C5a Receptors in Renal Transplantation
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C5L2 plays a Detrimental Role in Renal Ischemia/Reperfusion Injury: 
Indications for Differential Renal C5aR and C5L2 mediated Inflammatory Responses

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Manuscript in preparation
ABSTRACT

Renal ischemia/reperfusion injury (IRI) is inevitable in the clinical course of renal transplantation. The complement system, and specifically C5a, is known to be involved in IRI. There are two known receptors for C5a, C5aR and C5L2. Inhibition of C5aR protects kidneys from IRI. Although a decoy function for C5L2 has been suggested based on data from studies on leukocyte function, the role of C5L2 in renal IRI is unknown. Since C5aR and C5L2 have distinct expression patterns in the kidney, we hypothesized that C5L2 has an inflammatory function in renal IRI.

To this end, WT, C5aR−/− and C5L2−/− mice were subjected to 40 minutes of warm bilateral renal ischemia, followed by reperfusion for 1, 3 and 7 days. In contrast to WT mice, C5aR−/− and C5L2−/− mice showed attenuation of IRI-induced renal dysfunction, resulting in significant lower plasma creatinine and BUN levels compared to WT. In addition, C5L2−/− mice showed reduced acute tubular necrosis (ATN). In order to clarify the protective mechanism in C5L2−/− mice, systemic complement activation, leukocyte influx and renal gene expression profiles were analyzed. Baseline functional systemic complement activity was similar in all three strains, and activation of the complement system, especially the alternative pathway, was observed after IRI. Only subtle differences in quantities of infiltrated neutrophils, macrophages and T cells were found. However, differential gene expression profiles of inflammatory mediators were observed in kidneys from C5aR−/− and C5L2−/− compared to WT mice. Where C5aR−/− mice showed reduced IL-1β and MCP-1 expression after IRI, C5L2−/− mice showed reduced IL-6 and KC expression, and increased C3 expression.

These data indicate that renal C5L2 exerts pro-inflammatory functions of its own, rather than being a decoy receptor. In addition, the differential gene expression profiles suggest that distinct mechanisms are responsible for preservation of renal function in C5aR−/− and C5L2−/− mice after IRI.
INTRODUCTION

Renal ischemia/reperfusion injury (IRI) is inevitable in the clinical course of kidney transplantation. It is well known that the complement system is involved in IRI in various organs (1), including heart (2), liver (3), intestine (4), and the kidney (5,6). The complement system is part of the innate immune system and comprises over thirty soluble and membrane-bound proteins (7,8). This system can be activated via three different pathways, i.e. the classical (CP), lectin (LP) and alternative (AP) pathway. Activation of each of the three pathways results in activation of the central complement component C3, and subsequent activation of the common terminal pathway leading to formation of the membrane attack complex (MAC, C5b-9). Inherent to activation of the common terminal pathway is the generation of the anaphylatoxins C3a and C5a, of which C5a has the most potent chemotactic and pro-inflammatory properties.

There are two known receptors for C5a, namely C5a receptor (C5aR) (9) and C5a-like receptor 2 (C5L2) (10). These receptors have initially been identified on leukocytes, but expression of these two receptors has been shown on epithelial cells as well (11,12). We previously showed that both receptors are expressed in the kidney on distal tubular epithelial cells (13). Both receptors are seven-transmembrane receptors, and resemble a G-protein coupling receptor configuration (10). Indeed, C5aR has been shown to exert multiple pro-inflammatory functions via G-proteins. However, G-protein coupling seems virtually impossible for C5L2, since it lacks several receptor characteristics which are known to be essential for G-protein coupling (10,11,14-17). Therefore, C5L2 has been postulated to serve as a decoy receptor for C5a, preventing C5a-C5aR-mediated signaling. An alternative hypothesis suggests that C5L2 modulates C5aR-induced inflammation by interacting with C5aR-β-arrestin-complex (18). Both hypotheses implicate that C5L2 is not able to initiate intracellular signaling by itself, but only diminishes pro-inflammatory effects of the C5a-C5aR axis. In contrast, studies investigating the role of C5L2 in adipocytes suggest that C5L2 has C5aR-independent functions (19,20).

The role of C5aR in renal IRI has been studied using various animal models, showing that inhibition of the C5a-C5aR axis results in improved renal outcome after renal IRI (21-25). However, the role of C5L2 in renal IRI has not been investigated yet. Therefore, the aim of this study was to investigate the contribution of C5L2 in renal IRI-mediated inflammation. Using mice with targeted deletion of C5aR or C5L2, we assessed the effect of 40 min bilateral renal ischemia on impairment of renal function, renal histological damage, functional activity of the complement system, leukocyte infiltration and renal gene expression of inflammatory mediators.
MATERIAL AND METHODS

Experimental renal ischemia/reperfusion model
C57Bl/6 wildtype (WT), and C5aR−/− (26) and C5L2−/− (27) mice, both on C57Bl/6 background, were kindly provided by B. Lu, Harvard Medical School, Boston, USA. Mice were bred in the local animal facility of the University Medical Center Groningen. Animals were housed in groups in standard laboratory cages up to the experiments, and were housed individually after surgery up to sacrifice. Animals were allowed free access to food and water throughout the experiments. The studies were carried out under a protocol approved by the Institutional Animal Care Committee of the University of Groningen (project number 6377AA).

Male mice, aged 8 to 12 weeks, were anesthetized using 5% isoflurane/O₂. After induction, anesthesia was maintained by 2% isoflurane/O₂. Body temperature was maintained at 37°C by a heating pad during surgery, and in an incubator for neonates during ischemia. Under aseptic conditions, an abdominal midline incision was made and bilateral renal ischemia was induced by applying two non-traumatic vascular clamps per renal pedicle for 40 minutes. During ischemia, the abdominal cavity was covered with cotton soaked in sterile saline. After removal of the clamps, the kidneys were inspected for restoration of blood flow. The abdomen was closed in two layers and buprenorphine (0.1 mg/kg) was applied once subcutaneously for postoperative pain management. The animals were sacrificed at 1 day, 3 days or 7 days after ischemia/reperfusion surgery (n=8 per group). Sham operated animals were anesthetized and operated according to the protocol described above, but no vascular clamps were placed (n=8 per group). At time of sacrifice, blood and kidneys were collected for analysis.

Renal function
Creatinine and blood urea nitrogen (BUN) were measured in EDTA-plasma obtained at time of sacrifice, using a Roche Modular P system (Roche, Basel, Switzerland).

Renal morphology
Sections (4 µm) of formalin-fixed paraffin embedded left kidneys were stained with Periodic Acid Schiff. Renal damage was scored as percentage of necrotic tubuli (acute tubular necrosis, ATN) in the cortical area, by two individual observers using a semi-quantitative method. A scoring system ranging from 0 to 4 was applied (0 = 0% ATN, 1 = <10% ATN, 2 = 10-25% ATN, 3 = 25-50% ATN and 4 = >50% ATN).

Systemic complement consumption
Mouse complement pathway activity was assessed in EDTA-plasma with functional complement ELISAs for each of the three pathways, i.e. classical (CP), lectin (LP) and
alternative (AP) pathway, as recently developed by Kotimaa et al (Chapter 5). In brief, purified human IgM (produced in-house, Leiden University Medical Center (LUMC), Leiden, the Netherlands (28)) was coated at 1 µg/ml for CP ELISA, 10 µg/ml mannan for LP (Sigma-Aldrich, St. Louis, MO, USA) and 3 µg/ml LPS from Salmonella enteritis for AP (Hycult Biotechnology, Uden, the Netherlands). Each incubation step was 60 min at 37°C and after each step the plates were washed three times with PBS/0.05% Tween 20.

CP and LP plates were blocked with PBS/1% BSA and samples diluted 1/20 into ice-cold BVB++ buffer (Veronal buffered Saline/0.5 mM MgCl$_2$ / 2 mM CaCl$_2$ / 0.05% Tween 20 / 1% BSA, pH 7.5). AP plates were not blocked and samples were diluted 1/15 in ice-cold BVB++/MgEGTA buffer (BVB++/10mM EGTA/5 mM MgCl$_2$). Deposition of mouse C9 on functional pathway ELISAs was quantified with Digoxigenin conjugated rabbit anti-mouse C9 (developed in-house, LUMC) and anti-DIG-POD, Fab fragments (Roche) diluted in PBT (PBS / 1% BSA / 0.05% Tween20), and colorimetric reaction developed with TMB XTRA (Kem-En-Tek, Taastrup, Denmark). The colorimetric substrate step was 15-30 min at RT and stopped with 50 µl 1 M H$_2$SO$_4$ and read at 450 nm with a BioRad 550 microplate reader (Tokyo, Japan).

Immunohistochemistry
Sections (4 µm) from formalin fixed paraffin embedded left kidneys were analyzed for infiltration of neutrophils, macrophages and T cells. Sections were deparaffinized and antigen retrieval was performed using 0.4% pepsin for neutrophils, 0.1% protease for macrophages and 0.1M Tris/HCl pH 9 for T cells. Endogenous peroxidases were blocked with 0.3% H$_2$O$_2$ in PBS for 30 min at RT. Sections were incubated with primary antibodies directed against Ly-6G (eBioscience, San Diego, CA, USA) for neutrophils, F4/80 (Serotec, Oxford, UK) for macrophages or CD3 (Dako, Glostrup, Denmark) for T cells. Sections were incubated with appropriate horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako). Antibodies were diluted in PBS with 1% bovine serum albumin (Sanquin, Amsterdam, the Netherlands) and, if appropriate, 1% normal mouse serum. The reaction was developed by addition of 3-amino-9-ethylcarbazole (AEC) and 0.03% H$_2$O$_2$. Sections were embedded in Aquatex mounting agent (Merck, Darmstadt, Germany). Quantification of infiltrating cells, scored as percentages of total area, was performed using Aperio ImageScope (Leico Biosystems, Nussloch, Germany) and ImageJ software (National Institutes of Health, Bethesda, MD, USA).

RNA isolation and cDNA synthesis
Total RNA was isolated from left kidneys, snap frozen at time of sacrifice. Cryosections were lysed in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Chloroform (Merck) was added
to separate the RNA from DNA and protein content. Subsequently, isopropanol (Biosolve, Dieuze, France) was added to precipitate the RNA and next, the pellet was washed thrice with 75% ethanol (Fresenius Kabi, Schelle, Belgium). RNA pellet was air dried and finally dissolved in sterile water (Fresenius Kabi). RNA samples were treated with DNase Amplification Grade (Sigma-Aldrich) following the manufacturer’s instructions. Absence of contamination with genomic DNA was verified by performing RT-PCR reaction, in which addition of reverse transcriptase was omitted, using β-actin primers. For cDNA synthesis 1 µl oligo-dT (0.5 µg/µl, Invitrogen, Carlsbad, CA, USA) and 1 µg mRNA were incubated for 10 min at 70°C and cooled directly after that. cDNA was synthesized by adding a mixture containing 0.5 µl sterile water (Fresenius Kabi), 4 µl 5x First strand buffer (Invitrogen), 2 µl DTT (Invitrogen), 1 µl 20 mM dNTP’s (Invitrogen), 0.5 µl RNaseOut Ribonuclease inhibitor (Invitrogen) and 1 µl M-MLV Reverse Transcriptase (Invitrogen). The mixture was incubated for 50 min at 37°C. Subsequently, reverse transcriptase was inactivated by incubating the mixture for 15 min at 70°C. Samples were stored at -20°C.

### Table 1: Primer sequences

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<td>5’- GTTGGGTCATATGCAGCGTTAG-3’</td>
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### Real-Time PCR

mRNA transcripts were amplified with the primer sets outlined in Table 1. Real-Time PCR was carried out in reaction volumes of 15 µl containing 10 µl SYBR Green mastermix (Applied biosystems, Foster City, USA), 0.4 µl of each primer (50 µM stock concentration), 4.2 µl nuclease free water and 10 ng cDNA. In each sample, genes of interest were analyzed in triplicate. Primer sequences are depicted in Table 1. Thermal cycling was performed on
the Taqman Applied Biosystems 7900HT real-time PCR System with a hot start for 2 min at 50°C, followed by 10 min 95°C. Second stage started with 15 sec at 95°C (denaturation) and 60 sec at 60°C (annealing and elongation). The latter stage was repeated 40 times. Stage 3 was included to detect formation of primer dimers (melting curve) and began with 15 sec at 95°C, followed by 60 sec at 60°C and 15 sec at 95°C. CT values were corrected for β-actin (ΔCT) and a plate calibrator (ΔACT). Results were expressed as 2^{ΔΔCT} (CT: Threshold Cycle).

**Statistical analysis**
Statistical analysis was performed with GraphPad Prism 5.00 (GraphPad Software Inc, La Jolla, CA, USA). For comparison of more than two groups, a Kruskall Wallis test was performed, followed by a Mann Whitney post-test. All statistical tests were 2-tailed with P < 0.05 regarded as significant. Results are presented as mean values ± SD.

**RESULTS**

*C5L2* \(^{-/-}\) mice are protected against renal ischemia/reperfusion injury

To investigate the role of C5L2 in renal IRI, WT, C5aR\(^{+/+}\) and C5L2\(^{-/-}\) mice were subjected to 40 min bilateral renal warm ischemia followed by reperfusion, and were sacrificed 1, 3 or 7 days after surgery. All mice survived up to termination, except for one animal in the C5aR\(^{+/+}\) IRI group, due to a technical error. This animal was excluded from further analysis. Renal function, as measured by plasma creatinine and BUN levels, was assessed at all three time points. Both C5aR\(^{+/+}\) and C5L2\(^{-/-}\) mice showed attenuation of IRI-induced renal dysfunction at 1 day and 3 days after surgery, compared to WT mice (Figure 1, Table 2). For each mouse strain, plasma creatinine and BUN levels were significantly increased 1 day after IRI compared to sham-operated animals of the same strain. C5aR\(^{+/+}\) IRI and C5L2\(^{-/-}\) IRI mice tended to have lower plasma creatinine and BUN levels compared to WT 1 day after IRI (Table 2). At 3 days after IRI, plasma creatinine and BUN levels were significantly increased in all IRI groups compared to sham-operated animals of the same strain (Figure 1A and 1B). However, both C5aR\(^{+/+}\) and C5L2\(^{-/-}\) IRI groups now showed significant lower plasma creatinine and BUN levels compared to WT IRI. Moreover, C5L2\(^{-/-}\) IRI mice even had a significantly lower plasma creatinine level compared to C5aR\(^{+/+}\) IRI mice. At 7 days after IRI, plasma creatinine and BUN levels in all IRI mice reduced to values observed in sham-operated animals, with a significant difference remaining between the BUN levels of WT sham-operated and WT IRI mice (Table 2).
C5a Receptors in Renal IRI

Chapter 6

C5aR\(^{-/-}\) mice show reduced tubular damage after renal ischemia/reperfusion injury

To evaluate whether attenuation of IRI-induced renal dysfunction observed in C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice was in line with renal morphological damage, histological analysis was performed on kidneys collected at 1 day after IRI (Figure 2). Acute tubular necrosis (ATN) was observed in kidneys following IRI (Figure 2B), whereas sham-operated animals showed no ATN (Figure 2A). ATN was scored using a semi-quantitative scale, ranging from 0 to 4 (0 = 0% ATN, 1 = <10% ATN, 2 = 10-25% ATN, 3 = 25-50% ATN and 4 = >50% ATN). Representative pictures of score 1, 2 and 3 are shown in Figure 2C, 2D and 2E respectively. Score 4 was not observed in kidneys following IRI. In line with attenuation of IRI-induced renal dysfunction observed in C5L2\(^{-/-}\) mice, significantly less ATN was observed in C5L2\(^{-/-}\) mice compared to WT and C5aR\(^{-/-}\) mice 1 day after IRI (Figure 2F).

Table 2: Plasma creatinine levels (µM) and Blood Urea Nitrogen (mM) at day 1, 3 and 7 after bilateral renal ischemia/reperfusion injury

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<th>Day 1</th>
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<th>Day 7</th>
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<td>IRI</td>
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<td>Creatinine</td>
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<td>63.8 ± 44.6*</td>
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<td>C5aR(^{-/-})</td>
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<td>C5L2(^{-/-})</td>
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<td>BUN</td>
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<tr>
<td>WT</td>
<td>7.2 ± 0.8</td>
<td>44.3 ± 21.5*</td>
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Data are expressed as mean ± SD.

* Significant difference compared to sham-operated animal of the same strain
# Significant difference compared to WT IRI

Systemic complement activation in WT, C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice

Figure 1: Renal function 3 days after renal ischemia/reperfusion injury in WT, C5aR\(^{+/+}\) and C5L2\(^{+/+}\) mice.

(A) Plasma creatinine and (B) blood urea nitrogen levels at 3 days after bilateral renal ischemia/reperfusion injury in WT, C5aR\(^{+/+}\) and C5L2\(^{+/+}\) mice. Data are shown as mean ± SD (*P<0.05, **P<0.01, ***P<0.001). C5L2\(^{+/+}\) mice show reduced tubular damage after renal ischemia/reperfusion injury.

To evaluate whether attenuation of IRI-induced renal dysfunction observed in C5aR\(^{+/+}\) and C5L2\(^{+/+}\) mice was in line with renal morphological damage, histological analysis was performed on kidneys collected at 1 day after IRI (Figure 2). Acute tubular necrosis (ATN) was observed in kidneys following IRI (Figure 2B), whereas sham-operated animals showed no ATN (Figure 2A). ATN was scored using a semi-quantitative scale, ranging from 0 to 4 (0 = 0% ATN, 1 = <10% ATN, 2 = 10-25% ATN, 3 = 25-50% ATN and 4 = >50% ATN). Representative pictures of score 1, 2 and 3 are shown in Figure 2C, 2D and 2E respectively. Score 4 was not observed in kidneys following IRI. In line with attenuation of IRI-induced renal dysfunction observed in C5L2\(^{+/+}\) mice, significantly less ATN was observed in C5L2\(^{+/+}\) mice compared to WT and C5aR\(^{+/+}\) mice 1 day after IRI (Figure 2F).

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Data are expressed as mean ± SD.

* Significant difference compared to sham-operated animal of the same strain
# Significant difference compared to WT IRI
Chapter 6

Differences in activity of the systemic complement system have been observed between mouse strains. To investigate whether differences in the systemic complement system could have caused attenuation of IRI-induced renal dysfunction in C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice compared to WT mice, we analyzed complement pathway activity of the three individual pathways using functional ELISA’s. In these assays, measuring decreased complement pathway activity in plasma of certain mice implicates systemic consumption of complement components in these mice, and therefore in vivo systemic complement activation. Complement pathway activity was similar in sham-operated animals of all three mouse strains at 1 day (Figure 3A, 3B).

Figure 2: Tubular damage 1 day after renal ischemia/reperfusion injury in WT, C5aR\(^{+/+}\) and C5L2\(^{+/+}\) mice.
Tubular damage was scored as percentage of necrotic tubuli (acute tubular necrosis, ATN) in the cortical area using a semi-quantitative method (0 = 0% ATN, 1 = <10% ATN, 2 = 10-25% ATN, 3 = 25-50% ATN and 4 = >50% ATN). (A) Normal renal morphology was observed in sham-operated kidneys (score 0), while (B) IRI kidneys showed ATN. Computerized representative images of (C) score 1, (D) score 2 and (E) score 3 are shown, area with ATN marked in red. (F) Tubular damage scored at day 1 after IRI. Data are shown as mean ± SD (*P<0.05).
and 3C, white bars), 3 days (not shown) and 7 days (Figure 3D, 3E and 3F, white bars) after surgery. This implicates that WT, C5aR⁻/⁻ and C5L2⁻/⁻ mice all show similar baseline systemic complement activity for each of the pathways. Therefore, functional systemic complement activity does not explain attenuation of IRI-induced renal dysfunction in C5aR⁻/⁻ and C5L2⁻/⁻ mice.

Activation of the systemic complement system was observed after IRI in all three strains. Comparison of sham-operated animals with IRI animals revealed decreased complement activity (and therefore increased systemic complement activation in vivo) for all three pathways in all three mouse strains 1 day after IRI, reaching statistical significance for WT and C5L2⁻/⁻ mice in CP, LP and AP and for C5aR⁻/⁻ mice in AP (Figure 3A, 3B and 3C, black bars). At 1 day after IRI, reduced consumption of complement components was measured for AP in C5aR⁻/⁻ and C5L2⁻/⁻ compared to WT mice (Figure 3C). Systemic complement activity was fully restored to baseline levels in all animals at 7 days after IRI (Figure 3D, 3E and 3F, black bars).

Subtle differences in infiltrated leukocytes in C5aR⁻/⁻ and C5L2⁻/⁻ mice after renal IRI

Figure 3: Systemic complement consumption for the classical, lectin and alternative pathway in WT, C5aR⁻/⁻ and C5L2⁻/⁻ mice after renal ischemia/reperfusion injury.

Conservation of systemic complement components was analyzed for each of the three individual pathways (i.e. classical (CP), lectin (LP) and alternative (AP)) by functional ELISA's. Results for day 1 (A-C) and day 7 (D-F) after IRI are shown as mean ± SD. Significant differences of IRI group compared to Sham group of the same strain is indicated as *P<0.05, **P<0.01, ***P<0.001. Significant differences of IRI group of a knockout strain compared to IRI group of WT is indicated as #P<0.01.
Since C5a is a potent chemoattractant and C5aR is involved in leukocyte migration, infiltrating neutrophils, macrophages and T cells were quantified by immunohistochemistry (Figure 4). As these types of leukocytes are known to display different infiltration dynamics (29), infiltrated neutrophils were scored at 1 day after renal IRI, while macrophages and T cells were scored at 3 and 7 days after renal IRI.

Influx of neutrophils was observed in WT mice after IRI (Figure 4A), but did not reach statistical significance due to variation (Figure 4B). C5L2−/− mice showed significant influx of neutrophils after IRI, but C5aR−/− mice did not. Although both C5aR−/− and C5L2−/− strains tended towards lower mean neutrophil infiltration at 1 day after renal IRI compared to WT mice, no statistical significance was reached (Figure 4B).

Infiltration of macrophages was observed at 7 days after IRI, reaching statistical significance.

**Figure 4: Infiltrated leukocytes after renal ischemia/reperfusion injury in WT, C5aR−/− and C5L2−/− mice.**

Infiltrated leukocytes, i.e. (A) neutrophils, (C) macrophages and (F) T cells, were scored in paraffin embedded kidney sections at 1, 3 or 7 days after IRI. (B) Neutrophils 1 day after IRI, (D) macrophages 3 days after IRI, (E) macrophages 7 days after IRI, (G) T cells 3 days after IRI and (H) T cells 7 days after IRI. (*P<0.05, **P<0.01).
for WT and C5L2\(^{-/-}\) mice (Figure 4C and 4E). However, although not significant, this trend was also observed in C5aR\(^{-/-}\) mice. No significant infiltration of macrophages was observed in IRI kidneys at 3 days after IRI (Figure 4D).

No significant infiltration of T cells was observed in IRI kidneys at 3 days after IRI (Figure 4G). However, a significant influx of T cells was observed in kidneys from WT and C5L2\(^{-/-}\) mice at 7 days after IRI (Figure 4F and 4H). In C5aR\(^{-/-}\) mice, no influx of T cells was not observed at 7 days after IRI. Moreover, the quantity of T cells in C5aR\(^{-/-}\) IRI kidneys was significantly lower than in WT I/R kidneys (Figure 4H).

**Differential inflammatory gene expression in C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice after renal I/R injury**

To characterize the inflammatory profile of IRI kidneys from WT, C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice, gene expression analysis was performed on kidneys at 1 day and 3 days after renal IRI (Figure 5 and Figure 6 respectively). Since plasma creatinine and BUN levels restored to levels observed in sham-operated animals at 7 days after IRI, renal gene expression profiles at 7 days after IRI were not analyzed.

At 1 day after renal IRI, a significant induction of IL-1\(\beta\), IL-6, KC, TNF\(\alpha\), MCP-1, C3 and P-selectin was observed in WT mice (Figure 5A-5G). Although induction of IL-6, KC, TNF\(\alpha\), MCP-1 and P-selectin was observed in C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice as well, no significant induction of C3 was found in C5aR\(^{-/-}\) mice (Figure 5F) and induction of IL-1\(\beta\) was absent in both C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice (Figure 5A). In addition, expression of IL-1\(\beta\) was significantly lower in C5aR\(^{-/-}\) IRI kidneys compared to C5L2\(^{-/-}\) IRI kidneys. When comparing WT IRI kidneys with knockout IRI kidneys, C5aR\(^{-/-}\) mice showed decreased MCP-1 expression (Figure 5E), while C5L2\(^{-/-}\) mice showed significantly lower IL-6 expression (Figure 5B) and significantly higher C3 expression (Figure 5F). At 1 day after renal IRI, expression of IFN\(\gamma\) could not be detected.

At 3 days after renal IRI, significant induction of IL-6, KC, TNF\(\alpha\), MCP-1, C3 and P-selectin remained in WT, C5aR\(^{-/-}\) and C5L2\(^{-/-}\) mice (Figure 6). Significant differential expression of IL-6, MCP-1 and C3 between the three strains disappeared. In contrast, expression of KC was significantly lower in C5L2\(^{-/-}\) IRI kidneys compared to WT I/R kidneys at 3 days after IRI, while this difference was not observed at 1 day after IRI (Figure 6C).

In addition, KIM-1 expression was analyzed at 1 day and 3 days after renal IRI (Figure 5H and 6H). In line with plasma creatinine and BUN levels at 1 day and 3 days after IRI, KIM-1 expression was significantly induced in IRI kidneys compared to their sham-operated counterparts from the same strain at both time points. However, at 3 days after IRI, KIM-1 expression was significantly lower in C5L2\(^{-/-}\) mice compared to WT mice.
Figure 5: Renal gene expression of inflammatory mediators 1 day after renal ischemia/reperfusion injury.

Gene expression of inflammatory markers in kidneys 1 day after renal ischemia/reperfusion injury. (A) IL-1β, (B) IL-6, (C) KC, (D) TNFα, (E) MCP-1, (F) C3, (G) P-selectin, (H) KIM-1. Data are shown as expression relative to β-actin (*P<0.05, **P<0.01, ***P<0.001).
DISCUSSION

In the present study, we investigated the role of C5L2 in renal IRI by subjecting WT, C5aR\(^{-}\) and C5L2\(^{-}\) mice to 40 min of bilateral warm ischemia and examined the effects at 1, 3 and 7 days after reperfusion. We are the first to show that, next to C5aR\(^{-}\) mice, C5L2\(^{-}\) mice are protected against renal IRI as well. In addition, we propose that the underlying protective mechanism may be different in the two knockout strains.

Our observation that C5aR\(^{-}\) mice are protected against renal IRI is in line with data from literature. Previous studies using various renal IRI animal models showed that inhibition of
the C5a-C5aR axis attenuates IRI-induced renal dysfunction (21-25). Moreover, inhibition of the C5a-C5aR axis has not only been shown to be protective in IRI injury of the kidney, but also of the heart (2,30), liver (31), intestine (32) and brain (33). Also in renal transplantation models, C5aR inhibition has been shown to result in improved allograft survival and better renal function (34-36).

In addition to the protection in C5aR−/− mice, we showed that C5L2−/− mice are protected against renal IRI as well, resulting in attenuation of IRI-induced renal dysfunction and decreased tubular damage. Since activation of the systemic complement system is known to aggravate tubular damage after IRI, and functional systemic complement activity can differ between mouse strains, we determined systemic complement activity in WT, C5aR−/− and C5L2−/− mice. Hypothetically, diminished functional complement activity in the knockout strains could explain attenuation of IRI-induced renal dysfunction and reduced tubular damage compared to WT mice. However, baseline functional complement activity was similar in sham-operated animals at all three time points. Therefore, protection against renal IRI in C5aR−/− and C5L2−/− does not seem to be mediated by reduced activity of the systemic complement system. In addition, we observed that complement activation occurs in response to renal IRI. All three pathways were activated after IRI. As has been reported previously, the alternative pathway seemed to be the main pathway involved in renal IRI (37). Alternative pathway activation after IRI was significantly enhanced in WT mice compared to C5aR−/− and C5L2−/− mice, which is in line with the attenuation of IRI-induced renal dysfunction in these knockout strains.

Next to systemic complement activation, leukocytes are involved in IRI-mediated damage. C5aR is known to mediate chemotaxis of PMNs and PBMCs (38,39), but the role of C5L2 in leukocyte migration is unknown. Indeed, C5aR−/− mice tended to show reduced neutrophil and macrophage infiltration compared to WT mice, but no statistical significance was reached. Kidneys from C5L2−/− mice did show a significant influx of neutrophils and macrophages after IRI, implicating that C5L2 is not involved in leukocyte migration. However, further research is required to determine the role of C5L2 in leukocyte chemotaxis.

Previously we have shown that C5a stimulation of renal tissue induced a local inflammatory response (40). In the present study we observed differential expression profiles of inflammatory genes in kidneys from C5aR−/− and C5L2−/− compared to WT mice. Where C5aR−/− mice showed reduced IL-1β and MCP-1 expression after IRI, C5L2−/− mice showed reduced IL-6 and KC expression, and increased C3 expression. These results implicate that C5L2, like C5aR, has pro-inflammatory properties. With both pro- and anti-inflammatory properties reported for C5L2, Li et al posed that C5L2 might be the great masquerader in complement biology: its function depends on cell type, species and disease context.
(15). Interestingly, most studies implicating anti-inflammatory functions for C5L2 seemed to be performed in isolated leukocytes, transfected cell lines or in disease models which originate by activating leukocytes (mainly granulocytes) in various ways. On the contrary, multiple studies implicating pro-inflammatory functions for C5L2 are using models in which inflammation is initiated by inflicting damage directly to organs (41,42), including our study.

Initially, C5L2 was postulated to serve as a non-signaling decoy receptor for C5a, thereby preventing C5a-C5aR-mediated signaling and thus exhibiting an anti-inflammatory function. Although both C5aR and C5L2 are seven-transmembrane receptors and C5aR has been shown to function in a G-protein coupled manner, intracellular signalling by G-proteins seems virtually impossible for C5L2. It lacks both the DRY motif in the third transmembrane segment, which is essential for G-protein coupling (11), and it lacks a NPXXY motif, which has been demonstrated to be important for phospholipase C (PLC) and mitogen-activated protein kinase (MAPK) activation in receptors belonging to the same receptor superfamily (14,15). Although phosphorylation of C5L2 upon C5a stimulation has been observed in transfected cells, C5L2 seemed unable to induce receptor internalization, ERK1/2 phosphorylation, intracellular calcium mobilization, degranulation, and mRNA expression of inflammatory genes in transfected cells, upon stimulation with C5a or C5a_{desArg} (11,16,17). In addition to the decoy hypothesis, a secondary anti-inflammatory mechanism for C5L2 was proposed. Studies performed in human neutrophils by Bamberg et al showed that C5L2 reduces C5aR-mediated phosphorylation of ERK1/2 in a G-protein independent manner via a C5aR-C5L2-β-arrestin complex (18), which was supported by others (19,43). In this model, C5L2 does not function by scavenging C5a, but by negative modulating signalling downstream from C5aR. The potential anti-inflammatory property of C5L2 is supported by several animal studies. In a murine model of immune complex induced lung injury, C5L2^{-/-} mice show enhanced inflammation indicated by increased leukocyte lung infiltration and elevated serum levels of TNFα and IL-6 (27). In a murine model of allergic contact dermatitis, C5L2^{-/-} mice showed increased inflammation parameters (44). The only other study investigating C5L2 in the kidney was performed by Xiao et al, where crescentic glomerulonephritis was induced by anti-MPO injections. The investigators found that C5L2^{-/-} mice displayed aggravated disease, suffering from an increased percentage of crescents compared to WT and C5aR^{-/-} mice (45). In these animal studies, disease was initiated by activation of leukocytes, i.e. immune-complex-induced lung injury, T cell-mediated skin inflammation or kidney injury-mediated via anti-MPO stimulated neutrophils.

If C5L2 would only serve as a decoy receptor or would negatively modulate C5a-C5aR-mediated signaling, we would have observed an aggravated inflammatory phenotype in C5L2^{-/-} mice following renal IRI compared to WT and C5aR^{-/-} mice. However, C5L2^{-/-} mice were
protected against IRI suggesting a pro-inflammatory role for C5L2 in the kidney. In addition, in a previous study we showed distinct renal expression patterns for C5aR and C5L2, making a role for C5L2 as decoy receptor or negative modulator of C5a-C5aR-mediated signaling virtually impossible (13). In addition to our study, pro-inflammatory functions for C5L2 have been reported in other animal studies as well. Induction of sepsis using cecal ligation and puncture, resulted in increased survival rates in C5L2−/− mice compared to WT mice (46). In an ulcerative colitis model, C5L2−/− mice showed lower disease severity scores and less morphological damage of the colon compared to WT mice (47). Like in our studies, these animal studies initiated disease models by inflicting damage directly to organs, i.e. renal IRI, mechanical damage to the cecum and chemical damage to the colon.

In addition to pro-inflammatory functions for both C5aR and C5L2, our results indicate that, at least partly, distinct mechanisms are responsible for preservation of renal function in C5aR−/− and C5L2−/− mice after IRI. Although numerous studies investigated C5aR-mediated signaling (14,48), it has to be taken into account that almost all of these studies were performed in leukocytes or leukocytic cell lines and that C5aR-mediated signaling might be different in non-immune cells. Concerning C5L2-mediated signaling, further elucidation of downstream signaling pathways is required on both immune and non-immune cells. In addition, ligands other than C5a, like C3a desArg, should be included when investigating C5L2-mediated signaling, since C3a desArg was observed to have downstream effects on glucose and triglyceride metabolism in a C5L2 dependent manner (19,43).

In conclusion, our data show that C5L2 plays a significant detrimental role in renal IRI. Although the interaction between C5aR and C5L2 in this model remains to be investigated, these data suggest that distinct mechanisms mediate the protection against renal IRI in C5aR−/− and C5L2−/− mice. In addition, we suggest that C5L2 might have different roles in leukocyte-induced versus end-organ damage-induced inflammation, and future animal experiments using inflammatory models should take this into consideration. In the clinical setting, the C5L2 receptor might be considered as target for intervention before induction of ischemia, i.e. in the donor before organ retrieval, during cold storage or perhaps even early after reperfusion. It remains to be investigated whether inhibition of C5L2 during the renal transplant procedure will be beneficial for renal allograft outcome.

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