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TITLE:

Identification of the Source of Secreted Proteins in the Kidney by Brefeldin A Injection

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SUMMARY:

Identifying the cell type responsible for secreting cytokines is necessary to understand the pathobiology of kidney disease. Here, we describe a method to quantitatively stain kidney tissue for cytokines produced by kidney epithelial or interstitial cells using brefeldin A, a secretion inhibitor, and cell-type-specific markers.

ABSTRACT:

Chronic kidney disease (CKD) is one of the top ten leading causes of death in the USA. Acute kidney injury (AKI), while often curable, predisposes patients to CKD later in life. Kidney epithelial cells have been identified as key signaling nodes in both AKI and CKD, whereby the cells can determine the course of the disease through the secretion of cytokines and other proteins. In CKD especially, several lines of evidence have demonstrated that maladaptively repaired tubular cells drive disease progression through the secretion of transforming growth factor-beta (TGF-β), connective tissue growth factor (CTGF), and other profibrotic cytokines. However, identifying the source and the relative number of secreted proteins from different cell types *in vivo* remains challenging.

This paper describes a technique using brefeldin A (BFA) to prevent the secretion of cytokines, enabling the staining of cytokines in kidney tissue using standard immunofluorescent techniques. BFA inhibits endoplasmic reticulum (ER)-to-Golgi apparatus transport, which is necessary for the secretion of cytokines and other proteins. Injection of BFA 6 h before sacrifice leads to a build-up of TGF-β inside the proximal tubule cells (PTCs) in a mouse cisplatin model of AKI and a mouse aristolochic acid (AA) model of CKD. Analysis revealed that BFA + cisplatin or BFA + AA increased TGF-β-positive signal significantly compared to BFA + saline, cisplatin, or AA alone. These data suggest that BFA can be used to identify the cell type producing specific cytokines and quantify the relative amounts and/or different types of cytokines produced.

INTRODUCTION:

It is estimated that >10% of the world's population have some form of kidney disease[1]. Defined by its rapid onset, AKI is largely curable; however, an episode of AKI can predispose patients to develop CKD later in life[2,3]. Unlike AKI, CKD is marked by progressive fibrosis and worsening kidney function, leading to end-stage renal disease requiring renal replacement therapy. Most injuries to the kidneys target the specialized epithelial cells, such as podocytes or proximal tubule cells, that make up the nephron[4,5]. Following injury, the surviving epithelial cells help coordinate the repair response through the secretion of cytokines and other proteins. In this way, the surviving cells can modulate the immune response, direct extracellular matrix remodeling, and aid organ recovery.

Cytokines are small, secreted proteins essential for modulating the maturation, growth, and responsiveness of multicellular organisms[6,7]. They function as signal messengers among various cell types, including immune and epithelial cells[8]. Although cytokines are thought to be secreted mainly by immune cells, long-standing research has demonstrated that kidney epithelial and interstitial cells also secrete cytokines as signals for other resident kidney cells, such as tubule cells, interstitial cells, and immune cells[9,10]. PTCs, in particular, play an important role in the initiation and recovery phase after AKI[11]. However, maladaptively repaired PTCs are known to secrete profibrotic cytokines such as transforming growth factor-β (TGF-β), platelet-derived growth factor-D (PDGF-D), and connective tissue growth factor (CTGF), contributing to CKD progression[12]. Thus, kidney epithelial cells use secreted cytokines to modulate kidney injury.

While it is known that kidney epithelial cells secrete cytokines, the exact source and relative contribution of each cell type have been difficult to determine due to the technical challenges of studying secreted proteins[13]. Flow cytometry, a common approach used to measure cytokines, is challenging to perform on injured kidneys, especially in highly fibrotic ones. With Cre recombinase driven by a cytokine promoter, cytokine reporter mice are often used to identify the cell type that expresses a given cytokine. However, the use of reporter mice is limited because of the requirement to cross reporter mice into various knockout backgrounds, the lack of suitable reporters, and the fact that only one cytokine can be analyzed at a time. Thus, it is necessary to develop a simple, versatile, and affordable technique for detecting cytokine-releasing kidney cells.

We hypothesized that injection of BFA, a secretion inhibitor that blocks endoplasmic reticulum–Golgi transport *in vivo* would allow the staining of secreted proteins in kidney tissue (**Figure 1A,B**), as shown with flow cytometry-based assays[14,15]. Along with cell-type-specific makers, this technique could be used to identify the source and relative contribution of cytokine-producing cells in injured kidneys. Unlike samples for flow cytometry, fixed tissues can be kept long-term with preservation of proteins and cellular structures, allowing for a more thorough investigation of the secretory cells. To test this hypothesis, mouse kidneys were injured with a model of AKI (cisplatin) and a model of CKD (aristolochic acid nephropathy (AAN)), injected with BFA, and stained using standard immunofluorescent techniques.

PROTOCOL:

All animal experiments were performed in accordance with the animal use protocol approved by the Institutional Animal Care and User Committee of Vanderbilt University Medical Center.

1. Animals

1.1. Use 8–12-week-old BALB/c male mice (body weight: ~ approximately 25 g) for cisplatin- or aristolochic acid-induced nephropathy.

1.2. Ensure the mice are healthy and have no obvious signs of distress or wounds from fighting.

NOTE: Wounds, especially to the tail, could interfere with the protocol described here. BALB/c mice were chosen because it is easier to visualize the tail vein for injection in these mice. The protocol described here works for other mouse strains; however, the dosage of cisplatin or aristolochic acid may differ from strain to strain.

2. Cisplatin injection

2.1. Dissolve cisplatin in sterile saline to a final concentration of 1 mg/mL.

NOTE: Cisplatin will not dissolve completely at room temperature. It should be handled in a fume hood.

2.2. Warm the cisplatin solution in a water bath at 37 °C and vortex repeatedly until the cisplatin has dissolved completely.

2.3. Weigh the mice and calculate the volume of cisplatin solution needed to inject 20 mg/kg body weight (bw).

2.4. Disinfect the abdominal skin using povidone-iodine (7.5%) and alcohol (70%) swabs alternating 3x each.

2.5. Using an insulin syringe with a 25 G needle, inject the cisplatin solution intraperitoneally.

2.6. Proceed to section 4 on day 3 after the injection.

3. Aristolochic acid (AA) injection

3.1. Dissolve aristolochic acid-I in phosphate-buffered saline (PBS) at a final concentration of 0.5 mg/mL.

NOTE: AA should be handled in a fume hood. AA-I should be used as it is the dominant form inducing kidney injury.

3.2. Warm the AA solution in a water bath at 37 °C and vortex repeatedly until dissolved completely.

3.3. Weigh the mice and calculate the volume of the AA solution to inject 5 mg/kg bw.

3.4. Disinfect the abdominal skin using povidone-iodine (7.5%) and alcohol (70%) swab 3x.

3.5. Using an insulin syringe with a 25 G needle, inject the AA solution intraperitoneally.

3.6. Inject AA every other day for a total of 3 injections.

3.7. Proceed to section 4 on day 42 after the last injection.

4. Preparation of BFA solution

4.1. Dissolve BFA in dimethylsulfoxide at a concentration of 20 mg/L to make a stock solution.

4.2. Store the stock solution at -20 °C.

4.3. Dilute the BFA stock solution with sterile PBS at the final working concentration of 1.25 mg/mL

NOTE: Prepare a fresh working solution each time immediately before the injection.

5. Tail vein injection of BFA

5.1. Prior to injection, put the cage half on, half off the heating pad for 10 min to ensure the mice are warm to prevent a drop in body temperature, which can cause vasoconstriction of vessels in the tail and interfere with the injection.

NOTE: Place the cage on the heating pads so that half of the cage is on the pad while half is not. That way, when the mice feel warm, they can move to the other side of the cage and vice versa.

5.2. Restrain the mice using commercially available restraint devices of appropriate size.

5.3. Disinfect the tail using povidone-iodine and alcohol swab three times as described above.

5.4. Hold the tail horizontally and visualize the lateral tail veins (**Figure 1C**). Use a light source under the tail to help visualize the veins.

5.5. Insert a 28 G needle, keeping the needle and syringe parallel to the vein towards the direction of the head.

5.6. Inject 200 µL of the 1.25 mg/mL BFA solution (0.25 mg BFA). Wait for the vein to become clear as the blood is replaced with the injection solution, indicating that the injection was successful.

5.7. Remove the needle and press the tail gently until the bleeding stops.

5.8. Return the mice to the cage and monitor them for additional bleeding or signs of distress.

6. Sacrifice and harvest of the kidneys

6.1. Euthanize the mice with an overdose of isoflurane followed by cervical dislocation 6 h after BFA injection.

NOTE: The 6-h time point was chosen based on literature demonstrating that 6 h of BFA treatment in other organs allows for enough accumulation of cytokines within cells to visualize them by immunofluorescent staining[16].

6.2. Immediately after sacrifice, expose the abdomen and heart of the mouse by a ventral midline incision.

6.3. Collect 100–500 µL of blood for a blood urea nitrogen (BUN) assay by cardiac puncture. Use 25 G needles with 1 mL insulin syringes to collect the blood. To prevent coagulation, add 5 µL of heparin solution (100 mg/15 mL of dH_2O) to each sample.

NOTE: A minimum of 20 mL of blood is needed for the BUN assay; however, approximately 500 mL can be collected by cardiac puncture after euthanasia, which could be useful for other assays. Collect as much blood as possible.

6.4. Store the blood samples on ice until the kidneys are collected in section 7 below.

6.4.1. Centrifuge the blood samples at 1,300 × *g* for 10 min.

6.4.2. Isolate the plasma gently and store it at -20 °C until ready to perform the BUN assay in section 17 below. Alternatively, store the plasma samples for creatinine assays.

7. Perfusion and removal of the kidneys

7.1. Perfuse the mouse with 10–20 mL of PBS through the left ventricle using a 20 mL syringe at a flow rate of 2–4 mL/min until the perfusate becomes clear.

7.2. Remove the kidneys by holding the renal artery and vein close to the papilla and cutting the vessels on the side away from the kidney.

7.3. Gently remove the kidney capsule by peeling it off by hand or with a pair of fine, sterile

forceps.

7.4. Depending on the antibody, proceed to section 8 for preparation of fixed paraffin-embedded tissue or section 10 for preparation of fixed frozen tissue.

NOTE: Tissue processing will need to be optimized for each antibody to be used for staining.

8. Paraffin-embedded tissue for TGF-β and PDGF-D staining

8.1. Bisect the kidney by placing it on a clean glass slide and cutting it horizontally with a new razor blade. Place it in 10 mL of 4% paraformaldehyde (PFA) in PBS for 24 h on an end-over-end rotator at a speed of 10 rotations per min (rpm).

NOTE: The whole kidney is not needed for sectioning. One half of the kidney can be stored or used for other assays.

8.2. Replace the PFA with 70% ethanol.

8.3. Submit half of the kidney for processing and paraffin-embedding at this stage or section the paraffin-embedded kidney tissues at 4–6 µm using a microtome and mount them on precleaned, charged slides[17,18].

NOTE: Kidneys were processed and embedded in paraffin by the Vanderbilt University Medical Center Translational Pathology Shared Resource and stored at room temperature.

9. Deparaffinization and rehydration

9.1. Place the slides into a slide-staining rack and dunk them into a staining well containing D-limonene for 5 min, ensuring that the tissue is completely submerged. Repeat in a well containing fresh D-Limonene.

9.2. Rehydrate the tissues by dunking the slides in serial dilutions of ethanol 100% (2x), 95%, 90%, and 70% for 5 min each.

9.3. Wash the slides in flowing dH_2O for 5 min.

9.4. Perform antigen retrieval by incubating the sections in citrate buffer (pH 6.0) in a pressure cooker at 121 °C and 15 psi for 45 min.

9.5. Wash the slides in flowing dH_2O for 20 min.

9.6. Proceed to section 12.

10. Preparation of frozen tissue for CTGF staining

10.1. Bisect the kidney along the horizontal axis using a fresh razor blade.

NOTE: The whole kidney is not needed for sectioning. One half of the kidney can be stored or used for other assays.

10.2. Put half of the kidney in 10 mL of 0.5% PFA in a 15 mL tube on a rotator for 2 h at 4 °C at a speed of 10 rpm.

10.3. Decant the PFA into a container for proper disposal and add 10 mL of 0.1 M glycine in PBS for 1 h at 4 °C at a speed of 10 rpm.

10.4. Decant the glycine, add 10 mL of 15% sucrose dissolved in PBS, and place the tube on a rotator overnight at 4 °C and 10 rpm.

10.5. Decant the 15% sucrose and replace with 10 mL of 30% sucrose dissolved in PBS for 1 h at 4 °C and 10 rpm.

10.6. Fill the embedding mold with optimal cutting temperature compound (OCT) and embed the half of the kidney from step 10.5 with the cut surface of the kidney facing down.

10.7. Place the mold containing the half-kidney and OCT carefully in a pool of liquid nitrogen to freeze.

NOTE: Do not let the liquid nitrogen directly contact the OCT as this can result in bubble formation. Proper personal protective equipment must be worn when using liquid nitrogen, such as goggles/face shield, cryogenic gloves, and lab coat. It is best to use long, ~25 cm, forceps to place the molds in liquid nitrogen.

10.8. Once frozen solid, store the mold at -80 °C.

11. Frozen sectioning

11.1. Ensure the cryostat is at -20 °C.

11.2. Store molds containing specimens in OCT in the cryostat at -20 °C for 2 h to equilibrate the temperature.

11.3. Remove the mold by holding the tabs and pressing from the bottom.

11.4. Put fresh OCT onto a specimen holder and place the frozen specimen block on top, with the tissue side facing away from the specimen holder.

11.5. Place the specimen holder and specimen on the freezing shelf.

11.6. Place a weighted heat extractor on the top of the block to flatten the surface. Keep the cryostat cover closed when not in use to prevent temperature fluctuations.

11.7. Once the fresh OCT between the specimen block and specimen holder is frozen, check to ensure the connection is secure.

11.8. Clamp the specimen holder onto the cryostat microtome head.

11.9. Begin sectioning until the tissue is visible in the specimen block.

11.10. Section the kidney tissue at 4–6 mm and pick the sections up onto a room temperature-charged slide[19].

11.11. Once the tissue is picked up, store the slide at -20 °C to -80 °C until ready to stain. Do not allow it to thaw until ready to begin staining.

11.12. Prior to staining, remove the slide(s) from storage and allow to warm to room temperature. Do not allow the sections to dry.

11.13. Once at room temperature, immediately wash the sections with PBS for 5 min twice at room temperature to eliminate the OCT compound.

11.14. Proceed to section 12.

12. Immunofluorescence staining

12.1. Outline the tissue sections with a hydrophobic barrier marker pen. Maintain at least 5 mm distance from the tissue to the hydrophobic barrier outline.

12.2. Add 50 mL of blocking buffer containing 3% donkey serum and 0.1% Triton in 1% bovine serum albumin/Tris-buffered saline (TBS) on top of the section and incubate for 1 h at room temperature in a humidified chamber.

12.3. Dilute the primary antibodies with PBS at the appropriate concentration. For detection of cytokines, use 50 mL of solutions of primary antibodies directed against TGF-β1 (1:200), PDGF-D (1:400), and CTGF (1:200). For labeling myofibroblasts, use 50 mL of a solution of the primary antibody directed against alpha-smooth muscle actin (α-SMA) conjugated with Cy3 at 1:200.

12.4. Remove the blocking solution and add the primary antibodies to the section, ensuring it does not leak out of the circular hydrophobic outline and incubate overnight at 4 °C in a humidified chamber. Reapply the hydrophobic barrier if leakage occurs.

12.5. Wash 3x with PBS for 5 min.

12.6. Dilute the appropriate secondary antibodies at 1:200 with PBS.

12.7. Incubate the samples with 50 mL of secondary antibody solutions for 1 h at room temperature in a humidified chamber.

12.8. Wash with PBS for 5 min.

12.9. Dilute lotus tetragonolobus lectin (LTL) conjugated with fluorescein in PBS with Ca^{2+} and Mg^{2+} at the concentration of 1 mg/mL.

NOTE: $Ca^{2+}$and Mg^{2+} are necessary for LTL binding.

12.10. Incubate the tissues with 50 mL of LTL solution for 30 min at room temperature in a humidified chamber.

12.11. Wash with PBS with Ca^{2+} and Mg^{2+} for 5 min.

12.12. Incubate with 50 mL of 4',6-diamidino-2-phenylindole (DAPI, 5 mg/mL in water) to stain DNA/nuclei for 5 min at room temperature.

12.13. Mount the coverslips by adding 20 mL of antifade mounting reagent on the tissue and slowly placing the coverslip. Wait for 24 h for the antifade reagent to solidify before imaging.

NOTE: Lectin binding can degrade over time, resulting in a loss of signal. It is best to image lectin-stained samples soon after the mounting reagent is set up. If loss of signal is observed, Ca^{2+} and Mg^{2+} can be added to the mounting reagent to preserve the staining.

13. Image acquisition

13.1. Turn on the inverted microscope (see the **Table of Materials**) with an automated XY stage.

13.2. Select the 20x objective.

13.3. Open the image acquisition software (see the **Table of Materials**).

13.4. Click on **Live** to open the live view window.

13.5. Find the tissue section and ensure it is in focus.

13.6. Click on the **Acquire** menu and select **Scan Large Image**.

13.7. In the **Scan Large Image** window, set up the area to scan by moving the stage using the joystick to the leftmost part of the tissue section and click the **left arrow**. Repeat for uppermost, rightmost, and bottom tissue segments.

13.8. Click on the **acquisition** menu.

13.9. Ensure that the checkbox for **large image** is **checked**.

13.9.1. If capturing multichannel images, click on the **Lambda** tab.

13.9.2. Click on each **channel** and set the **exposure time** to a level where the staining is apparent without saturation of any part of the image.

NOTE: These settings need to be consistent within one experimental group. Changing acquisition settings between samples will lead to inaccurate results.

13.10. Repeat for each channel to be collected.

13.11. Click on **Run Now**.

14. Image analysis

14.1. Open the image acquisition software.

14.2. Click **File | Open** and select the **image**.

14.3. Right-click on the image window and choose **polygonal region-of-interest (ROI)**.

14.4. Outline the ROI with the **freehand** tool. Outline the LTL-positive tubule cells or α-SMA-positive interstitial cells.

14.5. Click on the **Analysis** tab **| Threshold**.

14.6. Set up the upper and lower limits of the threshold by adjusting the sliders to either side of the positive signal area.

14.7. Click on the **ROI** tab.

14.8. Click on the **Export** icon to save the values. Use spreadsheet software to calculate the percentage of positive signal area/ROI area.

15. Alternative: Image analysis with free software (ImageJ)

15.1. Open **ImageJ**.

15.2. Click **File | Open** to view an image.

15.3. Click **freehand selections**.

15.4. Select the **ROI** by outlining with the **freehand** tool. Outline the LTL-positive tubule cells or α-SMA-positive interstitial cells.

15.5. Click the **Edit** menu and select **Clear outside**.

15.6. Click **Analyz**e and select **Measure** to determine the area of ROI.

15.7. Go to **Image | Color | Split Channels**.

15.8. Adjust the upper and lower limits of the threshold to detect the positive signal area.

15.9. Click **Analyze** and select **Measure**.

15.10. Save the data.

15.11. Open the data with spreadsheet software and calculate the ratio of positive signal area/ROI area.

16. Optional: Imaging with laser scanning confocal microscope

NOTE: To obtain higher resolution images for publication, scanning confocal microscopy provides clearer images and reduced background in kidney tissue.

16.1. Turn on the laser scanning confocal microscope (see the **Table of Materials**).

16.2. Select 40x objectives (see the **Table of Materials**).

16.3. Click **Locate** tab and find the tissues by adjusting the focus.

16.4. Go to the **Acquisition** tab, click **Channels**, and choose **1 AU** in **pinhole setting**.

16.5. Adjust the laser gain intensity in each channel such that the positive signal is visible but not saturated. Ensure that settings within a set of experimental samples remain constant.

16.6. Click **Snap** and save the image in the desired format.

17. Plasma BUN level

17.1. Remove the samples from -20 °C and thaw them on ice.

17.2. Dilute the plasma with dH_2O in a 1:10 ratio.

17.3. For each sample, set up three separate reactions in duplicate in a 96-well plate.

17.3.1. For sample plus standard, add 5 µL of 200 mg/dL urea and 20 µL of diluted plasma.

17.3.2. For sample alone, add 5 µ L of dH_2O and 20 µL of diluted plasma.

17.3.3. For sample blank, add 5 µL of dH_2O and 20 µL of diluted plasma.

17.4. Mix 85 µL of the reagent + 1 µL of urease per sample to prepare the working solution.

17.5. Add 80 µL of the working solution to the 'sample plus standard' and 'sample alone' wells.

17.6. Add 80 µL of the reagent (no urease) to the 'sample blank' well from step 17.3 above.

17.7. Tap the plate gently to mix and incubate it for 5 min at room temperature.

17.8. Read OD_{560} with a plate reader.

17.9. Calculate the urea concentration and convert it to BUN using Eq (**1**).

BUN (mg/dL) = Urea concentration/2.14 (**1**)

NOTE: This assay measures the urea (molecular weight: 60) content of serum; however, BUN references only the nitrogen content of urea (molecular weight: 28). Thus, a correction of 2.14 (60/28) is needed to convert urea concentration to BUN.

REPRESENTATIVE RESULTS:

To examine the role of tubular epithelial cells in cytokine production following cisplatin-induced AKI, cisplatin was injected at a concentration of 20 mg/kg followed by an intravenous injection of 0.25 mg of BFA on day 3 after the cisplatin injection. The kidneys were harvested 6 h later. Paraffin-embedded kidneys were sectioned and stained with TGF-β, PDGF-D, and CTGF, representative cytokines responsible for tissue repair in AKI. As shown in **Figure 2A**, TGF-β^+ vesicles are observed in PTCs labeled with LTL in cisplatin-treated kidneys in the presence of BFA. Meanwhile, TGF-β^+ vesicles were not observed in uninjured or BFA-untreated kidneys.

Quantification revealed an increase in TGF-$\beta1^+$ area with BFA treatment in cisplatin-induced AKI (**Figure 2B**). Similar to TGF-β, PDGF-D^+ or $CTGF^+$ vesicles also accumulated with BFA treatment in cisplatin-induced AKI (**Figure 2C** and **Figure 2E**). Quantification demonstrated that PDGF-D^+ and $CTGF^+$ areas were significantly increased with BFA in LTL^+ PTCs (**Figure 2D** and **Figure 2F**). To study the effect of BFA treatment on renal function, plasma BUN levels were measured on day 3 after cisplatin injection. As shown in **Figure 2G**, BFA injection did not significantly increase plasma BUN

levels.

Next, to investigate which cytokines are secreted by interstitial cells in cisplatin-induced AKI, the kidneys were stained for TGF-β or CTGF, and interstitial myofibroblasts were labeled by α-SMA. As shown in **Figure 3A**, TGF-β^+ vesicles were observed in α-SMA-labeled interstitial cells in cisplatin-AKI with BFA treatment (**Figure 3A**). To quantify the signal within the α-SMA^+ cells, the α-SMA^+ area was outlined and the positive cytokine and α-SMA signals were quantified inside the area. Quantification revealed that the TGF-β^+ area in the α-SMA^+ area was significantly increased with BFA treatment in cisplatin AKI (**Figure 3B**). $CTGF^+$ vesicles also increased with BFA treatment (**Figure 3C**), and quantitation demonstrated that BFA treatment enhanced the ratio of $CTGF^+$ area/α-SMA^+ area in cisplatin-induced AKI (**Figure 3D**).

To determine whether BFA treatment can be used in chronic kidney injury models, kidney injury was induced via three doses of AA, which induces AKI that develops into a chronic injury with mature renal fibrosis and is clinically relevant to human CKD. BFA was injected on day 42 after AA injection, and the kidneys were harvested 6 h later. Compared to cisplatin-induced injury, TGF-β^+ vesicles in PTCs were much smaller in the chronic phase of AA. There was minimal positive staining in LTL^+ PTCs; however, the signal intensity of TGF-β in kidney injury molecule-1-positive (KIM-1^+) PTCs was increased with BFA treatment (**Figure 3E**). The mean intensity level of TGF-β was 3 times higher with BFA injection than without BFA (**Figure 3F**). This finding indicates that *in vivo* BFA injection can be used for immunofluorescence staining and in combination with other markers to evaluate cytokine production in a cell-type-specific manner.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of BFA mode of action. (**A**) BFA blocks ER-to-Golgi transport of vesicles containing proteins to be secreted, such as TGF-β. (**B**) BFA induces a build-up of intracellular cytokines, such as TGF-β, in kidney tubular epithelial cells. (**C**) Schematic cross-section of a mouse tail. The lateral veins are the most accessible for injection. Abbreviations: BFA = brefeldin A; ER = endoplasmic reticulum; TGF-β = transforming growth factor-beta; BFA- = BFA-negative; BFA+ = BFA-positive.

Figure 2: Immunofluorescence with BFA injection detects cytokine-rich vesicles in tubular epithelial cells in cisplatin-induced acute kidney injury. (**A**) Representative images of TGF-β^+ vesicles on day 3 after cisplatin (20 mg/kg) or saline administration. Scale bar = 20 μm. Arrows indicate TGF-β^+ vesicles in PTCs. (**B**) Quantification of TGF-β^+ vesicles/tubules in saline (n = 5), saline + BFA (n = 5), Cis (n = 5), Cis + BFA (n = 5). (**C**) Representative images of PDGF-D-vesicles on day 3 after cisplatin (20 mg/kg) or saline administration. Scale bar = 20 μm. (**D**) Quantification of PDGF-D^+ vesicles/tubules in saline (n = 4), saline + BFA (n = 4), Cis (n = 4), Cis + BFA (n = 4). (**E**) Representative images of $CTGF^+$ vesicles on day 3 after cisplatin (20 mg/kg) or saline administration. Scale bar = 20 μm. (**F**) Quantification of $CTGF^+$ vesicles/tubules in saline (n = 4), saline + BFA (n = 4), Cis (n = 4), Cis + BFA (n = 4). (**G**) Plasma BUN level on day 3 after cisplatin (20 mg/kg) administration: Cisplatin (Cis) (n = 7) and cisplatin + BFA (Cis + BFA) (n = 3). Data are presented as means ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Regions bounded by dashed white boxes are shown in higher magnification inset panel. Abbreviations: BFA =

brefeldin A; TGF-β = transforming growth factor-beta; Cis = cisplatin; PDGF-D = platelet-derived growth factor-D; CTGF = connective tissue growth factor; BUN = blood urea nitrogen; LTL = lotus tetragonolobus lectin; DAPI = 4',6-diamidino-2-phenylindole; HM = High magnification.

Figure 3: Cytokine-rich vesicles observed in myofibroblasts in cisplatin-induced acute kidney injury and tubular epithelial cells in chronic phase of aristolochic acid nephropathy. (**A**) Representative images of TGF-β^+ vesicles on day 3 after cisplatin (20 mg/kg) or saline administration. Scale bar = 20 µm. Arrows indicate TGF-β^+ vesicles in a-SMA$^+$ interstitial cells. (**B**) Quantification of TGF-β^+ area/α-SMA$^+$ area in saline (n = 4), saline + BFA (n = 4), Cis (n = 4), Cis + BFA (n = 4). (**C**) Representative images of CTGF$^+$ vesicles on day 3 after cisplatin (20 mg/kg) or saline administration. Scale bar = 20 µm. Arrows indicate CTGF$^+$ vesicles in a-SMA$^+$ interstitial cells. (**D**) Quantification of CTGF$^+$ area/α-SMA$^+$ area in saline (n = 4), saline + BFA (n = 4), Cis (n = 4), Cis + BFA (n = 4). (**E**) Representative images of TGF-β-stained (red) kidney in chronic aristolochic acid nephropathy. Scale bar = 20 µm. (**F**) Corresponding data of TGF-β^+ signal intensity / KIM-1$^+$ PTCs in AAN (n = 6) and AAN + BFA (n = 6). Data presented as means ± SD. * $p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. Regions bounded by dashed white boxes are shown in higher magnification inset panel. Abbreviations: BFA = brefeldin A; TGF-β = transforming growth factor-beta; α-SMA = alpha-smooth muscle actin; Cis = cisplatin; CTGF = connective tissue growth factor; KIM-1 = kidney injury molecule-1; LTL = lotus tetragonolobus lectin; AAN = aristolochic acid nephropathy; DAPI = 4',6-diamidino-2-phenylindole; HM = high magnification.

DISCUSSION:

Kidney PTCs are known to regulate AKI and CKD through the secretion of TGF-β, TNF-α, CTGF, PDGF, vascular endothelial growth factor, as well as many other proteins[20-23]. Similarly, glomeruli, distal tubules, and other kidney epithelial cells, as well as interstitial cells, secrete these and/or other proteins during injury[24-26]. The relative contribution of each of these cell types in cytokine secretion is difficult to elucidate as cytokines are secreted shortly after they are produced. While *in situ* hybridization and other RNA staining techniques can be used to stain the RNA of secreted proteins, it is difficult to combine these techniques with staining for cell-type-specific markers or injury markers. Alternatively, many studies combine *in vivo* findings with *in vitro* experiments performed in cultured kidney cells. In this case, kidney injury data are associated with cytokine secretion data from cultured cells to draw conclusions, which has limited translatability to the *in vivo* situation.

The advent of next-generation sequencing and single-cell RNAseq has enabled the classification of cell types by gene expression and identification of other genes expressed by each cell type[27]. While this provides exquisite detail on a population basis, it often leaves out much of the anatomical and pathological data that can be gleaned from kidney sections. In addition, single-cell sequencing often only identifies the top 3,000–7,000 genes expressed in each cell, which may not provide enough depth to screen all the cytokines of interest[28]. This paper offers an alternative to these approaches. Using BFA to block protein secretion, kidney tissue can be directly stained for cytokines and other proteins of interest, including cell type markers using standard immunofluorescent techniques.

TGF-β, PDGF-D, and CTGF are among the most widely studied cytokines in kidney injury. All three are known to act as double-edged swords, promoting recovery following AKI while contributing to fibrosis progression in CKD[29,30]. While it is known that kidney tubule epithelial cells and interstitial cells can potentially secrete TGF-β, PDGF-D, and CTGF in kidney injury, directly identifying which cell type and the relative production remains challenging. In the current study, we chose to stain TGF-β, PDGF-D, and CTGF in kidneys during cisplatin-induced AKI, and TGF-β in the AAN CKD model, along with the PTC marker LTL, PTC injury marker KIM-1, or the myofibroblast marker α-SMA. This will allow the determination of whether PTCs or myofibroblasts produce these cytokines and the relative expression levels between experimental groups.

In mice treated with BFA alone, there was little to no positive staining of either cytokine. Likewise, cisplatin treatment only induced a marginal increase in cytokine staining. The combination of cisplatin injury with BFA treatment for 6 h resulted in a dramatic increase in intracellular positive signals of all three cytokines. Similarly, only the cisplatin + BFA group showed a significant increase of TGF-β and CTGF signal in α-SMA$^+$ interstitial cells. In the chronic AAN model, in which TGF-β is associated with a pathological response, AA injection alone induced some TGF-β staining, particularly in injured KIM-1$^+$ PTCs. BFA treatment increased the positive intracellular signal in KIM-1$^+$ PTCs (PTCs are the only kidney tubule cells known to express KIM-1[31]). Interestingly, LTL$^+$ KIM-1$^+$ or LTL$^+$ KIM-1$^-$ PTCs did not show significant TGF-β staining, whereas LTL$^-$ KIM-1$^+$ PTCs had more TGF-β positivity. The loss of LTL staining suggests that the PTCs expressing higher levels of TGF-β are both injured and dedifferentiated in the chronic phase of injury. This phenotype is likely like the maladaptive repair described previously[32,33]. Thus, BFA treatment demonstrates that TGF-β is expressed in PTCs following AKI and during CKD.

While the current study is focused on immunofluorescent staining, it is important to note that BFA treatment can be combined with other assays. For instance, if the experiment does not call for identifying the cell type secreting the protein of interest, one could inject BFA and perform an immunoblot or ELISA on whole kidney lysates in control versus experimental groups. Another assay that could be considered is flow cytometry. BFA treatment has been combined with flow cytometry in immunological studies to determine the relative production of cytokines in different cell types[15]. While separating kidney epithelial cells from kidney tissue into single cells can be challenging, flow cytometry may be an alternative in laboratories that have the technique established. Additionally, BFA treatment can be used for *in vitro* cell culture studies in all the assays listed above.

The current study outlines two of the most common tissue preparation methods, fixed paraffin-embedded tissue and frozen tissue, that work well for the antibodies used in the study. Given the variable nature of antibodies, however, it is likely that researchers adopting this technique will be required to standardize staining protocols or tissue processing further, depending on the antibody. As cytokines are normally secreted, few anticytokine antibodies are tested for staining in tissue. To expedite the standardization of the protocol, we recommend generating positive controls in which organs known to secrete the cytokines of interest are harvested post BFA treatment. For instance, spleens from lipopolysaccharide- and BFA-treated animals could serve

as positive control tissue for many different cytokines. Harvesting spleens and processing as paraffin-embedded or frozen tissue would allow for quicker standardization of antibody concentration and buffers. Moreover, if the anticytokine antibodies have been characterized to stain cytokines in their native conformation *in vitro*, these antibodies are more likely to recognize their target in the less-processed frozen tissue.

While BFA treatment can be a powerful tool when measuring secreted proteins, it is not without limitations. The most apparent limitation is that the secreted protein being analyzed must follow the ER–Golgi secretion route; otherwise, BFA may have limited effect. Another limitation is that animals treated with BFA may have altered results in other experimental assays. For instance, some kidney injury biomarkers, such as KIM-1, rely on ER–Golgi transport to be presented on the cell surface. Thus, KIM-1 levels in the urine will likely be reduced in BFA-treated animals. Additionally, there is a concern that BFA may lead to increased cellular stress. While this concern can be mitigated by reducing the time of BFA treatment, it should be a consideration. No significant increase in BUN was observed in this study; however, there was ~10% increase. Thus, care must be taken while deciding other targets or markers to be analyzed in BFA-treated animals.

This study demonstrates that BFA can be utilized to block the secretion of proteins, leading to the intracellular build-up of cytokines, which can be stained by standard immunofluorescence techniques. This enables the identification of the cell types secreting specific cytokines or other proteins, and the quantification of cytokine production by these cells. The other advantage of this protocol is that histological and pathological data can be preserved and analyzed in the same or neighboring serial sections. Thus, BFA treatment provides a cost-effective and relatively simple approach to study cytokine production in kidney tissue.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

REFERENCES:

1 GBD Chronic Kidney Disease Collaboration. Global, regional, and national burden of kidney disease, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet*. **395** (10225), 709–733 (2020).

2 Canaud, G., Bonventre, J. V. Cell cycle arrest and the evolution of chronic kidney disease from acute kidney injury. *Nephrology Dialysis Transplantation.* **30** (4), 575–583 (2015).

3 Chawla, L. S., Eggers, P. W., Star, R. A., Kimmel, P. L. Acute kidney injury and chronic kidney disease as interconnected syndromes. *New England Journal of Medicine.* **371** (1), 58–66 (2014).

4 Berger, K., Moeller, M. J. Mechanisms of epithelial repair and regeneration after acute kidney injury. *Seminars in Nephrology.* **34** (4), 394–403 (2014).

5 Bonventre, J. V., Yang, L. Cellular pathophysiology of ischemic acute kidney injury. *The Journal of clinical investigation.* **121** (11), 4210–4221 (2011).
6 Kurts, C., Panzer, U., Anders, H. J., Rees, A. J. The immune system and kidney disease: basic concepts and clinical implications. *Nature Reviews. Immunology.* **13** (10), 738–753 (2013).
7 Panzer, U., Steinmetz, O. M., Stahl, R. A., Wolf, G. Kidney diseases and chemokines. *Current Drug Targets.* **7** (1), 65–80 (2006).
8 Commins, S. P., Borish, L., Steinke, J. W. Immunologic messenger molecules: cytokines, interferons, and chemokines. *The Journal of Allergy and Clinical Immunology.* **125** (2 Suppl 2), S53–72 (2010).
9 Jaber, B. L. et al. Cytokine gene promoter polymorphisms and mortality in acute renal failure. *Cytokine.* **25** (5), 212–219 (2004).
10 Black, L. M., Lever, J. M., Agarwal, A. Renal inflammation and fibrosis: A double-edged sword. *Journal of Histochemistry and Cytochemistry.* **67** (9), 663–681 (2019).
11 van Kooten, C., Woltman, A. M., Daha, M. R. Immunological function of tubular epithelial cells: the functional implications of CD40 expression. *Experimental Nephrology.* **8** (4–5), 203–207 (2000).
12 Canaud, G. et al. Cyclin G1 and TASCC regulate kidney epithelial cell G2-M arrest and fibrotic maladaptive repair. *Science Translational Medicine.* **11** (476), eaav4754 (2019).
13 Burton, C. J., Combe, C., Walls, J., Harris, K. P. Secretion of chemokines and cytokines by human tubular epithelial cells in response to proteins. *Nephrology, Dialysis, Transplantation.* **14** (11), 2628–2633 (1999).
14 Ripley, C. R., Fant, J., Bienkowski, R. S. Brefeldin A inhibits degradation as well as production and secretion of collagen in human lung fibroblasts. *Journal of Biological Chemistry.* **268** (5), 3677–3682 (1993).
15 Kovacs, S. B., Oh, C., Aachoui, Y., Miao, E. A. Evaluating cytokine production by flow cytometry using brefeldin A in mice. *STAR Protocols.* **2** (1), 100244 (2021).
16 Fujiwara, T., Oda, K., Yokota, S., Takatsuki, A., Ikehara, Y. Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum. *Journal of Biological Chemistry.* **263** (34), 18545–18552 (1988).
17 Sadeghipour, A., Babaheidarian, P. Making formalin-fixed, paraffin embedded blocks. *Methods in Molecular Biology.* **1897**, 253–268 (2019).
18 Qin, C. et al. The cutting and floating method for paraffin-embedded tissue for sectioning. *Journal of Visualized Experiments: JoVE.* (139), 58288 (2018).
19 Honvo-Houeto, E., Truchet, S. Indirect immunofluorescence on frozen sections of mouse mammary gland. *Journal of Visualized Experiments: JoVE.* (106), 53179 (2015).
20 Chung, S. et al. TGF-β promotes fibrosis after severe acute kidney injury by enhancing renal macrophage infiltration. *JCI Insight.* **3** (21), e123563 (2018).
21 Meng, X. M., Nikolic-Paterson, D. J., Lan, H. Y. TGF-β: the master regulator of fibrosis. *Nature Reviews. Nephrology.* **12** (6), 325–338 (2016).
22 Kok, H. M., Falke, L. L., Goldschmeding, R., Nguyen, T. Q. Targeting CTGF, EGF and PDGF pathways to prevent progression of kidney disease. *Nature Reviews. Nephrology.* **10** (12), 700–711 (2014).
23 Engel, J. E., Williams, E., Williams, M. L., Bidwell, G. L., 3rd, Chade, A. R. Targeted VEGF (vascular endothelial growth factor) therapy induces long-term renal recovery in chronic kidney

disease via macrophage polarization. *Hypertension.* **74** (5), 1113–1123 (2019).
24 Liu, B. C., Tang, T. T., Lv, L. L., Lan, H. Y. Renal tubule injury: a driving force toward chronic kidney disease. *Kidney International.* **93** (3), 568–579 (2018).
25 de Haij, S., Woltman, A. M., Bakker, A. C., Daha, M. R., van Kooten, C. Production of inflammatory mediators by renal epithelial cells is insensitive to glucocorticoids. *British Journal of Pharmacology.* **137** (2), 197–204 (2002).
26 Hong, S., Healy, H., Kassianos, A. J. The emerging role of renal tubular epithelial cells in the immunological pathophysiology of lupus nephritis. *Frontiers in Immunology.* **11**, 578952 (2020).
27 Hwang, B., Lee, J. H., Bang, D. Single-cell RNA sequencing technologies and bioinformatics pipelines. *Experimental & Molecular Medicine.* **50** (8), 1–14 (2018).
28 See, P., Lum, J., Chen, J., Ginhoux, F. A single-cell sequencing guide for immunologists. *Frontiers in Immunology.* **9**, 2425 (2018).
29 Gewin, L. et al. Deleting the TGF-β receptor attenuates acute proximal tubule injury. *Journal of the American Society of Nephrology.* **23** (12), 2001–2011 (2012).
30 Nlandu-Khodo, S. et al. Blocking TGF-β and β-catenin epithelial crosstalk exacerbates CKD. *Journal of the American Society of Nephrology.* **28** (12), 3490–3503 (2017).
31 Brooks, C. R. et al. KIM-1-/TIM-1-mediated phagocytosis links ATG5-/ULK1-dependent clearance of apoptotic cells to antigen presentation. *EMBO Journal.* **34** (19), 2441–2464 (2015).
32 Kishi, S. et al. Proximal tubule ATR regulates DNA repair to prevent maladaptive renal injury responses. *The Journal of Clinical Investigation.* **129** (11), 4797–4816 (2019).
33 Yu, S. M., Bonventre, J. V. Acute kidney injury and maladaptive tubular repair leading to renal fibrosis. *Current Opinion in Nephrology and Hypertension.* **29** (3), 310–318 (2020).

Figure 1

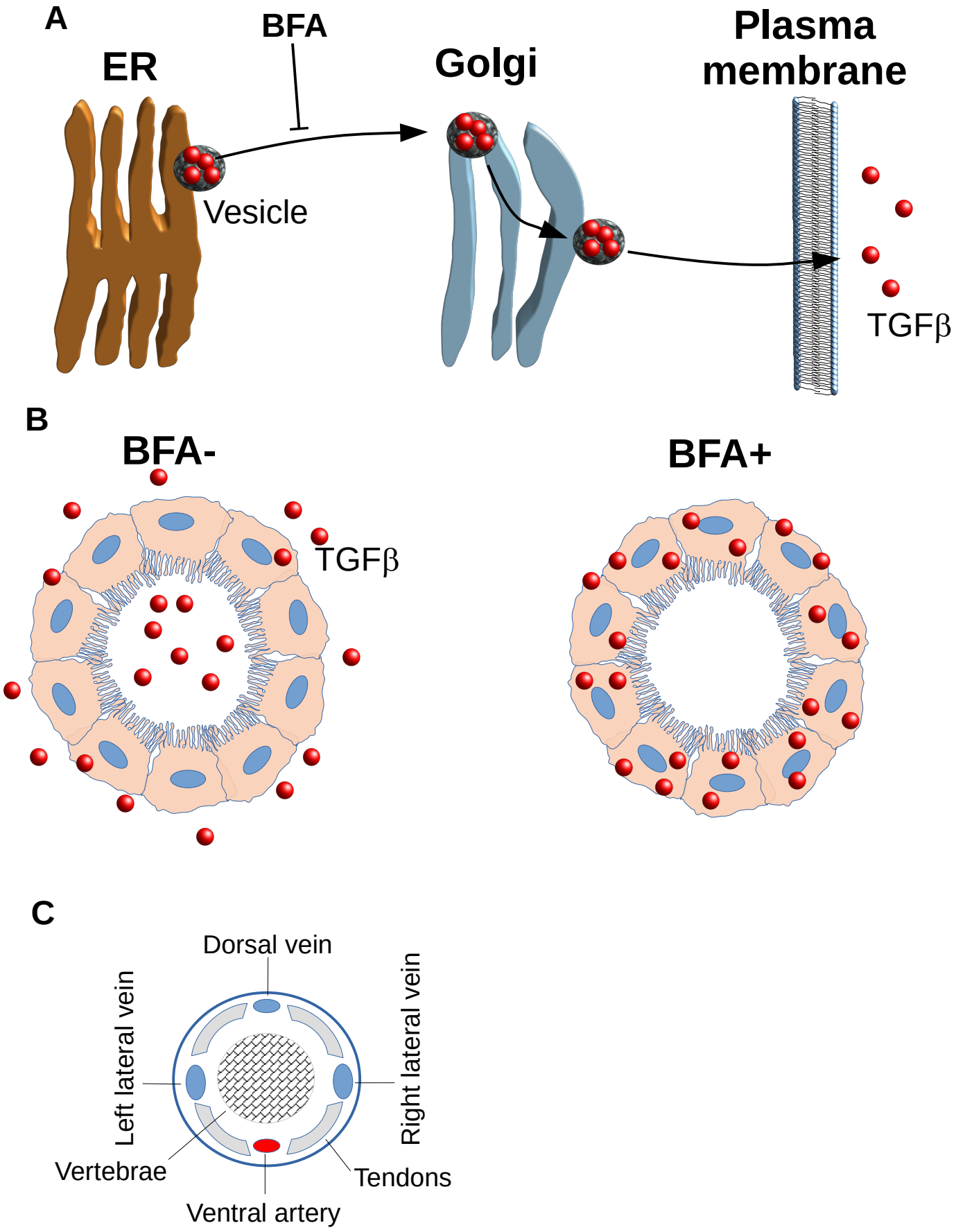


Figure 2

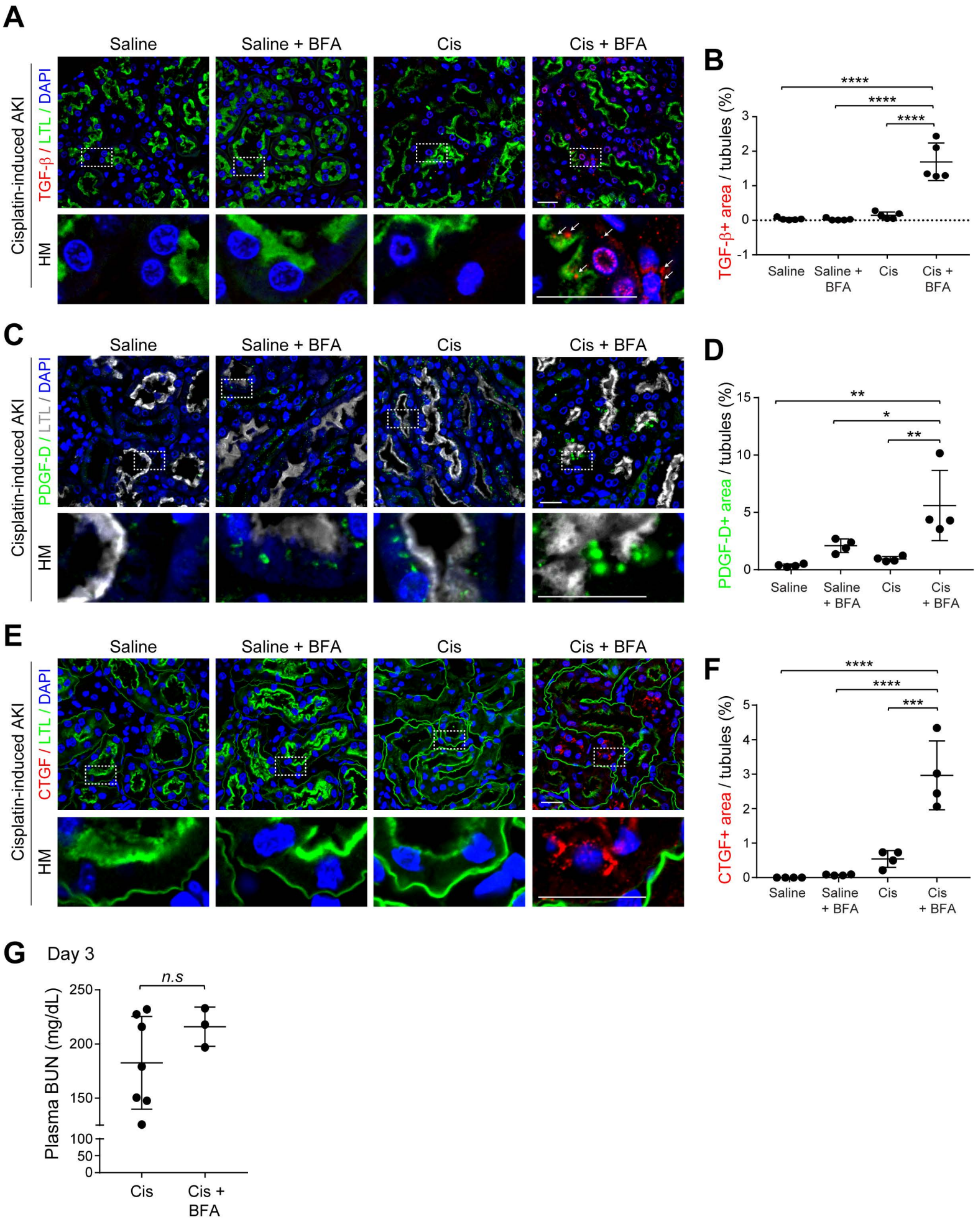
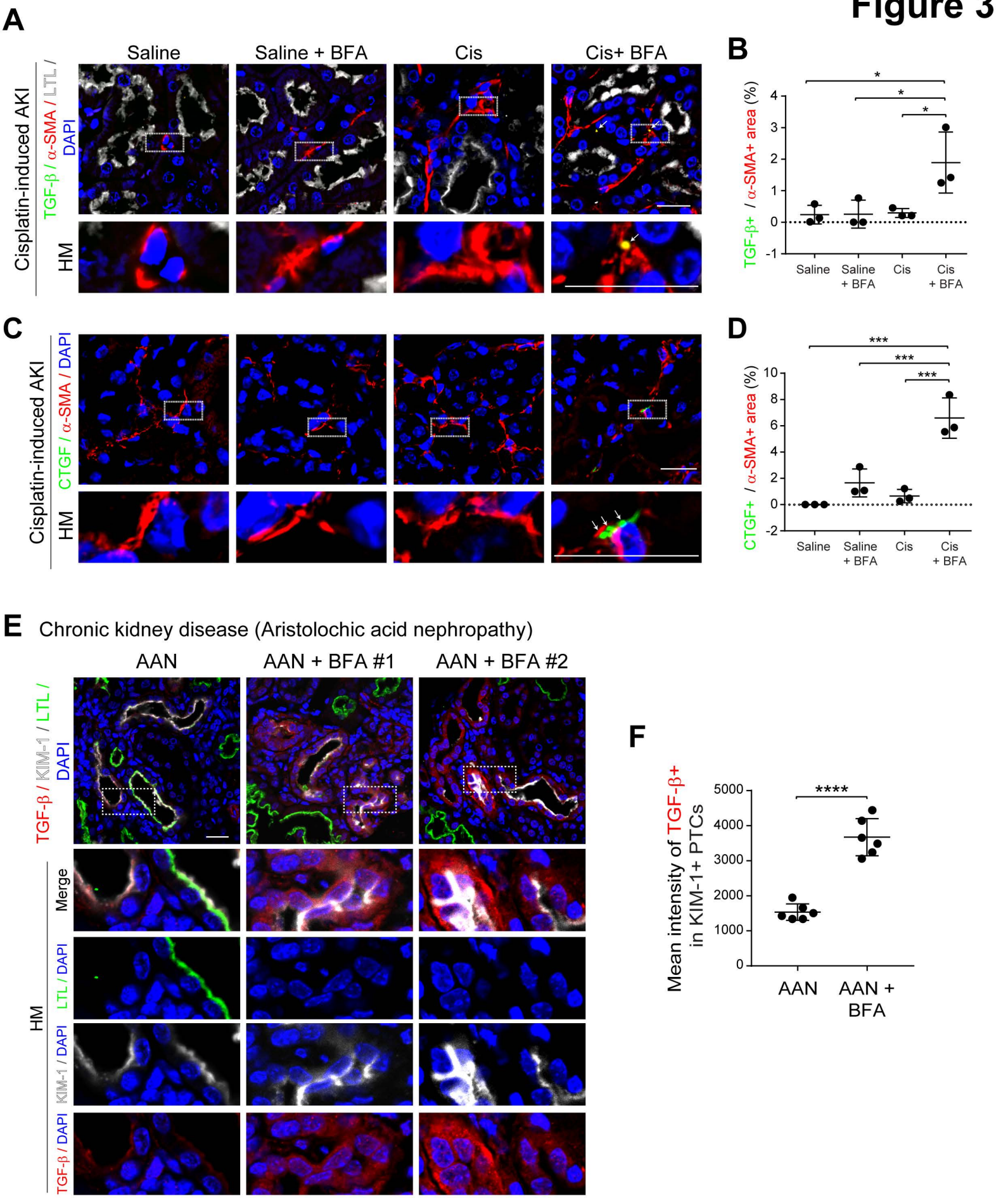


Figure 3



October 12, 2021

To JoVE Editorial Office:

Dear Dr. Iyer,

Thank you for giving us the opportunity to resubmit the enclosed manuscript: “Brefeldin A injection identifies the source of secreted proteins in the kidney.” We are pleased to resubmit this original article for consideration for publication in JoVE. We believe we have addressed all of the Reviewer and Editor concerns through editing the manuscript to add more details concerning the protocol and providing additional experimental data, resulting in improved manuscript.

In the revised manuscript, we have improved the study by:

- Editing the text to provide more detailed experimental steps.
- Including additional experimental data to demonstrate the versatility of the protocol in identifying multiple cytokines.
- Demonstrating the protocol can be used in different kidney cell types, such as epithelial or interstitial cells.
- Adding data to demonstrate the protocol can be used with paraffin embedded or frozen tissue.

All authors have read and approved the manuscript. The manuscript has not been published or submitted in any format in part or in it’s entirely elsewhere.

Thank you for your consideration.

Respectfully yours,

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Below are responses to the Reviewer and Editor comments. The comments are listed individually with responses indented below each comment. Changes to the manuscript text are marked in red in the manuscript file. Yellow highlighting in the manuscript file indicates the sections of the protocol to include in the video.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors try to visualize the precise localization of production of cytokines that are upregulated after kidney injury in mice using intravenous injection of brefeldin A.
This convenient and simple technique enhances the intensity of immunostaining for certain cytokines, and multiple staining may help the identification of specific cell populations that release those.
However, the major criticism of this paper is lacking the generalization of this technique to the other cytokines than TGF beta, and to the other cell populations than tubular epithelial cells. Generally, the shape of epithelial cells is cuboidal, and its cytoplasm is relatively easy to visualize. However, in the spindle shaped cells, like endothelial cells or fibroblasts, it is hard to visualize the intracellular organella in vivo. Nevertheless, even if this technique can be applied for visualizing only the cytokine-releasing epithelial cells, it may help the better understandings of intercellular crosstalk in various kidney disease models.

Major Concerns:
1. Authors only assessed the TGF beta expression in vivo. In order to generalize this technique to the other cytokines, additional immunostaining for the others like PDGF or CTGF should be evaluated.

> Thank you for pointing out the need to expand the data to demonstrate the versatility of this approach. In the revised manuscript, we have provided data demonstrating the staining of PDGF and CTGF in kidney tubular cells following injury and BFA treatment. This necessitated expanding the protocol somewhat to include fixed paraffin embedded tissue sections and frozen sections, to find conditions where the different antibodies stained well. Overall, these changes help further highlight the versatility of this technique.

2. The cytokines released from the other cell populations like endothelial cells or interstitial fibroblasts also should be analyzed. Otherwise, authors have to describe this limitation.

> Thank you for pointing out this limitation. To address this concern, we analyzed the accumulation of CTGF and TGFβ in αSMA positive interstitial cells. αSMA positive cells upregulated both CTGF and TGFβ with BFA treatment.

Reviewer #2:
Manuscript Summary:

In this study, Taguchi and others demonstrated an easy and convenient method to prevent secretion of cytokines (and other proteins) and thereby enhance the ability to detect their expression by immunohistochemical staining methods. Overall, the method is unique to the kidney and presents itself as a useful tool to examine the cell-specific expression of proteins during injury. Pros and cons to using this method have been discussed well.

Thank you for the positive comments.

Major Concerns:
All volumes, concentrations of stock and working solutions) should be listed - eg: missing volume in step 5.6; concentration in 11.7

Thank you for pointing out these omissions. These have been corrected.

Why were 6 hours after BFA administration chosen for analysis? If readers would like to incorporate this method, it would be important to state the rationale for the time chosen.

We have added a note explaining that the "6 hour timepoint was chosen based on literature demonstrating 6 hours of BFA treatment in other organs allows for enough accumulation of cytokines within cells to visualize by immunofluorescent staining."

Minor Concerns:
Figure 2 is not required and should be removed.

We agree that much of Figure 2 is not necessary. We have removed the original Figure 2. However, we kept the cross-section diagram of the mouse tail, because we felt it would be beneficial to people performing tail vein injections for the first time.

2.6. Proceed to step 4 on day 3 after .... (change from step 3 to step 4)

Thank you for pointing out this typo. It has been corrected.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use

We performed additional proofreading.

2. Please provide an email address for each author.

Email addresses have been added.

3. Please revise the following lines to avoid previously published work: 24, 75-77.

These have been revised.

4. Please use SI unit denotation for all units throughout the manuscript: L, mL, µL, cm, kg, etc. Hours, minute, and seconds can be written as h, min, s, respectively.

SI units have been added.

5 JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example: NDC 67618-151-17, Histo-clear, Nikon Eclipse Ti2-E, CMOS camera (DS-Qi-2), NIS Elements, ZEN imaging software, Nikon Plan Apo 20x/0.75, Nikon NIS software, LCM) 710, 1.10 LD C-Apochromat WATER etc.

We have corrected this based on the instructions below, mentioning the brand on the first use and using a generic term afterwards. Please let us know if additional edits are needed.

Obviously, if you have optimized your protocol with certain instruments/reagents/software, you will need to mention them, but include just the bare minimum information (e.g., name of product) in the manuscript and come up with a generic term to refer to the product (include this in the comments column in the Table of Materials) after the first mention so that you don't keep repeating commercial terms throughout the paper. Comparison of your equipment or software to other commercially available ones is allowed in the discussion, but without unnecessary repetition of these names and only for scientific discussion.

We have corrected this. Please let us know if additional edits are needed.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have updated the tense and added additional information about the use of safety equipment. Please let us know if additional edits are needed.

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Animal use statement has been added.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please remember that our scripts are directly derived from the protocol text. Please include all actions associated with each step.

We added additional details to the protocol steps.

Step 2.4, 3.4: What is the povidone-iodine and alcohol concentration used? How many times was this step done—3 times or just once?

The concentration and number of applications has been updated.

Step 5.2: Please mention how animals are anesthetized and how proper anesthetization is confirmed. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

We mentioned anesthesia is an option, but it was not used in the protocol. Therefore, we decided to remove this sentence.

Step 6.3: What is the volume of blood collected?

The volume has been added.

Step 6.4, 6.4.2, 16.6: Please provide the correct step numbers.

Step numbers have been corrected.

Step 6.4.1: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

The centrifuge speed has been corrected.

Step 7.1: What is the perfusion flow rate? What syringe size was used here? Please specify.

The perfusion flow rate has been added.

Step 8.1: How were the kidneys and kidney capsule removed?

Additional explanations have been added

Step 8.2: How was the bisection done?

Additional details have been added.

Step 9: How was processing and paraffin embedding done? You can also cite a published protocol if one is available.

We added a reference for this step.

Step 10: How were the slides prepared?

The slides we list in the Table of Materials are precleaned and charged. No additional preparation is needed.

Step 11.1, 11.6, 11.16: How was this done?

These steps have been updated.

Step 11.8: How much primary antibody was used?

We have added volume of antibody used.

Step 11.10: Which secondary antibody was used here? What was its final concentration? How much of this was added to the sample?

We have added volume and concentration of antibody used.

Step 11.13: How much LTL was added? What was the incubation temperature?

We have added volume and temperature of antibody used.

Step 11.15: How much DAPI was used and at what concentration?

The concentration has been added.

Step 12, 13, 14, 15, 16.8: Please include all the button clicks, command lines, etc. in the software and on the instrument. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc.). If using long scripts, please include as a supplementary file.

Button clicks for software have been added.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Please do not highlight any steps describing euthanasia.

We highlighted the first step for the steps we want to include in the video, as not to overwhelm the Reviewers with a lot of highlighting.

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique

We have included the following paragraphs: One paragraph to comparing the presented protocol to other techniques. One paragraph discussing how to interpret the results. One paragraph discussing troubleshooting/standardization of the staining. One paragraph discussing potential modifications of the protocol to use different assays for readout. One paragraph discussing potential limitations of the technique. One conclusion paragraph.

11. Figures 3A & 4A: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Please consider deleting Figure 2.

Scale bars have been added.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

We utilized the JoVE style within Endnote. Please let us know if we should use a different Endnote style.

13. Please ensure the inclusion of all items (tubes, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

Materials Table has been updated and sorted.