 for WT and MASP-2KO control mice and N=8 for WT and MASP-2KO AN mice. Bars represent Mean±SEM.

5.2.5 Renal histopathological changes in WT and MASP-2KO mice after AN

Adriamycin nephropathy in BALB/c mice is characterised by focal segmental sclerosis among other intense renal damage phenotypes. Observation of kidney sections from AN mice stained with H&E under light microscope showed several histopathological changes in both treated groups. These changes included focal intratubular cast formations, reabsorption droplets in tubular cells, deteriorated tubular brush border and overloaded tubules, glomeruli appeared increased in size and some of them contained vacuoles and hyaline depositions. These lesions were observed in both treated strains as shown in Figure 83 to similar extent. As examination under light microscopy is not a quantitative evaluation method, it was not possible to precisely evaluate the extent of injury in the examined tissues.
5.2.6 Renal inflammation in WT and MASP-2KO mice after two weeks of AN

To examine the degree of renal inflammation in WT and MASP-2KO mice after AN, sections from each group were stained with F4/80 macrophage specific antibody. Examination of these sections under light microscope showed a prominent increase in the macrophage influx in WT AN mice compared to MASP-2KO mice at the same level of treatment (Figure 84). Across the sections, dense macrophage populations were observed frequently in WT mice and to a much less extent in MASP-2KO mice. Quantitation of the stained area in these
sections showed that the macrophage influx in the WT group is prominently more than that of the MASP-2KO group (Figure 85). Statistical comparison using one-way ANOVA showed a highly significant difference between groups (****P<0.0001). Bonferroni’s correction method also showed a highly significant difference between AN WT and MASP-2KO groups (WT 2.68 ± 0.2 % vs. MASP-2KO 1.9 ± 0.14 %). This suggests that absence of the lectin pathway functional activity in MASP-2KO mice could be the reason behind the reduced macrophage infiltration.

Figure 84: Mouse kidney sections stained with F4/80 macrophage specific antibody after two weeks of AN. (A) WT control, (B) WT AN mice, (C) MASP-2KO control and (D) MASP-2KO AN mice. Examination under light microscope showed an increased macrophage infiltration rates in WT mice compared to MASP-2KO mice at the same level of injury.
Figure 85: Estimation of the F4/80 macrophage infiltration in WT and MASP-2\textsuperscript{KO} mouse kidney sections after two weeks of AN. While both AN groups showed a remarkable increase in macrophage infiltration compared to respective control groups, the WT group showed a statistically highly significant increase when compared to MASP-2\textsuperscript{KO} mice at the same level of treatment (**\textit{P}=0.003).

5.2.7 Renal fibrosis in WT and MASP-2\textsuperscript{KO} mice after two weeks of AN
Kidney sections from all experimental groups were stained with Sirius Red to examine the effect of the absence of the lectin pathway on the development of fibrosis. Examination of these tissues showed an increased deposition of collagen in AN WT mice compared to AN MASP-2\textsuperscript{KO} mice (Figure 86). Quantitative analysis of these slides using one-way ANOVA showed a highly significant difference between groups (****\textit{P}<0.0001). Comparison between AN groups showed an increase in the collagen deposition in WT AN group compared to AN MASP-2\textsuperscript{KO} group (WT 17.18 ± 1.3 % vs. MASP-2\textsuperscript{KO} 11.84 ± 0.86 %), (Figure 87). Bonferroni’s post hoc analysis also showed a highly significant difference between these two groups. Sham control mice from both strains showed similar baseline staining patterns with no significant difference between groups.
Figure 86: Collagen depositions stained with Sirius Red in WT and MASP-2\(^{\text{KO}}\) mouse kidney sections after two weeks of AN. (A) WT control, (B) WT AN mice, (C) MASP-2\(^{\text{KO}}\) control and (D) MASP-2\(^{\text{KO}}\) AN mice.

![Figure 86: Collagen depositions stained with Sirius Red in WT and MASP-2\(^{\text{KO}}\) mouse kidney sections after two weeks of AN.](image)

Figure 87: Analysis of the collagen deposition in WT and MASP-2\(^{\text{KO}}\) mouse kidney sections stained with Sirius Red after two weeks of AN. Bonferroni’s correction test showed a highly significant difference between AN WT and MASP-2\(^{\text{KO}}\) mice (**P=0.0013**).
5.3 Discussion

Adriamycin nephropathy is a model of focal segmental glomerulosclerosis that is well studied in mice and characterised by progressive glomerulosclerosis, proteinuria and tubulointerstitial injury (Lee & Harris, 2011; Yasuda et al., 2010). Many authors reported that complement activation plays a basic role in the development of the injury and that induction of AN in complement deficient mice lead to significant reduction in the tubulointerstitial inflammation and fibrosis (Lenderink et al., 2007; Turnberg et al., 2006a; Tang et al., 2009).

This study aimed to evaluate the role of the LP of complement activation in AN using the only available murine model with complete lectin pathway deficiency; a mouse strain with a targeted deletion of the lectin pathway key enzyme MASP-2 (Schwaebel et al., 2011). Induction of AN in WT and MASP-2KO mice was performed using the same dose of adriamycin hydrochloride, 10.5 mg/kg, and renal inflammation and fibrosis were estimated and compared between groups. The results of this study indicate that activation of the LP significantly contributes to the injury. Lack of the LP functional activity led to significant reduction in renal inflammation and fibrosis.

The results obtained from this study showed that AN established in WT mice led to progressive renal injury characterised by severe body weight loss, proteinuria and hypoproteinaemia. These three disease features were evidently observed in week 1 of the injury, progressed in week 2 and started to recover in weeks 3 and 4. These data are in agreement with the findings reported by (Wang et al., 2000; Lee & Harris, 2011; Jeansson et al., 2009). Renal inflammation measured by macrophage infiltration was also observed in week 1, progressed in week 2 and reached higher levels in week 4. Likewise, several renal histopathological changes were observed in week 1 mice and were progressed and worsened in week 2 and 4. These data are in agreement with Lee & Harris, 2011 who also reported that C57BL/6 mice are highly resistant to AN while BALB/c mice are not.
Comparison between the data obtained from the mice of weeks 1, 2 and 4 after AN suggested that the renal injury occurred in week 2 is suitable to further investigate the activation of the complement system yet the mice welfare is considered. AN were established for two weeks in WT and MASP-2KO mice along with controls from each genotype to evaluate the role of the LP in the injury.

Induction of AN in WT and MASP-2KO mice showed comparable body weight loss in both groups during the two weeks of the experiment. Both genotypes also produced similar proteinuria of about four fold increase compared to controls and similar hypoproteinaemia. In addition, AN kidney sections from both genotypes stained with H&E showed comparable renal histopathological lesions when examined by light microscopy with no observable differences between groups. These data indicate that both genotypes received similar insult due to administration of adriamycin and that differences in renal inflammation and fibrosis are not explained by reduced proteinuria in complement KO animals.

AN in this experiment resulted in significant renal inflammation and renal fibrosis in both WT and MASP-2KO mice compared to controls. These disease markers were measured by estimation of F4/80 macrophage infiltration and deposition of collagen respectively. No significant differences were observed between WT and MASP-2KO controls. This underlines that WT and MASP-2KO mice in this experiment feature similar tissue characteristics as they are on the same genetic background, BALB/c, and differences in renal inflammation and fibrosis are not explained by genetic variations other than the homozygous presence or absence of the disrupted targeted MASP-2 allele in the MASP-2KO mice and their WT controls.

Interestingly, WT AN-treated mice in this experiment showed a highly significant increase in macrophage infiltration in the kidney compared to AN MASP-2KO mice. This observation suggests that deficiency in the LP functional activity significantly reduced the renal inflammation. Many authors previously
reported similar findings when they studied the role of complement activation in proteinuric nephropathies (Fearn & Sheerin, 2015; Turnberg et al., 2006a; Hara et al., 1991) and previous reports speculated that the injury is basically mediated via the alternative pathway of complement activation (Lenderink et al., 2007; Morigi et al., 2016). The current study highlights that the LP is a key element in the development of AN and Lack of its functional activity led to significant reduction in renal injury.

Similarly, kidneys of WT AN mice showed a highly significant increase in the deposition of collagen compared to MASP-2\textsuperscript{KO} AN kidneys. This is in line with Turnberg et al., 2006a; Boor et al., 2007 and Eddy et al., 2012 who reported that complement activation mediated tubulointerstitial fibrosis. However, these authors investigated the activation of the complement system through models of C5 and C3 deficiencies which are not specific to certain pathway. The current study highlights for the first time the role the LP plays in the development of renal inflammation and fibrosis in AN.

5.4 Conclusions

- The data obtained so far from this on-going study suggest that the LP plays an essential role in the development of renal injury in AN
- The data showed a significant degree of protection from renal inflammation and fibrosis due to deficiency of the LP functional activity
- These data are in line with the data obtained from the POP and the UUO models in this study
- These promising data suggest further investigations of the model
Establishment of a Novel Mouse Model of Complement Deficiency with Targeted Defective Genes for Key Components of both the Classical and the Lectin Pathways of Complement

6.1 Introduction

The complement system is a critical component of the immune system, playing an important role in the inflammatory response and becomes activated as a result of tissue damage or microbial infection. The activation of this system is mediated through one or more of three pathways, named the classical, the lectin and the alternative pathways.

C1rA, C1qA and MASP-2 are three key proteins of the complement system. C1rA and C1qA is the essential recognition subcomponent of the classical pathway activation route while MASP-2 is the essential effector enzyme of the lectin pathway. Deficiency of either component leads disrupts their respective pathway. Several publications involving the use of either MASP-2 or C1qA deficient mice highlighted significant roles of both the lectin and the classical pathways in mediating a variety of inflammatory diseases (Schwaebel et al., 2011; Cervera et al., 2010). Establishment of a mouse line deficient in both the lectin pathway and the classical pathway would generate a unique animal model to investigate a possible synergistic co-operation between both the LP and the CP in mediating inflammatory renal pathology.

C1qKO and MASP-2KO mice show no gross abnormalities and are healthy and fertile. Previous attempts to cross both lines failed because both genes are closely linked on mouse chromosome 4 and are located too close to each other, less than 100 centi Morgan, to make a cross-over event between both targeted alleles likely. We know from existing complement deficient mouse strains, in particular the C3 deficient line, that even a total deficiency of complement does not predispose gross abnormalities and that the mice breed well. It is therefore likely that a mouse deficient in both C1qA and MASP-2 genes would be viable.
Two approaches were tried to resolve the challenge to achieve a mouse line with a combined deficiency of the classical and the lectin pathways: i) targeting C1rA gene located on mouse chromosome 6 instead of C1q; and ii) targeting the MASP-2 gene by using CRISPR/Cas9 technology in the C1q$^{KO}$ deficient mouse line.

6.2 Generation of a conditional targeting construct to target the murine C1rA gene using a recombineering-based technology

Recombineering is an *in vivo* method of genetic engineering used primarily in *E. coli* that utilizes relatively short base homologies. Because recombineering is based on homologous recombination it allows insertion, deletion or alteration of any sequence precisely and is not dependent on the location of restriction sites.

One of the major limitations for generating conditional knockout (CKO) mice is the difficulty and time it takes to make a CKO targeting vector. The conventional approach is to find appropriate restriction enzyme sites that are located in or near the gene. These sites are then used to ligate together LoxP sites and various other DNA fragments such as homology arms, a positive selection marker such as PGKNeo, and a negative selection marker such as MC1TK. The problem with this approach is that restriction sites are not always located in convenient places, and this can severely limit where LoxP sites can be placed. A newer and much simpler approach makes use of homologous recombination to construct the targeting vector. This new form of chromosome engineering, termed recombineering (Liu *et al.*, 2003; Muyrers *et al.*, 2001), makes it possible to introduce LoxP sites and selectable markers anywhere in a gene, and greatly shortens the time it takes to make a targeting vector.

6.2.1 Distribution of the expression levels of C1rA and C1rB genes in male and female mouse tissues

The high homology of the duplicated C1rA and C1rB genes indicates that they are probably both capable to assemble within the C1 complex. This suggested the need to study the expression levels of both C1rA and C1rB in various male and
female mouse tissues to determine which of the two genes is expressed and where. Targeting the correct gene that encodes the C1r component within the C1 complex is essential to successfully knockout of the classical activation pathway of complement.

Results obtained from qPCR analysis of different mouse tissues confirmed that C1rA gene is expressed in all tissues tested. C1rA is most abundantly expressed in the liver of both male and female mice, followed by abundant expression in the small intestine, seminal vesicle, spleen and ovarian fat pad. Results showed that the C1rB gene is exclusively expressed in the prostate gland and coagulating gland of the male mouse whereas the expression of C1rB was undetectable in any other tissues tested including liver, lung, spleen, heart, kidney, small intestine and brain (Figure 88).
Figure 88: Tissue distribution of C1rA and C1rB mRNA expression levels measured by qPCR in male (A) and female (B) mice. Expression levels were estimated relative to the expression of the house keeping gene GAPDH.

6.2.2 Design of the C1rA targeting construct

PL451 vector is a conditional knockout vector, which offers the option to keep the targeted gene operational. Once targeted, gene interruption can be carried out at a later stage through targeted digestion using site-specific recombinases. This valuable feature enables to even knockout genes that fulfil roles during
Establishment of a CP/LP Double Deficient Mouse

embryogenesis and allow them to work during that period but switch off these genes once the mice reached adulthood. Construct design with this feature requires the selection of two cassettes with no affected restriction sites in the exons of the targeted gene. One of these cassettes should contain a part of the gene that if deleted the gene is disrupted. Figure 89 shows the A and B cassettes chosen in accordance to these requirements. The A cassette is 840 bp segment falls in the intron between the second and the third exons and the B cassette is approximately 2 Kb containing the exon 3. Figure 90 shows the construct design with two LoxP sequences surrounding exon 3 of the C1rA gene. These two LoxP recombination sites are responsible for the conditional deletion once the gene is digested by Cre enzyme. This event deletes casB1 containing the exon 3 of the C1rA gene. Knocking out exon 3 causes a frame shift mutation that deforms the rest of the gene. This leads to the translation of fragmented polypeptides, which are probably meaningless as shown in Figure 91.

Figure 89: Schematic diagram represents the murine C1rA gene and showing the locations of the construct homologous cassettes A and B
Establishment of a CP/LP Double Deficient Mouse

Figure 90: Schematic diagram for the design of a conditional knockout construct for a targeted deletion of exon 3 from the murine C1rA gene. Red arrows represent the locations of LoxP sites before and after exon 3.

Figure 91: Estimation of the truncated protein product from the targeted C1rA gene after deletion of exon 3. Black letters represent the unchanged amino acids. Blue letters represent the changed amino acids after the frame shift mutation. Red asterisks represent stop codons.

6.2.3 Restriction fragment length polymorphism gene targeting screening

The C1r gene is duplicated in the mouse genome and present as two highly homologous genes C1rA and C1rB (Garnier et al., 2003). Although the construct
is designed to accurately target the C1rA gene; there is a possibility that the construct recombines with the homologous C1rB gene. To have a reliable method to confirm a targeted recombination event, three restriction enzymes were determined to produce variable length of DNA restriction fragments by digestion of the targeted genomes. The pattern of restriction fragment length polymorphism (RFLP) indicates the location of the recombination event occurred. In this case, the chosen three restriction enzymes that generated informative RFLP pattern are XhoI, BamHI and PstI. The calculated lengths of the genomic DNA fragments are shown in Table 3.

Table 3: Sizes of the restriction fragment length polymorphism of the murine C1rA and C1rB genes and their predicted targeted recombinants. Sizes are in Kb.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>C1rA gene</th>
<th>Recombinant C1rA</th>
<th>C1rB Gene</th>
<th>Recombinant C1rB</th>
</tr>
</thead>
<tbody>
<tr>
<td>XhoI</td>
<td>5.113</td>
<td>6.964</td>
<td>13.466</td>
<td>15.317</td>
</tr>
<tr>
<td>BamHI</td>
<td>2.214</td>
<td>4.065</td>
<td>4.574</td>
<td>6.425</td>
</tr>
</tbody>
</table>

6.2.4 Assembly of the construct homology arms in bacterial cloning vector

The construct was designed with three arms of homology that needed to be assembled and cloned in bacteria before they transferred to the mammalian expressing vector PL451. The bacterial cloning vector pGEM®-T Easy was used.

6.2.4.1 PCR amplification of the construct cassettes

To generate the construct three arms of homology, named CasA, CasB1 and CasB2, combinations of modified PCR primer pairs were designed in house and used to amplify the cassettes. These primers were designed with added restriction
sites to facilitate the insertion of the produced cassettes into the cloning and the targeting vectors. These restriction enzymes were selected because they are not contained in the backbone of any of the two vectors used or in the cassettes themselves. Restriction sites for these enzymes are present only in the insertion sites of the vectors. Therefore restriction digestion reactions with these enzymes cut exclusively in desired locations. The sequences of the primers used and the added restriction enzymes are shown in Table 4. Figure 92 shows an example of thermal gradient PCR performed to optimize the annealing temperature of the primers. In this reaction, optimum annealing temperature determined to produce CasB1 was 65°C (Figure 92, Lane 8). The three cassettes were amplified using a high-fidelity DNA polymerase (Thermo Fisher Scientific cat number F530L) (Figure 93) and purified from the PCR reaction buffer using QIAquick® nucleotide removal kit. Figure 94 shows the sequence of the leading strand of cassette A and the modified primers that were used to amplify it.
Table 4: PCR primer pairs used to amplify the homologous cassettes of the C1rA gene targeting construct

<table>
<thead>
<tr>
<th>Cassette</th>
<th>Forward primer name, sequence and (modified restriction enzyme)</th>
<th>Reverse primer name, sequence and (modified restriction enzyme)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cassette A</strong> (CasA)</td>
<td>Sall_F_CasA gtcgACGAGAAAAACTCCACACTTC (Sall)</td>
<td>HindIII_R_CasA aagcTTCTGCTTCGTCCCAATTC (HindIII)</td>
</tr>
<tr>
<td><strong>Cassette B1</strong> (CasB1)</td>
<td>NotI_F_CasB1 ggggcgCCTCCCCATGTATTCCCTGTAA (NotI)</td>
<td>XbaI_R_CasB1 tctAGGTGTGTATACAGGGAGG TAGAT (XbaI)</td>
</tr>
<tr>
<td><strong>Cassette B2</strong> (CasB2)</td>
<td>XbaI_F_CasB2 tctAGATAGCTTTAATCAGACTGG (XbaI)</td>
<td>SacII_R_CasB2 cggcgGTCCAAGGTCCCAAGGAAG GA (SacII)</td>
</tr>
<tr>
<td><strong>LoxP</strong></td>
<td>XbaI_LoxP_Sense ctag ATAACTTCGTATA ATGTATGC TATACGAAAGTTAT (XbaI)</td>
<td>XbaI_LoxP_Antisense ctag ATAACTTCGTATA GCATACT TATACGAAAGTTAT (XbaI)</td>
</tr>
</tbody>
</table>
Establishment of a CP/LP Double Deficient Mouse

Figure 92: Agarose gel image showing a thermal gradient PCR to optimize the annealing temperature of the CasB1 primers pair. Annealing temperatures from 1 to 8 were 56.3, 57.1, 58.4, 59.8, 61.3, 62.7, 63.9, 65.1 and 66.5°C respectively.

Figure 93: Agarose gel images showing the PCR amplicons of CasA (A), CasB1 (B) and CasB2 (C)
6.2.4.2 Bacterial cloning of the construct homology arms

For bacterial cloning of the construct cassettes, the purified PCR products were A-tailed and were ligated into the pGEM®-T Easy vector following standard ligation protocol from the manufacturer. Ligates were transfected into a chemically receptive (competent) *E. coli* bacteria of the strain TOP10F as described in the materials and methods section and bacteria were cultured on LB agar plates containing X-gal/IPTG. Successful cloning of DNA inserts into pGEM®-T Easy vector was confirmed by blue/white colony screening. White bacterial colonies indicated the successful insertion of the ligated cassette into the vector (leading to the disruption of the β-galactosidase gene that catalyses X-gal) while blue coloured colonies indicated absence of inserts within the multiple cloning site inserted within the β-galactosidase gene in the vector.
White bacterial colonies were isolated from the cloning reactions of each cassette and were cultured further in LB medium. Plasmids were retrieved from the cultured bacteria using the Wizard® Plus SV Minipreps DNA purification system and the sequences of the ligated cassettes were confirmed by DNA sequencing to ensure proper PCR amplification and cloning.

6.2.4.3 Assembly of the two arms of cassette B

The construction of the conditional knockout vector required the assembly of the two arms of cassette B, i.e. CasB1 and CasB2, with a LoxP site in the sense direction in-between them; Figure 95 shows a schematic representation for the sequence of events. Figure 96 shows the leading strand sequences of CasB1, LoxP and CasB2 ligated to each other in the right order. It also shows the primers used to amplify the cassettes, green highlight, and the nucleotide modifications added to the primers in order to insert specific restriction sites, the red highlight small letters. Exon 3 is contained in CasB1 (fuchsia highlight).

Assembly of the cassettes was performed by digesting out the cloned CasB2 by a double restriction digestion reaction using the enzymes SacII and XbaI. This digestion reaction removed the CasB2 from the pGEM®-T Easy vector (Figure 97, lanes 1 and 2) and it was purified from the agarose gel using QIAquick® Gel Extraction Kit. The same two enzymes were used to digest the CasB1/pGEM®-T Easy construct. This digestion reaction linearized the vector but did not remove CasB1 from it, as both the restriction sites are located on the same side of the cassette (Figure 97, lanes 3 and 4).

The linearized CasB1 vector was cleaned out from the digestion reaction buffers using the QIAquick® nucleotide removal kit. The use of the same restriction enzymes to excise the CasB2 and to open up the CasB1 vector allowed the ligation of CasB2 into the CasB1 vector using a standard ligation reaction. Ligates were transfected into TOP10F competent bacteria and were cloned and the proper ligation of the two cassette B arms was confirmed by restriction digestion analysis.
Confirmation of ligation of CasB2 into the CasB1 vector was performed by digesting selected plasmids with the enzyme XbaI to produce a single band at the size of 5 Kb which resembles the vector, CasB1 and CasB2 all together. Also, double digestion of the plasmids with the enzymes NotI and SacII produced two bands at the sizes 3 Kb (the vector only) and 2 Kb (CasB1 and CasB2 ligated to each other). Figure 98 shows the analysis of four plasmids, two of them showed the proper ligation of the cassettes (Figure 98, lanes 3 and 4 digested with XbaI and lanes 7 and 8 digested with NotI and SacII) and two showed the absence of CasB2 (Figure 98, lanes 1 and 2 digested with XbaI and lanes 5 and 6 digested with NotI and SacII).

Figure 95: Schematic representation of the construction of Cassette B elements in pGEM®-T Easy vector. Numbers indicate the sequence of events; first, insertion of casB1 then CasB2 and lastly the LoxP recombination site.
Figure 96: The leading strand sequences of CasB1, LoxP and CasB2 ligated to each other in the right order. Colour code: (Red small letters) nucleotide modifications added to the primers in order to insert specific restriction sites, (Green) primers, (Yellow) cassettes sequences, (Fuchsia) exon 3 of the C1rA gene, (Grey) LoxP recognition region and (Light blue) LoxP spacer region.
Figure 97: Agarose gel image showing the DNA bands of the digestion of CasB2 out of the pGEM®-T Easy vector (lanes 1 and 2) and CasB1 ligated into the vector (lanes 3 and 4).

Figure 98: Agarose gel image showing the restriction digestion analysis of CasB1 and CasB2 ligated into the pGEM®-T Easy vector.
6.2.4.4 Insertion of a LoxP recombination site into cassette B

A LoxP recombination site was synthesised with two XbaI restriction sites at the beginning and the end of its sequence. This allowed the insertion of this LoxP site in the desired location in-between CasB1 and CasB2 as this is the restriction enzyme the cassettes were designed with, at the end of CasB1 and the beginning of CasB2. The insertion of the LoxP site was performed by digesting the vector with XbaI to cut between the two cassettes and ligating the LoxP site in a DNA ligation reaction. To minimise relegation of the vector after digestion with XbaI, the vector was dephosphorylated using the Rapid DNA Dephos Kit, Roche. After ligation of the LoxP site, ligates were transfected into TOP10F competent bacteria and were cloned.

In this ligation reaction, the insertion of the LoxP site could happen in the sense or the anti-sense direction with equal possibilities as both sides contained the same sequence. Also more than one LoxP site could be inserted in this ligation. To validate that the insertion of LoxP was in the sense direction and one LoxP site only was inserted, two PCR reactions were performed. In the first reaction, the forward PCR primer of CasB1 (NotI_F_CasB1) was used with the single strand antisense sequence of the LoxP. This positive confirmation reaction should produce a PCR product at approximately 0.7 Kb only if one or more LoxP sites are in the sense direction. In the second PCR reaction, the single strand antisense sequence of the LoxP was used with the reverse PCR primer of CasB2 (SacII_R_CasB2). This negative confirmation reaction should produce a PCR product at approximately 1.2 Kb only if one or more LoxP sites are in the antisense direction.

Eight plasmids were examined using these PCR reactions (Figure 99). Preparations 4, 5, 6 and 7 showed bands at 0.7 Kb in the positive confirmation reaction (Figure 99A) and did not show bands in the negative confirmation reaction (Figure 99B). This suggested that the LoxP site was inserted in these plasmids in the desired sense direction. Ligate 1 and 3 showed bands in the negative confirmation reaction and not in the positive suggesting the LoxP site
was inserted in the antisense direction. Ligate 2 did not show any band in either of the reactions suggesting no LoxP sites were inserted and ligate 8 showed bands in the two reactions suggesting the insertion of LoxP sites in both the sense and the antisense directions in this ligate.

To confirm the insertion of the LoxP site in the desired place and direction and to validate that no more than one LoxP site was inserted, PCR positive samples were sequenced. Sequencing data confirmed the insertion of only one LoxP site and was in the desired sense direction (Figure 100), boxed in blue in the figure. Sequencing also confirmed that the two arms of cassette B are in the proper orientation.
Figure 99: Agarose gel images showing PCR products of positive (A) and negative (B) insertion of LoxP site in the sense direction.
Establishment of a CP/LP Double Deficient Mouse

Figure 100: Sequencing data of a cassette B ligate with a single LoxP recombination site (blue box) inserted in the desired place and in the sense direction.

6.2.5 Assembly of the C1rA targeting construct in the vector PL451
Successful generation of the homology arms in pGEM®-T Easy led to the assembly of these homology arms into the mammalian conditional knockout vector PL451. To perform that, properly assembled cassette B containing the LoxP recombination site was digested out from the bacterial pGEM®-T Easy vector by double digestion using NotI and SacII enzymes and purified. The PL451 vector was also double digested with these two enzymes and the cassette B was ligated into it. Plasmids were transfected into TOP10F competent bacteria and were cloned. Figure 101 shows the purified plasmids after cloning at approximately 7 Kb, this is the total size of the PL451 vector (approximately 5 Kb) and the cassette B (Approximately 2 Kb) ligated to it.

To add cassette A to the PL451 construct, cassette A and the pGEM®-T Easy/Cassette B plasmid were double digested with the enzymes SalI and HindIII and ligated to each other. Ligates were cloned in bacteria and were purified using Wizard® Plus SV Minipreps DNA purification system. The total size of the
construct containing the homology arms is approximately 8 Kb. Purified plasmids were examined on agarose gel and two of the preparations showed the right size band (Figure 102, lanes 6 and 8).

Figure 101: Agarose gel image showing the ligation of cassette B to the mammalian vector PL451 (lanes 1, 2, 3, 5 and 6). Lane 4 is showing the vector only.
6.2.6 Confirmation of proper assembly of the construct by DNA sequencing

To confirm the proper assembly of the construct, positive samples were sequenced. The data confirmed the proper assembly of all the cassettes in the vector. Figure 103 shows parts of the sequenced cassettes in the PL451 vector and the LoxP site in-between cassette B arms is showed boxed in green (Figure 103B).
6.2.7 Electroporation of the C1rA construct into 129/Sv ES cells

Embryonic stem cells from the mouse line 129/Sv were obtained from the Danish Genetically Modified Animals Resource (DAGMAR), Aarhus University, Denmark. ES cells were trypsinised and washed twice in PBS and approximately $1 \times 10^7$ cells were suspended in 0.8 ml PBS and transferred into the electroporation cuvette. 25 µg of linearized construct (1 µg/µl) were added to the cell suspension in the electroporation cuvette and mixed. Using the GenePulser BioRad electroporator, the ES cells were electroporated with 240 V, 500 µF electric pulses for 50 mSec. Cells were then washed twice in ES medium and split in four 60 mm culture dishes with mouse embryonic feeder cell layer (MEFs). The next day media were changed with a neomycin supplemented medium for selection. The selection medium was changed daily and survived colonies of ES cells (Figure 104) were picked after a week and cultured in 96-well plates.
Establishment of a CP/LP Double Deficient Mouse

6.3 Establishment of a novel mouse model of complement deficiency through combined disruption of the C1q and the MASP-2 genes

Since the conventional procedure to cross two mouse lines with defined single deficiencies does not work, the proposed strategy is to target MASP-2 gene in the C1q\(^{\text{KO}}\) mouse line through pronuclear injection of CRISPR/Cas9 reagents.

6.3.1 Design of the CRISPR based targeting of the MASP-2 gene

The online database used to design this CRISPR-based gene targeting experiment was the Harvard University web tool ChopChop\(^{\text{®}}\) (Montague \textit{et al.}, 2014). Figure 105 Shows the MASP-2 gene with all predicted gRNAs on it. The deletion event was designed to excise a portion of exon 9, exon 10 and most of exon 11 of the MASP-2 gene, approximately 2.3 Kb in total, by Non-Homologous End Joining.

The work on this project was put on hold at this stage due to the implementation of a new approach to establish the targeted transgenic mouse model, i.e. the CRISPR approach.

Figure 104: 129/Sv ES cells transfected with the C1rA conditional knockout construct and growing on MEF cells in a neomycin-supplemented selection medium
Establishment of a CP/LP Double Deficient Mouse

approach NHEJ. This resembles the deletion strategy previously used for the establishment of MASP-2\textsuperscript{KO} mouse line (Schwaeble \textit{et al.}, 2011). To achieve that, four gRNAs were chosen, two on each side of the targeted deletion, from high-rank guides predicted by ChopChop. Two gRNAs were used on each side of the targeted deletion to improve the CRISPR activity (Personal contact with Dr Brendan Doe, Wellcome Trust Sanger Institute, Cambridge, UK). All the gRNA guides used are on the leading strand of the MASP-2 gene and showed no predicted off-target activity on the mouse genome (Table 5). Figure 105A shows a schematic diagram of the MASP-2 gene and Figure 105B shows the targeted part of the gene and the locations of the used gRNAs.
Table 5: CRISPR gRNAs used to target the murine MASP-2 gene

<table>
<thead>
<tr>
<th>Given name</th>
<th>Ranking</th>
<th>Target sequence</th>
<th>Genomic location</th>
<th>Exon</th>
<th>Strand</th>
<th>GC content (%)</th>
<th>Off-targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>M_MASP2_L1</td>
<td>23</td>
<td>TGACCTACCCAATGCGCATGTGG</td>
<td>chr4:148612057</td>
<td>9</td>
<td>+</td>
<td>57</td>
<td>0 0 0</td>
</tr>
<tr>
<td>M_MASP2_L2</td>
<td>24</td>
<td>GGTAAGTACCTTCTTCATGGAGG</td>
<td>chr4:148612166</td>
<td>9</td>
<td>+</td>
<td>48</td>
<td>0 0 0</td>
</tr>
<tr>
<td>M_MASP2_R1</td>
<td>29</td>
<td>TGTGCTGGCTTAGAGACTGGTGG</td>
<td>chr4:148614312</td>
<td>11</td>
<td>+</td>
<td>57</td>
<td>0 0 0</td>
</tr>
<tr>
<td>M_MASP2_R2</td>
<td>32</td>
<td>GGGTTCCATTAATTGTGGGCGG</td>
<td>chr4:148614425</td>
<td>11</td>
<td>+</td>
<td>57</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>
Figure 105: MASP-2 gene showing the gRNAs predicted by the web tool ChopChop® (A) and part of the gene showing exon 9, 10 and 11 (B) showing the location of the gRNAs used in this study

6.3.2 Validation of the C1q^{KO} mouse line sequence preservation

Due to short sequences of CRISPR gRNAs which is approximately 23 nucleotides, mismatches to the targeted sequence in as little as one nucleotide could inhibit the gRNA activity. This suggested the need to sequence the genomic DNA for the predicted gRNA targets in the C1q^{KO} mice beforehand. This is to ensure that no mutations occurred in the C1q^{KO} mice in these loci. Cassettes from the genomic DNA of the C1q^{KO} mice containing the targeted sequences were amplified by PCR and sequenced (Figure 106). Sequencing data confirmed that all the targeted sequences are completely matching the predicted gRNAs.
Figure 106: Sequencing of the targeted loci in C1qKO mice. (A) sequence targeted by the gRNA M_MASP2_L1; (B) sequence targeted by the gRNA M_MASP2_L2; (C) sequence targeted by the gRNA M_MASP2_R1 and (D) sequence targeted by the gRNA M_MASP2_R2
6.3.3 Synthesis of the CRISPR gRNAs

The four chosen gRNAs were synthesised in house by *in vitro* transcription using the GeneArt™ Precision gRNA Synthesis Kit as described in the methods. The integrity of the gRNAs produced was checked by spectrophotometric analysis and by running samples of them on agarose gel (Figure 107). All the four gRNAs produced a band at approximately 140 bp size as expected suggesting the success of the gRNA synthesis.

![Figure 107: In vitro transcribed gRNAs. Samples were run on the gel to check the integrity of the synthesized gRNAs, (1) M_MASP2_L1; (2) M_MASP2_L2; (3) M_MASP2_R1 and (4) M_MASP2_R2](image)

6.3.4 Microinjection of the CRISPR reagents into C1qKO embryos and embryo transfer

C1qKO 1-cell stage embryos were produced after super ovulating C1qKO females and mating them with C1qKO stud males (Botto, 1998). The four synthesised gRNAs along with the Cas9 mRNA enzyme (TriLink Biotechnologies cat number L-6125) were microinjected intracytoplasmic into the embryos. Embryos
were pre-washed and cumulous cells were removed by hyaluronidase digestion before microinjection. Up to 15 of the surviving embryos were transferred into the tubular ampullae of CD-1 foster mothers mated with vasectomised males the night before the embryo transfer. Foster mothers were kept in standardised conditions and pregnancies were confirmed by the third week after embryo transfer. Table 6 shows the numbers of produced, injected, surviving and transferred embryos and the numbers of born pups.

16 pups were born alive after these experiments, 12 of them survived the postnatal care and 4 of them died shortly after birth. 9 of these pups were genotyped and 3 of them were born recently and the genotyping of them is ongoing (Table 6).
Table 6: Outcomes of 6 microinjection sessions performed to inject the CRISPR reagents into C1q\textsuperscript{KO} embryos. Total of 12 CRISPR targeted pups were obtained.

<table>
<thead>
<tr>
<th>Microinjection session</th>
<th>No donor females</th>
<th>Copulatory plugs</th>
<th>Confirmed fertilization</th>
<th>Total Embryos harvested</th>
<th>Fertilized 1 cell</th>
<th>Embryos injected</th>
<th>Embryos Lysed (post injection)</th>
<th>Embryos transferred</th>
<th>No of foster mothers</th>
<th>Pregnancy confirmation</th>
<th>No of pups born</th>
<th>No of pups survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>96</td>
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<td>33</td>
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<td>65</td>
<td>33</td>
<td>30</td>
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<td>26</td>
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<td>228</td>
<td>201</td>
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<td>8</td>
<td>16</td>
<td>12</td>
</tr>
</tbody>
</table>
6.3.5 Genotyping of the CRISPR targeted pups

Successful CRISPR activity of all the injected gRNAs is expected to produce a 2.3 Kb deletion from the targeted location on the MASP-2 gene. However, the double strand break caused by any of the injected gRNAs could be repaired by many possible courses of action in the non-homologous end joining DNA repair pathway. In addition, the activities of the injected gRNAs are not guaranteed to work simultaneously in all cases. Therefore, several genotyping outcomes could be detected in the CRISPR targeted animals.

Several primer pairs were used to amplify the targeted sequence and were optimized in WT mice (Figure 108). A pair of them, flanking the expected deletion, produced a high quality PCR band on the gel. This pair produced a PCR amplicon of approximately 2.6 Kb in the wild type and should produce a 0.3 Kb if the targeted cassette was deleted. This primer pair was further optimized by thermal gradient PCR (Figure 109) to determine the optimal PCR conditions to produce a highly specific PCR amplicon and the optimal annealing temperature determined was 67ºC. This step allowed the production of well-defined and sharp PCR bands on the agarose gel to permit the detection of possible small size deletions.
Establishment of a CP/LP Double Deficient Mouse

Figure 108: Optimization of the CRISPR genotyping PCR primer pairs

Ear snips were collected from all the 9 pups and genomic DNA was extracted. PCR genotyping was performed using the optimised pair of primers and amplicon sizes were compared to the PCR amplicons of WT mice (Figure 110).

Figure 109: Thermal gradient PCR to determine the optimum annealing temperature for the CRISPR genotyping PCR primer pairs. (1) 66°C; (2) 67°C and (3) 68°C.
PCR amplicons produced from all the targeted pups were at the size of WT mice, approximately 2.6 Kb, and none of them showed the targeted 2.3 Kb deletion, this suggested the need to examine the sequence of these DNA amplicons.

Figure 110: Genotyping of the CRISPR targeted pups. Lanes 1 to 9 are pups IDs 141, 142, 143, 153, 161, 162, 171, 172 and 173. Lanes 10 to 12 are wild type control samples. None of the CRISPR targeted pups showed the targeted deletion.

6.3.6 Confirmation of CRISPR pups genotyping by DNA sequencing

Sequencing of the PCR products showed that 8 of the samples are WT, i.e. not showing any CRISPR activity, and one of them is showing heterozygous DNA sequence in the loci of two of the gRNAs. These are the far left and the far right gRNAs given the names M_MASP2_L1 (Figure 111) and M_MASP2_R2 (Figure 112), no frame shift mutations or deletions were detected. Compared to WT mice, the DNA sequences in the expected cut loci of these two gRNAs showed unrecognised nucleotides (N), boxed in red in Figure 111 and Figure 112, suggesting a mixed signal as would result from a heterozygous allele in this mouse, the gRNAs sequences are showed boxed in green in these two figures. These data suggested the need to backcross breed this mouse with WT littermates in order to split the heterozygous allele and analyse it further.
Figure 111: DNA sequencing of the mouse ID 141 (A) compared to a WT mouse (B). Analysis showed a CRISPR activity in the cut location of the forward gRNA M_MASP2_L1 (boxed in red). The sequence of the gRNA is boxed in green on the WT sequence.
Figure 112: DNA sequencing of the mouse ID 141 (A) compared to a WT mouse (B). Analysis showed a CRISPR activity in the cut location of the reverse gRNA M_MASP2_R2 (boxed in red). The sequence of the gRNA is boxed in green on the WT sequence.
6.4 Discussion

The currently available transgenic models of complement deficiency provide a useful tool to study the mechanisms underlying complement activation in inflammatory diseases. Models of complete deficiency of the LP and the CP are currently available and used in this study. However, a model of collective deficiency of both the CP and the LP is not available. This mouse line will be very useful to establish a possible synergistic co-operation between both the LP and the CP in mediating inflammatory renal pathology. To establish this novel mouse model, two transgenic technologies were approached in this study; a) recombineering technology was applied to target the C1rA gene in WT ES cells; b) CRISPR/Cas9 technology was applied to target the MASP-2 gene in C1qKO mice.

C1rA is an essential protein for the activation of the CP. The interruption of its gene is expected to produce a mouse line completely deficient in the CP. This mouse line is expected to establish a CP/LP double deficient mouse line by backcrossing with the currently available MASP-2KO mice. Establishment of this model of double CP/LP deficiency by backcrossing the already available C1qKO and MASP-2KO mice is unlikely to happen because the C1q and the MASP-2 genes are closely linked, less than a 100cM away from each other, on the mouse chromosome 4. The C1rA gene is located on the mouse chromosome number 6 and backcrossing with the MASP-2 gene, located on chromosome 4, should be achievable.

Previous attempts to establish the CP/LP double deficient mouse model were not successful. C4KO is one of the models established for this purpose and is deficient in the complement C4 protein which is one of the basic components of the C3 convertase in both the CP and LP. However, previous reports defined a unique MASP-2–dependent, C4-independent route of C3 activation (Schwaebel et al., 2011; May & Frank, 1973). Therefore, this model is completely deficient in the CP but not the LP. C1sA+- mouse line was also established to permit
backcrossing with MASP–2KO mice and produce CP/LP double deficient mice. However, homozygous interruption of the C1sA gene was lethal (Jassal, 2010) therefore C1sA−/− mouse line could not be established. Therefore, targeting the C1rA gene was attempted in the current study.

In humans, gene mapping and genomic DNA sequencing data showed that C1r serine protease gene is encoded by a single gene and is primarily expressed, but not exclusively, in hepatic cells (Nakagawa et al., 2003). However, a previous report showed that the murine C1rA serine protease gene is duplicated forming two different gene clusters, i.e. C1rA and C1rB (Garnier et al., 2003). These data suggested the need to investigate the expression levels of C1rA and C1rB genes in various mouse tissues in order to determine the active isoform of these two copies of the gene. Targeting the active isoform of the gene is essential for successful knockout of the CP.

qPCR analysis of the expression levels of the C1rA and C1rB genes in various tissues from male and female mice showed that C1rA gene is expressed in all the studied tissues. In contrast to that, the data showed that the C1rB gene in the male mouse is exclusively expressed in the reproductive glands, i.e. the prostate and the coagulating glands, and not expressed in any of the studied female tissues. These data are in agreement with Garnier et al., 2003 who reported that C1rA gene in mouse is expressed in various tissues while the C1rB gene is expressed exclusively in the male reproductive tissues. These data suggested that C1rA is the active isoform of the gene and therefore interruption of it should lead to deficiency of the CP.

A specific vector was designed and constructed to flank exon 3 of the murine C1rA gene with two LoxP recombination sites. These LoxP site were used to mediate the deletion of the DNA sequence in-between them when they are recombined with Cre enzyme, this allows the interruption of the gene when desired (Liu et al., 2003; Newman et al., 2015). The data presented in this thesis demonstrate the protocols that were performed to construct this vector. After
making this construct, murine 129/Sv ES cells were targeted with this construct by electroporation and positively transfected cells were selected by means of antibody selection. However, during the course of this research, a recently discovered gene targeting technology, i.e. CRISPR/Cas9 technology, was suggested and I adapted in the experimental design of this project. Several authors reported that, compared to conventional gene targeting protocols including recombineering, CRISPR technology is more efficient, more cost effective and time saving approach to target murine genes (Lin et al., 2016; Miano et al., 2016; Williams et al., 2016; Bishop et al., 2016; Kalebic et al., 2016; Carroll et al., 2016; Schwank & Clevers, 2016). This growing body of evidence strongly recommended the implementation of this technology to establish the desired mouse model of CP/LP double deficiency therefore the work on the generated C1rA construct was held.

Application of the CRISPR technology in this project allowed targeting the MASP-2 gene in the C1qKO mouse line. Targeting the MASP-2 gene using CRISPR technology provided several advantages over targeting the C1rA gene in WT mice using recombineering technology. This included overcoming the need to backcross manipulated mouse lines since targeting the MASP-2 gene can be performed directly in the C1qKO mice. This overcomes a number of the technical challenges of the project concerning the feasibility of backcrossing uncharacterised mouse lines and the cost and time needed. Moreover, a previous report showed that interruption of MASP-2 gene produced a healthy and fertile mouse model that is completely deficient in LP (Schwaeble et al., 2011). In contrast to that, no data are available to show whether interruption of the C1rA gene would produce healthy and fertile mice or not, therefore interruption of the MASP-2 gene in C1qKO mice was preferred.

To achieve that, four MASP-2 specific gRNAs were designed and synthesized in house. These gRNAs along with Cas9 mRNA were injected into the cytoplasm of C1qKO one cell embryos produced in a series of precisely timed experiments. These embryos were transferred into pseudo pregnant foster mothers to complete
their due course of embryonic development and the foster mothers were kept in standardized conditions. Out of all the conducted experiments, 16 pups were born. However, four of these pups did not survive the postnatal care (25%) and died shortly. Loss of some pups after embryo transfer procedures is frequently observed due to several factors including small litter sizes and mothers sometimes abandon the pups. Some studies reported the loss of 50% of the born pups after embryo transfer (Sasaki et al., 1994; Rose et al., 2012). Genotyping of the manipulated pups revealed that one of them is showing heterozygous allele in two loci of the targeted sequences. I succeeded to establishing this novel founder mouse line towards the end of my PhD program and this line will provide a most valuable tool to establish the role of the alternative activation pathway in absence of both, the lectin and the alternative pathway in renal disease in on-going future studies.

6.5 Conclusions

- The results obtained from this study confirmed that the C1rA gene is dominantly expressed in various tissues in male and female mice while the C1rB gene expression was detected only in the male genital glands
- A conditional gene targeting vector was constructed to target the mouse C1rA gene
- A new technology, i.e. CRISPR/Cas9 approach, was adopted during the due course of this study to refine and improve the outcomes of the study
- One of the targeted mice potentially showed a CRISPR-based manipulation of its genome in two of the targeted loci
7 Summary and Future Work

7.1 Summary

This thesis presents the results of three inter-related studies aimed to establish the role of complement activation, mainly the lectin pathway, in progressive kidney disease. It also presents the technologies I approached to establish a novel mouse line of complement deficiency with targeted defective genes for key components of both the classical and the lectin activation pathways. To achieve this, I applied CRISPR technology to disrupt the MASP-2 locus in a C1q deficient mouse line. This was necessary since both the murine MASP-2 and the murine C1q genes are closely linked (less than a 100cM away from each other on the mouse chromosome 4. This mouse line will be very useful to establish a possible synergistic co-operation between both the LP and the CP in mediating inflammatory renal pathology. Since this mouse line was established towards the end of my PhD program, I could not analyse its phenotype in models of renal pathology but used for these studies the already established LP deficient mouse line with a targeted deletions of MASP-2 gene.

Two mouse strains have been used in this study; BALB/c and C57BL/6. In the three models of kidney injuries investigated in this study, data suggested that the development of the renal scarce in each model was strain dependent. Unlike C57BL/6 mice, BALB/c mice were found to be more susceptible to proteinuria and associated renal inflammatory responses both in POP and AN models (Wang et al., 2000, Ishola et al., 2006). On the other hand, C57BL/6 mice were found to be more susceptible to renal fibrosis and development of CKD in UUO studies when compared to BALB/c mice (Puri et al., 2010). This comes in line with the results generated in the current study.

Specific genetic and epigenetic factors leading to these variable susceptibilities in mice are not well defined and data available are limited. Some mouse strains are polymorphic for renin genes having either one gene (Ren-1) expressed in the
kidney or two (Ren-1 and Ren-2), suggesting differences in the regulation mechanisms of the glomerular filtration (Sigmund & Gross, 1991). However, this genetic difference was not established between C57BL/6 and BALB/c mice as both having only Ren-1 expressed in the kidney (Ishola et al., 2006). Also no ultrastructure differences in the glomerular filtration barriers or in the systemic blood pressure have been noticed between these two strains (Ishola et al., 2006). Defining the basic factor for this strain-based susceptibility of CKD in mice may prove useful in elucidating why some ethnic groups of humans are more susceptible to develop CKD than others.

Three renal injury models have been investigated in this study; each adding some more details to the understanding of the role the complement system plays in different aetiologies of renal diseases. First, POP is a model of proteinuric nephropathy characterised by leak of excessive amount of serum proteins leading to kidney tubular damage along with inflammation and scarring in the interstitium of the kidney. UUO on the other hand is a model of obstructive non-proteinuric renal inflammations and is associated with elevated interstitial hydrostatic pressure in the kidney and regression of normal blood circulation. AN is also a model of proteinuric nephropathy that is characterised by podocyte injury followed by glomerulosclerosis, interstitial inflammation and fibrosis. In common, all models encounter tubulointerstitial inflammation, however each model is unique in the cascade of events that develop the disease. The hypothesis of the current study was examined in these three models to investigate whether the complement activation plays a role in the inflammation cascade per se or is driven by particular injury mechanisms. Despite the different sets of mechanisms that lead to the development of renal scarring in each model, results from the current study showed significant reduction in the renal inflammation in all the models when the complement components MASP-2 or C1q were absent. This suggests that the classical and/or the lectin pathways of the complement system play an essential role in the development of the renal inflammation per se and this may not be driven by the mechanisms that cause or lead to developing the disease.
The first part of this study was conducted to establish the role of the lectin pathway of complement in protein overload proteinuria, an experimental model of proteinuric kidney disease that originates in the tubular compartment of the kidney. The results of this study showed that the LP contributes significantly to the development of renal tubulointerstitial injury in this model. Importantly, deficiency of the LP functional activity led to a measurable protection from renal injury. Proteinuric WT mice showed a significant increase in all inflammation markers examined compared to MASP-2\textsuperscript{KO} mice. These included macrophage infiltration, expression of TGF-β, TNF-α and IL-6 and apoptosis. In addition, the expression level of the renal fibrosis related gene Col4α1 was significantly reduced in MASP-2\textsuperscript{KO} mice compared to WT mice. Most importantly, the results showed that antibody mediated inhibition of the LP in mice resulted in reduced severity of tubulointerstitial injury. Apoptotic cells were significantly less in the mice treated with an anti-MASP-2 antibody compared to relevant isotype control groups. Similarly, the expression levels of TGF-β were also significantly reduced in the MASP-2 inhibited mice compared to isotype controls; however the macrophage infiltration, TNF-α and IL-6 expression levels were not changed by the antibody treatment. Statistical analysis showed no significant differences between MASP-2 inhibited group and the isotype control group in any of the studied genes. However, examined genes showed lower expression levels that were not up to statistical significance. This might suggest the need to investigate this model in large group sizes to allow better understanding of the results. Also the antibody administration method might need to be refined to achieve better access to the kidney tissues. Collectively, these results might suggest that the antibody targeting of the lectin pathway might provide a helpful avenue to abrogate renal injury.

In the second part of this project, the roles of the lectin and the classical pathways of complement were studied in UUO; a mouse model of obstructive kidney disease. The results showed that deficiency of either the LP or the CP leads to significant protection from renal fibrosis and inflammation. Compared to
WT mice, both MASP-2\textsuperscript{KO} and C1q\textsuperscript{KO} mice showed lower macrophage infiltration and collagen deposition in their kidneys after UUO. MASP-2\textsuperscript{KO} mice, but not the C1q\textsuperscript{KO} mice, showed biologically observable reductions in the expression levels of the inflammation related genes Il6 and Ifn-\(\gamma\), and the fibrosis related genes TGF\(\beta\)-1 and Col4\(\alpha\)1 when compared to WT mice. However, the differences in this gene expression study were not up to statistical significance except for Ifn-\(\gamma\) suggesting the need to reproduce the data in larger mouse group sizes. Furthermore, antibody mediated inhibition of the lectin pathway did not show significant differences in this model.

The data obtained from the MASP-2\textsuperscript{KO} mice experiments in these two models, POP and UUO, showed a significant reduction in renal inflammation and tissue scarring. However, the data obtained after treatment with either of the anti-MASP-2 antibodies, OMS721 or 721-SGMI-H2, did not show comparable levels of reduction in the kidney inflammation. This highlights that antibody-mediated inhibition of the MASP-2 in these models of renal injury may not be as efficient as complete absence of the MASP-2 protein at genetic level in MASP-2\textsuperscript{KO} mice. This could be looked at from several angels; first, despite the efficient inhibition of the lectin pathway in the sera of the treated mice after administration of 721-SGMI-H2, the inhibition of the LP in the kidneys may not be fully acquired. This could be due to the short time of the antibody administration prior to experimentation (7 days) compared to prenatal absence of MASP-2 enzyme in MASP-2\textsuperscript{KO} mice. This also could be due to limited penetration of the antibody into renal tissues especially in the UUO model. Moreover, due to the lack of published data on the use of these antibodies in these particular models of renal injury to suggest antibody dosing regimen, therefore, the dosing of the antibody in the current study was based on the available data from stroke mouse models (Orsini \textit{et al.}, 2016). However, the penetration of the antibody through the brain-blood barriers may not be similar to that in the kidney glomerular filtration barriers. This suggest the need to further optimize the dose to suite these particular models of renal injury.
The third part of this thesis demonstrates the early data obtained from a study aimed to evaluate the role of the LP of complement activation in AN; a model of focal segmental glomerulosclerosis. The data generated so far suggested that the LP is essential to mediate the injury. Interestingly, WT mice in this study showed a highly significant increase in macrophage infiltration and collagen deposition when compared to MASP-2$^{ko}$ mice; this underlines that activation of the LP in this model significantly adds to the injury. These early data are promising and further analysis of the model is highly recommended.

Both POP and AN are models of proteinuric renal diseases where excessive amounts of serum proteins, including complement components, are infiltrated through compromised glomerular filtration barriers and reach the tubular compartment of kidney. Normally, most of the serum-circulating complement proteins are not filtered in the kidney and are maintained in the blood stream. There are some evidence that the brush border of the tubular cells lack many of the membrane bound complement regulators (Zhou et al., 2014) and exposure to complement proteins drove these cells to epithelial-to-mesenchymal transition. Interaction between complement proteins and the relatively-lacking complement regulators brush borders of the tubular cells is suggested as one reason driving complement activation in the tubular compartment.

Initiation of the complement activation cascade in the tubular section of the kidney might lead to the production of anaphylatoxins and deposition of sub-lytic concentrations of the MAC. Injured cells at this point might be driven into cascades of initiating inflammation by producing pro-inflammatory proteins and recruitment of macrophages. This could be supported by the elevated levels of macrophage infiltration, TNF-$\alpha$ and interleukin 6 expressions observed in the kidneys of POP mice in this study. Exposure to anaphylatoxins also can stimulate cells to express C3a and C5a receptors (Braun et al., 2004; Zahedi et al., 2000) and increase collagen synthesis and expression of TGF-$\beta$. This also could be supported by elevated TGF-$\beta$ levels measured in the POP kidneys in this study and collagen deposition measured in AN mice.
Taken together, the results obtained from all the kidney disease models investigated in this project clearly demonstrate that activation of the LP contributes significantly to the renal injury in various phenotypes of kidney disease. Moreover, the results obtained from the UUO model also indicate a possible role of CP in mediating the injury. Most importantly, antibody mediated inhibition of the lectin pathway in POP model reduced the level of tissue injury in some of the studied parameters. This might suggest a novel therapeutic approach to relief renal injury in proteinuric nephropathies by targeting the activation of the lectin pathway.

The fourth part of this thesis demonstrate the technical procedures I followed to establish a novel mouse line of complement deficiency with targeted defective genes for key components of both the classical and the lectin activation pathways. The model has a wide scope of potential applications to study a variety of microbial and inflammatory diseases including kidney disease. To establish this model, a conditional KO construct was made to target the C1rA gene using a recombineering-based technology. Conditional KO technology allows the interruption of the gene at a desired time point of the mouse development or in certain tissues specifically. The conditional KO strategy was approached due to a previous report suggested that the interruption of the C1rA gene could be lethal (Jassal, 2010). However, after the generation of the conditional KO construct, no further steps were performed to target the C1rA gene in the mouse due to implementation of a recent gene targeting approach that proven more efficient. This implemented approach was to target the MASP-2 gene in C1q\(^{\text{KO}}\) mice using CRISPR/Cas9 technology. CRISPR gRNAs, specific to the murine MASP-2 gene, were designed and synthesised in house. These gRNAs along with Cas9 mRNA were microinjected into the cytoplasm of C1q\(^{\text{KO}}\) embryos and embryos were transferred into foster mothers. 12 manipulated pups were born and 9 of them were genotyped. Out of these 9 pups, analysis of one pup showed heterozygous interruption of the MASP-2 gene. Further breeding of this pup to separate and analyse the heterozygous allele is ongoing.
7.2 Future work

The data from this thesis demonstrate for the first time the protective effects of lacking the LP functional activity on renal tubulointerstitial inflammation, tubular cell injury, pro-fibrotic cytokine release and scarring in three models of kidney disease. Moreover, antibody mediated inhibition of the LP led to some protection in POP model. This might suggest a novel therapeutic approach to relief renal injury by targeting the activation of the lectin pathway. Further investigations are needed to include a more thorough analysis of the extent of the renal protection in the mouse and/or other animal models.

The data presented in the first part of the results showed that, in POP WT mice, inhibition of the LP using 721-SGMI-H2 antibody reduced the expression of some of the studied inflammatory markers. This suggests the need to further investigate the activation and cell type characterisation of the infiltrated macrophages. This investigation should allow better understanding of the mechanisms that led to accumulation of macrophages in the tissue while the expression levels of the other studied inflammation markers were comparable to healthy controls. In addition to that, the data presented in this study showed that unlike BALB/c mice, C57BL/6 mice were resistant to develop proteinuria. Due to the unavailability of BALB/c C1q\textsuperscript{KO} mice during the course of this research, determination of the role of the CP in POP remains an unmet goal of this study. Therefore, the use of BALB/c C1q\textsuperscript{KO} mice to investigate the role of the CP in this model of POP is a recommended future work.

The data obtained from the UUO model showed that antibody mediated inhibition of the LP did not show significant difference compared to isotype controls while the LP deficient mice, i.e. MASP-2\textsuperscript{KO} mice, were protected from the renal injury to some extent. This suggests the need to further investigate the pharmacodynamics of the 721-SGMI-H2 antibody in the renal tissue to assess the likelihood of the antibody to enter the renal tubule compartment in obstructive renal disease. This should shed more light on the effectiveness of targeting the activation of the LP by administration of this antibody.
The early data obtained from the third model of kidney disease studied in this project, i.e. the adriamycin nephropathy, are promising. However, this interesting avenue of the research was suggested toward the end of my project and time restraints did not allow me to investigate it further. More elaborate studies on this model are recommended, particularly to examine the effects of the inhibition of the LP in WT mice.
8 Appendix I

Automated image analysis macro code.

This code was written using the Java programming language of the free image processing software NIH ImageJ v1.51d (Schneider et al., 2012). I gratefully allow the use of this code for free for research purposes. Acknowledging the source would be appreciated.

//The code starts:

dir1 = getDirectory("Choose source directory");
list = getFilePath(dir1);
dir2 = getDirectory("Choose destination directory");
setBatchMode(true);
for (i=0; i<list.length; i++) {
    path = dir1+list[i];
    open(path);
    title1 = getTitle;
    title2 = File.nameWithoutExtension;
    run("Subtract Background...", "rolling=50 light");
    run("8-bit");
    setThreshold(0, 180);
    run("Convert to Mask");
    run("Set Measurements...", "area min max mean area_fraction redirect=None decimal=2");
    run("Measure");
    saveAs("tiff", dir2+"Threshold of "+title2+".tiff");
} selectWindow("Results");
run("Summarize");
saveAs("txt", dir2+title2+".xls");
run("Close")
run("Close All");

//End of the code.
9 References


References


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