C3a and C5a Promote Renal Ischemia-Reperfusion Injury

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ABSTRACT

Renal ischemia reperfusion injury triggers complement activation, but whether and how the small proinflammatory fragments C3a and C5a contribute to the pathogenesis of this injury remains to be elucidated. Using C3aR−, C5aR−, or C3aR/C5aR-deficient mice and models of renal ischemia-reperfusion injury, we found that deficiency of either or both of these receptors protected mice from injury, but the C3aR/C5aR- and C5aR-deficient mice were most protected. Protection from injury was associated with less cellular infiltration and lower mRNA levels of kidney injury molecule-1, proinflammatory mediators, and adhesion molecules in postischemic kidneys. Furthermore, chimera studies showed that the absence of C3aR and C5aR on renal tubular epithelial cells or circulating leukocytes attenuated renal ischemia-reperfusion injury. In vitro, C3a and C5a stimulation induced inflammatory mediators from both renal tubular epithelial cells and macrophages after hypoxia/reoxygenation. In conclusion, although both C3a and C5a contribute to renal ischemia-reperfusion injury, the pathogenic role of C5a in this injury predominates. These data also suggest that expression of C3aR and C5aR on both renal and circulating leukocytes contributes to the pathogenesis of renal ischemia-reperfusion injury.


Ischemia reperfusion (IR) injury occurs upon reperfusion of vascularized tissue after an extended period of ischemia. It is a common source of illness and death in a wide variety of conditions, including myocardial infarction and stroke. IR injury is also an unavoidable event in organ transplantation and has a major effect on short- and long-term graft survival.1–4 The pathogenesis of IR injury is complex, and multiple factors (i.e., complement activation, the coagulation system, leukocytes, cytokines, chemokines, and adhesion molecules) are all thought to contribute to its development.5–9

The complement system consists of a set of distinct plasma proteins that react in a cascade manner upon triggering by various stimuli, such as infection and tissue injury. Complement activation generates a set of effector molecules, including the large fragment C3b and its metabolites (iC3b, C3dg); the small fragments (C3a, C5a); and the terminal product C5b-9, also called the membrane attack complex (MAC). These effector molecules have diverse biologic functions for example, C3b mediates opsonization of pathogens, C3a and C5a induce local inflammation and cell activation, and MAC mediates direct lysis of pathogens and tissue damage.10

Previous studies using mice deficient in complement components (e.g., C3, C4, C5, C6, factor B)5,11 or complement inhibitors (e.g., CD55, CD59)12,13

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have demonstrated that complement activation contributes to the pathogenesis of renal IR injury. Several studies have also suggested that the formation of MAC (C5b-9) in renal tubular epithelial cells is a critical effector mechanism through which complement mediates renal IR injury.\textsuperscript{13,14} In addition, the small fragments (C3a, C5a) could be involved in renal IR injury. Renal IR injury is a nonspecific inflammatory process; C3a and C5a, which are released during complement activation, play an important role in the initiation and regulation of inflammatory responses. Both renal cells and circulating leukocytes express C3aR and C5aR.\textsuperscript{15–20} Elevated C3a levels were detected in the circulation of mice after renal IR.\textsuperscript{21} Blocking C5aR signaling using a small interfering RNA technique or a receptor-specific antagonist reduced renal functional impairment or decreased tissue inflammation within the kidneys after renal IR,\textsuperscript{22–24} suggesting a pathogenic role for C5aR in renal IR injury.

Several important questions still remain unanswered, however: Does C3a, like C5a, participate in the development of the renal IR injury via receptor signaling? Does combined C3aR and C5aR signaling have a greater effect on the injury than does signaling of each receptor alone (because the ligands for C3aR and C5aR are likely to coexist)? How do C3a and C5a participate in the development of the injury? Which types of cells are likely to contribute to C3aR- and C5aR-mediated renal IR injury? To address these questions, we used in vivo and in vitro models of renal IR injury. Using mice with targeted deletions of C3aR and C5aR or of C3aR alone or C5aR alone and bone marrow chimeras (wild type [WT] ↔ C3aR/C5aR double knockout [DKO]), we assessed the effect of single or double deficiency of C3aR and C5aR on renal functional impairment, renal tissue damage and cellular infiltration, and production of inflammatory mediators in postischemic kidneys. We also evaluated the contribution of C5aR/C3aR-bearing renal and circulating cells to this injury.

RESULTS

Renal Expression of C3aR and C5aR Is Upregulated after Renal Ischemic Insult

Upregulation of C3aR or C5aR expression has been observed in certain pathologic conditions, such as asthma and prolonged cold ischemia.\textsuperscript{14,25} To assess whether expression of C3aR and C5aR in kidney tissues is upregulated after renal IR in our system (a warm ischemia model), we performed quantitative real-time RT-PCR to measure C3aR and C5aR expression in both noninjured and injured kidney tissues (collected 24 hours after reperfusion). Both C3aR and C5aR were detected in noninjured kidney tissues; however, expression of both receptors was significantly increased after renal ischemic insult (Figure 1, A and B). Thus, upregulation of C3aR and C5aR expression in ischemic kidneys, together with the observation of elevated C3a in the circulation after renal IR,\textsuperscript{21} would support the notion that C3aR- and C5aR-ligand interaction participates in the process of renal IR injury.

Deficiency of C3aR and C5aR Protects Mice from Renal IR Injury

To assess the effect of C3a and C5a receptor deficiency on renal IR injury, we induced renal IR injury in four groups of mice WT, C3aR\textsuperscript{−/−}, C5aR\textsuperscript{−/−}, and DKO. Renal function was assessed at 24 and 48 hours after reperfusion by measuring BUN. All three knockout groups had significantly lower levels of BUN at both time points compared with WT mice, indicating that renal function was protected in all three knockout groups (Figure 2A). To assess renal tubular damage, histologic analysis of kidneys collected at 24 and 48 hours after reperfusion was performed. In agreement with renal functional impairment, tubular injury consisting of tubule thinning, dilatation, loss of proximal brush border, and protein casts was reduced in all three knockout groups compared with measures in WT mice; the reduction of tubular damage was greater in C5aR\textsuperscript{−/−} or DKO mice (Figure 2, B–D and SuppFigure 1).

Kidney injury molecule-1 (KIM-1) has been described as a biomarker for acute tubular injury because its expression increases dramatically after injury in proximal tubule epithelial cells in postischemic rodent kidneys.\textsuperscript{26} We therefore assessed mRNA expression of KIM-1 in injured kidneys of WT, C3aR\textsuperscript{−/−}, C5aR\textsuperscript{−/−}, and DKO mice. In correlation with renal function impairment and tubule injury, renal expression of KIM-1 was significantly lower in the knockout groups of mice than in the WT group at 24 hours after reperfusion, with about 25% (C3aR\textsuperscript{−/−}), 50% (C5aR\textsuperscript{−/−}), and 60% (DKO) reduction in the WT expression levels (Figure 2E). Significantly low renal expression of KIM-1 was also observed at 48 hours after reperfusion in the C5aR\textsuperscript{−/−} and DKO tissue compared with WT tissue (Supplemental Figure 2). Taken together, these observations...
Figure 2. Deficiency of C3aR and C5aR protects mice from renal IR injury. Renal IR injury was induced in four groups of mice: WT, C3aR−/−, C5aR−/−, and DKO. Serum samples and kidney tissues were collected at 24 and 48 hours after reperfusion. (A) BUN levels at 24 and 48 hours after reperfusion. Number of mice studied per group is included in parentheses. The dotted line indicates a normal BUN level, which was obtained from pooled results of 10 normal mice. (B) Normal kidney histologic features. Light microscopic images of hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) staining of kidney from naive WT mouse. (C) Histopathologic features of injured kidneys. Representative light microscopic images of H&E and PAS staining of kidneys from the four groups, at 24 hours after reperfusion. (D) Histologic scores of renal tubule injury. Tissue injury was scored in cortical medullary junction areas at magnification of ×200 (n=6 per group three fields of each kidney). (E) KIM-1 gene expression in postischemic kidneys. The dotted line indicates KIM-1 gene expression level in normal kidney tissues. Quantitative real-time RT-PCR was performed in postischemic kidneys (n=4 per group), at 24 hours after reperfusion. Data in parts A, D, and E are shown as mean ± SEM and were analyzed by Mann-Whitney test *P<0.05, **P<0.005, ***P<0.0001 for WT versus C3aR−/− or C5aR−/− or DKO mice.
demonstrate that the lack of C3aR or C5aR receptors provides functional and structural protection against renal IR injury; however, protection is better with the lack of C5aR or both C3aR and C5aR.

Deficiency of C3aR and C5aR Reduces Renal Production of Inflammatory Mediators after Renal Ischemic Insult

Inflammatory mediators have been suggested to contribute to AKI after renal ischemic insult;5,9,27 we therefore assessed the production of inflammatory mediators in postischemic kidneys from WT, C3aR−/−, C5aR−/−, and DKO mice. Using quantitative real-time RT-PCR, we analyzed the gene expression of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ), chemokines (keratinocytes chemoattractant [KC, murine IL-8 homologous], monocyte chemoattractant protein-1 [MCP-1], macrophage inflammatory protein [MIP]-1α, MIP-1β) and adhesion molecules (intercellular adhesion molecule [ICAM]-1, CD146, von Willebrand factor) in kidney tissues collected at 24 and 48 hours after reperfusion. Renal tissue derived from C5aR−/− or DKO mice exhibited significantly lower levels of proinflammatory cytokines (TNF-α, IL-1β, IFN-γ) and chemokines (KC, MCP-1) compared with WT renal tissue at both 24 and 48 hours after reperfusion (Figure 3, A and B and Supplemental Figure 3, A and B). C3aR−/− mice had less reduction in renal synthesis of these cytokines and chemokines; only TNF-α was significantly reduced at 24 hours after reperfusion (Figure 3, A and B and Supplemental Figure 3, A and B).

In addition, we observed reduced levels of chemokines MIP-1α and MIP-1β as well as adhesion molecules ICAM-1, CD146, and von Willebrand factor in postischemic kidneys from C3aR−/− or C5aR−/− or DKO mice compared with WT mice however, significant reduction was observed on only some of those molecules (i.e., MIP-1α, MIP-1β, ICAM-1) in DKO mice (Figure 3, B and C and Supplemental Figure 3, B and C).

Thus, our data indicate that the presence of C3aR or C5aR is required for renal production of inflammatory mediators after renal ischemic insult, particularly at 24 hours after reperfusion.

Deficiency of C3aR and C5aR Reduces Cellular Infiltration in Postischemic Kidneys

In addition to tubule damage, renal interstitial cellular infiltration is a characteristic pathologic change in renal IR injury. To assess the influence of deficiency of C3aR and C5aR on cellular infiltration, we performed flow cytometry analysis (for CD45, Gr-1, and F4/80) and immunochemical staining (for Gr-1 and F4/80) in kidney tissues collected at 24 hours after reperfusion. Immunochemical staining suggested lower levels of Gr-1+ or F4/80+ cells in postischemic kidneys from C3aR−/−, C5aR−/−, and DKO mice compared with the levels in WT kidneys (Figure 4A). Flow cytometry results confirmed the reduction of cellular infiltration in all receptor knockout mice. The percentage of three types of cells (CD45+, Gr-1+, F4/80+) in single-cell suspension prepared from individual postischemic kidneys was lower in C3aR−/−, C5aR−/−, and DKO mice than in WT mice (Figure 4B). To investigate whether the observed lower number of renal CD45+, Gr-1+, and F4/80+ cells in the receptor knockout mice after renal IR injury; however, protection is better with the lack of C5aR or both C3aR and C5aR.
Figure 4. Deficiency of C3aR and C5aR reduces cellular infiltration in postischemic kidneys. Renal IR injury was induced in WT, C5aR, C3aR, and DKO mice (n=5 per group). Kidney tissues were harvested at 24 hours after reperfusion. Frozen sections and single cell suspensions were prepared from the kidneys and used for immunohistochemistry and flow cytometry, respectively. (A) Immunohistochemical staining of Gr-1 and F4/80. Representative light microscopic images at original magnification of × 200 from each group are shown. (B) Flow cytometric analysis of CD45⁺, Gr-1⁺, and F4/80⁺ cells in single-cell suspensions prepared from individual kidneys. Left four panels: representative dot plots from each group. Right panel: quantified percentages of CD45⁺, Gr-1⁺, and F4/80⁺ cells from each group (n=5 per group). Data are shown as mean ± SEM and were analyzed by Mann-Whitney test *P<0.05, **P<0.005, ns not significant, comparing WT and C3aR⁻/⁻ or C5aR⁻/⁻ or DKO mice.
is due to the effect of the intrinsic cell defect, we evaluated renal CD45+, Gr-1+, and F4/80+ cells in naive C3aR−/−, C5aR−/−, DKO, and WT mice. The proportions of the three cell types are similar in all the receptor knockout mice and WT mice, suggesting that the observed differences in cellular infiltration between WT and the receptor-deficient mice depend on ischemic insult (Supplemental Figure 4). Thus, our data demonstrate that single or double deficiency of C3aR and C5aR reduces cellular infiltration of leukocytes (mainly consisting of neutrophils and monocytes or macrophages) in kidneys at 24 hours in response to renal ischemic insult, although the deficiency of C3aR alone has a relatively small effect on this process.

Contribution of C3aR and C5aR on Renal Cells and Circulating Leukocytes to Renal IR Injury

Because both renal cells (e.g., tubular epithelial cells) and circulating leukocytes (e.g., neutrophils, monocytes, or macrophages) express C3aR and C5aR, we next determined whether C3aR and C5aR on both groups of cells can contribute to the development of renal IR injury. To do this, we generated murine chimeras between WT and DKO mice by bone marrow transplantation. We generated four groups of mice: (1) WT mice reconstituted with WT bone marrow (WT/WT, both renal cells and circulating leukocytes express C3aR and C5aR), (2) DKO mice reconstituted with DKO bone marrow (DKO/DKO), (3) WT mice reconstituted with DKO bone marrow (WT/DKO, renal cells lack C3aR and C5aR while circulating leukocytes express C3aR and C5aR), and (4) WT mice reconstituted with DKO bone marrow (DKO/WT,renal cells express C3aR and C5aR while circulating leukocytes lack C3aR and C5aR). We induced renal IR injury in these mice at 8 weeks after transfusion and evaluated renal function impairment at 24 hours after reperfusion. BUN levels were significantly lower in the DKO/DKO, DKO/WT, and WT/WT groups than in WT/WT mice after injury (Figure 5), indicating that the lack of C3aR and C5aR expression in renal cells or circulating leukocytes protected against renal IR injury and further confirms that expression of C3aR and C5aR on both renal intrinsic cells and infiltrating leukocytes contributes to renal IR injury.

C3a/C5a Stimulation Increases Proinflammatory Cytokine/Chemokine Production by Macrophages under Hypoxia-Reoxygenation Conditions

Our data presented so far suggest that C3aR and C5aR signaling is a critical pathogenic factor in the development of renal IR injury; this involves both renal cells and infiltrating leukocytes. Macrophages are important sources of proinflammatory mediators. To further investigate the mechanisms by which C3aR and C5aR on infiltrating cells contribute to local inflammation, we evaluated whether engagement of C3aR and C5aR on macrophages under hypoxia-reoxygenation conditions enhances their production of inflammatory mediators.
are also capable of producing several inflammatory cytokines and chemokines in response to pathogenic stimuli. We therefore evaluated whether engagement of C3aR and C5aR on primary cultured PTECs under hypoxia-reoxygenation conditions enhances their production of inflammatory mediators. Similar to our observations on macrophages, secretion of TNF-α and KC by PTECs was significantly increased by the stimulation of C3a or C5a or C3a/C5a (Figure 7, A and B). In addition to cytokines and chemokines, we also evaluated whether engagement of C3aR and/or C5aR on PTECs under hypoxia-reoxygenation conditions enhances their expression of KIM-1 (Figure 7C). Consistent with the observation in postischemic kidneys that KIM-1 expression was significantly lower in all receptor knockout mice, KIM-1 expression in PTECs was upregulated by the stimulation of C3a or C5a or C3/C5a. Taken together the data suggest a direct activation of renal epithelial cells by C3a and C5a during renal IR.

**DISCUSSION**

Previous studies have suggested that complement activation is a key mediator of renal IR injury and that the terminal product of complement activation (C5b-9) contributes to the effect of complement on mouse renal IR injury. More recently, several studies have also suggested a pathogenic role for the small complement fragment C5a in renal IR injury. However, it is unclear whether C3a also contributes to the injury, whether integration of the actions of C3a and C5a is required for the pathogenesis, and how they participate in the development of renal IR injury. These questions are important because they have implications for therapeutic strategies. This study has addressed these issues. The use of receptor knockout mice (C3aR−/−, C5aR−/−, DKO) allowed us to define the role of C3aR and C5aR signaling in renal IR injury in a more specific manner than that used with the pharmaceutical targeting of C5 or C5aR described in previous studies. The use of chimera mice allowed us to evaluate the contribution of C3aR and C5aR on renal cells and circulating leukocytes to renal IR injury. In addition, an in vitro model of hypoxia-reoxygenation helped us to investigate the potential mechanisms by which expression of C3aR and C5aR on renal tubular epithelial cells and macrophages could contribute to renal IR injury. The results presented here provide definitive evidence supporting a predominant role for C5a and a minor role for C3a in the pathogenesis of renal IR injury. Our study also identified several processes of renal IR injury that depend on C3aR and C5aR signaling, such as renal tissue inflammation, upregulation of KIM-1, activation of renal tubular epithelial cells, cellular infiltration, and endothelial activation.

An important question arising from our observation that the lack of C3aR and C5aR protects mice from renal IR injury is how C3a and C5a participate in the development of renal IR injury. The pathologic hallmark of renal IR injury, during the acute phase, is renal tubule destruction and cellular

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**Figure 6.** C3a and C5a stimulation increases proinflammatory cytokine and chemokine production by macrophages under hypoxia-reoxygenation conditions. Peritoneal macrophages were prepared from naive C57BL/6 mice and exposed to hypoxia for 24 hours, followed by 24 hours of reoxygenation, in the presence or absence of C5a (10 and 20 nM) and/or C3a (20 and 50 nM). The supernatants were harvested and used for analysis of cytokines and chemokine. Data are shown as mean ± SEM of pooled results from four individual experiments and were analyzed by t test *P<0.05 **P<0.005 for nonstimulation versus C3a or C5a or C3a/C5a stimulation under hypoxia-reoxygenation conditions.
infiltration of neutrophils, monocytes, and macrophages. Given that significant expression of C3aR and C5aR is observed on both renal tubular epithelial cells and granulocytes, and that the biologic functions of C3a and C5a in cell activation and C5a in chemotaxis, it is therefore possible that C3a and C5a participate in the pathogenesis of renal IR injury through promoting local inflammation by production of cytokines and chemokines by both renal tubular epithelial cells and infiltrating cells after engagement with C3aR and C5aR.

The observations made in this study strongly support this hypothesis. First, C3a and C5a stimulation increased TNF-α and KC production by PTECs and macrophages, in response to hypoxia-reoxygenation. Production of TNF-α and KC may mediate epithelial cell injury by autocrine and paracrine signaling, whereas KC promotes leukocyte chemotaxis to the renal tissue. C3a and C5a stimulation also increased IL-6 production by macrophages, which could stimulate innate IL-17-producing cells leading to local inflammation. Second, consistent with the above observations, renal expression of inflammatory mediators (e.g., TNF-α, IFN-γ, IL-1β, KC, MCP-1) after renal IR was significantly reduced in C5aR−/− and DKO mice and slightly reduced in C3aR−/− mice when compared with WT mice, as was the infiltration of neutrophils, monocytes, and macrophages in postischemic kidneys. Furthermore, renal injury was reduced in mice that lack C3aR and C5aR on renal intrinsic cells or circulating leukocytes, suggesting that C3aR C5aR on both renal cells and circulating leukocytes contribute to renal IR injury.

Our in vivo and in vitro studies showed an important effect of C3aR and C5aR signaling on KIM-1 expression (Figure 2, E and C). Although the exact function of this molecule still remains unclear, recent evidence has suggested that KIM-1 is not only a specific marker for acute renal tubule injury but also a functional molecule in the process of renal injury. It has been shown that KIM-1 can act as an endogenous ligand for leukocyte mono-immunoglobulin-like receptor 5 expressed on myeloid cells such as neutrophils, engagement of which leads to activation of cells and increased cell migration and chemokine production in the process of renal IR injury, thereby contributing to local inflammation. Therefore, our observation that increased KIM-1 expression in response to IR or hypoxia-reperfusion depends on C3aR and C5aR signaling suggests that C3a and C5a could participate in the pathogenesis of renal IR injury by modulating KIM-1 expression, consequently promoting local inflammation and renal tissue injury.

With regard to the involvement of endothelial cells in renal IR injury, our previous studies have found no apparent signs of vascular abnormalities (e.g., complement deposition, thrombosis) in postischemic kidney by histologic and immunohistochemical analysis, suggesting that endothelial injury is not the major feature in murine renal IR injury. In this study, to investigate whether there are some more subtle changes in endothelium and whether such changes would depend on C3aR and C5aR signaling, we analyzed renal expression of several adhesion molecules and endothelial activation associated molecules in postischemic kidneys. Our data suggest that

![Figure 7](image-url)

Figure 7. C3a and C5a stimulation increases proinflammatory cytokine and chemokine production and KIM-1 expression by PTECs under hypoxia-reoxygenation conditions. Primary PTECs were prepared from WT kidneys and exposed to hypoxia for 24 hours, followed by 24 hours of reoxygenation, in the presence or absence of C5a (10 and 20 nM) and/or C3a (20 and 50 nM). The supernatants were used for analyzing cytokine and chemokine secretion, and the cell pellets were used for analyzing gene expression. (A and B) Secretion of TNF-α and KC. (C) mRNA expression of KIM-1. Data are shown as mean ± SEM of pooled results from four individual experiments and were analyzed by t test *P<0.05, **P<0.005, ***P<0.0001 for nonstimulation versus C3a or C5a or C3a/C5a stimulation under hypoxia/reoxygenation conditions.
renal endothelial activation occurs in response to renal IR, this being more notable in the presence of C3aR/C5aR signaling. However, given that expression of C3aR and C5aR is more abundant in both renal tubular epithelial cells and inflammatory cells than in renal endothelial cells,15,23,29 and the relative lack of endothelium involvement in renal IR injury,5 it remains possible that the activation of renal tubular epithelial cells and the infiltration or activation of inflammatory immune cells are the major mechanisms of C3aR- and C5aR-mediated renal IR injury; C3a- and C5a-mediated local inflammation leads to endothelial activation, enabling leukocyte recruitment.

C3a and C5a are commonly described as inflammatory mediators with similar profiles of activity; however, differential or opposing effects of C3a and C5a have been reported in several other disease models, such as asthma,30,31 systemic lupus erythematosus,32,33 gram-negative bacteremia, and shock.34 In renal IR injury, previous studies have suggested that C5a is a pathogenic factor. Although the contribution of C3a to renal IR injury is unclear, studies in other organs (heart, intestine, brain) have demonstrated that the severity of IR injury in these tissues was reduced in the absence of C3aR signaling.35–38 We have now clearly shown that both C3aR and C5aR signaling can contribute to renal IR injury; multiple pathogenic processes (e.g., KIM-1 expression, cellular infiltration, generation of proinflammatory mediators in ischemic kidneys) depend on signaling by both receptors. Together, these studies suggest that in contrast to the opposing effect observed in other disease models, in the setting of renal IR, both C3a and C5a contribute to the acute renal injury despite a predominant role for C5a in the pathogenesis. The different roles of C3a in different disease models could be explained by different types of inflammation (e.g., with or without involvement of infectious agent or antigen, acute or chronic) and different types of target cells (e.g., lymphocytes or granulocytes and parenchymal cells). It is worth noting that although our in vitro experiments indicate that C3a and C5a have similar effects on activation of renal tubular epithelial cells and macrophages to produce inflammatory mediators, our in vivo and ex vivo experiments show that renal functional impairment, renal expression of inflammatory mediators, and the infiltration of leukocytes in postischemic kidneys are more dependent on C5aR signaling. This finding suggests that although C3a contributes to renal IR injury, the pathogenic role of C5a in renal IR injury predominates over that of C3a.

With regard to the chemotactic activity of C3a and C5a, although it is well known that C5a is a potent chemoattractant for leukocytes, the role for C3a in leukocyte chemotaxis is less clear. Several lines of evidence have suggested that C3a can contribute to leukocyte chemotaxis. Studies in C3ar−/− mice have found that absence of C3aR attenuates the spinal cord-infiltrating macrophages and CD4+ T cells in an experimental autoimmune encephalomyelitis model and reduces airway eosinophils, neutrophils, and lymphocytes in a model of allergic bronchopulmonary inflammation.39,40 In the present study, we observed that absence of C3aR reduces cellular infiltration in posts ischemic kidneys. A question raised from these studies is how C3a could contribute to leukocyte chemotaxis. Several in vitro studies have shown that C3a induces the expression of cytokines and chemokines (e.g., IL-1, IL-6, IL-8, MCP-1, and RANTES [regulated upon activation, normal T cell expressed and secreted]) by endothelial cells and astroglia cells,41,42 whereas selective antagonism of C3aR significantly attenuates production of MIP-2 and KC by a renal proximal tubular epithelial cells line.25 These observations, together with our findings that C3a stimulation increases proinflammatory cytokine and chemokine production by both renal tubular epithelial cells and macrophages under hypoxia-reoxygenation conditions, strongly suggest that C3a can contribute to leukocyte chemotaxis, possibly through inducing the production of other chemoattractants (e.g., IL-8, KC, MCP-1, or MIP) by kidney cells and immune cells at the site of injury. However, whether C3a has direct chemotactic effect on neutrophils and monocytes in renal IR injury remains to be elucidated.

On the basis of our findings, together with published literature about the role of C3a and C5a in local inflammation and cell activation, we postulate the mechanisms by which C3a and C5a contribute to the pathogenesis of renal IR injury through acting on renal and circulating cells. As illustrated in Figure 8, complement activation in response to renal ischemic insults results in the production of C3a and C5a. Binding of both C3a and C5a to specific receptors (C3aR and C5aR) on renal tubular epithelial cells and granulocytes, such as monocytes and macrophages, leads to cell activation and upregulation of proinflammatory cytokine and chemokine production. These inflammatory mediators lead not only to tubular epithelial cell injury but also to recruitment of more leukocytes into the interstitium. In addition, C3a and C5a cause renal endothelial activation and thereby enable leukocyte recruitment, which can be mediated by C5a and other chemoattractants. Therefore, in addition to C5b–9–mediated direct injury of renal tubule cells,5,14 C5a- (and possible C3a-) induced inflammatory responses also contribute to renal tissue destruction. Our findings offer new insight into the effector molecules of complement activation involved in the pathogenesis of renal IR injury, which has important implications for therapeutic strategies needed to treat this disease.

**CONCISE METHODS**

**Reagents**

Recombinant mouse C5a was from Hycult (Uden, The Netherlands). Human C3a was purchased from Fitzgerald Industries International (Concord, MA). Hydrocortisone, triiodothyronine, collagenase II, and deoxyribonuclease I were from Sigma-Aldrich (Poole, United Kingdom). The antibodies for CD45, Gr-1, and F4/80 were from Antibodies conjugates (Cambridge, United Kingdom). FcR-blocking antibody (CD16/32) and mouse.
cytometric bead array kit for KC, IL-6, and TNF-α were from BD Biosciences (Cowley, United Kingdom). Collagenase D and Dispase II were from Roche (Welwyn, United Kingdom). GoTaq Hot Start Polymerase was from Promega (Southampton, United Kingdom). Cell culture medium, FCS, and insulin-transferrin-selenium solution were purchased from Invitrogen (Paisley, United Kingdom).

**Mice**
Homzygous C3aR−/− and C5aR−/− mice were derived by homologous recombination in embryonic stem cells43,44 and backcrossed on to the C57BL/6 (H-2b) parental strain for at least 12 generations. C5aR and C3aR DKO mice were generated from cross-breeding of C3aR−/− and C5aR−/− mice. WT C57BL/6 mice were purchased from Harlan Laboratories (United Kingdom). Male mice (8–12 weeks old) were used in all experiments. Animal procedures adhered to criteria outlined by the Animals (Scientific Procedures) Act of 1986.

**Induction of Renal IR Injury and Assessment of Renal Function and Pathology**
Renal IR injury was induced as we described previously with a modified ischemic time.5 In brief, mice were anesthetized with isoﬂurane and the body temperature was kept constant by placing a warm pad beneath the animals during surgery. Using a midline abdominal incision, renal arteries and veins were bilaterally occluded for 30 minutes with microaneurysm clamps. After removal of the clamps, 0.8 ml of warm saline was placed in the abdomen, the incision sutured, and animals returned to their cages. Blood samples were taken at 24 hours and 48 hours after reperfusion by tail bleeding (or cardiac puncture for terminal mice). In some experiments, mice were euthanized at 24 or 48 hours to collect the kidneys. Renal function was assessed by measuring concentrations of BUN using a Urea Assay Kit from Thermo Fisher (Epsom, United Kingdom). To assess renal pathologic features, the injured kidneys were fixed in a solution of 4% formalin in PBS for 24 hours and embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin and periodic acid–Schiff. Histologic score was performed in a blinded fashion by two persons as described previously,3 with some modiﬁcation. The percentage of tubules in the corticomedullary junction showing epithelial injury (i.e., loss of proximal tubule brush border, cell blebbing or vacuolization, and cell necrosis) was quantiﬁed using a 7-point scale (0, no injury; 1, 0%–15%; 2, 15%–30%; 3, 30%–45%; 4, 45%–60%; 5, 60%–75%; 6, >75%) at a magniﬁcation of ×100; three ﬁelds per kidney were assessed.

**Quantitative Real-Time RT-PCR**
Total RNA was extracted from the kidney tissues using an SV total RNA isolation system and used for cDNA synthesis using an RT kit according to manufacturer’s instructions from Promega (Southampton, United Kingdom). Quantitative PCR was performed with a DyNaMo HS SYBR Green qPCR kit from Thermo Fisher (Epsom, United Kingdom) and an MJ Research PTC-200 Peltier Thermal Cycler from Bio-Rad (Hemel Hempstead, United Kingdom). Amplification was performed according to the manufacturer’s cycling protocol and done in duplicate. Gene expression was expressed as 2−ΔΔCt, where Ct is cycle threshold, ΔΔCt (Ct) is testing samples Δ (Ct) − control samples Δ (Ct), and Δ (Ct) is testing gene (Ct) −18s (Ct). The control samples were normal kidney tissues (in the experiments of postischemic kidneys) or unstimulated cells (in the experiments of epithelial cells and macrophages). The information for primer sequences is given in Supplemental Table 1.

**Immunocytochemical Staining**
Frozen sections (4 μm) of optimal cutting temperature–embedded kidneys were air-dried for a minimum of 20 minutes at room temperature and then acetone fixed. Sections were incubated with rat antimouse Gr-1 or F4/80 antibody, followed by horseradish peroxidase–conjugated rabbit antirat antibody. Stained kidney sections were visualized under light microscopy and photographed at a magnification of X200.
Flow Cytometric Analysis

Single renal cell suspension was prepared using the method described elsewhere, with some modification. In brief, the whole kidney was minced and digested with a cocktail containing collagenase D (0.25 mg/ml), Dispase II (1.5 mg/ml), deoxyribonuclease (0.01 mg/ml), and FCS (10%) for 40 minutes at 37°C. The digested tissue mixture was passed through a 40-μm nylon sieve, and the cells were used for flow cytometry. Renal suspension cells (3×10^6) were preincubated with FcR-blocking antibody (CD16/32) and stained with rat antimouse allophycocyanin–conjugated CD45 and phycoerythrin–conjugated Gr-1 or F4/80 antibodies or the appropriate isotype control antibodies at 4°C for 20 minutes, and then fixed in 400 μl of 1% paraformaldehyde in PBS. All flow cytometric analyses were performed using Calibur Flow Cytometer (BD) and Flowjo software (Tree Star, Ashland, OR).

Generation of Chimeric Mice

Chimeric mice were generated using a method established in our laboratory. Mice were first irradiated at a dose of 9 Gy and reconstituted with an intravenous administration of 7.5×10^6 donor bone marrow cells within 3 hours after irradiation. At 8 weeks after bone marrow transplantation, mice were used for induction of renal IR.

Induction of Hypoxia-Reoxygenation Injury in Naive Macrophages and Primary Renal Tubular Epithelial Cells

Peritoneal macrophages were harvested from naive male C57BL/6 mice (two or three mice per experiment) and cultured for 1 hour to remove nonadhesive cells. Macrophages were placed in duplicate in a 24-well plate (1×10^6 cells per well). Primary tubular epithelial cell cultures were prepared from kidneys of naive male C57BL/6 mice as we described previously. In brief, minced cortex was digested with 0.1% collagenase II and then passed through a 40-μm nylon sieve. The cells and tubules were collected and cultured in a DMEM-12 medium that contained 2% FCS, insulin (40 ng/ml), and tri-iodothyronine (10^{-6} M). Nonpassaged 7-day cultured cells were used. Both cells were cultured under hypoxic conditions (5% CO2 and 95% N2) in a gas chamber (Billups-Rothenberg, Del Mar, CA) for 24 hours, followed by reoxygenation (21% O2 and 5% CO2) for 24 hours in the presence of C5a or C3a. The supernatants were collected and used for measuring cytokines (TNF-α, IL-6) and chemokine (KC) by cytometric bead array assay (according to the manufacturer’s instructions); in some experiments, total RNA was extracted from mouse PTECs and quantitative RT-PCR was performed to assess KIM-1 expression.

Statistical Analyses

Statistical analyses between different experimental groups were performed using the Mann-Whitney test or t test. All the analyses were performed using Graphpad Prism software.

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DISCLOSURES

None.

REFERENCES


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