C5a Receptors in Renal Transplantation
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C5L2 is not involved in *in vivo* Neutrophil Migration in Response to C5a and C5a_{desArg}

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Manuscript in preparation
ABSTRACT

Complement activation products C3a and C5a are major mediators of leukocyte migration, with C5a having the most potent chemoattractant properties. Today, two receptors for C5a and its degradation product C5a$_{desArg}$ have been described, namely C5a receptor (C5aR) and C5a-like receptor 2 (C5L2). An increasing number of studies investigated the function of the recently discovered C5L2 in inflammatory processes, but controversy about its role in inflammation exists. Both receptors are abundantly expressed on leukocytes, mainly 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, we performed an in vivo migration study in which wildtype (WT), C5aR$^{-/-}$ and C5L2$^{-/-}$ mice were injected intraperitoneally with PBS, recombinant C3a, C5a or C5a$_{desArg}$. Subsequently, leukocyte migration towards the peritoneal cavity in response to these stimuli was analyzed six hours after injection by peritoneal lavage. Neutrophil, monocyte and lymphocyte migration capacity was determined. We observed that C5aR, but not C5L2, is involved in neutrophil migration in response to C5a and C5a$_{desArg}$. No major differences were observed in monocyte or lymphocyte migration in response to the complement ligands. Our results indicate that C5L2 is not involved in C5a and C5a$_{desArg}$-mediated neutrophil migration.
INTRODUCTION

Leukocytes play a key role in inflammatory processes, including elimination of microorganisms, removal and regeneration of damaged tissue, and in disease processes like autoimmune disease and allograft rejection after solid organ transplantation. The recruitment of leukocytes to sites of inflammation has been studied extensively and the complement system has been shown to be a major mediator of leukocyte migration. Activation of the complement system results in formation of anaphylatoxins, namely C3a and C5a, of which C5a has been shown to be the most powerful chemoattractant (1).

C5a is a 74 amino acid peptide which is formed upon C5 cleavage by C5 convertases (2,3). This ~11 kDa peptide has a potential 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 arginyl residue at position 74 (4-6). This C-terminal arginine can be cleaved off by carboxypeptidase N present in serum (4,7) or by bacterial proteases (8), yielding its degradation product desarginated C5a or C5a_{desArg}. Late 1960s, C5a was shown to induce responses resembling anaphylactic shock, like contraction of ileal smooth muscle cells (which was histamine dependent), and increase of vascular permeability in skin, whereas C5a_{desArg} did not possess this capacity (9). However, both C5a and C5a_{desArg} have shown to be effective chemoattractants for leukocytes (2,10,11).

To date, two receptors are known that bind both C5a and C5a_{desArg}, being the C5a receptor (C5aR, also known as CD88) (12) and C5a-like receptor 2 (C5L2, also known as GPR77) (13). Both receptors are abundantly expressed on leukocytes, especially on neutrophils, but expression has been shown on non-immune cells as well (14-16). C5aR and C5L2 have more or less the same binding affinity for C5a, but C5L2 has been reported to have a 10-50 fold higher affinity for C5a_{desArg} compared to C5aR (15,17). In neutrophils, the C5a-C5aR interaction exerts multiple pro-inflammatory functions like chemotaxis, and release of superoxides, granula and cytokines. Although C5a_{desArg} has been shown to induce neutrophil migration (2,18) and that C5a_{desArg}-C5aR interaction induces cell activation in C5aR-transfected rat basophilic leukemia cells (5), it remains to be proven that C5a_{desArg} is indeed able to induce neutrophil migration via C5aR ligation.

Considerably less is known about the role of C5L2 in leukocyte migration. Indirect evidence from inflammatory disease models indicates that C5L2 inhibits C5aR-mediated neutrophil migration (19,20), which would support the hypothesis that C5L2 serves as a decoy receptor for C5a and C5a_{desArg} (21). However, the direct involvement of C5L2 in leukocyte migration has not been studied.
Most studies investigating leukocyte migration used (a modification of) the in vitro Boyden chamber assay (22). Although this method is widely accepted, an in vivo model may resemble the physiological setting more closely. In addition, activated serum, with or without addition of a carboxypeptidase N inhibitor, was often used as source for C5a or C5a\textsubscript{desArg}. In these studies, presence of trace amounts of C5a in C5a\textsubscript{desArg} isolates, or vice versa, can not be excluded. Therefore, to investigate the role of C5aR and C5L2 in leukocyte migration in response to C5a and C5a\textsubscript{desArg}, we performed an in vivo migration study in wildtype (WT), C5aR\textsuperscript{-/-} and C5L2\textsuperscript{-/-} mice following treatment with recombinant C5a and C5a\textsubscript{desArg}.

**MATERIALS AND METHODS**

*Animals*

C57Bl/6 wildtype (WT), and C5aR\textsuperscript{-/-} (23) and C5L2\textsuperscript{-/-} (19) 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 at the University Medical Center Groningen. Animals were housed in groups in standard laboratory cages up to the experiments, and were housed individually after intraperitoneal injection up to sacrifice (see below). Animals were allowed free access to food and water throughout the experiment. The studies were carried out under a protocol approved by the Institutional Animal Care Committee of the University of Groningen (project number 6810AB).

*In vivo migration model*

Male mice, aged 8 to 12 weeks, were injected intraperitoneally with 200 µl of PBS, 2 µM mouse recombinant C5a (Hycult Biotechnology, Uden, The Netherlands), 2 µM mouse recombinant C5a\textsubscript{desArg} (Hycult Biotechnology), or 2 µM mouse recombinant C3a (MyBioSource, San Diego, CA, USA). Recombinant C5a and C5a\textsubscript{desArg} were reconstituted in sterile water (B. Braun Melsungen AG, Melsungen, Germany), and recombinant C3a was reconstituted in sterile PBS (Pharmacy University Medical Center Groningen, Groningen, The Netherlands), according to the manufacturer’s instructions. For the injections, sterile BD Micro-Fine+ insulin syringes (BD, Franklin Lakes, NJ, USA) were used.

Peritoneal lavage (PL) was performed 6 hours after injection under general anesthesia (2% isoflurane/O\textsubscript{2}), after which mice were sacrificed by exsanguination. PL was carried out as follows. Using a 23G needle, 5 ml ice cold PBS was injected into the peritoneal cavity, after which the belly was gently massaged to distribute the PBS throughout the peritoneal cavity. The peritoneal fluid was collected via a small midline abdominal incision. Thereafter, blood was collected via heart puncture and transferred to EDTA-collection tubes.
**Cell counts**
Different subsets of leukocytes in blood and PL were determined using Sysmex XN-10 and XN-20 cell counters in combination with SP-10 slide maker and stainer (Sysmex, Kobe, Japan).

**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 individual values and their means.

**RESULTS**

*Increased circulating neutrophils in C5aR⁻/⁻ and C5L2⁻/⁻ mice*
Baseline characteristics of circulating red blood cells, leukocyte subtypes and platelets were determined for the three mouse strains. As can be observed in Table 1, WT, C5aR⁻/⁻ and C5L2⁻/⁻ mice contained similar levels of circulating red blood cells, total white blood cells, monocytes, lymphocytes and platelets. However, elevated levels of circulating neutrophils were observed in C5aR⁻/⁻ and C5L2⁻/⁻ mice compared to WT mice.

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Wildtype mice (n=5)</th>
<th>C5aR⁻/⁻ mice (n=5)</th>
<th>C5L2⁻/⁻ mice (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red blood cells</td>
<td>10¹²/L</td>
<td>9.12 ± 0.59</td>
<td>9.63 ± 0.26</td>
<td>9.40 ± 0.36</td>
</tr>
<tr>
<td>White blood cells</td>
<td>10⁹/L</td>
<td>4.02 ± 1.80</td>
<td>4.78 ± 0.62</td>
<td>6.26 ± 1.83</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>10⁹/L</td>
<td>0.29 ± 0.09</td>
<td>0.58 ± 0.21*</td>
<td>0.54 ± 0.11*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>10⁹/L</td>
<td>3.71 ± 1.73</td>
<td>4.18 ± 0.56</td>
<td>5.71 ± 1.79</td>
</tr>
<tr>
<td>Monocytes</td>
<td>10⁹/L</td>
<td>0.02 ± 0.01</td>
<td>0.01 ± 0.01</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>Blood platelets</td>
<td>10⁹/L</td>
<td>980 ± 219</td>
<td>1186 ± 71</td>
<td>1104 ± 176</td>
</tr>
</tbody>
</table>

* P < 0.05 compared to wildtype mice

*Total white blood cells*
To investigate the effect of C5aR and C5L2 on leukocyte migration in response to different complement ligands, WT, C5aR⁻/⁻ and C5L2⁻/⁻ mice were injected intraperitoneally with PBS, C3a, C5a or C5a₉Arg⁻/⁻. Leukocytes migrated into the peritoneal cavity were harvested by peritoneal lavage (PL) 6 hours after injection. Total numbers of white blood cells in the peritoneal cavity were identical between PBS injected WT, C5aR⁻/⁻ and C5L2⁻/⁻ mice (Figure 1). In both WT and C5L2⁻/⁻ mice, injections with C3a, C5a or C5a₉Arg increased the total amount of white blood cells in the peritoneal cavity, reaching statistical significance for C3a and C5a₉Arg (Figure 1A and 1C). On the contrary, while a significant increase of total white blood cells was also observed for C3a injected C5aR⁻/⁻ mice, this increase in total white blood
cells was completely absent in C5a or C5a\textsubscript{desArg} injected C5aR\textsuperscript{-/-} mice (Figure 1B).

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**Neutrophils**

Subsequently, we determined which leukocyte subtypes migrated into the peritoneal cavity upon intraperitoneal injection with complement ligands. Neutrophils turned out to compose the major subset of leukocytes migrating towards C3a, C5a and C5a\textsubscript{desArg}. The percentage of neutrophils from total white blood cells present in the peritoneal cavity increased from 3.5% ± 1.0 in PBS WT injected mice, to 62.8% ± 3.4 in C3a, 46.0% ± 17.5 in C5a, and 44.8% ± 16.1 in C5a\textsubscript{desArg} injected WT mice respectively. Although elevated levels of circulating neutrophils were observed in C5aR\textsuperscript{-/-} and C5L2\textsuperscript{-/-} mice (Table 1), PBS injected WT, C5aR\textsuperscript{-/-} and C5L2\textsuperscript{-/-} mice all showed similar low numbers of neutrophils in the peritoneal cavity. Injection with C3a, C5a or C5a\textsubscript{desArg} resulted in significantly increased numbers of neutrophils in both WT and C5L2\textsuperscript{-/-} mice (Figure 2A and 2C), and no significant differences were observed in neutrophil migration between WT and C5L2\textsuperscript{-/-} mice in response to any of the complement ligands. Also in C5aR\textsuperscript{-/-} mice, injection with C3a significantly increased numbers of neutrophils in the peritoneal cavity. However, neutrophil migration in response to C5a and C5a\textsubscript{desArg} was...

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**Figure 1: Total white blood cells in the intraperitoneal cavity after complement ligand injection.**

PBS, C3a, C5a or C5a\textsubscript{desArg} (5ARG) were injected intraperitoneally in (A) WT, (B) C5aR\textsuperscript{-/-} and (C) C5L2\textsuperscript{-/-} mice. Leukocytes were harvested 6h post injection by peritoneal lavage (PL). Data are shown as individual values and their mean (*P<0.05, **P<0.01).

**Figure 2: Neutrophils in the intraperitoneal cavity after complement ligand injection.**

PBS, C3a, C5a or C5a\textsubscript{desArg} (5ARG) were injected intraperitoneally in (A) WT, (B) C5aR\textsuperscript{-/-} and (C) C5L2\textsuperscript{-/-} mice. Leukocytes were harvested 6h post injection by peritoneal lavage (PL). Data are shown as individual values and their mean (*P<0.05, **P<0.01).
completely abrogated in C5aR\(^{+/−}\) mice (Figure 2B).

**Monocytes**

PBS injected WT, C5aR\(^{+/−}\) and C5L2\(^{+/−}\) mice all showed similar numbers of monocytes in the peritoneal cavity (Figure 3). In our model, intraperitoneal injections with C3a, C5a or C5a\(_{\text{desArg}}\) did not result in migration of monocytes into the peritoneal cavity in any of the mouse strains.

**Lymphocytes**

In accordance with the results found for neutrophils and monocytes, PBS injected WT, C5aR\(^{+/−}\) and C5L2\(^{+/−}\) mice showed similar levels of lymphocytes in the peritoneal cavity (Figure 4). C3a and C5a were unable to induce migration of lymphocytes into the peritoneal cavity. Remarkably, injection of C5a\(_{\text{desArg}}\) resulted in increased numbers of lymphocytes in the peritoneal cavity in C5L2\(^{+/−}\) mice, but not in WT and C5aR\(^{+/−}\) mice (Figure 4C, 4A and 4B respectively). With the techniques used, no discrimination between B and T lymphocytes could be made.

**Figure 3: Monocytes in the intraperitoneal cavity after complement ligand injection.**

PBS, C3a, C5a or C5a\(_{\text{desArg}}\) (5ARG) were injected intraperitoneally in (A) WT, (B) C5aR\(^{+/−}\) and (C) C5L2\(^{+/−}\) mice. Leukocytes were harvested 6h post injection by peritoneal lavage (PL). Data are shown as individual values and their mean.

**Figure 4: Lymphocytes in the intraperitoneal cavity after complement ligand injection.**

PBS, C3a, C5a or C5a\(_{\text{desArg}}\) (5ARG) were injected intraperitoneally in (A) WT, (B) C5aR\(^{+/−}\) and (C) C5L2\(^{+/−}\) mice. Leukocytes were harvested 6h post injection by peritoneal lavage (PL). Data are shown as individual values and their mean (*P<0.05).
DISCUSSION

Leukocyte migration starts with cell polarization induced by chemoattractant-receptor interaction. Complement-activation products C3a, C5a, and C5a\textsubscript{desArg} have been demonstrated to be strong inducers of leukocyte chemotaxis. For C5a and C5a\textsubscript{desArg}, leukocyte chemotaxis and activation has been attributed to interaction of these ligands with C5aR (24, 25), although both ligands bind to C5L2 as well (17). In addition, C3a has been reported to bind to C5L2 (17). C5L2 is thought to modulate C5aR-induced leukocyte activation responses (24, 26), but less is known about the role of C5L2 in leukocyte chemotaxis. Using an \textit{in vivo} migration assay, we showed that C5L2 is not involved in leukocyte, and more specific in neutrophil migration.

Both C5aR and C5L2 are known to be abundantly expressed on diverse subsets of leukocytes, with expression of both receptors being reported on neutrophils (26), monocytes (15), natural killer cells (27), and dendritic cells (13, 28). Additionally, C5aR expression has been reported on T lymphocytes (28, 29) and hematopoietic stem/progenitor cells (30). However, most research involving these receptors has been performed on neutrophils. Previously, Gerard et al suggested that deficiency in C5L2 might result in enhanced neutrophil chemotaxis (19). In a model of ovalbumin-induced immune complex lung injury, increased neutrophil infiltration was observed in C5L2\textsuperscript{-/-} mice compared to WT mice. In line with these findings, we observed that C5a and C5a\textsubscript{desArg} were able to induce neutrophil chemotaxis in WT and C5L2\textsuperscript{-/-} mice. However, in our \textit{in vivo} assay, neutrophil chemotaxis in response to these complement ligands was not enhanced in C5L2\textsuperscript{-/-} mice compared to WT mice. Therefore, the enhanced neutrophil infiltration observed by Gerard et al might be mediated by non-complement derived chemotactic factors generated in the inflammatory disease model. In our model, C5a and C5a\textsubscript{desArg} were able to induce neutrophil migration to the same extend in WT and C5L2\textsuperscript{-/-} mice, but not in C5aR\textsuperscript{-/-} mice. In addition, we observed significant infiltration of neutrophils after renal ischemia/reperfusion (IRI) injury in C5L2\textsuperscript{-/-} mice, but not in C5aR\textsuperscript{-/-} mice (Chapter 6). Taken together, our data indicate that neutrophil migration in response to C5a or C5a\textsubscript{desArg} depends on C5aR, but not on C5L2.

Although C3a has been reported to bind to C5L2, we did not observe decreased C3a-induced neutrophil migration in C5L2\textsuperscript{-/-} mice. Most likely, C3a-induced neutrophil migration occurred through interaction with C3a receptor (C3aR), and not via C5L2.

In contrast to neutrophils, no influx of monocytes into the peritoneal cavity was observed in response to any of the complement ligands. Although C3a and C5a are known to be important in monocyte and macrophage chemotaxis (24), the short 6h time frame might explain our findings. It is known that different subsets of leukocytes display different infiltration
dynamics (31). Indeed, Chen et al observed peritonitis induced migration of macrophages at 72h, but not on the short term (19,24). In line with these findings, we observed renal infiltration of macrophages at 7 days, but not at 3 days after renal IRI (Chapter 6). Since we observed attenuation of IRI-induced renal dysfunction in C5L2−/− mice as early as 1 day after IRI (Chapter 6), we focused on neutrophils.

T lymphocytes have been shown to express C3aR and C5aR. Together, C3a and C5a are reported to regulate T cell differentiation, maturation and proliferation (reviewed in (32)), but not migration. In concordance with these findings, C5L2 was not involved in C3a-mediated migration of bone marrow derived lymphocytes (33). Surprisingly, we observed increased numbers of lymphocytes in the peritoneal cavity of C5L2−/− mice after injection with C5a des-Arg. Hypothetically, C5a des-Arg might alter the apoptosis/proliferation balance in C5L2−/− mice. In addition, C5L2 in lymphocytes might function as a decoy receptor or as negative modulator of C5aR-induced responses, as has been described for neutrophils (15,21,26). C5L2 deficiency might enhance C5a des-Arg-C5aR-induced migration. Whether the increased numbers of C5L2−/− lymphocytes in PL in response to C5a des-Arg are due to an altered apoptosis/proliferation balance, an actual migration effect or another mechanism remains to be investigated.

Taken together, the current study indicates that C5L2 is not involved in C5a and C5a des-Arg-mediated neutrophil migration.

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