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C5L2 promotes Local Renal Inflammation after Donor Brain Death

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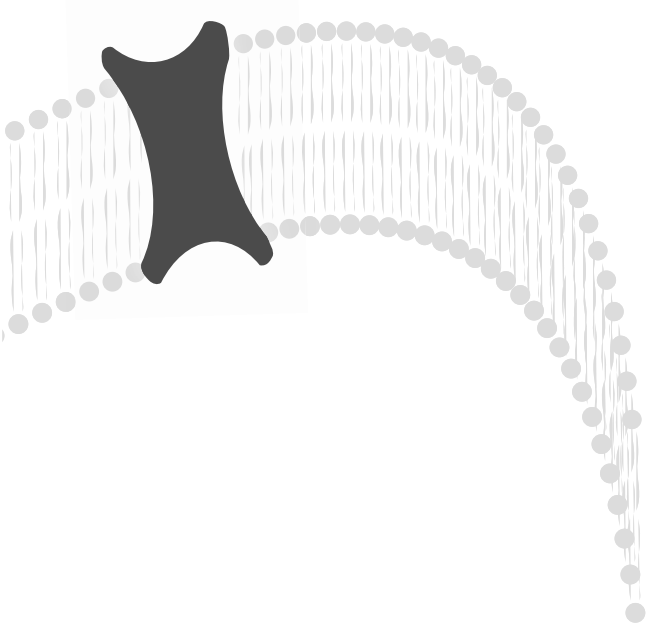

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ABSTRACT

Kidneys retrieved from brain-dead donors show inferior post-transplant renal function and survival rates compared to kidneys from living donors. Brain death in itself triggers activation of the immune system. Possibly, the systemic inflammatory response induced upon donor brain death affects organ viability prior to organ retrieval, resulting in reduced post-transplant function and survival. As part of the inflammatory response, the complement system is activated, reflected by elevated levels of the anaphylatoxin C5a circulating in the potential organ donor. The two known receptors for C5a, C5aR and C5L2, are expressed in the kidney and we previously reported that expression of C5aR is increased in kidney biopsies from brain-dead donors compared to living donors. In the present study, we investigated the contribution of C5aR and C5L2 to donor brain death-induced renal inflammation. In order to achieve this, we first developed a mouse brain death model, in which a stable 3h brain death period combined with continuous blood pressure monitoring was accomplished. Subsequently, WT, $C5aR^{-/-}$ and $C5L2^{-/-}$ mice were subjected to this brain death procedure. We confirmed that the complement system was activated in brain-dead mice using a C3 fragment ELISA. We observed that, compared to WT mice, $C5L2^{-/-}$ mice, but not $C5aR^{-/-}$ mice, show reduced renal gene expression levels of KC, TNFα, MCP-1 and P-selectin upon donor brain death. These results suggest that C5L2, instead of C5aR, is involved in brain death-induced renal inflammation, and therefore C5L2 might be a target for intervention in order to prevent renal allograft priming in brain-dead donors.

INTRODUCTION

Renal transplantation is the first choice of treatment for end-stage renal disease. An increasing number of kidney transplantations are performed each year, with renal allografts originating from living, deceased heart-beating brain-dead (DBD), and deceased cardiac dead (DCD) donors. Although the numbers of renal grafts derived from living and DCD donors are increasing, a substantial number of renal grafts is derived from DBD donors. Kidneys derived from DBD donors show inferior renal function and poorer graft survival after transplantation when compared to their living counterparts (1-4). It is now well established that donor brain death in itself triggers hemodynamic instability, hormonal changes, and activation of the immune system (5-7). Most likely, these processes affect organ viability before transplantation and could be responsible for the inferior results of DBD-derived renal allografts.

The systemic inflammation occurring upon donor brain death is characterized by elevated circulating levels of general inflammatory markers like IL-6 (5,8), IL-8 (8), MCP-1 (8), soluble TNF receptor II (5), and soluble IL-2 receptor (5), but also triggers activation of the complement system. The complement system contains over thirty membrane-bound and soluble proteins (9-11). This complex cascade can be activated via three pathways: the classical (CP), the alternative (AP) and the lectin (LP) pathway. Activation of each of the three pathways results in cleavage and activation of central complement component C3. Subsequent cleavage of C5 initiates formation of the polymeric C5b-9 or membrane attack complex (MAC). Inherent to complement terminal pathway activation is formation of the anaphylatoxins C3a and C5a, of which C5a possesses the most potent chemotactic and pro-inflammatory properties. To reflect complement activation upon donor brain death, we observed increased circulating levels of C3d, C5a and C5b-9 in DBD donors compared to living donors (12-14). In addition, local renal production of complement components is observed in brain-dead rats (7) and in humans (13,15).

There are two known receptors for the powerful anaphylatoxin C5a, namely C5a receptor (C5aR) and C5a-like receptor 2 (C5L2). These receptors have initially been identified on leukocytes, but expression has been shown on non-immune cells as well (16,17). We previously showed that both receptors are expressed in the kidney on distal tubular epithelial cells (18). However, renal C5aR and C5L2 expression were found to be confined to different segments of the distal nephron. Both receptors are seven-transmembrane receptors, and resemble a G-protein coupling receptor configuration (19). 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 (16,19-23). Therefore, C5L2 has been postulated to serve as a decoy receptor for C5a, preventing C5a-C5aR-mediated signaling and its subsequent pro-inflammatory effects. An alternative hypothesis suggests that C5L2 modulates C5aR-induced inflammation by interacting with the C5aR-β-arrestin-complex (24). 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 (25,26).

In addition to elevated circulating levels of C5a in DBD donors, we showed that renal protein expression of C5aR is increased in biopsies from DBD donors compared to living donors (13). In addition, we observed that stimulation of human renal tissue with C5a leads to local production of inflammatory cytokines, like IL-1, IL-6 and IL-8 (13). Combining systemically generated C5a and induction of renal C5aR expression in DBD donors, with local production of inflammatory cytokines upon C5a stimulation of human renal tissue, we hypothesized that the C5a-C5aR interaction in the kidney induces priming of the renal allograft to be. Possibly, renal inflammation upon C5a-C5aR stimulation initiated by donor brain death could explain the inferior function and graft survival of DBD donor-derived renal allografts after transplantation.

Studies investigating donor brain death are almost exclusively carried out in rats using a gradual onset brain death model (27), in which efficacy of interventions can be analyzed. In order to expand the possibilities in donor brain death research, and investigate the role of specific complement components in donor brain death, development of a mouse brain death model would be of great benefit, since it could be applied to knock-out mice. We here describe the development of a mouse brain death model, in which a stable 3h brain death period in combination with continuous blood pressure monitoring is achieved, adapted from a previously described model (28). Subsequently, we subjected WT, $C5aR^{-/-}$ and $C5L2^{-/-}$ mice to this brain death procedure to investigate the contribution of these receptors to donor brain death-induced local renal inflammation.

MATERIALS AND METHODS

Animals

C57Bl/6 wildtype (WT), and C5aR$^{-/-}$ (29) and C5L2$^{-/-}$ (30) 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 in the University Medical Center Groningen. Animals were housed in groups in standard laboratory cages, with *ad libitum* access to food and water. The studies were carried out under a protocol approved by the Institutional Animal Care Committee of the University of Groningen (project number 6279AF).

In the experiments, male mice, aged 8 to 12 weeks, with a weight of 25-28 grams were used. Per mouse strain, 8 animals were subjected to 3h of brain death as described below. At time of sacrifice, blood and kidneys were collected for analysis.

Anesthesia, monitoring and ventilation

Mice were anesthetized using 5% isoflurane / 100% O_2. Body temperature was monitored using a rectal probe for mice (RET-3, Physitemp Instruments, Cliften, NJ, USA) and maintained at 37°C by a heating pad. Under aseptic conditions, a midline incision was made in the neck of the mice, ranging from just caudal of the mandibula to the manubrium. In supine position, right carotid artery and left jugular vein were cannulated using fine bore polythene tubing (0.28 mm ID, 0.61 mm OD; Smiths medical, Kent, UK), to monitor blood pressure and administer saline respectively. Coagulation was inhibited by 12.5 µg/ml lepirudin (Celgene, Summit, NJ, USA) in saline (Baxter, Utrecht, the Netherlands). Intubation by tracheostomy was performed using a shortened 22G i.v. catheter (BD insyte-W, Franklin Lakes, NJ, USA). Ventilation was performed using mouse ventilator minivent type 845 (Harvard apparatus, Holliston, MA, USA), with breathing frequency of 200 breaths / min, tidal volume of 225 µl / stroke, and PEEP of 1 cm. During preparation for and induction of brain death, ventilation was performed with 2% isoflurane / 100% O_2.

Brain death procedure

The mice were changed to prone position. On the scull, a sagittal midline incision was made and bupivacaine (2.5 mg/ml, Actavis, Hafnarfjordur, Iceland) was administered locally. A hole was drilled in the left parietal part of the skull, just caudal to the bregma, using a microdrill (Minicraft MB730, Elmatik AS, Tallinn, Estonia) and 2.4 mm engraving cutter (Dremel Europe, Breda, the Netherlands). A balloon catheter (Fogarthy Arterial Embolectomy Catheter, 2F, Edwards Lifesciences Corporation, Irvine, CA, USA) was inserted into the extradural space with the tip pointing caudally. The balloon catheter was fixed with Evicel human fibrin sealant (Omrix Biopharmaceuticals, Diegem, Belgium). Induction of brain death was started by increasing the intracranial pressure by inflating the balloon with 14 µl saline per minute, to a

total volume of 70 µl, using a 100 µl syringe (Hamilton, Reno, NV, USA) and Harvard Pump 11 plus Single Syringe (MA1 70-2208, Harvard apparatus). The balloon was kept inflated until the end of the experiment. Henceforth, isoflurane was switched off. Brain death was confirmed by a positive apnea test, 10 minutes after inflation of the balloon catheter.

Hemodynamic management

During brain death, a mean arterial pressure (MAP) above 60 mmHg was considered to be normotensive. To maintain stable blood pressure, 50 µl saline with lepirudin was administered every 15 minutes via the jugular vein cannula. When blood pressure dropped below 60 mmHg, an additional 50 µl of saline was administered. A maximum of 1200 µl of saline was administered during the 3 hours brain death period. 30 minutes after declaration of brain death, ventilation was switched to a mixture of oxygen and medical air (50%-50%). Body temperature was continuously monitored and maintained at 37°C as described above. At time of sacrifice, blood and kidneys were collected for analysis. Venous blood gas analysis was performed using the i-STAT system and i-STAT CG4+ cartridge (Abbott Point of Care, Princeton, NJ, USA).

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).

Mouse C3b/C3c/iC3b ELISA

Mouse C3 fragments were measured in EDTA-plasma by a C3b/C3c/iC3b ELISA, as recently developed by Kotimaa et al (Chapter 5). The mouse C3 fragments were captured with rat anti-mouse monoclonal specific to C3b/C3c/iC3b (Hycult Biotechnology, Uden, the Netherlands), coated on Nunc Maxisorp plates (Thermo Fisher Scientific, NY) with CB buffer (100 mM Na_2CO_3 /$NaHCO_3$, pH 9.6) 16h at room temperature (RT). Assay volume was 50 µl/well and each incubation step was 1h at 37°C, except for sample incubation which was performed at 4°C. After each step the wells were washed 4 x 5 min with PT (PBS / 0.05% Tween 20). First, plates were blocked with PB (PBS / 1% BSA) and samples diluted in PTB/E (PBS / 1% BSA / 0.05% Tween20 / 10 mM EDTA). C3b/C3c/iC3b was detected with biotinylated rabbit anti-mouse C3 pAb (Hycult Biotechnology), Streptavidin-HRP (Hycult Biotechnology) and TMB Plus2 (Kem-En-Tek, Denmark). The colorimetric substrate of all ELISAs was 15-30 min at room temperature and stopped with 50 µl 1M H_2SO_4 and read at 450 nm with a BioRad 550 instrument (Tokyo, Japan). Standard for the assay was prepared incubating CD1 NMS (IMSCD1-COMPL, Innovative Research) with 4 mg/ml zymosan (Z4250, Sigma-Aldrich, MO) for 2h at 37°C, centrifuged at 3000g for 10 min, and stored at -20°C. The undiluted standard was set to 100 arbitrary units per ml (AU/ml).

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.

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. 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 (ΔCT). Results were expressed as $2^{-\Delta\Delta CT}$ (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. In addition, Dixon's Q test for outliers was used.

Table 1: Primer sequences

Gene	Primers	Amplicon size (bp)
β-actin	5'- ACACCCTTTCTTTGACAAAACCTAA-3' 5'-GCCATGCCAATGTTGTCTCTTAT-3'	67
IL-1β	5'-GGACCCATATGAGCTGAAAGCT-3' 5'- TGGTTGATATTCTGTCCATTGAGGT-3'	51
IL-6	5'-ACATAAAATAGTCCTTCCTACCCCAATT-3' 5'-TTAGCCACTCCTTCTGTGACTCC-3'	76
KC	5'- GTGTCTAGTTGGTAGGGCATAATGC-3' 5'- TGTCCCGAGCGAGACGAG-3'	76
C3	5'-AGCTGTTGGATGATTTTGATGAGTAC-3' 5'-GAGCCTGACTTGATGACCTGCT-3'	60
TNFα	5'-AGGACCCAGTGTGGGAAGCT-3' 5'-CAAAAGAGGAGGCAACAAGGTAGAG-3'	101
IFNγ	5'-CCAAGCGGCTGACTGAACTC-3' 5'-TCACTGCAGCTCTGAATGTTTCTTAT-3'	78
MCP-1	5'-GGTCTTCAGCACCTTTGAATGTG-3' 5'-TGAGGTGGTTGTGGAAAAGGTA-3'	79
KIM-1	5'-TGCATTCCCGAGCTGAAGA-3' 5'-CAGAGGGCCACTGGTACTCATT-3'	78
P-selectin	5'-CCTCACAGCCACCTAGGAACA-3' 5'-GTTGGGTCATATGCAGCGTTAG-3'	55

RESULTS

Experimental mouse brain death model

Development of the mouse brain death model was based on experience with the rat brain death model (27). Detailed description of the mouse brain death model is given in the Materials and Methods section, and a representative overview of the setup and a close up picture are provided in Figure 1A and 1B respectively. In brief, WT mice were anesthetized using isoflurane/O_2. In supine position, right carotid artery and left jugular vein were cannulated (Figure 1, #2 and #4), to monitor blood pressure (Figure 1, #3) and administer saline respectively. Intubation by tracheostomy (Figure 1, #1) was performed, and ventilation was done using a mouse ventilator (Figure 1, #6). In prone position, a balloon catheter (Figure 1, #5) was inserted into the extradural space with the tip pointing caudally. Induction of brain death was started by increasing the intracranial pressure by inflating the balloon with saline using a syringe and a single syringe pump (Figure 1, #7). Thereafter, isoflurane was switched off, and brain death was confirmed by a positive apnea test.

As can be observed in Figure 1C, real-time blood pressure monitoring was achieved. During brain death, MAP above 60 mmHg was considered normotensive. To maintain stable blood pressure, 50 µl saline was administered every 15 minutes via the jugular vein cannula. When the MAP dropped below 60 mmHg, an additional 50 µl of saline was administered. With this protocol, WT mice were kept stable during the 3h time span of brain death (Figure 2A).

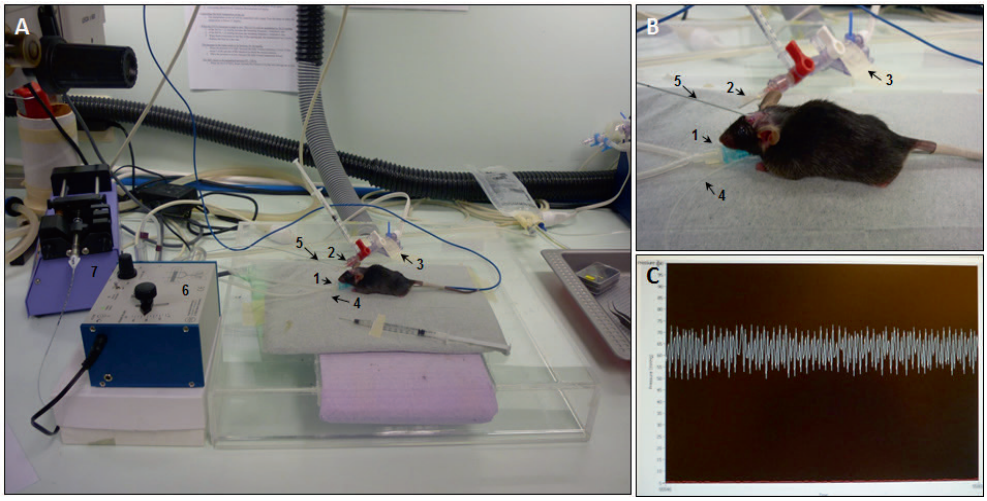

Figure 1: Experimental setup of mouse brain death model
(A) Overview picture and (B) close up picture of the experimental set up of the mouse brain death model. In (A) & (B) the following equipment is marked: (1) Trachea cannula inserted via tracheostomy; (2) cannula in right carotid artery for blood pressure registration, connected to (3) sphygmomanometer; (4) cannula in left jugular vein for administration of saline; (5) balloon catheter into the extradural space; (6) mouse ventilator minivent type 845; (7) Harvard Pump 11 plus. (C) Representative picture of blood pressure registration observed during mouse brain death.

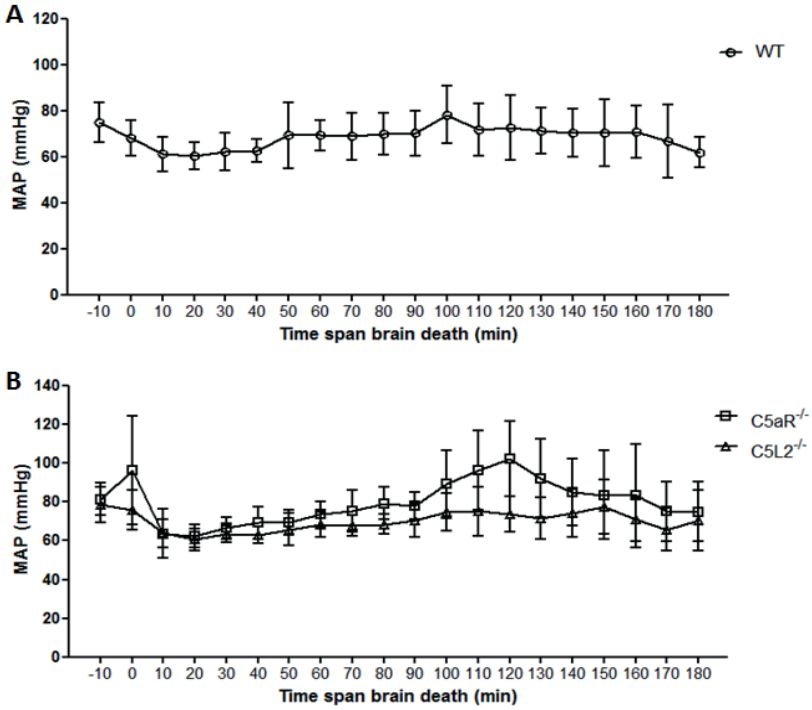

Figure 2: Mean arterial pressure during brain death
Registration of mean arterial pressure during 3h of mouse brain death in (A) WT mice and (B) C5aR$^{-/-}$ and C5L2$^{-/-}$ mice.

At the beginning of the experiment, mice were anesthetized with 5% isoflurane / 100% O_2. Directly after, baseline venous blood gas analysis was performed (Table 2). Lactate levels of 5.36 ± 0.33 mmol/L were observed before any of the procedures had been initiated. After intubation, ventilation was performed with a breathing frequency of 200 breaths / min, tidal volume of 225 µl / stroke, and PEEP of 1 cm. During preparation for and induction of brain death, ventilation was performed with 2% isoflurane / 100% O_2, and 30 minutes after declaration of brain death, ventilation was switched to a mixture of oxygen and medical air (50%-50%). After 3h of brain death, venous blood gas analysis was repeated to examine the effects of mechanical ventilation (Table 2). Despite reduced O_2 saturation and pO_2, pH and pCO_2 remained stable during the 3h brain death period with these ventilation settings. In addition, lactate levels restored to 1.54 ± 0.22 mmol/L.

Since complement is known to be activated during donor brain death in humans, we analyzed whether the complement system was also activated in the brain dead mice. Since activation of C3 results in production of C3 fragments, the presence of these fragments would indicate activation of the complement system. Therefore, C3 fragment levels were measured in EDTA plasma obtained after 3h of brain death (Figure 3). In WT mice, C3 fragments of 2.4 ± 0.5 AU/ml were detected.

Table 2: Venous blood gas analysis

		Baseline values (n=5)	3h after BD (n=5)
pH		7.29 ± 0.01	7.29 ± 0.03
pCO_2	(mmHg)	47.8 ± 3.0	43.6 ± 4.4
pO_2	(mmHg)	82 ± 8	48 ± 6**
Base excess	(mmol/L)	-4 ± 1	-5 ± 2
HCO_3^-	(mmol/L)	23.2 ± 1.0	20.8 ± 1.5*
tCO_2	(mmol/L)	25 ± 1.1	22 ± 2*
O_2 saturation	(%)	94 ± 2	78 ± 5*
Lactate	(mmol/L)	5.36 ± 0.33	1.54 ± 0.22**

Data are expressed as mean ± SD (*P<0.05, **P<0.01)

Brain death in C5aR$^{-/-}$ and C5L2$^{-/-}$ mice

Subsequently, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice were subjected to brain death as described above. As can be observed in Figure 2B, adequate MAP was achieved during brain death in C5aR$^{-/-}$ and C5L2$^{-/-}$ mice, alike WT mice. The amount of administered saline was not significantly different between WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice, with on average 856 ± 124 µl administered to WT, 850 ± 71 µl to C5aR$^{-/-}$ and 844 ± 78 µl to C5L2$^{-/-}$ mice. In addition, all mice showed urine retention in the bladder at time of sacrifice after 3h of brain death (0.54 ± 0.24 ml in WT mice, 0.38 ± 0.16 ml in C5aR$^{-/-}$ and 0.39 ± 0.18 ml in C5L2$^{-/-}$ mice).

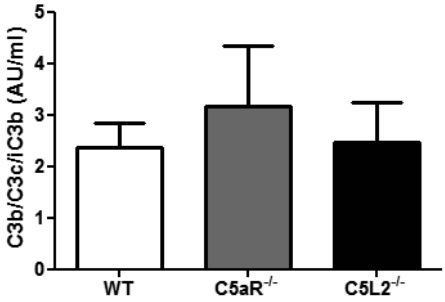

Figure 3: C3 fragment levels after 3h of brain death in WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice
Systemic complement activation was measured as C3 fragments (C3b/C3c/iC3b) in EDTA plasma obtained after 3h of brain death in WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice. Results are shown as mean ± SD.

To compare systemic complement activation upon brain death in C5aR$^{-/-}$ and C5L2$^{-/-}$ mice with WT mice, C3 fragment levels were determined in these mouse strains as well (Figure 3). In C5aR$^{-/-}$ mice and C5L2$^{-/-}$ mice, C3 fragment levels of 3.2 ± 1.2 AU/ml and 2.5 ± 0.8 AU/ml were detected respectively. No significant differences in C3 fragment levels between WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice were detected.

Renal function after brain death in WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice

To examine whether absence of C5aR or C5L2 affects renal function upon brain death, renal function was measured as plasma creatinine and blood urea nitrogen (BUN) in EDTA plasma collected after 3h of brain death (Figure 4). Plasma creatinine level of one C5L2$^{-/-}$ mouse was confirmed to be an outlier using the Dixon's Q test, and therefore excluded from analysis. As can be observed in Figure 4A, C5aR$^{-/-}$ mice showed a significant increase in plasma creatinine at 3h after brain death compared to WT mice, which was not observed in C5L2$^{-/-}$ mice. Plasma creatinine levels in C5L2$^{-/-}$ mice did not differ from those in WT mice. This difference was not observed for BUN levels (Figure 4B).

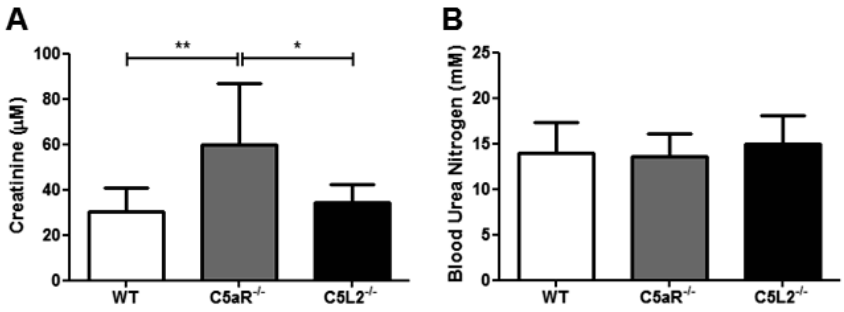

Figure 4: Renal function after 3h of brain death in WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice
(A) Plasma creatinine and (B) blood urea nitrogen levels after 3h after mouse brain death in WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice. Data are shown as mean ± SD (*P<0.05, **P<0.01).

Renal gene expression profiles after 3h of mouse brain death

Next, we investigated the local renal inflammatory response induced by donor brain death in WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice (Figure 5). We observed reduced renal inflammation in kidneys from C5L2$^{-/-}$ mice compared to WT and C5aR$^{-/-}$ mice. Although not significant for IL-1β (Figure 5A), C5L2$^{-/-}$ mice showed reduced gene expression levels of keratinocyte-derived chemokine (KC; Figure 5C), TNFα (Figure 5D), MCP-1 (Figure 5E) and P-selectin (Figure 5G) compared to either WT mice, or both WT and C5aR$^{-/-}$ mice. In contrast, IL-6 (Figure 5B) levels were significantly increased in C5aR$^{-/-}$ mice compared to WT and C5L2$^{-/-}$ mice, and KIM-1 (Figure 5H) levels were significantly lower in C5aR$^{-/-}$ mice compared to WT mice.

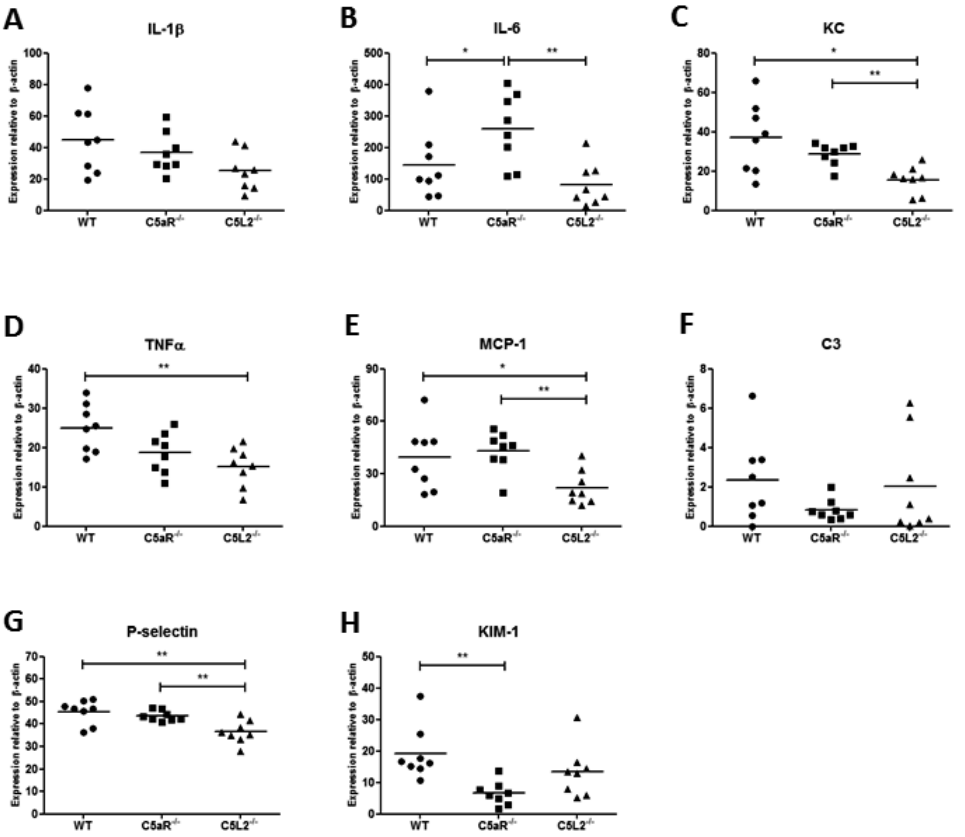

Figure 5: Renal gene expression of inflammatory mediators after 3h of brain death
Gene expression of inflammatory markers in kidneys after 3h of mouse brain death. (A) IL-1β, (B) IL-6, (C) KC, (D) TNFα, (E) MCP-1, (F) C3, (G) P-selectin, (H) KIM-1. Data are shown as expression relative to β-actin (*P<0.05, **P<0.01).

DISCUSSION

In the present study, we investigated the contribution of C5aR and C5L2 to donor brain death-induced renal inflammation. In order to achieve this, we first developed a mouse brain death model, in which a stable 3h brain death period and continuous blood pressure monitoring were accomplished. By subjecting WT, C5aR$^{-/-}$ and C5L2$^{-/-}$ mice to this brain death procedure, we showed that C5L2 promotes renal inflammation upon donor brain death. These results suggest that C5L2 is a potential target for intervention to prevent renal allograft priming in DBD donors.

In our mouse brain death model, continuous blood pressure measurement was used to monitor the mice during the 3h brain death period. In addition, venous blood gas analysis was performed. Before induction of brain death, we observed an effect of anesthesia on venous pH and lactate levels. The observed lower pH and increased lactate concentrations could be explained by the use of isoflurane, since isoflurane has repeatedly been reported to cause respiratory acidosis in mice (31). Although lactate levels were considerably reduced 3h after brain death, the slight acidosis remained. The slight acidosis after 3h of brain death could not be ascribed to isoflurane, since after inflation of the balloon, isoflurane was switched off, and ventilation was continued with a mixture of oxygen and medical air 30 min after declaration of brain death. If ventilation settings during donor management would have been inadequate, increased pCO_2 levels would have been observed in an attempt to compensate this slight metabolic acidosis. The reduced pO_2 and O_2 saturation in the venous blood gas analysis 3h after brain death compared to baseline can be explained by the use of 100% oxygen during the beginning of the procedure compared to the use of 50% oxygen during donor management. This, in combination with equal levels of pCO_2 at baseline and 3h after brain death and reduced lactate levels 3h after brain death, implicate that ventilation settings were rather sufficient.

Brain death induces systemic inflammation and activation of the complement system. To validate that indeed the complement system is activated in our mouse brain death model, we analyzed C3 fragment levels in plasma from brain-dead mice. Studies described in Chapter 5 and 6 showed that baseline C3 fragment levels in WT mice are around 1.2 ± 0.1 AU/ml. In addition, C3 fragment levels significantly increased 1 day after renal ischemia/reperfusion injury to 1.8 ± 0.4 AU/ml in WT mice (Chapter 5). Therefore, C3 fragment levels of 2.4 ± 0.5 AU/ml in brain-dead WT mice, 3.2 ± 1.2 AU/ml in brain-dead C5aR$^{-/-}$ mice, and 2.5 ± 0.8 AU/ml in brain-dead C5L2$^{-/-}$ mice indicate that indeed systemic complement activation takes place in this mouse brain death model.

With systemic activation of the complement system upon mouse brain death, one may assume that C5a is generated systemically as well. The two known receptors for C5a are C5aR and C5L2 and we recently showed that both receptors are expressed in the distal region of the nephron (18). Moreover, we observed that C5aR protein expression was increased in kidney biopsies from brain-dead donors compared to living donors (13). In addition, we showed that C5a stimulation of human renal tissue induced local renal expression of pro-inflammatory mediators like IL-1, IL-6 and IL-8 (13). Therefore, we previously hypothesized that systemically generated C5a interacts with renal C5aR, resulting in increased immunogenicity of the renal allograft to-be. Hence, inhibition of the C5a-C5aR interaction would reduce brain death-induced renal allograft priming. Surprisingly, we found that C5aR$^{-/-}$ mice have a reduced renal function, as reflected by increased plasma creatinine levels, upon donor brain death compared to WT mice. In contrast, C5L2$^{-/-}$ mice had similar plasma creatinine levels compared to WT mice. Although plasma creatinine and BUN usually both reflect renal function, we observed differences in plasma creatinine levels after donor brain death, but not in BUN levels. Assuming that the difference in plasma creatinine in C5aR$^{-/-}$ mice indeed reflected a reduction in renal function, it is unlikely to be caused by pre-renal or post-renal factors. Blood pressure and systemic fluid administration were similar in all mouse strains making a pre-renal factor less likely. Since all mice contained urine in their bladder at time of termination, ureteral obstruction as a post-renal cause seems unlikely as well. Therefore, these data might suggest that C5aR has a non-harmful or even protective function in the kidney as well, depending on the disease model. Recently, the C5a-C5aR axis has been suggested to be beneficially involved in regeneration, as in bone repair after fracture (32), and in neural tube formation during embryogenesis (33). In addition, we observed increased expression of IL-6 and reduced expression of KIM-1 in C5aR$^{-/-}$ mice, compared to WT mice. A hypothetical protective effect for C5aR, additional to the pro-inflammatory properties of C5aR, might explain the increased expression of the pro-inflammatory cytokine IL-6 in C5aR$^{-/-}$ mice. Although KIM-1 is generally accepted as damage marker for acute kidney injury (34), KIM-1 has also been hypothesized to contribute to cell survival and repair (35). Therefore, the reduced gene expression of KIM-1 in C5aR$^{-/-}$ mice would be compatible with a potential protective function of C5aR in the kidney in specific disease models, including mouse brain death. Although we previously assumed that inhibition of the C5a-C5aR axis in brain-dead donors would be beneficial for renal allograft quality, the current data indicate that C5aR should not be inhibited during donor brain death. Nevertheless, further research is required to elucidate the role of C5aR in kidneys from brain-dead donors.

In addition, we observed that C5L2$^{-/-}$ mice display reduced local renal inflammation upon donor brain death compared to WT mice. Moreover, for some pro-inflammatory mediators C5L2$^{-/-}$ mice even showed reduced gene expression compared to C5aR$^{-/-}$ mice. In a

previous study, we observed that C5a stimulation of human renal tissue resulted in local renal expression of pro-inflammatory cytokines (13). The data from the current study suggest that not C5aR, but C5L2 mediates this local renal-induced expression of pro-inflammatory mediators upon C5a stimulation. Therefore, inhibition of renal C5L2 in the brain-dead donor might reduce local renal inflammation and renal allograft priming prior to organ retrieval.

Unfortunately, selective C5L2 antagonists do not exist to date. Although the ligand binding sites of C5aR and C5L2 for C5a are almost identical, PMX53 does not prevent C5a binding to C5L2, as it does for C5aR (36). In 2004, the C5a mutant $A8^{\Delta 71\text{-}73}$ was reported to prevent binding of C5a to both C5aR and C5L2 (36). However, where inhibition of C5L2 in the brain-dead donor might be beneficial, our results suggest that inhibition of C5aR might be detrimental for the renal allograft to-be. Therefore, other options like C5L2 blocking antibodies or novel development of specific small molecule C5L2 antagonists should be explored.

In conclusion, we described the development of a mouse brain death model, in which a stable 3h brain death period and continuous blood pressure monitoring was accomplished. In addition, we propose that inhibition of C5L2 might be a potential strategy in brain-dead donors in order to reduce local renal inflammation induced by donor brain death and prevent renal allograft priming prior to organ retrieval. Although the effect of C5L2 inhibition on non-renal donor organs should be investigated as well, C5L2 inhibition in brain-dead donors might improve post-transplant renal allograft outcome.

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Part B

C5aR and C5L2 in Renal Ischemia/Reperfusion injury