Pharmacologic Blockade of $\alpha v\beta 1$ Integrin Ameliorates Renal Failure and Fibrosis In Vivo

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ABSTRACT

Activated fibroblasts are deemed the main executors of organ fibrosis. However, regulation of the pathologic functions of these cells in vivo is poorly understood. PDGF receptor $\beta$ (PDGFR$\beta$) is highly expressed in activated pericytes, a main source of fibroblasts. Studies using a PDGFR$\beta$ promoter–driven Cre system to delete $\alpha v$ integrins in activated fibroblasts identified these integrins as core regulators of fibroblast activity across solid organs, including the kidneys. Here, we used the same PDGFR$\beta$-Cre line to isolate and study renal fibroblasts ex vivo. We found that renal fibroblasts express three $\alpha v$ integrins, namely $\alpha v\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$. Blockade of $\alpha v\beta 1$ prevented direct binding of fibroblasts to the latency-associated peptide of TGF-$\beta 1$ and prevented activation of the latent TGF-$\beta$ complex. Continuous administration of a recently described potent small molecule inhibitor of $\alpha v\beta 1$, compound 8, starting the day of unilateral ureteral obstruction operation, inhibited collagen deposition in the kidneys of mice 14 days later. Compound 8 also effectively attenuated renal failure, as measured by BUN levels in mice fed an adenine diet known to cause renal injury followed by fibrosis. Inhibition of $\alpha v\beta 1$ integrin could thus hold promise as a therapeutic intervention in CKD characterized by renal fibrosis.


CKD is an increasingly significant public health concern affecting 26 million Americans.¹ Currently, no effective agent exists that can directly halt disease progression.

Regardless of the cause, renal fibrosis is a final common pathway of CKD. Fibroblasts are key effectors of fibrosis and contribute to excessive production of extracellular matrix. We recently reported that fibroblast-derived $\alpha v$ integrins contribute importantly to fibrosis.² Selective $\alpha v$ deletion using PDGF receptor $\beta$ (PDGFR$\beta$)-Cre protected mice from renal fibrosis after UUO, bleomycin-induced lung fibrosis, and carbon tetrachloride–induced liver fibrosis.² Integrins are transmembrane receptors with an $\alpha$ and a $\beta$ subunit. There are five $\alpha v$ integrins, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, and $\alpha v\beta 8$. Global deletion of $\alpha v\beta 3$ or $\alpha v\beta 5$ or fibroblast-specific deletion of $\alpha v\beta 8$ (mice with global $\beta 8$ deletion die in utero) did not protect mice against liver fibrosis.² Therefore, either multiple fibroblast $\alpha v$ integrins contribute to tissue fibrosis or the protection is mostly due to loss of $\alpha v\beta 1$. Until now, it was impossible to study the in vivo role of $\alpha v\beta 1$ because mice lacking $\beta 1$ on fibroblasts do not survive. To address this, we developed a highly potent small molecule inhibitor of $\alpha v\beta 1$ called Compound 8 (C8). Our colleagues Reed et al. recently published that C8 significantly reduced cell adhesion through $\alpha v\beta 1$ and protected mice from pulmonary and hepatic fibrosis.³ Here, using the same C8 as described by Reed et al.,³ and $\alpha v$ blocking antibodies, we studied the contribution of fibroblast $\alpha v$ integrins to the development of renal fibrosis. We found that renal fibroblasts express three of five $\alpha v$ integrins ($\alpha v\beta 1$, $\alpha v\beta 3$, and $\alpha v\beta 5$). Among them, $\alpha v\beta 1$ is the main $\alpha v$ integrin used by renal fibroblasts to directly bind to latency-associated peptide (LAP) of TGF-$\beta 1$. Inhibition of either $\alpha v\beta 1$ or $\alpha v\beta 3$ reduced activation of latent TGF-$\beta$ in vitro, but the effects of $\alpha v\beta 3$ inhibition appeared to be an in vitro artifact mediated by loss of adhesion to serum-coated tissue culture plates. Therapeutic delivery of $\alpha v\beta 1$ inhibitor C8 significantly attenuated UUO-induced renal fibrosis in vivo, whereas global $\alpha v\beta 3$ genetic deletion did not protect mice from renal fibrosis.

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More importantly, C8 partially rescued renal failure in mice fed an adenine diet known to cause elevated BUN, renal tubular toxicity, and interstitial fibrosis. These results identified αvβ3 integrin as a promising target for antifibrosis therapy.

We first examined the expression profile of αv integrins in activated renal fibroblasts. Fibroblasts were isolated from PDGFRβ-Cre, Ai14tdtomatoFlox/Flox mouse kidneys using Fluorescence Activated Cell Sorting (FACS) and then differentiated in culture for 1 week before experiments. Flow cytometry with specific antibodies showed that these cells express αvβ3 and αvβ5 (Figure 1A). They do not express αvβ6, an epithelium-restricted integrin, or αvβ8 (Figure 1A). Flow cytometry using an antibody against mouse αvβ1 was not done because no such antibody exists. However, we were able to detect αvβ1 expression by coimmunoprecipitation (co-IP) followed by western blot (WB) (Figure 1B). Co-IP experiments also confirmed the presence of αvβ3 and αvβ5 on these cells (Figure 1B).

We and others have shown that αv integrins can bind LAP through the RGD motif, thereby activating latent TGF-β. To investigate whether renal fibroblasts can directly activate TGF-β and, if so, which αv integrins are responsible for that action, we performed TGF-β activation assays in the presence of specific blocking antibodies against mouse αvβ1 was not done because no such antibody exists. However, we were able to detect αvβ1 expression by coimmunoprecipitation (co-IP) followed by western blot (WB) (Figure 1B). Co-IP experiments also confirmed the presence of αvβ3 and αvβ5 on these cells (Figure 1B).

Figure 1. Renal fibroblasts express multiple αv integrins and activate TGF-β. (A) Tdtomato-positive kidney cells isolated from PDGFRβ-Cre, Ai14tdtomatoFlox/Flox mice and differentiated in culture were subjected to flow cytometry analysis. Cells were incubated with either secondary antibody alone (control, in black) or first with mouse mAbs (in gray) for αvβ3 (Axum-4), αvβ5 (Alula), αvβ6 (3G9), or αvβ8 (Adwa11), all generated by immunizing knockout mice with integrin subunits. (B) Co-IP and WB of cell lysates of isolated Tdtomato-positive mouse kidney cells show that these cells express αvβ1, αvβ3, and αvβ5. Cell lysates were immunoprecipitated with anti-αv antibodies (RMV-7) or isotype control rat IgG1k. Each immunoprecipitate was divided in half and each half was subjected to WB with antibodies against αv and a respective β integrin subunit. TL, total lysates. (C) Isolated Tdtomato-positive cells were used in coculture TGF-β activation assay in the presence or absence of blocking antibodies against αv (rat monoclonal IgG1k), β1 (hamster monoclonal IgG1k), β3 (hamster monoclonal IgG1k), or αvβ5 (mouse monoclonal IgG). *P < 0.05, specific antibody versus corresponding isotype control-treated samples). hlgG, hamster IgG1k; mlgG, mouse IgG; rlgG, rat IgG1k. (D) TGF-β activation assays with isolated Tdtomato-positive cell in the presence of C8 (line with black markers) or inactive control compound C16 (line with gray markers) at indicated concentrations. No Rx, no treatment. For both (C and D), relative TGF-β activation is expressed as percentage luminescence of each treatment sample compared with untreated samples after subtraction of TGF-β-independent luminescence. Data shown are the mean ± SEM from three experiments.

We next used the αvβ1 inhibitor C8 described by Reed et al. C8 attenuated TGF-β activation by fibroblasts starting at 0.01 nM concentration (Figure 1D). At 1 nM, C8 reduced TGF-β activation by >50%, consistent with the described IC50 for this compound. These data demonstrated that renal fibroblasts can activate TGF-β in vitro and that blockade of either αvβ1 or αvβ3 reduces that capacity.

We next examined the effects of in vivo αvβ1 or αvβ3 inhibition on renal fibrosis. To inhibit αvβ1 in vivo, C8 (or the inactive control, C16) was delivered by subcutaneous Alzet pump, placed on the day of UUO surgery, at 70 mg/kg per day. Collagen deposition in the kidneys was evaluated 14 days after surgery by two methods. First, we measured tissue content of hydroxyproline, a major component of collagen (Figure 2A). We next compared picrosirius red staining of
fixed kidney tissue sections (Figure 2, B and C). C8 did not alter baseline collagen content. UUO surgery increased collagen content compared with sham operation in C16-treated groups. C8 treatment reduced collagen deposition after UUO by 38% as measured by hydroxyproline assay (Figure 2A) and by 59% as measured by quantification of picrosirius red staining (Figure 2B), respectively. Neither C16 nor C8 had any effect on sham-operated mouse kidneys (Supplemental Figure 1). Thus, C8 treatment does not directly cause structural changes in the kidneys at baseline, a finding that is encouraging because C8 may be a prototype for development of clinically potent antiﬁbrotics.

Because αvβ3 inhibition in vitro also decreased TGF-β activation, we examined the consequences of αvβ3 deletion in vivo using Igba3−/− mice. In contrast to αvβ1 blockade, genetic deletion of Igba3 had no effect on collagen deposition in the kidneys after UUO as measured by hydroxyproline assays (Figure 3A) and picrosirius red staining of collagen (Figure 3B and C).

To examine whether αvβ1 blockade in vivo can rescue renal failure in addition to reducing tissue ﬁbrosis, we treated mice with C8 after induction of renal failure by adenoine diet. Adenine diet has been shown to cause renal injury characterized by increase in BUN levels and tubulointerstitial ﬁbrosis.8 Mice were fed adenine diet for 28 days total. By day 12, mice developed signiﬁcant renal failure and renal ﬁbrosis as evidenced by elevated BUN levels and tissue collagen deposition in adenine diet groups (data not shown). On day 14, Alzet pumps containing C8 (or C16) were placed to deliver compounds continuously from days 14–28. By day 28, BUN levels were highly elevated in control compound C16-treated mice. In contrast, C8 treatment reduced the increase in BUN by 28% (BUN 240 ± 27 mg/dl in C16 group, 171 ± 11 mg/dl in C8 group; P = 0.04) (Figure 4A). Although it does not completely reverse renal failure, αvβ1 blockade by C8 can reduce the severity of renal failure. Similar to the UUO model, we observed that C8 treatment attenuated the degree of renal ﬁbrosis on the basis of tissue collagen deposition (Figure 4, B and C). Quantitative RT-PCR showed that expression of COL1a1 and αSMA was also lower in C8-treated mouse kidneys compared with C16-treated ones after adenine diet (Figure 4D). Thus, αvβ1 inhibition both reduced the degree of renal ﬁbrosis and effectively preserved residual renal function in a therapeutic model where intervention was begun after the onset of renal failure.

One well described mechanism to activate TGF-β is integrin binding to the RGD motif of LAP, thereby causing a conformational change of the latent TGF-β complex and freeing active TGF-β1.9 We thus determined whether isolated renal ﬁbroblasts could directly bind LAP using either αvβ1 or αvβ3. Isolated renal ﬁbroblasts were plated on LAP-coated dishes with or without speciﬁc antibodies or inhibitors. Cell adhesion to LAP was signiﬁcantly reduced by pan-αv antibody or β1 antibody (Figure 5A), but not by β3 blocking antibody. C8 also caused dose-dependent inhibition of adhesion of αvβ3 and β1 suffering worst inhibition both reduced the degree of renal ﬁbrosis and pre- serving renal function in vivo. This lead compound provides an excellent basis for designing pharmacologic agents to treat renal ﬁbrosis and CKD.

In summary, our study identiﬁed fbroblast αvβ1 as a critical regulator of renal ﬁbrosis. It is the only αv integrin on renal ﬁbroblasts that can bind and activate TGF-β. Small molecule inhibitor C8 was highly effective in blocking αvβ1 in vitro and in attenuating renal ﬁbrosis and preserving renal function in vivo. This lead compound provides an excellent basis for designing pharmacologic agents to treat renal ﬁbrosis and CKD.

The signaling pathways engaged by αvβ1 to promote ﬁbrosis remain to be fully explored. One such pathway may involve interplay between αvβ1 and other integrins. Hartner et al. described de novo α8β1 expression on renal ﬁbroblasts after UUO.10 They found that mice lacking α8β1 suffered worse UUO-induced renal ﬁbrosis compared with wild-type controls, suggesting a possible protective effect of α8β1. α8β1 has been reported to bind LAP but does not activate TGFβ1.11 As mentioned in our earlier study, C8 is highly selective for αvβ1 versus αvβ3 (IC50 approximately 1 nM for αvβ1, IC50 > 100,000 nM for αvβ3).12 It is conceivable that, by blocking αvβ1, C8 indirectly clears the way for α8β1 to bind to LAP-TGFβ1 thereby trapping TGFβ1 in its latent form. To test this hypothesis, kidney ﬁbrosis models can be established in itga8−/− mice in future studies to examine the effects of C8.

CONCISE METHODS

Mice

Ai14 (Rosa-CAG-LSL-tTomato-WPRE) mice12 were obtained from Jackson Laboratory and crossed with PDGFβ-Cre mice13.
(obtained from Ralf Adams, University of Münster, Germany). They were maintained on C57BL/6 background. $\text{Itgb3}^{-/-}$ mice on 129/sv background were obtained from Richard Hynes (Massachusetts Institute of Technology, Cambridge). Wild-type 129/sv littermates were used as control in UUO experiments. $\text{Itgb8}^{-/-}$ mice were described previously and were maintained on CD1 background. Genotyping of all mice was performed by PCR as described previously. Wild-type C57/BL6 mice were purchased from Jackson Laboratory. Mice used for all experiments were 8–12 weeks old and were housed under specific pathogen-free conditions in the Animal Barrier Facility of the University of California San Francisco (UCSF) and University of California Irvine (UCI). All experiments were approved by the Institutional Animal Care and Use Committee of UCSF and UCI.

**Figure 2.** Specific $\alpha\beta_1$ blockade with small molecule inhibitor C8 ameliorates UUO-induced renal fibrosis in mice. (A) Hydroxyproline assay of kidney tissues harvested from wild-type mice 14 days after compound treatment. In both compound treatment groups, mice were further divided to undergo either UUO or sham operations (n=4–7 in each group). There is a significant increase in hydroxyproline content after UUO compared with sham operation. This increase is largely attenuated with C8 treatment ($*P=0.001$, C8 versus C16 treatment in UUO groups). (B) Percentage of sirius red–positive area was quantified. UUO kidneys treated with C8 showed 59% less sirius red positivity compared with C16 treatment group ($**P=0.03$, C8 versus C16 treatment in UUO groups). (C) Representative images of picrosirius red staining of kidney sections from C16 and C8 treatment groups are shown here. All images are original magnification, ×200. All kidneys were harvested 14 days postsham or -UUO operation.

**Figure 3.** $\alpha\beta_3$ gene deletion does not protect mice from UUO-induced renal fibrosis. (A) Hydroxyproline assay of kidney tissues from wild-type and $\text{Itgb3}^{-/-}$ mice 14 days after sham or UUO operations (n=4–10 in each group). (B) Percentage of sirius red–positive area was quantified. There is no significant difference in hydroxyproline content ($P=0.33$) or percentage sirius red positivity ($P=0.44$) between UUO kidneys of wild-type and $\text{Itgb3}^{-/-}$ mice. NS, not significant. (C) Representative images of picrosirius red staining of kidney sections from wild-type and $\text{Itgb3}^{-/-}$ mice after sham or UUO operations are shown here. All images are original magnification, ×200. All kidneys were harvested 14 days postsham or -UUO operation.

**Unilateral Ureteral Obstruction**
For renal fibrosis, UUO was induced by ligation of the left ureter in 8–12-week-old male mice as described previously. Sham-operated mice underwent an identical surgical procedure except that ligation of the ureter was not performed. Kidneys were harvested 14 days after surgery. To treat mice with small molecule inhibitors, Alzet pumps containing C8 or C16 were placed subcutaneously on the day of surgery and continued to release compounds for 14 days at a rate of 70 mg/kg per day. The chemical composition and production of the compounds was described in detail in our recent publication.

**Adenine Diet–Induced Renal Injury Model**
Mice were fed 0.2% adenine diet to induce renal injury as described previously. Wild-type C57/BL6 mice 10–12 weeks old were fed with either 0.2% adenine diet or regular diet as control for a total of 28 days. On day 14, Alzet pumps containing C8 or C16 were placed subcutaneously on the day of surgery and continued to release compounds for 14 days at a rate of 70 mg/kg per day. Mice continued to receive adenine diet from days 14–28 while receiving compound treatment. BUN levels were measured on day 12 (before Alzet pump placement) to confirm establishment of renal injury and on day 28. Kidneys were harvested on day 28 of the experiment. Again, the
Figure 4. αvβ1 blockade with small molecule inhibitor C8 reduces adenine diet–induced renal failure and tissue fibrosis in mice. (A) Mice fed an adenine diet had increased BUN levels. This increase was attenuated in mice treated with C8 compared with C16 treatment (average BUN: 240 ± 27 mg/dl in C16 group with adenine diet, n=7; 170 ± 11 mg/dl in C8 group with adenine diet, n=6; *P=0.04, C8 versus C16 treatment in adenine diet groups). (B) Adenine diet induced significant interstitial fibrosis in mouse kidneys as evidenced by increased picrosirius red–positive area. This was significantly reduced with C8 treatment (average picrosirius red–positive area: 5.30% ± 0.25% in C16 group and 2.82% ± 0.2% in C8 group after adenine diet, **P<0.001). (C) Shown here are representative images of picrosirius red staining of kidney sections from C16- and C8-treated mice fed an adenine diet. All images are original magnification, ×200. (D) Kidney tissue expression of Col1a1 and αSMA was measured using qRT-PCR. Both markers increased after adenine diet but the increase was significantly smaller in the C8 treatment group (***P=0.02 for Col1a1 and P=0.04 for αSMA, C8 versus C16 treatment in adenine diet groups). Kidneys and blood samples were obtained 28 days after initiation of adenine diet. Mice were treated with C8 (or C16) on days 14–28.

Figure 5. αvβ1, but not αvβ3, mediates direct interaction of renal fibroblasts to TGF-β1 LAP. Inhibitory effects of β3 blocking antibodies are due to inhibition of cell tethering. (A) Adhesion assays of isolated Tdtomato-positive mouse kidney cells to LAP. Anti-αv and anti-β1 antibodies, but not anti-β3, significantly inhibited cell adhesion to LAP (*P<0.001, specific antibody versus its isotype control). αvβ1 inhibitor C8 also
Among the properties of the compounds described in our recent publication, the chemical composition and production of the compounds was described in detail. The hydroxyproline assay and the other half was fixed in 10% formalin for histology studies. Hydroxyproline assay was described previously. Briefly, half kidneys were weighed, homogenized, and then precipitated with trichloroacetic acid. The precipitants were baked overnight at 110°C in HCl. Samples were reconstituted in water, and hydroxyproline content was measured using a colorimetric chloramine T assay.

**Primary Cell Isolation and FACS**

Mice were perfused with PBS through the left ventricle to remove blood cells. The kidneys were excised and the capsules removed. They were then minced with scissors into pieces 2 mm in size and digested in Dulbecco Modified Eagle Medium (DMEM; Invitrogen) containing 0.13 IU/ml Liberase (Roche) and 0.5 mg/ml collagenase 4 (Sigma). The cell suspension was shaken (200–250 rpm) at 37°C for 30 minutes and then gently vortexed for 30 seconds. The cell suspension was passed through a 40 μm cell strainer and centrifuged at 1000 rpm for 5 minutes to form a pellet. To remove residual red blood cells, the cell pellet was resuspended in red blood cell lysis buffer and left to sit at room temperature for 10 minutes. The cells were washed with DMEM twice to remove cell debris and resuspended in FACS buffer (PBS supplemented with 3% FCS). After live/dead staining with sytox blue (Invitrogen), live Tdtomato-positive cells from PDGFRβ-Cre, Ai14tdtomato Flox/Flox mice were sort using FACSaria (BD Biosciences). Tdtomato-positive cells were cultured in DMEM supplemented with glucose, L-glutamine, penicillin/streptomycin, and 15% FCS.

**Flow Cytometry**

Cell-sorted Tdtomato-positive cells from kidneys of PDGFRβ-Cre, Ai14tdtomato Flox/Flox mice were plated on tissue culture plastic for 7 days before experiments. Cells were harvested with trypsin, washed twice with PBS, and resuspended in FACS buffer containing 10% normal goat serum (Jackson Laboratory). Cells were then washed twice with PBS and sequentially incubated with primary antibodies and secondary antibodies (or secondary antibodies only for control) at 4°C for 30 minutes each. At the end of incubation, the cells were washed with PBS and analyzed on a Becton Dickinson FACS Canto II. The primary antibodies used were mouse mAbs generated in our laboratory: Axum-4 (αvβ3)18 Alula (αvβ5),19 3G9 (αvβ6),20 and Adwa11 (αvβ8). Mouse monoclonal αvβ8 antibody Adwa11 was generated by injecting fbgb8/−/− mice with recombinant human αvβ8 protein (Cat#1435-AV-050; R&D Systems). The specificity of Adwa11 to αvβ8 was tested on 293T cells stably expressing human αvβ8 (293T-β8 cells21) and mouse astrocytes (obtained from ATCC) known to express αvβ8 (Supplemental Figure 2). APC-conjugated goat anti-mouse (Invitrogen) was used as secondary antibody.

**Immunoprecipitation and WB**

Isolated and cultured Tdtomato-positive cells were solubilized in HBSM buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM CaCl2, 5 mM MgCl2) containing 1% Triton-100 detergent and protease inhibitors. Cleared lysates from 2 x 106 cells were divided into two tubes. Cell lysates in each tube were incubated with rotation with 10 μg of either αv integrin antibody (clone RMV-7; the hybridoma was a generous gift from Dr. Hideo Yagita, Juntendo University, Japan) or rat IgG1k isotype control (BioLegend) for 2 hours at 4°C. Immune complexes were collected with protein G agarose (GE Health Care) and eluted with reducing Laemmli sample buffer by boiling for 5 minutes. The samples were then subjected to SDS-PAGE and WB. Antibodies used for WB were mouse anti-αv integrin mAb (clone 21/CD51, Cat#611012; BD Biosciences), rabbit anti-β1 integrin polyclonal antibody (Cat#ab1952; Millipore), rabbit anti-β3 integrin mAb (clone EPR2417Y, Cat#ab75872; Abcam), and rabbit anti-β3 integrin polyclonal antibody (Cat#ab15459; Abcam).

**TGF-β Activation Assay**

Isolated Tdtomato-positive cells were cultured for 7 days before experiments. For TGF-β activation assay, cells were plated at 50,000 cells/well density in 96-well plates with mink lung epithelial cells (TMLC) expressing firefly luciferase downstream of a TGF-β-sensitive portion of the PAI-1 promoter (15,000 cells/well). Cells were cocultured with or without specific antibodies or compounds for 16 hours before lysis. TGF-β activity was calculated by measurement of luminescence of cell lysates from each well. There was baseline TGF-β-independent PAI-1 activity in samples treated with TGF-β blocking antibody (1D11, generated in our laboratory from a hybridoma obtained from ATCC). We subtracted the luminescence of 1D11 treatment samples decreased cell binding to LAP starting at 1 nM concentration (P<0.001, C8 versus C16 treatment at the same concentrations). All antibodies were used at 40 μg/ml. C16 and C8 were used at the concentrations indicated (1–100 nM). hlgG, hamster IgG1k; no Rx, no treatment; rlgG, rat IgG1k. (B) Adhesion assays of the same cells to vitronectin (VN) or collagen 1 (Col1). C8 did not alter cell adhesion to either VN or Col1. Anti-β3 decreased cell adhesion to VN but not Col1 (P<0.001, anti-β3 versus hlgG). All antibodies were used at 40 μg/ml concentration. C8 and C16 were used at 100 nM concentrations. (C) Confocal microscopy images of cells plated on VN. Anti-β3 blocking antibodies reduced β3 recruitment to focal contacts and stress fiber formation (β3 staining in red, phalloidin staining in green, DAPI in blue). C8 had no effect on the formation of either focal contacts or stress fibers of cells adherent to VN. All images shown are original magnification, ×400. (D) TGF-β activation assays of isolated Tdtomato-positive cells plated on dishes that were uncoated (UC), or coated with VN or Col1. Cells were either untreated (No Rx) or treated with increasing concentrations of C16 or C8. C8 reduced cell activation of TGF-β regardless of the matrix they attached to (P<0.001, C8 versus C16 treatment at the same concentrations). (E) TGF-β activation assays of cells either untreated (No Rx) or treated with anti-β3 antibody and its isotype control (hlgG). Anti-β3 antibody inhibited TGF-β activation by cells plated on uncoated dishes or dishes coated with VN (P<0.001, anti-β3 versus hlgG). Anti-β3 antibodies did not affect TGF-β activation by cells adhering to Col1. Antibodies were used at 40 μg/ml. Data shown in (A, B, D, and E) are the mean±SEM from three experiments.
(TGF-β–independent activity) from the luminescence of other treated or untreated conditions and consider the remaining portion as specific TGF-β activation. The specific functional blocking antibodies used were rat anti-αβ1 mAb (clone RMV-7, Cat#CBL1346Z-1; Millipore), hamster anti-β1 mAb (clone HMB11-1, Cat#102209; BioLegend), hamster anti-β3 monoclonal (clone 2C9.G2, Cat#104309; BioLegend), and mouse anti-αvβ5 mAb (Alula, produced in our laboratory18). Isotype control antibodies used were rat IgGlκ, mouse IgGl, and hamster IgGlκ (all from BioLegend).

Immunohistochemistry and Immunofluorescence
Paraffin-embedded sections were processed for immunohistochemistry as described previously.23 Five-micrometer sections were stained with picrosirius red or hematoxylin and eosin. To quantify picrosirius red positivity, a Leica CTRS000 microscope was used to capture ten nonoverlapping fields of each kidney section at a final magnification of 200× and analysis was performed using ImageJ as described previously.23,24 The same microscope was used to capture images from hematoxylin and eosin–stained kidney sections at a final magnification of 200×. For immunofluorescence staining, cells were plated on either uncoated cover slips or cover slips coated with 1 μg/ml vitronectin (R&D Systems), collagen I (BD Biosciences), LAP (recombinant human LAP TGF-β1), or BSA as controls, as described previously.2 Briefly, cells were allowed to adhere for 60 minutes, washed with PBS, and fixed and stained with solution containing 0.5% crystal violet, 1% formaldehyde, and 20% methanol. Cells were then extracted with 2% Triton X-100 before luminescence was read at 595 nm.

Statistical Analyses
All data are presented as mean ± SEM. Statistical significance was calculated using a two-tailed paired t test. Differences with a P value of <0.05 were considered statistically significant.

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DISCLOSURES
D.S., W.F.D., H.J., and N.I.R. are inventors of US Patent application 61/884,583 related to this work. D.S. and W.F.D. are cofounders of Plant Therapeutics, Inc.

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Supplemental Figure 1. Sham-operated kidneys from mice treated with C16 or C8 do not demonstrate any apparent histologic differences. Kidneys were harvested 14 days after sham operation. Tissue sections were stained with hematoxylin and eosin (H&E). Shown here are representative images from the medulla and cortex regions of sham-operated kidneys of mice treated with either C16 or C8. All images are 200x magnification.
Supplemental Figure 2. Adwa11 is specific for human and mouse αvβ8. We performed flow cytometry analysis on three cell types: human embryonic kidney 293T cells that express αv but not β8 endogenously (293T cells); 293T cells stably transfected with human β8 and thus express αvβ8 dimers (293T-β8 cells); mouse astrocytes that express endogenous αvβ8. Incubation with anti-αvβ8 antibodies Adwa11 caused a shift in fluorescence intensity of 293T-β8 cells but not that of 293T cells (left and middle panels) demonstrating the antibody’s specific activity against αvβ8. Furthermore, Adwa11 also recognized endogenous αvβ8 on mouse astrocytes. Thus, Adwa11 is specific for αvβ8 integrin and cross reacts with both human and mouse αvβ8.