Epithelial Cell TGFβ Signaling Induces Acute Tubular Injury and Interstitial Inflammation

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ABSTRACT

TGFβ signaling plays a central role in the development of acute and chronic kidney diseases. Previous in vivo studies involved systemic alteration of TGFβ signaling, however, limiting conclusions about the direct role of TGFβ in tubular cell injury. Here, we generated a double transgenic mouse that inducibly expresses a ligand-independent constitutively active TGFβ receptor type 1 (TβR1) kinase specifically in tubular epithelial cells, with expression restricted by the Pax8 promoter. In this model, activation of TGFβ signaling in the tubular epithelium alone was sufficient to cause AKI characterized by marked tubular cell apoptosis and necrosis, oxidative stress, dedifferentiation and regenerative cell proliferation, reduced renal function, and interstitial accumulation of inflammatory cells. This tubular injury was associated with mitochondrial-derived generation of reactive oxygen species (ROS), but cell damage and apoptosis were partially independent of mitochondrial-derived ROS. TβR1 signaling-induced tubular injury also associated with significant leukocyte infiltration consisting of F4/80+ macrophages, CD11c+ F4/80+ dendritic cells, CD11c+ F4/80− Ly6Chigh dendritic cells/monocytes, and T cells. Inhibition of mitochondrial-derived ROS significantly reduced accumulation of CD11c+ F4/80+ dendritic cells and T cells, suggesting a role for ROS in the activation and recruitment of the adaptive immune response to tubular injury. Taken together, these results suggest that TGFβ signaling in the tubular epithelium alone is sufficient to cause acute tubular injury and inflammation; therefore, TGFβ may be a mechanistic link between acute injury and chronic progression of kidney disease.


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TGFβ signaling plays a central role in the development of acute and chronic kidney diseases through regulation of cell differentiation and proliferation, apoptosis, immune responses, and extracellular matrix remodeling.1 TGFβ promotes heterodimerization of TGFβ receptor type 1 (TβR1) and type 2 as well as consequent activation of TβR1 kinase with canonical signaling via phosphorylation and activation of Smad2 and 3, which mediate transcription of target genes.2,3 Inhibiting TGFβ signaling in vivo by neutralizing antibody or disruption of the Smad3 gene attenuates tubular epithelial apoptosis and atrophy, inflammatory leukocyte infiltration, and interstitial fibrosis in murine unilateral ureteral obstruction (UUO) models.4,5 Conversely, transgenic mice overexpressing TGFβ develop progressive glomerulosclerosis, tubular atrophy, and interstitial fibrosis,6–8 strongly suggesting that TGFβ signaling is central to inducing renal diseases. However, overexpression of TGFβ, disruption of Smad3, or administration of a neutralizing TGFβ antibody all had systemic effects on TGFβ signaling, affecting multiple renal and extrarenal cell types, thereby limiting interpretation of
TGF-β’s renal cell type-selective actions. TGF-β can also induce generation of reactive oxygen species (ROS) \textit{in vitro},\textsuperscript{9–12} and mitochondrial dysfunction and ROS generation are important in tubular injury and apoptosis in acute and chronic kidney injury.\textsuperscript{13–15} Initial reports suggested a role for TGF-β predominantly in activation of interstitial (myo)fibroblasts manifesting with progressive fibrogenesis and extracellular matrix accumulation.\textsuperscript{16} However, it remains unclear whether interstitial cell activation and fibrogenesis are direct targets or secondary phenomena dependent on primary epithelial damage induced by TGF-β. For example, tubular cell injury and ROS generation result in local leukocyte accumulation, predominantly mononuclear phagocytes (including macrophages, dendritic cells, and monocytes).\textsuperscript{17,18} Dendritic cells and macrophages can mediate direct tissue injury through generation of ROS and secretion of proinflammatory mediators,\textsuperscript{17–24} but also play an important role in the repair process by promoting survival and proliferation of tubular epithelial cells, and clearance of cell and matrix debris.\textsuperscript{22,23,25,26}

The aim of this study was to determine the \textit{in vivo} role of TGF-β signaling restricted specifically to tubular epithelial cells. To this aim, we generated a double transgenic mouse system for inducible expression of ligand-independent, constitutively active TbR1 kinase\textsuperscript{26} specifically in tubular epithelial cells. Induction of TGF-β signaling in the tubular epithelium rapidly resulted in an AKI characterized by tubular injury, apoptosis, necrosis, ROS generation, interstitial inflammatory cell infiltration, and reduced renal function. Although inhibition of mitochondrial-derived ROS significantly reduced the inflammatory cell response, the overall tubular injury including tubular epithelial cell apoptosis and necrosis remained largely unaffected. Thus, activation of TGF-β signaling restricted to epithelial cells is sufficient to

\begin{figure}[h]
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\caption{Pax8-rTATA/TetO-TbR1(AAD) animals inducibly express constitutively active TbR1 kinase in the tubular epithelium after doxycycline treatment. (A) The Pax8 promoter drives expression of rTATA in the tubular epithelium, which in the presence of doxycycline (Dox), binds to the tetracycline response element and drives expression of constitutively active TbR1 kinase (AAD). (B) Fold change in whole kidney TbR1(AAD) transgene and PAI mRNA expression in Pax8-rTATA/TetO-TbR1(AAD) animals treated with doxycycline for 1–6 days relative to untreated Pax8-rTATA/TetO-TbR1(AAD) (control) animals is determined by quantitative real-time PCR. Data are the mean ± SD of at least five animals per group and one-way ANOVA with Tukey post-test is used to statistically compare control and doxycycline treatments, with $P<0.05$ considered significant. (C) The presence of phospho-Smad in whole kidney lysates from untreated control and doxycycline-treated animals is detected by Western blot. Tubulin is included as a loading control. (D) Representative images of double immunofluorescence staining for Smad2/3 (green) and DAPI (blue) showing nuclear localization for Smad2/3 after induction of TbR1(AAD) expression in Pax8-rTATA/TetO-TbR1(AAD) animals treated with doxycycline for 1, 3, or 5 days but not for control untreated Pax8-rTATA/TetO-TbR1(AAD) or TbR1(AAD) single transgenic animals. (E) Tubular localization of Smad2/3+ nuclei is confirmed with triple immunofluorescence staining for the following: (upper) Smad2/3 (red), proximal tubular marker LTG (green), and DAPI (blue); (lower) Smad2/3 (green), distal tubular marker DBA (red), and DAPI (blue). *$P<0.05$; **$P<0.01$; ***$P<0.001$. Original magnification, ×400 in D.}
\end{figure}
cause a full-blown picture of acute tubular injury with progression toward tubular atrophy. Interstitial inflammatory cell infiltration was partially, specifically dendritic cells and T cells, dependent on epithelial injury–associated mitochondrial-derived ROS. Our findings, that TGFβ signaling in tubular epithelial cells 
per se can initiate acute and progressive tubular injury, may represent a direct link for the development of AKI to CKD.

RESULTS

Generation of a Transgenic Mouse Model for Inducible, Tubular Epithelium–Specific Expression of a Constitutively Active TβR1
To define the role of TGFβ signaling in tubular injury, we generated Pax8-rtTA/TetO-TβR1(AAD) double transgenic mice that express a constitutively active, ligand-independent TβR1 kinase mutant (TβR1 (AAD)) selectively in tubular epithelial cells in a doxycycline-inducible manner under the control of the Pax8 promoter27,28 (summarized in Figure 1A). Significant induction of TβR1(AAD) mRNA was detected within 1–2 days of doxycycline treatment compared with untreated double transgenic animals (Figure 1B). Rapid (within 1–2 days) and sustained (3–6 days) activation of TβR1 was confirmed by increased mRNA expression of the TGFβ-Smad target gene plasminogen activator inhibitor 1 (PAI-1) (Figure 1B), Western blot for phosphorylated (TβR1-activated) Smad2 on whole kidney lysates (Figure 1C), and nuclear staining for Smad2/32 (Figure 1D). Combining Smad2/3 staining with markers of proximal and distal tubular segments—Lotus tetragonolobus (LTG) lectin and Dolichos biflorus agglutinin (DBA), respectively—confirmed that expression and activity of TβR1(AAD) was limited to tubular epithelial cells (and not seen in infiltrating immune cells at early time points), with approximately 50% of proximal and 35% of distal tubular nuclei staining for nuclear Smad2/3 (Figure 1E).

Induction of TβR1 Signaling in the Tubules Results in Marked Tubular Cell Damage, Apoptosis, and Proliferation
To assess histologic features of tubular injury, sections stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) were examined by an experienced renal pathologist (V.D.D.) in a blinded manner. Induction of TβR1(AAD) rapidly resulted in tubular injury with swelling and vacuolization, prominent epithelial apoptosis, and necrosis (Figure 2, Supplemental Figure 1, and Table 1). Induction of TβR1 signaling in the tubular epithelium increased the number of apoptotic cells comparable with what is observed in human and experimental kidney diseases.29 Increased rate of apoptosis was confirmed by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (Figure 3, A and B). However, although histologic examination clearly distinguishes tubular and interstitial inflammation, sections stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) were examined by an experienced renal pathologist (V.D.D.) in a blinded manner. Induction of TβR1(AAD) rapidly resulted in tubular injury with swelling and vacuolization, prominent epithelial apoptosis, and necrosis (Figure 2, Supplemental Figure 1, and Table 1). Induction of TβR1 signaling in the tubular epithelium increased the number of apoptotic cells comparable with what is observed in human and experimental kidney diseases.29 Increased rate of apoptosis was confirmed by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (Figure 3, A and B). However, although histologic examination clearly distinguishes tubular and interstitial inflammation, sections stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) were examined by an experienced renal pathologist (V.D.D.) in a blinded manner. Induction of TβR1(AAD) rapidly resulted in tubular injury with swelling and vacuolization, prominent epithelial apoptosis, and necrosis (Figure 2, Supplemental Figure 1, and Table 1). Induction of TβR1 signaling in the tubular epithelium increased the number of apoptotic cells comparable with what is observed in human and experimental kidney diseases.29 Increased rate of apoptosis was confirmed by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (Figure 3, A and B). However, although histologic examination clearly distinguishes tubular and interstitial inflammation, sections stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) were examined by an experienced renal pathologist (V.D.D.) in a blinded manner. Induction of TβR1(AAD) rapidly resulted in tubular injury with swelling and vacuolization, prominent epithelial apoptosis, and necrosis (Figure 2, Supplemental Figure 1, and Table 1). Induction of TβR1 signaling in the tubular epithelium increased the number of apoptotic cells comparable with what is observed in human and experimental kidney diseases.29 Increased rate of apoptosis was confirmed by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining (Figure 3, A and B).

Figure 2. Induction of constitutively active TβR1 kinase signaling results in marked tubular epithelial cell damage, apoptosis, necrosis, and interstitial inflammation. (A) Representative images of H&E- and PAS-stained kidney sections from Pax8-rtTA/TetO-TβR1(AAD) mice treated with doxycycline for 5 days demonstrate that induction of constitutively active TβR1 signaling results in marked interstitial inflammation and tubular cell injury compared with untreated (control) mice. (B) High-power images further demonstrate considerable tubular epithelial apoptosis (open arrows, middle and right panel), necrosis (closed arrows, left panel), and mitosis (arrowhead, center panel), as well as tubular vacuolization (asterisk, middle panel) and the early stages of tubular atrophy (white circle, right panel) after 5 days on doxycycline. Original magnification, ×200 in A; ×600 in B.
cell apoptosis/necrosis (Table 1), quantitation of apoptosis by TUNEL staining is confounded by indistinguishable tubular and interstitial TUNEL-positive cells (Figure 3, A and B).

Furthermore, tubular TβR1 signaling reduced E-cadherin but increased vimentin mRNA levels (Supplemental Figure 2), consistent with the observed epithelial dedifferentiation (Figure 2),30,31 Whereas serum creatinine was not significantly elevated compared with controls, BUN became significantly elevated after 5–6 days of doxycycline treatment (Figure 3C).

In AKI, tubular regeneration occurs in parallel with injury and involves proliferation of surviving epithelial cells and their precursors.30,31 We observed proximal and distal tubular regeneration by epithelial cell mitosis (Figure 2B). Costaining with markers for proximal (LTG) and distal (DBA) tubular cells confirmed that proliferation occurred within both injured proximal and distal tubules (Supplemental Figure 3, A and B).

Consistent with selective activation of TβR1(AAD) within the tubules, no lesions were observed within the glomeruli (Figure 2A) and urine albumin/creatinine ratios were unchanged (0.10±0.08 for control and 0.19±0.33 for day 5, n=9 for each).

### Epithelial Injury Induced by TβR1 Signaling Is Associated with Oxidative Stress and Recruitment of Inflammatory Leukocytes

Because cell injury is associated with oxidative stress, we stained for 3-nitrotyrosine residues and 8-oxoguanine (8-oxoG) as known oxidative stress-induced lesions to proteins and DNA.32,33 Induction of TβR1 signaling resulted in robust staining for both 3-nitrotyrosine and 8-oxoG at days 3 and 5 compared with controls (Figure 4A). 8-oxoG staining was punctate and did not colocalize with nuclear 4,6-diamidino-2-phenylindole (DAPI) staining, suggesting that it is mitochondrial. This was supported by costaining for mitochondrial transcription factor A, which colocalized with 8-oxoG staining (Supplemental Figure 4). Measurement of 8-oxo-deoxyguanosine (8-oxodG) in the urine was used to quantify oxidative damage, because 8-oxodG bases are excised and directly excreted as part of the repair process.34,35 Consistent with tissue staining, urinary 8-oxodG was increased by induction of TβR1 signaling from 4.6 nmol 8-oxodG/mg creatinine in controls to 28.1 after 3 days and 71.2 after 5 days on doxycycline (Figure 4C, data shown on a logarithmic scale). Thus, TβR1 signaling in the tubular epithelium is associated with ROS generation and oxidative mitochondrial DNA damage.

### Table 1. Histologic assessment of tubular cell injury in Pax8-rtTA/TetO-TβR1(AAD) animals, after induction with doxycycline for 3–4 or 5–6 days

<table>
<thead>
<tr>
<th>Histologic Feature</th>
<th>Control</th>
<th>Days 3–4</th>
<th>Days 5–6</th>
</tr>
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<tbody>
<tr>
<td>Tubular swelling</td>
<td>0.0±0.0</td>
<td>15.7±2.8*</td>
<td>9.0±1.0b</td>
</tr>
<tr>
<td>Tubular vacuolization</td>
<td>0.0±0.0</td>
<td>20.0±4.2c</td>
<td>19.0±4.0p</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>0.0±0.0</td>
<td>14.3±1.7c</td>
<td>15.0±3.5c</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0.0±0.0</td>
<td>11.4±0.9a</td>
<td>17.0±2.0a</td>
</tr>
<tr>
<td>Interstitial inflam.</td>
<td>0.0±0.0</td>
<td>25.7±2.3c</td>
<td>39.0±8.1a</td>
</tr>
</tbody>
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Data are mean ± SEM % of total cortical tubules exhibiting each pathologic feature and % total cortical area with interstitial inflammation (n=4 for controls, n=7 for days 3–4, and n=5 for days 5–6).

*P<0.001 compared with control.

bP<0.05 compared with control.

aP<0.01 compared with control.

**Figure 3.** Constitutively active TβR1 signaling induces tubular epithelial cell apoptosis. Pax8-rtTA/TetO-TβR1(AAD) mice are treated with doxycycline for 1–6 days. Controls include both single transgenic animals treated with doxycycline and untreated double transgenic animals. (A) Representative images of TUNEL staining of kidney sections. (B) TUNEL staining is quantified by direct counting of the number of positively staining cells (tubular and interstitial) per ×400 field. Each dot represents a single animal and is the mean of 10 randomly selected cortical fields counted for that animal. Line and bars represent the mean ± SEM for each treatment group (n=9 per group). (C) Serum creatinine and BUN levels (mg/dl) from at least eight mice per group. One-way ANOVA with Tukey post-test is used to statistically compare control and doxycycline treatments, with P<0.05 considered significant. *P<0.05; **P<0.01; ***P<0.001. Original magnification, ×400 in A and B.
Tissue damage and ROS generation cause recruitment of leukocytes to the site of injury. \(^{17,36}\) Staining for F4/80, CD11c, and CD3 demonstrated interstitial accumulation of macrophages, dendritic cells, and T cells after TβR1(AAD)-induced tubular injury (Figure 4, A and C). F4/80 is expressed on the surface of most renal macrophages and dendritic cells and CD11c is expressed on dendritic cells,\(^{18,37}\) whereas CD3 is expressed on T cells. Interstitial F4/80\(^+\) and CD11c\(^+\) staining was present in control animals, consistent with the steady-state renal network of dendritic cells and macrophages,\(^{18,38}\) but rose 4- and 9-fold, respectively, after induction of TβR1 signaling (Figure 4, A and C). Due to the irregular, stellate morphology of F4/80\(^+\) and CD11c\(^+\) cells, the percentage of positively staining area was calculated by morphometric quantification.\(^{39}\) CD3\(^+\) T cells were directly counted (Figure 4C) and increased 3-fold compared with controls. Ki67 staining demonstrated that up to 10% of F4/80\(^+\) and 25% of CD11c\(^+\) cells were proliferating at day 5 (Supplemental Figure 3, C and D).

**Characterization of Leukocyte Infiltrate by Flow Cytometry**

Leukocyte populations were characterized further by flow cytometry.\(^{19,40}\) Whole kidney cell suspensions were stained for expression of CD45, B220, CD3, NK1.1, and Ly6G as markers of total leukocytes, B cells, T cells, natural killer (NK) cells, and neutrophils, respectively. CD4 and

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**Figure 4.** Constitutively active TβR1 kinase signaling induces oxidative stress and recruitment of inflammatory cells, including macrophages, dendritic cells, and T cells. Pax8-rTA/TetO-TβR1(AAD) mice are treated with doxycycline for 1–6 days. Controls include both single transgenic animals treated with doxycycline, and untreated double transgenic animals. (A) Representative images of the kidney cortex are stained as follows: (top row) immunohistochemical staining for nitrotyrosine (brown) with hematoxylin counterstain (blue); (second row) double immunofluorescence staining for 8-oxoG (green) and DAPI (blue), and inset depicts an enlarged area from the same image; (third row) double immunofluorescence staining for dendritic cell macrophage marker F4/80 (red) and DAPI (blue); (fourth row) triple immunofluorescence staining for dendritic cell marker CD11c (red), T cell marker CD3 (green), and DAPI (blue). CD11c\(^+\) area increases 9-fold from 0.14% to 1.254% and T cell numbers increase 3-fold compared with controls. F4/80\(^+\) dendritic cells/macrophages are the largest population increasing 4.5-fold from 2% F4/80\(^+\) area in controls to 9% by day 5 of doxycycline administration. (B) Urinary 8-oxodG is quantified by high-performance liquid chromatography and is expressed relative to urine creatinine as Log nmol 8-oxodG/mg creatinine (at least 9 per group; lines and bars represent mean and SEM). (C) Quantification of F4/80, CD11c, and CD3 staining as percentage positive area for F4/80 or CD11c or the number of positive cells per \( \times 200 \) field for CD3. Each dot represents a single animal and is the mean of 10 randomly selected cortical fields counted for that animal. Line and bars represent the mean ± SEM for each treatment group (at least 6 per group). One-way ANOVA with Tukey post-test is used to statistically compare control and doxycycline treatments, with \( P < 0.05 \) considered significant. \( * P < 0.05; ** P < 0.01; *** P < 0.001 \). Original magnification, \( \times 630 \) (top row) and \( \times 400 \) (remaining rows) in A; \( \times 200 \) in C.
Figure 5. Flow analysis of infiltrating leukocytes recruited by constitutively active TβR1 signaling in the tubules, which reveals strong macrophage infiltration and a switch in the predominant dendritic cell subtype. (A) Representative example of the proportions of individual CD45⁺ populations within whole kidney cell suspension of a Pax8-rtTA/TetO-TβR1(AAD) mouse treated with doxycycline for 5 days. The following gates are used: CD45⁺ leukocytes (gate 1), CD45⁺ B220⁺ B cells (gate 2), CD45⁺ CD3⁺ T cells (gate 3), CD45⁺ CD3⁺ CD4⁺ helper T cells (gate 4), CD45⁺ CD3⁺ CD8⁺ cytotoxic T cells (gate 5), and CD45⁺ CD11c⁻ (gate 6) from which NK cells (CD45⁺ CD11c⁻ NK1.1⁺; gate 7), neutrophils (CD45⁺ CD11c⁻ Ly6G⁺; gate 8), and macrophages (CD45⁺ CD11c⁺ F4/80⁺; gate 9) are defined. Dendritic cells are defined as CD45⁺ CD11c⁺ (gate 10) and further subdivided into F4/80⁻ (gate 11) and F4/80⁺ (gate 12). (B)
CD8 expression was used to further subdivide T cells as \( T_{\text{helper}} \) or cytotoxic T cells. CD11c and F4/80 expression was used to define dendritic cell and macrophage populations. Figure 5A illustrates the gating strategy for a representative example from a Pax8-rtTA/TetO-T\( ^{R1}(AAD) \) animal treated with doxycycline for 5 days. The leukocytes made up approximately 1.5% of the kidney cell suspension, of which approximately 30% were T cells (65% \( T_{\text{helper}} \) and 35% cytotoxic T cells), 15% were B cells, 14% were NK cells, 2% were neutrophils, and 36% were dendritic cells/macrophages. The relative proportions of T cells, B cells, and neutrophils were not changed in day 5 doxycycline-treated animals compared with controls (data not shown). In contrast, the dendritic cell/macrophage populations changed significantly after tubular injury (Figure 5B). Of the three main dendritic cell/macrophage populations, the proportion of CD11c\(^{-} \) F4/80\(^{-} \) macrophages was strongly increased from 6% of leukocytes in controls to 17% at day 3 and 30% at day 5 of doxycycline administration. CD11c\(^{+} \) F4/80\(^{-} \) dendritic cells increased from 1.4% in controls to 4% at days 3 and 5. In contrast, CD11c\(^{-} \) F4/80\(^{+} \) dendritic cells were proportionally reduced from 6% of leukocytes in controls to 3.7% at day 3 and 2.4% at day 5 (Figure 5B). Both dendritic cell populations expressed CD11b (not shown). CD11c\(^{-} \) F4/80\(^{-} \) dendritic cells were Ly6C\( ^{\text{intermediate}} \), whereas CD11c\(^{+} \) F4/80\(^{-} \) dendritic cells were Ly6C\( ^{\text{high}} \) (Figure 5C) and expressed lower levels of MHC class II and the maturation markers CD80 and CD86 compared with CD11c\(^{-} \) F4/80\(^{+} \) dendritic cells (not shown), suggesting that CD11c\(^{-} \) F4/80\(^{+} \) dendritic cells are less mature and represent cells differentiating from monocytes recruited in response to tubular injury.42,43

**Scavenging Mitochondrial ROS Modulates Dendritic Cell and T Cell Recruitment, But Not Macrophage Infiltration or Tubular Epithelial Cell Apoptosis and Proliferation**

Because T\( ^{R1}(AAD) \)-induced tubular injury was associated with marked mitochondrial oxidative stress in tubular epithelial cells, we tested the role of mitochondrial-derived oxidative stress with systematic treatment with the mitochondrially targeted antioxidant MitoTEMPO (a SOD mimetic).\(^{44-46} \) Pax8-rtTA/TetO-T\( ^{R1}(AAD) \) mice were implanted with osmotic mini-pumps containing saline or MitoTEMPO 1 day before induction of T\( ^{R1}(AAD) \) with doxycycline. Staining for 8-oxoG and measurement of 8-oxodG in the urine confirmed that oxidative damage to DNA was significantly reduced, but not completely prevented, in mice treated with MitoTEMPO for 5 days (10.6 nmol 8-oxodG/mg creatinine compared with 29.1 for saline-treated animals (Figure 6, A and B) and 5 nmol 8-oxodG/mg creatinine for untreated healthy control animals). By histologic examination, features of tubular cell injury including vacuolization, apoptosis, and necrosis were approximately 50% reduced in MitoTEMPO-treated, compared with saline-treated animals, but this improvement did not reach statistical significance (Figure 6C and Table 2). However, infiltration of leukocytes by histology was significantly reduced by MitoTEMPO treatment (Table 2). Immunofluorescence staining demonstrated that specifically CD11c\(^{-} \) dendritic cells and CD3\(^{+} \) T cells were significantly reduced by MitoTEMPO treatment, but that there was no significant change in F4/80\(^{+} \) macrophage infiltration (Figure 7, A and B, and Table 2). Flow cytometry confirmed that MitoTEMPO treatment did not prevent the increase in proportion of CD11c\(^{-} \) F4/80\(^{-} \) macrophages or CD11c\(^{-} \) F4/80\(^{-} \) dendritic cells. The proportion of CD11c\(^{-} \) F4/80\(^{+} \) dendritic cell population from 12.4% in saline-treated animals to 6.1% with MitoTEMPO treatment (Figure 7C). MitoTEMPO had no effect on surface MHC class II or CD86 expression on either population (not shown). Together, these results suggest that mitochondrial oxidative DNA damage and oxidative injury induced by T\( ^{R1} \) signaling in the tubular epithelium contributes to tubular cell apoptosis and tubular injury and is selectively required for recruitment of dendritic cells and T cells, but not macrophages.

Different macrophage functions have been associated with specific markers; inducible nitric oxide synthase (iNOS) expression is associated with inflammatory M1 type macrophages, whereas mannose receptor (MR) and arginase-1 are associated with M2 macrophages, which are linked with limiting inflammation and favoring repair.\(^{21,22,24} \) Induction of epithelial T\( ^{R1} \) signaling did not change renal iNOS mRNA levels, whereas MR mRNA was increased compared with controls (Supplemental Figure 5A). Arginase-1 mRNA was also increased but this did not reach significance, probably due to the large variability between animals (Supplemental Figure 5). Immunofluorescence staining confirmed that MR\(^{+} \) cells were increased after 5 days compared with controls (Supplemental Figure 5, B and C). Comparison of F4/80 and MR staining on consecutive sections indicated that MR positivity accounted for approximately 10% of the total F4/80 area (Supplemental Figure 5B). MitoTEMPO had no effect on either MR staining or mRNA levels for MR or arginase-1, suggesting that mitochondrial-derived ROS does not influence M2 marker expression.

Because chemokines are instrumental in attracting leukocytes to the site of injury,\(^{47} \) we examined mRNA levels of the
major chemokines involved in macrophage (CCL2 and CCL5) and T cell (CXCL9 and CXCL10) recruitment into the kidney (Supplemental Figure 6). Renal CCL2 mRNA levels were significantly increased after 5 days of doxycycline treatment compared with controls and MitoTEMPO had no effect on CCL2 mRNA, consistent with the unaltered F4/80+ macrophage infiltrate after MitoTEMPO treatment. Likewise, macrophage colony-stimulating factor (M-CSF) mRNA transcripts were significantly increased after induction of epithelial TβR1 signaling. Local generation of M-CSF may have contributed to the accumulation of the macrophages by enhancing their survival and local proliferation. Based on the absence of an effect of MitoTEMPO administration on mRNA levels for CCL-2 and M-CSF and the macrophage infiltration, we conclude that mitochondrial ROS generation has little effect on these processes. Consistent with reduced T cell infiltration in this model, CXCL9 and CXCL10 transcripts tended to be reduced by MitoTEMPO, although this was not statistically significant.

DISCUSSION

We demonstrate that activation of TGFβ signaling in the tubular epithelium alone is sufficient to cause tubular injury, apoptosis, oxidative stress, regenerative cell proliferation, and accumulation of interstitial inflammatory cells, leading to a picture that is similar to experimental or human AKI. This AKI-like phenotype was completely unexpected, because numerous studies have concentrated on a critical role for TGFβ in CKD and progressive renal fibrosis rather than on acute tubular injury. Inhibition of TGFβ signaling in vivo with neutralizing antibodies or Smad3 gene disruption resulted in attenuated tubular atrophy, interstitial inflammation, and fibrosis after UUO, whereas transgenic overexpression of TGFβ resulted in development of progressive glomerulosclerosis, tubular atrophy, and interstitial fibrosis. However, in each of these studies TGFβ signaling was altered systemically, affecting multiple renal and extrarenal cell types. Our study is the first to look at the effect of TGFβ signaling specifically in the tubular epithelium in vivo and directly demonstrates that activation of TGFβ signaling restricted to epithelial cells is sufficient to induce tubular injury associated with mitochondrial oxidative damage and inflammatory cell infiltration. Our findings are also consistent with data recently obtained by Gewin et al. in a complementary system where TβR2 is deleted in proximal tubular cells, which resulted in decreased tubular damage and apoptosis after mercuric chloride-induced AKI.

Thus, TGFβ signaling alone is sufficient to cause tubular epithelial cell dedifferentiation, apoptosis, and necrosis. This is consistent with in vitro experiments showing that TGFβ can...
induce apoptosis via transcriptional regulation of apoptotic factors. TGFβ signaling can also increase ROS levels in vitro, and this can precede apoptosis. Consistent with a role for TGFβ in increasing ROS, we observed increased nitration of tyrosine residues and oxidative DNA damage (8-oxoG) after activation of TGFβ signaling in the tubules. The 8-oxoG staining colocalized with mitochondrial DNA, suggesting a role for mitochondrial-derived ROS. Indeed, reduction of mitochondrial-derived ROS with the mitochondrial-targeted antioxidant MitoTEMPO resulted in a considerable, albeit not significant, 50% reduction of tubular injury. These results appear to be consistent with studies demonstrating that reducing ROS with antioxidants can reduce tubular injury, apoptosis, and reduction in renal function after ischemia reperfusion injury. Thus, our results suggest that TβR1 signaling induces injury and apoptosis, possibly via a concerted activation of transcriptional programs of apoptotic genes and mitochondrial-derived ROS.

TβR1(AAD)-induced tubular injury was associated with a significant leukocyte infiltrate that included T cells and three populations of mononuclear phagocytes; CD11c+ F4/80+ macrophages, CD11c+ F4/80+ dendritic cells, and CD11c+ F4/80−Ly6Chigh cells. The latter may represent peripheral blood monocytes recruited to the injured kidney, where they subsequently exhibit considerable plasticity in terms of differentiation and function. Accumulation of dendritic cells and macrophages after tubular injury may represent both recruitment in response to chemokines such as CCL2 and local proliferation and differentiation by increased M-CSF levels with a proportion of F4/80+ (up to 10%) and CD11c+ cells (up to 25%) containing for Ki67.

Ablation studies have demonstrated that dendritic cells and macrophages have both pathogenic and renal-protective functions during AKI depending on the type of injury, disease stage, and tissue microenvironment. These cells can mediate direct tissue injury through ROS generation, secretion of proinflammatory mediators, antigen presentation, and subsequent T cell activation, but also play an important role in the repair process by promoting survival and proliferation of tubular epithelial cells, and clearance of cell and matrix debris. For macrophages, different markers have been associated with these different functions; iNOS expression is associated with inflammatory M1 type macrophages, whereas MR expression denotes more repair-oriented M2 macrophages. In spite of the marked increase in macrophages after tubular injury, mRNA levels of M1 marker iNOS did not increase, while both mRNA levels and staining for the M2 marker MR did increase. Presence of these M2 macrophages is consistent with the robust tubular epithelial proliferative response observed after activation of TβR1(AAD)-induced tubular injury, because M2 macrophages promote healing through cell proliferation and cytokine production, as well as antigen presentation and T cell activation in the case of dendritic cells.

Because many acute and chronic human diseases are characterized by TGFβ activation and tubular injury, our findings using the Pax8-rtTA/TetO-TβR1(AAD) double transgenic system to active TGFβ signaling specifically within the tubular epithelium have wide-reaching implications in elucidating the molecular mechanisms involved in pathogenesis of these renal diseases. Furthermore, our model has the advantage of an “on-off switch,” which will allow future studies to explore the tubular repair process and the regression of the inflammatory infiltrate after switching off tubular expression of the TβR1(AAD) transgene. This on-off switch will also allow determination of the “point of no return” at which AKI becomes irreversible or evolves into a progressive form of CKD, considerations of great interest in view of the accumulating evidence that a considerable proportion of AKI eventually progress to CKD.

**Table 2. Histologic assessment of MitoTEMPO on TβR1 signaling-induced tubular epithelial cell injury**

<table>
<thead>
<tr>
<th>Histologic Feature</th>
<th>Day 5 Dox + Saline</th>
<th>Day 5 Dox + MitoTEMPO</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubular swelling</td>
<td>7.2 ± 2.9</td>
<td>3.1 ± 1.4</td>
<td>0.22</td>
</tr>
<tr>
<td>Tubular vacuolization</td>
<td>10.8 ± 6.3</td>
<td>16.4 ± 4.5</td>
<td>0.47</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>13.3 ± 4.2</td>
<td>7.1 ± 1.5</td>
<td>0.17</td>
</tr>
<tr>
<td>Necrosis</td>
<td>12.5 ± 4.0</td>
<td>6.0 ± 1.6</td>
<td>0.14</td>
</tr>
<tr>
<td>Interstitial infiltration</td>
<td>28.3 ± 7.2</td>
<td>12.1 ± 2.4</td>
<td>0.04</td>
</tr>
</tbody>
</table>

All data are mean ± SEM % of cortical tubular cell area (n = 6 for day 5 Dox + Saline and n = 7 for day 5 Dox + MitoTEMPO). The t-test was used to statistically compare MitoTEMPO- and saline-treated group and P values are given. Dox, doxycycline.

**CONCISE METHODS**

**Mice**

We generated a tubule-specific TβR1(AAD) expression mouse model by intercrossing doxycycline-inducible tet-O-TβR1(AAD) transgenic mice with Pax8-rtTA transgenic mice. Tet-O-TβR1(AAD) mice (on a FVB/N background) were generated by placing the constitutively active, ligand-independent TβR1 AAD mutant construct under the control of the Tet-On promoter. Genotyping was carried out using PCR primers for the TβR1(AAD) transgene.

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TβR1(AAD) expression was induced in 7- to 10-week-old double transgenic mice by replacing regular chow with doxycycline chow containing 2000 ppm doxycycline in regular control AIN-76A food pellets (Research Diets). Mito-TEMPO (10 mg/kg per day in saline; Enzo Life Sciences) or saline was administered either by daily subcutaneous injection or by Alzet micro-osmotic pump (model 1007D; Alzet-Durect). Pumps were placed subcutaneously under light anesthesia 1 day before administration of the doxycycline chow. Similar results were seen with mini-pumps and subcutaneous injection. All data shown were obtained using mini-pumps except for the flow cytometry data in Figure 6, in which subcutaneous injection was used. All mouse protocols were approved by the Mount Sinai School of Medicine Institutional Animal Care and Use Committee.

**Figure 7.** The mitochondrial antioxidant mitoTEMPO decreases dendritic cells and T cells, but not macrophage infiltration after tubular injury. Single transgenic (TβR1) control and double transgenic Pax8-rtTA/TetO-TβR1(AAD) mice are treated with doxycycline (Dox) for 5 days in the presence or absence of 10 mg/kg per day MitoTEMPO administered by micro-osmotic pump. (A) Representative images of the kidney cortex stained as follows: (upper panel) double immunofluorescence staining for dendritic cell macrophage marker F4/80 (red) and DAPI (blue); (lower panel) triple immunofluorescence staining for dendritic cell marker CD11c (red), T cell marker CD3 (green), and DAPI (blue). (B) Quantification of F4/80 and CD11c staining as the percentage positive area and CD3 staining as the number of positive cells per 3200 field. Each dot represents a single animal and is the mean of 10 randomly selected cortical fields counted for that animal. Line and bars represent the mean ± SEM for each treatment group (at least 6 per group). The t test was used to statistically compare MitoTEMPO- and saline-treated groups, with P<0.05 considered significant. (C) Proportions of CD45+CD11c− F4/80+ macrophages and CD45+CD11c+ F4/80− and F4/80+ dendritic cells as a percentage of the total CD45+ leukocytes. Data are representative of three animals per treatment group. Changes in each group examined are comparable with the representative data shown. Original magnification, ×400 in A; ×200 in B.
Microscopy

Histology
We stained 10% formalin-fixed, paraffin-embedded sections (4 μm) of bisected kidneys with H&E and PAS using standard protocols. The morphologic features in Tables 1 and 2 were assessed by a renal pathologist blinded to the experimental treatments. The percentage of total cortical tubules showing tubular epithelial cell swelling, vacuolization, apoptosis, and necrosis and the percentage of total cortical area with interstitial inflammation were estimated in whole kidney cross-sections.

Immunohistochemistry
Paraffin-embedded sections were stained for nitrotyrosine (3NT), using a monoclonal anti-nitrotyrosine, clone 2A8.2 (Millipore) with a biotinylated anti-mouse secondary (Vectastain Kit; Vector Labs). We used 3,3′-diaminobenzidine for visualization as well as hematoxylin as a counterstain.

Immunofluorescence
Cryosections were postfixed in ice-cold acetone, air-dried, rehydrated, blocked with 2% BSA-PBS, and then stained overnight with hamster anti-CD11c (HL3), rat anti-CD3 (17A2; BD Biosciences), rat anti-F4/80 (A3-1; AbD Serotec), rabbit anti-Ki67 (Abcam), or rabbit anti-Smad2/3 (Cell Signaling Technology). Staining was then visualized using an appropriate fluorescent-labeled secondary antibody: rabbit anti-rat IgG-Alexa-488 or -594, donkey anti-rabbit IgG-Alexa-488 or -594, or goat anti-Armenian hamster IgG-DyLight-594 (Jackson ImmunoResearch Laboratories). FITC conjugated LTG lectin was used to stain for proximal tubules, whereas distal tubules were stained with rhodamine-conjugated DBA (both Vector Labs). All sections were counterstained with DAPI (0.1 μg/ml for 10 minutes) before mounting in ProLong Gold antifade reagent (Invitrogen). For 8oxoG staining, cryosections were postfixed in ice-cold methanol, rehydrated, incubated in 1 mM RNase (Qiagen) for 1 hour at 37°C, blocked with 2% BSA, 2% PBS, 0.2% fish gelatin, and PBS, and then stained with mouse anti-8-oxoG mAb (N45.1; Japan Institute for the Control of Aging), followed by chicken anti-mouse IgG-Alexa-488 (Invitrogen).

TUNEL Assay
Apoptotic nuclei were detected using DAPI staining in combination with the ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Chemicon).

Quantitative Digital Image Analyses
Images were taken as TIFF files with a Zeiss Axioplan 2, equipped with a Q-imaging MP3.3 RTV color camera running QED capture software (provided by the Mount Sinai School of Medicine microscopy core facility). All analyses were performed blinded to treatment received. Number of CD3⁺ cells, Ki67⁺ nuclei, or TUNEL⁺ nuclei per field were quantified by direct counting of 10 randomly selected cortical fields per animal at ×400 (Ki67 and TUNEL) or ×200 (CD3) magnification, using the cell counter tool from ImageJ (version 1.44o; National Institutes of Health). Proportions of Ki67⁺ or Smad⁻ nuclei in relation to tubular markers were quantified by the same method. Due to the irregular cell shape for F4/80⁺, CD11c⁺, and MR⁺ cells, the percentage positive area was determined for these markers by MetaMorph (version 6.3r3; Molecular Devices) threshold analysis³ on 10 randomly selected cortical fields per animal at ×200 magnification.

Urinary 8-oxodG Measurement
Aliquots of urine (50 μl) were loaded onto 500-μg, low hydrocarbon, C18-OH solid phase extraction Bond Elut columns (Agilent). The analyte was eluted with 50% methanol in water. Urine 8-oxodG was measured by high-performance liquid chromatography with electrochemical detection³⁵,³⁷ using a Waters amperometric detector with a glassy-carbon working electrode and an Ag/AgCl reference electrode. The oxidation potential was 0.4 V. Levels of 8-oxodG in the samples were calculated against 8-oxodG standards and expressed relative to urine creatinine of each sample. Urinary creatinine was measured using the creatinine companion kit (Exocell Laboratories).

Serum Creatinine and BUN
Serum creatinine was measured by acetonitrile deproteinization, followed by isocratic, cation exchange high-performance liquid chromatography, as described previously.⁵⁸ BUN was measured using the DIUR-500-QuantiChromUrea Assay (Gentaur).

Quantitative Real-Time PCR
Total kidney RNA was prepared from whole kidney lysates using Qiagen RNeasy mini columns and then reverse transcribed with Superscript II RT (Invitrogen Life Technologies). Quantitative amplification of the cDNA was performed on an ABI-Prism 7900HT Sequence Detection system and evaluated using SDS software (version 2.0; Applied Biosystems). Results were normalized to β-actin content and relative mRNA levels are expressed as fold change compared with the untreated control animals. The following primers were used: TBJR1(AAD) forward: 5′-AAAGCTGACCCCTGGCC TTA-3′ and reverse: 5′-TTCTCTAAATCAGCCTTTCG-3′; PAI-1 forward: 5′-ATGTTTAGTGCAACCCTGGC-3′ and reverse: 5′-TTTGAGTGTGGTGTCTAC-3′; and β-actin forward: 5′-ACC GTGAAAAAGATGACCACCCAG-3′ and reverse: 5′-AGCTCTGGATGGCT ACGTACA-3′.

Flow Cytometry
Whole kidney single cell suspensions were prepared as described by Vielhauer et al., with some modifications. In brief, finely minced kidney was digested with 1 mg/ml Collagenase D (Roche) and 100 U/ml DNase I (Sigma) for 30 minutes at 37°C. The digestion was then passed through a 70-μm cell strainer, washed with flow buffer (PBS containing 0.5% BSA and 2 mM EDTA), re-suspended in 30% Percoll-flow buffer, and centrifuged at 250 × g, in order to remove cellular debris that does not pass through the Percoll layer. The resulting cell pellet was re-suspended in flow buffer, blocked with 5 μg/ml anti-mouse CD16/32 (eBioscience) on ice for 30 minutes and then divided into individual tubes and stained with combinations of fluorochrome-labeled antibodies for 1 hour on ice, before washing and fixation with 1% PFA. Antibodies used were CD45 (Clone 30-F11), CD3 (17A2), CD4 (RM4-5), CD8 (53-6.7), CD11c (N418), B220 (clone 40.11; BioLegend), and MR (clone 39.11; BioLegend), followed by a secondary antibody labeled with PE, Alexa-488, and DyLight-594.
Statistical Analyses

All analyses were carried out using GraphPad Prism software (GraphPad Software Inc). One-way ANOVA with Tukey post-test was used to compare control animals to animals treated for 3–4 or 5–6 days. The unpaired, two-tailed t test was used to compare MitoTEMPO-treated animals to animals treated with saline as a vehicle control.

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DISCLOSURES

None.

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