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Graphical abstract

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The C5a/C5aR2 axis promotes renal inflammation and tissue damage

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Disclosure

All the authors have declared that no conflict of interest exists.
Abstract

C5a is a potent inflammatory mediator, which binds C5aR1 and C5aR2. Although pathogenic roles of C5a/C5aR1 axis in inflammatory disorders are well-documented, the roles for C5a/C5aR2 axis in inflammatory disorders and underlying mechanisms remain unclear. Here, we show that C5a/C5aR2 axis contributes to renal inflammation and tissue damage in a mouse model of acute pyelonephritis. Compared with WT littermates, C5ar2−/− mice had significantly reduced renal inflammation, tubular damage and renal bacterial load following bladder inoculation with uropathogenic E. coli. The decrease in inflammatory responses in the kidney of C5ar2−/− mice was correlated with reduced intrarenal levels of high mobility group box 1 protein (HMGB1), NLRP3 inflammasome components, cleaved caspase-1 and IL-1β. In vitro, C5a stimulation of macrophages from C5ar1−/− mice (lacking C5aR1 but expressing C5aR2) led to significant upregulation of HMGB1 release, NLRP3/caspase-1 inflammasome activation and IL-1β secretion. Furthermore, blockade of HMGB1 significantly reduced C5a-mediated upregulation of NLRP3/caspase-1 inflammasome activation and IL-1β secretion in the macrophages, implying a HMGB1-dependent upregulation of NLRP3/caspase-1 inflammasome activation in macrophages. Our findings demonstrate a pathogenic role for C5a/C5aR2 axis in renal injury following renal infection and suggest that C5a/C5aR2 axis contributes to renal inflammation and tissue damage through up-regulation of HMGB1 and NLRP3/caspase-1 inflammasome.

Key words: C5aR2, Renal inflammation, HMGB1, NLRP3 inflammasome, Pyelonephritis
Introduction

The complement system is a key component of innate immunity. Complement activation occurs rapidly in response to different insults (e.g. infection, tissue stress). Complement activation generates a set of molecules with diverse biological functions. The small fragments (C3a, C5a) mediate inflammation through interacting with their specific receptors (C3aR, C5aR), the large fragments (e.g. C3b, C4b) mediate opsonisation through interacting with complement receptors (CR 1-4), and the terminal product C5b-9 mediates direct killing of pathogens (1).

C5a is one of most potent inflammatory mediators, which mediates leukocyte chemotaxis, activates leukocytes and endothelial cells, and drives production of inflammatory mediators (e.g. histamine, cytokines, chemokines). C5a binds two C5aRs namely C5aR1 and C5aR2 (2). C5a/C5aR1 interaction-mediated inflammatory responses have been shown to play critical roles in the pathogenesis of many inflammatory and immunological diseases including renal injuries (3-8). However, the roles of C5a/C5aR2 interactions in inflammatory process and its involvement in the pathophysiology of disease are complex, and both anti-inflammatory and proinflammatory effects have been reported, this seems depending on circumstances, such as disease models and types of cells (9). On the one hand, C5aR2 was suggested as a functional receptor, and C5a/C5aR2 interactions can mediate proinflammatory responses through intracellular signals contributing to organ and systemic inflammatory diseases (10-17). On the other hand, C5aR2 was suggested as a decoy receptor which suppresses C5a/C5aR1 mediated responses, thus functioning as a negative regulator of pro-inflammatory responses (18-21).

Pyelonephritis is inflammation of the kidney, often caused by ascending uropathogenic Escherichia coli (UPEC) from the lower urinary tract. The development of pyelonephritis can be influenced both by properties of the infecting pathogens and host responses to pathogens, in addition to other factors such as anatomical abnormality (22). Although innate immune responses play essential roles in the first line of host defense against pathogens, most human UPEC strains are resistant to complement-mediated killing (23, 24). In addition, excessive or dysregulated inflammatory responses to the pathogens represent an important pathogenic mechanism in urinary tract infections (UTI) (22). For examples, in the acute conditions, uroepithelial cells and inflammatory cells, in response to UPEC stimulation, produce a number of pro-inflammatory mediators (e.g. IL-6, TNF-α and IL-8), which (if present in excess) cause epithelial inflammation/damage, allowing bacteria to enter the underlying tissue. In addition, if the activation of neutrophils is not tightly regulated, the reactive oxygen species and cytotoxic enzymes and ingested bacteria could be released into the surrounding areas causing tissue destruction and pathogen dissemination. Previous researches in experimental acute pyelonephritis have found that Tlr4−/−, Il-1β−/− or mice treated with forskolin (which has anti-inflammatory properties) had attenuated tissue inflammation and less severe acute kidney infection.
This suggests that excessive inflammatory responses could be harmful, instead of beneficial for the host in this model (25, 26).

Furthermore, our recent studies have shown that C5a/C5aR1 axis, as a potent driver of inflammation, contributes significantly to renal injury (i.e. tissue damage, tubulointerstitial fibrosis) in experimental pyelonephritis, thus suggesting that C5a/C5aR1 represents a potential target for therapeutic intervention of kidney injury in this disorder (6, 7). Because C5aR2 is often co-expressed with C5aR1 in myeloid cells and has high affinity binding sites for C5a, C5a/C5aR2 interactions could potentially involve in the pathogenesis of renal disorders. The role of C5aR2 in renal injury remains less explored. Such information will improve our understanding of the roles for C5a in the pathogenesis of renal injury, which has implications for therapeutic strategies to treat renal inflammation and tissue damage as drugs targeting C5 or C5aR1 or both C5aR1/C5aR2 have been developed (27, 28).

In the present study, we employed a murine model of acute pyelonephritis in combination with $C5ar2^{-/-}$ mice to investigate the role of C5aR2 in renal inflammation and tissue damage and the underlying mechanisms. We show that $C5ar2^{-/-}$ mice had significantly reduced tissue damage, bacterial load and inflammatory signals (e.g. HMGB1, NLRP3 inflammasome, IL-1β) in the kidney, compared with WT littermates. We show that in vitro engagement of C5aR2 with C5a mediated upregulation of HMGB1 release, NLRP3/caspase-1 inflammasome activation and IL-1β secretion in macrophages.

**Results**

$C5ar2^{-/-}$ mice have reduced tissue damage and bacterial load in the kidney following inoculation with UPEC

To determine the role of C5aR2 in renal injury, we induced the pyelonephritis in WT and $C5ar2^{-/-}$ mice by bladder inoculation of human UPEC strain J96 and analysed renal tissue injury and bacterial load at 24 and 48 hours post the inoculation (hpi). Renal injury and tissue damage were assessed by evaluating renal histopathologic changes and measuring renal tissue levels of kidney injury molecule-1 (KIM-1) (a renal injury marker) (29). Renal histopathologic changes (i.e. cellular infiltration, bacterial patchiness, abscess, and tubule destruction) within the papilla/medulla and cortex were evaluated by performing histologic scoring in individual mice based on the images of H&E and PAS stained histology slides. Our results showed that the histopathologic changes were significantly attenuated in $C5ar2^{-/-}$ mice compared with WT mice (24phi) (Figure 1A-1C). Renal KIM-1 levels were significantly lower in $C5ar2^{-/-}$ mice than those in WT mice (24phi, 48phi) (Figure 1D). Beside renal injury and tissue damage, renal bacterial loads were significantly reduced in $C5ar2^{-/-}$ mice compared with WT mice (24phi, 48phi) (Figure 1E). These results demonstrate that $C5ar2^{-/-}$
mice have reduced tissue damage and bacterial load in the kidney following inoculation with UPEC, which is similar to that seen in C5ar1<sup>-</sup> mice previously reported in this model (6).

Inflammatory response to UPEC is one of the hallmarks of acute pyelonephritis and can lead to tissue damage and subsequent bacterial entering the underlying tissue. We assessed the impact of C5aR2 deficiency on renal tissue inflammation following the inoculation of UPEC by analysing leukocyte infiltration and inflammatory mediator expression in the kidney. Flow cytometric analysis showed that C5ar2<sup>-</sup> mice had fewer infiltrating cells including leukocytes (CD45<sup>+</sup>), neutrophils (Ly6G<sup>+</sup>), monocytes/macrophages (MO/MΦ) (Ly6G<sup>-</sup>CD11b<sup>+</sup>), and inflammatory monocytes (ratio of Ly6C<sup>+</sup>/Ly6C<sup>low</sup>) in the kidney, compared with WT controls (24hpi) (Figure 2A, 2B, sFigure 1). Renal tissue lysate ELISA showed that IL-1β, TNF-α and CXCL-1 levels were significantly decreased in C5ar2<sup>-</sup> mice compared with WT mice (6hpi, 24hpi) (Figure 2C).

Taken together, these results demonstrate that C5ar2<sup>-</sup> mice have reduced renal inflammation, tissue damage and bacterial load in the kidney following inoculation with UPEC, which is similar to that seen in C5ar1<sup>-</sup> mice previously reported in this model (6).

**C5ar2<sup>-</sup> mice have reduced intrarenal HMGB1 expression and NLRP3/caspase-1 inflammasome activation following inoculation with UPEC**

High mobility group box-1 (HMGB1) is an intracellular protein which can translocate to the nucleus where it binds DNA and regulates gene expression. It can also be released from cells to the extracellular space in response to diverse insults (e.g. stress, infection), where it can interact with pattern recognition receptors (PRRs) (e.g. TLRs, RAGE), thus playing a key role at the intersection of sterile and infectious inflammatory responses (30). NLR family pyrin domain containing 3 (NLRP3) inflammasome is a multiprotein intracellular complex (consisting of NLRP3, apoptosis-associated speck-like protein [ASC], caspase-1), which detects pathogenic microorganisms and sterile stressors, and results in the processing and the release of IL-1β and IL-18, thus playing important roles in the innate immune responses (31). Given the observation that kidney inflammation and intrarenal IL-1β production being significantly reduced in C5ar2<sup>-</sup> mice following the inoculation (Figure 2C), we reasoned that the attenuated inflammatory responses in the kidney of C5ar2<sup>-</sup> mice could be involved in down regulation of HMGB1 and NLRP3 inflammasome in the kidney. Accordingly, we assessed protein levels of HMGB1, NLRP3, ASC and cleaved caspase 1 in kidney tissue of C5ar2<sup>-</sup> mice and WT mice following the inoculation. Renal tissue lysate ELISA showed that HMGB1 levels were significantly lower in C5ar2<sup>-</sup> mice than that in WT mice at both 6hpi and 24hpi (Figure 3A). Western blotting on renal tissue lysate (24hpi) further confirmed the reduction of intrarenal levels of HMGB1 in C5ar2<sup>-</sup> mice, and showed a reduction of NLRP3, ASC and cleaved caspase-1 in C5ar2<sup>-</sup> mice, compared with WT mice (Figure 3B, 3C). Uninfected mice exhibited very
low levels of these proteins, which were comparable between the WT and C5aR2−/− mice (Figure 3B, 3C). We also assessed intrarenal protein levels of HMGB1 and caspase-1 in C5ar1−/− mice following the inoculation. Interestingly, there was no apparent reduction in HMGB1 and cleaved caspase-1 in the kidney of C5ar1−/− mice compared with WT mice (sFigure 2). Taken together, these results indicate that C5ar2−/− mice, but not C5ar1−/− mice, have reduced intrarenal HMGB1 expression and NLRP3/caspase-1 inflammasome activation following inoculation with UPEC.

C5ar2−/− mice have reduced intrarenal p-AKT, p-ERK1/2 and p-JNK following inoculation with UPEC

In addition to HMGB1 expression and NLRP3/caspase-1 inflammasome, we also assessed the impact of C5aR2 deficiency on TLR signalling pathways in the kidney following the inoculation. Western blotting on renal tissue lysate (24hpi) showed a clear reduction of p-AKT, p-ERK1/2 and p-JNK levels in C5ar2−/− mice compared with WT mice (Figure 4A, 4B). However, the level of p-IκB was not affected by C5aR2 deficiency (Figure 4C,4D). Uninfected mice exhibited very low levels of p-AKT, p-ERK1/2 and p-JNK, which were comparable between the WT and C5ar2−/− mice (Figure 4A, 4B). Thus, C5aR2 deficiency leads to a reduction of AKT, ERK, JNK signalling, but has no effect on NF-κB signalling following inoculation with UPEC. We also assessed the activities of these intracellular signalling pathways in renal tissue lysate of WT and C5ar1−/− mice (24hpi). Intrarenal levels of p-AKT, p-ERK1/2 and p-JNK in C5ar1−/− mice were significantly reduced compared with WT mice, which is consistent with that observed in C5ar2−/− mice. However, the levels of p-IκB in C5ar1−/− mice were also reduced, which is not the same as that observed in C5ar2−/− mice (sFigure 3).

C5aR2 is primarily detected in inflammatory cells in murine kidney

C5aR2 expression has been reported in myeloid cells (e.g. neutrophils, macrophages), however, little is known about its expression in murine kidney (32, 33), we therefore examined C5aR2 expression and its location in the kidney. RT-PCR showed that C5aR2 mRNA was detected in normal kidney tissue, and the levels were increased following the inoculation (6hpi, 24hpi) (Figure 5A, 5B). Immunohistochemistry revealed that C5aR2 was hardly detectable in normal kidney sections and the positive staining was mainly detected in interstitial inflammatory cells (and few tubular epithelial cells) in cortex and medullar region following the inoculation (24hpi) (Figure 5C). Co-staining of CD11b and C5aR2 showed that most CD11b+ cells were positive for C5aR2 in the infected kidneys (Figure 5D). Flow cytometric analysis revealed that majority of renal infiltrating leukocytes were CD11b+ cells following the inoculation in this model (sFigure 4). Together, these results indicate that C5aR2 is primarily expressed on inflammatory cells in the kidney and suggest that C5aR2 contribute to renal injury in this model mainly through modulating inflammatory cell function.
Inflammatory cells from C5ar2<sup>−/−</sup> mice have reduced ability to produce proinflammatory cytokines

Next, we assessed the impact of C5aR2 deficiency on inflammatory molecule production in inflammatory cells in vitro. Peritoneal exudate cells were prepared from WT and C5ar2<sup>−/−</sup> mice d1 and d3 after thioglycolate injection. We then analyzed proinflammatory molecule production in these cells under both with /without LPS stimulation by RT-PCR and ELISA. RT-PCR results showed that d3 WT cells exhibited much higher expression of IL-1β, TNF-α and CXCL-1, particularly under with LPS stimulation condition, compared with d3 C5aR2<sup>−/−</sup> cells (Figure 6A). d1 WT cells also exhibited higher expression of IL-1β and to a lesser extent TNF-α and CXCL-1 compared with d1 C5aR2<sup>−/−</sup> cells (Figure 6B). ELISA results showed that IL-1β, TNF-α and CXCL-1 levels in d3 WT cell culture supernatants were significantly higher than that in d3 C5aR2<sup>−/−</sup> cell culture supernatants (Figure 6C). These results demonstrate that inflammatory cells from C5ar2<sup>−/−</sup> mice have reduced ability to produce proinflammatory cytokines, supporting a proinflammatory role for C5aR2 in immune modulation and inflammation.

Engagement of C5aR2 mediates upregulation of HMGB1 and NLRP3/caspase-1 inflammasome activation in macrophages

Having demonstrated that C5aR2 is required for intrarenal expression of HMGB1 and NLRP3 inflammasome activation upon infection, and proinflammatory molecule production in inflammatory cells, we next investigated whether engagement of C5aR2 regulates HMGB1 expression/secretion and NLRP3 inflammasome activation in macrophages in vitro. We prepared macrophages from peritoneal exudates of C5ar1<sup>−/−</sup> mice (lacking C5aR1 but expressing C5aR2) and stimulated the cells with C5a alone or in combination with a small dose of LPS (2 ng/ml) for up to 24h. Intracellular protein levels of HMGB1, NLRP3, ASC and cleaved caspase-1 were measured by Western blotting. HMGB1, IL-1β and TNF-α concentrations in the culture supernatants were measured by ELISA. Western blotting showed that, intracellular levels of HMGB1, NLRP3, ASC and cleaved caspase-1 were significantly increased following stimulation with C5a (2 nM, 10 nM) alone or in combination with LPS (2 ng/ml) for 6h. In the presence of LPS, the overall intracellular levels of these proteins, particularly for NLRP3 were elevated, compared with no LPS (Figure 7A, 7B). ELISA showed that HMGB1 and IL-1β levels in both 6h and 24h culture supernatants were significantly increased following C5a/LPS stimulation (Figure 7C, 7D). TNF-α levels were only increased in the 24h (but not 6h) culture supernatants (Figure 7E). The levels of HMGB1, IL-1β, TNF-α in the supernatants were very low in the absence of LPS, which make it difficult to assess the effect of C5a alone on the release of
these proteins. These results demonstrate that engagement of C5aR2 mediates upregulation of HMGB1 expression/secretion and NLRP3/caspase-1 inflammasome activation in macrophages.

Previous studies in non-immune cells (smooth muscle cell, hepatocyte) have suggested that HMGB1 can promote NLRP3 inflammasome activation (34, 35). We reasoned that C5aR2-mediated upregulation of HMGB1 may promote NLRP3 inflammasome activation in macrophages. To test this, we employed a HMGB1 protein and a HMGB1 neutralising antibody in C5a stimulation experiments of macrophages as described above. We first assessed the impact of HMGB1 on NLRP3 inflammasome activation in the macrophages and found that addition of HMGB1 protein to the culture medium resulted in increased secretion of cleaved caspase-1 and IL-1β from the macrophages (Figure 8A-8C). We then assessed whether blockade of HMGB1 could have an impact on C5a-mediated upregulation of NLRP3 inflammasome activation in the macrophages and found that addition of the anti-HMGB1 antibody to the culture medium significantly inhibited C5a-upregulated NLRP3 expression, caspase-1 activation and IL-1β secretion (Figure 8D-8F).

Discussion

Although it is well-recognised that the C5a/C5aR1 axis promotes proinflammatory responses to stress or infection which contributes to the pathogenesis of many acute and chronic diseases including renal injuries, the role of C5a/C5aR2 axis in inflammation and disease is relatively less studied and the literature is also controversial. In the present study, we employed a murine model of acute pyelonephritis that we previously used for studying the role of C5a/C5aR1 axis in renal injury to determine the role of C5a/C5aR2 axis in this pathology. Our study provides evidence that C5aR2 (same as the C5aR1) is required for the development of acute pyelonephritis, as mice with C5aR2 deficiency have reduced renal injury and bacterial load in the kidney, thus supporting a pathogenic role for C5a/C5aR2 axis in this pathology. Our findings are consistent with the findings from previous reports in the context of pathogenic roles of C5aR2 in other types of renal injury (15, 36).

In addition to demonstrating a pathogenic role for C5aR2 in acute pyelonephritis, we also made several important observations regarding the molecular mechanisms that C5aR2 promotes renal inflammation and tissue damage. In our in vivo experiments, we found that C5ar2−/− mice had lower levels of HMGB1 and decreased NLRP3/caspase-1 inflammasome activity in the kidney following the inoculation compared with WT mice, suggesting a C5aR2-dependent upregulation of HMGB1 and NLRP3/caspase-1 inflammasome in the kidney upon infection. To assess whether C5aR2-dependent upregulation of HMGB1 and NLRP3 inflammasome is mediated by the C5a/C5aR2 interaction, we performed a series of in vitro experiments using C5ar1+/− macrophages (lacking C5aR1 but expressing C5aR2). Results from C5a stimulation experiments clearly showed that C5a,
particularly when combined with a low-dose of LPS, led to the upregulation of HMGB1 expression/secretion, NLRP3/ASC expression, caspase-1 activation and IL-1β secretion in the C5aR2 expressing macrophages, thus supporting an argument of that C5a/C5aR2 interaction mediates upregulation of HMGB1 and NLRP3/caspase-1 inflammasome in macrophages, which could contribute to renal inflammation and tissue damage.

Regarding the relationship between the upregulation of HMGB1 and NLRP3/caspase-1 inflammasome activation, we assessed the possibility of HMGB1-dependent NLRP3/caspase-1 inflammasome activation in macrophages by HMGB1 blockade. Results from HMGB1 blockade experiments clearly showed that C5a/C5aR2 interaction-mediated upregulation of caspase-1 activation/IL-1β secretion was sufficiently inhibited by anti-HMGB1 antibody, which strongly suggest that HMGB1 driven by C5a/C5aR2 interaction can act as an upstream effector of NLRP3/caspase-1 inflammasome in macrophages. C5aR2 could contribute to proinflammatory responses through upregulation of HMGB1 expression/release and subsequent activation of NLRP3/caspase-1 inflammasome. A previous study with C5ar2−/− mice and macrophages has also shown that C5aR2 deficiency restricts activation of NLRP3 inflammasome and release of HMGB1 in vivo and in vitro (17), our findings in this study align well with their findings, indicating that C5aR2 is required for activation of NLRP3 inflammasome and release of HMGB1. With regard to the molecular mechanisms of C5aR2-dependent regulation of NLRP3 inflammasome, the previous study has suggested that upregulation of protein kinase B expression is responsible for C5aR2 dependent NLRP3 inflammasome activation. The results of the present study suggest a different upstream molecule (HMGB1) responsible for C5aR2 dependent NLRP3 inflammasome activation. Taken together, these results therefore highlight the possibility of C5aR2 mediating NLRP3 inflammasome activation in inflammatory cells through multiple signalling pathways.

In our in vivo experiments we also found that C5ar2−/− mice had lower intrarenal levels of phosphorylated forms of AKT, ERK and JNK than WT mice following the inoculation, in addition to the reduction of intrarenal levels of HMGB1 and NLRP3/caspase-1 inflammasome. These findings agree with previous studies showing that C5aR2 is required for C5a-induced ERK1/2 and AKT signalling in murine macrophages (11, 12), and the release of HMGB1 from macrophages is dependent on C5a/C5aR2 axis-induced MAPK and AKT signalling (13). This leads to a suggestion that engagement of C5aR2 induces the activation of AKT and MAPK signalling pathways which could mediate upregulation of HMGB1 expression/release. Given that the released HMGB1 from cells can activate TLRs and RAGE to induce inflammatory signalling (30, 37), C5a/C5aR2 interaction-caused HMGB1 release can mediate a number of cellular processes including gene transcription of cytokines and inflammasome components (e.g. NLRP3, ASC) (38, 39), ultimately enhancing cytokine production and NLRP3/caspase-1 inflammasome activation. Therefore, it is
conceivable that C5aR2 induced-signalling may not only mediate upregulation of HMGB1, but also play an important role in bridging HMGB1 and NLRP3/caspase-1 inflammasome activation.

Based on our findings in this study and previous literature we propose a molecular mechanism by which C5a/C5aR2 axis promotes proinflammatory responses in macrophages upon infection. As illustrated in Figure 9, engagement of C5aR2 with C5a in macrophages upon infection induces upregulation of HMGB1 expression/release through intracellular signaling (e.g. MAPK, AKT), which upregulates NLRP3/caspase-1 inflammasome activation and IL-1β secretion, possibly through engagement of PRRs inducing proinflammatory signaling, and as yet unidentified mechanisms. This, together with proinflammatory signaling-mediated cell death and upregulation of proinflammatory cytokine genes (e.g. TNF-α) contributes to renal inflammation and tissue damage. Bacterial endotoxins amplify the C5a/C5aR2 axis-mediated upregulation of HMGB1/NLRP3/inflammasome during the infection.

Besides investigating the role for C5a/C5aR2 axis in acute pyelonephritis, we also performed some experiments to make a comparison between the C5a/C5aR1 axis and C5a/C5aR2 axis in this model, in terms of the involved molecular signalling pathways. Our in vivo results showed that although a significant reduction of intrarenal levels of HMGB1, caspase-1 was observed in C5ar2−/− mice following the inoculation, no apparent reduction of these protein levels in C5ar1−/− mice. On the other hand, NF-κB signalling (a well-established signalling driven by engagement of C5ar1 (40, 41)) was not affected by C5aR2 deficiency. Furthermore, our in vitro results showed that despite C5a stimulation increased both IL-1β and TNF-α secretion in C5aR2 expressing macrophages at 24h time point, C5a stimulation only increased IL-1β secretion, but not TNF-α secretion at an earlier time point (6h) (which is mainly regulated by gene expression). These results support the notion that upregulation of HMGB1/NLRP3 caspase-1 inflammasome activation and IL-1β release, but not the NF-κB activation and TNF-α secretion, is the primary process involving in inflammatory responses driven by C5a/C5aR2 interactions in this model. Therefore, our findings in this study, together with previously reported role for C5aR1 in this model (6), suggest that both C5a/C5aR1 and C5a/C5aR2 axes promote proinflammatory responses contributing to renal injury, however they promote proinflammatory responses through different dominate signalling pathways, despite sharing some signalling pathways. C5a/C5aR1 axis promotes proinflammatory responses mainly through inflammatory signalling pathways leading to NF-κB activation, whereas C5a/C5aR2 promotes proinflammatory responses mainly through HMGB1 and NLRP3 inflammasome pathways.

In conclusion, the present study demonstrates a pathogenic role for C5aR2 in renal injury mediated by infection and suggests that C5a/C5aR2 axis can contribute to renal inflammation/tissue damage through up-regulation of HMGB1 and NLRP3/caspase-1 inflammasome in inflammatory cells.
Furthermore, the findings of this study, together with the pathogenic roles of C5aR1 in renal injury previously reported, suggest that both C5aR1 and C5aR2 are potential therapeutic targets in renal inflammatory disorders.

Materials and Methods

Materials

We used the following reagents and materials: tryptone, yeast extract, cystine lactose electrolyte deficient (CLED) agar (from Oxoid, Basingstoke, UK); monoclonal rat anti-mouse CD45 (30-F11, APC), Ly6G (1A8, PE), Ly6C (HK1.4, PE/Cy7), purified rat anti-mouse/human CD11b (M1/70, FITC), recombinant mouse HMGB1 (from Biolegend, San Diego, USA); monoclonal mouse anti-human C5aR2 (E-8, Santa Cruz Biotechnology, Dallas, USA); Alexa Fluor 488 goat anti-rat IgG, Cy™3-AffiniPure goat anti-mouse IgG, Alexa Fluor 594 donkey anti-mouse IgG (from Jackson Immuno Research Lab., West Grove, USA); fluorescein-labelled Lens Culinaris Agglutinin (LCA) (Vector Laboratories, Burlingame, USA); anti- HMGB1, -NLRP3, -Cleaved Caspase-1 (Asp296), anti-phospho-ERK1/2 (Thr202/Tyr204), -Akt (Ser473), -JNK (Thr183/Tyr185), -IκB (Ser 32), and anti-ERK1/2, -Akt, -JNK and -IκB antibodies used for Western blot and signalling pathway studies (from Cell Signaling Technology, Danvers, USA); cell culture medium, fetal calf serum, TRIzol, countBright™ absolute counting beads, Fast SYBR® Green Master Mix, M-PER mammalian protein extraction reagent, RIPA lysis buffer and BCA protein assay kit (from Thermo Fisher, Waltham, USA); recombinant mouse C5a (HyCult Biotechnology, Uden, the Netherlands); thioglycollate (TG), LPS (derived from E. coli with serotype O55:B5, contains O antigen) (from Sigma-Aldrich, St. Louis, USA); Collagenase D (from Roche, Basel, Switzerland); collagenase II (from Worthington Biochemical Corp., Lakewood, USA); FcR-blocking antibody (CD16/32, 2.4G2), mouse ELISA kits for TNF-α (from BD Biosciences, Franklin Lakes, USA); mouse HMGB1 antibody, chicken IgY control antibody, mouse ELSA kit for KIM-1, CXCL-1 and IL-1β (from R&D Systems, Minneapolis, USA); mouse ELISA kit for HMGB-1 (Westang, Shanghai, China); M-MLV, RQ1 RNase-free DNase, GoTaq® DNA polymerase, RQ1 RNase-free DNase (from Promega, Fitchburg, USA).

Mice

Homozygous C5ar1<sup>−/−</sup>, C5ar2<sup>−/−</sup> mice were generated by homologous recombination in embryonic stem cells (18, 42) (provided by Dr. Bao Lu, Harvard Medical School, Boston) and backcrossed onto the C57BL/6 (H-2b) parental strain for at least 10 generations. Wildtype littermate mice were used as controls. Female mice (8-10 weeks old) were used in all experiments. All mice were maintained in specific pathogen-free conditions.
**Genotyping of C5ar2−/− mice**

Tail biopsies were collected from WT and C5ar2−/− mice, and genomic DNA were prepared and used for genotyping by PCR using primers for gDNA C5aR2 and neomycin. The information for primer sequences as follows: C5aR2: forward 5'-CCACACCACCAGCGAGTATTATG-3'; Reverse 5'-TCTATGCCACACAAGTGG-3'. Neomycin: forward 5'-ATACTTTTCGCGCAGGACA-3'; reverse 5'-AGACAATCGGCTGCTCTGAT-3'. C5aR2 gene deficiency was confirmed by the deletion of C5aR2 specific DNA and expression of neomycin gene (Figure 5). C5aR2 deficiency was further confirmed by RT-PCR analysis for C5aR mRNA expression (refer to later section) in kidney tissue and peritoneal inflammatory cells of WT and C5ar2−/− mice. Results show that C5aR2 mRNA was negatively detected in kidney tissue and inflammatory cells of C5ar2−/− mice, but which was positively detected in the tissue and cells of WT mice (Figure 6).

**Induction of pyelonephritis**

Murine UTI, a model of ascending urinary tract infection leading to pyelonephritis, was induced in female mice by bladder inoculation with human uropathogenic E. coli strain J96 (serotype O4; K6) (2x10^8 cfu in 50 μl PBS) per urethra as previously described. Before anesthetisation, urine were collected and plated on CLED agar plates to test sterility. Mice were killed at different time points (up to 48 hpi) for evaluation of renal histopathology, tissue inflammation and bacterial load.

**Measurement of bacterial load in the kidney**

Total bacterial load in kidney tissue was determined by bacterial plate count assay as previously described. After incubation of plates for 24 h at 37°C, bacterial colony forming units (cfu) on the plates were manually counted and expressed as an average cfu per gram of kidney tissue.

**Assessment of kidney histopathology**

Kidney paraffin sections (4μm) were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The severity of renal histopathology (i.e., tissue destruction, cellular infiltration, bacterial patchiness, and presence of abscesses) was graded using a 7-point scale, in which 0, 1, 2, and 3 indicated normal, mild, moderate, and severe pyelitis, respectively (pathological changes were mainly located within the medulla); while 4, 5, and 6 indicated mild, moderate, and severe pyelonephritis, respectively (pathological changes spread to more parts of the kidney), as previously described. The assessment was performed in a blinded fashion by two experienced researchers. Kidney sections (2-3 per mouse) were viewed and average histopathological score was presented.

**Assessment of inflammatory cell infiltration in the kidney**

Single renal cell suspensions were prepared using a method described previously. The cells were preincubated with FcR-blocking antibody, then stained with rat anti-mouse APC-conjugated CD45, PE-conjugated Ly6G, PEcy7-conjugated Ly6C, and FITC-conjugated CD11b antibodies, or the
appropriate isotype control antibodies at 4°C for 20 min. In order to quantify absolute cell counts in kidney tissue, we used CountBright™ absolute counting beads in our flow cytometry assays, according to the manufacturer's instructions. All flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and Flowjo software (Tree Star, Ashland, OR, USA).

**Immunofluorescence microscopy**

To detect C5aR2 expression, frozen sections (4µm) from normal or infected mouse kidneys were stained with anti-C5aR2 monoclonal antibody at 4°C overnight and followed by Alex Fluor 594 labelled donkey anti-mouse IgG, DAPI (for detection of nuclei) and fluorescein labelled LCA (for identification of renal structure). Sections were viewed and imaged with the confocal laser microscope system (Leica TCS SP8). 3-4 viewing fields at inner medullar, cortical-medullar junction and outer cortex for each kidney were examined. C5ar2−/− mice kidney sections were used as control.

In some experiment, to show the colocalization of C5aR2 and CD11b positive inflammatory cells, after staining for C5aR2, sections were counterstained with purified rat anti-mouse/human CD11b antibody (M1/70) and followed by Cy3-AffiPure goat anti-mouse IgG and Alexa Fluor 488 labelled goat anti-rat IgG and fluorescein-labelled goat anti-rat IgG and then stained with DAPI, and then viewed and imaged with the Leica SP8 system.

**Macrophage preparation and stimulation**

Peritoneal macrophages were prepared using the protocol as described previously (44). In brief, elicit peritoneal cells were harvested from mice which had given a 1 mL of 3% Brewer thioglycollate broth intraperitonealy 72 h prior collection. The peritoneal cells were seeded into 6-well plate at the density of 4x10⁶ cells/mL and cultured in serum-free medium for 1 hour at 37°C. Nonadherent cells were removed by gently washing three times with warm PBS. The adherent cells were kept in RPMI-1640 medium (containing 10% of fetal calf serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin). To assess the effect of C5a/C5aR2 interaction on HMGB1 expression/secretion, NLRP3 inflammasome activation and IL-1β release, macrophages were stimulated with recombinant mouse C5a (0, 2, 10 nM) in the presence or absence of LPS (2 ng/ml) for 6h or 24h. In some experiments, anti-HMGB1 neutralising antibody or control IgY (2 µg/ml) were added 1h before C5a stimulation. To assess the effect of HMGB1 on caspase-1 activation and IL-1β release, macrophages were stimulated with recombinant HMGB1 (0-100 ng/ml) for 6h. At the end of the treatment, cells were collected for Western blot and supernatants were collected for ELISA.

**Western blotting**

Mouse kidney tissue lysates were prepared by homogenising kidney tissue in a lysis buffer (Radioimmune precipitation assay buffer) containing proteinase and phosphatase inhibitor cocktail mixture on ice. Macrophage cell lysate was prepared using M-PER mammalian protein extraction
reagent containing proteinase on ice. Supernatants of the tissue or cell lysates were collected after centrifugation at 14,000g at 4°C for 15 min. Protein concentrations were determined by BCA protein assay kit according to the manufacturer’s instruction. Equal amounts of protein (30 μg per lane) were subjected to SDS-PAGE electrophoresis. After separation, the proteins were transferred from the gel on to PVDF membrane. The membranes were incubated with primary antibodies for overnight at 4°C and followed by incubation with HRP-conjugated secondary antibody for 1h. Protein bands were visualized by Amersham ECL Select™ detection reagent (GE Healthcare Life Sciences, USA). Quantification of protein bands on the gel was performed by measuring the intensity of individual band using Image J software (National Institutes of Health, USA). The relative amount of phosphorylated-AKT, -ERK1/2, -JNK, or -Iκb was generated by normalisation to the total protein of respective molecules. The relative amount of HMGB1, NLRP3, ASC or caspase-1 was generated by normalisation to β-actin.

**ELISA assay**
Levels of KIM-1, IL-1β, TNF-α, IL-6, CXCL-1 and HMGB1 in the supernatants (prepared from kidney tissue lysates or cell cultures) were determined using commercial ELISA kits according to the manufacturer’s instructions. The supernatants of renal tissue lysates were prepared as previously described (45), individual kidney was weighed and homogenised in PBS containing 1% Triton X-100, 1mM EDTA and 1% protease inhibitor cocktail then spun clear at 10,000g for 10 min.

**Quantitative and conventional RT-PCR**
Total RNA was purified from kidney tissue or peritoneal cells using TRIZol reagent, followed by cDNA synthesis. To exclude genomic DNA contamination, DNase was used before reverse-transcription. The conventional PCR was performed using C5aR2 and GAPDH primers, the reaction consisted 35 cycles of 1min at 94°C, 1min at 60°C, and 30s at 72°C. The real-time PCR was performed with Fast SYBR® Green Master Mix on a Step One™ Real-time PCR instrument (ThermoFisher, Waltham, MA, USA) using C5aR2 and 18S primers. The 2̶ΔΔCt method with normalization to 18S and controls was used for calculation (46). The controls were normal kidney tissues. The information for primer sequences as follows: C5aR2: forward 5’-ACCAGGAACACCAAGATGATG-3’, reverse 5’-TCACGGCATCCTCAACCGG-3’ (306 bp, NM_176912.4); IL-1β: forward 5’-GCTCTCCACCTCAATGGACA-3’, reverse 5’-TTGGGATCCACACTCTCCAG-3’ (182 bp, NM_008361); TNF-α: forward 5’-TGAGCACAGAAAGCATGATCC-3’, reverse 5’-GCCATTTGGGAACTTCTCATC-3’ (200bp, NM_013693.3); CXCL-1: forward 5’-TTGAAGGTGTGGCCCTCAG-3’, reverse 5’-ACAGGTGCCIACATGACG1-3’ (181bp, NM_008176.3); GAPDH: forward 5’-ACCACGTCCATGCCATCAG-3’, reverse 5’-TCCACCACCTTGTGTGCTGA-3’ (453 bp, NM_001289726.1); 18S: forward 5’-ATCCCTGAGAAGTGTCCAGCA-3’, reverse 5’-CCTCTTGGTAAGGTCAAGCAGCT-3’ (153 bp, NM_011296.1).
Statistical analysis
Data are shown as mean ± SD or the readout of individual mice. t test and One-way or Two-way ANOVA were used where appropriate to determine significant differences between samples. All the analyses were performed using Graphpad Prism 8 Software (Graphpad Software, La Jolla, CA, USA). P<0.05 was considered significant.

Study approval
The study was approved by the school Ethics Review Committee for Animal Experimentation at Xi’an Jiaotong University, Xi’an, China. The Ethics Review Committee approved and oversaw all mouse experiments.

Author contributions
KL and WZ conceived and designed the study and supervised the project. TZ, KYW, NM and LLW performed experiments. KL, TZ, and KYW analysed data. GM contributed to interpretation of results and helped with experimental design. TZ, KL and WZ wrote the manuscript.

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Figure legends

**Figure 1. C5ar2\(^{-}\) mice have reduced tissue damage and bacterial load in the kidney following inoculation with UPEC**

(A, B) Representative images of PAS (A) and H/E (B) staining of kidney sections of WT and C5ar2\(^{-}\) mice (24hpi). Middle and bottom panels: high magnification images that correspond with the boxed area. Arrows indicate histopathologic changes (i.e. cellular infiltration, bacterial patchiness, abscess, and tubule destruction) within the papilla/medulla and cortex. Scale bars, 250 μm. (C) Histological scores of kidney sections of WT and C5ar2\(^{-}\) mice (24hpi). Data were analyzed by Unpaired 2-tailed Student’s t test (n = 14 mice/group). (D) KIM-1 levels in the infected kidney tissues of WT and C5ar2\(^{-}\) mice at 24hpi and 48hpi, determined by ELISA. The dotted line across each graph represents the level of KIM-1 in normal kidney tissue, which is similar between WT and C5ar2\(^{-}\) mice. Data were analyzed by Mann-Whitney test (n = 12 mice/group for 24hpi, n = 8 mice/group for 48hpi). (E) Bacterial loads in kidney tissues of WT and C5ar2\(^{-}\) mice at 24hpi and 48hpi, determined by colony-forming units (CFU) assay. Data were analyzed by analysed by Mann-Whitney test (n = 10 mice/group). Error bars represent standard deviation.

**Figure 2. C5ar2\(^{-}\) mice have reduced inflammatory responses in the kidney following inoculation with UPEC**

(A, B) Quantification of inflammatory cells in infected kidney tissues of WT and C5ar2\(^{-}\) mice at 24hpi, determined by flow cytometry. The gating strategy of analysing inflammatory cells by flow cytometry was given in supplementary figure 1a. The dotted line across each graph represents the levels of inflammatory cells in normal kidney tissue, which is similar between WT and C5ar2\(^{-}\) mice. Data were analyzed by Unpaired 2-tailed Student’s t test (n = 10 mice/group). (C) Cytokine levels in the infected kidney tissues of WT and C5ar2\(^{-}\) mice at 24hpi, determined by ELISA. The dotted line across each graph represents the levels of cytokine in normal kidney tissue, which is similar between WT and C5ar2\(^{-}\) mice. Data were analyzed by Two-way ANOVA with multiple comparisons test (n = 10 mice/group). Error bars represent standard deviation. *P < 0.05, **P < 0.005, ****P < 0.0001. Mo/MΦ, monocyte/macrophage.

**Figure 3. C5ar2\(^{-}\) mice have reduced intrarenal HMGB1 expression and NLRP3/caspase-1 inflammasome activation following inoculation with UPEC**

(A) Intrarenal levels of HMGB1 in WT and C5ar2\(^{-}\) mice at 6phi and 24phi, determined by ELISA. Data were analyzed by Two-way ANOVA with multiple comparisons test (n=10 mice/group). (B) Representative Western blots showing HMGB1, NLRP3, ASC and cleaved caspase-1 and b-actin in kidney lysates from WT and C5ar2\(^{-}\) mice (i.e. uninfected [normal] and infected [24phi]). (C) Relative amounts of HMGB1, NLRP3, ASC and cleaved caspase-1, corresponding with the blots in (B),
quantified as described in Materials and methods. Data were analyzed by Unpaired 2-tailed Student’s t test (n= 4-6 mice/group, pooled from two experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.005, ****P < 0.0001.

Figure 4. Effects of C5aR2 deficiency on intracellular signaling pathways in the kidney following inoculation with UPEC

(A, C) Representative Western blots showing total and phosphorylated several proteins (as indicated) in kidney tissues from WT and C5ar2−/− mice (i.e. uninfected [normal] and infected [24phi]). (B) Relative amounts of p-AKT, -ERK1/2 and -JNK, corresponding with the blots in (A). (D) Relative amounts of p-IκB, corresponding with the blot in (C). (B, D) The amounts were quantified as described in Materials and methods. Data were analyzed by Unpaired 2-tailed Student’s t test (n= 3-6 mice/group, pooled from two experiments). Error bars represent standard deviation. ns, no significant difference.

Figure 5. Expression and distribution of C5aR2 in murine kidney

(A, B) RT-PCR were performed in normal and infected kidney tissues of WT mice. (A) The agarose gel of conventional PCR showing the PCR products for C5aR2 and internal control GAPDH. The 100 bp DNA markers are shown alongside the gel. (B) Results of qPCR. Data are expressed as were analyzed by One-way ANOVA with multiple comparisons test (n=6 mice/group). Error bars represent standard deviation. (C) Immunochemical staining for C5aR2 in (WT) normal and infected mouse kidney sections (24hpi). Images were taken from cortex, corticomedullary junction, medulla of the kidney sections, stained with anti-C5aR2 (red), LCA (green) (used for identifying renal structure) and DAPI (blue). Boxed regions corresponding to the images at the bottom panel. Arrows indicate cells stained positive for C5aR2. Scale bars, 100 μm (top two panels), 50 μm (bottom panel). (D) Immunochemical staining for CD11b and C5aR2 in (WT) infected kidney sections (24hpi). Images were taken from corticomedullary junction. CD11b (green), C5aR2 (red) and DAPI (blue) were shown. Arrows indicate cells stained positive for both CD11b and C5aR2. Scale bars: 50 μm. A representative of three experiments is shown.

Figure 6. Inflammatory cells from C5ar2−/− mice have reduced ability to produce proinflammatory cytokines

(A) RT-PCR were performed in d3 peritoneal exudate cells derived from WT and C5ar2−/− mice with or without LPS (2 ng/ml) stimulation for 6 h. (B) RT-PCR were performed in d1 peritoneal exudate cells derived from WT and C5ar2−/− mice with or without LPS (2 ng/ml) stimulation for 6 h. (A, B) Data were analyzed by Two-way ANOVA with multiple comparisons test (n = 4/group, resulting from 4 independent experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001. (C) d3 peritoneal exudate cells derived from WT and C5ar2−/− mice were cultured in the presence of LPS (2 ng/ml) for 6h. Supernatant levels of proinflammatory cytokines

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were measured by ELISA. Data were analyzed by paired 2-tailed Student’s t test (n=4/group, resulting from 4 independent experiments).

**Figure 7. C5a/C5aR2 interaction mediates upregulation of HMGB1 and NLRP3/caspase-1 inflammasome in macrophages**
(A, B) Peritoneal macrophages derived from *C5ar1*−/− mice were incubated with C5a with or without LPS (2 ng/ml) for 6 hours and subjected to Western blotting analysis. (A) Representative Western blots showing HMGB1, NLRP3, ASC, cleaved caspase-1 and b-actin in the 6h cell lysates. (B) Relative amounts of HMGB1, NLRP3, ASC and cleaved caspase-1, corresponding with the blots in (A), quantified as described in Materials and methods. Data were analyzed by paired 2-tailed Student’s t test (n=4/group, resulting from four independent experiments). (C-E) Peritoneal macrophages derived from *C5ar1*−/− mice were incubated with C5a, in the absence or presence of LPS (2 ng/ml) for 6h and 24h. Supernatant levels of HMGB1, IL-1β and TNF-α were measured by ELISA. Data were analyzed by paired 2-tailed Student’s t test (n=3/group, resulting from 3 independent experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.01, ***P < 0.005.

**Figure 8. Blockade of HMGB1 reduces C5a/C5aR2 interaction-mediated caspase 1 activation and IL-1β secretion in macrophages**
(A-C) Peritoneal macrophages derived from *C5ar1*−/− mice were incubated with HMGB1 in the presence of LPS (2ng/ml) for 6 hours and subjected to Western blotting analysis and ELISA. (A) Representative Western blots showing cleaved caspase-1 and β-actin in the cell lysates. (B) Relative amount of caspase-1, corresponding with the blots in (A), quantified as described in Materials and methods. (C) Supernatant levels of IL-1β, determined by ELISA. (D-F) *C5aR1*−/− macrophages were incubated with C5a (10 nM) alone or C5a plus anti-HMGB1 antibody, or C5a plus control Ig (2 mg/ml) for 6 hours, in the presence of LPS (2 ng/ml) and subjected to Western blotting analysis and ELISA. (D) Representative Western blots showing NLRP3, cleaved caspase-1 and β-actin in the cell lysates. (E) Relative amounts of NLRP3 and cleaved caspase-1, corresponding with the blots in (D), quantified as described in Materials and methods. (F) Supernatant levels of IL-1β determined by ELISA. (B, C, E, F) Data were analyzed by One-way ANOVA with multiple comparisons test (n=4/group, resulting from four independent experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.01.

**Figure 9. Proposed molecular mechanism by which C5a/C5aR2 axis promotes proinflammatory responses in inflammatory cells upon infection**
Based on our findings in this study and literature, we propose a molecular mechanism by which C5a/C5aR2 axis promotes proinflammatory responses in macrophages. Engagement of C5aR2 with C5a in macrophages upon infection induces upregulation of HMGB1 expression and release through
intracellular signaling (e.g. MAPK, AKT), which upregulates NLRP3/caspase-1 inflammasome activation and IL-1β secretion, possibly through engagement of PRRs inducing proinflammatory signaling. This, together with proinflammatory signaling-mediated upregulation of cytokine genes (e.g. TNF-α) contributes to renal inflammation and tissue damage. Bacterial endotoxins amplify the C5a/C5aR2 axis-mediated upregulation of HMGB1/NLRP3/inflammasome during the infection.
Figure 1. *C5ar2*−/− mice have reduced tissue damage, bacterial load and inflammatory responses in the kidney following inoculation with UPEC.
Figure 1. *C5ar2* mice have reduced tissue damage and bacterial load in the kidney following inoculation with UPEC.
Figure 1. *C5ar2*⁺⁺ mice have reduced tissue damage and bacterial load in the kidney following inoculation with UPEC

(A, B) Representative images of PAS (A) and H/E (B) staining of kidney sections of WT and C5ar2⁻/⁻ mice (24hpi). Middle and bottom panels: high magnification images that correspond with the boxed area. Arrows indicate histopathologic changes (i.e. cellular infiltration, bacterial patchiness, abscess, and tubule destruction) within the papilla/medulla and cortex. Scale bars, 250 μm. (C) Histological scores of kidney sections of WT and C5ar2⁻/⁻ mice (24hpi). Data were analyzed by Unpaired 2-tailed Student's t test (n = 14 mice/group). (D) KIM-1 levels in the infected kidney tissues of WT and C5ar2⁻/⁻ mice at 24hpi and 48hpi, determined by ELISA. The dotted line across each graph represents the level of KIM-1 in normal kidney tissue, which is similar between WT and C5ar2⁻/⁻ mice. Data were analyzed by Mann-Whitney test (n = 12 mice/group for 24hpi, n = 8 mice/group for 48hpi). (E) Bacterial loads in kidney tissues of WT and C5ar2⁻/⁻ mice at 24hpi and 48hpi, determined by colony-forming units (CFU) assay. Data were analyzed by Mann-Whitney test (n = 10 mice/group). Error bars represent standard deviation.
Figure 2. C5ar2⁻/⁻ mice have reduced inflammatory responses in the kidney following inoculation with UPEC

(A) Quantification of inflammatory cells in infected kidney tissues of WT and C5ar2⁻/⁻ mice at 24hpi, determined by flow cytometry. The gating strategy of analysing inflammatory cells by flow cytometry was given in supplementary figure 1a. The dotted line across each graph represents the levels of inflammatory cells in normal kidney tissue, which is similar between WT and C5ar2⁻/⁻ mice. Data were analyzed by Unpaired 2-tailed Student’s t test (n = 10 mice/group). (B) Cytokine levels in the infected kidney tissues of WT and C5ar2⁻/⁻ mice at 24hpi, determined by ELISA. The dotted line across each graph represents the levels of cytokine in normal kidney tissue, which is similar between WT and C5ar2⁻/⁻ mice. Data were analyzed by Two-way ANOVA with multiple comparisons test (n = 10 mice/group). Error bars represent standard deviation. *P < 0.05, **P < 0.005, ****P < 0.0001. Mo/MΦ, monocyte/macrophage.
Figure 3. *C5ar2*−/− mice have reduced intrarenal HMGB1 expression and NLRP3/caspase-1 inflammasome activation following inoculation with UPEC

A

![Graph showing HMGB1 expression over time](image1)

B

| Normal | 24hpi |
|--------|-------|
| WT     | C5ar2−/− | WT | C5ar2−/− |
| HMGB1  | ![Image of HMGB1 blots](image2) | ![Image of HMGB1 blots](image2) | ![Image of HMGB1 blots](image2) |
| NLRP3  | ![Image of NLRP3 blots](image3) | ![Image of NLRP3 blots](image3) | ![Image of NLRP3 blots](image3) |
| ASC    | ![Image of ASC blots](image4) | ![Image of ASC blots](image4) | ![Image of ASC blots](image4) |
| Cleaved caspase-1 | ![Image of Cleaved caspase-1 blots](image5) | ![Image of Cleaved caspase-1 blots](image5) | ![Image of Cleaved caspase-1 blots](image5) |
| β-Actin | ![Image of β-Actin blots](image6) | ![Image of β-Actin blots](image6) | ![Image of β-Actin blots](image6) |

C

![Bar charts showing relative amount](image7)
Figure 3. *C5ar2*−/− mice have reduced intrarenal HMGB1 expression and NLRP3/caspase-1 inflammasome activation following inoculation with UPEC

(A) Intrarenal levels of HMGB1 in WT and C5ar2−/− mice at 6phi and 24phi, determined by ELISA. Data were analyzed by Two-way ANOVA with multiple comparisons test (n=10 mice/group). (B) Representative Western blots showing HMGB1, NLRP3, ASC and cleaved caspase-1 and b-actin in kidney lysates from WT and C5ar2−/− mice (i.e. uninfected [normal] and infected [24phi]). (C) Relative amounts of HMGB1, NLRP3, ASC and cleaved caspase-1, corresponding with the blots in (B), quantified as described in Materials and methods. Data were analyzed by Unpaired 2-tailed Student’s t test (n= 4-6 mice/group, pooled from two experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.005, ****P < 0.0001.
Figure 4. Effects of C5aR2 deficiency on intracellular signaling pathways in the kidney following inoculation with UPEC.

A

|          | Normal 24hpi | 24hpi |
|----------|--------------|-------|
| WT       | C5ar2−/−     | WT    | C5ar2−/− |
| p-AKT    |              |       |          |
| AKT      |              |       |          |
| p-ERK1/2 |              |       |          |
| ERK1/2   |              |       |          |
| p-JNK    |              |       |          |
| JNK      |              |       |          |
| β-Actin  |              |       |          |

(kDa)

B

- **p-AKT**
  - Normal 24 hpi: WT (0.005)
  - WT C5ar2−/−: 24 hpi (0.0041)

- **p-ERK**
  - Normal 24 hpi: WT (0.0024)
  - WT C5ar2−/−: 24 hpi

- **p-JNK**
  - Normal 24 hpi: WT (0.0041)
  - WT C5ar2−/−: 24 hpi
Figure 4. Effects of C5aR2 deficiency on intracellular signaling pathways in the kidney following inoculation with UPEC

(A, C) Representative Western blots showing total and phosphorylated several proteins (as indicated) in kidney tissues from WT and C5ar2−/− mice (i.e. uninfected [normal] and infected [24phi]). (B) Relative amounts of p-AKT, -ERK1/2 and -JNK, corresponding with the blots in (A). (D) Relative amounts of p-IκB, corresponding with the blot in (C). (B, D) The amounts were quantified as described in Materials and methods. Data were analyzed by Unpaired 2-tailed Student's t test (n= 3-6 mice/group, pooled from two experiments). Error bars represent standard deviation. ns, no significant difference.
Figure 5. Expression and distribution of C5aR2 in murine kidney

A

B

C

LCA/C5aR2/DAPI
Figure 5. Expression and distribution of C5aR2 in murine kidney

(A, B) RT-PCR were performed in normal and infected kidney tissues of WT mice. (A) The agarose gel of conventional PCR showing the PCR products for C5aR2 and internal control GAPDH. The 100 bp DNA markers are shown alongside the gel. (B) Results of qPCR. Data are expressed as were analyzed by One-way ANOVA with multiple comparisons test (n=6 mice/group). Error bars represent standard deviation. (C) Immunochemical staining for C5aR2 in (WT) normal and infected mouse kidney sections (24hpi). Images were taken from cortex, corticomedullary junction, medulla of the kidney sections, stained with anti-C5aR2 (red), LCA (green) (used for identifying renal structure) and DAPI (blue). Boxed regions corresponding to the images at the bottom panel. Arrows indicate cells stained positive for C5aR2. Scale bars, 100 μm (top two panels), 50 μm (bottom panel). (D) Immunochemical staining for CD11b and C5aR2 in (WT) infected kidney sections (24hpi). Images were taken from corticomedullary junction. CD11b (green), C5aR2 (red) and DAPI (blue) were shown. Arrows indicate cells stained positive for both CD11b and C5aR2. Scale bars: 50 μm. A representative of three experiments is shown.
Figure 6. Inflammatory cells from C5ar2-/- mice have reduced ability to produce proinflammatory cytokine

A

**IL-1β**

|         | No LPS | LPS   |
|---------|--------|-------|
| **WT**  | ![Graph](image) | ![Graph](image) |
| **C5ar2-/-** | ![Graph](image) | ![Graph](image) |

**TNF-α**

|         | No LPS | LPS   |
|---------|--------|-------|
| **WT**  | ![Graph](image) | ![Graph](image) |
| **C5ar2-/-** | ![Graph](image) | ![Graph](image) |

**CXCL-1**

|         | No LPS | LPS   |
|---------|--------|-------|
| **WT**  | ![Graph](image) | ![Graph](image) |
| **C5ar2-/-** | ![Graph](image) | ![Graph](image) |

B

**IL-1β**

|         | No LPS | LPS   |
|---------|--------|-------|
| **WT**  | ![Graph](image) | ![Graph](image) |
| **C5ar2-/-** | ![Graph](image) | ![Graph](image) |

**TNF-α**

|         | No LPS | LPS   |
|---------|--------|-------|
| **WT**  | ![Graph](image) | ![Graph](image) |
| **C5ar2-/-** | ![Graph](image) | ![Graph](image) |

**CXCL-1**

|         | No LPS | LPS   |
|---------|--------|-------|
| **WT**  | ![Graph](image) | ![Graph](image) |
| **C5ar2-/-** | ![Graph](image) | ![Graph](image) |

C

**IL-1β**

|         | WT | C5ar2-/- |
|---------|----|----------|
| **Cytokine levels (pg/ml)** | ![Graph](image) | ![Graph](image) |

**TNF-α**

|         | WT | C5ar2-/- |
|---------|----|----------|
| **Cytokine levels (pg/ml)** | ![Graph](image) | ![Graph](image) |

**CXCL-1**

|         | WT | C5ar2-/- |
|---------|----|----------|
| **Cytokine levels (pg/ml)** | ![Graph](image) | ![Graph](image) |
Figure 6. Inflammatory cells from C5ar2-/ mice have reduced ability to produce proinflammatory cytokine

(A) RT-PCR were performed in d3 peritoneal exudate cells derived from WT and C5ar2-/ mice with or without LPS (2 ng/ml) stimulation for 6 h. (B) RT-PCR were performed in d1 peritoneal exudate cells derived from WT and C5ar2-/ mice with or without LPS (2 ng/ml) stimulation for 6 h. (A, B) Data were analyzed by Two-way ANOVA with multiple comparisons test (n = 4/group, resulting from 4 independent experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.0001. (C) d3 peritoneal exudate cells derived from WT and C5ar2-/ mice were cultured in the presence of LPS (2 ng/ml) for 6h. Supernatant levels of proinflammatory cytokines were measured by ELISA. Data were analyzed by paired 2-tailed Student’s t test (n=4/group, resulting from 4 independent experiments).
Figure 7. C5a/C5aR2 interaction mediates upregulation of HMGB1 and NLRP3/caspase-1 inflammasome in macrophages

A

| C5a (nM) | no LPS | LPS | (kDa) |
|---------|--------|-----|-------|
| HMGB1   | ![Image](image1.png) | ![Image](image2.png) | 29    |
| NLRP3   | ![Image](image3.png) | ![Image](image4.png) | 110   |
| ASC     | ![Image](image5.png) | ![Image](image6.png) | 22    |
| Cleaved caspase-1 | ![Image](image7.png) | ![Image](image8.png) | 22    |
| β-Actin | ![Image](image9.png) | ![Image](image10.png) | 45    |

B

![Graphs showing the expression levels of HMGB1, NLRP3, ASC, and Caspase-1](image11.png)
Figure 7. C5a/C5aR2 interaction mediates upregulation of HMGB1 and NLRP3/caspase-1 inflammasome in macrophages

(A, B) Peritoneal macrophages derived from C5ar1−/− mice were incubated with C5a with or without LPS (2 ng/ml) for 6 hours and subjected to Western blotting analysis. (A) Representative Western blots showing HMGB1, NLRP3, ASC, cleaved caspase-1 and β-actin in the 6h cell lysates. (B) Relative amounts of HMGB1, NLRP3, ASC and cleaved caspase-1, corresponding with the blots in (A), quantified as described in Materials and methods. Data were analyzed by paired 2-tailed Student’s t test (n=4/group, resulting from four independent experiments). (C-E) Peritoneal macrophages derived from C5ar1−/− mice were incubated with C5a, in the absence or presence of LPS (2 ng/ml) for 6h and 24h. Supernatant levels of HMGB1, IL-1β and TNF-α were measured by ELISA. Data were analyzed by paired 2-tailed Student’s t test (n=3/group, resulting from 3 independent experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.01, ***P < 0.005.
Figure 8. Blockade of HMGB1 reduces C5a/C5aR2 interaction-mediated caspase 1 activation and IL-1β secretion in macrophages

A

HMGB1 - 10 100 (ng/ml)  
LPS + + +  
Cleaved caspase-1  
β-Actin

B

Cleaved caspase-1  
HMGB1 (ng/ml) - 10 100

C

IL-1β (pg/ml)  
HMGB1 (ng/ml) - 10 100

D

Anti-HMGB1 - - - +  
Control Ig - - + -  
C5a - + + +  
NLRP3  
Cleaved caspase-1  
β-Actin

E

NLRP3  
Relative amount  
Anti-HMGB1 - - - +  
Control Ig - - + -  
C5a - + + +

F

IL-1β (pg/ml)  
Anti-HMGB1 - - - +  
Control Ig - - + -  
C5a - + + +
Figure 8. Blockade of HMGB1 reduces C5a/C5aR2 interaction-mediated caspase 1 activation and IL-1β secretion in macrophages

(A-C) Peritoneal macrophages derived from C5ar1-/- mice were incubated with HMGB1 in the presence of LPS (2ng/ml) for 6 hours and subjected to Western blotting analysis and ELISA. (A) Representative Western blots showing cleaved caspase-1 and β-actin in the cell lysates. (B) Relative amount of caspase-1, corresponding with the blots in (A), quantified as described in Materials and methods. (C) Supernatant levels of IL-1β, determined by ELISA. (D-F) C5aR1-/- macrophages were incubated with C5a (10 nM) alone or C5a plus anti-HMGB1 antibody, or C5a plus control Ig (2 mg/ml) for 6 hours, in the presence of LPS (2 ng/ml) and subjected to Western blotting analysis and ELISA. (D) Representative Western blots showing NLRP3, cleaved caspase-1 and β-actin in the cell lysates. (E) Relative amounts of NLRP3 and cleaved caspase-1, corresponding with the blots in (D), quantified as described in Materials and methods. (F) Supernatant levels of IL-1β determined by ELISA. (B, C, E, F) Data were analyzed by One-way ANOVA with multiple comparisons test (n=4/group, resulting from four independent experiments). Error bars represent standard deviation. *P < 0.05, **P < 0.01.
Based on our findings in this study and literature, we propose a molecular mechanism by which C5a/C5aR2 axis promotes proinflammatory responses in macrophages. Engagement of C5aR2 with C5a in macrophages upon infection induces upregulation of HMGB1 expression and release through intracellular signaling (e.g. MAPK, AKT), which upregulates NLRP3/caspase-1 inflammasome activation and IL-1β secretion, possibly through engagement of PRRs inducing proinflammatory signaling. This, together with proinflammatory signaling-mediated upregulation of cytokine genes (e.g. TNF-α) contributes to renal inflammation and tissue damage. Bacterial endotoxins amplify the C5a/C5aR2 axis-mediated upregulation of HMGB1/NLRP3/inflammasome during the infection.