General Introduction and Scope of the Thesis
Renal transplantation is the first choice of treatment for end-stage renal disease, and increasing numbers 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. In the last decades, major advances in development of immunosuppressive drugs resulted in excellent one year graft survival of renal allografts. One year allograft survival rates improved from 88.8% in 1988 to 93.6% in 1996 for kidneys originating from living donors, and from 75.7% in 1988 to 87.7% in 1996 for kidneys originating from deceased donors (1). In 2012 in the Netherlands, one year renal allograft survival rates reached 98% and 96% for kidneys originating from living and deceased donors respectively (2). However, long term renal allograft survival did not benefit from these improvements to the same extent, and especially performances of kidneys originating from deceased donors lag behind compared to their living counterparts (1,3). In order to improve long term graft function and survival, identification of new targets for treatment of potential organ donors, the renal allograft itself and renal transplant recipients is crucial. Emerging evidence shows that the complement system is a potential target to serve this purpose, since complement is activated at all stages throughout the renal transplant procedure, e.g. during donor brain death, ischemia/reperfusion injury (IRI), and antibody-mediated rejection.

The complement system
Complement was first described in the 1890s as a heat-labile protein in serum that ‘complemented’ heat-stable antibodies in the killing of bacteria (4). Today, the complement system is known to be part of the innate immune system, and comprises over thirty membrane-bound and soluble proteins (5-7). This complex cascade can be activated via three pathways: the classical, the alternative and the lectin pathway. All pathways converge to cleavage and activation of central complement component C3 by C3 convertases. The classical pathway can be initiated by antibody-antigen complexes, apoptotic cell debris and acute phase proteins like C-reactive protein (CRP), whereas the lectin pathway can be initiated by mannose binding lectin (MBL) or ficolins interacting with their carbohydrate ligands. Activation of these two pathways leads to activation of C4 and C2, and subsequent formation of the C3 convertase C4b2a, resulting in cleavage of C3 in C3a and C3b. The alternative pathway consists of spontaneous low grade hydrolysis of systemic C3, resulting in C3(H2O), which can associate with factor B. This complex is subsequently activated by factor D, resulting in the C3 convertase C3bBb, leading to cleavage of C3 in again C3a and C3b. C3b can bind to cell-surface carbohydrates, thereby enhancing alternative pathway activation. In addition, the alternative pathway can serve as an amplification loop of all three pathways, when bound C3b formed in any of the pathways binds to factor B and yields extra C3 convertases.
Formation of the C3 convertases and cleavage of C3 results in formation of the C5 convertases C4b2a3b or C3bBb3b, depending on the pathway which initiated complement activation. C5 will be cleaved into C5a and C5b. When C5b associates with C6 and C7, formation of the terminal complement complex C5b-9 or membrane attack complex (MAC) is initiated, resulting in formation of a pore in the cell membrane followed by lysis of the cell.

**Complement component 5a**

Besides formation of C5b-9, C3a and C5a are generated during complement activation. Late 1960s, they were shown to induce anaphylactic shock-like responses, like spasmogenic activity of ileal (8) and bronchial (9) smooth muscle cells, increase of vascular permeability (8,10), decrease of peripheral vascular resistance (11) and vasoconstriction of large arteries (12), hence rendering their label as anaphylatoxins. In addition, both C3a and C5a are able to recruit leukocytes to the site of complement activation, but C5a is known to have the most potent chemotactic and pro-inflammatory properties (13).

C5a is a 74 amino acid (~11 kDa) peptide. It has a glycosylation site in asparagine at position 64, but glycosylation is not essential for its function. While the N-terminus of C5a is essential for binding to its receptors, the agonist activity of C5a is retained in the C-terminal amino acid residues 69-74, in particular the arginine residue at position 74 (14-16). This C-terminal arginine can be cleaved off by carboxypeptidase N present in serum (14,17) or by bacterial proteases (18), yielding its degradation product desarginated C5a or C5a\textsubscript{desArg}. Where C5a is able to induce anaphylactic shock-like responses, C5a\textsubscript{desArg} is not (8). However, C5a\textsubscript{desArg} is able to induce leukocyte chemotaxis as well (19-21).

**The receptors for C5a**

To date, two receptors for C5a have been identified: C5a receptor (C5aR, also known as CD88) (22,23) and C5a-like receptor 2 (C5L2, also known as GPR77) (24), which were first discovered in 1991 and 2000 respectively. Although C5aR was initially identified on neutrophils and C5L2 on dendritic cells, these receptors are now known to be abundantly expressed on diverse subsets of leukocytes, with expression of both receptors being reported on neutrophils (25-27), monocytes / macrophages (22,27,28), dendritic cells (24,25,29), natural killer cells (30), T-lymphocytes (25,29) and bone marrow / hematopoietic stem/progenitor cells (31). An overview of leukocyte subsets expressing C5aR and C5L2 is provided in Table 1.

C5aR and C5L2 are seven-transmembrane receptors, and genetically belong to the rhodopsine superfamily of G-protein coupled receptors (GPCRs) (16,22,24,32). Both receptors are encoded on the long arm of chromosome 19, with C5L2 being located downstream of C5aR, and the genes sharing 58% nucleotide sequence homology (16,33).
Table 1: Leukocyte subsets with reported mRNA or protein expression of C5aR and C5L2, or effects of receptor deficiency or inhibition

<table>
<thead>
<tr>
<th>Leukocyte Subset</th>
<th>mRNA expression</th>
<th>C5aR Protein expression</th>
<th>C5aR deficiency or inhibition affect cell function / response</th>
<th>mRNA expression</th>
<th>C5L2 Protein expression</th>
<th>C5L2 deficiency or inhibition affect cell function / response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basophils</td>
<td>Bamberg et al, 2010 (25)</td>
<td></td>
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</tbody>
</table>
Although the majority of GPCR genes appears intronless (14,34), C5aR and C5L2 genes both contain introns (14,16). The presence of introns could result in alternative splicing and receptor variants (35), although C5aR and C5L2 splice variants have not been reported to date. C5aR and C5L2 mRNA contain 350 and 337 codons respectively, yielding 42 kDa C5aR and 37 kDa C5L2 proteins, sharing 36-38% amino acid homology (14,16,24,32,33).

C5a binds to the extracellular N-terminus and third extracellular loop of C5aR (16). C5L2 has an N-terminal binding site for C5a almost identical to that of C5aR (36). Both receptors bind C5a with the same high affinity. However, C5L2 has a much higher affinity for C5a$_{desArg}$ (28,36). In addition, C5L2 has been shown to bind to C3a and its degradation product C3a$_{desArg}$ (37). It has been proposed that C5L2 has two distinct ligand binding sites: one for C5a and C5a$_{desArg}$ and one for C3a and C3a$_{desArg}$ (36,37). Since identical subsets of leukocytes are known to express C5aR or C5L2 (Table 1), and respond to C5a, C5a$_{desArg}$, C3a or C3a$_{desArg}$ (Table 2), it is tempting to hypothesize that both receptors mediate intracellular signaling, and influence inflammatory responses. Indeed C5a-C5aR interaction has been shown extensively to result in intracellular signaling. However, contribution of C5L2 to intracellular signaling remains debatable.

**Intracellular signaling mediated by C5a receptors**

In line with its genetic and structural features, C5aR has been shown to function in a G-protein coupled manner (reviewed in 46). This receptor associates with Ga$\alpha$ and Ga$\alpha$16 subunits (16,32,46,47). C5a stimulation of C5aR results in intracellular calcium mobilization (36) and activation of several signaling pathways including phosphatidylinositol 3-kinase (PI-3K) / Akt (48), phospholipase D (PLD) (49), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) (28) pathways. In multiple subsets of leukocytes, C5a exerts all kinds of pro-inflammatory effects via these pathways, including chemotaxis, expression of adhesion molecules, release of reactive oxygen species, granule enzymes, and cytokines, phagocytosis and inhibition of apoptosis (36,48-50).

In contrast to C5aR, intracellular signaling via G-proteins seems virtually impossible for C5L2. GPCRs contain a highly conservative DRY motif in the third transmembrane segment, which is essential for G-protein coupling (51). Substitution of the essential arginine residue in the DRY motif to a leucine residue makes C5L2 obligatory uncoupled from heterotrimeric G proteins (28). The third intracellular loop of GPCRs is also involved in binding of G-proteins, but the third intracellular loop of C5L2 is shortened and lacks PKC phosphorylation sites. On top, C5L2 seems to lack an NPXXY motif, which has been demonstrated to be important for phospholipase C (PLC) and MAPK activation in receptors of the same superfamily (16,33). However, C5L2 holds seven serine and threonine residues, which could serve as potential
Table 2: Leukocyte subsets with reported responsiveness to complement ligands C5a, C5a_{desArg}, C3a and C3a_{desArg}

<table>
<thead>
<tr>
<th>Subset</th>
<th>C5a</th>
<th>C5a_{desArg}</th>
<th>C3a</th>
<th>C3a_{desArg}</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-lymphocytes</td>
<td>Honczarenko et al, 2005 (42)</td>
<td>Honczarenko et al, 2005 (42)</td>
<td></td>
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<tr>
<td>B-lymphocytes</td>
<td>Honczarenko et al, 2005 (42)</td>
<td>Honczarenko et al, 2005 (42)</td>
<td></td>
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<tr>
<td>PMNs</td>
<td>Scola et al, 2009 (43)</td>
<td>Scola et al, 2009 (43)</td>
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<tr>
<td></td>
<td>Gao et al, 2005 (26)</td>
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<td>Chen et al, 2007 (27)</td>
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<tr>
<td></td>
<td>Bamberg et al, 2010 (25)</td>
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<tr>
<td>Eosinophils</td>
<td>Schraufstatter et al, 2002 (10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basophils</td>
<td>Bürgi et al, 1994 (45)</td>
<td>Bürgi et al, 1994 (45)</td>
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</tbody>
</table>

phosphorylation sites by G-protein coupled receptor kinases (GRKs) (24). Although C5a stimulation was shown to induce C5L2 phosphorylation in transfected cells (28), C5a or C5a_{desArg} seemed unable to induce C5L2 internalization, ERK1/2 phosphorylation, intracellular calcium mobilization, degranulation, or mRNA expression of inflammatory genes (28,36). These observations led to the hypothesis that C5L2 serves as a non-signaling decoy receptor, preventing C5a binding to C5aR and thereby inhibiting C5a-C5aR-mediated signaling (43). In addition to the decoy hypothesis, a secondary anti-inflammatory mechanism for C5L2 was proposed by Bamberg et al, suggesting that C5L2 acts as a negative modulator of C5aR-mediated signaling. They showed that in human neutrophils C5L2 reduces C5aR-mediated phosphorylation of ERK1/2 in a G-protein independent manner via a C5aR-C5L2-β-arrestin complex (25). This theory was supported by others (52,53). In this model, C5L2 does not function by scavenging C5a, but by negatively modulating signaling downstream from C5aR.

C5a in renal disease
The complement system is involved in antibody-mediated rejection (AMR) of renal allografts. This is most prominently reflected by C4d deposition in renal allografts as diagnostic tool for AMR. Although the function of C5aR and C5L2 in renal allograft rejection has not been investigated yet, emerging evidence suggests that C5a and/or C5a_{desArg} do play a role in AMR.
Eculizumab is a humanized monoclonal antibody directed against C5, preventing cleavage of C5 into C5a and C5b, thereby inhibiting generation of C5a, C5a_{desArg} and formation of C5b-9 (61). It is registered for treatment of paroxysmal nocturnal hemoglobinuria (PNH) (62). In addition, it has been shown to be effective in treatment of several kidney diseases like atypical hemolytic uremic syndrome (aHUS) (63-66), C3 glomerulopathies (65,67), and lupus nephritis (68). An increasing number of cases has been reported in which eculizumab has been used to treat biopsy proven, C4d-positive acute AMR of renal allografts, resulting in recovery of renal function and reduction of inflammatory markers (case reports: 69-74). Extrapolating these findings, Stegall et al showed that post-transplant treatment with eculizumab prevented early AMR, reducing incidence rates from 41.2% to 7.7% (75). To date, these effects of eculizumab have been attributed to inhibition of C5a-C5aR-mediated signaling.

C5aR and C5L2 in the kidney

Although C5aR and C5L2 were initially detected on immune cells, expression of both receptors has been demonstrated on non-immune cells as well. As shown in Table 3, C5aR and C5L2 mRNA expression has been reported in virtually all organs throughout the body. In addition, C5aR protein expression has been reported in the kidney (26,54,57-60), liver (26,56), heart (26), lung (26,56), brain (55) and bladder (54). In contrast, C5L2 protein expression has only been reported in heart, liver and lung (26).

In the kidney, C5aR expression has been investigated under diverse inflammatory conditions, and has been reported on both proximal and distal tubular epithelial cells. In contrast, renal C5L2 protein expression has not been reported so far. Therefore, in Chapter 2, the expression and localization of C5aR and C5L2 under healthy conditions in the kidney were studied. Subsequently, renal C5aR and C5L2 expression dynamics in human post-transplant kidney biopsies from patients suffering from acute tubular necrosis, acute cellular and vascular rejection or interstitial fibrosis and tubular atrophy were examined.

C5aR and C5L2 in donor brain death

Although the numbers of renal grafts derived from living and DCD donors are increasing, a substantial quantity of renal grafts is derived from DBD donors. It is well established that kidneys from DBD donors show inferior renal function and poorer graft survival after transplantation when compared to their living counterparts (1,3,76,77). This difference proved to be independent from HLA mismatching and cold ischemia times (3). Donor brain death in itself triggers hemodynamic instability, hormonal changes, and activation of the immune system (78-80), which could very well be responsible for the inferior results of DBD-derived renal allografts. In this way, organ viability is affected before transplantation.
Table 3: Tissues with reported mRNA or protein expression of C5aR or C5L2.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>C5aR mRNA expression</th>
<th>C5aR Protein expression</th>
<th>C5L2 mRNA expression</th>
<th>C5L2 Protein expression</th>
</tr>
</thead>
</table>
The systemic inflammation occurring upon donor brain death is characterized by elevated circulating levels of general inflammatory markers like IL-6 (78,81), IL-8 (81), MCP-1 (81), soluble TNF receptor II (78), and soluble IL-2 receptor (78). In addition, donor brain death triggers activation of the complement system, as reflected by increased circulating levels of C3d and C5b-9 in DBD donors compared to living donors (82,83). As a result, the systemic inflammation initiated by donor brain death induces local organ inflammation, priming the renal allograft to be. Kidneys from brain-dead rats show increased expression of pro-inflammatory molecules and influx of neutrophils, macrophages and T cells (76,77,79,80). In addition, local renal production of complement components is observed in brain-dead rats (80) and humans (84). Possibly, renal inflammation initiated by donor brain death could explain the inferior function and graft survival of DBD donor-derived renal allografts after transplantation. In Chapter 3, systemic release of C5a in human brain-dead donors and the expression dynamics of renal C5aR upon donor brain death were analyzed. Thereafter, direct effects of C5a on human renal tissue were investigated. To explore the contribution of C5aR and C5L2 to donor brain death-induced renal inflammation, a mouse brain death model was developed, in which a stable 3h brain death period and continuous blood pressure monitoring were accomplished. In Chapter 4, this mouse brain death model is described, and the role of C5aR and C5L2 in brain death-induced renal inflammation is studied by inducing brain death in C5aR−/− and C5L2−/− mice using this model.

C5aR and C5L2 in ischemia/reperfusion injury
Renal ischemia/reperfusion injury (IRI) is unavoidable when performing renal transplantation. Multiple components of the innate immune system, including the complement system, toll-like receptors (TLRs), NOD-like receptors and inflammasomes have been shown to be involved in renal IRI (85-87). Zhou et al demonstrated that C3-, C5- and C6-deficient mice were protected against renal IRI, where C4-deficient mice were not, implicating a role for the terminal pathway in renal IRI (88). In addition, involvement of C5aR in renal IRI has been shown extensively. Using different animal models including C5aR−/− mice, C5aR antagonists, siRNA directed against C5aR and antibodies directed against C5 preventing formation of C5a and C5b, and inhibition of the C5a-C5aR axis were shown to protect rodent kidneys against renal IRI (89-94). However, the role of C5L2 in renal IRI has not been investigated yet.

In order to characterize IRI-mediated complement activation in mice, assays measuring functional systemic complement pathway activity are essential. In addition, due to novel treatment options intervening in the complement system, there is an increasing interest in the monitoring of systemic complement activation in the experimental setting. However, measuring systemic complement activation in mice is still challenging. Therefore, we developed a sandwich ELISA detecting mouse C3 activation fragments, as well as functional
ELISAs detecting specific activities of the three complement pathways at the level of C3 and at the level of C9, as will be described in Chapter 5. Subsequently, in Chapter 6, the role of C5L2 to renal IRI-mediated inflammation was investigated by subjecting C5aR\textsuperscript{−/−} and C5L2\textsuperscript{−/−} mice to 40 minutes warm bilateral renal IRI, followed by reperfusion for 1, 3 and 7 days. In this study, the effect of IRI on renal function, renal morphology, systemic complement activation, leukocyte influx and renal gene expression profiles were analyzed.

Leukocytes are involved in inflammatory processes, like removal and regeneration of damaged tissue, and in allograft rejection after solid organ transplantation. C5aR and C5L2 are abundantly expressed on leukocytes, especially on neutrophils. Although the role of C5aR in leukocyte migration has been studied extensively, the role of C5L2 in leukocyte migration is largely unknown. Therefore, in Chapter 7, an in vivo migration study in C5aR\textsuperscript{+/−} and C5L2\textsuperscript{+/−} mice was performed and the role of these two receptors on the migration of neutrophils in response to C5a and C5a\textsubscript{desArg} was examined.

In Chapter 8, the role of renal-expressed C5aR and C5L2 versus leukocyte-expressed C5aR and C5L2 in the pathophysiology of renal IRI was investigated. Bone marrow chimeras were generated by replacing bone marrow of WT, C5aR\textsuperscript{+/−} and C5L2\textsuperscript{+/−} mice with WT bone marrow. In addition, bone marrow of WT mice was replaced with WT, C5aR\textsuperscript{−/−} or C5L2\textsuperscript{−/−} bone marrow. These bone marrow chimeras were subjected to 40 minutes warm bilateral renal IRI, and sacrificed 3 days after reperfusion. Subsequently, renal function and renal morphology were studied.

To conclude this thesis, in Chapter 9 the results described in chapters 2 to 8 are summarized and discussed in their context, and considerations for further research are provided.
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Part A

C5aR and C5L2 in Donor Brain Death