

Epithelial and Endothelial Adhesion of Immune Cells Is Enhanced by Cardiotonic Steroid Signaling Through Na⁺/K⁺-ATPase- α -1

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Background—Recent studies have highlighted a critical role for a group of natriuretic hormones, cardiotonic steroid (CTS), in mediating renal inflammation and fibrosis associated with volume expanded settings, such as chronic kidney disease. Immune cell adhesion is a critical step in the inflammatory response; however, little is currently understood about the potential regulatory role of CTS signaling in this setting. Herein, we tested the hypothesis that CTS signaling through Na⁺/K⁺-ATPase α -1 (NKA α -1) enhances immune cell recruitment and adhesion to renal epithelium that ultimately advance renal inflammation.

Methods and Results—We demonstrate that knockdown of the α -1 isoform of Na/K-ATPase causes a reduction in CTS-induced macrophage infiltration in renal tissue as well reduces the accumulation of immune cells in the peritoneal cavity in vivo. Next, using functional adhesion assay, we demonstrate that CTS-induced increases in the adhesion of macrophages to renal epithelial cells were significantly diminished after reduction of NKA α -1 in either macrophages or renal epithelial cells as well after inhibition of NKA α -1-Src signaling cascade with a specific peptide inhibitor, pNaKtide in vitro. Finally, CTS-induced expression of adhesion markers in both endothelial and immune cells was significantly inhibited in an NKA α -1-Src signaling dependent manner in vitro.

Conclusions—These findings suggest that CTS potentiates immune cell migration and adhesion to renal epithelium through an NKA α -1-dependent mechanism; our new findings suggest that pharmacological inhibition of this feed-forward loop may be useful in the treatment of renal inflammation associated with renal disease. (*J Am Heart Assoc.* 2020;9:e013933. DOI: 10.1161/JAHA.119.013933.)

Key Words: adhesion • cardiotonic steroids • macrophage • Na⁺/K⁺-ATPase • renal epithelium • renal inflammation

Inflammation is a major component of the potentially modifiable risk factors that drive renal disease.¹ Elevated levels of proinflammatory molecules predict both mortality and declines in renal function in patients with chronic kidney disease (CKD).² In fact, treatment strategies that use anti-inflammatory therapies have been shown to reduce mortality related to cardiovascular complications and other causes.³ Recruitment of immune cells and their entry into the renal tissue are fundamental in the inflammatory response, and it includes a series of molecular events, of which immune cell

adhesion plays a critical role.⁴ We have previously shown that cardiotonic steroids (CTSs) mediate proinflammatory responses in both renal epithelial cells and macrophages on binding and signaling through the Na⁺/K⁺-ATPase α -1-Src kinase (NKA α -1-Src) complex.^{5–7} CTS levels are increased in volume-expanded settings, such as CKD, where the major pathway of synthesis appears to be located in the adrenal cortex and involves the cytochrome P450 gene family enzyme CYP27A1 via the bile acid pathway.^{8,9} Our experimental data showed that CTSs activated multiple proinflammatory

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Clinical Perspective

What Is New?

- Cardiotonic steroids (CTSs) are volume-sensitive hormones that are increased in settings such as hypertension and chronic kidney disease and mediate proinflammatory responses in both renal epithelial cells and macrophages on binding and signaling through the Na⁺/K⁺-ATPase α -1-Src kinase complex
- CTSs enhance interactions of major cell types involved in renal inflammation, including monocytes/macrophages as well as renal endothelium and epithelium.
- The inflammatory response mediated by CTSs involves signaling through the Na⁺/K⁺-ATPase α -1 and increases immune cell recruitment and accumulation in renal tissue.

What Are the Clinical Implications?

- Although CTSs have been shown to promote natriuresis in settings of volume expansion, the current study supports the concept that long-term elevations of these natriuretic hormones may also promote kidney inflammation and injury.
- Identification of CTSs as mediators of the inflammatory response in chronic kidney disease suggests new diagnostic targets for monitoring disease progression in this setting.
- Modulation of CTS–Na⁺/K⁺-ATPase α -1 signaling may present a novel therapeutic target to reduce the inflammatory events that contribute to the initiation and progression of renal injury in volume-expanded settings, such as chronic kidney disease.

cytokines and chemokines in renal epithelial cells, whereas in immune cells, CTSs induced both oxidative burst and nuclear factor κ -light-chain enhancer of activated B cells activation.^{5–7,10} However, whether CTSs play a role in driving the adhesion process is unknown.

Cytokine and chemokine production within renal tissue make important contributions to immune cell adhesion and are a feature we previously found to be mediated through the CTS–NKA α -1-Src signaling cascade.^{7,11} Studies have shown the involvement of Src kinases in regulating some of the major events in immune response, including immune cell activation¹² and migration,¹³ in addition to triggering tubular epithelial and endothelial cell proinflammatory effects.^{12,14} As activation of Src kinase is central to the CTS–NKA α -1-Src signaling axis, we reasoned that this signaling pathway may also have a role in driving the immune cell adhesion process.

The inflammatory response is driven not only by activation of immune cells, but also their interactions with both endothelial and epithelial cells, a concept that has been thoroughly reviewed previously.^{15–17} This response involves a sequence of events that include expression of molecules both

on leukocytes as well as endothelium and epithelium.⁴ Tubular epithelial cells produce cytokines and chemokines that activate immune cells and enhance their migration toward the tissue.¹⁷ Leukocyte integrins, such as lymphocyte function-associated antigen 1 and macrophage-1 antigen, which are present on the cell surface of most leukocytes, interact with intercellular adhesion molecule 1 (ICAM-1), an endothelial cell surface glycoprotein; and this interaction is capable of mediating leukocyte–endothelial cell adhesion, immune cell migration, and inflammatory response.^{18–21} Immune cells, such as monocytes, can differentiate into macrophages and dendritic cells on infiltration into renal tissue.^{22,23} Cumulatively, these events enhance and potentiate the renal inflammatory response.²⁴ Regulation of these events is important to control cellular injury and repair in CKD. Despite advances in management strategies and patient care measures, CKD continues to be associated with high morbidity and mortality. Understanding of the cellular pathophysiological processes underlying inflammation and oxidative stress in CKD will hopefully result in the design of more targeted therapies to prevent kidney injury and reduce progression of the disease course. In the current study, we tested the hypothesis that CTS, through an NKA α -1-Src kinase-dependent signaling mechanism, enhances immune cell recruitment and adhesion to epithelium within the kidney that potentiates renal inflammation.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Reagents

The CTS telocinobufagin was purchased from Baoji Herbest Bio-tech (Baoji City, Shaanxi Province, China). pNaKtide was synthesized and purchased from Ohio Peptide (Columbus, OH). Materials for SDS-PAGE were purchased from Bio-Rad Laboratories. DMEM with L-glutamine, 4.5 g/L glucose, and sodium pyruvate were purchased from Fisher Scientific. Fetal bovine serum was purchased from Rocky Mountain Biologicals Inc. Trypsin-EDTA (0.25%) and penicillin-streptomycin (10 000 U/mL) were purchased from ThermoFisher Scientific (Waltham, MA).

Cell Culture

Tissue culture media and supplements were from Life Technologies (ThermoFisher Scientific). Peritoneal macrophages were obtained by lavage after 72 hours of thioglycolate injection, and adherent cells were maintained in culture,

as described.¹⁰ The human HK-2 and porcine LLC-PK1 renal proximal tubule cell lines were obtained from American Tissue Type Culture Collection (Manassas, VA). Sublines of LLC-PK1 cells expressing Na/K-ATPase- α -1 small interfering RNA to knock down expression by 90% (PY-17 cells) or control transfected cells (P-11) were cultured in the same manner as the parent cells.^{10,25}

HK2 cells were purchased from American Tissue Type Culture Collection (CRL-21900), and murine macrophage raw blue cells were purchased from Invivogen (San Diego, CA). For telocinobufagin treatment of adherent cells, on reaching 80% to 90% confluence, cells were serum starved for 16 hours. For experiments using NKA α -1-*Src* signaling antagonist, pNaKtide, 1 μ mol/L pNaKtide was added to the cells for 30 minutes before any telocinobufagin treatments. Cell lysate or conditioned media were collected, as we have previously published.^{10,26} Human coronary artery endothelial cells (Lonza, Basel, Switzerland; catalog No. CC-2585) were grown in endothelial basal medium-2 supplemented with endothelial growth factors and 5% fetal bovine serum at 37°C under humidified air (5% CO₂). Human monocytic cell line (THP-1) was maintained in RPMI 1640, supplemented with 10% fetal bovine serum, penicillin-streptomycin, and β -mercaptoethanol.²⁷ Human renal proximal tubular epithelial cells (catalog No. 930-05A) and human peripheral blood monocytes (catalog No. 6906-50A), along with the special cell culture media recommended by the vendor (Renaepi Growth Medium for human renal proximal tubular epithelial cells, catalog No. 911-500; Blood Cell Growth Medium for human peripheral blood monocytes, catalog No. 615-250), were all purchased from Millipore Sigma (Burlington, MA).

Animals

All animal experiments were conducted in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* under protocols approved by the Institutional Animal Care and Use Committee at the University of Toledo and the Cleveland Clinic Institutional Animal Care and Use Committee. Na/K-ATPase α 1 subunit heterozygous null mice (NKA α -1^{+/-}) and their wild-type littermate controls (NKA α -1^{+/+}) were generated as described.^{28,29} These mice were a generous gift from Professor Jerry Lingrel (Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati College of Medicine). Mice were randomly assigned to received either infusion with vehicle or telocinobufagin (4 weeks at 0.1 μ g/g per day; n=6/group) intraperitoneally via osmotic minipumps (Alzet model 1004). In separate studies, rats on the background of Dahl salt sensitive (S/Jr or S) were randomly assigned for IP injection with either telocinobufagin (0.1 μ g/g per day) or vehicle for 4 weeks (n=4/group).

In Vitro Adhesion Assays

A functional live cell assay was used to examine cell-cell interactions of macrophages with a renal epithelial monolayer under physiologically relevant conditions. Renal epithelial cells were coated on 6-well plates. Cells were treated with vehicle at 80% confluency or with 10-nmol/L telocinobufagin for 24 hours. Macrophages were then incubated with vehicle, or with 100-nmol/L telocinobufagin for 24 hours, and labeled with calcein-AM (4 μ mol/L) at 37°C for 30 minutes. Macrophages were washed twice with prewarmed PBS and resuspended in Hank's balanced salt solution. Macrophages suspended in Hank's balanced salt solution were added to the 6-well plate and incubated at 37°C for 1 hour on orbital shaker at 60 rpm. The experimental design for these experiments is outlined in a schematic (Figure S1). Nonadherent calcein-labeled macrophage cells were removed by careful washing, and fluorescence imaging was performed on live cell imaging fluorescence microscope (Cytation 5; BioTek, Winooski, VT). For each well, 10 images were taken randomly, and fluorescent calcein-labeled macrophages were quantified manually to indicate macrophage adhesion to the epithelium per field.

Histology

Kidneys were fixed in 4% formaldehyde (pH 7.2) paraffin embedded and cut into 4- μ m sections. The tissue sections were deparaffinized with xylene and rehydrated by sequential incubations in ethanol and water. Vectastain Elite-ABC kit (Vector Labs, Burlingame, CA) was used, following manufacturer's protocol. For each section, 10 images were randomly taken with a bright-field microscope with a 20 \times lens and quantitative morphometric analysis was performed using automated and customized algorithms/scripts for batch analysis (ImageIQ Inc, Cleveland, OH) written for Image Pro Plus 7.0, as we have described in detail.¹⁴

Immunofluorescence

Paraffin-embedded kidneys 4 μ m in thickness were deparaffinized, as described above, for immunohistochemistry and immunofluorescence staining. The slides were incubated with primary antibodies cluster of differentiation (CD) 11b monoclonal antibody (M1/70), Alexa Fluor 488, and ICAM-1 antibody (9HCLC), ABfinity Rabbit Oligoclonal (Thermo Scientific, Waltham, MA), overnight at 4°C, after blocking with 1% BSA for 1 hour at room temperature. Next, slides were washed with a tris-buffered saline-Tween solution, followed by incubating with a secondary antibody for 2 hours at room temperature. The slides were then incubated with mounting medium containing 4',6-diamidino-2-phenylindole for nuclear staining and mounted with coverslip. Fluorescent signals were

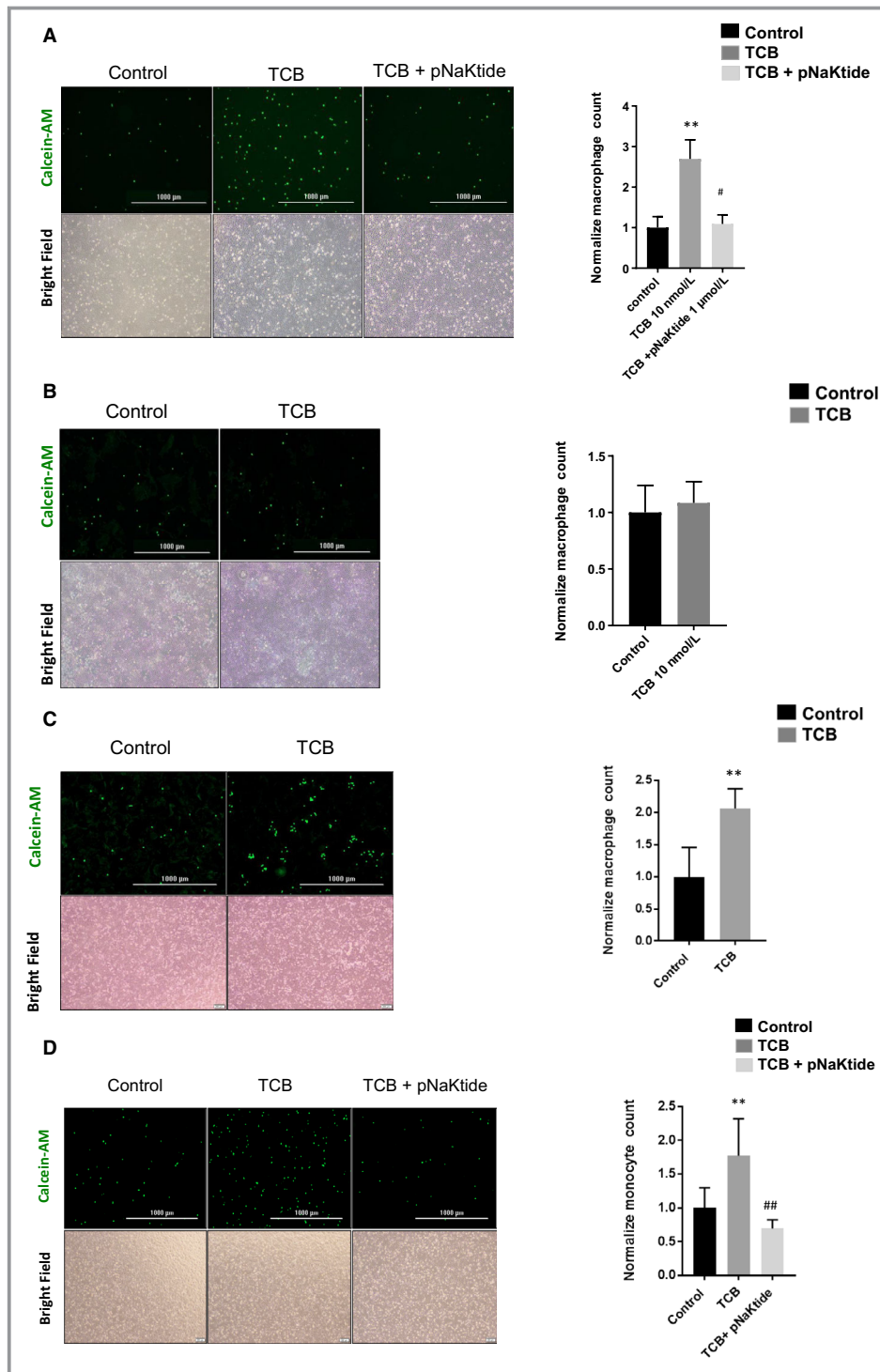


Figure 1. Reduction of $\text{Na}^+/\text{K}^+\text{-ATPase } \alpha\text{-1}$ (NKA $\alpha\text{-1}$) or inhibition of NKA $\alpha\text{-1}$ -Src kinase with specific inhibitor pNaKtide in renal epithelial cells attenuates telocinobufagin (TCB)–induced macrophage interaction with renal epithelial cells in vitro. **A**, Blocking Src kinase using pNaKtide (1 $\mu\text{mol/L}$) attenuates TCB (10 nmol/L; 24 hours) induced macrophage adhesion to the renal epithelial monolayer. **B**, NKA $\alpha\text{-1}$ knockdown in renal epithelial cells (PY17) attenuates TCB (10 nmol/L; 24 hours) induced macrophage adhesion to the renal epithelial monolayer compared with the control renal epithelial cells (P11). **C**, Rescue of Na/K-ATPase α1 expression in AAC-19 (which are PY-17 cells rescued with rat α1 cDNA) restores TCB (10 nmol/L; 24 hours) mediated immune cell adhesion. **D**, Blocking Src kinase using pNaKtide (1 $\mu\text{mol/L}$) attenuates TCB (10 nmol/L; 24 hours) induced monocyte adhesion to the renal epithelial monolayer in human primary cell lines. The data are presented as mean \pm SEM from 3 separate experiments. ** $P < 0.01$ vs control, # $P < 0.05$ vs pNaKtide, ## $P < 0.01$ vs pNaKtide.

visualized using microscope. Ten images were taken from each slide. Immunofluorescence for presence of monocyte/macrophage was performed using rat anti-monocyte/macrophage antibody (Abcam) as we have described.³⁰ Data from 4 to 5 animals in each group were analyzed by GraphPad software version 7.0.

Cellular Communication Network Factor 1 Measurement

Human Cyr61/cellular communication network factor 1 (CCN-1) in conditioned media was measured by ELISA purchased from R&D Systems (Minneapolis, MN) and performed in accordance with the manufacturer's protocol.

Western Blot Analysis

Proteins from kidney tissue were homogenized in ice-cold radioimmunoprecipitation assay lysis buffer (pH 7.0; sc-24948; Santa Cruz Biotechnology, Santa Cruz, CA) supplemented with freshly prepared Halt Protease and Phosphatase Inhibitor Cocktail (78446; Thermo Scientific). For in vitro experiments, cells were washed with ice-cold PBS before lysis in the same radioimmunoprecipitation assay lysis buffer with protease and phosphatase inhibitor cocktails. Cell lysates were vortexed briefly and then rotated at 4° for 15 minutes. The tissue homogenate or cell lysate was centrifuged at 15 000g for 15 minutes at 4°C. The supernatant was separated for protein quantification, and total of 30 µg protein was used for detection. Proteins were resolved via SDS-PAGE under reducing conditions, unless otherwise noted. After gel electrophoresis, the proteins were electrotransferred from the gel onto polyvinylidene difluoride (PVDF) membranes (0.45-µm PVDF Transfer Membrane; Thermo Scientific). Then, the membrane was blocked with Rapid Block TM solution (VWR Life Science, Radnor, PA) and probed with the indicated antibody.

ToxinSensor Chromogenic LAL Endotoxin Assay

The Genscript ToxinSensor Chromogenic LAL Endotoxin Assay Kit, purchased from Genscript (Piscataway, NJ), was used to assess the purity of the telocinobufagin preparation. This kit is capable of detecting endotoxin concentration in the range of 0.01 to 1 EU/mL and was performed in accordance with the manufacturer's protocol.

Bone Marrow Transplantation

To further assess the contribution of NKA α-1 on circulating immune cells to enhancement of CTS-mediated adhesion in the kidney, we analyzed renal tissue from a bone marrow transplantation study, which we have previously reported.³⁰ Briefly,

apolipoprotein E-null (ApoE^{-/-}; NKA α1^{+/+} wild-type) recipient mice were exposed to whole body γ-irradiation to eradicate endogenous bone marrow stem cells as well as bone marrow-derived cells. Next, bone marrow isolated from either ApoE^{-/-} NKA α1^{+/+} (wild-type) or ApoE^{-/-} NKA α1^{+/-} (NKA knockdown) mice was injected to recipient mice through retro-orbital injection (8 × 10⁶). Mice were maintained on normal chow diet for 4 weeks to allow bone marrow reconstitution and then diet was changed to a high-fat diet for additional 12 weeks to induce renal injury.³⁰ Histopathology was performed on kidney sections to assess the presence of macrophage-2 antigen-positive immune cells as well as renal fibrosis.

Statistical Analysis

Data are presented as the mean ± SEM. Student's unpaired *t* test was used to assess statistically significant differences between 2 groups. One-way ANOVA and post hoc multiple comparison tests were used when comparing >2 groups. Statistical significance was accepted as *P* < 0.05. All statistical analysis was performed using GraphPad Prism 7 software.

Results

CTS Signaling Through NKA α-1 and Src Kinase Enhances Functional Cell-Cell Interaction Between Macrophages and Renal Epithelial Cells

We examined the role of CTS signaling through NKA α-1 and Src kinase in mediating macrophage adhesion to renal epithelium using a functional assay (see Materials and Methods) to demonstrate cell-cell interactions of macrophages with a renal epithelial monolayer under physiologically relevant conditions. We found that although telocinobufagin treatment for 24 hours significantly enhanced macrophage adhesion to the renal epithelial monolayer, compared with vehicle, pretreatment with the NKA α-1/Src complex inhibitor pNaKtide (1 µmol/L; 30 minutes) attenuated telocinobufagin-induced increases in macrophage adhesion (Figure 1A).

Next, to assess whether the observed effect is specific to Na/K-ATPase α-1 isoform, we used sublines of LLC PK1 porcine proximal tubule cells expressing NKA α-1 small interfering RNA to knock down expression by 90% (PY-17 cells with 10% NKA α-1 expression) or control transfected cells (P-11 cells with 100% NKA α-1 expression).^{10,26} We found that knockdown NKA α-1 showed a statistically significant reduction in telocinobufagin-induced macrophage adhesion in PY-17 cells (*P* < 0.05; Figure 1B) compared with the control P11 parent cell line. Next, to further establish a role of the Na/K-ATPase α1 isoform in telocinobufagin-mediated immune cell adhesion, we performed a functional adhesion assay using

ACC19 cells (which are PY-17 cells rescued with rat $\alpha 1$ cDNA). LLC-PK1 (P11) and AAC-19 cells expressed comparable amounts of $\alpha 1$, whereas $\alpha 1$ expression is reduced by ~90% in PY-17 cells (see Figure 2A in the study by Xie et al²⁶). Although knockdown of the $\alpha 1$ isoform of Na/K-ATPase caused a statistically significant reduction in immune cell adhesion in PY-17 cells ($P < 0.05$), this reduction was fully recovered by rescue of Na/K-ATPase $\alpha 1$ in AAC-19 cells (Figure 1C). Similar results were obtained on examining human primary renal proximal tubule epithelial cells and human primary peripheral monocytes. Herein, we observed increased adhesion of immune cells to renal epithelial monolayer with telocinobufagin treatment of 10 nmol/L for 24 hours. The telocinobufagin effect was also diminished with pNaktide pretreatment of 1 μ mol/L (30 minutes; Figure 1D). Next, we investigated the role of macrophage NKA α -1-Src signaling in modulating functional cell-cell interactions. We used macrophages that were isolated from either wild-type NKA α -1^{+/+} or knockdown NKA α -1^{+/-} mice and treated them with 100 nmol/L telocinobufagin for 24 hours. We found that although telocinobufagin induced wild-type macrophage adhesion to a renal epithelial monolayer ($P < 0.05$; Figure 2A), the effect was abolished with pNaktide pretreatment of wild-type macrophages (Figure 2A) and in NKA α -1^{+/-} knockdown macrophages (Figure 2B).

These findings suggest that the NKA α -1-Src signaling complex is an important mediator of CTS-induced functional cell-cell interactions between macrophages and renal epithelial cells. More important, the telocinobufagin compound used in the current study was tested for lipopolysaccharide contamination using a highly sensitive assay that revealed no detectable lipopolysaccharide contamination.

CTS Signaling Through NKA α -1 and Src Kinase Enhances Adhesion Molecule Expression on Immune Cells and Endothelial Cells in Vitro

To test the role of CTS signaling on adhesion molecule expression in immune cells, we treated THP-1 cells, a human monocyte cell line, for 24 hours with 10 nmol/L telocinobufagin. We observed a statistically significant increase in both CD11b and CD18 protein expression with telocinobufagin treatment when compared with the control vehicle treated cells ($P < 0.01$; Figure 3A). Next, we tested the involvement of Src kinase signaling in telocinobufagin-induced adhesion molecule protein expression using biochemical approaches. We found that pretreatment with the NKA α -1/Src complex inhibitor pNaktide¹¹ (1 μ mol/L; 30 minutes) attenuated telocinobufagin-induced increase in adhesion molecule protein expression of CD11b and CD18 ($P < 0.01$; Figure 3A). Similar findings were observed when treating primary human coronary artery endothelial cells for 24 hours with 10 nmol/L

telocinobufagin. We found that although telocinobufagin induced increase in ICAM-1 and VCAM-1 (vascular cell adhesion protein 1) expression in endothelial cells, this effect was also diminished after inhibiting NKA α -1-Src signaling using pNaktide pretreatment (Figure 3B). We also demonstrated that telocinobufagin (10 nmol/L; 24 hours) upregulates the adhesion molecule VLA-4 (integrin $\alpha 4\beta 1$) protein expression in monocytes (THP-1) (Figure S2). Next, we examined the effect of CTS signaling on the expression of CCN-1 in human monocyte THP1 cells. Herein, our data showed a statistically significant increase CCN-1 expression with telocinobufagin treatment when compared with the control vehicle treated cells ($P < 0.01$; Figure 3C). This effect was attenuated after pretreatment with NKA α -1/ Src complex inhibitor pNaktide (1 μ mol/L; 30 minutes; Figure 3C). In addition, to assess telocinobufagin effects on adhesion molecule protein expression in endothelial cells (human coronary artery endothelial cells) and monocyte (THP-1), we also examined telocinobufagin's effect in mediating monocyte (THP-1) adhesion to endothelial monolayer under physiologically relevant conditions using a functional assay (see Materials and Methods). Our data demonstrated that telocinobufagin (10 nmol/L; 24 hours) significantly enhanced monocyte adhesion to the endothelial monolayer, compared with vehicle (Figure S3).

CTS Signaling Through NKA α -1 Kinase Enhances Adhesion Molecule Expression in Renal Tissue in Vivo

To further examine the role of the NKA α -1 in mediating CTS-induced adhesion molecule expression in vivo, we administered telocinobufagin (4 weeks telocinobufagin at 1 μ g/kg per day via osmotic minipump²⁹) in NKA α -1^{+/+} wild-type and NKA α -1^{+/-} knockdown mice and examined the effect of CTS signaling on adhesion molecule (CD11b and ICAM) expression in the kidneys. Herein, we observed significant decrease in the expression of both CD11b and ICAM-1 in renal tissue of NKA α -1^{+/-} knockdown versus NKA α -1^{+/+} wild-type mice (Figure 4A and 4B).

CTS Signaling Through NKA α -1 and Src Kinase Enhances Immune Cell Adhesion in Renal Tissue in Vivo

Next, to test the role of CTS signaling through NKA α -1 and Src kinase in mediating immune cell adhesion into renal tissue, we examined immune cell recruitment in kidneys collected from wild-type NKA α -1^{+/+} and NKA α -1^{+/-} knockdown mice after administration of telocinobufagin (4 weeks telocinobufagin at 1 μ g/kg per day via osmotic

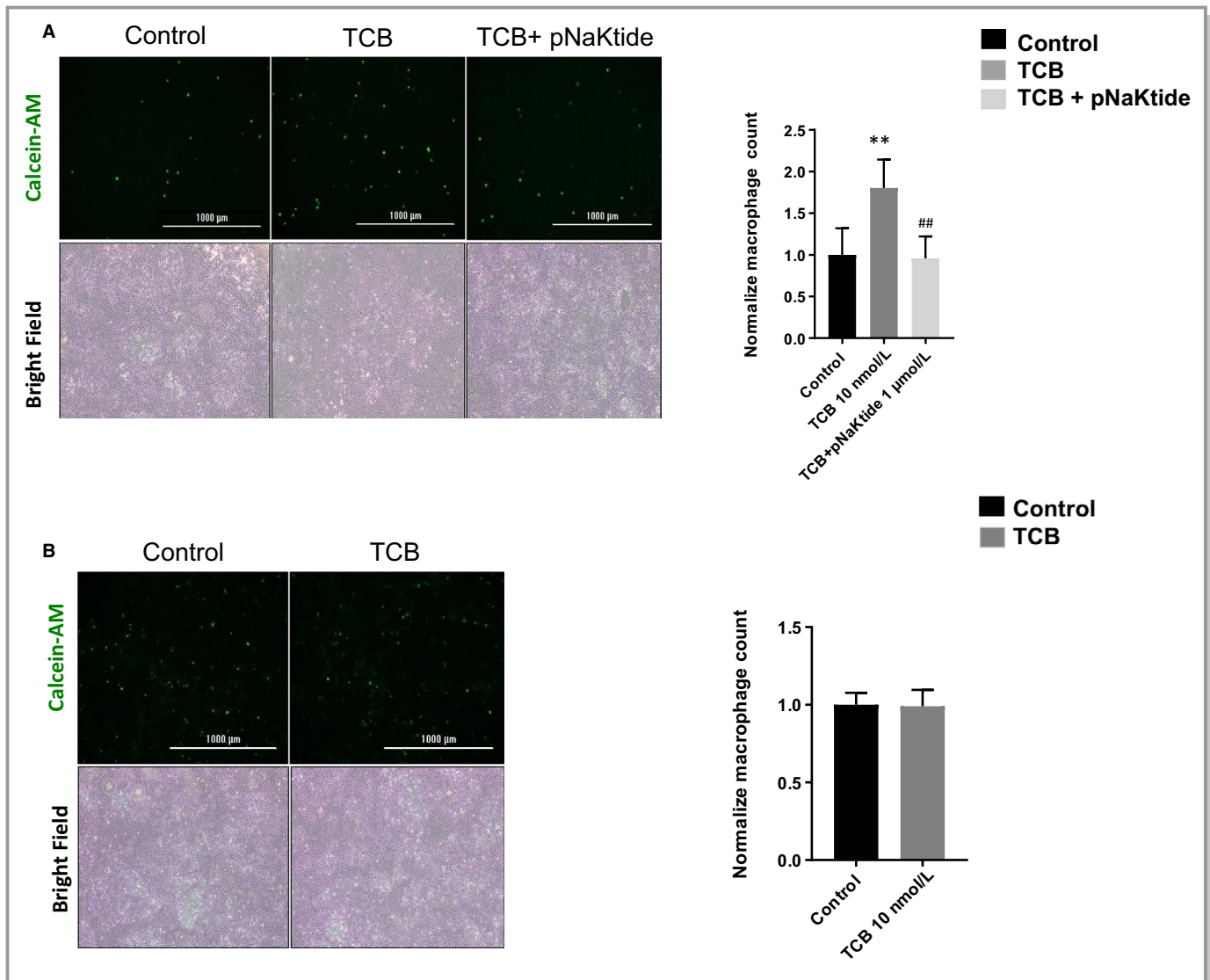


Figure 2. Reduction of Na^+/K^+ -ATPase α -1 (NKA α -1) or inhibition of NKA α -1-Src kinase with specific inhibitor pNaKtide in macrophages attenuates telocinobufagin (TCB)-induced macrophage interaction with renal epithelial cells in vitro. **A**, Blocking Src kinase using pNaKtide (1 $\mu\text{mol/L}$) attenuates TCB (10 nmol/L; 24 hours) induced macrophage adhesion to the renal epithelial monolayer. **B**, NKA α -1 knockdown in macrophages (NKA- α -1 $^{+/-}$) attenuates TCB (10 nmol/L; 24 hours) induced macrophage adhesion to the renal epithelial monolayer compared with the control wild-type macrophages (NKA- α -1 $^{+/+}$). The data are presented as mean \pm SEM from 3 separate experiments. ** P <0.01 vs control, ## P <0.01 vs pNaKtide.

minipump²⁹). We performed immunohistochemistry to examine for the presence of anti-monocyte/macrophage antibody-positive immune cells (monocytes/macrophages). Quantitative morphometry demonstrated that kidneys collected from the NKA α -1 $^{+/-}$ knockdown mice showed significantly less immune cell accumulation compared with the wild-type controls at 4 weeks (Figure 5A). Similar results were obtained when we stained for CD68-positive macrophages, where CTS-mediated macrophage accumulation in the kidney tissue was diminished in the NKA α -1 $^{+/-}$ knockdown mice compared with the NKA α -1 $^{+/+}$ wild-type mice (Figure 5B). These findings were noted in both the

tubulointerstitial compartment and glomeruli. Following, we examined the effect of telocinobufagin on different inflammatory cell types (eg, CD3 and CD11C-positive immune cells) in the kidney. On examining kidney sections, we found that telocinobufagin slightly increased CD3 and CD11C-positive immune cell recruitment in the kidney. However, there was no difference between NKA α -1 $^{+/+}$ wild-type mice and NKA α -1 $^{+/-}$ knockdown mice (Figure S4A and S4B).

Next, to further assess the contribution of NKA α -1 on circulating immune cells to enhancement of CTS-mediated adhesion in the kidney, we analyzed renal tissue from a bone marrow transplantation study performed in the setting of

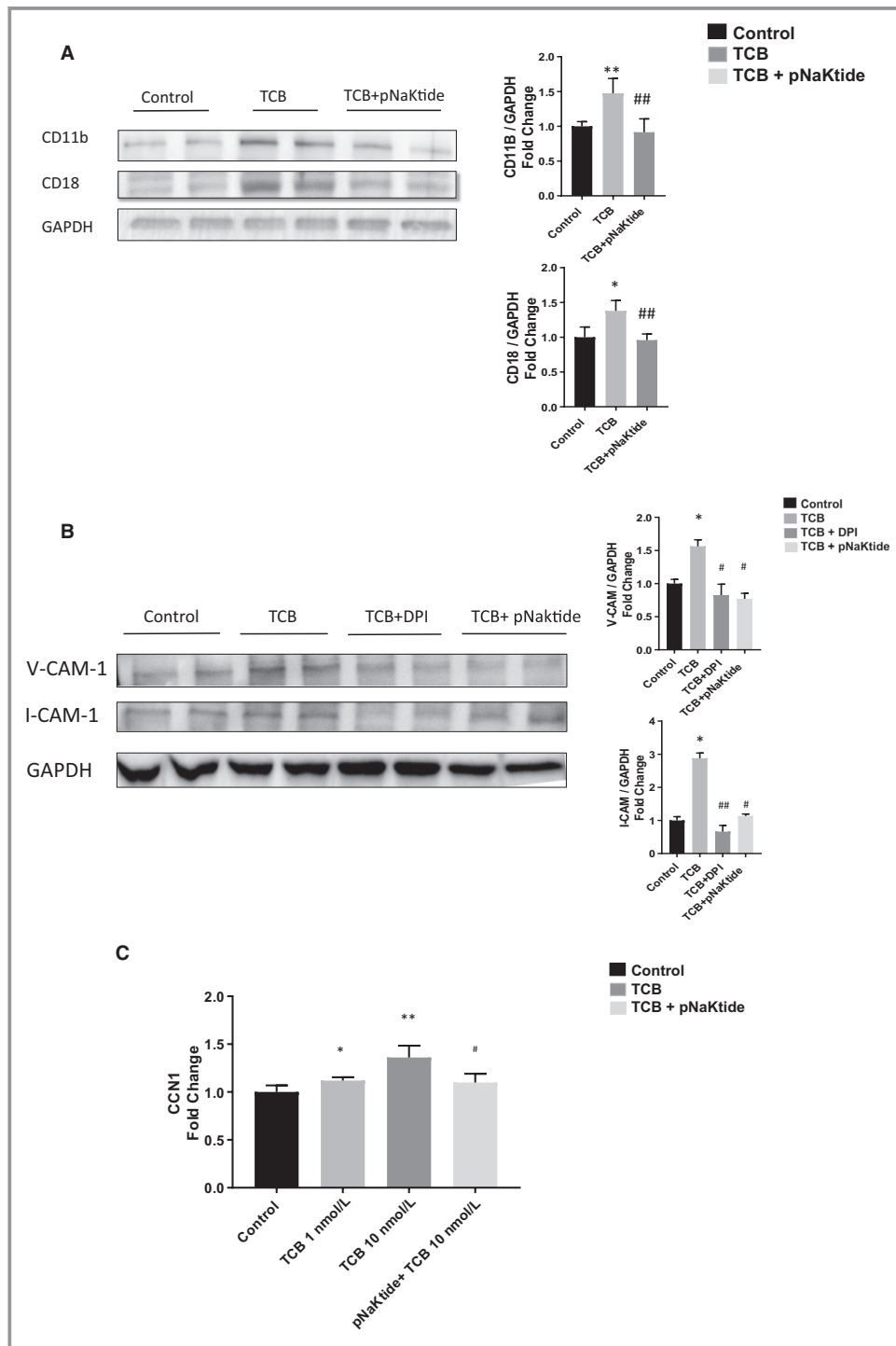


Figure 3. Telocinobufagin (TCB)–induced protein expression of inflammatory adhesion molecules in immune cells and endothelial cells is attenuated after treating with Src kinase inhibitor pNaKtide. **A**, Blocking Src kinase using pNaKtide 1 (μmol/L) attenuates TCB (10 nmol/L; 24 hours) induced cluster of differentiation (CD) 11b and CD18 expression in human monocytes (THP-1) cells. **B**, Blocking Src kinase using pNaKtide (1 μmol/L) attenuates TCB (10 nmol/L; 24 hours) induced intercellular adhesion molecule 1 (ICAM-1) and VCAM-1 (vascular cell adhesion protein 1) expression in human endothelial cells (human coronary artery endothelial cells) similar to inhibition with a known inhibitor of ICAM-1, diphenyleneiodonium (DPI). **C**, TCB (10 nmol/L; 24 hours) induced immune cell adhesion molecule cellular communication network factor 1 (CCN-1) expression in human monocytes (THP-1) is attenuated via pretreatment with Src kinase inhibitor pNaKtide (1 μmol/L). The data are presented as mean±SEM from 3 to 4 separate experiments. **P*<0.05 vs control, ***P*<0.01 vs control, #*P*<0.05 vs pNaKtide, ##*P*<0.01 vs pNaKtide.

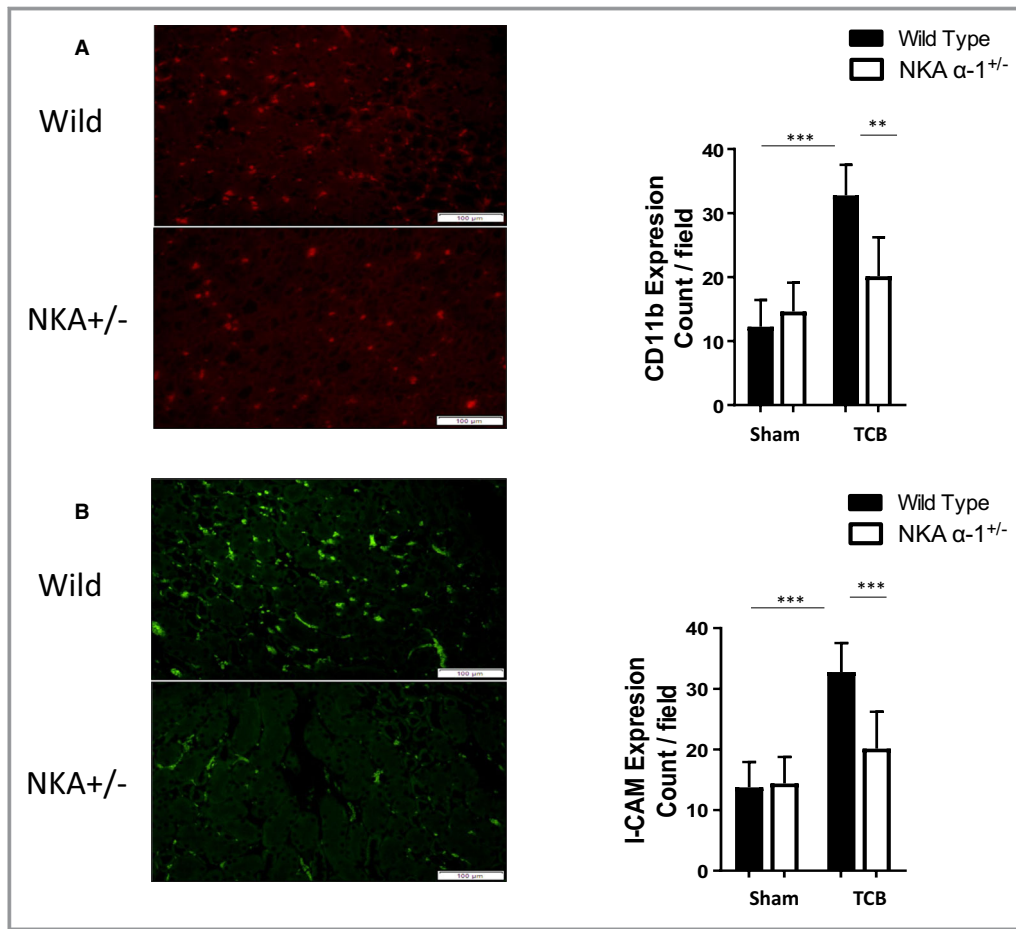


Figure 4. Na⁺/K⁺-ATPase α -1 (NKA α -1) knockdown attenuates telocinobufagin (TCB)-induced adhesion molecule expression in NKA α -1^{+/-} kidney compared with wild-type NKA α -1^{+/+} kidney. **A, Left**, Representative histologic images of intercellular adhesion molecule 1 (ICAM-1) expression in kidney from wild-type NKA α -1^{+/+} mice (**top**) and NKA α -1^{+/-} mice (**bottom**) with quantification (**right**). **B, Left**, Representative histologic images of immune cell adhesion molecule cluster of differentiation (CD) 11b expression in kidney from wild-type NKA α -1^{+/+} mice (**top**) and NKA α -1^{+/-} mice (**bottom**) with quantification (**right**). The data are presented as mean \pm SEM from n=6 kidneys per group. ** P <0.01, *** P <0.001.

high-fat diet induced renal injury on the ApoE^{-/-} background,³⁰ which is a model of elevated CTS levels.¹⁰ Analysis of the kidneys from these mice demonstrates that reduction of bone marrow-derived NKA leads to significant reduction in macrophage-2 antigen-positive immune cell infiltration in the kidney (Figure S5A) as well as decreased renal fibrosis (Figure S5B).

CTS Signaling Through NKA α -1 and Src Kinase Enhances Immune Cell Recruitment in Vivo

To examine the role of CTS signaling through NKA α -1 and Src kinase in mediating immune cell accumulation, we examined peritoneal lavage of wild-type NKA α -1^{+/+} and NKA α -1^{+/-} knockdown mice after administration of telocinobufagin for 4 weeks (1 μ g/kg per day) for immune cell accumulation. We observed that peritoneal lavage from telocinobufagin-

administered NKA α -1^{+/-} knockdown mice showed significantly less mononuclear cell number accumulation, as indicated by the presence of decreased CD11b-positive immune cells compared with wild type NKA α -1^{+/+} (Figure 6A). Next, to further examine CTS effects on immune cell accumulation using different model, we injected Dahl S rats intraperitoneally with either telocinobufagin (1 μ g/kg per day) or vehicle for 4 weeks, followed by lavage of the peritoneal cavity with PBS. We found that peritoneal lavage from telocinobufagin-injected rats showed increased immune cell accumulation, represented by the presence of more CD11b protein compared with the vehicle-treated group (Figure 6B). On further examination of the peritoneal lavage, we observed higher cellular content, represented by higher protein concentration and cell count, in peritoneal lavage from telocinobufagin-injected rats compared with the vehicle-treated group (Figure 6C).

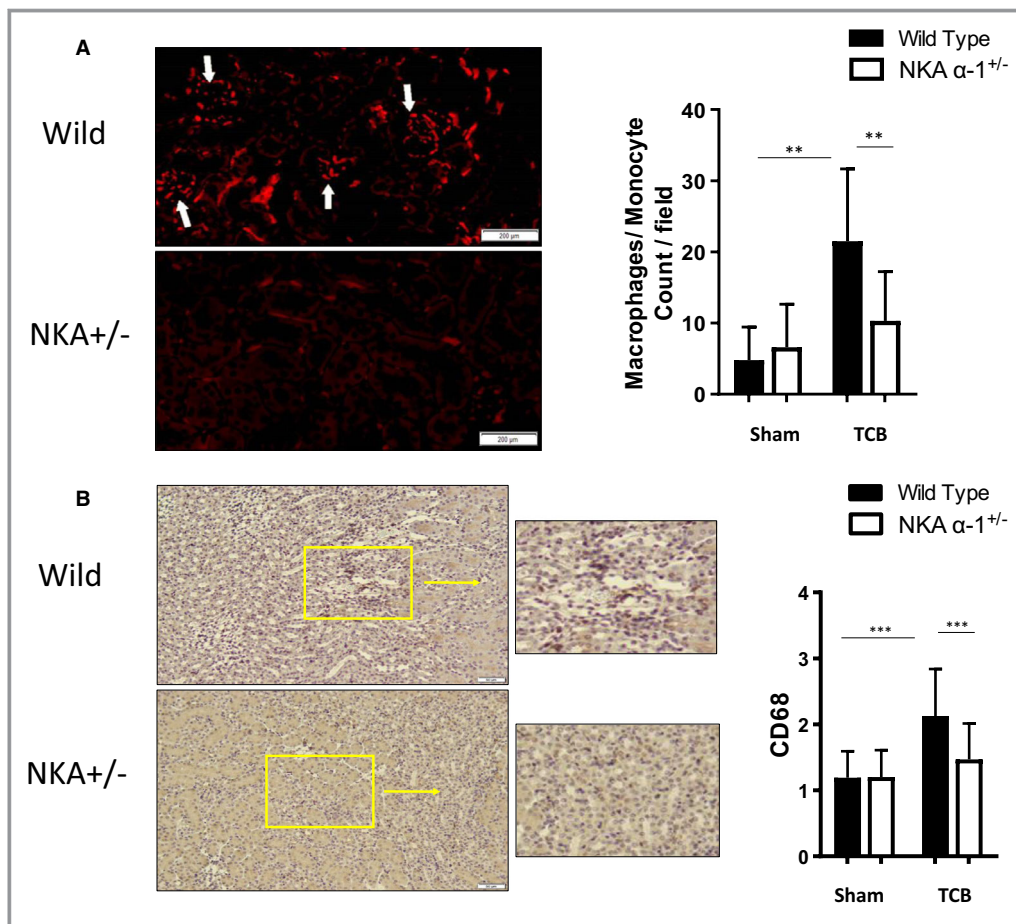


Figure 5. Na^+/K^+ -ATPase α -1 (NKA α -1) knockdown attenuates telocinobufagin (TCB)-induced immune cell accumulation in NKA α -1^{+/-} kidney compared with wild-type NKA α -1^{+/-} kidney. **A, Left,** Representative histologic images of monocyte/macrophage positive immune cells in kidney from wild-type NKA α -1^{+/-} mice (**top**) and NKA α -1^{+/-} mice (**bottom**) with quantification (**right**). **B, Left,** Representative histologic images of cluster of differentiation (CD) 68 positive immune cell (macrophage) recruitment in kidney from wild-type NKA α -1^{+/-} mice (**top**) and NKA α -1^{+/-} mice (**bottom**) with quantification (**right**). The data are presented as mean \pm SEM from n=6 kidneys per group. ** P <0.01, *** P <0.001.

Discussion

Inflammation is an essential component of renal disease and one of the core contributors in its pathophysiological characteristics. Although the exact pathways that initiate and advance CKD have not been fully clarified, it is known that a proinflammatory state exists in the initiation and progression of CKD.^{31,32} Substantial improvement has been made in identifying major elements of this process; nevertheless, the molecular and cellular interactions among epithelial cells and immune cells are poorly understood. We have identified a mechanistic link that may help to explain part of the inflammation and oxidative stress that mediate CKD progression in volume-expanded states, where levels of CTS are elevated.^{29,33–35} We have found that CTS signaling through the NKA α -1-Src signaling complex promotes a proinflammatory phenotype in both renal epithelial and immune cells.⁷ We

and others have also shown that elevated CTS levels are associated with nitrate stress and adverse clinical outcomes in patients with heart failure,^{14,36–38} and they mediate CKD progression.^{39–43} In fact, we have previously demonstrated that long-term CTS administration in rodents promotes renal fibrosis in an NKA α -1-dependent manner by enhancing interstitial fibroblast activation as well as increases in collagen expression and impairs renal function, as noted by increases in plasma cystatin C and urinary protein.^{29,44} Likewise, elevated levels of CTS have been shown to induce cardiac inflammation⁴⁵ as well as NKA α -1-Src-epidermal growth factor receptor mediated fibrosis.⁴²

The current study provides several lines of evidence that CTS enhances cell-cell interactions between immune cells and renal epithelial cells that may serve to potentiate renal inflammation. CTS signaling through Na/K -ATPase α -1 and Src kinase mediates critical steps in immune cell activation,

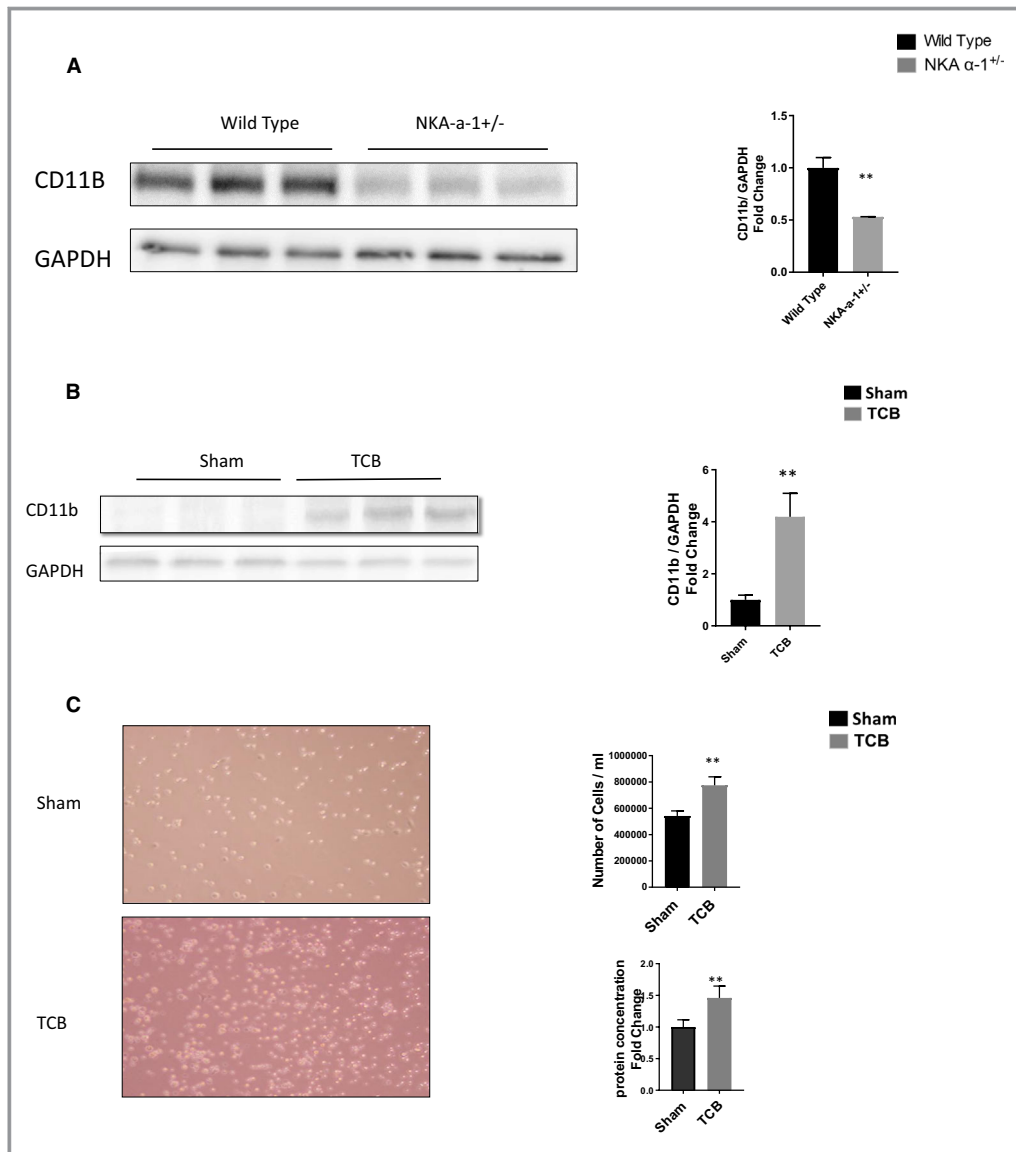


Figure 6. Na⁺/K⁺-ATPase α -1 (NKA α -1) knockdown attenuates telocinobufagin (TCB)-induced immune cell accumulation in peritoneal lavage in NKA α -1^{+/-} mice compared with wild-type NKA α -1^{+/+} mice. **A**, Cluster of differentiation (CD) 11b positive immune cells in peritoneal lavage, after 4 weeks TCB intraperitoneal administration, were analyzed by Western blot. CD11b-positive immune cell (macrophage) accumulation in peritoneal lavages collected from NKA α -1^{+/-} mice and wild-type NKA α -1^{+/+} mice. **Right**, Results were normalized using GAPDH as a loading control and are expressed as a fraction of the control (n=4). **B**, CD11b-positive immune cells in peritoneal lavage, after 4 weeks TCB intraperitoneal administration, were analyzed by Western blot. CD11b-positive immune cell (macrophage) accumulation in peritoneal lavages collected from TCB-injected rats and vehicle-treated rats. **Right**, Results were normalized using GAPDH as a loading control and are expressed as a fraction of the control. **C**, **Left**, Representative microscopic images of peritoneal lavage from TCB-injected rats (**bottom**) and vehicle-treated rats (**top**) and quantification (**right**) for cell number per mL (**top**) and protein concentration (**bottom**) (n=4). The data are presented as mean \pm SEM. ***P*<0.01.

including recruitment and adhesion, which advance the inflammatory response. Using a variety of in vivo studies, such as intraperitoneal administration of CTS in rodent models, we demonstrate enhanced accumulation of inflammatory cells in the peritoneal cavity as well as increased

monocytes/macrophages in the kidney, which was diminished in NKA α -1^{+/-} knockdown mice.

Our findings are in agreement with another published report that demonstrated that mice injected intraperitoneally with another CTS, ouabain, showed significant increase in

immune cell accumulation in the peritoneal cavity (compared with vehicle-injected mice), which was diminished in NKA α -1^{+/-} knockdown mice.⁴⁶ In addition, we found that although CTS stimulates the expression of adhesion molecules CD11b and ICAM-1 in kidney tissue collected from wild-type mice, this effect was diminished in the NKA α -1^{+/-} knockdown mice. These findings were in line with our in vitro studies that show that CTS-induced expression of adhesion molecules in immune and endothelial cells is reduced with inhibition of the NKA α -1/Src signaling complex using pNaKtide. We also demonstrated that the use of pNaKtide can attenuate CTS-induced expression of CCN-1 in human monocyte THP1 cells. CCN-1 is a matricellular protein known to play an important role in immune cell trafficking and adhesion.⁴⁷ This extracellular matrix associated signaling protein is expressed by immune cells on proinflammatory stimulus and mediates immune cell trafficking through locally immobilizing and attracting immune cells.⁴⁸ As these molecules (ie, CD11b,

ICAM-1, and CCN-1) are known to mediate immune cell activation, recruitment, and adhesion, this may contribute to the enhanced inflammatory cell accumulation initiated by CTS via the NKA α -1-Src signaling complex. In fact, our data showed that kidneys collected from wild-type mice demonstrated significant increases in immune cell infiltration after telocinobufagin administration; and this effect was significantly reduced in NKA α -1^{+/-} knockdown mice. Similarly, on examining peritoneal lavage for immune cell accumulation, we observed that peritoneal lavage from telocinobufagin-administered NKA α -1^{+/-} knockdown mice showed significantly less immune cell accumulation compared with wild-type NKA α -1^{+/+}.

Next, to examine this further, we performed experiments in specific cell types and signaling pathways using functional assay to assess CTS effects on functional cell-cell interactions between macrophages and renal epithelial cells. We found that CTS increases macrophage adhesion to renal epithelium

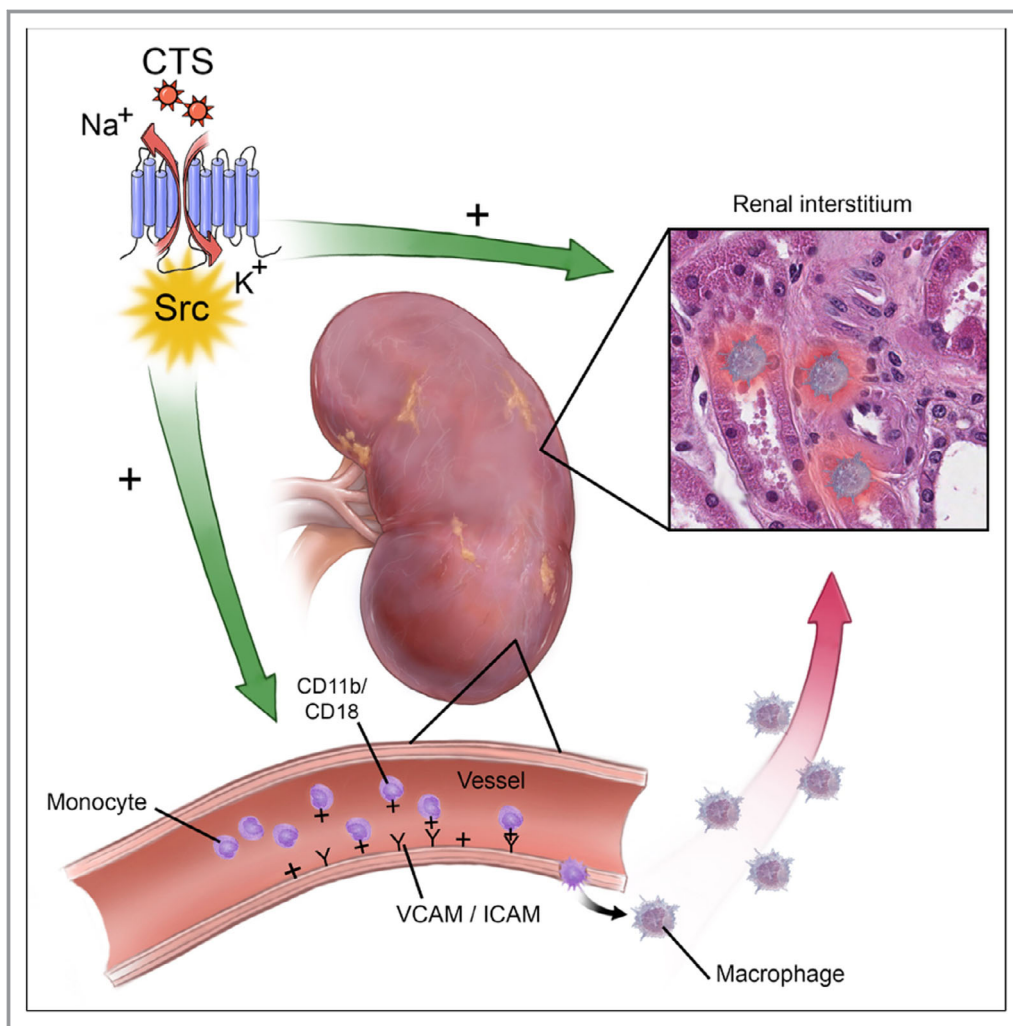


Figure 7. Schematic illustrating the role of the cardiotonic steroid (CTS)–Na⁺/K⁺-ATPase-Src kinase signaling axis in mediating immune cell adhesion to renal epithelium. CD indicates cluster of differentiation; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion protein.

through a signaling pathway mediated by the NKA α -1-Src signaling complex. Our experimental data showed that CTS-induced adhesion of macrophages to renal epithelial cells can be significantly attenuated by inhibition of the NKA α -1/Src complex signaling pathway with pNaktide or by reduction of NKA α -1 in either macrophages or renal epithelial cells. Although several lines of evidence point to the ability of CTS to initiate inflammatory signaling pathways, this is the first report that demonstrates that the stimulation of the NKA α -1-Src signaling pathway with CTS is also capable of activating the adhesion response, as summarized in Figure 7. Future studies will provide us with better understanding of the cellular and molecular aspects underlying the progression of renal disease, which will help in developing new therapeutic targets to modulate inflammation and oxidative stress associated with renal disease.

Initiation and resolution of inflammation in the kidney is a complex process that has both positive and negative consequences for overall end-organ function.⁴⁹ Thus, our results need to be interpreted within this context as there are likely both physiologic and pathologic implications for elevated levels of CTS. We speculate that similar to the “trade-off” between natriuresis and hypertension/fibrosis, which is seen with these hormones in volume-expanded settings (reviewed in Khalaf et al³⁵), a similar trade-off may exist with CTSs in regard to their capacity to induce inflammation. Indeed, other groups have noted beneficial anti-inflammatory effects of CTSs. In fact, the CTS bufalin exhibits anti-inflammatory effects in the lung, where treatment with bufalin leads to a significant reduction in total inflammatory cells as well cytokines interleukin-4, interleukin-5, and interleukin-13 in a rodent asthma model.⁵⁰ This may have relevance to the role CTSs play in other settings, such as cancer, where the antitumorigenic effects of CTS may involve modulation of the immune response.^{51,52} Thus, a fundamental mechanistic understanding of how CTS contributes to the development and resolution of inflammation will be critical to enhancing these hormones for diagnostic and therapeutic targets in a variety of physiologic and pathophysiologic settings.

Conclusions

Our studies have identified a central role for CTS activation of the NKA α -1-Src signaling pathway in enhancing interactions of key cell types involved in renal inflammation, including monocytes/macrophages as well as endothelium and epithelium. These findings are relevant given the elevated levels of CTS in settings such as CKD and the profibrotic role that these natriuretic hormones play in the trade-off associated with long-term volume expansion. Our studies demonstrate that CTS signaling not only mediates an inflammatory

response in renal epithelial and immune cells but also increases immune cell recruitment and accumulation in the renal tissue, a key step in renal inflammation. Our findings suggest that modulation of CTS levels or activity, via the NKA α -1-Src signaling, may present novel therapeutic opportunities to modulate the inflammatory events that contribute to the initiation and progression of renal disease in volume-expanded settings.

Acknowledgments

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Disclosures

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SUPPLEMENTAL MATERIAL

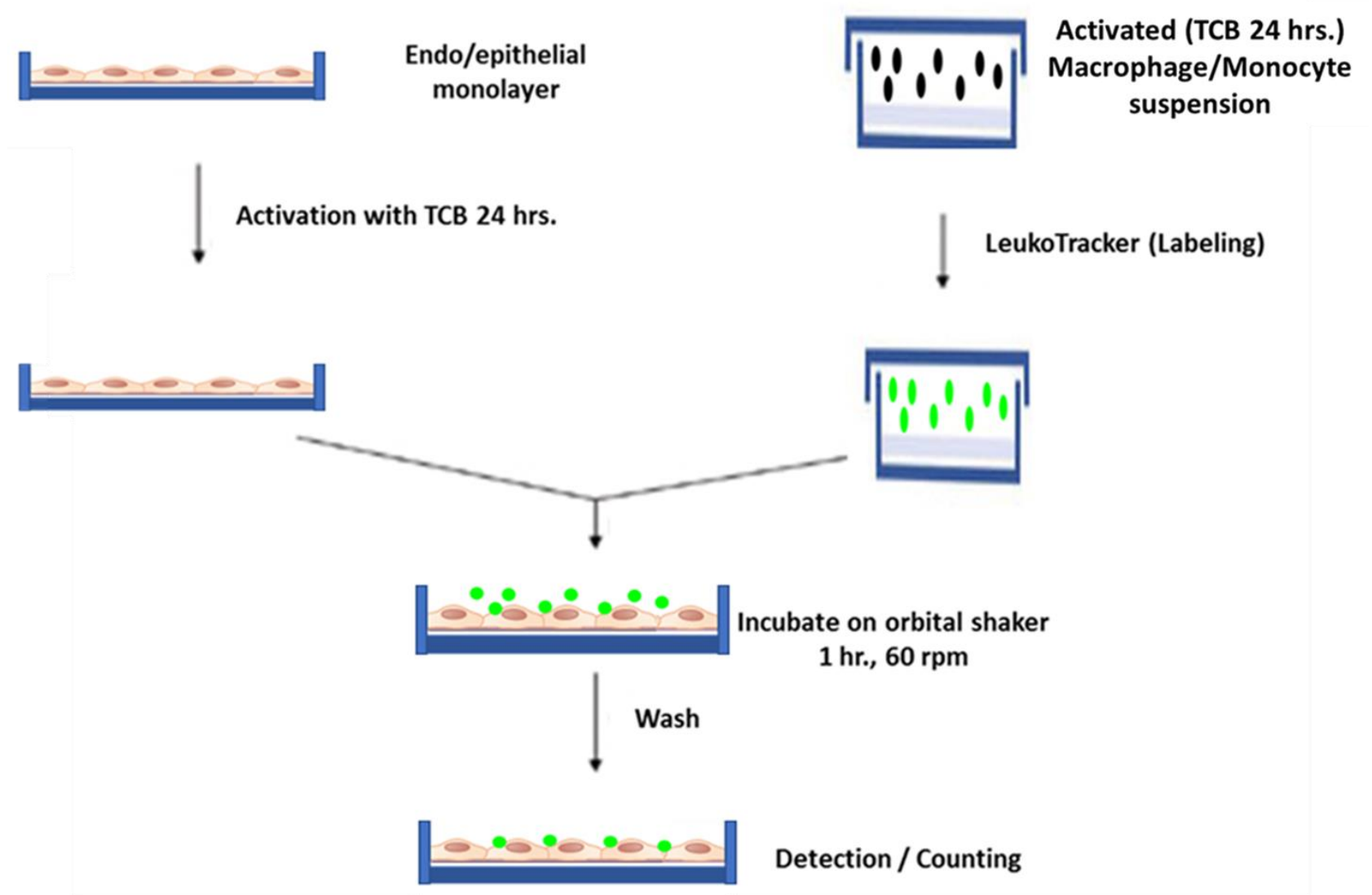


Figure S1. Schematic presentation outlined the experimental design of the functional adhesion assay

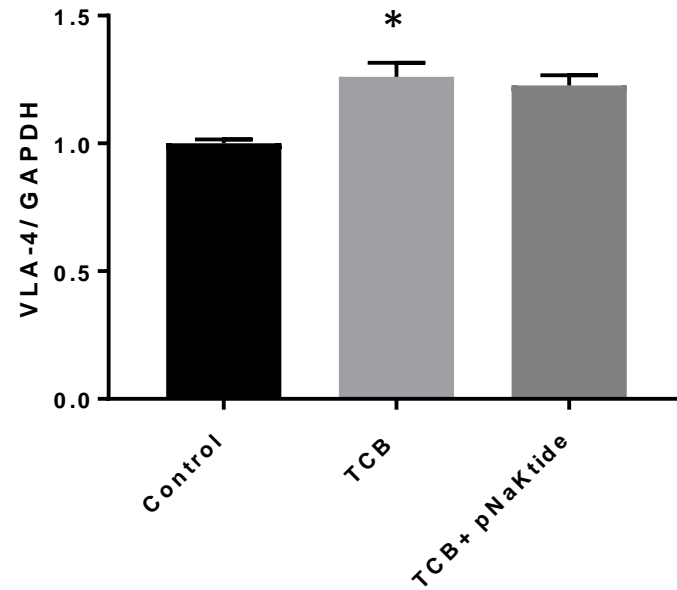
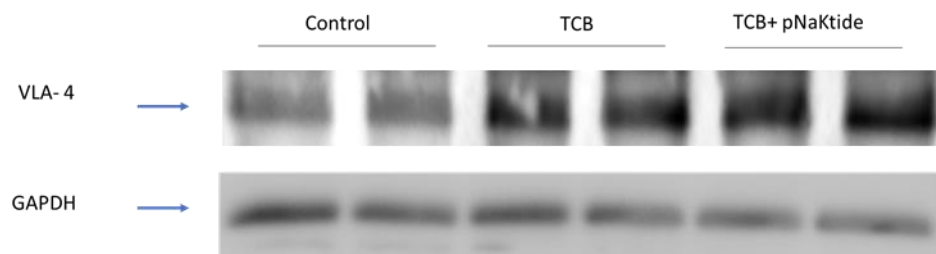


Figure S2. Telocinobufagin (TCB) (10 nM, 24hrs) induces the protein expression levels of the inflammatory adhesion molecules VLA-4 in human monocyte THP-1, TCB effect did not attenuate after treating with Src kinase inhibitor pNaKtide1 μ M. (Left) Western blot analysis showing protein expression levels and quantification (right). The data are presented as mean \pm SE from 4 separate experiments. * p <0.05 vs. control.

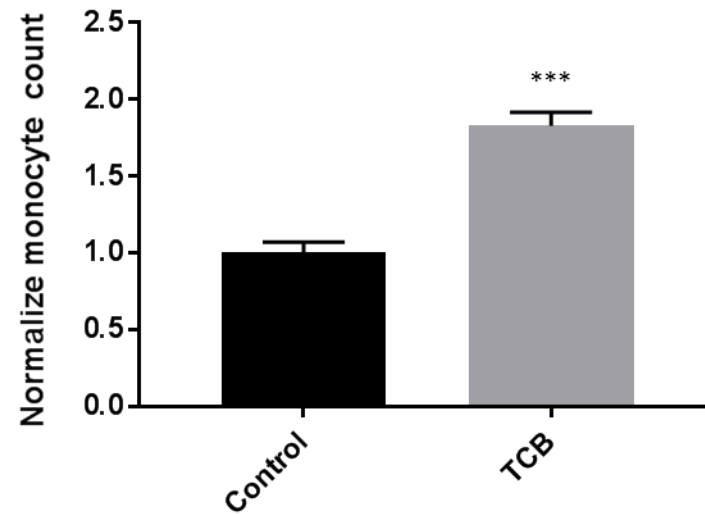
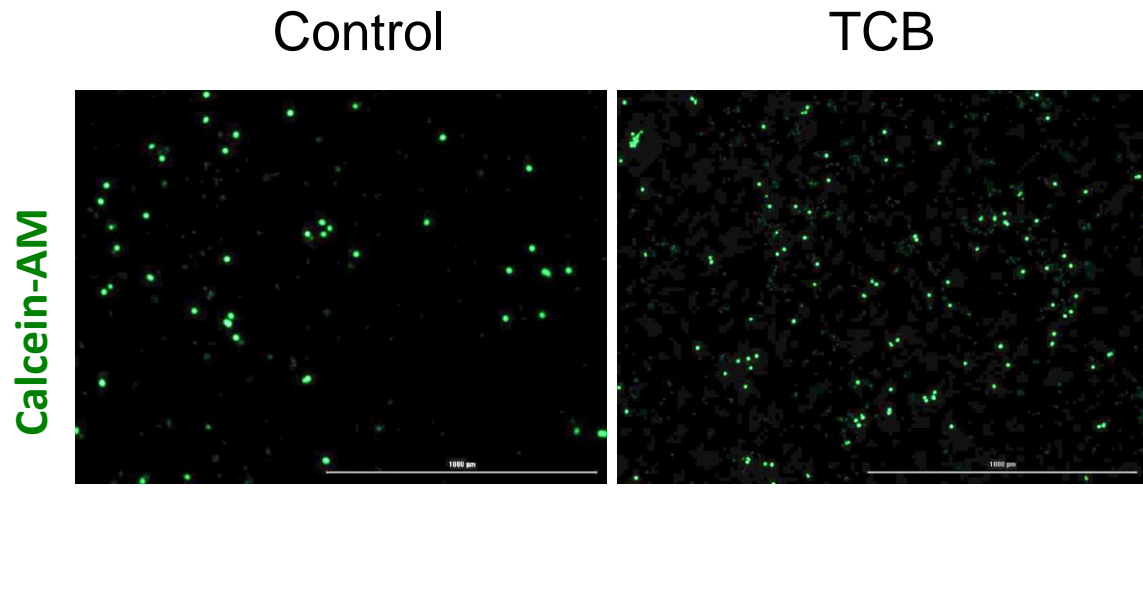


Figure S3. Telocinobufagin (TCB) (10 nM, 24 hrs) induces monocyte THP-1 interaction with endothelial cells monolayer HCAECS in vitro. The data are presented as mean \pm SE from 3 separate experiments. ** $p < 0.01$ vs. control.

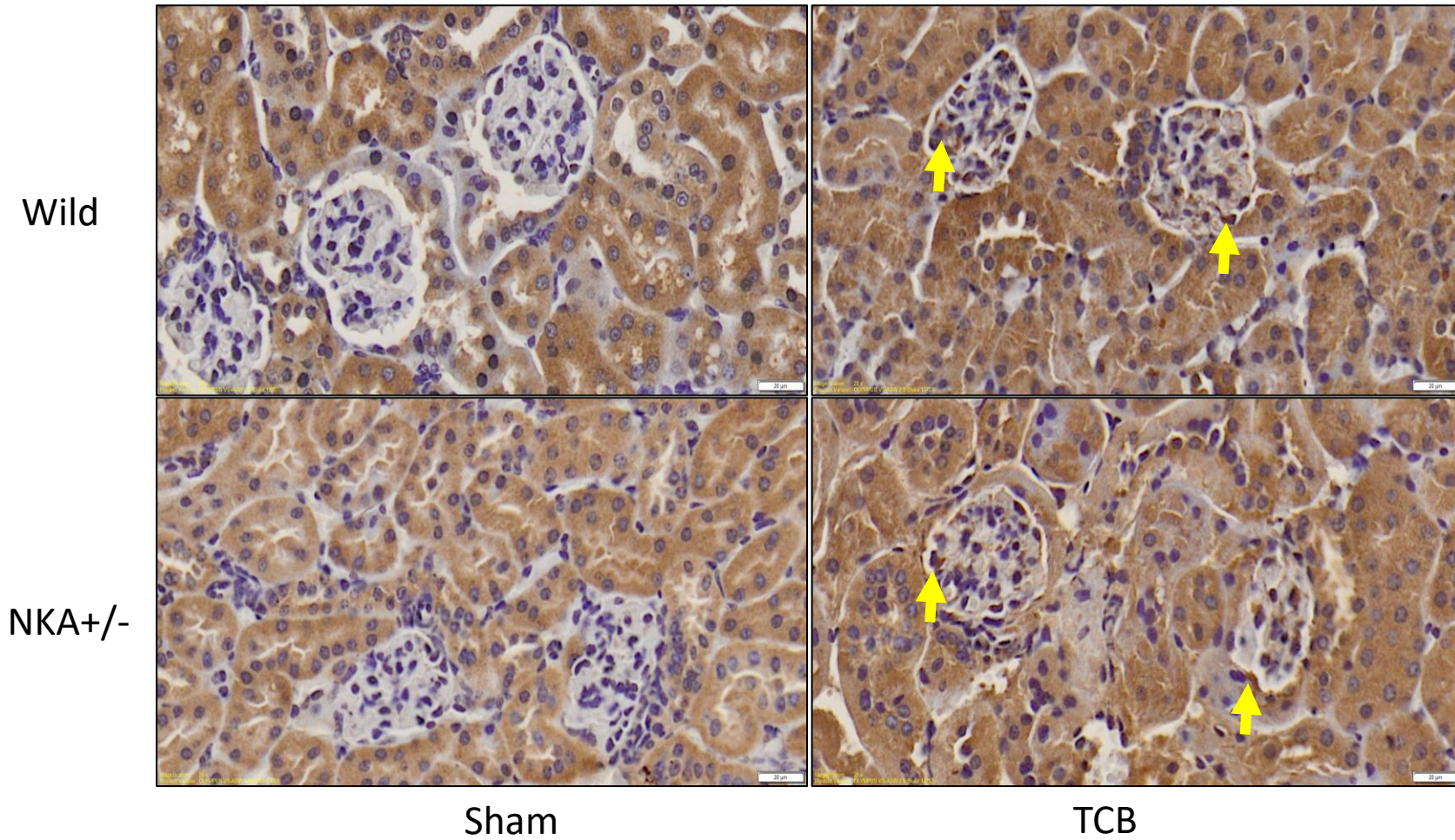


Figure S4 A. Telocinobufagin (TCB) administration for 4 wks induces CD3 positive immune cells infiltration in kidney in vivo. Representative histologic images in kidney from wild type NKA α -1 +/+ mice (above) and NKA α -1 +/- mice (below). (Left panel) vehicle treated mice, (right panel) TCB treated mice. TCB induced CD3 positive immune cells infiltration in wild type NKA α -1 +/+ mice just as in NKA α -1 +/- mice. The data are presented as mean \pm SE. from n=6 kidneys per group.

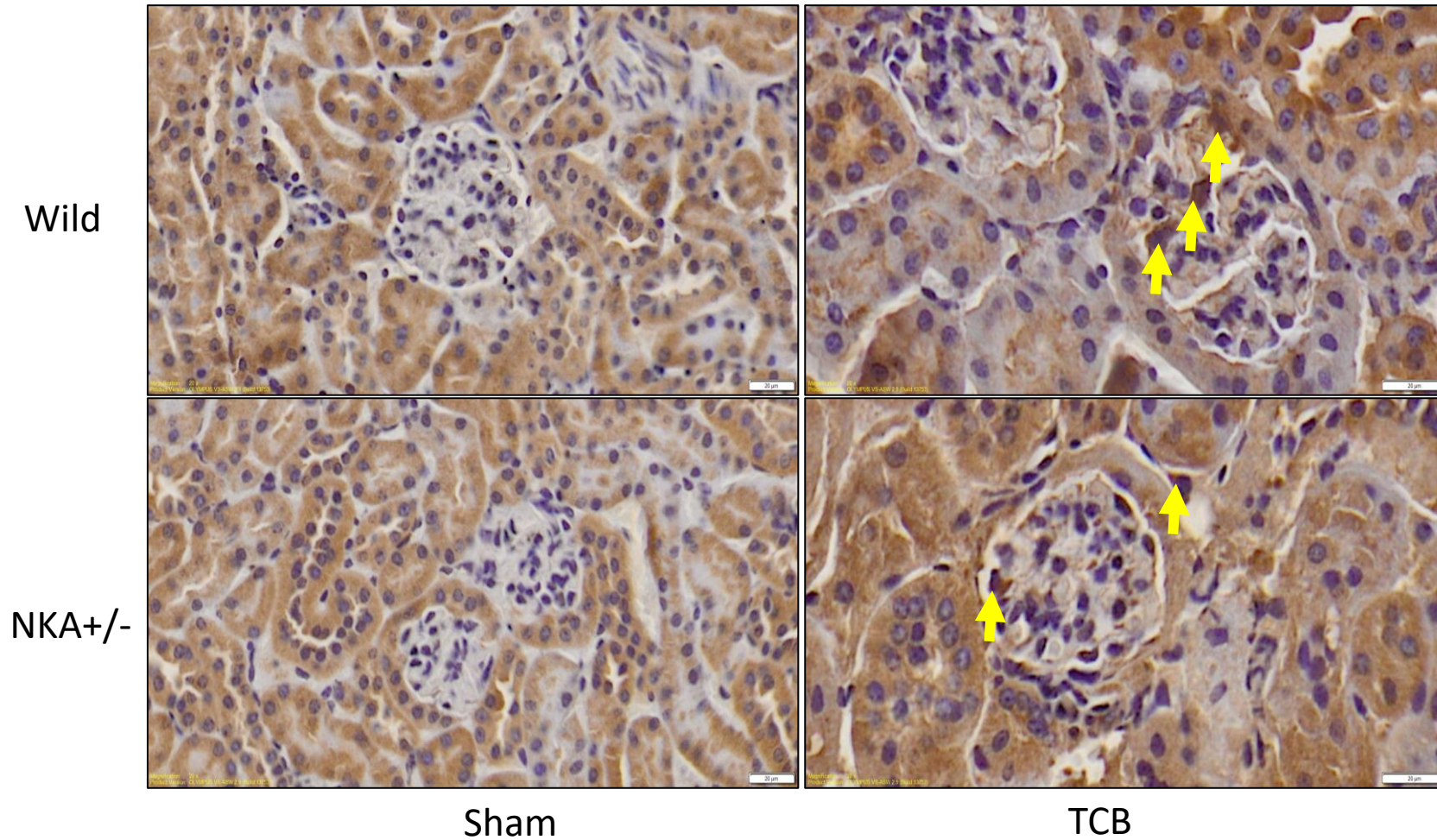


Figure S4 B. Telocinobufagin (TCB) administration for 4 wks induces CD11c positive immune cells infiltration in kidney in vivo. Representative histologic images in kidney from wild type NKA α -1 $+/+$ mice (above) and NKA α -1 $+/-$ mice (below). (Left panel) vehicle treated mice, (right panel) TCB treated mice. TCB induced CD11c positive immune cells infiltration in wild type NKA α -1 $+/+$ mice just as in NKA α -1 $+/-$ mice. The data are presented as mean \pm SE. from n=6 kidneys per group.

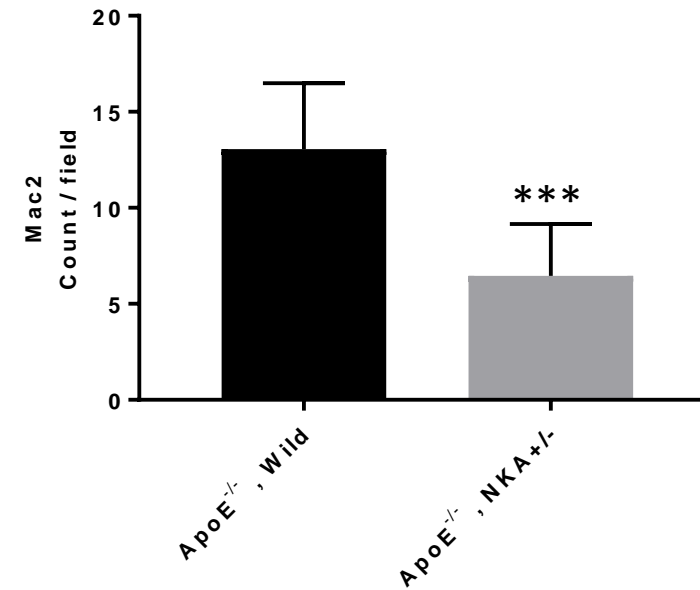
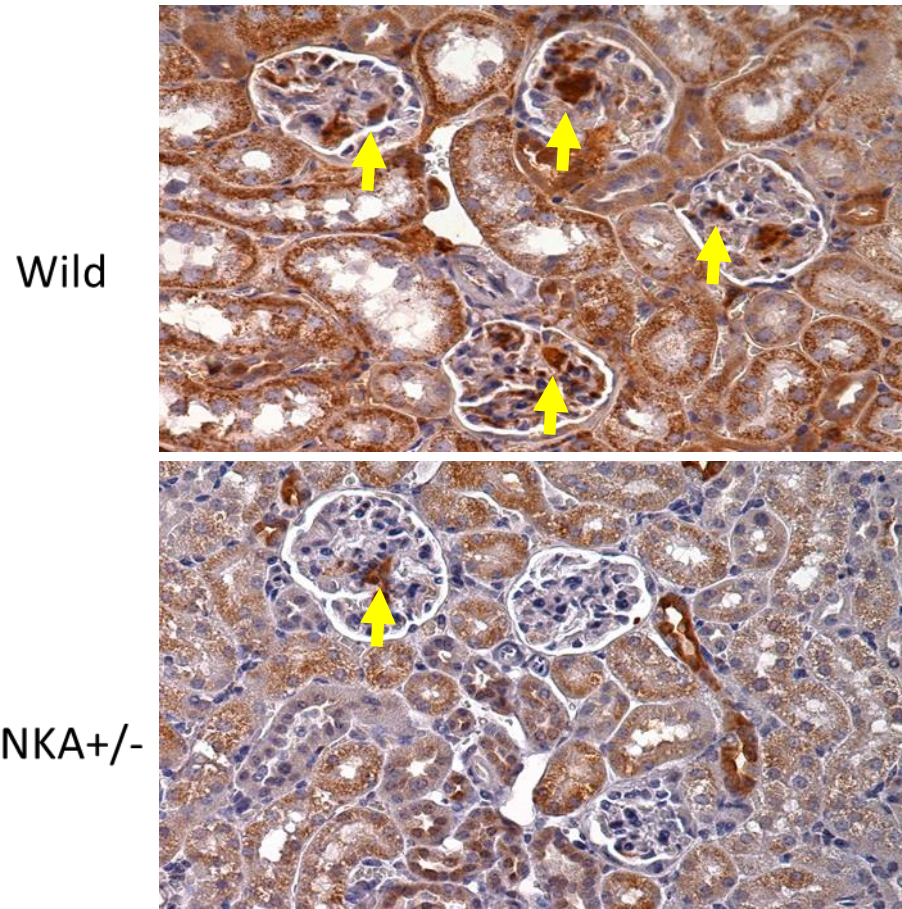


Figure S5 A. Reduction of bone marrow derived NKA leads to significant reduction in MAC2 positive immune cell infiltration in the kidney . (Left) Representative histologic images in kidney from mice injected with wild type NKA a1^{+/+} Bone marrow (above) or NKA a1^{+/-} Bone marrow (down), with quantification (right). The data are presented as mean \pm SE. from n=6 kidneys per group. **p<0.01

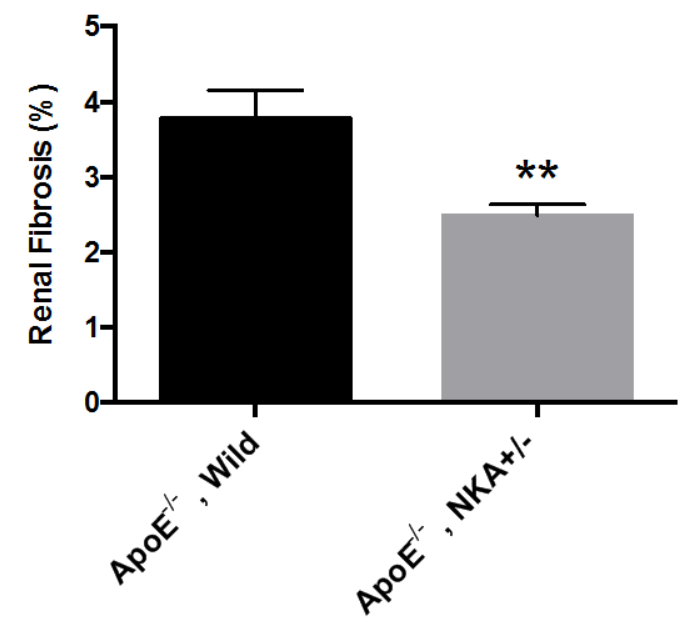
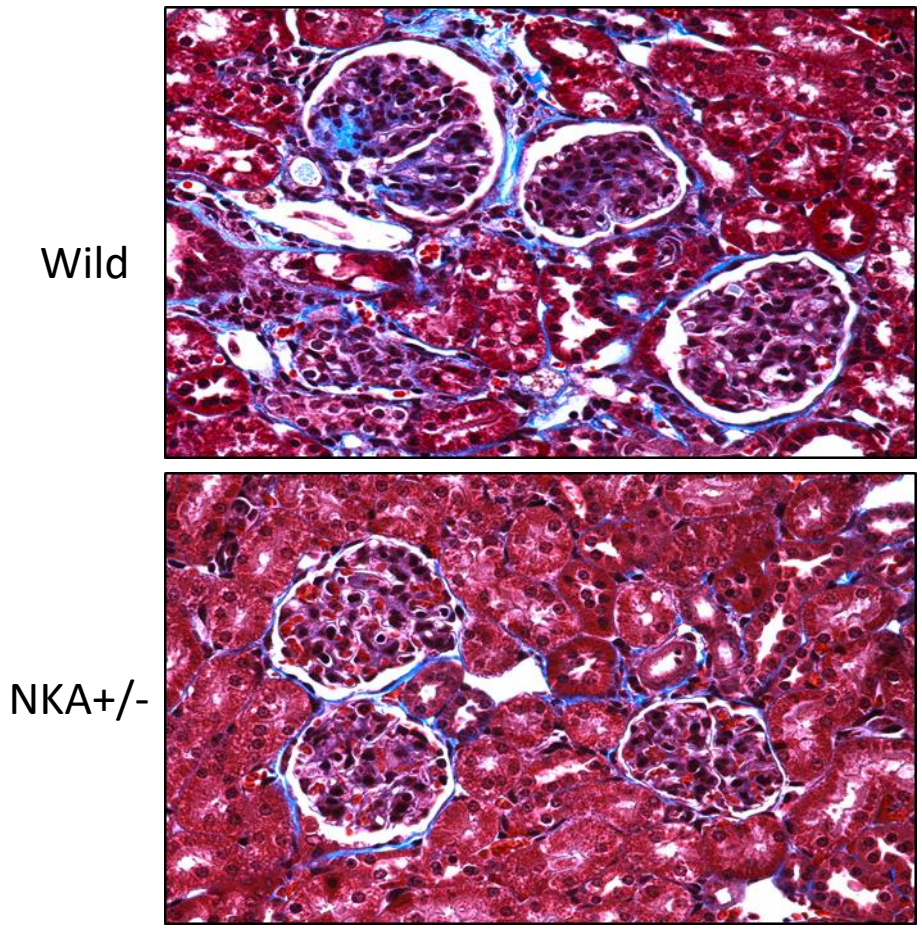


Figure S5 B. Reduction of bone marrow derived NKA leads to significant reduction in renal fibrosis. (Left) Representative histologic images in kidney from mice injected with wild type NKA a1^{+/+} Bone marrow (above) or NKA a1^{+/-} Bone marrow (down), with quantification (right). The data are presented as mean \pm SE. from n=6 kidneys per group. **p<0.01