Twist1 in T Lymphocytes Augments Kidney Fibrosis After Ureteral Obstruction

Jiafa Ren,¹ Xiaohan Lu,¹ Robert Griffiths,¹ Jamie R. Privratsky,² Steven D. Crowley¹

¹Division of Nephrology, Departments of Medicine, ²Department of Anesthesiology,

Durham VA and Duke University Medical Centers, Durham, NC

Corresponding author:

Steven D. Crowley, M.D.

Division of Nephrology, Department of Medicine

Durham VA and Duke University Medical Centers

DUMC Box 103015

Durham, NC 27710

E: steven.d.crowley@duke.edu
Key Points

- The transcription factor Twist1 acts in T lymphocytes to promote kidney fibrogenesis.
- T cell Twist1 limits the accumulation of TNF-producing CD8+ T cells in the injured kidney.
- TNF produced by T lymphocytes limits kidney scar formation after ureteral obstruction.

Abstract

Background Twist1 is a basic helix-loop-helix domain containing transcription factor that participates in diverse cellular functions including epithelial-mesenchymal transition and the cellular immune response. Although Twist1 plays critical roles in the initiation and progression of kidney diseases, the effects of Twist1 in the T lymphocyte on the progression of renal fibrosis require elucidation.

Methods 129/SvEv mice with a floxed allele for the gene encoding Twist1 or TNFα were bred with CD4-Cre mice to yield CD4-Cre Twist1\textsuperscript{flox/flox} (Twist1 TKO) or CD4-Cre TNF\textsuperscript{flox/flox} (TNF TKO) mice with robust but selective deletion of Twist1 or TNFα mRNA in T cells, respectively. Twist1 TKO, TNF TKO, and WT controls underwent UUO with assessment of kidney fibrosis and T cell phenotype at 14 days.

Results Compared with WT controls, obstructed kidneys from Twist1 TKO mice had attenuated extracellular matrix deposition. Despite this diminished fibrosis, Twist1 TKO obstructed kidneys contained more CD8\textsuperscript{+} T cells than in WTs. These intra-renal CD8\textsuperscript{+} T cells exhibited greater activation and higher levels of TNFα expression than those from WT obstructed kidneys. We found further that deletion of TNFα selectively from T cells exaggerated renal scar formation and injury after UUO, highlighting the capacity of T cell TNF to constrain fibrosis in the kidney.

Conclusions Twist1 in T cells promotes kidney fibrogenesis in part by curtailing the renal accumulation of TNF-elaborating T cells.
Introduction

Tubulointerstitial fibrosis is a common consequence of chronic kidney diseases leading to end-stage renal failure irrespective of the cause (1-3). Interstitial infiltration of T lymphocytes into the kidney is an early and characteristic feature of progressive renal fibrosis in patients with CKD and in animal models. T cell recruitment and activation following kidney injury can have profound effects on renal fibrogenesis (4). Under the influence of the local inflammatory microenvironment in injured tissues, T lymphocytes undergo polarization culminating in their elaboration of divergent constellations of cytokines (5, 6). Accordingly, T lymphocyte heterogeneity plays an important role in regulating tissue injury and scar formation. For example, type 1 T helper (Th1) CD4+ T cells can have opposing effects on organ fibrogenesis depending on the specific clinical context (4, 7-10). Type 2 T helper (Th2) CD4+ T cells have been shown to trigger renal fibrosis7(7). Finally, γδ T cells and CD4+ Th17 cells that produce IL-17A can also drive collagen deposition in the kidney (11). By contrast, Regulatory T cells (Tregs) suppress kidney injury and scar formation despite their elaboration of TGF-β12(12), and CD8+ effector T cells can similarly constrain fibrogenesis in kidneys or tumor (13-15). Although great efforts have been made to determine the molecular mechanisms governing T cell phenotype and activation in kidney fibrosis, they remain largely unknown. Elucidating these mechanisms could lead to immunomodulatory interventions to mitigate kidney fibrosis.

Twist1 regulates the progression of several forms of renal disease, and operates through distinct mechanisms in different cell lineages (16-18). Twist1 also shapes cellular immune responses and contributes to heterogeneity in T lymphocyte phenotype by modulating their elaboration of various inflammatory cytokines (19, 20). Accordingly, Twist1 is upregulated in chronically activated Th1, Th17, and T follicular helper cells (21). Twist1 negatively regulates cytokine production in several T cell subsets via diminishing NF-κB and Runx3 activation, limiting TNFα, IFNγ, or IL-2 expression in Th1 cells (22), or repressing IL-6/STAT3 activation,
leading to restricted IL-17 and IFN-γ production (20). However, the role of its actions in T lymphocytes during fibrogenesis has not been explored. Given that T lymphocytes accumulate in the injured kidney and that their secreted cytokines modulate fibrosis, the contribution of Twist1 in lymphocytes to kidney fibrogenesis warrants scrutiny.

In this study, we find that Twist1 expression is induced in T lymphocytes that infiltrate fibrotic kidneys. Using a mouse model with selective deletion of Twist1 in T lymphocytes, we demonstrate that Twist1 in T lymphocytes augments kidney fibrosis accruing from unilateral ureteral obstruction (UUO). Furthermore, T lymphocyte Twist1 curtails the renal accumulation of CD8+ T lymphocytes that elaborate TNFα. In turn we find that TNFα from T lymphocytes constrains kidney fibrosis and injury. Thus, Twist1 promotes T cell-dependent fibrosis in the kidney, possibly by constraining TNFα production in lymphocytes.

**Materials and Methods**

**Animals**

All mice were housed and bred in the animal facilities at the Durham Veterans’ Affairs Medical Center according to National Institutes of Health guidelines. All animal experiments were approved by the Durham Veteran’s Affairs Medical Center Institutional Animal Care and Use Committee. Homozygous Twist1 floxed mice (a kind gift from Richard Behringer) (23) backcrossed to the 129/SvEv background were bred with 129/SvEv CD4-Cre (24) mice to generate CD4-Cre+ Twist1fl/fl (Twist1 TKO) mice and WT Cre− control littermates. Similarly, 129/SvEv TNFfl/fl mice25(25) were bred with 129/SvEv CD4-Cre mice to yield CD4-Cre+ TNFfl/fl (TNF TKO) mice and WT control subjects. To map the distribution pattern of Cre recombinase expression, mT/mG mice from the Jackson Laboratory (Bar Harbor, ME) were crossed with the CD4-Cre recombinase transgenic lines; mT/mG mice normally express red fluorescent protein
in all tissues. When Cre is present, the mT cassette is deleted, triggering expression of membrane-targeted eGFP. Male mice, aged 8 to 12 weeks, were used in our experiments.

**UUO model of kidney fibrosis**

UUO was performed as previously described (17). In brief, mice were anesthetized with 4% isoflurane and maintained at 2% for the procedure lasting approximately 10 minutes, and the left ureter was isolated and ligated 3-5 mm below its origin. At 14 days after ligation, mice were euthanized and the obstructed and contralateral non-obstructed kidneys were harvested for analysis.

**Histological analysis and immunohistochemistry staining**

Mouse kidney samples were fixed in 10% formalin (Sigma-Aldrich) overnight and embedded in paraffin. Sections 5 μm in thickness were used for periodic acid–Schiff staining and immunohistochemistry staining. Renal damage including necrotic tubules, loss of brush borders, tubule dilatation, cast formation, tubular epithelial swelling, and vacuolar degeneration was semi-quantitatively scored. A score of 0 represents injury area less than 5%, whereas 1, 2, 3, and 4 connote damage involving 5% to 25%; 26% to 50%; 51% to 75%; and >75% of the whole kidney area, respectively. The investigator, who was blinded to experimental conditions, assigned a score to each field based on the degree of kidney injury. At least 10 random fields were assessed under the microscope (original magnification 200), and an average score was calculated for each mouse. To visualize interstitial collagen deposition, sections were stained with anti-type I collagen as previously described (18).

**Western blots**

Kidney tissues (20 mg) were homogenized in radioimmunoprecipitation assay buffer (Sigma-Aldrich). Concentrations of protein were quantitated using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of sample were subjected to electrophoresis
through 4% to 12% Bis-Tris gels and transferred to polyvinylidene difluoride membranes. After blocking with 5% milk in Tris-buffered saline solution with Tween 20, the blots were incubated with anti-collagen type I antibody (catalog No. 1310-01, Southern Biotech, Birmingham, AL), FN (catalog No. 2413, Abcam, Cambridge, UK) or anti-glyceraldehyde-3-phosphate dehydrogenase (catalog No. 2118, Cell Signaling Technology, Danvers, MA) overnight in 4°C. The blots were then washed and incubated for 1 hour at room temperature with individual secondary antibodies accordingly. Bands were detected using an enhanced chemiluminescence detection system (Bio-Rad). The detected bands were quantified by densitometry through ImageJ 1.38 for windows.

**Kidney flow cytometry and T cell isolation**

Kidney single-cell suspensions were prepared by mechanical and enzymatic digestion as described previously (18). To achieve homogenization, obstructed kidneys underwent mechanical dissociation and enzymatic digestion. Cell suspensions were then filtered through 70- and 40-μm cell strainers. The resulting single cells were incubated with Fc Block for 20 minutes at 4°C. For kidney flow cytometry, single cells were stained with CD45, CD8, CD4, CD44, CD62L, CD11b, Ly6G, CD64, TNFα and near-infrared dead cell indicator (cat: L34976, Life Technologies) for 30 minutes at 4°C. Cells were washed and fixed with Fix/Perm buffer (catalog No. 554655, BD Biosciences, San Jose, CA). Then, 20 μl of CountBright absolute counting beads (catalog No. C36950, Invitrogen, Carlsbad, CA) were added to cells, and samples were analyzed on an LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ). For T cell isolation, the single cells were stained with CD45, CD8, CD4 and DAPI, and then subjected to fluorescent cell sorting. The gating strategy was as follows: first gate on single live cells, second gate on CD45+ cells, and third gate on CD4 vs. CD8 cells. The single CD4 positive cells and single CD8 positive cells were isolated and saved for real-time PCR.
T lymphocyte chemotaxis assays

Naïve splenic T lymphocytes were isolated using the Mouse T cell Isolation Kit-negative Selection (cat: 19851A, STEMCELL). The purified T cells (1X10^5/ml) were resuspended in 100 μl medium (RPMI 1640 supplemented with 1% BSA) and added to each insert (diameter, 6.5 mm) with 8-μm pore size (cat: CLS3464, Corning) polyester membranes. Then vehicle or 100 ng/ml CCL5-containing migration medium (500 μl) was placed in the lower compartment of the well. After 3 hours, the number of migrated T cells was determined by using a hemocytometer to count the cells present in the lower well media.

Statistical Analyses

The values of each parameter within a group are expressed as the mean± SEM. For comparisons between groups with normally distributed data, statistical significance was assessed using the two-tailed unpaired t test. For comparisons between groups with non-normally distributed variables, we used the Wilcoxon test.

Results

Generation of mice with genetic deletion of Twist1 in T lymphocytes.

To examine the functions of Twist1 in T cells during kidney interstitial fibrosis, we ablated Twist1 in T lymphocytes by using a Cre-Lox gene targeting approach. During the double positive phase of maturation in the thymus, all thymocytes destined to become single positive CD4^+ or CD8^+ circulating T lymphocytes express the CD4 promoter. Accordingly, by breeding a CD4-Cre mouse line with a double fluorescence reporter mouse (mT/mG) we confirmed robust Cre expression marked by GFP within the thymus and spleen of CD4-Cre^+ mT/mG mice but the absence of Cre expression marked by red fluorescent protein. There were also sparse GFP-positive T cells around kidney tubular cells in naïve CD4-Cre^+ mT/mG kidneys (Figure 1A). We therefore bred the CD4-Cre mouse line with a Twist1 flox line harboring loxP sites on either side
of the coding region for the transcription factor Twist1 (Figure 1B). For our experiments, we used \( CD4-Cre^+ \) Twist1\(^{\text{flox/flox}} \) mice (Twist1 TKO) and \( CD4-Cre^- \) Twist1\(^{\text{flox/flox}} \) (WT) littermates (Figure 1C). To confirm T cell-specific deletion of Twist1 in our Twist1 TKO animals, we labeled splenocytes for T and B lymphocyte markers and isolated CD4\(^+\) T cells (CD4\(^+\)CD8\(^-\)CD19\(^-\)), CD8\(^+\) T cells (CD8\(^+\)CD4\(^-\)CD19\(^-\)), and B lymphocytes (CD19\(^+\)Thy1\(^-\)) via fluorescent cell sorting as in previous studies (26). Using mRNA from these immune cells and from kidney and heart, we then performed real-time PCR for the gene encoding Twist1 to confirm the degree and precision of T cell-specific deletion. Compared with their WT littermates, Twist1 TKO mice exhibited at least >85% deletion of Twist1 from both CD4\(^+\) and CD8\(^+\) T cells but preserved Twist1 expression in B cells and all other tissues examined (Figure 1D).

**Twist1-deficiency in T cells attenuates kidney interstitial fibrosis induced by UUO.**

Twist1 in both renal tubular cells and myeloid cells plays an important role in renal fibrogenesis, but the actions of Twist1 in lymphocytes during renal interstitial fibrosis have not been established. Studies from several groups show prominent accumulation of T lymphocytes in fibrotic kidney after UUO (4, 5, 7). We detected enhanced expression of Twist1 mRNA in renal T cells following UUO compared to Twist1 levels from splenic T cells in naïve animals (Supplemental Figure 1). WT and Twist1 TKO mice were therefore subjected to UUO. We then evaluated whether ablating Twist1 in T lymphocytes influences the progression of UUO-induced interstitial fibrosis. The weights of the UUO kidneys were similar between Twist1 TKO and WT groups (Supplemental Figure 2). We measured mRNA and protein levels for collagen, fibronectin (FN) and fibrosis mediators in UUO-induced fibrosis. First, immunohistochemistry staining for collagen I (Col-I) of kidney sections after UUO revealed diffuse interstitial collagen deposition in both groups. Twist1 TKO mice exhibited less severe kidney fibrosis at 2 weeks after UUO compared with WTs as quantified by Col-I staining area (Figure 2A-B). At day 14 after UUO, mRNA expressions for Col-I, FN, transforming growth factor β1 (TGF-β1) and...
plasminogen activator inhibitor-1 (PAI-1) were significantly less in Twist1 TKO kidneys compared with WT mice (Figure 2C). By Western blot analysis, UUO kidneys from Twist1 TKO animals showed markedly lower levels of Col-I and FN protein than WTs (Figure 2D-E).

Compared with kidneys from WT controls, obstructed kidneys from Twist1 TKO animals also showed less kidney injury based on blinded pathology scores (Figure 3A-B) and gene expression for the kidney injury marker, neutrophil gelatinase-associated lipocalin (NGAL) (Figure 3C). These data indicate that Twist1 in T cells exacerbates the severity of renal injury and fibrosis following ureteral obstruction.

**Twist1 in T lymphocytes constrains renal accumulation of activated CD8+ T cells**

Next, we examined leukocyte accumulation including T lymphocytes, neutrophils and monocytes/macrophages in the kidney after ureteral obstruction. At day 14 UUO, we isolated infiltrating CD45+ cells from the injured kidney via flow cytometry (Supplemental Figure 3) and quantified the numbers of CD4+ and CD8+ T lymphocytes, CD11b+Ly6G+ neutrophils, and CD11b+CD64+ monocytes/macrophages and, within these groups, naïve and effector memory T cells marked by CD62LhiCD44lo and CD62LloCD44hi expression, respectively (27-30). As shown in Figure 4A-B, the absolute numbers of CD8+ T lymphocytes from Twist1 TKO kidneys after UUO were significantly higher than from WT obstructed kidneys. While the numbers of CD4+ T cells were increased in the Twist1 TKO kidneys, this difference did not achieve statistical significance (p=0.11). Numbers of effector memory T cells marked by CD62LloCD44hi expression were markedly increased within the CD8+ T cell subset (Figure 4C-D) from the Twist1 TKO kidneys but were not significantly different from WTs in CD4+ T cell subset (Figure 4E-F). Numbers of Ly6G+ neutrophils and CD64+ monocytes/macrophages were also similar in the kidneys from the 2 groups (Supplemental Figure 4). We then investigated the role of Twist1 in T cell migration and splenic T cell numbers after UUO using an *in vitro* trans-well system and flow cytometry, respectively. No differences in migratory capacity of T cells *in vitro* or splenic T
cell numbers *in vivo* were found between the Twist1 TKO and WT groups (Supplemental Figures 5 and 6). Prior studies have demonstrated that Twist1 attenuates proinflammatory cytokine expression by suppressing NF-κB activation. TNFα, IL-1β and IL-17A, all regulated by Twist1, have each been implicated in renal fibrogenesis (19, 20, 23). We therefore investigated the effects of Twist1 on the cytokine expression in CD4+ and CD8+ T lymphocytes infiltrating the obstructed kidney. To this end, we used fluorescent cell sorting to isolate CD4+ and CD8+ T cells from UUO kidney at day 14 and measured mRNA expression for these cytokines (Figure 5A). As shown in Figure 5B-D, we were able to detect mRNA for TNFα (TNF), IL-1β and IL-17A in these T cells. We found mRNA expression for TNF, but not IL-1β or IL-17A, were significantly increased in the CD8+ T cells isolated from the Twist1 TKO kidneys compared to WT controls. By contrast, CD4+ T cell expression of TNF, IL-1β and IL-17A were all similar in the Twist1 TKO and WT kidneys after UUO (Figure 5B-D). To further confirm the expression of TNF at the protein level, we used flow cytometry to perform intracellular stains for TNF in CD8+ T cells isolated from the obstructed kidney (Figure 5E). By this metric, the absolute numbers of CD8+TNF+ T lymphocytes from Twist1 TKO kidneys after UUO were significantly higher than from WT obstructed kidneys (Figure 5F), and fluorescent stains of obstructed kidney corroborated this finding (Figure 5G). TNFα can promote renal fibrogenesis as global inhibition of TNF curtails renal scar formation (31). On the other hand, TNF can inhibit fibroblast activation or promote matrix metallopeptidase production (32), and may act selectively within T cells to restrain renal damage (33). We therefore posited that TNFα produced by CD8+ T cells may play a protective role in kidney fibrogenesis.

**Deletion of TNFα in T cells enhances UUO-induced kidney fibrosis and injury.**

To investigate the actions of TNFα in T cells in the pathogenesis of kidney fibrosis, we deleted TNFα in T cells using a Cre-Lox approach (Supplemental Figure 7) and subjected TNF TKO
and WT control mice to UUO (Figure 6A). The extent of fibrosis was examined after 14 days by scoring of collagen-1 stains and Western blot of kidney fibrosis markers. Blinded scoring of collagen-1 stains revealed that the obstructed kidneys from TNF TKO mice exhibited significantly more deposition of collagen than WT littermates (Figure 6B-C). mRNA expression for TGF-β1 was significantly higher in obstructed TNF TKO kidneys compared with WT mice (Figure 6D). Western blot analysis confirmed that the TNF TKO obstructed kidneys expressed higher levels of FN and Col-I (Figure 6E-F). Moreover, blinded injury scores indicated that tubular injury in TNF TKO kidneys after UUO was more severe than that in WTs (Figure 7A-B). These findings were confirmed at the mRNA level for NGAL (Figure 7C). Collectively, these data suggest that TNFα from T cells in Twist1 TKO kidneys attenuates UUO-induced kidney fibrosis and injury.

Discussion

Regardless of etiology, renal fibrosis typically features a robust inflammatory response, leading to the accumulation of large numbers of T cells and myeloid cells in the injured kidney. T lymphocyte-elicited immune responses exert critical roles in the initiation and development of kidney disease. CD8+ T cells in particular play a complex part in regulating kidney scar formation (14, 15, 28). In the current study we find that Twist1 in lymphocytes promotes kidney fibrogenesis by limiting CD8+ T cell accumulation, activation, and TNFα production after UUO.

CD4+ and CD8+ T lymphocytes play divergent roles in kidney fibrogenesis. Transfer of CD4+ but not CD8+ T cells into lymphocyte-deficient recipients exacerbates kidney scar formation (7), likely due to the profibrotic effects of multiple CD4+ T cell subpopulations. For example, the transcription factor T-bet that drives differentiation of pro-inflammatory CD4+ “Th1” cells is required to mediate full fibrotic response in the injured kidney (4). Despite elaborating a completely different cytokine profile, anti-inflammatory CD4+ “Th2” cells also worsen the
progression of renal fibrosis (34). By contrast, CD8$^+$ T cells may constrain kidney fibrosis. For example, recruiting CD8$^+$ T cells into the kidney inhibits activation of the myofibroblasts that produce collagen and in turn protects against renal fibrosis (13). Inversely, depleting CD8$^+$ T cells augments renal fibrosis following ureteral obstruction by facilitating CD4$^+$ T cell differentiation toward a Th2 phenotype (14). As another mechanism for CD8$^+$ T cell protection from renal fibrosis, Wang et al. reported that CD8$^+$ T cells co-expressing CD11c could induce fibroblast death via the secretion of several cytotoxic mediators in the setting of kidney fibrosis (15). Analogously cytotoxic CD8$^+$ T cells can limit cancer growth by killing tumor-associated fibroblasts (35, 36). In our current studies, to determine the impact of Twist1 on the accumulation and activation of T lymphocytes after UUO injury, we measured the numbers of CD62L$^{lo}$CD44$^{hi}$ effector memory T cells in the UUO kidneys by flow cytometry. These data indicate that Twist1 in T cells limits the accumulation and activation of CD8$^+$ T cells in the kidney and aggravates renal fibrosis. However, in our studies the augmented renal infiltration of protective CD8$^+$ T cells was not associated with clear alterations in the phenotype of accompanying renal CD4$^+$ T cells. Rather, Twist1 blunted the generation of TNF$\alpha$ in the infiltrating CD8$^+$ T cells, consistent with its capacity to restrain cytokine production by mononuclear cells during chronic disease (19, 37). As we detected no difference in migratory capacity between WT and Twist1 knockout T cells or T cell numbers in WT and Twist1 TKO spleens after UUO, we speculate that the enhanced CD8$^+$ T cell accumulation in Twist1 TKO obstructed kidneys may relate to effects of Twist 1 on T cell survival in the obstructed kidney or rates of T cell egress from the injured kidney. In this regard, TNF$\alpha$ has been shown to promote T cell survival (38, 39).

In our studies, Twist1 restrained expression of TNF$\alpha$, but not IL-17A or IL1$\beta$, in CD8$^+$ T cells infiltrating the obstructed kidney. In other systems, Twist1 can also regulate IL-17A and IL1$\beta$ expression, so this isolated effect of Twist1 on T cell TNF may be specific to the UUO
microenvironment (19, 20). TNFα is a pleiotropic cytokine exhibiting a dual effect on fibrogenesis that may depend on its cellular source or site of injury, so we posited that TNF in T cells could be mediating renal protection in our model. Consistent with this notion, we found that deletion of TNFα selectively from T lymphocytes augmented extracellular matrix deposition and kidney injury after UUO. Our studies cannot distinguish whether the primary effect of TNF in this context was to limit tubular injury, interstitial fibrosis, or both. Regardless, this finding contrasts with a multitude of studies implicating TNF in tissue fibrogenesis. For example, treatment with TNFα antagonists attenuate silica- and bleomycin induced pulmonary fibrosis (40, 41), whereas continuous infusion of recombinant TNFα enhances scar formation in the lung (40), and overexpression of TNF in the lungs promotes local fibroblast accumulation and collagen deposition (42). On the other hand, inhibiting TNFα worsened lung fibrosis in a patient with systemic sclerosis and led to progression of fibrosis (43). Moreover, TNFα inhibits the synthesis of type I collagen, type III collagen and fibronectin in cultured dermal fibroblasts (44, 45), and reduces the numbers of fibrocytes and deposition of collagen I in the obstructed kidney (8). On a molecular level, TNF may blunt collagen accumulation through interactions with the classical NF-κB and TGF-β1/Smad signaling pathways or matrix degrading enzymes system (32). Our current studies corroborate a protective function for TNF in T lymphocytes during renal fibrogenesis after UUO and are in agreement with our finding that TNF in T cells can restrain inflammatory injury to the glomerulus (33).

Collectively, these data indicate that Twist1 in T lymphocytes promotes kidney injury and fibrosis potentially via Twist1’s effects to suppress TNFα generation in infiltrating T cells. Our studies suggest that blocking TNFα directly in T lymphocytes could exacerbate kidney scar formation, warranting further scrutiny to identify targets that act downstream of Twist1 and TNF in T cells to regulate the pathogenesis of kidney fibrosis.
Disclosures
J. Privratsky reports Scientific Advisor or Membership: Life Sciences journal; Association of University Anesthesiologists scientific advisory board. All remaining authors have nothing to disclose.

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Authors Contributions
J Ren: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Writing - original draft; Writing - review and editing
X Lu: Data curation; Project administration
R Griffiths: Investigation; Methodology; Project administration
J Privratsky: Data curation; Formal analysis; Methodology; Writing - review and editing
S Crowley: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision; Writing - review and edit

Supplemental Material
Supplemental Figure 1. mRNA levels of Twist1 in CD3+ T lymphocytes from naïve spleens and obstructed kidneys (“UUO”).
Supplemental Figure 2. The weights of UUO kidneys from WT and Twist1 TKO mice.
Supplemental Figure 3. The gating strategy for excluding cellular debris and analyzing viable CD4+ and CD8+ T cells from UUO kidneys at day 14.
Supplemental Figure 4. Absolute numbers of neutrophils and monocytes/macrophages from Twist1 TKO and WT UUO kidneys are similar.
Supplemental Figure 5. No differences in migratory capacity between WT and Twist1 TKO T cells (n=3-6).
Supplemental Figure 6. Splenic T cells numbers in WT and Twist1 TKO mice after UUO are similar.
Supplemental Figure 7. Verification of TNF deficiency in TNF T cell knockout (TNF TKO) mice.
References


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Figure 1. Verification of Twist1 deficiency in Twist1 T cell knockout (Twist1 TKO) mice. (A) Representative histology of thymus, spleen, and kidney in CD4-Cre+ mT/mG and control (CD4-Cre- mT/mG) mice. Green fluorescence indicates the presence of CD4 Cre expression whereas red fluorescence indicates the absence of CD4-Cre expression. Blue fluorescence in kidney is a nuclear DAPI stain. Scale bar, 20 μm (B) Strategy for generating mice with T lymphocyte-specific deletion of Twist1. (C) Genotyping the mice by PCR analysis of genomic DNA. (D) mRNA expression for Twist1 in T lymphocyte subsets, CD4+ and CD8+ T cells, B cells, whole kidney, and heart from WT and Twist1 TKO animals (n= 6). Data represent the mean± SEM. *p<0.05. fl, flox; Twist1 TKO, CD4-Cre+ Twist1fl/fl. WT, wild-type.

Figure 2. Twist1 deficiency in T cells attenuates kidney fibrosis after ureteral obstruction. (A) Representative sections from obstructed WT and Twist1 TKO kidneys stained with Col-I at day 14 after UUO. Scale bar, 100 μm (B) Blinded morphometric quantitation of Col-I (n=7). (C) mRNA levels for Col-I, FN, PAI-1 and TGF-β1 in contralateral (“CTL”) and obstructed (“UUO”) kidneys (n=7-11). (D) Western blot for FN and Col-I protein in contralateral (“CTL”) and obstructed (“UUO”) kidneys from WT and Twist1 TKO mice at day 14. (E) Semiquantitative determination of FN and Col-I protein levels (n=7). Data represent the mean± SEM. *p<0.05, #p<0.05. Col-I, type I collagen; FN, fibronectin; TGF-β1, transforming growth factor-β1; PAI-1, plasminogen activator inhibitor 1 Twist1 TKO, CD4-Cre+ Twist1fl/fl. WT, wild-type.

Figure 3. Twist1 deletion in T lymphocytes ameliorates UUO-induced kidney injury. (A) Representative images of PAS-stained sections of obstructed kidneys from WT and Twist1 TKO mice. Scale bar, 100 μm (B) WT and Twist1 TKO obstructed kidney pathology scores (n= 7-11). (C) mRNA levels for NGAL in contralateral and obstructed kidneys at day 14. Data represent the mean± SEM. *p< 0.05. PAS, Periodic acid-Schiff; CTL, contralateral; NGAL, neutrophil gelatinase-associated lipocalin; Twist1 TKO, CD4-Cre+ Twist1fl/fl. WT, wild-type.

Figure 4. Twist1 in T cells constrains CD8+ T cell accumulation and activation in the fibrosing kidney. (A) Representative plots for CD4+ and CD8+ T cell subsets from WT and Twist1 TKO UUO kidneys. (B) Absolute numbers of CD4+ and CD8+ T cells per mg of WT and Twist1 TKO kidney after UUO (n= 7-8). (C, E) Representative plots for CD44 versus CD62 staining from CD8+ (C) and CD4+ (E) T cell subset of WT and Twist1 TKO UUO kidneys. (D, F) Absolute numbers of CD8+CD44+CD62L- (D) and CD4+CD44+CD62L- (F) T cells per gram of WT and Twist1 TKO kidney after UUO (n= 7-8). Data represent the mean± SEM. *p< 0.05. Twist1 TKO, CD4-Cre+ Twist1fl/fl. WT, wild-type.

Figure 5 CD8+ T cells from UUO kidneys of Twist1 TKO animals have augmented TNFα expression. (A) Representative flow plot of CD4 versus CD8 staining on single cell suspensions from obstructed kidney of a WT mouse at day 14. (B-D) mRNA levels for TNFα (B), IL-17A (C) and IL-1β (D) in CD4+ and CD8+ T cells from WT and Twist1 TKO UUO kidneys (n= 7-9). (E) Representative plots for TNFα staining in CD8+ T cells from WT and Twist1 TKO UUO kidneys. (F) Absolute numbers of CD8+TNFα+ T cells per mg of WT and Twist1 TKO kidney after
UUO (n= 6). (G) Representative sections from obstructed WT and Twist1 TKO kidneys stained for TNF at day 14 after UUO. Scale bar, 40 µm. Data represent the mean± SEM. *p< 0.05.
Twist1 TKO, CD4-Cre Twist1fl/fl. WT, wild-type.

**Figure 6 TNF in T lymphocytes protects against kidney fibrosis after UUO.** (A) Genotyping the mice by PCR analysis of genomic DNA. (B) Representative sections from obstructed WT and TNF TKO kidneys at day 14 stained with Col-I. Scale bar, 100 µm. (C) Blinded morphometric quantitation of Col-I (n= 6) from groups as indicated. (D) mRNA levels for TGF-β1 in contralateral (CTL) and obstructed (“UUO”) kidneys (n=8). (E) Western blot for FN and Col-I protein in contralateral (“CTL”) and obstructed (“UUO”) kidneys from WT and TNF TKO mice at day 14. (F) Semiquantitative determination of FN and Col-I protein in Figure D (n= 6). Data represent the mean± SEM. *p< 0.05, #p< 0.05. FN, fibronectin; Col-I, type I collagen; UUO, unilateral ureteral obstruction; TNF TKO, CD4-Cre Twist1fl/fl. WT, wild-type.

**Figure 7 TNF deletion in T cells enhances UUO-induced kidney injury.** (A) Representative images of PAS-stained sections of obstructed kidneys from WT and TNF TKO mice. Scale bar, 100 µm. (B) WT and TNF TKO obstructed kidney pathology scores (n=8). (C) Renal mRNA levels for NGAL from contralateral and obstructed kidneys (n=8). Data represent the mean± SEM. *p< 0.05. UUO, unilateral ureteral obstruction; TNF TKO, CD4-Cre Twist1fl/fl. WT, wild-type; CTL, contralateral; NGAL, neutrophil gelatinase-associated lipocalin.
Figure 4

A

B

C

D

E

F

CD8

CD8

CD4

CD4

CD4

CD62L

CD62L

CD62L

CD4

CD4

CD4

CD8+ T cells

CD4+ T cells

CD8+CD44+CD62L− T cells

CD4+CD44+CD62L− T cells

WT

Twist1 TKO

p=0.11

*
Figure 6

A) Western blot analysis showing flox = 295 bp, wt = 254 bp, Cre = 445 bp, and wt = β-actin = 322 bp.

B) Images comparing WT and TNF TKO for Collagen I.

C) Graph showing Col-I area score (% per field) for UUO.

D) mRNA fold change graph comparing TGF-β1 expression in WT and TNF TKO.

E) Western blot analysis for FN and Col-I with KO comparison.

F) Protein fold change graphs for FN and Col-I comparing CTL and UUO.