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ORIGINAL RESEARCH

Succinate Facilitates CD4$^+$ T Cell Infiltration and CCL1 Production to Promote Myofibroblast Activation and Renal Fibrosis in UUO Mice

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Aim: Obstructive nephropathy is a leading cause of kidney injury and fibrosis, which is always associated with metabolic aberrations and chronic inflammation. Succinate is an important intermediate metabolite involved in inflammatory responses and various diseases. However, the precise pathogenic mechanisms of succinate in obstructive nephropathy remain to be elucidated.

Methods: Succinate was supplemented in the drinking water to study its impact on the pathogenesis of obstructive nephropathy induced by unilateral ureteral obstruction (UUO) in mice. Kidney fibrosis, injury, inflammatory cytokines, and infiltrated immune cells were analyzed. Transcriptome analysis and in vitro studies were performed to study the cellular and molecular mechanisms by which succinate regulates CD4$^+$ T cells and renal fibrosis.

Results: Kidney proteomics revealed that the tricarboxylic acid (TCA) cycle and mitochondrial dysfunction were the hallmarks of obstructive nephropathy. Succinate was significantly accumulated in the obstructed kidneys. Succinate supplementation promoted UUO-induced renal fibrosis, injury, and inflammation. Moreover, succinate facilitated renal infiltration of CD4$^+$ T cells by upregulating the T-cell chemokines CXCL9 and CXCL10. Transcriptome analysis suggested that succinate promoted CD4$^+$ T cell activation and induced the production of CCL1, which mediated the transition of fibroblasts to myofibroblasts through the ERK signaling pathway. Recombinant CCL1 treatment promoted UUO-induced renal fibrosis and inflammation.

Conclusion: Our study uncovers the important role of succinate in mediating T-cell response that orchestrates the pathogenesis of obstructive nephropathy. Targeting succinate accumulation may be a therapeutic strategy for the treatment of obstructive nephropathy.

Keywords: obstructive nephropathy, renal fibrosis, succinate, CD4$^+$ T cell, CCL1

Introduction

Obstructive nephropathy, a prominent cause of renal injury and fibrosis in pediatric patients, often stems from ureteropelvic junction obstruction (UPJO).[1–3] UPJO-induced hydronephrosis elevates intrapelvic and intratubular hydrostatic pressure, leading to compromised blood supply, hypoxia, tubular injury, and persistent renal inflammation.[4,5] These cascading effects contribute to myofibroblast activation, aberrant extracellular matrix accumulation, and interstitial fibrosis.[4] Nonetheless, the precise cellular and molecular pathways driving these processes remain largely elusive.

Succinate is an important tricarboxylic acid (TCA) cycle metabolite catalyzed from succinyl CoA, which is oxidized to fumarate by succinate dehydrogenase (SDH).[6] However, in hypoxic microenvironments like obstructive nephropathy, SDH can operate in reverse and reduce fumarate to succinate, and SDH inhibition decreases succinate abundance.[7,8] Mounting evidence has suggested that succinate plays crucial roles in metabolism, mitochondrial homeostasis, inflammation, signal transduction, and redox balance.[9,10] Importantly, studies have reported that succinate is associated with the pathogenesis of kidney diseases. In acute

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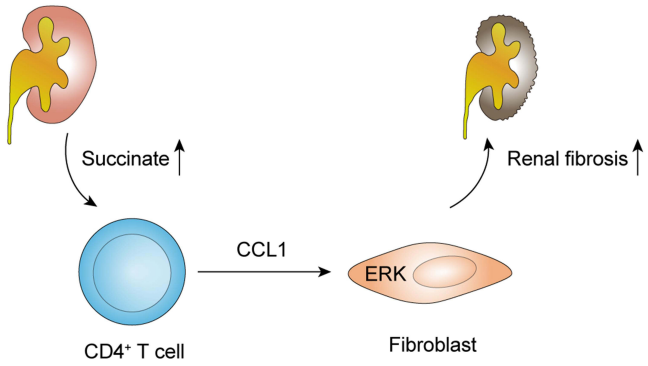
Graphical Abstract



kidney injury (AKI) induced by ischemia and reperfusion, succinate significantly accumulates and promotes tissue injury by damaging mitochondrial function and regulating inflammation.[11] Succinate accumulation also drives ischemia and reperfusion injury in kidney transplantation.[12] Dimethyl malonate (DMM)-mediated SDH inhibition decreases succinate content and preserves renal and mitochondrial functions in ischemic AKI.[13,14] Moreover, urinary succinate is identified as an independent indicator of diabetic kidney disease and albuminuria in patients,[15] which is associated with succinate-mediated oxidative stress.[16] Furthermore, succinate facilitates the infiltration and activation of M2 macrophages during renal interstitial fibrosis.[17] However, whether and how succinate regulates T cells during renal fibrosis remains to be elucidated.

Chemokines are a class of cytokines regulating the infiltration of immune cells into inflamed tissue. Chemokine (C-C motif) ligand 1 (CCL1), which always signals through the receptor CCR8, participates in various inflammatory diseases.[18,19] For instance, reduced numbers of CCL1-positive macrophages are associated with liver metastasis of colorectal cancer.[20] Fibroblast-derived CCL1 has been reported to induce T regulatory cell differentiation which contributes to the establishment of the pre-metastatic niche in lung.[21] Notably, CCL1 expression positively correlates with the development of pulmonary fibrosis.[22,23] CCL1 facilitates lung fibrosis by activating fibroblasts via the AMFR-ERK pathway[23] or promoting M2 macrophage polarization.[22] Moreover, CCL1 blockade can alleviate pulmonary fibrosis in a sclerodermatous graft-versus-host disease model.[24] However, the cellular source and role of CCL1 in renal fibrosis are unclear.

We previously performed a comparative proteomic study of obstructed kidneys from UPJO patients and UUO mice.[25] Here, using kidney proteomics, we revealed that dysfunction of mitochondria and the TCA cycle was the hallmark of obstructive nephropathy. Importantly, we found that succinate, a TCA cycle metabolite, was significantly accumulated in UUO kidneys. Supplementation with succinate promoted UUO-induced renal fibrosis and inflammation. Moreover, succinate facilitated the infiltration and activation of $CD4^+$ T cells, which produced CCL1 to drive fibroblast activation and fibrosis. Thus, our study revealed an important role of succinate in regulating $CD4^+$ T cell response and obstruction-induced renal fibrosis.

Materials and Methods

Mice and UUO Model

The C57BL/6 background mice were obtained from Spfbiotech (Beijing). Our study complied with the Chinese National Standard for Ethical Review of Laboratory Animal Welfare (GB/T 35823-2018) and was approved by the Institutional Animal Care and Use Committee (IACUC) of Chinese PLA General Hospital. The mice were housed in a specific pathogen-free environment. Male mice aged 8 weeks were used for the UUO model. Following anesthesia (Avertin, Sigma, T48402), a median abdominal incision was made, and the left ureter was double ligated. The sham group underwent the same surgical procedure without ureteral ligation. Mice were sacrificed 14 days post-surgery, and their kidneys were collected for further analysis. A 4% succinate solution (Sigma, 136441) was added to the drinking water for 14 days. DMM (Sigma, 136441, 40mg/kg) was administrated intraperitoneally every other day until day 14 after the UUO surgery. To investigate the in vivo

functional role of CCL1, we administered recombinant murine CCL1 (MCE, HY-P73913, 1 mg/kg every 3 days) and a CCL1-neutralizing antibody (Invitrogen, PA5-47952, 50 μg/kg every 3 days) into UUO mice.

Proteomic Analysis

The proteomics data used in the present study were from our previous work, which was deposited in the ProteomeXchange Consortium (PXD039314).[25] We performed Gene Set Enrichment Analysis (GSEA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of the downregulated proteins in patients with obstructive nephropathy and UUO mice.

Kidney Leukocyte Isolation and Flow Cytometry Analysis

Mice were anesthetized using Avertin (Sigma, T48402). The kidneys were minced and subjected to digestion with 0.05% collagenase IV and 2 mM $CaCl_2$ at 37 °C for 25 min. The tissue was filtered through a 70 μm nylon mesh, followed by centrifugation of the cell suspension at 500 g for 5 min. The cells were then treated with a Fcγ receptor blocker (101320, BioLegend) for 10 min. The following fluorescent antibodies (all from BioLegend) were used: CD45-BV421 (103134), CD11b-FITC (101206), Ly6G-APC/Cyanine7 (127624), Ly6C-PE (128008), F4/80-APC (123116), CD206-PE/Cyanine7 (141720), CD3-PE (100206), CD4-PE/Cyanine7 (116016), CD8a-APC/Cyanine7 (100713), NK1.1-FITC (156508), and CD20-APC (152107). Flow cytometry analysis was conducted using a FACSCanto II (BD Biosciences). The data were analyzed using FlowJo software 10.4.

Western Blots

Kidney protein extracts were prepared according to standard protocols. The tissue lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The following primary antibodies were used: anti-KIM1 (R&D, AF1817), anti-αSMA (Proteintech, 67735-1-lg), anti-Col I (Abcam, ab260043), anti-Col I (Proteintech, 14695-1-AP), anti-cleaved Caspase 3 (CST, 9661S), anti-IKK-β (CST, 2678T), anti-NF-κB p65 (CST, 8242T), anti-CD38 (Santa Cruz, sc-374650), anti-ERK1/2 (Proteintech, 11257), anti-p-ERK1/2 (Proteintech, 80031), anti-CCL1 (R&D, MAB845), and anti-β-actin (Huaxingbio, HX1827). Quantification was conducted using Image J software.

Real-Time Quantitative PCR (qPCR)

Mouse kidneys were homogenized, and total RNA was extracted utilizing an RNA Extraction kit following the manufacturer's instructions (Huaxingbio, HXR8075). Subsequently, complementary DNA was synthesized using a Reverse Transcription kit (Takara, RR037A). Real-time quantitative PCR was performed on the iCycler iQ5 Real-Time PCR detection system (Bio-Rad). The target gene's expression was standardized against the housekeeping gene, *Gapdh*. Relative gene expression levels were determined using the standard $2^{-\Delta\Delta Ct}$ method. The following primers were used:

Genes	Forward	Reverse
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Col1a2	GCAGGTTCACCTACTCTGTCCT	CTTGCCCCATTCATTTGTCT
Acta2	ACTGCCGAGCGTGAGATTGT	TGATGCTGTTATAGGTGGTTTCG
Il1b	TGTAATGAAAGACGGCACACC	TCTTCTTTGGGTATTGCTTGG
Il6	CTGCAAGTGCATCATCGTTGTTC	CTGCAAGTGCATCATCGTTGTTC
Tnf	TCCAGGCGGTGCCTATGT	CACCCCGAAGTTCAGTAGACAGA
Kim1	CTATGTTGGCATCTGCATCG	AAGGCAACCACGCTTAGAGA
Ccl1	GCTTACGGTCTCCAATAGCTGC	GCTTTCTCTACCTTTGTTCAGCC
Ccl3	ACTGCCTGCTGCTTCTCCTACA	ATGACACCTGGCTGGGAGCAAA

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Masson Staining and Histological Analysis

The mouse kidneys were fixed in 4% paraformaldehyde and embedded in paraffin. The kidney sections were stained with Masson's trichrome. Images of the kidney slides were obtained using a Nano Zoomer Slide Scanner (Hamamatsu Photonics).

Succinate Detection

Mouse kidneys were harvested and homogenized, followed by centrifuging at 10,000 rpm for 5 min. The supernatants were subjected to succinate detection using a Succinate Colorimetric Assay Kit (Shanghai Rayzbio, LZ-S63771) following the manufacturer's instructions.

Renal Function Analysis

Mouse serum was collected through centrifugation. The serum levels of blood urea nitrogen (BUN) and creatinine (Cr) were measured using a Urea Assay Kit (C013-2-1, Nanjing Jiancheng, China) and a Creatinine Assay Kit (C011-2-1, Nanjing Jiancheng, China) following the manufacturer's instructions.

$CD4^+$ T Cell Isolation and Treatment

Spleen $CD4^+$ T cells were isolated using a Mouse Naïve $CD4^+$ T Cell Isolation Kit (19852, Stemcell) following the manufacturer's instructions. $CD4^+$ T cells (1×10^6 cells/mL) were activated with Mouse T-Activator CD3/CD28 (11452D, Gibco) and 5 ng/mL IL-2 (212-12, Peprotech) in 1640 supplemented with 10% fetal bovine serum. Subsequently, $CD4^+$ T cells were treated with the cell membrane permeable dimethyl succinate (Sigma, 112402, 0.5 μmol/mL). After 3 days of stimulation, the expression levels of CCL1 and CCL3 was analyzed. For adoptive transfer experiment, $CD4^+$ T cells were treated with dimethyl succinate for 2 days, which were then transferred into mice through tail vein 3 days after UUO (2×10^6 cells/mouse).

Cell Culture and Treatment

The murine fibroblast cell line NIH-3T3 was obtained from the Cell Resource Center at the Institute of Basic Medical Sciences in Beijing, China. The cells were cultured in 1640 medium (Gibco) supplemented with 10% fetal bovine serum. Fibroblast activation was induced using recombinant human TGF-β1 (MCE, HY-P7118, 10ng/mL) and recombinant mouse CCL1 (MCE, HY-P73913, 10ng/mL). For co-culture experiment, a transwell system (Corning) was used. NIH-3T3 fibroblasts were seeded in the lower chamber, while $CD4^+$ T cells pretreated with or without dimethyl succinate were cultured in the upper compartment. After 48 hours of co-culture, fibroblast activation was assessed by quantifying *Col1a2* and *Acta2* mRNA expression using qPCR.

Bulk RNA Sequencing

Spleen $CD4^+$ T cells were isolated and treated with or without dimethyl succinate for 3 days. RNA was extracted using an RNA Extraction kit following the manufacturer's instructions (Huaxingbio, HXR8075). RNA quality and quantity were analyzed using a NanoDrop and Agilent 2100 bioanalyzer. The RNA was then reverse transcribed to generate cDNA for library preparation. Sequencing was performed on a NovaseqX Plus platform (Novogene, China). The data were aligned to the mouse reference genome (version mm10).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.0c. The data are presented as mean ± SEM. Two-tailed Student's *t*-test was used to compare the two groups. One-way ANOVA was used to analyze three or more comparisons. A p-value less than 0.05 was considered significant.

Results

Kidney Proteomics Revealed Metabolic Aberrations in UPJO Patients and UUO Mice, and Succinate was Accumulated in the Obstructed Kidneys

We previously characterized the proteomic landscape of obstructed kidneys from UPJO patients and UUO mice.[25] We revealed that mitochondrial damage was the key feature of obstructive nephropathy. Notably, the downregulated

proteins in the human obstructed kidneys were significantly enriched in the TCA cycle and mitochondrial organization (Figure 1A and B). Consistently, KEGG enrichment analysis showed that the proteins downregulated in UUO mice were associated with several metabolic pathways, such as fatty acid biosynthesis, pyruvate

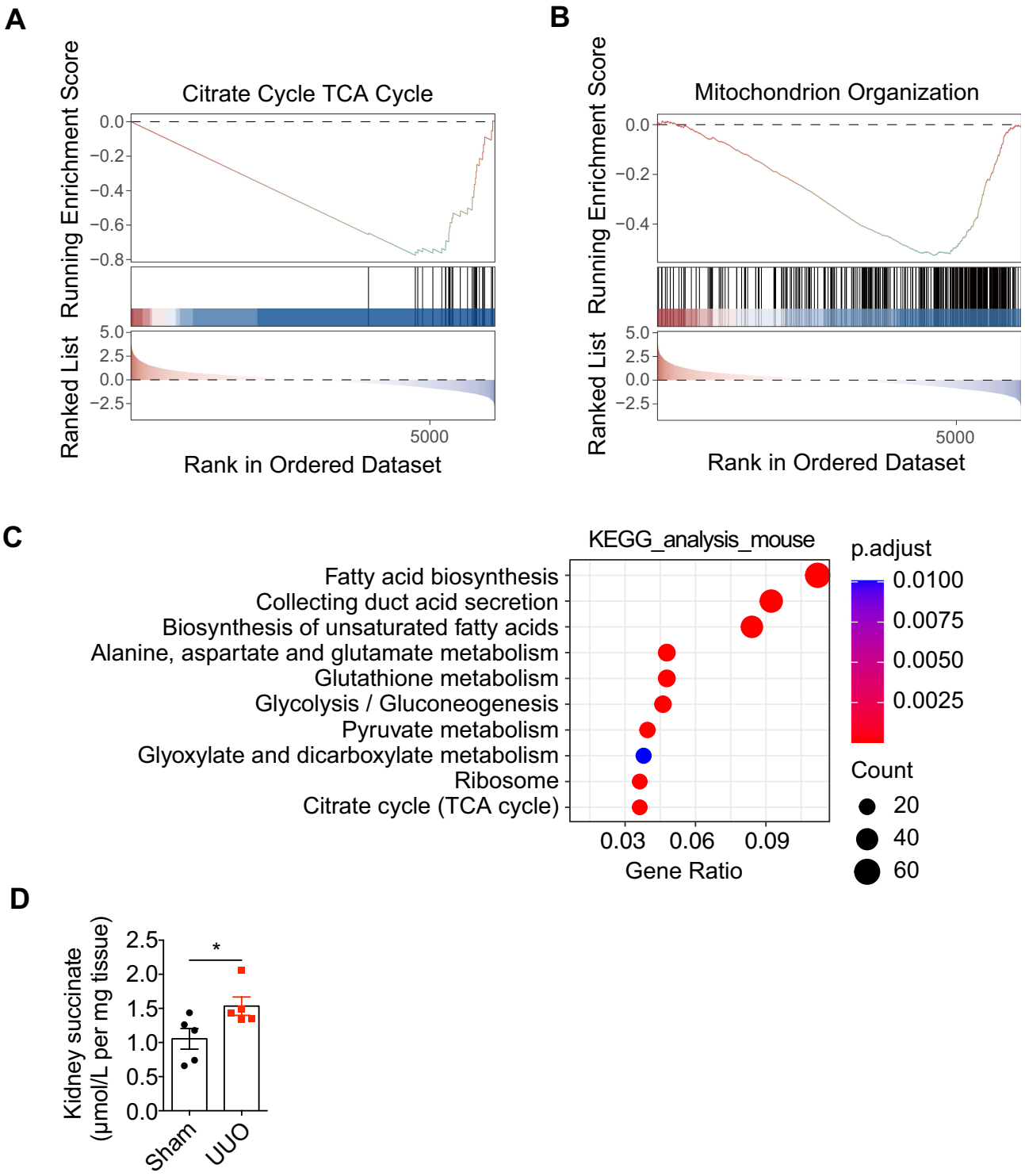

Figure 1 Kidney proteomics revealed metabolic aberrations in UPJO patients and UUO mice, and succinate was accumulated in the obstructed kidneys. GSEA analysis showed that the downregulated proteins in UPJO patients were significantly enriched in (**A**) the TCA cycle and (**B**) mitochondrion organization. (**C**) KEGG analysis showed the top 10 downregulated pathways enriched in UUO kidneys in mice. (**D**) The succinate content was analyzed in kidney tissues from sham or UUO mice (n = 5). The results represent mean ± SEM. *p < 0.05.

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metabolism, the TCA cycle, and oxidative phosphorylation (Figure 1C). These data suggest that ureteral obstruction resulted in metabolic aberrations in the kidney, especially mitochondrial dysfunction. Hypoxia in the tissue microenvironment is always associated with the production of succinate.[7] As obstruction led to hypoxia and TCA cycle damage in the obstructed kidneys, we reasoned that succinate might accumulate in kidney tissues. Indeed, we found significantly elevated levels of succinate in UUO kidneys compared to sham control (Figure 1D). These findings suggest that ureteral obstruction triggers dysregulated metabolism and succinate accumulation in the kidneys.

Succinate Supplementation Exacerbated UUO-Induced Renal Fibrosis and Injury

To understand the pathogenic roles of succinate in the development of obstructive nephropathy, we supplemented UUO mice with succinate in their drinking water (Figure 2A). This supplementation led to elevated levels of succinate in the obstructed kidneys (Figure 2B). Of note, succinate administration resulted in upregulated expression of the fibrosis markers collagen I (Col I) and α-smooth muscle actin (αSMA) at both the mRNA and protein levels (Figure 2C–E). Moreover, succinate-treated mice exhibited larger collagen-positive areas in the obstructed kidneys (Figure 2F), indicating that succinate promotes renal fibrosis.

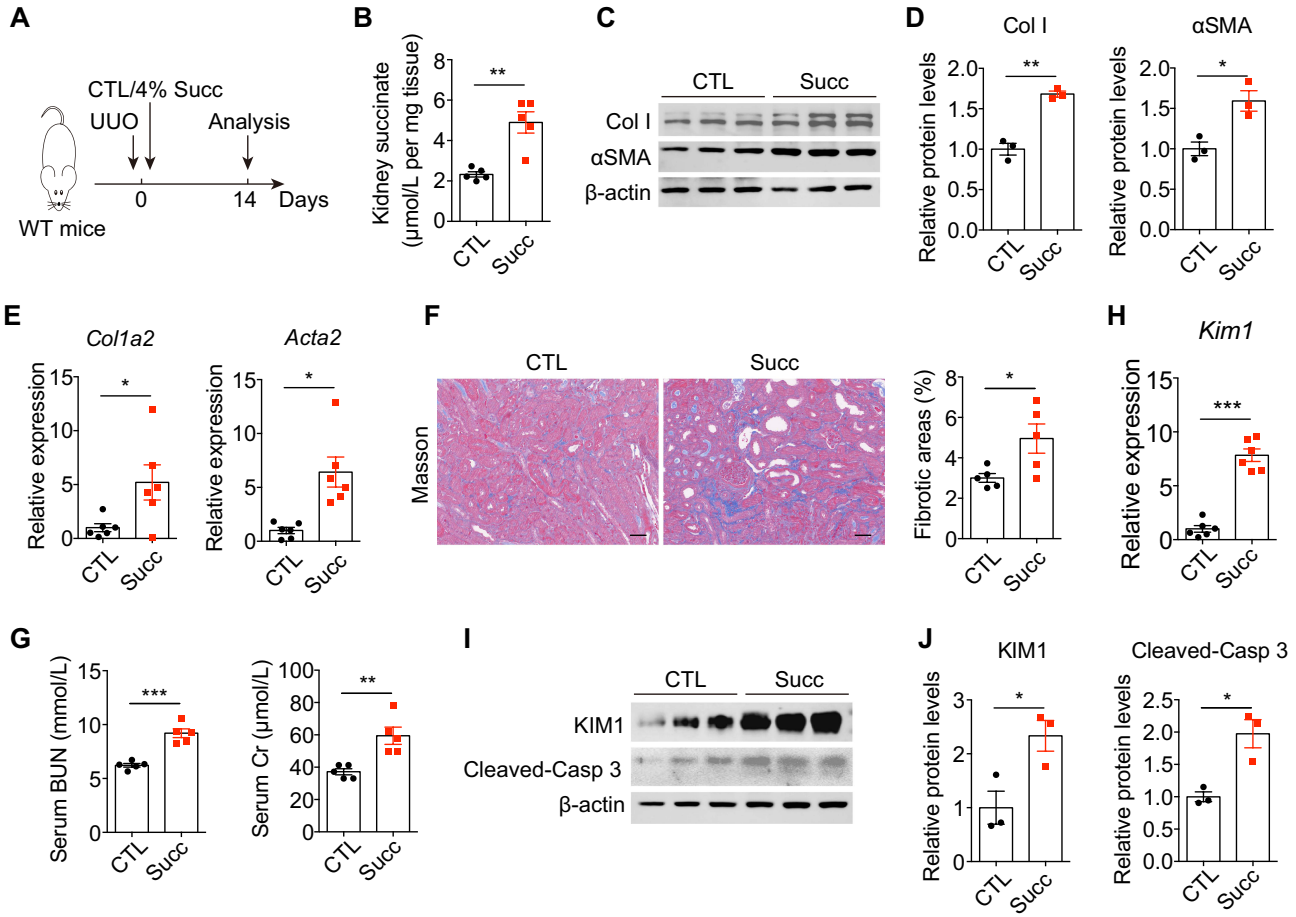

Figure 2 Succinate supplementation exacerbated UUO-induced renal fibrosis and injury. (**A**) The schematic of the experimental design. Wild-type (WT) C57BL/6 mice were used. (**B**) The succinate content in UUO kidneys from control (CTL) and succinate-supplemented (Succ) mice (n = 5). (**C**) Immunoblot and (**D**) quantification analysis of Col I and αSMA expression in UUO kidneys from control and succinate-supplemented mice (n = 3). (**E**) qPCR analysis for *Col1a2* and *Acta2* in UUO kidneys from control and succinate-supplemented mice (n = 6). (**F**) Masson staining and quantification of UUO kidneys from control and succinate-supplemented mice (n = 5), scale bar = 40 μm. (**G**) Blood urea nitrogen (BUN) and serum creatinine (Cr) were analyzed in control and succinate-supplemented mice subjected to UUO surgery (n = 5). (**H**) qPCR analysis for renal *Kim1* in control and succinate-treated mice (n = 6). (**I**) Immunoblot and (**J**) quantification analysis of KIM1 and cleaved Caspase 3 in UUO kidneys from control and succinate-treated mice (n = 3). The results represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

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In addition, we found that succinate supplementation contributed to higher levels of serum BUN and creatinine in UUO mice (Figure 2G), suggesting a detrimental role of succinate in obstructive nephropathy. The well-established kidney injury marker, KIM1, was also upregulated at the mRNA and protein levels in succinate-treated mice compared to the control (Figure 2H–J). Furthermore, succinate supplementation led to enhanced apoptosis in obstructed kidneys as revealed by the upregulated expression of cleaved Caspase 3 (Figure 2I and J). Collectively, these results support a pathogenic role of succinate in UUO-induced kidney injury and fibrosis.

Succinate Supplementation Promoted Kidney Inflammation

Given that succinate is a critical regulator of inflammation which is central to the pathogenesis of renal fibrosis,[26–28] we asked whether succinate affected kidney inflammation during obstructive nephropathy. Importantly, succinate upregulated renal expression of IKK-β and NF-κB p65 (Figure 3A and B), an important inflammatory signaling in renal fibrosis.[26] We previously demonstrated that CD38 is a crucial promoter of renal inflammation and fibrosis.[25] Interestingly, CD38 expression was dramatically elevated in response to succinate treatment (Figure 3A and B). Moreover, compared to the control, succinate-treated mice displayed upregulated expression of pro-inflammatory cytokines, including *Il1b, Il6*, and *Tnf* (Figure 3C). These data suggest that succinate promoted inflammation in UUO kidneys.

Succinate Contributed to $CD4^+$ T Cell Accumulation in UUO Kidneys

We then analyzed immune cell infiltration in the obstructed kidneys by flow cytometry. Interestingly, succinate supplementation did not affect the accumulation of monocytes, macrophages, neutrophils, B cells, natural killer (NK) cells, or natural killer T (NKT) cells (Figure 4A–D). However, it significantly facilitated the renal infiltration of T cells (Figure 4D). We further analyzed the impact of succinate on T cell subsets and noted that succinate specifically promoted the infiltration of $CD4^+$ T cells instead of $CD8^+$ T cells (Figure 4E). Moreover, the T-cell recruiting chemokines CXCL9 and CXCL10 were increased in UUO kidneys upon succinate supplementation

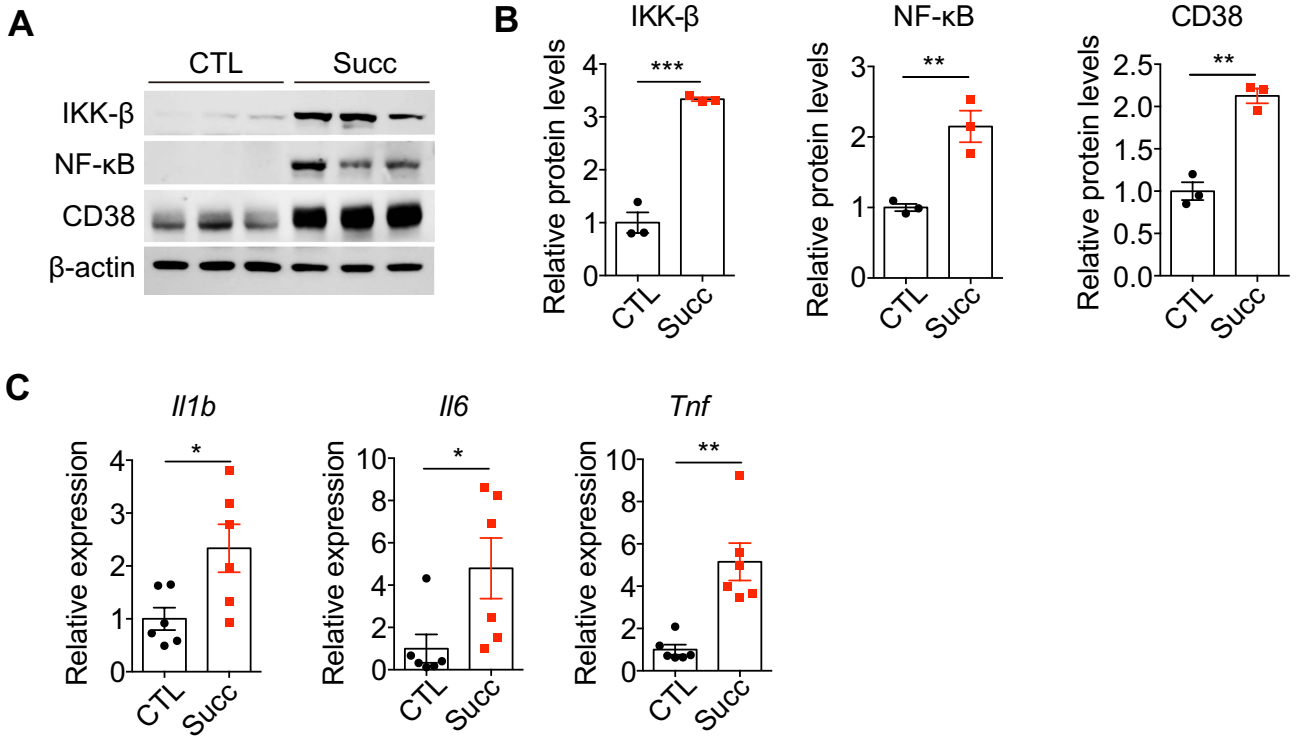

Figure 3 Succinate supplementation promoted kidney inflammation. (**A**) Immunoblot and (**B**) quantification analysis of IKK-β, NF-κB p65, and CD38 in UUO kidneys from control and succinate-treated mice (n = 3). (**C**) qPCR analysis for renal *Il1b, Il6* and *Tnf* in control and succinate-treated mice (n = 6). The results represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

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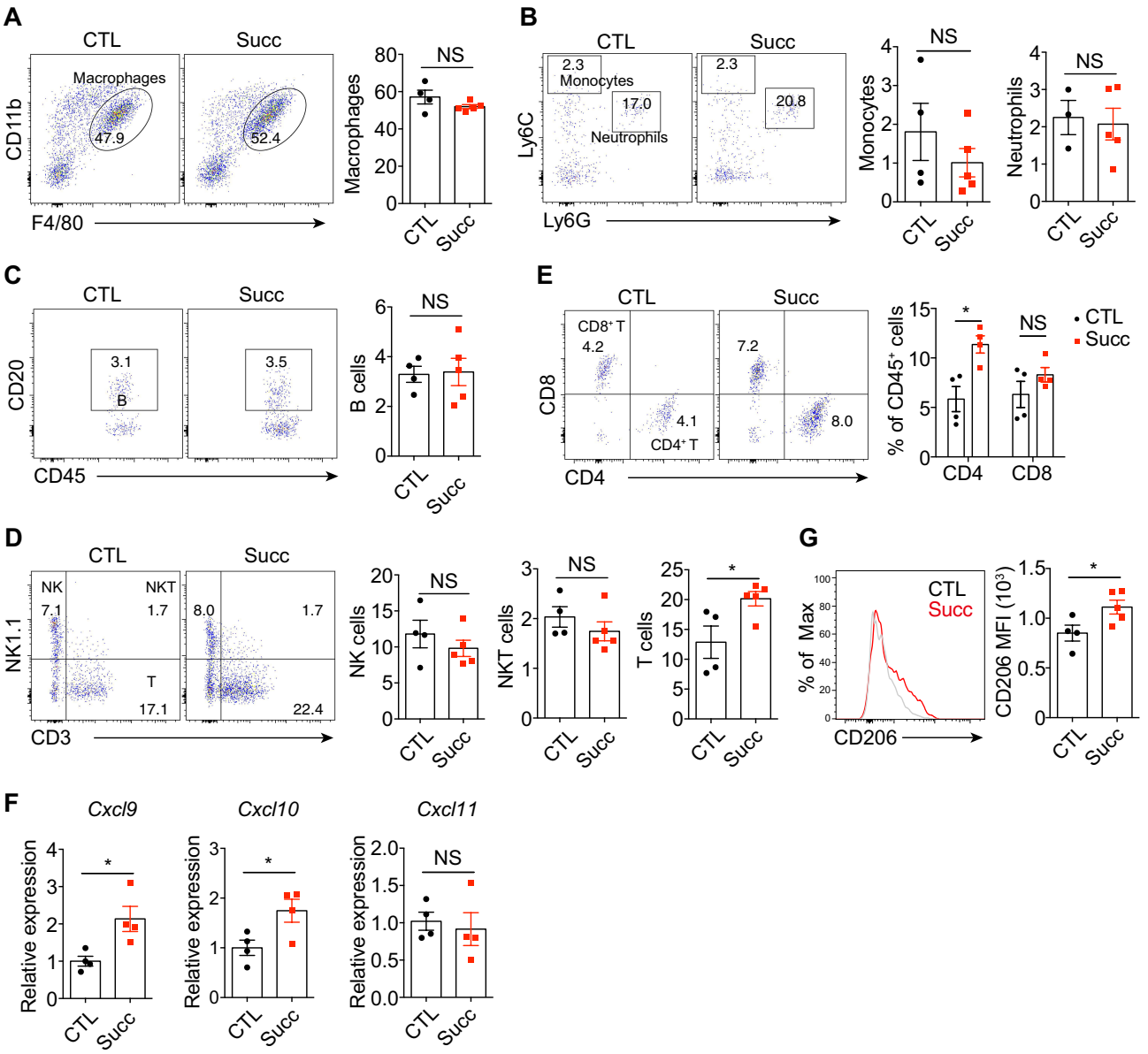

Figure 4 Succinate contributed to CD4$^+$ T cell accumulation in UUO kidneys. Representative flow cytometry plots and quantification of (**A**) macrophages, (**B**) monocytes and neutrophils, (**C**) B cells, (**D**) NK, NKT, and T cells, and (**E**) CD4$^+$ T and CD8$^+$ T cells (n = 4/5). (**F**) qPCR analysis for renal *Cxcl9*, *Cxcl10*, and *Cxcl11* in control and succinate-treated mice (n = 4). (**G**) Representative flow cytometry plots and quantification of CD206 expression in renal macrophages (n = 4/5). The results represent mean ± SEM. *p < 0.05, NS no significance.

(Figure 4F). Studies have suggested that succinate induces M2 macrophages, which play a critical role in renal fibrosis.[17] Consistently, we found that the M2 marker CD206 was significantly induced by succinate in kidney macrophages (Figure 4G). Together, these data suggest that succinate contributes to the accumulation of CD4$^+$ T cells and the polarization of M2 macrophages in obstructive nephropathy.

Transcriptome Analysis of CD4$^+$ T Cells Treated with Succinate

To dissect the mechanisms underlying succinate regulation on CD4$^+$ T cells and renal fibrosis, we performed bulk RNA sequencing (RNA-seq) analysis of isolated spleen CD4$^+$ T cells treated with or without cell membrane permeable dimethyl succinate, which can enter into T cells and increase intracellular succinate concentration.[29] RNA-seq analysis revealed 169 upregulated and 105 downregulated genes in succinate-treated T cells ($p < 0.05$, fold change > 1.5) (Figure 5A). Gene Ontology (GO) enrichment analysis showed that the upregulated genes in succinate-treated CD4$^+$

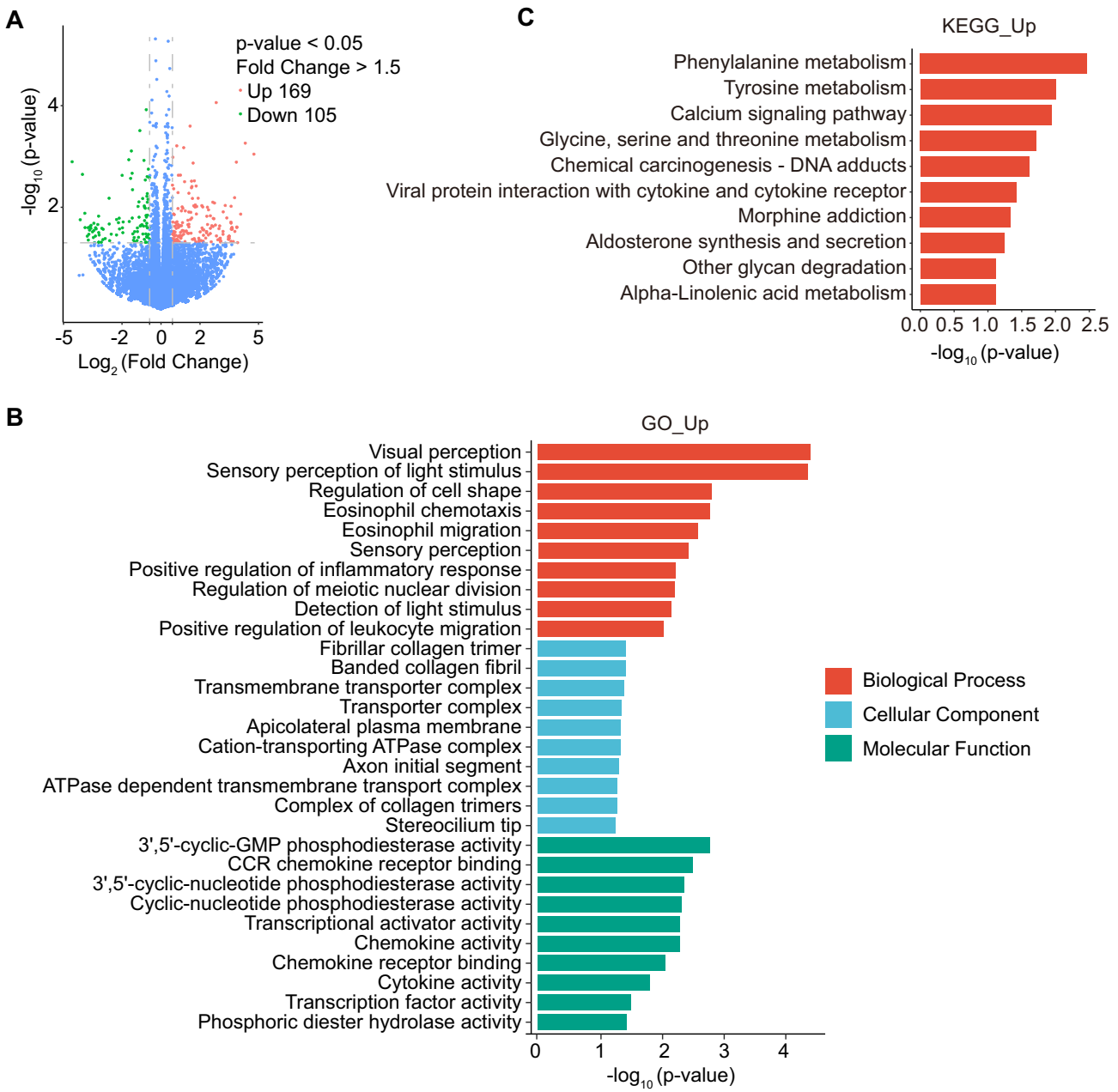

Figure 5 Succinate promoted CD4$^+$ T cell activation. (**A**) Volcano plot showing the differential expressed genes in control and succinate-treated spleen CD4$^+$ T cells ($p < 0.05$, fold change > 1.5). (**B**) GO analysis of the upregulated genes in succinate-treated CD4$^+$ T cells, including the enrichment of biological process, cellular component, and molecular function. The top 10 items were shown. (**C**) KEGG analysis of the upregulated genes in succinate-treated CD4$^+$ T cells. The top 10 items were shown.

T cells were significantly associated with the biological processes involved in T cell activation, such as regulation of cell shape, positive regulation of inflammatory response, regulation of meiotic nuclear division, and positive regulation of leukocyte migration (Figure 5B). Moreover, GO molecular function analysis showed that transcription activity, chemokine activity, and cytokine activity were significantly elevated in succinate-treated T cells (Figure 5B). Consistently, KEGG analysis displayed significant upregulation of pathways that were shown to be associated with T cell activation, including calcium signaling and several metabolic pathways (Figure 5C). Therefore, the transcriptome analysis demonstrates that succinate contributes to the activation of CD4$^+$ T cells.

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Succinate-Mediated CD4$^+$ T Cell-Derived CCL1 Promoted Myofibroblast Activation

To elucidate the molecular mechanisms by which succinate-mediated T cell activation contributes to renal fibrosis, we mined the RNA-seq data to identify the secreting cytokines that might participate in the fibrotic process. It has been reported that the chemokine CCL1 promotes the differentiation of lung fibroblasts into myofibroblasts during pulmonary fibrosis.[23] Interestingly, we found that *Ccl1* was among the upregulated genes in succinate-treated T cells, along with *Ccl3* (Figure 6A). *Klb*, which encodes the fibrosis inhibitor Klotho,[30] was also decreased in T cells after succinate treatment (Figure 6A). To confirm the increase of *Ccl1*, we treated CD4$^+$ T cells with dimethyl succinate and found that *Ccl1* and *Ccl3* mRNA levels were dramatically elevated upon succinate stimulation (Figure 6B). Succinate treatment also upregulated the protein level of CCL1 in UUO kidneys (Figure 6C and D). We then took advantage of DMM to inhibit SDH, which drives the production of succinate within the hypoxic microenvironment. As expected, DMM-mediated succinate inhibition led to reduced CCL1 levels in UUO kidneys (Figure 6C and D). Collectively, these results suggest that succinate promotes CCL1 expression in CD4$^+$ T cells.

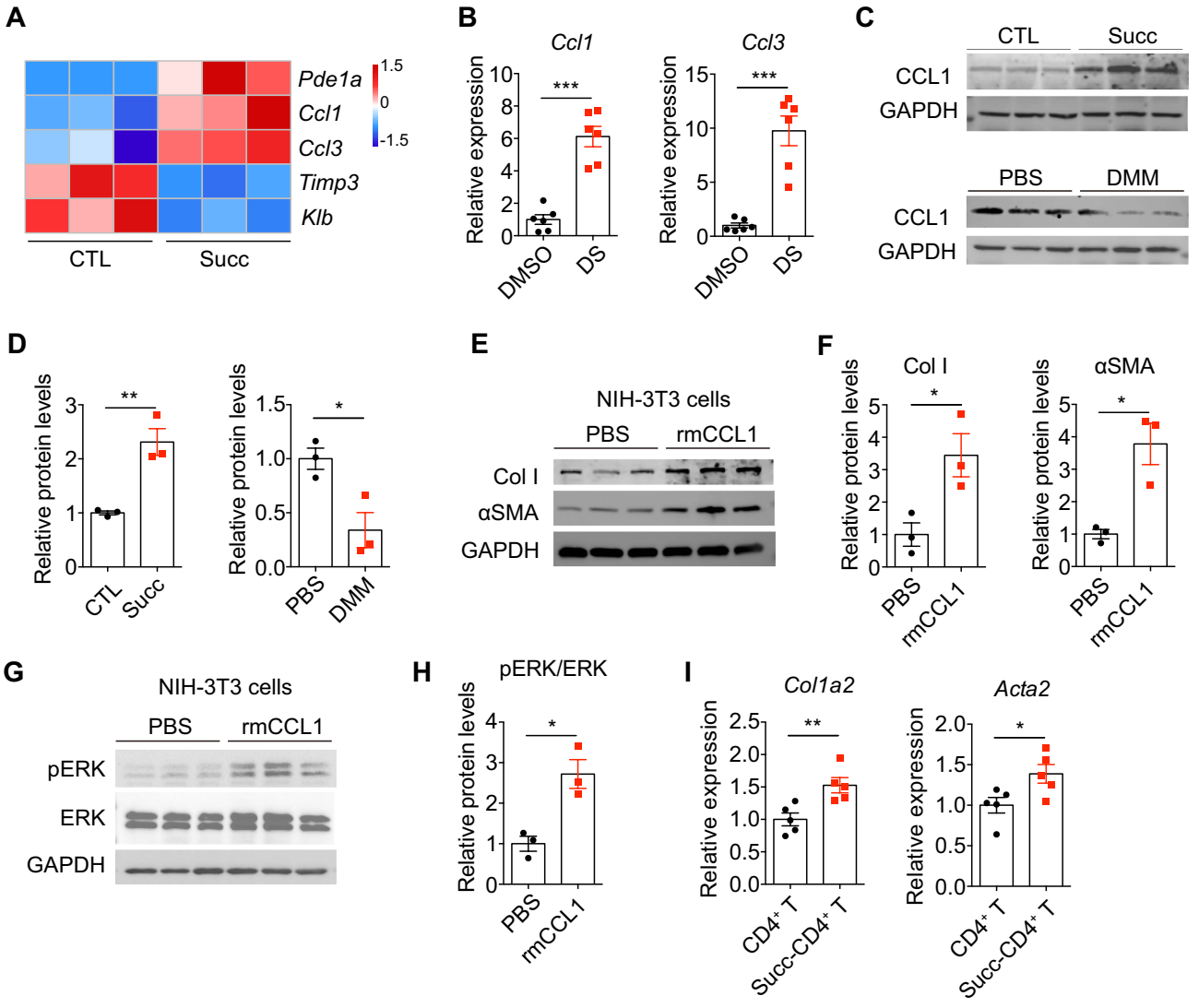

Figure 6 Succinate potentiated the production of CCL1 in CD4$^+$ T cells to promote myofibroblast activation. (**A**) Heatmap showing the expression of *Pde1a*, *Ccl1*, *Ccl3*, and *Klb* in control and succinate-treated CD4$^+$ T cells. (**B**) qPCR analysis for *Ccl1* and *Ccl3* in CD4$^+$ T cells treated with DMSO or dimethyl succinate (DS) (n = 6). (**C**) Immunoblot and (**D**) quantification analysis of CCL1 in the UUO kidneys from mice treated with succinate or DMM (n = 3). (**E**) Immunoblot and (**F**) quantification analysis of Col I and αSMA in NIH-3T3 cells treated with PBS or rmCCL1 (n =3). (**G**) Immunoblot and (**H**) quantification analysis of pERK and ERK in NIH-3T3 cells treated with PBS or rmCCL1 (n =3). (**I**) qPCR analysis for *Col1a2* and *Acta2* in NIH-3T3 fibroblasts co-cultured with succinate-preconditioned CD4$^+$ T cells or untreated CD4$^+$ T cells (n = 5). The results represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

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Given that myofibroblasts are the primary source of extracellular matrix during the fibrotic process[31] and CCL1 can mediate myofibroblast activation in pulmonary fibrosis,[23] we asked whether $CD4^+$ T cell-derived CCL1 was able to activate fibroblasts during renal fibrosis. Thus, we treated the mouse fibroblast cell line NIH-3T3 with recombinant mouse CCL1 (rmCCL1). We found that Col I and αSMA were dramatically increased in the presence of rmCCL1 (Figure 6E and F), suggesting that CCL1 facilitated myofibroblast activation. Moreover, CCL1 stimulation was associated with enhanced phosphorylation of ERK (Figure 6G and H) as previously reported.[23] Moreover, the expression levels of *Col1a2* and *Acta2* were significantly higher in NIH-3T3 fibroblasts co-cultured with succinate-preconditioned $CD4^+$ T cells compared to those co-cultured with untreated $CD4^+$ T cells (Figure 6I). Therefore, CCL1 drives myofibroblast activation in vitro.

CCL1 Promoted UUO-Induced Renal Fibrosis and Inflammation

Then, we sought to understand the pathogenic role of CCL1 in vivo (Figure 7A). Consistent with in vitro results, CCL1-treated mice exhibited increased expression of myofibroblast markers Col I and αSMA compared to PBS-treated mice (Figure 7B and C).

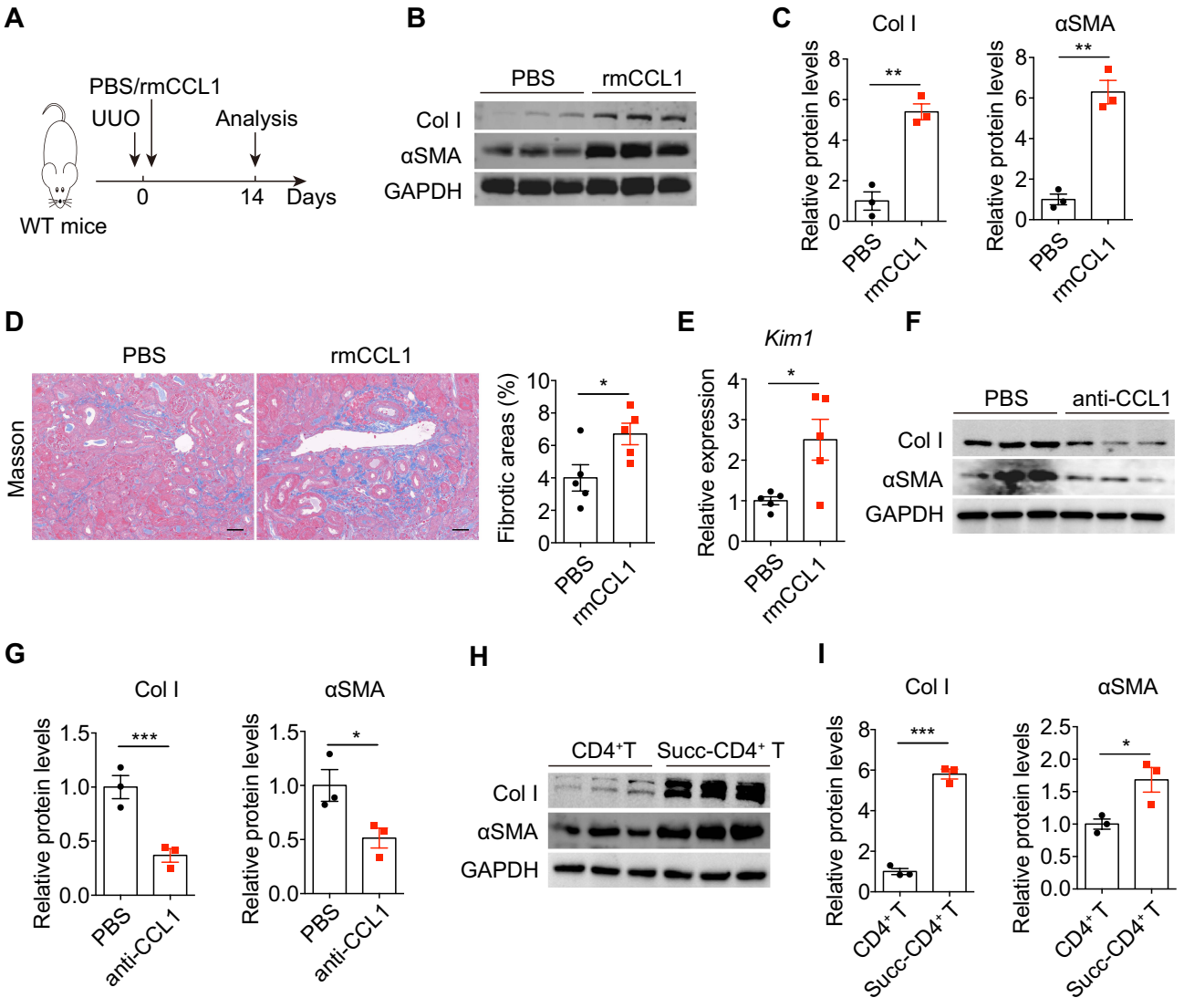

Figure 7 CCL1 promoted UUO-induced renal fibrosis. (**A**) The schematic of the experimental design. (**B**) Immunoblot and (**C**) quantification analysis of Col I and αSMA expression in UUO kidneys from control and rmCCL1-treated mice (n = 3). (**D**) Masson staining and quantification of UUO kidneys from control and rmCCL1-treated mice (n = 5), scale bar = 40 μm. (**E**) qPCR analysis for *Kim1* in the UUO kidneys from control and rmCCL1-treated mice (n = 5). (**F**) Immunoblot and (**G**) quantification analysis of Col I and αSMA expression in UUO kidneys from control and anti-CCL1-treated mice (n = 3). (**H**) Immunoblot and (**I**) quantification analysis of Col I and αSMA expression in UUO kidneys from mice transferred with untreated or succinate-treated $CD4^+$ T cells (n = 3). The results represent mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

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Moreover, CCL1-treated mice displayed larger collagen-positive areas (Figure 7D) and higher *Kim1* levels (Figure 7E) in UUO kidneys compared to the control. We also treated UUO mice with PBS or a CCL1-neutralizing antibody. The results demonstrated that anti-CCL1 treatment significantly suppressed the expression of Col I and αSMA compared to the PBS-treated group (Figure 7F and G). These findings support the profibrotic role of CCL1 in renal fibrosis progression. Furthermore, UUO mice receiving succinate-primed $CD4^+$ T cells exhibited elevated levels of Col I and αSMA expression relative to those receiving PBS-treated $CD4^+$ T cells, indicating that succinate-activated $CD4^+$ T cells promote fibrotic processes (Figure 7H and I).

Discussion

The roles and associated mechanisms of succinate in obstructive nephropathy remain largely unknown. Here, we showed that succinate induced CXCL9/10 expression and promoted the infiltration and subsequent activation of $CD4^+$ T cells, which produce CCL1 to drive the activation of fibroblasts and kidney inflammation during obstructive nephropathy.

The TCA cycle is a metabolic hub and also a signaling hub in the cell. Succinate is a critical intermediate metabolite that is always confined to the mitochondria but leaks into the cytoplasm and extracellular space when cells are stressed and the TCA cycle is disrupted.[6] We and others have repeatedly demonstrated that mitochondrial and TCA dysfunction is the hallmark of kidney diseases, including obstructive nephropathy.[25,32–34] The levels of succinate are tightly controlled by the enzyme SDH, also known as mitochondrial Complex II. Obstruction-induced hydronephrosis creates a hypoxic microenvironment, in which the TCA cycle is damaged and SDH operates in reverse to produce succinate.[8] Here, we showed that succinate promotes UUO-induced renal fibrosis and $CD4^+$ T cell-associated inflammation. It is worth noting that several studies have recently documented the involvement of succinate in the fibrotic process, a common pathogenic mechanism leading to organ failure in many diseases. For instance, reduced succinate content prevents lung fibrosis and respiratory dysfunction.[8] In liver fibrosis, excessive intracellular succinate goes out of the cells and binds to GPR91 (also known as succinate receptor 1, SUCNR1), leading to the migration and proliferation of hepatic stellate cells, the primary source of extracellular matrix in the liver.[35,36] Moreover, increased succinate levels in the serum and GRP91 expression are observed in intestinal tissue of Crohn's disease patients, and genetic deletion of GRP71 protects mice against intestinal fibrosis.[37] Together with our study, these findings support the critical role of succinate in the pathogenesis of organ fibrosis.

Emerging evidence has demonstrated that succinate is a critical regulator of immune cells, including macrophages, dendritic cells (DCs), and T cells.[38,39] Succinate is not only a marker but also contributes to the activity of pro-inflammatory macrophages. Lipopolysaccharide-stimulated macrophages exhibit an accumulation of succinate, resulting in HIF-1α stabilization and IL-1β induction.[40,41] The interaction between succinate and SUCNR1on DCs activates intracellular calcium and ERK1/2 signaling, promoting DC maturation and IL-1β secretion.[42] Of note, succinate is also involved in T cell response. It has been reported that SDH deficiency and succinate accumulation induce a pro-inflammatory gene signature in T cells by regulating chromatin accessibility and pro-inflammatory transcription factors.[29] In the experimental autoimmune uveitis model, succinate has been shown to enhance the frequencies of Th1/Th17 cells.[43] Interestingly, our study showed that succinate-induced T-cell chemokines CXCL9/10 promote $CD4^+$ T cell infiltration, which leads to the production of CCL1 and the activation of myofibroblasts. Thus, we provided new evidence that succinate regulates T cell response in the scenario of obstructive nephropathy.

The UUO mouse model is a well-established tool for studying renal fibrosis, owing to its rapid and reproducible induction of interstitial fibrosis and its utility in elucidating molecular pathways underlying fibrotic progression. However, this model primarily recapitulates acute obstructive injury rather than chronic, multifactorial human kidney diseases, such as hypertensive nephropathy or diabetic nephropathy. It fails to fully capture the complexity of human chronic kidney disease, where metabolic dysregulation, dysregulated immune responses, and comorbid conditions dynamically interact.

Moreover, our study has several limitations. First, the precise mechanisms underlying succinate-mediated upregulation of renal CXCL9/10 expression require further investigation, particularly regarding their cellular origins. Second, the molecular pathways through which succinate enhances CCL1 production in $CD4^+$ T cells remain to be fully elucidated. Third, while GPR91 has been identified as a succinate receptor, its specific role in mediating succinate-induced T cell activation warrants validation through receptor knockout models. Finally, the potential heterogeneity of succinate's immunomodulatory effects across distinct $CD4^+$ T cell subpopulations remains unclear.

Conclusion

We uncovered an important role of succinate in mediating T cell response during obstructive nephropathy. Succinate promoted $CD4^+$ T cell activation and CCL1 expression, which orchestrated renal fibrosis by inducing the transition of fibroblasts to myofibroblasts. Our study thus provided new insight into the cellular and molecular mechanisms involving the interaction between metabolism and immunity in obstructive nephropathy and renal fibrosis.

Data Sharing Statement

The RNA-seq datasets were deposited in the GEO database under accession code GSE278379.

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Disclosure

The authors declare that there is no conflict of interest in this work.

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