Friend or Foe? The Role of Transforming Growth Factor-β (TGFβ) Signaling in Calcineurin Inhibitor-Induced Renal Damage

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FRIEND OR FOE?

THE ROLE OF TRANSFORMING GROWTH FACTOR-β (TGFβ) SIGNALING IN CALCINEURIN INHIBITOR-INDUCED RENAL DAMAGE

A Dissertation submitted in partial fulfillment of requirements for the degree of

Doctor of Philosophy

By

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February 23, 2023

I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Adaku Ume ENTITLED Friend or Foe? The Role of Transforming Growth Factor-β (TGFβ) Signaling in Calcineurin Inhibitor-Induced Renal Damage BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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ABSTRACT

Ume, Adaku. PhD. Biomedical Sciences PhD. Program, Wright State University, 2023. Friend or Foe? The Role of Transforming Growth Factor-β (TGFβ) Signaling in Calcineurin Inhibitor-Induced Renal Damage.

With its incorporation into clinical practice in the early 1980s, the class of pharmacological agents known as calcineurin inhibitors (CNIs) quickly became the cornerstone of immunosuppressive therapy post-organ transplantation. However, its use is limited by irreversible kidney damage in the form of renal fibrosis. The molecular mechanism by which CNIs induce renal fibrosis remains to be better understood, and to date, there are no specific therapeutic strategies to mitigate this damage. This dilemma presents a critical need to explain mechanisms by which CNIs cause renal damage.

Kidneys of patients on chronic CNI therapy show increased expression of the proinflammatory cytokine Transforming Growth Factor β (TGFβ). TGFβ is a multipotent regulator of cell survival, differentiation, proliferation, and extracellular matrix (ECM) production in a variety of tissues. Renal biopsy samples from patients with tacrolimus nephrotoxicity showed both increased mRNA and protein expression of TGFβ along with fibronectin and collagen, additional profibrotic markers. However, the role of TGFβ signaling in CNI-induced renal damage remains to be defined and this gap in knowledge prompts further investigation. To this end, this dissertation will I) determine the role of
TGFβ signaling in CNI-induced renal damage (Aim 1) and II) establish whether disruption of TGFβ signaling ameliorates renal damage with CNI-induced immunosuppression (Aim 2). This insight will direct development of newer generation CNI immunosuppressants exhibiting reno-preservative potential.

Our group reported that aberrant Transforming Growth Factor-β (TGFβ)/Smad signaling drives the profibrotic effects induced by CNIs. Specifically, we demonstrated that 1) tacrolimus inhibits the calcineurin/NFAT axis while inducing TGFβ ligand secretion and receptor activation in renal fibroblasts, 2) aberrant TGFβ receptor activation stimulates Smad-mediated production of myofibroblast markers, notable features of fibroblast-to-myofibroblast transition (FMT) and 3) FMT contributes to extracellular matrix (ECM) expansion in tacrolimus-induced renal fibrosis. These findings spurred follow-up studies investigating the feasibility in inhibiting TGFβ receptor activation to maintain CNI-mediated immunosuppression while ultimately preserving kidney health.

We found that inhibition of TGFβ signaling 1) promotes positive effects such as the attenuation of tacrolimus-induced interstitial fibrosis and fibroblast activation. However, disruption of TGFβ signaling also exacerbates CNI-related nephrotoxic effects such as disruption of both 2) glomerular and 3) tubular functions. Taken together, these results indicate that renal TGFβ signaling exerts both beneficial and detrimental effects, which establish its role as both a friend and foe in the kidney. Given this revelation, directly
targeting TGFβ signaling is not a suitable approach to prevent the renal damage associated with CNI therapy.
# Table of Contents

**Introduction** ............................................................................................................................. 1

**Chapter I: Tacrolimus Induces Fibroblast to Myofibroblast Transition via a TGFβ-Dependent Mechanism to Contribute to Renal Fibrosis** ............................................................................. 7

**Chapter II: Friend or Foe? Inhibition of the TGFβ Receptor Attenuates Tacrolimus-Induced Renal Fibrosis While Exacerbating Kidney Dysfunction** ................................................................. 57

**Chapter III: Conclusions & Future Directions** ........................................................................... 97

**Appendix A: Abbreviations** ........................................................................................................ 112
## Introduction

**Calcineurin inhibitors (CNIs) blunt the immune response by inhibiting calcineurin phosphatase activity.**

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### Chapter I: Tacrolimus Induces Fibroblast to Myofibroblast Transition via a TGFβ-Dependent Mechanism to Contribute to Renal Fibrosis

1. *Tacrolimus induces kidney damage in the form of renal fibrosis in mice.*

   24

2. *Tacrolimus-induced renal fibrosis is accompanied by fibroblast activation in mice.*

   26

3. *Tacrolimus inhibits calcineurin activity/NFAT activation in renal fibroblasts.*

   29

4. *Tacrolimus induces TGFβ receptor activation and downstream Smad 2/3 phosphorylation in mouse kidneys.*

   32

5. *Inhibition of renal TGFβ signaling exacerbates disruption of the glomerular barrier.*

   35

6. *TGFβ signaling mediators Smad 2/3 drive tacrolimus-induced FMT and ECM production in renal fibroblasts.*

   38

7. *Tacrolimus induced fibroblast to myofibroblast (FMT) transition via a TGFβ-dependent mechanism to contribute to renal fibrosis.*

   41

---

### Chapter II: Friend or Foe? Inhibition of the TGFβ Receptor Attenuates Tacrolimus-Induced Renal Fibrosis While Exacerbating Kidney Dysfunction
LY21 inhibits tacrolimus-induced renal TGFβ receptor activation ..............................................................68

TGFβ receptor inhibition does not disrupt tacrolimus-induced IL-2 suppression in the kidneys ..........................................................71

TGFβ receptor inhibition attenuates tacrolimus-induced renal fibrosis and fibroblast activation ..........................................................74

TGFβ signaling is accompanied by enhanced mesangial matrix expansion ........................................................................78

Inhibition of renal TGFβ signaling exacerbates disruption of the glomerular barrier .................................................................80

Inhibition of TGFβ signaling also exacerbates tacrolimus-induced renal tubular dysfunction ..........................................................83

Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis while promoting kidney dysfunction ................................................86-88

CHAPTER III: CONCLUSIONS & FUTURE DIRECTIONS

1 Tacrolimus induced fibroblast to myofibroblast (FMT) transition via a TGFβ-dependent mechanism to contribute to renal fibrosis .........................99

2 Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis .................................................................101

3 Inhibition of TGFβ receptor activation exacerbates glomerular barrier dysfunction .................................................................102

4 Inhibition of TGFβ receptor activation further exacerbates tubular dysfunction .................................................................103

5 CnAα and CnAβ are two catalytic calcineurin (CnA) isoforms expressed in the kidneys .................................................................105

6 CnAα and CnAβ possess divergent signaling functions in
the kidneys

7 CNI-induced nephropathy may be isoform-specific
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chapter I: Tacrolimus Induces Fibroblast to Myofibroblast Transition via a TGFβ-Dependent Mechanism to Contribute to Renal Fibrosis</strong></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Body and kidney weights of vehicle and tacrolimus-treated mice</em></td>
</tr>
</tbody>
</table>

| **Chapter II: Friend or Foe? Inhibition of the TGFβ Receptor Attenuates Tacrolimus-Induced Renal Fibrosis While Exacerbating Kidney Dysfunction** | |
| 1 | *Overall health was preserved with LY21 treatment* | 66 |
| 2 | *Low kidney mass induced by tacrolimus treatment remains with TGFβ receptor inhibition* | 76 |
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This Dissertation is dedicated to my Lord and Savior, Jesus Christ.

“Trust in the Lord with all of your heart and lean not on your own understanding; In all your ways acknowledge Him and He will make your paths straight.”
-Proverbs 3:5-6

“Call on me, and I will answer you and tell you great and unsearchable things that you did not know.”
-Jeremiah 3:33

“Unless the Lord builds this house, those who build it labor in vain. Unless the Lord watches the city, the guards stand watch in vain.”
-Psalms 127:1

“But He knows the way that I take; When He has tested me, I will come forth as gold.”
-Job 23:10
INTRODUCTION

CALCINEURIN INHIBITORS: MEDICINE OF THE EARTH

With its incorporation into clinical practice in the early 1980s, the class of pharmacological agents known as *calcineurin inhibitors (CNIs)* quickly became the cornerstone of immunosuppressive therapy post-organ transplantation (1,2). The first and prototype of its class, *cyclosporine A (CsA)*, was isolated from the soil fungus *Tolypocladium inflatum* in 1970 by Sandoz Laboratory (now Novartis) scientists, Dr. Sandor Lazary and Dr. Jean-Francois Borel (3). Successful preliminary human studies fast-tracked immediate use of CsA in renal transplant recipients in 1980 (4). After dosage adjustments, initial reports citing CsA’s lethal side effects decreased—with post-transplantation survival rates increasing significantly (5,6).

In 1985, an additional immunosuppressive compound identified as *FK-506* was isolated from the soil microbe *Streptomyces tsukubaensis*. FK-506 also demonstrated immunosuppressive effects according to scientists at Fujisawa Pharmaceuticals in Japan, and the compound was later renamed *tacrolimus* (acronym *tsukuba macrolide immunosuppressive*) (7). Due to better efficacy and a milder range of side effects, tacrolimus largely outpaced CsA in success, with majority of solid organ transplant
MECHANISM OF ACTION

Studies attribute CsA and tacrolimus’ immunosuppressive effects to inhibition of calcineurin, a proinflammatory enzyme critical to lymphocyte activation (3,13) (Figure 1). Upon immune cell stimulation, calcineurin subunits associate with Ca\(^{2+}\)/calmodulin to form an active enzyme complex (14). Activation of this complex leads to the dephosphorylation of well-recognized downstream calcineurin targets, such as Nuclear Factor of Activated T-cells (NFAT) proteins. Once dephosphorylated, NFAT proteins translocate into the nucleus to promote

recipients placed on tacrolimus treatment protocols post-operatively (2,8). The success of CNIs in transplant medicine broadened the clinical scope of use to also include management of inflammatory conditions such as lupus nephritis (9), rheumatoid arthritis (10), vitiligo (11) and atopic dermatitis (12).

Figure 1: Calcineurin Inhibitors (CNIs) blunt the immune response by inhibiting calcineurin phosphatase activity. CNIs bind to cytosolic proteins called immunophilins (I) (step 1) enabling the CNI-I complex to bind calcineurin active site, thereby inhibiting phosphatase activity (step 2). Upon inhibition, transcription factors such as nuclear factor of activated T-cells (NFAT) are unable to become activated by dephosphorylation (step 3). This prevents NFAT translocation into the nucleus to increase IL-2 transcription (step 4), thereby blunting immune activation.
transcription of proinflammatory cytokines, such as Interleukin-2 (IL-2) (15). CNIs exert an immunosuppressive effect by complexing with cytosolic immunophilins (step 1) (CsA: cyclophilin; Tacrolimus: FK506 binding protein 12, FKBP12) to noncompetitively inhibit calcineurin (step 2) (13,16–18). Thus, CNIs severely limit calcineurin-mediated dephosphorylation of target proteins (step 3). However, due to the ubiquitous expression of calcineurin in various tissues (17,19), calcineurin inhibition in immune cells is often accompanied by the induction of collateral damage in other organ systems, particularly the kidneys (2,20,21).

TROUBLE IN PARADISE: RENAL DAMAGE

The initial success of CNIs in transplant medicine was quickly followed up with early clinical evidence of CNI nephropathy, leading to renal failure in some cases (22,23). In one group of patients, CNI nephropathy was observed as early as 3 months post-operatively (24) with increased incidence in around 20-25% of patients at 1 year (24,25) and 50% at 2 years. In a cohort of patients assessed using a chronic allograft renal damage index, fibrosis was present in around 70-85% of grafts at 1 year, making it the most common sign of nephropathy (26,27). Renal fibrosis is a pathological process characterized by increased extracellular matrix (ECM) protein deposition coupled with reduced matrix turnover, thereby promoting ECM expansion. These matrix disturbances contribute to the disruption of renal architecture and functional capacity. Detailed histopathological analyses describe irreversible damage by CNI treatment, with fibrotic
lesions noted within the blood vessels (arteriolar hyalinosis) (28), glomeruli (glomerulosclerosis) (6,29), and between tubular epithelial cells (tubulointerstitial fibrosis) (29,30). CNI-induced nephropathy was later noted as progressive interstitial fibrosis, tubular atrophy, arteriolar hyalinosis, glomerulosclerosis and irreversible deterioration of renal function (22,31). Lesions partially attributable to CNI nephrotoxicity can be seen in virtually all histological sections ten years after transplantation (32). These structural changes were not exclusive to long-term use of CsA but also observed with tacrolimus treatment (29). The molecular mechanism by which CNIs induce renal fibrosis remains to be better understood, and to date, there are no specific therapeutic strategies to mitigate this damage. Moreover, the emergence of newer generation CNIs such as the CsA analogue voclosporin (also known as ISA\textsubscript{TX}247) highlight the real need to investigate this phenomenon further (9,33–36). In light of this dilemma, the objectives of my dissertation research are to 1) identify initiating events by which CNIs cause irreversible renal damage and 2) explore approaches to achieve CNI-mediated immunosuppression without irreversible renal damage. This insight will advance understanding of the pathophysiologic mechanisms underlying CNI-induced renal damage while providing approaches to attain immunosuppression without the associated renal damage.

**A MOLECULAR CULPRIT: TRANSFORMING GROWTH FACTOR β (TGFβ)**

Kidneys of patients on chronic CNI therapy show increased expression of the proinflammatory cytokine Transforming Growth Factor β (TGFβ) (26,37–39). TGFβ is
a multipotent regulator of cell survival, differentiation, proliferation, and extracellular matrix (ECM) production in a variety of tissues (40,41). Renal biopsy samples from patients with tacrolimus nephrotoxicity showed both increased mRNA and protein expression of TGFβ along with fibronectin and collagen, additional profibrotic markers (38). CNIs have also been reported to increase both in vivo and in vitro TGFβ expression and receptor activity in experimental models (42,43). However, the role of TGFβ signaling in CNI-induced renal damage remains to be defined and this gap in knowledge prompts further investigation. To this end, this dissertation seeks I) to determine the role of TGFβ signaling in CNI-induced renal damage (Aim 1) and II) to establish whether disruption of TGFβ signaling ameliorates renal damage with CNI-induced immunosuppression (Aim 2). This insight will direct development of newer generation CNI immunosuppressants exhibiting reno-preservative potential.

Chapter I provides compelling evidence identifying a role of TGFβ signaling in CNI-induced renal damage in a recently submitted manuscript entitled “Tacrolimus induces fibroblast to myofibroblast transition via a TGFβ-dependent mechanism to contribute to renal fibrosis.” This manuscript was accepted for publication by the American Journal of Physiology- Renal Physiology. Chapter I will not only incorporate renal fibroblasts into the growing list of cell types negatively impacted by CNIs, but also identifies renal fibroblast-to-myofibroblast transition as a process mediated via a TGFβ-dependent mechanism. This insight will direct future studies investigating the feasibility
in inhibiting TGFβ signaling to maintain CNI-mediated immunosuppression while ultimately preserving kidney health.
CHAPTER I

Tacrolimus induces fibroblast to myofibroblast transition via a TGFβ-dependent mechanism to contribute to renal fibrosis

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Running Title: FMT drives tacrolimus-induced renal fibrosis

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ABSTRACT

Use of the immunosuppressants calcineurin inhibitors (CNIs) are limited by irreversible kidney damage, hallmarked by renal fibrosis. CNIs directly damage many renal cell types. Given the diverse renal cell populations, additional targeted cell types and signaling mechanisms warrant further investigation. We hypothesize that fibroblasts contribute to CNI-induced renal fibrosis and propagate profibrotic effects via the transforming growth factor β (TGFβ)/suppression of mothers against decapentaplegic (Smad) signaling axis. To test this, kidney-damage resistant mice (C57BL/6) received tacrolimus (10mg/kg) or vehicle for 21 days. Renal damage markers and signaling mediators were assessed. To investigate their role in renal damage, mouse renal fibroblasts were exposed to tacrolimus (1nM) or vehicle for 24 hrs. Morphological and functional changes in addition to downstream signaling events were assessed. Tacrolimus-treated kidneys displayed evidence of renal fibrosis. Moreover, α-smooth muscle actin (α-SMA) expression was significantly increased, suggesting the presence of fibroblast activation. TGFβ receptor activation and downstream Smad2/3 signaling were also upregulated. Consistent with in vivo findings, tacrolimus-treated renal fibroblasts displayed a phenotypic switch known as fibroblast-to-myofibroblast transition (FMT), as α-SMA, actin stress fibers, cell motility and collagen IV expression were significantly increased. These findings were accompanied by concomitant induction of TGFβ signaling. Pharmacological inhibition of the downstream TGFβ effector Smad3 attenuated tacrolimus-induced phenotypic changes. Collectively, these findings suggest
that 1) tacrolimus inhibits the calcineurin/nuclear factor of activated T-cells (NFAT) axis while inducing TGFβ1 ligand secretion and receptor activation in renal fibroblasts, 2) aberrant TGFβ receptor activation stimulates Smad-mediated production of myofibroblast markers, notable features of FMT and 3) FMT contributes to extracellular matrix (ECM) expansion in tacrolimus-induced renal fibrosis. These results incorporate renal fibroblasts into the growing list of CNI-targeted cell types and identify renal FMT as a process mediated via a TGFβ-dependent mechanism.
NEW & NOTEWORTHY

Renal fibrosis, a detrimental feature of irreversible kidney damage, remains a sinister consequence of long-term calcineurin inhibitor (CNI) immunosuppressive therapy. Our study not only incorporates renal fibroblasts into the growing list of cell types negatively impacted by CNIs, but also identifies renal fibroblast-to-myofibroblast transition as a process mediated via a TGFβ-dependent mechanism. This insight will direct future studies investigating the feasibility in inhibiting TGFβ signaling to maintain CNI-mediated immunosuppression while ultimately preserving kidney health.
INTRODUCTION

The influence of calcineurin inhibitors (CNIs) such as cyclosporine (CsA) and tacrolimus bolstered the field of organ transplantation with their immunosuppressive effects (1–3). However, the overall success of these immunosuppressant drugs is limited by nephropathy during chronic use. Long-term CNI therapy causes irreversible kidney damage, hallmarked by interstitial fibrosis and glomerulosclerosis (4–8). CNI-induced renal fibrosis is a pathological process characterized by increased extracellular matrix (ECM) protein deposition coupled with reduced matrix turnover, thereby promoting ECM expansion. These matrix disturbances contribute to the disruption of renal architecture and functional capacity. Consequently, approximately 15% of patients on chronic CNI therapy experience an eventual progression to end stage renal disease (ESRD) and organ failure (9,10). Mechanisms underlying CNI-induced nephropathy remains a poorly understood dilemma in the field, and to date, there are no specific therapeutic strategies to mitigate this damage. Kidneys of patients on chronic CNI therapy show increased expression of the profibrotic cytokine Transforming Growth Factor β (TGFβ). However, the precise role of TGFβ signaling in mediating renal damage induced by CNIs remains to be defined. These existing gaps in knowledge along with the detrimental outcomes present a critical need to identify the driving mechanisms by which CNIs induce renal damage. This insight will direct development of newer generation CNI immunosuppressants exhibiting reno-preservative potential.
Clinicians utilize CNIs based on their profound immunosuppressive effects. Upon immune cell stimulation, calcineurin subunits associate with Ca\textsuperscript{2+}/calmodulin to form an active enzyme complex (11). Activation of this complex leads to the dephosphorylation of well-recognized downstream calcineurin targets, such as Nuclear Factor of Activated T-cells (NFAT) proteins (11–14). Once dephosphorylated, NFAT proteins translocate into the nucleus to promote transcription of proinflammatory cytokines (15). CNIs exert an immunosuppressive effect by complexing with cytosolic immunophilins (CsA: cyclophilin; Tacrolimus: FK506 binding protein 12, FKBP12) to noncompetitively inhibit calcineurin (14,16–18). Thus, CNIs severely limit calcineurin-mediated dephosphorylation of target proteins. However, due to the ubiquitous expression of calcineurin in various tissues (17,19), calcineurin inhibition in immune cells is often accompanied by the induction of collateral damage in other organ systems, particularly the kidneys (1,10,20). CNIs directly induce damage to many renal cell types including epithelial, endothelial and mesangial cells (21–23). However, given the diverse cell populations in the kidney (24), additional targeted cell types and signaling mechanisms behind CNI-induced renal damage remains to be uncovered.

In this study, we aimed to identify renal fibroblasts as a contributor to CNI-induced fibrosis through a phenotypic switch known as fibroblast-to-myofibroblast transition (FMT). FMT occurs following injury, where the quiescent fibroblast state is halted in favor of proliferation into an activated fibroblast (myofibroblast) state (25).
Myofibroblasts have enhanced motility via upregulation of contractile markers (i.e. α-smooth muscle actin (α-SMA), etc.) in addition to forming collagen-rich extracellular matrix (ECM) components that fill the interstitium (25). Progression of these events contribute to nephron loss and declining kidney function. Therefore, an abundance of myofibroblasts is recognized as a predictor of fibrosis in both human and animal models of renal disease (26). The myofibroblast cell type arises from circulating progenitors, tubular epithelial cells and resident fibroblasts (27). While the role of myofibroblasts in tacrolimus-induced renal damage is widely accepted (28), their origin is still a matter of debate.

Given their role as the primary matrix-producing cells in the kidney, we investigated renal FMT as a molecular mechanism inducing renal fibrosis with CNI use. Specifically, using both in vivo and in vitro models of CNI-induced nephropathy, we demonstrate that 1) tacrolimus inhibits the calcineurin/NFAT axis while inducing TGFβ1 ligand secretion and receptor activation in renal fibroblasts and 2) aberrant TGFβ receptor activation stimulates Smad-mediated transcription of myofibroblast markers, notable features of FMT and 3) FMT contributes to ECM expansion in tacrolimus-induced renal fibrosis. Taken together, these results not only incorporate renal fibroblasts into the growing list of cell types targeted by CNIs, but also identifies renal FMT as a process mediated via a TGFβ-dependent mechanism.
**EXPERIMENTAL DESIGN**

**CNI Models**

*In vivo:* Although gender is not considered a risk factor for CNI-induced nephropathy in humans (29), male rats exhibited greater CNI-induced nephropathy than female rats (30). In light of these findings, male WT C57Bl/6 mice (n=16 total; n=8/group; 8 weeks old; Jackson Laboratory) were randomly assigned to receive intraperitoneal injections of FK-506 (tacrolimus, 10 mg/kg/day) (Enzo) or vehicle (ethanol/DMSO/saline) for 21 days. Factoring in the drug pharmacokinetics in mice (31), this animal dose is consistent with the human equivalent tacrolimus dosing of organ transplant recipients (0.15 to 3mg/kg/day) (32–34). Further, similar doses (35) and time points (32,35) were shown to induce upregulation of profibrotic markers in experimental animal models. The order of daily injections was alternated between treatment groups to minimize potential confounding variables. For this study, mice were socially housed in standard cages with a regular 12hr light/dark cycle and fed standard rodent chow. Furthermore, all animal protocols and procedures were approved by the Animal Care and Use Committee (IACUC) at Wright State University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

*In vitro:* The renal fibroblast cell line was kindly gifted to the Williams’ Laboratory by Dr. Jennifer Gooch. These spontaneously immortalized cells were generated from
kidneys of WT male mice from a mixed genetic background as previously described (20,36). Furthermore, cell lines were authenticated by IDEXX BioResearch. For *in vitro* experiments, renal fibroblasts were maintained in growth medium (DMEM, F-10 Hams, 10% fetal bovine serum, penicillin/streptomycin and Plasmocin™) at standard conditions (37°C, 5% CO₂). At 85% confluence, culture medium was changed to serum-free medium for 24 hours. After, cells were exposed to serum-free medium containing either 1nM tacrolimus or 0.01% vehicle (ethanol) for 24 hours. Previous studies have shown that similar doses (37) and time points (12,20,38,39) were sufficient to induce upregulation of profibrotic markers. In a separate set of experiments, both vehicle and tacrolimus-treated cells were pre-incubated with 1µM ionomycin (a Ca²⁺ ionophore known to induce calcineurin/NFAT signaling(40)) (STEMCELL Technologies; Vancouver, Canada; catalog #73722), for 15 min. A final set of experiments consisted of fibroblasts treated with either 1nM tacrolimus or 0.01% vehicle (ethanol) in the presence or absence of Smad3 inhibitor SIS3 (MedChemExpress; Monmouth Junction, NJ, catalog #HY-13013; 500nM). SIS3 acts as a selective inhibitor of Smad3 phosphorylation (activation).

**Histology and Immunocytochemistry**

Kidney samples were fixed in freshly prepared 4% paraformaldehyde and routine histology was performed on 5 µm-thick sections. Alterations in kidney morphology were initially assessed by examination of hematoxylin and eosin (H&E)-stained sections.
(conducted by AML Laboratories; St. Augustine, FL). Images of glomerular and tubular changes were captured using a Keyence BZ-X800 Fluorescence Microscope (60X magnification, 1/45s exposure time). Extracellular matrix accumulation (fibrosis) was assessed by collagen deposition using both Masson Trichrome (Abcam, Cambridge, United Kingdom, catalog #ab150686) and Picrosirius Red protocols (Abcam, catalog #ab150681). Images were acquired using a Keyence BZ-X800 Fluorescence microscope (60X magnification, 1/80s exposure time).

To assess myofibroblast cell abundance, fluorescent detection of the myofibroblast marker α-smooth muscle actin (α-SMA) was performed. Sections were blocked in 10% goat serum (Life Technologies; Carlsbad, CA; catalog #16210-072) in TBS-tween for 30 min. Sections were then incubated with α-SMA-specific primary antibody (Cell Signaling Technology (CST), Danvers, Massachusetts, catalog #19245S, 1:100 dilution). After incubation with Alexa Fluor-555 anti-rabbit secondary antibody (Invitrogen, Waltham, MA, catalog #A32794, 1:1000 dilution), images (5-6 sections from each treatment group) were captured using a Keyence BZ-X800 Fluorescence Microscope (60X magnification, 1/80s exposure time). The Zen 3.1 software program was utilized to quantify α-SMA intensity.

To assess expression of the phosphorylated type I TGFβ receptor subunit (phospho-TGFβRI), chromogen staining was performed. Briefly, endogenous peroxidases were quenched in deparaffinized sections by incubation in 0.3% H$_2$O$_2$ (Abcam; Cambridge,
United Kingdom; catalog #ab64218) for 15 minutes. Sections were then blocked in 10% goat serum (Life Technologies) in TBS-tween for 30 min and incubated with phospho-TGFβRI-specific antibody (Invitrogen; catalog #PA5-40298; 1:100). Sections were incubated with HRP-conjugated secondary antibody (Invitrogen, catalog #A32794, 1:200 dilution). Bound antibody was identified by 3,3'-diaminobenziidine (DAB) staining according to the manufacturer’s instructions (Vector Laboratories; Burlingame, CA; catalog #SK-4100). In addition, sections were counterstained with hematoxylin according to the manufacturer’s instructions (Abcam; Cambridge, United Kingdom; catalog #220365). Phospho-TGFβRI abundance was visualized using a Keyence BZ-X800 Fluorescence Microscope and brightfield images at 60X magnification and 1/45s exposure time were captured.

Treated fibroblasts were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton-PBS before incubation with the following antibodies: α-SMA (CST; dilution 1:1000), Col IV (Santa Cruz Biotechnology (SCBT); Dallas, TX; catalog #sc-398655; 1:500 dilution), NFATc1 (CST; Danvers, MA; catalog #5861S; dilution 1:500), and phospho-TGFβRI (Invitrogen; 1:500 dilution). Subsequently, cells were incubated with either Alexa Fluor™ 488 (Invitrogen; Waltham, MA; catalog #A32790; 1:1000 dilution) or Alexa Fluor-555 secondary antibody (Invitrogen; 1:1000 dilution) and DAPI (ThermoFisher; catalog #D1306; 1:1000 dilution). Moreover, a subset of cells was also incubated with the Alexa-Fluor™ 555-conjugated phalloidin probe (Invitrogen; catalog #A30106; 1:1000 dilution) and DAPI (ThermoFisher; 1:1000 dilution). Representative
images were acquired using a Keyence BZ-X800 Fluorescence Microscope (at both 20X and 60X magnification; Depending on antibody, exposure times ranged from 1/40s to 1/80s). The relative intensities from 5-6 images (per treatment group) were quantified utilizing both CellProfiler™ and ImageJ™ software programs. To quantify nuclear NFATc1 intensity, the following pipeline was used: the “IdentifyPrimaryObjects” module was used to identify DAPI-stained nuclei. The “MeasureObjectIntensity” module was then used to quantify the mean intensity of nuclear NFAT per cell. For quantification of α-SMA, Col IV, phalloidin and phospho-TGFβRI intensity, the respective pipeline in CellProfiler was utilized: Initially, the “IdentifyPrimaryObjects” module was used for identification of DAPI-stained nuclei. Next, the “IdentifySecondaryObjects” module propagation method was used to identify and select all cells present in the image. Finally, the “IdentifyTertiaryObjects” module was used to subtract the outlined nuclei from the cell selections, leaving only the cytoplasmic portion of each cell to be quantified. For a field of view of 282x182 μm, the total intensity for all fluorescent images was calculated, expressed as arbitrary light units (alu), and displayed on the respective image. Lastly, a heat map of images was generated using ImageJ.

Motility Assessment

Scratch wound assays were utilized to assess fibroblast motility. Upon formation of an ~100% confluent monolayer, a midline linear scratch (“wound”) was generated using a sterile pipette tip. After a sterile PBS wash to remove cellular debris, cells were
treated with either 1nM tacrolimus or 0.01% vehicle (ethanol) for 12 hours. Images of initial scratches (time 0) and respective scratch closures (time 12 hours) were captured using an Invitrogen™ EVOS™ XL Core Imaging System (10x magnification). Images of three focal areas per well were captured for each experiment. Differences in scratch closures between groups were quantified and averaged by a blinded reviewer using ImageJ software™. The migration rate percentage was calculated using an established formula (41):

\[
\text{Migration Rate} \% = \frac{\text{Average distance of scratch (time 0)} - \text{Average distance (time 12 hours)}}{\text{Average distance of scratch (time 0)}} \times 100
\]

**Morphological changes**

To visualize morphological changes such as cell size and shape, brightfield images of treated cells were acquired using a Keyence BZ-X800 Fluorescence Microscope at 60X magnification (1/50s exposure time). Images of four focal areas per well were captured for each experiment. ImageJ™ software was also used to quantify both cell number and morphological changes by a blinded reviewer. Mean number of myofibroblasts were calculated based on cytoplasmic granule intensity compared to quiescent fibroblasts by an established method (27,42).

**Western blotting**
Briefly, whole cell protein was extracted from both tissue and cells using RIPA lysis buffer, as previously described. In separate experiments, nuclear proteins were collected using a nuclear extraction kit (Activ Motif; Carlsbad, CA, catalog #40410), as described by the manufacturer. Both whole cell (40μg) and nuclear protein (100μg) were separated by 7.5% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were then incubated in either 3% nonfat milk or 5% BSA in either PBS or TBS-tween before incubation with primary antibodies specific for either: α-SMA (CST; 1:1,000 dilution), total TGFβRI (Invitrogen; catalog #PA5-14959; 1:1000 dilution), phospho-TGFβRI (Invitrogen; 1:1000 dilution), Col IV (SCBT; 1:500 dilution), phospho-Smad 2/3 (CST; catalog #8828; 1:500 dilution), β-actin (CST; catalog #8457L; 1:10,000 dilution) or histone deacetylase 1 (HDAC1, Novus Biologicals; Centennial, CO; catalog #NB100; 1:5000 dilution). After incubation in either rabbit (Sigma Aldrich; St. Louis, MO; catalog #A0545; 1:5,000 dilution) or mouse secondary antibody (Invitrogen; catalog #62-6520; 1:5,000 dilution), immunoreactive bands were detected using Azure Biosystems c500 Infrared Imaging System and cSeries Capture Software. Densitometric values were obtained using AzureSpot Analysis Software. Values of proteins of interest were normalized to either β-actin (whole cell) or HDAC1 (nuclear extracts). Data were expressed as fold change of vehicle.

**ELISA**
To examine NFAT activation, NFAT DNA binding activity was assessed by a TransAM® NFATc1 Transcription Factor Assay Kit (Active Motif; Carlsbad, CA; catalog #40796), as described by the manufacturer. This ELISA-based assay measures binding of active NFAT to its DNA recognition sequence. Briefly, nuclear cell extracts were incubated with an immobilized oligonucleotide containing a 5’-AGGAAA-3’ DNA motif. Bound NFAT was detected and quantified. Data were expressed as fold change of vehicle.

To assess TGFβ type 1 (TGFβ1) ligand secretion, ELISAs were also conducted according to manufacturer’s instructions (R&D Systems, Minneapolis, MN, catalog #DY167905). Using culture media collected from both vehicle- and tacrolimus-treated cells, active TGFβ1 concentrations were quantified by extrapolating the absorbance values of experimental samples from a standard curve of recombinant TGFβ1 proteins.

**Statistical Analysis**

Graph creation and statistical analyses for all experiments were performed using GraphPad software (Prism, San Diego, CA). Statistical tests were conducted utilizing either t-test or a two-way analysis of variance to detect differences between the means of the experimental groups. A p-value of <0.05 was considered statistically significant.
RESULTS

Overall animal health was preserved with tacrolimus treatment. There were no significant differences in body weights between tacrolimus and vehicle-treated animals (Table 1). Although kidney weights are lower in tacrolimus-treated animals compared to control, this difference was not statistically significant. A similar trend was observed for kidney weight-to-body weight ratios.

<table>
<thead>
<tr>
<th></th>
<th>Vehicle (n=8)</th>
<th>Tacrolimus (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>23.2 ± 0.6</td>
<td>23.0 ± 0.2</td>
</tr>
<tr>
<td>Kidney weight, mg</td>
<td>377 ± 15.2</td>
<td>338 ± 32</td>
</tr>
<tr>
<td>Kidney/body weight</td>
<td>16 ± 0.6</td>
<td>15 ± 1.4</td>
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Table 1: Body and kidney weights of vehicle and tacrolimus-treated mice. Values are means of with ± SEM of 8 animals per group.

Tacrolimus induces kidney damage in the form of renal fibrosis in mice. To establish that our in vivo model recapitulates the clinical morphological features of CNI-induced nephropathy, wild-type mice were treated with either tacrolimus (10mg/kg) or vehicle (ethanol/DMSO) for 21 days (Fig 1A). Tacrolimus and vehicle-treated mice
kidneys were assessed for histologic lesions via H&E staining (Fig 1B). Vehicle-treated kidneys retain a normal renal architecture that consisted of healthy glomeruli and tubules. However, tacrolimus-treated kidneys show notable glomerular and tubular changes consistent with CNI nephrotoxicity (43). Particularly, glomeruli from tacrolimus-treated kidneys display thrombotic microangiopathy, characterized by the presence of hyaline thrombi in glomerular capillaries (box). Additionally, the reduction of interstitial spacing in tacrolimus-treated kidneys is indicative of ECM expansion, another characteristic of CNI nephropathy (6). To confirm histologic characteristics of chronic CNI use, kidneys were further assessed for collagen (ECM) accumulation via Masson Trichrome staining (Fig. 1C). In tacrolimus-treated kidneys, ECM deposition (blue) was present in peri-glomerular and interstitial areas (arrows). These findings were confirmed with picrosirius red staining (Fig. 1D), an additional method to detect collagen abundance. Collectively, these findings establish that tacrolimus induces kidney damage in the form of renal fibrosis.
Figure 1: Tacrolimus induces kidney damage in the form of renal fibrosis in mice.
To establish that our in vivo model recapitulates the morphological features of CNI-induced nephropathy, wild-type mice were treated with either 10mg/kg tacrolimus or vehicle (ethanol/DMSO/saline) for 21 days (A). Tacrolimus-induced histologic lesions were assessed by H&E staining (B; square). ECM accumulation (collagen) was assessed by both Masson Trichrome staining (blue) (C; arrows) and Picrosirius red staining (D). Representative images shown of 8 animals per group.

Tacrolimus-induced renal fibrosis is accompanied by fibroblast activation in mice. While our findings establish that tacrolimus induces kidney fibrosis, the cellular source of ECM deposition was unknown. To examine whether fibroblast activation accompanies tacrolimus-induced renal fibrosis, myofibroblast marker α-smooth actin (α-SMA, red) was assessed via immunofluorescence and quantified (Fig. 2A). Vehicle-treated kidneys localize α-SMA expression exclusively in the renal vasculature, with low glomerular expression. However, α-SMA expression in tacrolimus-treated kidneys is limited not only to vascular cells, but also now present in the interstitium, lining nearby tubular and glomerular cells. These findings were confirmed via Western blot (Fig. 2B), with tacrolimus-treated kidneys showing increased α-SMA expression (2.2 ± 0.42 au) compared to vehicle-treated kidneys (1.0 ± 0.11 au). Collectively, these findings suggest
that fibroblasts, the primary matrix-producing cells in the kidney, serve as a culprit of CNI-induced renal fibrosis.

**Figure 2**: Tacrolimus induces fibroblast activation in mice. To investigate whether fibroblast activation accompanied renal fibrosis, wild-type mice were treated with either 10mg/kg tacrolimus or vehicle (ethanol/DMSO/saline) for 21 days. Myofibroblasts were visualized via immunofluorescence (A; red) and quantified with Zen 3.1 software (expressed as arbitrary light units, alu). Western blot (B) further quantified α-SMA expression. Representative images shown. Values are means ± SEM with 8 animals/group. Statistical tests conducted using a t-test to detect differences between the means of experimental groups. *p<0.05 and **p<0.005 vs vehicle.
Figure 2: Tacrolimus-induced renal fibrosis is accompanied by fibroblast activation in mice. To investigate whether fibroblast activation accompanied renal fibrosis, wild-type mice were treated with either 10mg/kg tacrolimus or vehicle (ethanol/DMSO/saline) for 21 days. Myofibroblast marker α-smooth muscle actin (α-SMA) was also visualized via immunofluorescence (A, red) and quantified with Zen 3.1 software (expressed as arbitrary light units, alu). Western blot (B) further quantified α-SMA expression. Representative images shown. Values are means ± SEM with 8 animals per group. Statistical tests conducted using a t-test to detect differences between the means of experimental groups. *p<0.05 and **p<0.005 vs vehicle.

Tacrolimus inhibits calcineurin activity/NFAT activation in renal fibroblasts.

The kidney contains a diverse population of cell types (epithelial tubular cells, mesangial cells, immune cells, etc.), some of which have been identified as contributors to renal fibrosis (12,32,38,44–47). Given their role as the primary matrix-producing cells in the kidney, we investigated whether renal fibroblasts were a cellular source of tacrolimus-induced renal fibrosis. In this endeavor, we initially assessed whether calcineurin (Cn) activity/NFAT activation was inhibited in fibroblasts treated with tacrolimus. To this end, resting kidney fibroblasts (quiescent cells that have not transitioned into motile ECM-producing myofibroblasts) were treated with either tacrolimus (1nM) or vehicle (0.01% ethanol) for 24 hours (Fig. 3A). We then assessed activation of well-known Cn...
substrate, NFAT1. While basal levels of nuclear NFAT (green) were present in vehicle-treated cells (Fig. 3B, i), tacrolimus-treated cells (3B, ii) showed decreased nuclear NFAT localization compared to vehicle. In contrast, a surge of nuclear NFAT is induced in cells exposed to ionomycin (Fig. 3B, iii), a Ca\(^{2+}\) ionophore known to induce Cn activation and subsequent NFAT nuclear translocation. However, tacrolimus treatment attenuated the surge in nuclear NFAT induced by ionomycin (3B, iv). Confirming these qualitative findings, NFAT activation assays (Fig. 3C) showed a statistically significant reduction in NFAT DNA binding in cells treated with tacrolimus compared to vehicle (tacrolimus: 0.74 ± 0.07 au vs vehicle: 1.0 ± 0.00 au). In contrast, NFAT binding significantly increased with vehicle and ionomycin treatment (1.4 ± 0.13 au), an effect attenuated in tacrolimus-treated fibroblasts (0.87 ± 0.06 au). Collectively, these findings demonstrate that tacrolimus effectively inhibits the Cn/NFAT axis in renal fibroblasts.
Figure 3: Tacrolimus inhibits calcineurin activity/NFAT activation in renal fibroblasts. To investigate whether tacrolimus inhibits calcineurin (Cn) signaling in vitro, renal fibroblasts were treated with either 1nM tacrolimus or vehicle for 24 hours (A). Activation of a well-known Cn substrate—Nuclear Factor of Activated T-cells (NFAT)
was assessed. To confirm NFAT activation, both vehicle and tacrolimus-treated cells were incubated with 1µM ionomycin (a Ca\textsuperscript{2+} ionophore known to induce Cn/NFAT signaling) for 15 min. Nuclear NFAT abundance was visualized by immunofluorescence and quantified by Cell Profiler software (B) (expressed as arbitrary light units, alu), while NFAT activation was assessed via NFAT transcription factor assays (C). Representative images shown. Values are means ± SEM from 3-4 independent studies. Statistical tests conducted using two-way analysis of variance to detect variances between experimental groups. *p<0.05, **p<0.005 and ***p<0.0005 vs vehicle; ##p<0.005 and ###p<0.0005 vs vehicle + ionomycin.

**Tacrolimus induces TGFβ receptor activation and downstream Smad 2/3 phosphorylation in mouse kidneys.** Next, we aimed to identify the signaling mediators driving tacrolimus-induced FMT. Kidneys of patients on long-term CNI therapy show increased expression of TGFβ1 ligand. However, the precise role of TGFβ signaling in mediating renal damage induced by CNIs remains to be defined. We then investigated whether tacrolimus induces TGFβ receptor activation. Western blot analysis (Fig. 4A) showed a decrease in total TGFβ receptor type 1 (TGFβRI) expression in tacrolimus-treated kidneys compared to vehicle (tacrolimus: 0.66 ± 0.09 au vs vehicle: 1.0 ± 0.05 au). TGFβRI activation was then examined by assessing phospho-TGFβRI expression, also via Western blot (Fig. 4B). Although tacrolimus
treatment reduced total TGFβRI expression, phosphorylation of the present receptors is increased (tacrolimus: 1.4 ± 0.15 au vs vehicle: 1.0 ± 0.12 au). These findings were confirmed with immunohistochemistry (Fig. 4C), as tacrolimus-treated kidneys showed greater phospho-TGFβRI expression (dark brown) in selected tubular epithelial cells. To assess the activation of downstream TGFβ receptor signaling mediators, phospho-Smad 2/3 expression was quantified via Western blot. Compared to vehicle, phospho-Smad 2/3 abundance was significantly increased with tacrolimus treatment (tacrolimus: 1.17 ± 0.05 vs vehicle: 1.0 ± 0.05 au) (Fig. 4D). Collectively, these findings demonstrate that tacrolimus activates the TGFβ/Smad signaling axis.
Figure 4: Tacrolimus induces TGFβ receptor activation and downstream Smad2/3 phosphorylation in mouse kidneys. To investigate whether tacrolimus induces TGFβ
receptor activation, wild-type C57BL6 mice were treated with either 10mg/kg tacrolimus or vehicle (ethanol/DMSO/saline) for 21 days. Mice kidneys were assessed for total TGFβRI expression via Western blot (A). TGFβRI activation was examined by assessing phospho-TGFβRI expression both via Western blot (B) and immunohistochemistry (C). To assess whether tacrolimus induces downstream TGFβ receptor signaling, phospho-Smad2/3 abundance (D) was assessed via Western blot. Representative images shown. Values are means ± SEM with 7-8 animals per group. Statistical tests conducted using a t-test to detect differences between the means of experimental groups. *p<0.05 and **p<0.005 vs vehicle.

Tacrolimus promotes fibroblast-to-myofibroblast transition (FMT) and ECM production. Next, we investigated whether renal fibroblasts were a cellular source of tacrolimus-induced renal fibrosis (Fig. 5A). As assessed by immunofluorescence, heat map analysis (Fig. 5B) showed enhanced α-SMA expression in tacrolimus-treated cells and revealed high luminescence around the cell perimeter (red). Consistently, Western blotting showed an increase in α-SMA protein abundance (tacrolimus: 1.6 ± 0.08 au vs. vehicle: 1.0 ± 0.00 au) (Fig. 5C). To further confirm the myofibroblast phenotype, as indicated by the presence of actin stress fibers, phalloidin abundance was assessed by immunofluorescence. Heat map analysis revealed that tacrolimus-treated cells showed increased phalloidin staining compared to vehicle (Fig. 5D). These findings establish
the presence of activated renal myofibroblasts with tacrolimus treatment. To provide evidence of enhanced motility of these cells, the rate of fibroblast migration (%) was assessed via scratch test assays (Fig. 5E). Compared to vehicle, tacrolimus-treated cells show a 17% increase in migration rate after 12 hours (tacrolimus: 75.5 ± 5.3% vs vehicle: 58.2 ± 4.4%). Taken together, these findings suggest that tacrolimus directly stimulates renal fibroblast differentiation into myofibroblasts with enhanced motility. Lastly, ECM production was examined in these activated myofibroblasts. Heat map analysis revealed increased Col IV protein expression in tacrolimus-treated cells compared to control (Fig. 5F). Consistently, Western blot quantification confirmed elevated Col IV expression (Tacrolimus: 1.2 ± 0.02 au vs vehicle: 1.0 ± 0.00 au) (Fig. 5G). Collectively, these findings demonstrate tacrolimus as an inducer of FMT and ECM production in the kidney.
Figure 5: Tacrolimus promotes fibroblast to myofibroblast transition (FMT) and ECM production. To investigate fibroblasts as a source of tacrolimus-induced renal fibrosis, renal fibroblasts were treated with either tacrolimus (1nM) or vehicle (0.01% ethanol) for 24 hours (A). Myofibroblast marker—α-SMA protein was visualized via
immunofluorescence (B) and quantified with Cell Profiler software (expressed as arbitrary light units, alu). Western blot (C) also quantified α-SMA expression. To further assess FMT, actin stress fiber content was visualized by immunofluorescent staining of phalloidin (D) and quantified with Cell Profiler software (expressed as alu). To evaluate the effect of tacrolimus on fibroblast motility, scratch test assays were conducted and cell motility rates (%) were calculated (E). ECM production was assessed by both immunofluorescent detection and quantification of collagen IV (Col IV) protein (using Cell Profiler software, expressed as alu) (F). Western blot further quantified Col IV expression (G). Representative images of at least 3-4 independent studies shown. Values are means ± SEM. Statistical tests conducted using a t-test to detect differences between experimental groups. *p<0.05, ***p<0.0005 and ****p<0.00005 vs vehicle.

**TGFβ signaling mediator Smad3 drives tacrolimus-induced FMT and ECM production in renal fibroblasts.** Finally, we examined the role of TGFβRI signaling in tacrolimus-induced FMT ([Fig. 6A](#)). As assessed by ELISA ([Fig. 6B](#)), increased TGFβ1 ligand abundance is observed in media from tacrolimus-treated cells compared to control (tacrolimus: 421.1 ± 27.3 vs vehicle: 107.9 ± 23.2). TGFβ receptor activation was then examined by visualizing phospho-TGFβRI expression via immunofluorescence ([Fig. 6C](#)). Compared to vehicle, tacrolimus-treated cells showed increased luminescent expression of phospho-TGFβRI, which was also confirmed by
Western blot (Fig. 6D) (tacrolimus: $1.31 \pm 0.11$ au vs. vehicle: $1.0 \pm 0.00$ au). Western blot quantification also showed enhanced nuclear abundance of the TGFβ signaling mediators Smad 2/3 in tacrolimus-treated cells compared to control (Fig. 6E) (tacrolimus: $1.3 \pm 0.10$ au vs. vehicle: $1.0 \pm 0.00$ au). To assess the role of TGFβ receptor signaling in tacrolimus-induced FMT, cellular morphological changes were visualized in fibroblasts treated with either tacrolimus (1nM) or vehicle (0.01% ethanol) in the presence or absence of the selective Smad3 inhibitor– SIS3 (500nM) for 24 hrs (Fig. 6F). While fibroblasts maintain their normal spindle-like resting morphology with vehicle treatment (Fig. 6F; i, iii), tacrolimus treatment induces resting fibroblasts to adopt a more myofibroblast-like phenotype (Fig. 6F; ii), which are greater in size, containing ruffled membranes and enlarged endoplasmic reticulum. Interestingly, pre-treatment of fibroblasts with SIS3 significantly attenuated the tacrolimus-induced myofibroblast phenotype (Fig. 6F; iv). Western blot quantification confirmed these morphological findings, as α-SMA (tacrolimus: $2.3 \pm 0.13$ au vs. vehicle: $1.0 \pm 0.00$ au) and Col IV expression (tacrolimus: $1.5 \pm 0.11$ au vs. vehicle: $1.0 \pm 0.00$ au) both increased with tacrolimus treatment (Fig. 6G). Notably, SIS3 cotreatment attenuated these tacrolimus-induced profibrotic effects (α-SMA, tacrolimus+SIS3: $1.4 \pm 0.09$ au vs. tacrolimus: $2.3 \pm 0.13$ au), (Col IV, tacrolimus+SIS3: $1.1 \pm 0.03$ au vs. tacrolimus: $1.5 \pm 0.11$ au). Taken together, our results indicate that 1) fibroblasts are a source of
tacrolimus-induced TGFβ secretion and 2) TGFβRI signaling mediator Smad 2/3 drives tacrolimus-induced FMT and ECM production.

Figure 6: TGFβ signaling mediators Smad2/3 drive tacrolimus-induced FMT and ECM production in renal fibroblasts. To examine the role of TGFβ receptor signaling in tacrolimus-induced FMT, renal fibroblasts were treated with either tacrolimus (1nM) or
vehicle (0.01% ethanol) for 24 hours (A). TGFβ1 ligand secretion was quantified in media collected from both tacrolimus- and vehicle-treated cells by ELISA (B). TGFβRI activation was examined by visualizing phospho-TGFβRI expression via immunofluorescence (C) and quantified with Cell Profiler software (expressed as arbitrary light units, alu). Western blot (D) further quantified phospho-TGFβRI expression. Nuclear abundance of the TGFβ signaling mediators Smad2/3 was assessed by Western blot (E). To investigate the relevance of the TGFβ/Smad signaling pathway in tacrolimus-induced FMT and ECM accumulation, cellular morphological changes were visualized and quantified with ImageJ software in fibroblasts treated with either tacrolimus (1nM) or vehicle (0.01% ethanol) in the presence or absence of Smad3 inhibitor—SIS3 (500nM) (F). To confirm these morphological findings, α-SMA and Col IV abundance were assessed via Western blot (G). Representative images of at least 3 independent studies shown. Values are means ± SEM. Statistical tests conducted using either two-way analysis of variance or a t-test to detect differences between the experimental groups. *p<0.05, **p<0.005, ***p<0.0005 and ****p<0.00005 vs vehicle; #p<0.05, ##p<0.005 and ####p<0.00005 vs tacrolimus.
DISCUSSION

It is well-established that chronic CNI use invokes irreversible kidney damage in the form of renal fibrosis (10,36,48). CNIs are directly toxic to various renal cell types including vascular (21) mesangial, glomerular and tubular epithelial cells (22,23). However, given the diverse population of cells in the kidney (24), additional targeted cell types and signaling mechanisms behind this progressive disease state continues to be defined. In this study, we identified renal fibroblasts as a contributor to CNI-induced fibrosis. The overall findings of our study reveal that the TGFβ/Smad pathway is a pivotal signaling event underlying FMT and subsequent CNI-induced renal fibrosis. Specifically, using both in vivo and in vitro models of CNI-induced nephropathy, we demonstrate that 1) tacrolimus inhibits the Cn/NFAT axis while inducing TGFβ1 ligand secretion and receptor activation, 2) aberrant TGFβ receptor activation stimulates Smad-mediated production of myofibroblast markers, notable features of FMT and 3) FMT contributes to ECM expansion in tacrolimus-induced renal fibrosis. Taken together, these results not only incorporate renal fibroblasts into the growing list of cell types impacted by CNIs, but also identify renal FMT as a process mediated via a TGFβ-dependent mechanism (Figure 7).
Figure 7: Proposed Schema. Tacrolimus induced fibroblast to myofibroblast (FMT) transition via a TGFβ-dependent mechanism to contribute to renal fibrosis: 1) tacrolimus inhibits the calcineurin (Cn)/NFAT axis while inducing TGFβ1 ligand secretion and receptor activation in renal fibroblasts, 2) aberrant TGFβ receptor activation stimulates
Smad-mediated production of myofibroblast markers, notable features of FMT and 3) FMT contributes to extracellular matrix (ECM) expansion in tacrolimus-induced renal fibrosis.

Previous experimental studies characterize features of CNI-induced renal damage using animal models susceptible to kidney damage (including CD-1, BALB/c, etc.) (22,49). However, we provide additional evidence of CNI nephropathy using C57BL/6 mice—one of the most commonly used mouse strains in kidney injury research (50,51). Given that C57BL/6 mice are resistant to chronic kidney disease (CKD)-associated pathologies (50,51), we demonstrate the profuse capacity of tacrolimus to induce histological signs of chronic renal damage, particularly interstitial fibrosis. Fibrosis was characterized by increased collagen deposition, accompanied by the appearance of α-SMA-positive myofibroblasts in damaged areas (Figure 2A). Given our findings, the next step became to identify the cellular source of the tacrolimus-induced renal damage in addition to the signaling mechanism that drives this chronic (and irreversible) process.

**Tacrolimus inhibits the Cn/NFAT axis while inducing TGFβ1 ligand secretion and receptor activation.** In mesangial cells, TGFβ (partially via the Cn/NFAT axis) upregulates ECM production (52). To determine whether renal
fibroblasts were also a cellular source of tacrolimus-induced renal fibrosis, we initially assessed whether Cn/NFAT activation was inhibited in tacrolimus-treated fibroblasts. Our findings demonstrated that tacrolimus inhibited the Cn/NFAT axis in renal fibroblasts (Figure 3). This study along with previous reports (52,53) suggests that either activation or suppression of NFAT could mediate CNI-induced nephropathy. Given the capacity of TGFβ to induce ECM production in both mesangial cells (52) and tubular epithelia (12), this study aimed to investigate TGFβ signaling as a driver of tacrolimus-induced FMT. TGFβ1 has been reported as a lead signaling mediator of CsA-induced nephrotoxicity (36,45,54). We found that tacrolimus treatment also resulted in elevated renal fibroblast secretion of TGFβ1 ligand (Figure 6B), which becomes available to bind receptors in both a paracrine and autocrine fashion (55). Previous reports were consistent with our findings, as increased TGFβ1 expression was observed in renal biopsies obtained from transplant recipients with CNI nephropathy (32,56) and in kidneys of tacrolimus-treated Sprague-Dawley rats (57).

In the presence of TGFβ1 ligand binding, membrane-bound type II TGFβ receptor subunits (TGFβRII) phosphorylate and thereby activate type I TGFβ receptor subunits (TGFβRI) to initiate downstream TGFβ signaling (58). In addition to promoting extracellular TGFβ1 ligand release, we show both in vivo and in vitro that TGFβRI activation increased with tacrolimus treatment (Figure 4B-C; 6C). Our findings illustrate that tacrolimus reduced total TGFβRI protein expression in vivo (Figure 4A), however,
this observation could be attributed to a compensatory downregulation of the total TGFβ receptor pool—to prevent further activation of the receptor. It is worth noting that tacrolimus can potentially induce TGFβRI activation through association with its cytosolic immunophilin, FKBP12. TGFβRI activation is usually inhibited by FKBP12 binding at the cytoplasmic tail of the receptor, blocking access to activation signals (57). However, in the presence of tacrolimus (FK506), FKBP12 binds tacrolimus, prompting release from the receptor to cause downstream kinase activation and subsequent TGFβR signaling (21).

Aberrant TGFβ receptor activation stimulates Smad-mediated production of myofibroblast markers, notable features of fibroblast-to-myofibroblast transition (FMT). TGFβ receptor activity prompts downstream recruitment and activation of intracellular mediators Smad2/3, which translocate to the nucleus to regulate gene transcription (24). A previous in vitro study reported that tacrolimus upregulated Smad2 expression in mesangial cells (38). However, we expanded these findings by demonstrating that tacrolimus treatment increased Smad2/3 activation both in kidneys and in renal fibroblasts (Figure 4E, 6E). We further demonstrated that Smad signaling drives tacrolimus-induced FMT (Figure 6F-G). Assessing morphological features of FMT, tacrolimus treatment induced phenotypic changes indicative of a transition into contractile myofibroblasts (27) (Figure 5; 6F-G). Morphological changes were accompanied with upregulation of established myofibroblast markers (Figure 6F-G). On
the contrary, pharmacological inhibition of Smad3 activation attenuated these tacrolimus-induced phenotypic changes (Figure 6F-G). Our findings reveal that the TGFβ/Smad signaling axis mediates tacrolimus-induced FMT in kidneys, which is consistent with both observations from patient renal biopsies (56) and studies conducted with human kidney fibroblasts (44).

**FMT contributes to ECM expansion in tacrolimus-induced renal fibrosis.**

The progression of kidney disease towards end-stage renal failure is facilitated by pathological changes such as ECM accumulation (47). In CKD, the primary cell source responsible for ECM accumulation is the myofibroblast, a mesenchymal cell type possessing the phenotypic characteristics of both fibroblasts and smooth muscle cells (27). Myofibroblasts arise from circulating progenitors, tubular epithelial cells and resident fibroblasts (27). While the role of myofibroblasts in tacrolimus-induced renal damage is widely accepted, their origin is still a matter of debate (28). Human renal proximal tubular epithelial cells (RPTECs) have been utilized extensively in the investigation of both CsA and tacrolimus nephropathy (32,45,54,59). However, given their role as the primary matrix-producing cells in the kidney, we selected mouse renal fibroblasts to further investigate mechanisms underlying renal fibrosis with CNI use. Resting renal fibroblasts exposed to tacrolimus for 24 hours exhibited profound morphological alterations consistent with FMT (27), which was confirmed by a significant increase in de novo α-SMA and Col IV protein expression (Figure 5B-C and
The myofibroblast phenotype was further validated by detection of abundant stress fibers in tacrolimus-exposed cells, enabling the acquisition of enhanced cell motility and contractibility (Figure 5D-E). In line with our in vivo findings, these data indicate that tacrolimus treatment is sufficient to induce FMT, a process capable of causing sinister consequences in the kidney.

It is worth noting that epithelial-to-mesenchymal transition (EMT) is recognized as a contributor to the myofibroblast population in renal interstitial fibrosis and is well known to be present in CNI-treated kidneys (12,45,54). EMT is the phenomenon characterized by disruption of polarized tubular epithelial cell morphology into characteristic features of contractile fibroblast cells (27,60). However, given its capacity to release the profibrotic factor TGFβ into the extracellular environment, our data suggests that activated fibroblasts may be initiators of tacrolimus-induced EMT (12,44). This conclusion is based on our 1) in vivo data showing a lack of α-SMA-positive tubular epithelial cells and 2) in vitro data demonstrating that tacrolimus significantly increased α-SMA protein levels after only 24 hours, while a previously published study showed tacrolimus-induced expression of α-SMA mRNA in tubular epithelial cells after 48 hours (12). Moreover, 3) because our in vivo findings also show increased TGFβRI activation in select nephron segments, we suspect that this occurrence is due to fibroblast secretion of TGFβ1 ligand, binding and stimulating nearby epithelial cell receptors. Fibroblast activation preceding EMT is quite possible, as this phenomenon has been
noted in the pathogenesis of tumor invasion (61), acute renal injury (62), and pulmonary fibrosis (63,64). Fibroblast activation is associated with chronic renal disease, consisting of progressive interstitial scarring and tubular cell atrophy. Our findings indicate that tacrolimus triggers TGFβ1 signaling to induce FMT, initiating a cascade of downstream profibrotic events that cause further damage to the renal parenchyma (Figure 7).
**PERSPECTIVES AND SIGNIFICANCE**

This study expands understanding of the many cell types and central signaling mediators driving CNI-induced renal fibrosis. Not only does our work incorporate renal fibroblasts into the growing list of cell types negatively impacted by CNIs, but it also identifies renal fibroblast-to-myofibroblast transition as a process mediated via a TGFβ-dependent mechanism. Ultimately, our findings provide a more comprehensive picture of how calcineurin inhibition and profibrotic pathways intersect in the kidneys. Considering the impact of aberrant TGFβ signaling on multiple cell types in the kidney, it is now necessary to demonstrate the feasibility of inhibiting TGFβ signaling as a means of preventing the renal fibrosis associated with chronic CNI use. Understanding the cellular and molecular mechanisms of tacrolimus-induced renal damage and their role in the initiation of fibrosis will inform the development of novel therapeutic strategies to ameliorate the nephropathy observed in patients requiring long-term CNI therapy.
ACKNOWLEDGEMENTS:

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CHAPTER II

Friend or Foe? Inhibition of the TGFβ receptor attenuates tacrolimus-induced renal fibrosis while exacerbating kidney dysfunction

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**ABSTRACT**

Use of the immunosuppressants calcineurin inhibitors (CNIs) are limited by irreversible kidney damage, hallmarked by renal fibrosis. We reported that aberrant TGFβ/Smad signaling drives the profibrotic effects induced by CNIs. However, controversy surrounds the feasibility of targeting this aberrant TGFβ signaling to prevent the renal damage associated with CNI therapy. We hypothesize that disruption of TGFβ signaling ameliorates renal damage while maintaining CNI-mediated immunosuppression. To test this, C57BL/6 mice received tacrolimus (1mg/kg) or vehicle for 28 days. A subset of tacrolimus-treated animals also received concomitant injections of LY2109761 (inhibitor of TGFβ receptor activation, 10mg/kg) also for 28 days. At the end of the study, renal morphological and functional changes were assessed. While tacrolimus-treated kidneys display evidence of extracellular matrix (ECM) accumulation, inhibition of TGFβ receptor activation by LY2109761 attenuates this tacrolimus-induced effect. These findings are accompanied by a decrease in α-smooth muscle actin (α-SMA; myofibroblast marker) and collagen IV (Col IV; ECM protein) expression. Conversely, inhibition of TGFβ signaling also disrupts the glomerular filtration barrier as evident by enhanced mesangial matrix expansion and albuminuria. Moreover, inhibition of TGFβ signaling promotes electrolyte disturbances, a characteristic of tubular dysfunction. Collectively, these findings indicate that inhibition of TGFβ signaling promotes positive effects such as the attenuation of interstitial fibrosis and fibroblast activation. However, disruption of TGFβ signaling also promotes nephrotoxic effects such as disruption of
both 2) glomerular and 3) tubular functions. Taken together, these results indicate that renal TGFβ signaling exerts both beneficial and detrimental effects, which establish its role as both a friend and foe in the kidney. Given this revelation, directly targeting TGFβ signaling is not a suitable approach to prevent the renal damage associated with CNI therapy.
INTRODUCTION

Incorporation of calcineurin inhibitors (CNIs) tacrolimus and cyclosporine into clinical practice has revolutionized post-transplantation immunosuppression. However, their nephrotoxic side effects pose a major concern (1). Long-term CNI therapy causes irreversible damage to the kidneys in the form of renal fibrosis. CNI-induced renal fibrosis is a pathological process characterized by increased extracellular matrix (ECM) protein deposition coupled with reduced matrix turnover, thereby promoting ECM expansion (2). These matrix disturbances ultimately lead to a decline in renal function and can progress to end-stage renal failure, ultimately requiring a transplant for survival (3). The molecular mechanism by which CNIs induce renal fibrosis remains to be better understood, and to date, there are no specific therapeutic strategies to mitigate this damage. This dilemma presents a critical need to understand the pathophysiologic mechanisms underlying CNI-induced renal damage. More importantly, approaches to maintain CNI-mediated immunosuppression without the associated renal damage must also be explored.

Kidneys of patients on chronic CNI therapy show increased expression of the proinflammatory cytokine Transforming Growth Factor β (TGFβ) (4). TGFβ is a multipotent regulator of cell survival, differentiation, proliferation, and extracellular matrix (ECM) production in a variety of tissues (5,6). Our group established that aberrant TGFβ activation and downstream signaling contribute to tacrolimus-induced renal fibrosis.
(Chapter I). We demonstrated that aberrant TGFβ receptor activation stimulates Smad-mediated fibroblast transition into myofibroblasts that bolster production of extracellular matrix proteins, which contribute to tacrolimus-induced renal fibrosis. Given these findings, we set out to demonstrate the feasibility of targeting aberrant TGFβ receptor signaling to prevent the renal damage associated with CNI therapy. We tested the hypothesis that disruption of TGFβ activation ameliorates renal damage while maintaining CNI-mediated immunosuppression. Using an in vivo model of CNI-induced nephropathy, we demonstrate that inhibition of TGFβ signaling 1) promotes positive effects such as the attenuation of interstitial fibrosis and fibroblast activation. However, inhibition of TGFβ signaling also promotes nephrotoxic effects such as disruption of both 2) glomerular and 3) tubular functions. Taken together, these results indicate that renal TGFβ signaling exerts both beneficial and detrimental effects, which establish its role as both a friend and foe in the kidney.
EXPERIMENTAL DESIGN

CNI Model

*In vivo:* Two *independent* studies were conducted where male WT C57Bl/6 mice (n=22 total; n=7-8/group; 13 weeks old; Jackson Laboratory) were randomly assigned to receive subcutaneous injections of FK-506 (tacrolimus, 1 mg/kg/day) (Enzo) or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals also received subcutaneous injections of LY2109761 (inhibitor of TGFβRI activation; MedChem Express; catalog# HY-12075, 10 mg/kg/day) for 28 days. Order of daily injections alternated between treatment groups to minimize potential confounding variables. Mice were socially housed in standard cages with a regular 12hr light/dark cycle and fed standard rodent chow. All animal protocols and procedures were approved by the Animal Care and Use Committee (IACUC) at Wright State University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Urine Analysis

Metabolic cage studies were performed to collect 24-hour urine samples for further analysis. To assess renal tubular function, total urinary electrolytes were calculated using an electrolyte analyzer (EasyLyte, Medica). Briefly, Na, K, and Cl concentrations (mmol/L) were measured in urine samples and normalized to 24-hour urine volume.
Renal Atrophy Assessment

Renal atrophy was determined from calculation of total kidney mass-to-body weight ratios.

Histology

Kidney samples were fixed in freshly prepared 4% paraformaldehyde and routine histology was performed on 5 µm-thick sections. Extracellular matrix accumulation (fibrosis) was initially assessed by collagen deposition using both Masson Trichrome (Abcam, Cambridge, United Kingdom, catalog #ab150686) and Picrosirius Red protocols (Abcam, catalog #ab150681). Images were acquired using a Keyence BZ-X800 Fluorescence microscope (60X magnification, 1/80s exposure time). Alterations in kidney morphology were further assessed by examination of both hematoxylin and eosin (H&E)- and Periodic Acid-Schiff (PAS)-stained sections (conducted by AML Laboratories; St. Augustine, FL). Images of glomerular and tubular changes were captured using a Keyence BZ-X800 Fluorescence Microscope (60X magnification, 1/45s exposure time).

To assess expression of IL-2, chromogen immunohistochemistry staining was also performed. Briefly, endogenous peroxidases were quenched in deparaffinized sections by incubation in 0.3% H₂O₂ (Abcam; Cambridge, United Kingdom; catalog# ab64218)
for 15 minutes. Sections were then blocked in 10% goat serum (Life Technologies) in TBS-tween for 30 min and incubated with IL-2-specific antibody (Rockland; catalog# 209401B95; dilution: 1:100). Sections were incubated with HRP-conjugated secondary antibody (Invitrogen, catalog# A32794, 1:200 dilution). Bound antibody was identified by 3,3'-diaminobenziidine (DAB) staining according to the manufacturer’s instructions (Vector Laboratories; Burlingame, CA; catalog# SK-4100). In addition, sections were counterstained with methyl green according to the manufacturer’s instructions (Vector Laboratories; catalog# H-3402-500). IL-2 abundance was visualized using a Keyence BZ-X800 Fluorescence Microscope and brightfield images at 60X magnification and 1/30s exposure time were captured.

**Western blotting**

Briefly, whole cell protein was extracted from tissues using RIPA lysis buffer. Whole cell or urinary protein (40μg) protein was separated by 7.5-15% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were then incubated in either 3% nonfat milk or 5% BSA in either PBS or TBS-tween before incubation with primary antibodies specific for either: phospho-TGFβRI (Invitrogen; 1:1000), IL-2 (Rockland; 1:1000), α-SMA (CST; 1:1,000 dilution), Col IV (SCBT; 1:500 dilution), albumin (Invitrogen; catalog# PA5-89332;1:1,000 dilution). After incubation in either rabbit (Sigma Aldrich; St. Louis, MO; catalog# A0545;1:5,000 dilution) or mouse
secondary antibody (Sigma Aldrich; St. Louis, MO; catalog# A0545; 1:5,000 dilution), immunoreactive bands were detected using Azure Biosystems c500 Infrared Imaging System and cSeries Capture Software. Densitometric values were obtained using AzureSpot Analysis Software. Values of proteins of interest were normalized to total protein. Data expressed as fold change of vehicle.

**Statistical Analysis**

Graph creation and statistical analyses for all experiments were performed using GraphPad software (Prism, San Diego, CA). Statistical tests were conducted utilizing a one-way analysis of variance followed by a Tukey post-hoc analysis. A p-value of <0.05 was considered statistically significant.
RESULTS

Overall animal health was preserved with LY21 treatment. Similar to our previous findings, animal health was preserved with tacrolimus administration (Table 1). No signs of distress were observed as there were no significant differences in both body weights and food intake between vehicle-, tacrolimus-, or tacrolimus+LY2109761 (abbreviated as LY21)-treated groups. Therefore, no animals were excluded from the study.

<table>
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<th>Vehicle ((n=8))</th>
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<th>Tacrolimus + LY21 ((n=7))</th>
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<td>Food Intake, g</td>
<td>2.2 ± 0.2</td>
<td>1.9 ± 0.2</td>
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Table 1: Overall animal health was preserved with LY21 treatment. Body weights (grams, g) and food intake (g) of vehicle, tacrolimus, and tacrolimus+LY21-treated mice after 28 days of treatment. Values are means ± SEM of 7-8 animals per group.

LY21 inhibits tacrolimus-induced renal TGFβ receptor activation. We previously reported that aberrant TGFβ receptor activation and downstream signaling contribute to tacrolimus-induced renal fibrosis. However, the feasibility of targeting aberrant TGFβ signaling to prevent the renal damage associated with chronic
tacrolimus therapy remains under investigation. Utilizing the pharmacological agent LY2109761 (abbreviated as LY21), which inhibited TGFβ receptor activation in both the pancreas and liver (7), we assessed whether LY21 was sufficient to inhibit tacrolimus-induced renal TGFβ signaling. To this end, wild-type C57BL6 mice were treated with either 1 mg/kg tacrolimus or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals was treated with 10 mg/kg LY21 for 28 days. Mice kidneys were assessed for phosphorylated (activated) TGFβ receptor type 1 subunit (TGFβRI) expression via Western blot (Fig. 1A). Consistent with our previous findings, tacrolimus-treated kidneys show an increased abundance in phospho-TGFβRI expression compared to vehicle (tacrolimus: 1.5 ± 0.1 – au vs vehicle: 1.0 ± 0.08 – au) (Fig. 1B). However, treatment with LY21 prevented this tacrolimus-induced effect (tacrolimus+LY21: 1.1 ± 0.08 – au vs tacrolimus: 1.5 ± 0.1 – au) (Fig. 1B). Collectively, these findings demonstrate the feasibility of LY21 to attenuate tacrolimus-induced TGFβ receptor activation in the kidneys.
Figure 1: **LY21 inhibits tacrolimus-induced renal TGFβ receptor activation.** To investigate whether LY2109761 inhibits tacrolimus-induced renal TGFβ receptor activation, wild-type C57BL6 mice were treated with either 1 mg/kg tacrolimus or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals was treated with 10 mg/kg LY2109761 (inhibitor of TGFβ receptor activation) for 28 days. Mice kidneys were assessed for phosphorylated (activated) TGFβ receptor type 1 subunit (TGFβRI) expression via Western blot (A) and quantified (B). Representative images shown. Values are means ± SEM with 7-8 animals/group. Statistical tests conducted using one-way analysis of variance and all pair-wise comparisons carried out with Tukey programs. **p<0.005 vs vehicle; #p<0.05 vs tacrolimus.

**TGFβ receptor inhibition does not disrupt tacrolimus-induced IL-2 suppression in the kidneys.** In targeting TGFβ signaling as a therapeutic approach to prevent CNI-induced renal fibrosis, it is essential to establish that downregulation of TGFβ signaling will maintain/ not prevent/ not interfere with the vital immunosuppression that necessitates CNI use. To investigate whether tacrolimus-induced immunosuppression is maintained, mice kidneys were assessed for expression of the proinflammatory cytokine interleukin-2 (IL-2) via immunohistochemistry (Fig. 2A). Compared to vehicle, tacrolimus-treated kidneys exhibited lower IL-2 expression (dark brown) in both glomeruli and selected tubular epithelial cells. Moreover, IL-2 expression
remained low with LY21 treatment. These findings were confirmed via Western blot analysis (Fig. 2B) (vehicle: 1.0 ± 0.07 – au vs tacrolimus: 0.72 ± 0.04 – au vs tacrolimus+LY21: 0.73 ± 0.05 – au). Collectively, these findings demonstrate that inhibition of TGFβ signaling maintains tacrolimus-induced IL-2 suppression in the kidneys.
Figure 2: TGFβ receptor inhibition does not disrupt tacrolimus-induced IL-2 suppression in the kidneys. To investigate whether renal TGFβ receptor inhibition maintains tacrolimus-induced immunosuppression, wild-type C57BL6 mice were treated with either 1 mg/kg tacrolimus or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals was treated with 10 mg/kg LY2109761 (inhibitor of TGFβRI
activation) also for 28 days. Mice kidneys were assessed for IL-2 expression via immunohistochemistry (A) and Western blot (B). Representative images shown. Values are means ± SEM with 3-4 animals/group. Statistical tests conducted using one-way analysis of variance and all pair-wise comparisons carried out with Tukey programs. *p<0.005 vs vehicle.

**TGFβ receptor inhibition attenuates tacrolimus-induced renal fibrosis and fibroblast activation.** To investigate whether TGFβ receptor inhibition prevents tacrolimus-induced renal fibrosis, mice kidneys were assessed for ECM (collagen) accumulation via Masson Trichrome staining (Fig. 3A, top). In tacrolimus-treated kidneys, ECM accumulation (blue) was present in the interstitium. However, interstitial ECM accumulation was significantly reduced with LY21 treatment. These findings were confirmed by Picrosirius red staining, an additional method to detect collagen accumulation (Fig. 3A, bottom). Additionally, Western blot analysis quantified Collagen IV (Col IV) abundance (Fig. 3B), as tacrolimus-treated kidneys show increased Col IV expression compared to vehicle-treated kidneys (tacrolimus: 1.5 ± 0.28- au vs vehicle: 1.0 ± 0.15 – au). With LY21 treatment, this tacrolimus-induced effect was attenuated (tacrolimus+LY21: 0.5 ± 0.06 – au vs tacrolimus: 1.5 ± 0.28– au). We recently reported that fibroblast activation was a contributor to the ECM accumulation induced by tacrolimus treatment. To examine whether TGFβ receptor inhibition prevents tacrolimus-induced fibroblast activation, myofibroblast marker α-smooth actin (α-SMA,
red) was assessed via Western blot (Fig. 3B). Consistent with previous findings, tacrolimus-treated kidneys showed increased α-SMA expression compared to vehicle-treated kidneys (tacrolimus: 2.2 ± 0.20- au vs vehicle: 1.0 ± 0.12 – au). However, these effects were attenuated with LY21 treatment (tacrolimus+LY21: 1.1 ± 0.04 – au vs tacrolimus: 2.2 ± 0.20- au). Collectively, these findings establish that TGFβ receptor inhibition attenuates both tacrolimus-induced ECM accumulation and fibroblast activation.
A

Trichrome

Sirius Red

B

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<th>Vehicle</th>
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<th>Tacrolimus + LY21</th>
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<tr>
<td>α-SMA</td>
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<tr>
<td>Total protein</td>
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**Col IV/Total protein (Fold vs Vehicle)**

**α-SMA/Total protein (Fold vs Vehicle)**

# # #

*** ***
Figure 3: TGFβ receptor inhibition attenuates tacrolimus-induced renal fibrosis and fibroblast activation. To investigate whether TGFβ receptor inhibition attenuates tacrolimus-induced renal fibrosis, wild-type C57BL6 mice were treated with either 1 mg/kg tacrolimus or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals was treated with 10 mg/kg LY21 also for 28 days. Mice kidneys were assessed for ECM (collagen) accumulation via Masson Trichrome (blue) and Picrosirius Red staining (red) (A). Collagen IV (Col IV) abundance was then quantified via Western blot (B). To assess whether inhibition of TGFβ signaling abrogates tacrolimus-induced fibroblast activation, α-smooth actin (α-SMA, myofibroblast marker) expression was quantified by Western blot (B). Representative images shown. Values are means ± SEM with 3-4 animals/group. Statistical tests conducted using one-way analysis of variance and all pair-wise comparisons carried out with Tukey programs. ***p<0.0005 vs vehicle, #p<0.05 and ##p<0.005 vs tacrolimus.

Low kidney mass induced by tacrolimus treatment remains with TGFβ receptor inhibition. CNI-induced nephropathy is uniformly characterized by the triad of glomerulosclerosis, interstitial fibrosis, and tubular damage, which contribute to lower kidney weights (8). Although TGFβ receptor inhibition attenuates the interstitial fibrosis induced by tacrolimus, mice kidney weights were assessed to further evaluate the presence of kidney damage (Table 2). Consistent with evidence of renal damage (2), both kidney mass and kidney mass-to-body weight ratios were reduced in tacrolimus-
treated kidneys compared to vehicle. Interestingly, kidney mass remained lower with LY21 treatment. Taken together, these findings suggest that damage to the renal parenchyma remain with TGFβ receptor inhibition.

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<th>Tacrolimus + LY21 (n=7)</th>
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<td>0.49 ± 0.02</td>
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**Table 2: Low kidney mass induced by tacrolimus treatment remains with TGFβ receptor inhibition.** Kidney mass (mg) and kidney mass-to-body weight ratios of vehicle, tacrolimus, and tacrolimus+LY21-treated mice. Values are means ± SEM of 7-8 animals per group. Statistical tests conducted using one-way analysis of variance and all pair-wise comparisons carried out with Tukey programs. *p<0.05, **p<0.005, ***p<0.0005, and ****p<0.00005 vs vehicle.

**Inhibition of renal TGFβ signaling is accompanied by enhanced mesangial matrix expansion.** To investigate whether inhibition of TGFβ signaling promotes damage to the renal parenchyma, mice kidneys were assessed for histological lesions via H&E staining (**Fig. 4A**). Consistent with our previous findings, tacrolimus-treated kidneys show notable glomerular and tubular changes compared to vehicle-treated kidneys. Particularly, glomeruli from tacrolimus-treated kidneys display thrombotic
microangiopathy, characterized by the presence of hyaline thrombi in glomerular capillaries (white box). Additionally, the reduction of interstitial spacing in tacrolimus-treated kidneys is indicative of ECM expansion, a hallmark of CNI nephropathy (9). Although LY21 treatment attenuates the tacrolimus-induced reduction in interstitial spacing, glomeruli of these animals display profound morphological changes. Particularly, the reduction in Bowman’s space (yellow boxes) suggests the presence of mesangial matrix expansion. To confirm these glomerular disturbances, kidneys were further assessed for mesangial matrix expansion via Periodic Acidic-Schiff (PAS) staining (Fig. 4B). Compared to vehicle, glomeruli of tacrolimus-treated animals display mesangial expansion (purple; arrows), an effect enhanced with LY21 treatment. Taken together, these findings determine that inhibition of renal TGFβ signaling is exacerbated tacrolimus-induced morphological disturbances, notably enhanced mesangial matrix expansion.
Figure 4: **Inhibition of renal TGFβ signaling is accompanied by enhanced mesangial matrix expansion.** To investigate whether inhibition of TGFβ signaling
promotes damage to the renal parenchyma, wild-type C57BL6 mice were treated with either 1 mg/kg tacrolimus or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals was treated with 10 mg/kg LY21 also for 28 days. Mice kidneys were assessed for histological lesions via H&E staining (A; boxes). PAS staining (B; arrows) was utilized to further assess disturbances in the mesangial matrix. Representative images shown of 3-4 animals/group.

Inhibition of renal TGFβ signaling disrupts the glomerular filtration barrier.

To evaluate disruption of glomerular barrier function with inhibition of TGFβ signaling, metabolic cage studies were performed to assess for urinary markers of renal damage. Particularly, evidence of urinary albumin (microalbuminuria) was investigated via Western blot (Fig. 5A). Compared to vehicle, urinary albumin was elevated in tacrolimus-treated mice (tacrolimus: 1.5 ± 0.2 – au vs vehicle: 1.0 ± 0.1- au), an effect also enhanced with LY21 treatment (tacrolimus+LY21: 2.7 ± 0.6 – au vs tacrolimus: 1.5 ± 0.2- au). Taken together, these findings suggest that inhibition of renal TGFβ signaling exacerbates disruption of the glomerular barrier.
Inhibition of renal TGFβ signaling exacerbates disruption of the glomerular barrier. To investigate whether further disruption of the glomerular barrier occurs with inhibition of renal TGFβ signaling, wild-type C57BL6 mice were treated with either 1 mg/kg tacrolimus or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals was treated with 10 mg/kg LY21 also for 28 days. Metabolic cage studies were performed to assess for urinary markers of renal damage. Particularly, evidence of urinary albumin (microalbuminuria) was investigated via
Western blot (A). Representative image shown. Values are means ± SEM with 7-8 animals/group. Statistical tests conducted using one-way analysis of variance and all pair-wise comparisons carried out with Tukey programs. *p<0.05 vs vehicle.

**Inhibition of TGFβ signaling also exacerbates tacrolimus-induced renal tubular dysfunction.** Albumin is filtered through the glomerulus with a sieving coefficient of 0.00062, which results in approximately 3 g of albumin filtered daily in human kidneys (10). Upon glomerular filtration, albumin is reabsorbed back into the blood by receptor-mediated endocytosis, a process orchestrated by renal epithelial tubular cells (10). Particularly, ~70% of albumin re-entry to the blood occurs via proximal convoluted tubular cell reabsorption while the distal nephron (loop of Henle, distal convoluted tubule, and collecting duct) accounts for ~30% (10). Thus, tubular dysfunction may accompany the enhanced microalbuminuria noted with inhibition of renal TGFβ signaling. To access tubular function, renal electrolyte handling was assessed by quantifying total urinary electrolytes (Fig. 6). Although total urinary Na levels are lower with tacrolimus treatment compared to vehicle (tacrolimus: 238 ± 20.3 mmol vs vehicle: 345 ± 26.3 mmol) (Fig. 6A), a further decline occurs with LY21 treatment (tacrolimus+LY21: 74 ± 6.9 mmol vs tacrolimus: 238 ± 20.3 mmol). Consistent with clinical reports noting the presence of hyperkalemia with tacrolimus treatment (11,12), urinary K levels were lower in tacrolimus-treated animals compared to vehicle.
(tacrolimus: 437 ± 64.6 mmol vs vehicle: 680.6 ± 56.4 mmol) (Fig. 6B). Interestingly, these levels were further lowered with LY21 treatment (tacrolimus+LY21: 169 ± 17 mmol vs tacrolimus: 437 ± 64.6 mmol). Lastly, there was no statistically significant difference between total urinary Cl levels between tacrolimus-treated animals compared to vehicle (tacrolimus: 393 ± 92 mmol vs vehicle: 475 ± 59 mmol) (Fig. 6C). However, a decline occurs with LY21 treatment (tacrolimus+LY21: 133 ± 14 mmol vs tacrolimus: tacrolimus: 393 ± 92 mmol). Collectively, these findings suggest that TGFβ receptor inhibition is accompanied by electrolyte disturbances, a feature of renal tubular dysfunction.
Figure 6: **Inhibition of TGFβ signaling also exacerbates tacrolimus-induced renal tubular dysfunction.** To access tubular function with inhibition of renal TGFβ signaling, wild-type C57BL6 mice were treated with either 1 mg/kg tacrolimus or vehicle (DMSO/sunflower oil) for 28 days. A subset of tacrolimus-treated animals was treated...
with 10 mg/kg LY21 also for 28 days. Metabolic cage studies were performed, and total urinary electrolytes were quantified (A-C). Values are means ± SEM with 3-4 animals/group. Statistical tests conducted using one-way analysis of variance and all pair-wise comparisons carried out with Tukey programs. *p<0.05, ***p<0.0005, and ****p<0.00005 vs vehicle; #p<0.05, ##p<0.005 vs tacrolimus.
DISCUSSION

Our group (Chapter I) and others (13) established that aberrant TGFβ activation and downstream signaling contribute to tacrolimus-induced renal fibrosis. We demonstrated that TGFβ signaling stimulates Smad-mediated fibroblast transition into myofibroblasts that upregulate production of extracellular matrix proteins, which contribute to tacrolimus-induced renal fibrosis. While we demonstrate that directly targeting TGFβ receptor signaling resolves renal fibrosis, is not a feasible approach to prevent the renal dysfunction associated with CNI therapy. Specifically, using an in vivo model of CNI-induced nephropathy, we demonstrate that inhibition of TGFβ signaling promotes positive effects such as the attenuation of interstitial fibrosis and fibroblast activation. However, inhibition of TGFβ signaling also exacerbates tacrolimus-induced nephrotoxic effects such as disruption of both the glomerular filtration barrier and tubular function. Taken together, these results indicate that renal TGFβ signaling exerts both beneficial and detrimental effects, which establish its role as both a friend and foe in the kidney (Figure 7).
Figure 7a: Proposed Schema. Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis while promoting kidney dysfunction. Inhibition of TGFβ signaling 1) promotes positive effects such as the attenuation of interstitial fibrosis and fibroblast activation.
Figure 7b: Proposed Schema. Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis while promoting kidney dysfunction. However, inhibition of TGFβ signaling also 2) exacerbates nephrotoxic effects such as disruption of the glomerular filtration barrier.
Figure 7c: Proposed Schema. Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis while promoting kidney dysfunction. Lastly, inhibition of TGFβ signaling further exacerbates tubular function.
Given the preponderance of evidence demonstrating a critical role of TGFβ in renal fibrosis (particularly types induced by CNIs) (2,8,9,14–21), studies conclude that inhibitors of TGFβ signaling may be important future drugs in controlling this condition (17,22). To this end, we utilized the pharmacological agent LY21 to inhibit tacrolimus-induced renal TGFβ signaling. First, we show that tacrolimus-induced renal TGFβRI activation was attenuated with LY21 treatment in vivo (Figure 1). Our findings are consistent with previous reports demonstrating that LY21 also inhibited TGFβ receptor activation in both the pancreas and liver (7). Next, we sought to establish that downregulation of TGFβ signaling does not interfere with the vital immunosuppression that necessitates CNI use. Our findings reveal that TGFβ inhibition maintains renal IL-2 suppression at levels similar to tacrolimus-only (Figure 2). This finding is significant as a relationship between TGFβ signaling and immune status has yet to be established in the kidneys. It is worth noting that TGFβ is known as a potent systemic immunosuppressant in vivo (23), and that this immunosuppressive effect is certainly likely to underlie the beneficial effects of systemic CNI administration (24,25). Given this insight, future analyses include assessment of systemic immunosuppression via examination of IL-2 abundance in splenic lymphocytes. Interestingly, a previous report cites that optimal TGFβ receptor antagonist dosing can prevent the profibrotic effects of cyclosporine (CsA) while maintaining its immunosuppressive activities (23); However, it remains to be determined whether this discovery also applies to tacrolimus. Further dose-response studies are certainly necessary to parse these out details.
Inhibition of TGFβ signaling attenuates interstitial fibrosis and fibroblast activation. Renal interstitial fibrosis occurs in ~50% of patients with CNI nephropathy after two years. This progressive kidney disease state is currently regarded as a reliable predictor of kidney failure (15). To disrupt the progression of kidney disease noted with chronic tacrolimus treatment, we demonstrate that inhibition of TGFβ signaling reduces histological signs of CNI-induced interstitial fibrosis (Figure 3A). Moreover, these findings are accompanied by the reduction of both ECM protein (collagen) and myofibroblast (α-SMA) abundance in tacrolimus+LY21 treated kidneys (Figure 3B). Our findings are consistent with previous reports that inhibition of TGFβ signaling suppressed the production of ECM proteins, dramatically attenuating the histological manifestations of both CsA-induced nephropathy (23) and glomerulonephritis (26).

However, inhibition of TGFβ signaling disrupts the glomerular filtration barrier. CNI-induced nephropathy is uniformly characterized by the triad of glomerular and tubular damage, in addition to interstitial fibrosis, which contribute to lower kidney weights (8). Although TGFβ receptor inhibition attenuates the interstitial fibrosis induced by tacrolimus, both kidney mass and kidney mass-to-body weight ratios remained low in these animals compared to vehicle (Table 2). Given our findings, the next step was to further investigate signs of renal damage accompanied by inhibition of
TGFβ signaling. Assessing histological features of renal damage, we demonstrate that inhibition of TGFβ signaling was accompanied by glomerular changes indicative of mesangial matrix expansion (Figure 4B). Because of its intricate architecture and filtration function, these morphological disturbances render the kidneys particularly susceptible to the consequences of mesangial matrix expansion. Therefore, we assessed glomerular barrier function with inhibition of TGFβ signaling. Our findings demonstrate that enhanced urinary albumin excretion was also observed with inhibition of TGFβ signaling (Figure 5).

**Moreover, inhibition of TGFβ signaling is also accompanied by tubular dysfunction.** Proteinuria is usually ascribed to disruptions of the glomerular filtration barrier, including podocyte detachment, glomerular basement membrane rupture, and slit diaphragm dysfunction in both acute and chronic kidney disease states (10). Meanwhile, dysfunction of albumin reabsorption in the proximal tubules (due to reduced megalin expression) also contributes to microalbuminuria (10). Thus, tubular dysfunction may accompany the enhanced microalbuminuria noted with inhibition of renal TGFβ signaling. Using electrolyte disturbances as an indicator of tubular dysfunction (27), our findings illustrate that total urinary electrolyte (Na, K and Cl) levels were the lowest with LY21 treatment (Figure 6).
The marathon continues: future steps. Attempts to target TGFβ signaling in the treatment of disease have resulted in conceptual advances in knowledge of its growth factor activities (28). Taken together, this study’s results indicate that renal TGFβ signaling exerts both beneficial and detrimental effects, which establish its role as both a friend and foe in the kidney. While a growing body of evidence implicates excessive action of TGFβ signaling driving the scarring noted in tissue damage, it is also a cytokine vital to tissue repair (17). These revelations support our conclusion that direct inhibition of TGFβ receptor activation is not a suitable approach to ameliorate the renal damage with CNI use. The following chapters of this dissertation provide future steps to investigate molecular mechanisms driving the renal damage induced by chronic CNI therapy.
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CONCLUSIONS & FUTURE DIRECTIONS

The molecular mechanism by which CNIs induce renal fibrosis remains to be better understood, and to date, there are no specific therapeutic strategies to mitigate this damage. In light of this dilemma, the objectives of this dissertation are to 1) identify initiating events by which CNIs cause irreversible renal damage and 2) explore approaches to achieve CNI-mediated immunosuppression without irreversible renal damage. To this end, aim 1 determines the role of TGFβ signaling in CNI-induced renal damage while aim 2 establishes whether disruption of TGFβ signaling ameliorates renal damage with CNI-induced immunosuppression.

AIM 1 HIGHLIGHTS:

Tacrolimus induces fibroblast to myofibroblast transition via a TGFβ-dependent mechanism to contribute to renal fibrosis

Our group reported that aberrant Transforming Growth Factor-β (TGFβ)/Smad signaling drives the profibrotic effects induced by CNIs (Ume et al; accepted American Journal of Physiology- Renal Physiology, 2023). Specifically, we demonstrated that 1) tacrolimus inhibits the calcineurin/NFAT axis while inducing TGFβ ligand secretion and
receptor activation in renal fibroblasts, 2) aberrant TGFβ receptor activation stimulates Smad-mediated production of myofibroblast markers, notable features of fibroblast-to-myofibroblast transition (FMT) and 3) FMT contributes to extracellular matrix (ECM) expansion in tacrolimus-induced renal fibrosis (Figure 1). This study expands understanding of the many cell types and central signaling mediators driving CNI-induced renal fibrosis. Not only does our work incorporate renal fibroblasts into the growing list of cell types negatively impacted by CNIs, but it also identifies renal fibroblast-to-myofibroblast transition as a process mediated via a TGFβ-dependent mechanism. Ultimately, our findings provide a more comprehensive picture of how calcineurin inhibition and profibrotic pathways intersect in the kidneys.
**Figure 1:** Tacrolimus induced fibroblast to myofibroblast (FMT) transition via a TGFβ-dependent mechanism to contribute to renal fibrosis: 1) tacrolimus inhibits the calcineurin (Cn)/NFAT axis while inducing TGFβ1 ligand secretion and receptor activation in renal fibroblasts, 2) aberrant TGFβ receptor activation stimulates Smad-mediated production of myofibroblast markers, notable features of FMT and 3) FMT contributes to extracellular matrix (ECM) expansion in tacrolimus-induced renal fibrosis.
**AIM 2 HIGHLIGHTS:**

Friend or Foe? Inhibition of the TGFβ receptor attenuates tacrolimus-induced renal fibrosis while exacerbating kidney dysfunction

Considering the impact of aberrant TGFβ signaling on multiple cell types in the kidney, our next studies investigated the feasibility of inhibiting TGFβ signaling as a means of preventing the renal fibrosis associated with chronic CNI use. We found that inhibition of TGFβ signaling 1) promotes positive effects such as the attenuation of tacrolimus-induced interstitial fibrosis and fibroblast activation (Figure 2). However, disruption of TGFβ signaling also *exacerbates* CNI-related nephrotoxic effects such as disruption of both 2) glomerular and 3) tubular functions (Figures 3-4). Taken together, these results indicate that *renal TGFβ signaling* exerts both beneficial and detrimental effects, which establish its role as both a *friend* and *foe* in the kidney. With this
revelation, directly targeting TGFβ signaling is not a suitable approach to prevent the renal damage associated with CNI therapy.

**Figure 2:** Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis while promoting kidney dysfunction. inhibition of TGFβ signaling 1) promotes positive effects such as the attenuation of interstitial fibrosis and fibroblast activation.
**Figure 3:** Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis while promoting kidney dysfunction. However, inhibition of TGFβ signaling also 2) exacerbates nephrotoxic effects such as disruption of the glomerular filtration barrier.
Figure 4: Inhibition of TGFβ receptor activation attenuates tacrolimus-induced renal fibrosis while promoting kidney dysfunction. Lastly, inhibition of TGFβ signaling 3) further exacerbates tubular function.
**FUTURE DIRECTIONS**

**A TALE OF TWO MAIN RENAL CALCINEURIN ISOFORMS: CnAα & CnAβ**

Given our previous findings, it is crucial that future studies identify additional mechanisms of chronic CNI-induced nephropathy in addition to approaches to mitigate this problem. An emerging body of evidence suggests that a potential solution to CNI-induced nephropathy lies in further characterization of the calcineurin enzyme. Full catalytic activation of calcineurin requires formation of a Ca$^{2+}$/Calmodulin-dependent subunit heterodimer consisting of catalytic subunit A (CnA) and regulatory subunit B (CnB) (1). Three major calcineurin A isoforms are expressed in humans: CnAα, CnAβ, and CnAγ (2,3). **CnAα and CnAβ** are ubiquitously expressed in tissues such as the kidneys, while CnAγ is mainly enriched in the both the testis and brain (2,3). (Figure 5)
**Figure 5:** CnAα and CnAβ are two catalytic calcineurin (CnA) isoforms expressed in the kidneys.

Although CnAα and CnAβ share a high degree of sequence homology (81% (4)), these isoforms contribute to a more precise regulation of diverse calcineurin functions in different tissues (40). The most striking difference between CnAα and CnAβ is the amino acid proline-rich region located in the N-terminus of CnAβ. It was later revealed that the proline-rich N-terminal sequence of CnAβ promotes NFAT binding (5). These structural differences contribute to differences in functions of these isoforms. In the kidneys, CnAα expression is predominant as this isoform plays an essential role in renal development.
Gooch and colleagues demonstrated that CnAα−/− mice on a mixed genetic background experienced kidney failure, with death occurring within weeks after birth (6). Our lab demonstrated that upregulation of CnAβ/NFAT signaling promotes kidney hypertrophy, a feature of diabetic nephropathy (45, 53). Taken together, findings indicate that CnA isoforms possess distinct functions in the kidneys (10) (Figure 6).
Figure 6: CnAα and CnAβ possess divergent signaling functions in the kidneys.

CNIs have the capacity to inhibit both CnAα and CnAβ (7). Gooch and colleagues later identified CnAα as a key player in CNI-induced renal fibrosis. They found that loss of this isoform reproduces features of CsA nephrotoxicity in vivo and in vitro. Particularly, loss of CnAα in vivo results in histopathologic changes including matrix expansion, whereas loss of the β isoform does not (8). Consistent with their in vivo findings, CnAα−/− renal fibroblasts exhibited increased fibronectin and TGFβ protein expression (8). A follow-up study demonstrated that both CsA treatment and loss of CnAα are accompanied by a significant increase in metalloproteinase-9 (MMP-9) expression and activity in renal fibroblasts (9). MMP-9 works in conjunction with TGFβ in extracellular matrix remodeling, a key feature of fibrosis.

Our work further expands on how loss of the CnAα isoform reproduces features of CsA nephrotoxicity (4). Particularly, we demonstrated that 1) CnAα loss stimulates Nox2 upregulation, 2) NFκB is a novel CnAα-regulated transcription factor and 3) NFκB mediates CnAα-induced Nox2 and ROS upregulation (10). Our results demonstrate that CnAα plays a key role in Nox2 and ROS generation. Further, these novel findings provide strong evidence of divergent CnA isoform signaling pathways in the kidneys.
Although these pivotal studies pave the way into a better understanding of calcineurin inhibition and the development of renal fibrosis, further investigation is necessary to address additional gaps in knowledge (Figure 7). Further studies are necessary to identify whether tacrolimus promotes a loss of CnAα. If so, strategies and therapies to preserve CnAα function could therefore reduce side effects and prevent subsequent kidney transplants because of CNI toxicity. Targeting oxidative stress also rises as a potential treatment for CNI-nephropathy. The key to taking advantage of the established experimental findings is designing studies that investigate how CNIs alter regulation of these isoforms, particularly CnAα. The emergence of systemic CsA analogues such as voclosporin in long-term treatment of autoimmune diseases such as lupus nephritis highlight the real need to investigate this phenomenon further (11–15). This insight will spur the development of newer generation CNIs that preserve CnAα effectively blunt the immune response while circumventing extensive renal damage noted with long-term CNI use.
Figure 7: CNI-induced nephropathy may be isoform-specific. Loss of the CnAα isoform reproduces features of cyclosporine nephrotoxicity, such as ECM expansion. Further studies demonstrate that loss of the CnAα isoform also induces oxidative damage, which also contributes to ECM accumulation. These findings trigger a cascade of additional questions to be answered.
REFERENCES


ABBREVIATIONS

α-SMA – α-smooth muscle actin
BER – Base Excision Repair
Cn – calcineurin
CnA - calcineurin A catalytic subunit
CnAα - calcineurin Aα isoform
CnAβ - calcineurin Aβ isoform
CNIs – calcineurin inhibitors
Col IV – collagen IV
CsA – cyclosporine A
ESRD – end stage renal disease
ECM – extracellular matrix
FKBP12 – FK506 binding protein 12
FMT – fibroblast-to-myofibroblast transition
H&E – hematoxylin & eosin
NER – Nucleotide Excision Repair
NCC – sodium-chloride co-transporter
NFAT – nuclear factor of activated T-cells
SOTR – solid organ transplant recipient
Smad – suppression of mothers against decapentaplegic
TGFβ – transforming growth factor β
TGFβRI – transforming growth factor β receptor type I subunit