CD226 promotes renal fibrosis by regulating macrophage activation and migration

Yun Song1†, Yazhen Wang1†, Juan Li2, Yuting Shen1, Yongli Hou1, Zhaoyue Fu1, Liang Fang1, Boquan Jin1, Lihua Chen1,2\*

*1Department of Immunology,* *Fourth Military Medical University, Xi’an 710032, ShaanXi, China*

*2College of Life Sciences, Northwest University, Xi’an 710069, ShaanXi, China*

**†**These authors contributed equally to this work.

**\* Correspondence:****Lihua Chen****[chenlh@fmmu.edu.cn](mailto:chenlh@fmmu.edu.cn)**

Abstract

It has been found that CD226 plays an important role in regulating macrophage function, but its expression and function on macrophage during renal fibrogenesis have not been studied. Our data demonstrated that CD226 expression in macrophages was obviously upregulated in the UUO model, while CD226 deficiency attenuated collagen deposition in renal inerestitum along with fewer number of M1 within renal cortex and renal medulla and a lower level of proinflammatory factors compared to control littermates. Further studies demonstrated that *Cd226-/-*-BMDMs transferring to *Cd226+/*+ mice could significantly reduce the tubular injury, collagen deposition and proinflammatory cytokines secretion compared with WT-BMDMs group and WT-PBS group in adoptively transferring assay. Mechanistic investigations revealed that CD226 could suppress KLF4 expression in macrophages, which subsequently promoted more proinflammatory M1 accumulation in the kidney of WT mice than that of CD226 deficient mice. *In vitro*, we silenced KLF4 expression in BMDMs deriving from WT or CD226 deficient mice and the trend that CD226 promting more numbers of M1 disappeared. Therefore, our results uncover a pathogenic role of CD226 during the development of CKD by promoting monocyte infiltration from peripheral blood into the kidney and enhancing macrophage activation towards to the inflammatory phenotype by suppressing KLF4 expression.

Keywords: CD226; macrophage homeostasis; inflammation; KLF4; renal fibrosis

# Introduction

Chronic kidney disease (CKD) is estimated to account for 8-16% of the worldwide population[1]. The progression of CKD is characterized by progressive loss of renal pathological injury and its functions, mainly including tubular atrophy or dilation, interstitial inflammatory cell infiltration and extracellular matrix deposition[2]. Interstitial fibrosis caused by destruction of the parenchyma is the main cause (*e.g.*, glomerular sclerosis, tubular atrophy) of CKD after the terminal stage[3]. Renal parenchyma injury and tissue repair are complicated dynamic processes, and excessive damage or inflammatory responses are closely related to fibrosis development. Following injury, ongoing inflammation, including the increase of infiltrated inflammatory cells and the production of proinflammatory cytokines, triggers the fibrotic process[4]. Tissue repair after renal injury is a process involving a variety of immune cells, such as macrophages, Tregs as well as Th cells[5]. In the kidney unilateral ureteral obstruction (UUO) model, a reduction in macrophage infiltration attenuated parenchyma injury and renal fibrosis via recruitment of inflammatory macrophages to the renal interstitium[6, 7].

The renal inflammatory microenvironment is thought to be an important factor in determining tissue destruction or repair, and the local inflammatory condition is dependent on inflammatory cell infiltration and the production of proinflammatory cytokines. Macrophages mainly polarize toward the M1 phenotype (proinflammatory phenotype) once tissue injury and the local inflammatory statement is a crucial factor to determine the quality of tissue repair[8]. In addition, macrophages recruited from bone marrow can differentiate into myofibroblasts contributing to renal fibrosis[9]. CD226 is expressed in various immune cells, and it mediates a broad spectrum of cell adhesion, immune synapse formation and cytotoxic effects after interacting with its ligand [10].

It is well known that many factors (*e.g.*, TNF-α, MMP-1, TIMP-1, IL-4, IL-10, TGF*-β*) responsible for macrophage polarization are also closely related to the development of kidney injury and fibrosis[11, 12]. Given the role of CD226 in cellular adhesion and immune cell activation during pathological inflammation, we speculated that CD226 may play an important role in macrophage activation and homeostasis. In our study, CD226-deficient mice (*Cd226-/-*) was used to assess macrophage numbers and their phenotype during UUO. Our data demonstrated that CD226-deficient macrophages have a lower ability to polarize into the M1 phenotype because of Krüppel-like factor 4 (KLF4) expression.

KLF4 is a DNA-binding transcriptional regulator with conserved zinc (Zn)-finger domains in its C-terminal regions, which is highly expressed in monocytes and the endothelium. It has been reported that KLF4 expression is markedly upregulated in M2-like macrophages and strongly reduced in M1-like macrophages, which suggests that KLF4 is a new regulator of macrophage polarization [13] In addition, KLF4 expression in myeloid cells alleviated tubular epithelial cell necroptosis and kidney interstitial fibrosis after UUO surgery mainly by suppressing TNF-α production in infiltrating myeloid cells[14]. It has been reported that KLF4 plays an anti-inflammation role involving acute kidney injury, atherogenesis and cell differentiation[15-17]. [However, the mechanism of which KLF4 is regulated during CKD remains elusive.](https://doi.org/10.1016/j.ebiom.2019.01.021" \t "_blank)

In the present study, CD226 aggravated renal dysfunction by increasing inflammatory cell infiltration after UUO, while macrophage polarization into M1-like macrophages was mainly dependent on inhibiting KLF4 expression via CD226. Our results underline that CD226 plays important roles in renal inflammation and fibrogenesis by impacting leukocyte infiltration and proinflammatory macrophage activation.

In this study, we first found CD226 expression on macrophages was enhanced significantly in renal tissue of UUO mice, CD226-deficient mice had less collagen deposition in renal inerestitum, along with fewer numbers of M1 and a lower level of proinflammatory cytokines. Then the adoptively transferred assay showed *Cd226-/-*-BMDMs transferring to *Cd226+/*+ mice could significantly reduce the tubular injury, collagen deposition and pro-inflammatory cytokines secretion compared with those of WT-BMDMs and WT group treated with PBS. Mechanistic investigations revealed that CD226 could suppress KLF4 expression in macrophages, which subsequently promoted more proinflammatory M1 accumulation in the kidney of WT mice than that of CD226 deficient mice. Our results underline that CD226 plays an important role in renal inflammation and fibrogenesis by impacting leukocyte infiltration and proinflammatory macrophage activation.

1. **Results**

## CD226-positive macrophage is accumulated in the kidney after UUO surgery

The tubulointerstitial fibrosis is characterized by the accumulation of lots of proinflammatory cells in local tissue. We subjected WT mice to ligature of the left ureter and then examined macrophage infiltration in the kidney using flow cytometry. And the accumulation of ECM (collagen deposition) was assessed by Masson staining in renal tissues of UUO mice. Interstitial collagen accumulation was increased in the fibrotic kidney (Fig. 1A). Flow cytometry analysis of renal cell suspensions showed that F4/80 expression in CD45-positive cells was significantly increased in the kidneys, especially at day 3 and day 7 (Fig. 1B, C). In addition, we examined CD226 expression on macrophages using flow cytometry and immunofluorescence. Flow cytometry analysis showed that macrophages with CD226 positive expression (F4/80+CD226+) were greatly upregulated during renal fibrosis compared with those in the sham group (Fig. 1D). An increase of CD226 positive macrophages (F4/80+CD226+) was quantified by immunofluorescence in the fibrotic kidneys at day 3 and day 7 (Fig. 1E). Taken together, these results showed that CD226+F4/80+ macrophages in renal tissue were increased in UUO induced renal fibrosis.

## Expression of CD226 promotes the injury of glomerular and tubular and the accumulation of ECM in the kidney after UUO

Since macrophage infiltration correlates with renal fibrogenesis and function, we next assessed the accumulation of ECM (collagen deposition) in the fibrotic kidneys. Interestingly, compared to WT UUO mice a significant reduction of interstitial extracellular matrix deposition was found at day 3 (17.5% vs. 12.9%), 7 (21.6% vs. 14.2%) and 14 (31.1% vs. 18.1%) in CD226-deficient UUO mice (Fig. 2A-B). Furthermore, reverse-transcription quantitative PCR (RT-qPCR) was performed to analyze the mRNA expression of ECM (*Col-I/IV*, *Pdgf*, *Vimentin*). Compared with WT UUO mice, lower mRNA expression of *Col-I*, *Col-IV*, *Pdgf*, and *Vimentin* were observed in the kidney of CD226-deficient UUO mice (Fig. 2C). To examine the effects of UUO on renal function, we evaluated the ratio of kidney to body weight and the ratio of kidney/body weight of UUO mice exhibited a dramatic increase at day 7 in WT UUO mice as depicted in Fig. 2D. In contrast, the ratio of kidney/body weight increased slowly in CD226-deficient UUO mice, with an approximately 50% decrease at day 7 (14.82 vs. 32.44) compared to the WT group (Fig. 2D). We further measured the BUN level of serum samples at day 3, 7 and 14 and found that there was a strikingly lower BUN level in CD226-deficient mice *versus* WT mice (Fig. 2E), suggesting CD226-deficient mice had less impairment of renal function. These data demonstrate that renal ECM deposition and functional impairment were alleviated in CD226-deficient UUO mice, indicating CD226 plays an important role during renal fibrogenesis in UUO.

## CD226 promotes inflammatory leukocyte migration into kidney tissue

Inflammatory cells [infiltration](file:///C:/Users/Administrator/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;)in in local tissue is one of the typical characters of tubulointerstitial fibrosis. Increasing evidence reveals that chronic tissue injury and continuous infiltration of macrophages can ultimately lead to additional pathology, including parenchymal tissue destruction, microvascular thinning, and irreversible fibrosis. Therefore, we examined inflammatory cell infiltration in the kidney of WT UUO mice and *Cd226-/-* UUO mice at day 7 by immunohistochemistry. The size of kidney was obviously greater after UUO surgery (Fig. 3A). The data showed that CD226-deficient mice had significantly lower numbers of CD45+ (leukocytes) and F4/80+ cells (macrophages) infiltrating into the kidney at day 7 than WT mice (Fig. 3B-C). The positive staining areas of CD45+ cells and F4/80+ cells were significantly downregulated in the CD226-deficient mice, and the numbers of CD45+ and F4/80+ cells in *Cd226-/-* mice were decreased by [approximately](file:///C:/Users/Administrator/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;) 53% and 69% respectively, compared with those in WT mice (Fig. 3C). Flow cytometry analysis showed that CD45+ (14.23% vs. 20.20%) and F4/80+ (5.58% vs. 21.36%) cells had a decline after CD226 deficiency compared with those in the WT mice (Fig. 3D). ELISA results confirmed that the ablation of CD226 significantly reduced the secretion of TNF-α, IL-6 and IFN-γ in the fibrotic kidneys, especially the level of IL-6 and IFN-γ (*p*<0.05; Fig. 3E). Moreover, the mRNA expression of *Il-6* was lower at day 3 and 7 in the fibrotic kidneys of CD226-deficient mice (Fig. 3F). While TGF-β expression had a decline at day 7 and was significantly downregulated in CD226-deficient mice at day 14 (Fig. 3F). Taken together, WT mice obviously manifested much more severe [renal](https://dict.cn/vasculogenesis) injury characterized by more inflammatory cell [accumulation](file:///C:/Users/Administrator/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;), especially macrophages.

## CD226 regulates the balance of M1(F4/80+iNOS+) and M2(F4/80+CD206+) macrophages in the kidney after UUO

Macrophages marked with different phenotypes have different roles, such as clearing damaged tissue, remodeling extracellular matrix or regulating inflammation during the later phase of tissue repair [18]. CD226 could also enhance the migration of peritoneal macrophages in mouse ulcerative colitis[19]. We further explored the phenotype of local macrophages in renal tissues of UUO mice by immunofluorescence staining. The results showed that F4/80+iNOS+ M1-like macrophages accumulated in the medulla and cortex of renal tissue of WT UUO mice at day 3, 7 and 14 compared with that in CD226-deficient UUO mice (Fig. 4A-B). Of note, in contrast 502 cells/mm2 F4/80+iNOS+M1-like macrophages in the medulla of WT UUO mice, only 87 cells/mm2 F4/80+iNOS+M1-like macrophages in that of CD226-deficient UUO mice at day 3. This indicates that CD226 increases proinflammatory macrophage accumulation at the early stage of UUO. Meanwhile, we also observed that notably fewer F4/80+CD206+ M2-like macrophages localized in the medullary and cortical parts of renal tissue in WT UUO mice at day 7 and 14 than in CD226-deficient UUO mice (Fig. 4C-D). Taken together, our results suggested that the expression of CD226 stimulates macrophages to prefer M1-like macrophages during kidney injury and contributes to fibrosis in UUO mice.

## CD226 deletion arrested more iMos within the peripheral blood

Ki-Wook et al. reported CD226+ macrophages derived from blood Ly6Chi monocytes and replenished circulating CCR2+ monocytes [20]. In addition, monocytes were treated with anti-DNAM-1 mAb, they could be suppressed at the apical surface of the endothelium over intercellular junctions [21]. We further analyzed monocytes in peripheral blood of UUO mice. Flow cytometry showed that larger numbers of monocytes (CD11b+Ly6C+) which around 35% increase at day 3, and 7 were found in the peripheral blood of the CD226-deficient mice, compared to the WT mice (Fig. 5A-B). Together, these data showed that CD226 deficient monocytes had lower ability of migration from peripheral blood.

## CD226 deletion macrophage modulates renal inflammatory statement and attenuates renal firbosis

To study the role of CD226 expression on macrophages in renal injury induced by UUO, we adoptively transferred BMDMs (M0) derived from WT mice or CD226-konckout (KO) mice into WT mice after 24 h of UUO surgery and harvested the renal tissue at day 7 (Fig. 6A). Furthermore, the ratio of UUO kidney/body weight was significantly reduced in the WT BMDM-injected group (Fig. 6B). Renal histopathologic changes (e.g., tissue destruction, proinflammatory cell infiltration and abscess) from the cortex area to the medulla area were assessed by histologic scoring based on the results of histological staining (H&E, Masson staining and immunohistochemistry). We observed that the Masson-positive stained area and histopathologic changes were greatly alleviated in the KO BMDM-injected mice than in the WT mice and WT BMDM-injected mice (Fig. 6C-D). Meanwhile, immunohistochemistry staining analyses showed that positive staining area was largely reduced in KO BMDM-injected group compared to those in WT BMDM-injected group in the fibrotic kidneys (Fig. 6E). Intrarenal expression of profibrogenic and proinflammatory cytokines (*Collagen-I, Collagen-IV, Kim-1, Vimentin, Tgf-β* and *Tnf-α*) was greatly down-regulated in the KO BMDM-injected group (Fig. 6F). Additionally, there was a significant difference changed in concentrations of TNF-α, IL-6 and IFN-γ in serum of the two groups and the mice transferring with KO-BMDMs had lower level of inflammatory cytokines, especially TNF-α which was less than half of the WT-BMDM transferring mice (Fig. 6G). The expression of chemokines of WT-BMDMs and KO-BMDMs was alos examined.

Together, these data show that CD226-knockout macrophage can attenuate renal inflammation and fibrogenesis after tissue injury.

## RNA-seq analysis discovers different transcriptome features of M1 based on CD226 expression

Our *in vivo* data presented in this article suggested an important role of CD226 in macrophage polarization of renal fibrosis after kidney UUO surgery. To explore the cellular basis of the effects of CD226 on macrophage polarization, we cultured bone marrow-derived macrophages (BMDMs) from WT and CD226-deficient mice. Then, we stimulated BMDMs with IFN-γ (20 ng/ml) and LPS (100 ng/ml) for 24 h to induce BMDM activation toward M1. To deeply explore the role of CD226 in M1-like macrophages, we performed RNA-seq analysis to reveal the transcriptome changes in M1-like BMDMs isolated from WT and CD226-deficient mice. RNA-seq revealed high-quality clean reads that were assembled into 16,350 unigenes. DESeq algorithm was performed to filter the differentially expressed genes. Genes displaying differences in expression with a *P* value of < 0.05 and a fold change (FC) cutoff of > 2 were considered DESeq. After removing mRNAs with low signal intensity, pairwise comparisons based on *t*-tests were conducted for gene expression identification between the CD226-deficient M1-like macrophages and controls, which revealed 571 transcripts with significant differences, accounting for 3.49% of the murine transcriptome on the HiSeqXten-based RNA-Seq platform. A heatmap and volcano map show the gene expression levels (Fig. 7A, B).

We further performed gene ontology (GO) analysis to examine the biological implications of differentially expressed genes in CD226-deficient M1-like macrophages *vs.* WT M1-like macrophages. Differentially expressed genes in CD226-deficient M1-like macrophages compared to the control groups were enriched in GO terms; the top 15 categories (*p*<0.0001) were involved in leukocyte chemotaxis, response to lipopolysaccharide and inflammatory response (Fig.7C). In addition, we analyzed the differentially expressed transcription factors of CD226-deficient M1-like macrophages compared to the control groups, accounting for 43.75% of the upregulated transcription factors belonging to the C2H2 zinc finger family (Fig. 7D). Furthermore, upregulated transcription factors associated with C2H2 zinc finger family include 2010315B03Rik, Klf4, Ikzf3, Hivep3, Bcl11a, Plagl1 and Ikzf4 (Fig. 7E, Supplemental Table 4), therein KLF4 plays a crucial role in anti-inflammation and cell differentiation. In brief, the data demonstrated that CD226 influences M1-like macrophage activation mainly by increasing the transcript levels of proteins involved in the response to lipopolysaccharide and C2H2 zinc finger domain binding.

Furthermore, KEGG pathway enrichment analysis revealed that the pathways “Cytokine-cytokine receptor interaction”, “Chemokine signaling pathway”, “PPAR signaling pathway”, “PI3K-Akt signaling pathway” and “TNF signaling pathway” were downregulated in CD226-deficient M1-like macrophages (Supplemental Table 3). These pathways play important roles in the inflammatory response, chemotaxis, inflammatory cytokines, and cell adhesion and differentiation. As deletion of KLF4 stimulates the production of TNF-α in infiltrating myeloid cells, TNF-α expression in macrophages favors their activation to the proinflammatory M1 phenotype[14]. Thus, these data suggested that CD226 deficiency suppressed monocyte migration from peripheral blood and promoted the proinflammatory M1 phenotype *via* KLF4-TNF-α signaling.

## CD226 deletion modulates macrophage activation by promoting KLF4 expression and attenuates renal injury

As krüppel-like factor 4 (KLF4), which is an important transcription factor with zinc-finger domains, is an essential regulator of macrophage polarization that acts by restraining inflammatory signals. We detected KLF4 expression in unactivated macrophages (M0) and M1-like macrophages of CD226-deficient mice and control groups. After treatment with IFN-γ (20 ng/ml) and LPS (100 ng/ml) for 24 h, the mRNA expression of KLF4 was upregulated in unactivated macrophages (M0) and M1-like macrophages of CD226-deficient macrophages, and M1-like CD226-deficient macrophages showed a twofold increase compared with M1-like WT macrophages (Fig. 8A). To evaluate the effect of CD226 on macrophage activation, we harvested the peritoneal macrophages of WT and CD226-deficient mice at day 7 after UUO surgery and assessed KLF4 expression in peritoneal macrophages isolated from WT and CD226-deficient mice by Western blot. Compared with peritoneal macrophages isolated from WT mice, the expression of KLF4 was obviously increased in macrophages isolated from CD226-deficient mice (Fig. 8B). The immunofluorescence staining analysis revealed there was a significant increase of F4/80+KLF4+ cells in the renal tissue of CD226-deficient mice compared to WT mice (Fig. 8C). These results indicated a predominant role of CD226 in macrophage polarization.

To examine whether the difference of macrophage polarization between WT and CD226-deficient macrophage caused by KLF4, we next performed siRNA against mouse *Klf4* (siKLF4), and the mRNA expression of *Klf4* was downregulated in WT peritoneal macrophages induced by siKLF4. Then, we applied siRNA, which had a better ability to silence *Klf4,* to peritoneal macrophages isolated from WT and CD226-deficient mice. Western blot results showed that KLF4 was markedly downregulated when macrophages were treated with siKLF4 (Fig. 8D). Furthermore, we stimulated WT and CD226-deficient peritoneal macrophages with IFN-γ (20 ng/ml) and LPS (100 ng/ml) for 12 h after treatment with siRNA. M1 marker genes involving *iNOS* was obviously downregulated in CD226-deficient mice, whereas the trend disappeared after siKLF4, and even the mRNA expression of *Tnf-α* was strongly heightened in CD226-deficient macrophages (Fig. 8E, F). Thus, these data demonstrated that CD226 deficiency could significantly alleviate renal fibrosis by promoting KLF4 expression in macrophages to modulate macrophage activation.

Together, these data show that, CD226 expression on macrophage aggravates renal fibrogenesis by influencing KLF4 expression to regulate macrophage activation. Therefore, CD226-deficient macrophages can attenuate renal inflamation and fibrogenesis after tissue injury.

1. **Discussion**

Although it is well known that myeloid cell infiltration contributes to both injurious and reparative functions in CKD, more importantly, the processes governing macrophages with proinflammatory and anti-inflammatory functions are very complex in the damaged kidney[22, 23]. The effects of CD226 on the AKI-CKD transition and inflammation are not well studied. Our study provides the evidence that CD226 is required in the development of renal injury, as CD226-deficient mice have markedly reduced proinflammatory myeloid cells, accompanied by less ECM deposition and better recovery of renal function, thus supporting a pathogenic role for CD226-positive myeloid cells in CKD. Overall, these findings reveal a pathogenic role of CD226 in the progression of renal fibrosis, suggesting a novel mechanism of CD226 involving in the profibrotic effects, videlicet, the promotion of leukocyte migration and accumulation of ECM.

An important findings from our study is that CD226 could enhance renal inflammation and renal interstitial fibrosis through macrophage polarization. Also, the proinflammatory and profibrotic molecules secreted by proinflammatory macrophages lead to ECM accumulation in the real interstitium. Then we focus on how CD226 contributes to renal chronic inflammation and renal interstitial fibrosis.

Less is known about the role of CD226 in CKD. CD226, named DNAX accessory molecule-1 (DNAM-1), is expressed in most of T cells, monocytes/macrophages and other immune cells and it has two ligands known as CD155 (PVR or poliovirus receptor) and CD112 (Nectin-2 or PVRL2) [21, 24-26]. Previous studies have demonstrated that CD226 plays a vital role in tumor diseases by mediating the cytotoxic and immune functions of T cells and NK cells[27, 28]. Recent studies have found that CD226 is involved in renal fibrosis[29]. To investigate the cellular mechanisms and underlying pathogenic role of CD226 in CKD, we first performed *in vivo* experiments. We found that CD226-positive macrophages (CD45+F4/80+) were greatly upregulated in renal cell suspensions under UUO conditions compared with that in the sham group, indicating that the expression of CD226 in macrophages has a close relationship with the development of CKD. Moreover, we performed a series of observations at different time points. The substantial evidences have been found to support a pathogenic role of CD226 during CKD. First, CD226-deficient mice exhibited a significant reduction in collagen deposition in the tubular interstitium after UUO accompanied by less profibrogenic and proinflammatory cytokines in renal chronic tissue, suggesting that CD226 has a direct effect on the kidney inflammatory state. As CD226 is an adhesion molecule and closely associated with the movement of leukocytes across the endothelium, our data implied that the performance of CD226 in CKD depends on its impact on inflammatory cell [infiltration](file:///C:/Users/Administrator/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;), especially macrophages. Second, we evaluated renal function at days 3, 7 and 14 by measuring the BUN level in serum and found that there were significantly lower BUN levels in CD226-deficient mice, indicating that CD226 expression exacerbates impairment of renal function. Therefore, these experiments elucidate a mechanism by which CD226 expression on leukocytes aggravates CKD inflammation and provides direct evidence that CD226 deficiency mice alleviates renal fibrosis with the fewer inflammatory cells [infiltration](file:///C:/Users/Administrator/AppData/Local/youdao/dict/Application/8.9.3.0/resultui/html/index.html#/javascript:;) after kidney injury.

* Our recent work in wound repair of a murine myocardial infarction (MI) model showed that CD226 deletion improved postinfarct healing and cardiac function. It was proposed that CD226 expression diminished macrophage activation toward M2 and increased M1 in the infarcted location after MI [30]. Macrophage differentiation from monocytes into tissues with physiological functions, such as secretion of inflammatory cytokines, antigen processing and presentation, proangiogenesis, and patrolling behavior, depends on the tissue microenvironment conditions[31]. The process is quite complicated and controversial. Macrophages also play a crucial role in wound healing by orchestrating proinflammatory and tissue repair in CKD[32, 33]. On the one hand, proinflammatory monocytes/M1 are thought to mediate tubular damage through upregulating the level of proinflammatory cytokines (*e.g.*, IL-1, IL-6, IL-12, and TNF-α) and oxidative metabolites (*e.g.*, nitric oxide). On the other hand, M2 macrophages (also termed alternatively activated) with highly expressed genes such as insulin-like growth factor-1 (Igf1), mannose receptor 1 (Mrc1, which encodes CD206), and arginase-1 (Arg1) tend to promote wound healing and contribute to the resolution of inflammation[34]. In the study, flow cytometry and immunohistochemical staining data confirmed that CD226-deficient mice had significantly smaller numbers of CD45+ (leukocytes), F4/80+ cells (macrophages), and Ly6G+ cells (neutrophils) infiltrating the kidney after UUO. Moreover, CD226 deletion restrained inflammatory monocyte mobilization from peripheral blood after UUO. Our findings are consistent with a recent analysis that confirmed that macrophages express proinflammatory genes in the kidney early after reperfusion (mainly CD11b+/Ly6Chigh cells), whereas macrophages within the kidney during the tubular repair phase (CD11b+/Ly6Cint cells) express more wound healing markers. Macrophage activation toward proinflammatory M1 aggrevates tubular cell death and renal fibrosis through TNF-α production[35]. Suppressing the proinflammatory M1 by activating type I angiotensin receptors ameliorates epithelial cell damage and kidney fibrosis after UUO[36]. Moreover, we observed that the influx of macrophages with fewer M1 infiltrating and lower level of inflammatory cytokines in CD226-deficient mouse kidney after UUO. Thus, it is plausible that inhibiting macrophage activation toward M1 could attenuate tissue inflammation, excessive accumulation of extracellular matrix and interstitial fibrosis[[42](#_ENREF_42),[43](#_ENREF_43)].

Regarding the relationship between macrophage activation and kidney function changes from our findings, we assessed RNA-seq analysis of the changes in the transcriptome profile of WT-M1 and CD226-deficient M1. RNA-seq analysis suggested that CD226 deficiency leads to significant decreases in leukocyte chemotaxis, cell adhesion, and the ability to respond to lipopolysaccharide. The transcription factor data revealed that the zf-C2H2 family was significantly increased after CD226 deletion in macrophages. KLF4 is an evolutionarily conserved zinc (Zn) finger-containing transcription factor of the C2H2 type that is also a critical regulator of monocyte differentiation and macrophage activation[37]. Zhang *et al* revealed that high level of KLF4 strongly suppress cerebrovascular endothelial inflammatory reactions in the acute ischemic stroke patients[38]. Conversely, the downregulation of KLF4 expression on macrophage induces TLR4/NF-κB signaling activation after palmitic acid treatment and prmotes the level of inflammatory cytokines, which serves a proinflammatory role[39]. In this light, KLF4 expression can reduce the degree of inflammation by orchestrating macrophage differenation and function. Here, we observed that KLF4 expression was upregulated in unactivated macrophages (M0) and M1-like macrophages with CD226 deficiency. Furthermore, accumulating evidence has demonstrated that CD226 expression promotes inflammatory monocyte infiltration into the kidney and induces their activation toward M1-like macrophages. To determine the underlying mechanism inducing macrophage activation, we downregulated KLF4 expression with siRNA. Then, RT-qPCR was performed to analyze macrophage activation after KLF4 silencing in WT and CD226-deficient macrophages. The trend in which M1 marker gene expression involving iNOS and TNF-α was obviously reduced in CD226-deficient macrophages disappeared, and the mRNA expression of iNOS was even strongly heightened after KLF4 silencing in CD226-deficient macrophages. Thus, KLF4 expression was accelerated by CD226 deficiency and downregulated KLF4 largely reversed the effect of CD226 deficiency on macrophage activation. In addition, the further experiments demonstrated that M1 macrophage inflammatory response was attenuated through KLF4 and it discovered a novel mechanism of inflammation resolution for maintaining homeostasis[40]. Taken together, these results suggest that KLF4 is an important regulator of macrophage activation in chronic inflammatory diseases. Thus, it is conceivable that CD226 expression suppressed M2 but increased M1 targeting KLF4 expression.

In conclusion, a key observation from our study is that renal interstitial fibrosis is dependent on CD226. we propose that CD226 contributes to tubulointerstitial fibrosis on the basis of our findings through three pathways: CD226 expression i) aggravates proinflammatory leukocyte infiltration, ii) suppresses KLF4 expression and induces proinflammatory M1 phenotype macrophages, and iii) elicits inflammatory cytokine production and increases ECM deposition and fibrogenesis in the kidney. We first demonstrate a pathogenic role of CD226 in the development of kidney fibrosis and uncover novel cellular and molecular mechanisms of CD226 that contribute to proinflammatory leukocyte infiltration into kidney tissue. Furthermore, these findings, together with new avenues of CD226 in modulating macrophage polarization in real fibrosis, support a potential therapeutic target in renal interstitial fibrosis.

# Materials and methods

## Animal Model and Adoptive Transfer Assay

CD226 knockout (*Cd226*-/- or KO) mice on a C57BL/6 background were gifted by Professor Marco Colonna (Washington University), and wild-type (WT) mice on a C57BL/6 background were purchased from Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). All mice were fed under specific pathogen-free (SPF) conditions at the animal center of the Fourth Military Medical University and were used at 6-8 weeks old. All animal experiments were performed under protocols approved by the Animal Welfare and Ethics Committee at The Fourth Military Medical University. Some of the WT mice treated with clodronate liposome to eliminate macrophages before BMDMs transferring[41].

The mice were randomly divided into three groups in the experiment (n=6) as follows:

(i) WT C57BL/6 mice aged 6–8 weeks underwent an operation of the unilateral ureteral obstruction (UUO) which the left ureter was exposed to tie off it at the level of the lower pole using suture threads, and the right kidneys were the corresponding sham group in each group.

(ii) WT UUO group + BMDMS derived from WT mice. In this group, 1×106 macrophages suspended in 100 µl of PBS were infused back intravenously into each one just 24 h after the UUO operation was performed.

(iii) WT UUO group + BMDMS derived from *Cd226*-/- mice. In this group, 1×106 macrophages suspended in 100 µl of PBS were infused back intravenously into each one just 24 h after the UUO operation was performed.

## Real Time Quantitative PCR (RT-qPCR)

RNA was extracted from renal tissue in TRIzol (Life Technologies, USA) according to the manufacturer’s recommendation. cDNA was synthesized with Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus) (Yeasen, China), and RT-qPCR was performed using SYBR PremixExTaqTM II (TaKaRa, Japan) according to the manufacturer’s instruction on a Step One Real-time PCR instrument (CFX96, BioRad). The relative gene expression was analyzed using the 2–ΔΔCt method with normalization to 18s and controls[42]. The primers used in the study are shown in Supplemental Table 1.

## Assessment of Inflammatory Cells in Blood and Infiltration in the Kidney

Single cells from peripheral blood were harvested, and red blood cells were removed with red cell lysis buffer. And resuspended in PBS supplemented with 2% FCS. The cell pellets were preincubated with FcR blocking antibody (CD16/32) and stained with antibodies specific for CD11b (PE/Cy7 anti-mouse CD11b, BioLegend, USA), Ly6C (FITC anti-mouse Ly6C, BioLegend, USA), Ly6G (PE anti-mouse Ly6G, BioLegend, USA) or the relevant isotype antibodies at 4°C for 20 min.

To determine the infiltration of macrophages and neutrophils in kidney sections, kidneys were weighed, cutted into samll pieces and incubated with collagenase D (1 mg/ml) for 10 min at 37°C with gentle stirring. Then the digested kidney mixture was treated with DMEM-F12 containing 10% FCS and passed through a 40 μm nylon sieve to collect cell pellets. Single cells were harvested and removed RBCs with red cell lysis buffer to in the same way with we treated peripheral blood. After that, the cells were washed with PBS containing 2% FCS for three times, preincubated with FcR blocking antibody (CD16/32) and then stained with antibodies including CD45 (PE anti-mouse CD45, BioLegend, USA), F4/80 (PE/Cy7 anti-mouse F4/80, BioLegend, USA) and CD226 (APC anti-mouse CD226, BioLegend, USA) or the appropriate isotype control antibodies. All data were acquired by a Novocyte flow cytometer (ACEA) and analyzed with NovoExpress software.

## Immunofluorescent and Immunohistochemical Staining

Renal sections from WT and *Cd226-/-* mice were removed post-UUO surgery on days 3, 7 and 14. Paraffin-embedded renal sections (4 μm thick) were deparaffinized, rehydrated. Then they were soaked in citrate buffer and heated by microwave for antigen retrieval. For immunofluorescence, kidney sections were then blocked with 1% BSA for 30 min and incubated with the following antibodies: anti-F4/80 (D2S9R, Cell Signaling, USA), anti-iNOS (Proteintech, USA), and anti-CD206 (Proteintech, USA) at 4°C overnight. The next day, after washing with PBS three times, tissues were stained with fluorescein-labeled secondary antibody for 1 h followed by DAPI (staining nuclei) at room temperature. The slides were viewed using an OLYMPUS inverted microscope. The assessment of the M1 to M2 macrophage ratio was monitored by counting the number of double-stained cells in the medulla or the cortex in 5-6 viewing fields. For immunohistochemistry, renal sections were soaked in 3% H2O2-methanol and blocking reagent for 10 min each. Subsequently, the following antibodies were used to stain the tissues at 4°C overnight: anti-CD45 (D9M8I, Cell Signaling, USA), anti-F4/80 (D2S9R, Cell Signaling, USA), and anti-Ly6G (P35461, Servicebio, China). The next day, after washing with PBS, the sections were incubated with anti-HRP-conjugated anti-rabbit antibody for 40 min at room temperature. The slides were visualized with a DAB detection kit and counterstained with hematoxylin to determine leukocyte infiltration after renal UUO.

## Masson Trichrome Staining

Reagents from Servicebio were used for Masson trichrome staining to determine collagen deposition in renal tissues according to the manufacturer’s protocol. Masson staining images are acquired by Pannoramic MIDI. We used ImageJ Software (ImageJ 1.41) to quantify and analyze the images, and the percentage of fibrotic area was showed by the ratio of blue-stained area to the area of the entire field.

## Transfection Assay

Negative control and KLF4 siRNA were transiently transfected using RNAFit according to the manufacturer’s instructions. The negative control and KLF4 siRNA were synthesized by HanBio Biotechnology. The sequences used in the experiment are shown in Supplemental Table 2.

## RNA-Seq

Total RNA was extracted from primary cultured M1-like macrophages from CD226 knockout (*Cd226-/-*) mice (n=3) and WT control mice (n=3) by TRIzol reagent (Life Technologies, USA) according to the manufacturer’s recommendation. RNA was qualified and quantified using an Agilent 2200 bioanalyzer (Santa Clara, CA, USA). The tagged cDNA libraries were pooled in equal ratios and used for 150 bp paired-end sequencing in a single lane of the Illumina HiSeqXTen system. Htseq was used to examine gene and lncRNA counts, and the RPKM method was used to detect gene expression[43]. We applied the EBSeq algorithm to filter the differentially expressed genes after significance analysis[44], *P* value calculations and FDR analysis with the following criteriafor mRNA: i) fold change>2 or <0.5; ii) FDR<0.05 [45].

## Statistical analysis

All data were analyzed with GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) and are shown as the mean ± SEM. The unpaired student’s *t-*test or one-way analysis of variance (ANOVA) was used according to the type of experiments. A *P* value < 0.05 was defined as statistically significant.

**References**

[1] Jha V, Garcia-Garcia G, Iseki K, et al. Chronic kidney disease: global dimension and perspectives[J]. Lancet. 2013, 382(9888): 260-272.

[2] Zeisberg M, Neilson E G. Mechanisms of tubulointerstitial fibrosis[J]. J Am Soc Nephrol. 2010, 21(11): 1819-1834.

[3] Webster A C, Nagler E V, Morton R L, et al. Chronic Kidney Disease[J]. Lancet. 2017, 389(10075): 1238-1252.

[4] Liu Y. Cellular and molecular mechanisms of renal fibrosis[J]. Nat Rev Nephrol. 2011, 7(12): 684-696.

[5] Lee S A, Noel S, Sadasivam M, et al. Role of Immune Cells in Acute Kidney Injury and Repair[J]. Nephron. 2017, 137(4): 282-286.

[6] Guiteras R, Sola A, Flaquer M, et al. Macrophage Overexpressing NGAL Ameliorated Kidney Fibrosis in the UUO Mice Model[J]. Cell Physiol Biochem. 2017, 42(5): 1945-1960.

[7] Wang Y, Wang Y, Cao Q, et al. By homing to the kidney, activated macrophages potently exacerbate renal injury[J]. Am J Pathol. 2008, 172(6): 1491-1499.

[8] Cao Q, Harris D C, Wang Y. Macrophages in kidney injury, inflammation, and fibrosis[J]. Physiology (Bethesda). 2015, 30(3): 183-194.

[9] Tang P M, Nikolic-Paterson D J, Lan H Y. Macrophages: versatile players in renal inflammation and fibrosis[J]. Nat Rev Nephrol. 2019, 15(3): 144-158.

[10] Huang Z, Qi G, Miller J S, et al. CD226: An Emerging Role in Immunologic Diseases[J]. Front Cell Dev Biol. 2020, 8: 564.

[11] Hesketh M, Sahin K B, West Z E, et al. Macrophage Phenotypes Regulate Scar Formation and Chronic Wound Healing[J]. Int J Mol Sci. 2017, 18(7).

[12] Sindrilaru A, Peters T, Wieschalka S, et al. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice[J]. J Clin Invest. 2011, 121(3): 985-997.

[13] Liao X, Sharma N, Kapadia F, et al. Kruppel-like factor 4 regulates macrophage polarization[J]. J Clin Invest. 2011, 121(7): 2736-2749.

[14] Wen Y, Lu X, Ren J, et al. KLF4 in Macrophages Attenuates TNFalpha-Mediated Kidney Injury and Fibrosis[J]. J Am Soc Nephrol. 2019, 30(10): 1925-1938.

[15] Rane M J, Zhao Y, Cai L. Krupsilonppel-like factors (KLFs) in renal physiology and disease[J]. EBioMedicine. 2019, 40: 743-750.

[16] Jiang F, Chen Q, Wang W, et al. Hepatocyte-derived extracellular vesicles promote endothelial inflammation and atherogenesis via microRNA-1[J]. J Hepatol. 2020, 72(1): 156-166.

[17] Zhu Y, Wang P, Zhang L, et al. Superhero Rictor promotes cellular differentiation of mouse embryonic stem cells[J]. Cell Death Differ. 2019, 26(5): 958-968.

[18] Wynn T A, Vannella K M. Macrophages in Tissue Repair, Regeneration, and Fibrosis[J]. Immunity. 2016, 44(3): 450-462.

[19] Li J, Zhao F, Qin Q, et al. The Effect of CD226 on the Balance between Inflammatory Monocytes and Small Peritoneal Macrophages in Mouse Ulcerative Colitis[J]. Immunol Invest. 2022, 51(6): 1833-1842.

[20] Kim K W, Williams J W, Wang Y T, et al. MHC II+ resident peritoneal and pleural macrophages rely on IRF4 for development from circulating monocytes[J]. J Exp Med. 2016, 213(10): 1951-1959.

[21] Reymond N, Imbert A M, Devilard E, et al. DNAM-1 and PVR regulate monocyte migration through endothelial junctions[J]. J Exp Med. 2004, 199(10): 1331-1341.

[22] Oishi Y, Manabe I. Macrophages in inflammation, repair and regeneration[J]. Int Immunol. 2018, 30(11): 511-528.

[23] Andrade-Oliveira V, Foresto-Neto O, Watanabe I, et al. Inflammation in Renal Diseases: New and Old Players[J]. Front Pharmacol. 2019, 10: 1192.

[24] Kojima H, Kanada H, Shimizu S, et al. CD226 mediates platelet and megakaryocytic cell adhesion to vascular endothelial cells[J]. J Biol Chem. 2003, 278(38): 36748-36753.

[25] Shibuya K, Shirakawa J, Kameyama T, et al. CD226 (DNAM-1) is involved in lymphocyte function-associated antigen 1 costimulatory signal for naive T cell differentiation and proliferation[J]. J Exp Med. 2003, 198(12): 1829-1839.

[26] Ma D, Sun Y, Lin D, et al. CD226 is expressed on the megakaryocytic lineage from hematopoietic stem cells/progenitor cells and involved in its polyploidization[J]. Eur J Haematol. 2005, 74(3): 228-240.

[27] Shibuya A, Campbell D, Hannum C, et al. DNAM-1, a novel adhesion molecule involved in the cytolytic function of T lymphocytes[J]. Immunity. 1996, 4(6): 573-581.

[28] Blake S J, Dougall W C, Miles J J, et al. Molecular Pathways: Targeting CD96 and TIGIT for Cancer Immunotherapy[J]. Clin Cancer Res. 2016, 22(21): 5183-5188.

[29] Mu Y, Zhang J, Liu Y, et al. CD226 deficiency on regulatory T cells aggravates renal fibrosis via up-regulation of Th2 cytokines through miR-340[J]. J Leukoc Biol. 2020, 107(4): 573-587.

[30] Li J, Song Y, Jin J Y, et al. CD226 deletion improves post-infarction healing via modulating macrophage polarization in mice[J]. Theranostics. 2020, 10(5): 2422-2435.

[31] Mosser D M, Hamidzadeh K, Goncalves R. Macrophages and the maintenance of homeostasis[J]. Cell Mol Immunol. 2021, 18(3): 579-587.

[32] Kumar S. Cellular and molecular pathways of renal repair after acute kidney injury[J]. Kidney Int. 2018, 93(1): 27-40.

[33] Chen T, Cao Q, Wang Y, et al. M2 macrophages in kidney disease: biology, therapies, and perspectives[J]. Kidney Int. 2019, 95(4): 760-773.

[34] Martinez F O, Helming L, Gordon S. Alternative activation of macrophages: an immunologic functional perspective[J]. Annu Rev Immunol. 2009, 27: 451-483.

[35] Liang Y, Sun X, Wang M, et al. PP2Acalpha promotes macrophage accumulation and activation to exacerbate tubular cell death and kidney fibrosis through activating Rap1 and TNFalpha production[J]. Cell Death Differ. 2021, 28(9): 2728-2744.

[36] Zhang J D, Patel M B, Griffiths R, et al. Type 1 angiotensin receptors on macrophages ameliorate IL-1 receptor-mediated kidney fibrosis[J]. J Clin Invest. 2014, 124(5): 2198-2203.

[37] Ghaleb A M, Yang V W. Kruppel-like factor 4 (KLF4): What we currently know[J]. Gene. 2017, 611: 27-37.

[38] Zhang X, Wang L, Han Z, et al. KLF4 alleviates cerebral vascular injury by ameliorating vascular endothelial inflammation and regulating tight junction protein expression following ischemic stroke[J]. J Neuroinflammation. 2020, 17(1): 107.

[39] Li J, Mao Y S, Chen F, et al. Palmitic acid up regulates Gal-3 and induces insulin resistance in macrophages by mediating the balance between KLF4 and NF-kappaB[J]. Exp Ther Med. 2021, 22(3): 1028.

[40] Wang X, Li H, Chen S, et al. P300/CBP-associated factor (PCAF) attenuated M1 macrophage inflammatory responses possibly through KLF2 and KLF4[J]. Immunol Cell Biol. 2021, 99(7): 724-736.

[41] Kozicky L K, Sly L M. Depletion and Reconstitution of Macrophages in Mice[J]. Methods Mol Biol. 2019, 1960: 101-112.

[42] Livak K J, Schmittgen T D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method[J]. Methods. 2001, 25(4): 402-408.

[43] Anders S, Pyl P T, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data[J]. Bioinformatics. 2015, 31(2): 166-169.

[44] Anders S, Huber W. Differential expression analysis for sequence count data[J]. Genome Biol. 2010, 11(10): R106.

[45] Benjamini Y, Drai D, Elmer G, et al. Controlling the false discovery rate in behavior genetics research[J]. Behav Brain Res. 2001, 125(1-2): 279-284.

# Acknowledgments

We were grateful to Dr. Jing Yi (Department of Transfusion Medicine, Xijing Hospital) for her help in immunohistochemistry staining.

# Financials

This study was supported by the National Natural Science Foundation of China (82071848) and the Fourth Military Medical University projects (No. 2018HKPY02).

# Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# Data Availability Statement

The data used or analyzed are available from the corresponding author on reasonable request. All data generated or analyzed during this study are available in the article or the supplementary file. The datasets of RNA-seq analyzed during the current study have been deposited in the Gene Exoression Omnibus (GEO) database. The Accession number is GSE181758.

**Figure 1. CD226-positive macrophage accumulation in kidney during renal fibrosis.**

(A) Representative images of Masson staining on the kidney section of WT mice at day 0, 3, 7, and 14 post-UUO, taken at the medullary section. Scale bars, 50 μm. Quantification of Masson staining areas corresponding to the kidney sections of WT mice in the left panels. (B, C) Stepwise gating strategy used in flow cytometry assessment of positive cells of CD45+ and CD45+F4/80+ from WT mice kidney sections after UUO surgery. (D) Representative flow cytometry graphs showing CD226 expression levels and quantification of CD226 positive F4/80+macrophages of kidney tissue at day 3, 7 and 14 after UUO surgery. Data are representative of three independent experiments (n=3-5 mice per group). (E) Representative immunofluorescence images of F4/80+ (green) and CD226+ (red) macrophages and quantitative analysis of F4/80+ CD226+macrophages in WT mice at day 0, 3, 7, and 14 after UUO surgery. DAPI was used for staining nuclear (blue). Scale bars, 15 μm (top). Scale bars, 30 μm (bottom). Error bars indicate SEM. Data were analyzed by one-way ANOVA or two-way ANOVA. *\*P<0.05; \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001.*

**Figure 2. CD226 deficiency alleviates collagen deposition and fibrogenesis in kidney during renal fibrosis.**

(A) Representative images of Masson staining on kidney of WT and *Cd226-/-* mice at day 3, 7, and 14 post-UUO, taken at the medullary section. Scale bars, 2000 μm (top). Scale bars, 100 μm (bottom). Arrows indicate postive staining of collagen fibrils. (B) Quantification of masson stained areas corresponding to the kidney sections of WT and Cd226*-/-* mice in the left panels. (C) Relative mRNA levels of profibrogenic: *Collagen-I, Collagen-IV, Pdfg* and *Vimentin* in the kidney of WT-sham, *Cd226-/-*-sham, WT-UUO and *Cd226-/-*-UUO at day 3, 7 and 14, determined by RT-qPCR. (D) The change of kidney to body weight ratio monitored in WT and *Cd226-/-* mice at day 3, 7 and 14 after UUO surgery. (E) Blood urea nitrogen (BUN) levels in WT and *Cd226-/-* mice at day 3, 7 and 14 post-UUO. All data are representative of three independent experiments. Data were analyzed by the unpaired, two-tailed *t* test (n=3–5 viewing fields from 4 mice/group). \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.0001.

**Figure 3. Deficiency of CD226 reverses renal tissue inflammation during renal fibrosis.**

(A) Gross observation of kidneys of WT and *Cd226-/-* mice at day 7 after UUO surgery. (B) Immunochemistry staining of CD45 and F4/80 in renal tissues from WT and *Cd226-/-* mice at day 7 post-UUO. Scale bars, 25 μm (top). Scale bars, 100 μm (bottom). (C) Quantification of immunochemistry staining areas corresponding to renal tissues of WT and *Cd226-/-* mice (B). (D) Representative flow cytometry histograms quantification of CD45+ and CD45+F4/80+ cells of kidney tissue at day 7 after UUO surgery. (E) ELISA analysis of TNF-α, IL-6 and IFN-γ in the serum of WT and *Cd226-/-*mice at day 7 after UUO surgery. (F) RT-qPCR assay of proinflammatory cytokines: *Il-6* and *Tgf-β* as described in kidney WT and *Cd226-/-* mice at day 3, 7 and 14 post-UUO. All data are representative of three independent experiments (n=3-4 mice/group). Data were analyzed by one-way ANOVA. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P* <0.0001.

**Figure 4. CD226 expression promotes M1 infiltration during renal fibrosis.**

(A, B) Representative immunofluorescence images of F4/80+ (red) and iNOS+ (green) macrophages and quantitative analysis of iNOS+M1 macrophages in the medulla or cortex area of WT and *Cd226-/-* UUO mice at day 3, 7 and 14. DAPI was used for nuclear staining (blue). Scale bars, 30 μm. (C, D) Representative immunofluorescence images of F4/80+ (red) and CD206+ (green) macrophages and quantitative analysis of F4/80+ CD206+ M2 macrophages in the medulla or cortex area of WT and *Cd226-/-* UUO mice at day 3, 7 and 14. Scale bars, 30 μm. All data are representative of three independent experiments (n=5 viewing fields from 3 mice/group). Data were analyzed by the unpaired, two-tailed *t* test. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P* <0.0001 vs. corresponding WT group.

**Figure 5. CD226 expression promotes renal leukocytes infiltration during renal fibrosis.**

(A) Representative flow cytometry graphs of inflammatory monocytes (CD11b+ Ly6Chi) in peripheral blood of WT and *Cd226-/-* mice at day 3, 7 and 14 after UUO. (B) Cumulative results calculating positive cells of inflammatory monocytes (CD11b+ Ly6Chi) in peripheral blood from WT mice at day 3, 7 and 14 after UUO. Data were repeated three times (n=3 mice/group) and analyzed by the unpaired, two-tailed *t* test. \**P*<0.05 vs. corresponding WT group.

**Figure 6. CD226 deficient BMDM ameliorates renal tissue injury and fibrosis.**

(A) Schematic of BMDM transfers from WT or *Cd226-/-* mice to WT-UUO mice. (B) Changes of kidney to body weight ratio monitored in WT BMDM-WT or KO BMDM-WT mice. (C, D) Representative images of Masson (C) and H&E (D) staining on kidney sections of WT mice injected with WT-BMDM or KO-BMDM after 24 h of UUO surgery, taken at kidney section at day 7. Scale bars, 2000 μm (top). Scale bars, 200 μm (bottom). Arrows indicate positive-stained areas or histopathologic changes (i.e., tissue destruction, proinflammatory cell infiltration, and tubule destruction). Histological scores of kidney sections of WT mice injected with WT-BMDM or KO-BMDM. (E) Immunochemistry staining of vimentin on renal tissues of WT mice injected with WT-BMDM or KO-BMDM after 24 h of UUO surgery, taken at kidney section at day 7. Scale bars, 50 μm. (F) Relative mRNA levels of profibrogenic and proinflammatory cytokines: *Collagen-I, Collagen-IV, Kim-1, Vimentin, Tgf-β* and *Tnf-α* in the kidney of WT-UUO mice treated by BMDM from WT or *Cd226-/-*mice determined by RT-qPCR. (G) ELISA analysis of TNF-α, IL-6 and IFN-γ in the serum of WT mice injected with WT-BMDM or KO-BMDM. Data were repeated three times and analyzed by the unpaired, two-tailed *t* test (n=3-4 mice/group). *\*P<0.05; \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001.*

**Figure 7.RNA-Seq analysis of global transcriptome changes in macrophages based on CD226 expression.**

(A) Heat map of more than 15,000 annotated genes expression in macrophages based on CD226 expression. The up-regulated mRNA expression in WT-M1 group with respect to *Cd226-/-* -M1 was represented by red color and down-regulated mRNA expression was presented by green color. The scale of color intensity was positively correlated to the fold change. (B) The volcano plot of *Cd226-/-* -M1 versus WT-M1. The red dots indicate genes with a fold change of higher than mean plus one standard deviation or smaller than mean minus one standard deviation and a local false discovery rate (LFDR) of < 0.05. (C) The bar chart of significantly decreased GO Cellular Component (CC) terms in the *Cd226-/-*-M1 versus WT-M1 datasets. (D) The bar chart shows transcription factors family with significantly enriched of *Cd226-/-*-M1 versus WT-M1. (E) Significant upregulated log2 fold changes of zf-C2H2 family are displayed. n=3 mice per group.

**Figure 8. Knockdown of KLF4 recovers macrophage polarization into M1 in *Cd226-/-* macrophage**

(A) Relative mRNA levels of KLF4 of WT-M0, *Cd226-/-*-M0, WT-M1, *Cd226-/-*-M1, determined by RT-qPCR of WT and *Cd226-/-* mice. (B) The expression of KLF4 in peritoneal macrophages of WT and *Cd226-/-* mice were examined by Western blot at day 7 after UUO surgery. (C) Representative immunofluorescence photographs of F4/80+ (green) and KLF4+ (red) macrophages and quantitative analysis KLF4 expression of macrophages in WT and *Cd226-/-* mice at day 7 after UUO surgery. DAPI was used for nuclear staining (blue). Scale bars, 100 μm. (D) The KLF4 expression in macrophages was examined by Western blot. Macrophages derived from WT or *Cd226-/-* mice were transfected with KLF4 vector or KLF4-siRNA. (E, F) The quantification of expression of *iNOS* and *Tnf-α* in WT or *Cd226-/-* mice before and after using KLF4-siRNA was analyzed by RT-qPCR. All the experiments were repeated three times (n=3 mice/group). Data were analyzed by the unpaired, two-tailed *t* test or one-way ANOVA. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001; \*\*\*\**P*<0.0001.