# Netrin-1 regulates the inflammatory response of neutrophils and macrophages, and suppresses ischemic acute kidney injury by inhibiting COX-2 mediated PGE2 production

**Punithavathi Vilapakkam Ranganathan**[1], **Calpurnia Jayakumar**[1], **Riyaz Mohamed**[1], **Zheng Dong**[2], and **Ganesan Ramesh**[1]

[1]Department of Medicine and Vascular Biology Center, Georgia Health Sciences University Augusta, GA 30912.

[2]Department of Cell Biology and Anatomy Georgia Health Sciences University Augusta, GA 30912.

## Abstract

Netrin-1 regulates inflammation but the mechanism by which this occurs is unknown. Here we explore the role of netrin-1 in regulating the production of the prostanoid metabolite PGE2 from neutrophils in *in vitro* and *in vivo* disease models. Ischemia reperfusion in wild-type and RAG-1 knockout mice induced severe kidney injury that was associated with a large increase in neutrophil infiltration and COX-2 expression in the infiltrating leukocytes. Administration of netrin-1 suppressed COX-2 expression, PGE2 and thromboxane production, and neutrophil infiltration into the kidney. This was associated with reduced apoptosis, inflammatory cytokine and chemokine expression, and improved kidney function. Treatment with the PGE2 receptor EP4 agonist enhanced neutrophil infiltration and renal injury which was not inhibited by netrin-1. Consistent with *in vivo* data, both LPS and IFNγ-induced inflammatory cytokine production in macrophages and IL-17-induced IFNγ production in neutrophils were suppressed by netrin-1 *in vitro* by suppression of COX-2 expression. Moreover, netrin-1 regulates COX-2 expression at the transcriptional level through the regulation of NFκB activation. Thus, netrin-1 regulates the inflammatory response of neutrophils and macrophages through suppression of COX-2 mediated PGE2 production. This could be a potential drug for treating many inflammatory immune disorders.

## Keywords

Netrin-1; inflammation; cyclooxygenase; PGE2

**Correspondence Address:** Ganesan Ramesh, Ph.D. Department of Medicine and Vascular Biology Center, CB-3330 Georgia Health Sciences University 1459 Laney-Walker Blvd Augusta, GA 30912 TEL. NO. (706) 721-9728 FAX. NO. (706) 721-9799 gramesh@georgiahealth.edu.

## Introduction

Acute kidney injury (AKI) due to ischemia is a frequent and serious complication of hospitalized patients. There is no effective therapy available to treat ischemic kidney diseases. A large body of evidence strongly suggests that ischemia reperfusion injury of the kidney is an inflammatory disease mediated by both adoptive and innate immune systems [1-8]. There is no effective therapy currently available for ischemic AKI. Thus, new therapeutic approaches are desperately needed. Netrin-1 is a laminin-related axon guidance cue known to regulate inflammatory cell migration and their functions in many diseases and suppresses ischemic AKI [9-12]. Recent studies suggest that netrin-1 suppresses leukocyte migration through an UNC5B receptor-mediated increase in cAMP and inhibition of chemotaxis *in vitro* [13] and *in vivo* [9]. Administration of netrin-1 to mice suppressed infiltration and inflammation in sepsis, AKI, acute lung injury, peritoneal inflammation, and whole body hypoxia [9;13-16]. In addition to inhibition of migration, netrin-1 also suppressed inflammatory cytokine and chemokine production [9]. However, the mechanism through which it suppresses immune cell function is not completely understood.

Arachidonic acid metabolites play a critical role in mediating inflammation and inflammatory cytokine production in many acute and chronic diseases [17]. Arachidonic acid is released from the plasma membrane by phospholipid A2, which is then metabolized by cyclooxygenase -1 and cyclooxygenase-2 (COX-1 and COX-2) into a series of prostaglandins, prostacyclins and thromboxanes. COX-1 is constitutively expressed whereas COX-2 expression is induced by inflammatory stimuli or mediators of inflammation [18;19]. Prostaglandin E2 (PGE2) is the most commonly studied prostanoid metabolite and is known to mediate a wide variety of functions, including stimulation of immune cell function, chemotaxis, and an increase in the production of inflammatory cytokines. Inhibition of inducible COX-2 expression or function suppressed inflammation and is currently used to treat many acute and chronic illnesses [18;20;21]. Another pro-inflammatory metabolite of COX-2 enzyme, thromboxane A2, also has been implicated in ischemia reperfusion injury [22;23]. Both prostaglandins and thromboxane are known to induce production of cytokines and chemokines, such as IFNγ and IL-17, and mediate neutrophils and monocyte activation [2;24-26]. Neutrophils, monocytes and inflammatory mediators released from these cells are known to cause ischemic injury of the kidney [2;21;27-29]. However, whether netrin-1 regulates arachidonic acid metabolism through regulation of COX-2 expression in neutrophils and macrophages, thereby suppressing inflammation and ischemia reperfusion injury are unknown.

The current study was carried out to investigate the hypotheses: 1. Netrin-1 regulates inflammation through suppression of COX-2-mediated PGE2 production in neutrophils and monocytes; 2. COX-2 metabolites mediate IL-17-mediated IFNγ production, neutrophil infiltration, IFNγ-induced activation of macrophages, and ischemic AKI; and 3. Netrin-1 regulates COX-2 expression through inhibition of NFκB activation in immune cells.

# Results

## Netrin-1 protects kidney against reperfusion injury in both Wild type and RAG-1 knockout mice

Several studies have demonstrated that neutrophils play a major role in mediating acute ischemic kidney injury [2;27]. Our earlier studies also showed that neutrophils are a major subset of that infiltrate after reperfusion injury [9]. However, it was not clear whether netrin-1-mediated protection against ischemia reperfusion injury and suppression of neutrophil infiltration occurs through direct or indirect action on T cells. To determine whether the netrin-1 effect on neutrophils and monocytes is direct and can protect kidney in the absence of T cells, RAG1 knockout mice were subjected to 26 minutes of ischemia followed by reperfusion. As shown in Figure 1, both wild-type (WT) and RAG1 knockout mice developed severe renal injury. Sham-operated WT and RAG1 knockout animals showed no renal dysfunction. Administration of recombinant netrin-1 to both WT and RAG1 knockout mice protected kidney, as shown by a significant reduction in serum creatinine (Figure 1A). Improved kidney function in netrin-1 treated WT and RAG-1 knockout animals was associated with preservation of kidney structure and reduction of tubular necrosis (Figure 1B).

**Netrin-1 suppresses inflammation and neutrophil infiltration independent of T-cells**—Because our previous studies demonstrated that netrin-1 regulates inflammation, we determined whether the protection of kidney function is due to the suppression of inflammation. As shown in Figure 2, neutrophil infiltration and cytokine and chemokine expression were increased in WT and RAG1 mice knockout treated with vehicle. However, administration of netrin-1 suppressed neutrophil infiltration and inflammatory cytokine and chemokine expression (Figure 2). Reduction in inflammation was associated with improvement in histological injury. Moreover, tubular apoptosis was also significantly suppressed (Figure 3). However, the pathway through which netrin-1 suppresses inflammation in ischemic AKI is unknown.

**Netrin-1 regulates COX-2 expression and PGE2 production *in vivo*—**We hypothesized that netrin-1 regulates neutrophil function and activation by suppressing COX-2-mediated PGE2 production, thereby suppressing inflammatory cytokine and chemokine production and infiltration of neutrophils. To determine this possibility, we examined the expression of COX-1 and COX-2 after reperfusion in WT and RAG-1 knockout mice. COX-1 expression was not altered significantly after reperfusion whereas the expression of COX-2 is significantly up-regulated (Figure 2). Administration of netrin-1 suppressed the expression of COX-2 in both WT and RAG1 knockout animals. Consistent with suppression of COX-2 expression, the ischemia reperfusion-induced increase in PGE2 levels in kidney were suppressed by administration of netrin-1 (Figure 4A). Excretion of PGE2 was also increased in both WT and RAG-1 knockout mice after ischemia reperfusion, which was suppressed by administration of netrin-1 (Figure 4B). To further confirm the netrin-1 effects on COX-2, we measured another COX-2 metabolite, thromboxane B2, which is a degradation product of thromboxane A2. Ischemia reperfusion induced an increase in thromboxane B2 excreation in urine, which was significantly suppressed by

administration of netrin-1 (Figure 4C). To determine specific cell type that expressed COX-2, immunolocalization was performed. As shown in Figure 4D, COX-2 expression was seen in the thick ascending limb, distal tubules (also in macula densa) and collecting ducts in sham-operated kidney, but not in proximal tubules. There is some staining in the interstitium, which may be associated with endothelial cells. Twenty four hours after reperfusion, COX-2 expression is highly induced in infiltrating cells. The increase in expression of COX-2 in infiltrating cells is inhibited in netrin-1-treated animals (Figure 4E). The infiltrating cells appear to be neutrophils. To confirm this flow cytometry analysis and co-localization of COX-2 expressing immune cells was performed 24hr after reperfusion. Over 86% of Gr-1 positive neutrophils and 80% of F4/80 positive monocyte/macrophage were positive for COX-2 expression. Very few CD4 T cells were seen in the kidney and 60% of them were positive for COX-2 expression. Consistent with flow cytometric analysis, neutrophils were co-localized for COX-2, confirming that COX-2-expressing infiltrating cells were indeed neutrophils and macrophages (Figure 5).

## Netrin-1 regulates inflammatory responses of macrophages and neutrophils through COX-2 expression

Since both monocytes and neutrophils are present in RAG-1 knockout animals and these two cell types are regulated by netrin-1 during ischemia reperfusion injury [9], we determined whether COX-2-mediated inflammation is regulated by netrin-1 in both macrophages and neutrophils. We hypothesized that netrin-1 regulates inflammatory responses of macrophages and neutrophils by suppressing COX-2-mediated PGE2 production, thereby suppressing inflammatory cytokine and chemokine production. To determine this possibility, we characterized the mechanism of netrin-1-mediated suppression of COX-2 and PGE2 production in a macrophage cell line. As shown in Figure 6, addition of either IFNγ or LPS treatment induced a large increase in COX-2 expression and PGE2 production, but not COX-1 expression (Figure 6A and C). Both IFNγ and LPS-induced COX-2 and PGE2 production were suppressed in netrin-1 treated macrophages. IFNγ also induced a large increase in MCP-1 and IP-10 production, which was inhibited by netrin-1 (Figure 6B and D). The netrin-1 mediated suppressive effect on chemokine production was abolished when PGE2 is added to the culture, suggesting that the netrin-1 effect is at the level of COX-2 expression.

To determine whether netrin-1-mediated suppression of the inflammatory response of neutrophils is through COX-2, we used IL-17 to induce an inflammatory response. IL-17 production in neutrophils in response to ischemia is known to initiate IFNγ production and infiltration into kidney [27]. Moreover, IFNγ also regulates IL-17 production in neutrophils. Previous studies have shown that PGE2 increases IL-17 production in both Th17 cells and neutrophils [30]. To determine directly that IL-17-induced IFNγ production is dependent on COX-2-mediated PGE2 and that netrin-1 suppresses this pathway, thereby suppressing IFNγ production and neutrophil infiltration into ischemic kidney, isolated neutrophils were stimulated with IL-17. As shown in Figure 6E & F, IL-17 increased COX-2 expression and IFNγ production. Increased COX-2 expression was associated with increased PGE2 production, but addition of netrin-1 suppressed both COX-2 expression and IFNγ production. The suppressive effect of netrin-1 on IFNγ production was abolished by addition

of PGE2, suggesting that the netrin-1 effect is at the level of production but is not at the level of its activity.

### PGE2 receptor subtype EP4 mediates inflammatory response and ischemia reperfusion injury

To determine whether netrin-1 suppresses not only COX-2 expression but also PGE2 activity *in vivo*, we administered PGE2 receptor EP4 selective agonist to WT mice and subjected them to ischemia reperfusion injury. Consistent with the *in vitro* data, administration of PGE2 receptor EP4 selective agonist (ONO-AE1-329) enhanced ischemic kidney injury and neutrophil infiltration, and netrin-1 did not suppress the EP4 agonist-induced increase in renal injury and neutrophil infiltration (Figure 7). These data suggest and confirm the *in vitro* findings that netrin-1 regulates COX-2 expression but not PGE2 activity.

### Netrin-1 suppresses COX-2 expression through suppression of NFkB activation

The signaling pathway through which netrin-1 suppresses COX-2 expression is unknown. The COX-2 promoter, however, has several NFkB binding sites and is up-regulated in response to NFkB activation. Since both IFN$\gamma$ and LPS are known to activate NFkB, we determined whether LPS-induced activation of NFkB is suppressed by netrin-1 in a macrophage cell line. As shown in Figure 8A, LPS induces COX-2 expression in a time-dependent manner, but addition of netrin-1 suppressed COX-2 expression. The induction of COX-2 is associated with a transient but rapid degradation of I$\kappa$B$\alpha$ (Figure 8A & B) which is significantly inhibited by netrin-1 treatment. Moreover, LPS induced increase in NFkB activation, as seen by nuclear translocation of the p65 subunit, was suppressed in netrin-1-treated cells (Figure 8D-E). Netrin-1 treatment alone did not alter NF$\kappa$B translocation. Moreover, LPS induced an increase in COX-2 promoter activity in the macrophage cell line, which was suppressed by netrin-1. These data suggest that netrin-1-mediated suppression of COX-2 expression may occur through IkB$\alpha$-mediated inhibition of NFkB.

## Discussion

Migration of inflammatory cells to the site of injury is a critical cellular response to initiate the removal of dead cells and induce a regeneration response. However, inappropriate excessive activation and migration of these cells into organs also can result in tissue destruction, and this abnormal influx is thought to be the mechanism involved in many ischemic injuries of organs. Barriers to aberrant immune cell activation and migration exist, for example, the production of immunosuppressive, chemorepellent molecules from endothelium and epithelial cells. One such molecule, netrin-1, is known to suppress inflammation and inflammatory cell migration into ischemic kidney. However, the mechanism as to how netrin-1 suppresses immune cell activation and migration has been unknown. Here, we show for the first time that the neuronal guidance cue netrin-1 regulates inflammation and inflammatory cell migration through suppression of COX-2-mediated PGE2 and thromboxane A2 production.

Consistent with our earlier studies [9], we have demonstrated that the administration of netrin-1 to WT mice suppressed inflammation and inflammatory cell infiltration. In addition, suppression of inflammation was also seen in RAG-1 knockout mice, suggesting that the effect of netrin-1 on neutrophil infiltration is independent of T and B cells. Consistent with improved renal function, the number of neutrophils that infiltrated the ischemic kidney is also reduced with netrin-1 treatment. COX-2-mediated production of PGE2 is a known inflammatory mediator in many disease processes. Our *in vivo* data shows that netrin-1-mediated suppression of COX-2 expression was associated with suppression of inflammatory cytokine production and reduction in infiltrating cells to the ischemic kidney. COX-2 is known to be induced in kidney epithelial cells and may mediate ischemic kidney injury [31-34]. Consistent with previous studies, netrin-1-mediated suppression of COX-2 expression also reduced ischemic kidney injury and inflammation.

PGE2 is known to bind and mediate its activity through four types of receptors (EP1-4). All four receptors are expressed in neutrophils [35]. Both EP2 and EP4 are highly inducible. EP4 receptor is known to mediate increased cytokine IL-8 and IL-17 production from endothelial cells and Th17 cells [26;30;36]. Since IL-17 is known to mediate IFNγ-regulated neutrophil infiltration in ischemic kidney, we tested an EP4 specific agonist on ischemia reperfusion injury and neutrophil infiltration. Netrin-1-mediated suppression of neutrophil infiltration is abolished by administration of the EP4 agonist, suggesting that the netrin-1 effect is at the level of PGE2 production, not at the level of PGE2 action. Earlier studies showed a mixed role of COX-2 in ischemia reperfusion injury. This could be due to use of the whole body knockout of COX-2 or global inhibition of COX-2 activity. It can be seen from our study that netrin-1 inhibits only the inducible form of COX-2 expression in response to ischemia reperfusion. The induction of COX-2 is specific and localized in infiltrating cells. Therefore, netrin-1-mediated inhibition of COX-2 expression may be more advantageous as compared to global inhibition of COX-2.

The signal for the increased production of IL-17 and IFNγ in neutrophils at early hours of reperfusion of the kidney is unknown. Our data suggest that COX-2-mediated PGE2 production may initiate IL-17 and IFNγ production, which will then mediate neutrophil infiltration and kidney injury. This view was supported by another study that showed that an IL.23.mediated neutrohil migration was increased by PGE2 through increasing the production of IL-17-Moreover, IL-17-mediated neutrophil infiltration depends on TNFα, leukotrienes, and IFNγ [27;30]. Consistent with this study, we demonstrate that the IL-17-induced production of TNFα, COX-2 expression, and IFNγ was suppressed by addition of netrin-1, which was abolished by addition of PGE2. Our results also suggest that PGE2 is not only necessary for IL-17 production but also its downstream effect on inducing cytokine expression and neutrophil infiltration.

COX-2 is highly induced after inflammation and hypoxia in many cells and tissues [37-40]. NFkB transcription factor-mediated increase in transcription of the COX-2 gene was responsible for the increase in COX-2 mRNA. COX-2 promoter analysis showed the presence of multiple NFkB binding sites. NFκB was sequestered in an inactive form in the cytoplasm by IkBα. Upon phosphorylation of inhibitory kappa kinase (IKK), IkBα is degraded, and the released NFkB is then translocated into nucleus for transactivation of

various genes including COX-2 [37]. Our results show that LPS-induced IkBα degradation is suppressed by netrin-1, which was associated with suppression of COX-2 expression. Moreover, LPS-induced COX-2 promoter activity and NFkB translocation into the nucleus are suppressed by netrin-1. These results suggest that netrin-1 may regulate COX-2 expression through inhibition of NFkB activation.

IFNγ was shown to play an important role in mediating neutrophil infiltration into ischemic kidney [2;27] and activation of monocyte/macrophages to induce chemokines. Our earlier studies had determined that administration of netrin-1 suppressed IFNγ-producing neutrophil infiltration into the kidney. Moreover, netrin-1 also suppressed monocyte infiltration into the kidney. Consistent with our earlier observation, netrin-1 suppressed neutrophil infiltration into ischemic kidney. In addition, our current study also documents that netrin-1 regulates IL-17-induced IFNγ production through the COX-2 – PGE2 pathways. Our result also shows that IFNγ-induced macrophage activation and chemokine production also depends on COX-2 expression and PGE2 production.

In summary, our study demonstrates that netrin-1 protects kidney from ischemia reperfusion injury in both WT and RAG-1 knockout mice by suppressing inflammation, infiltration of neutrophils, and apoptosis. In addition, netrin-1 also suppressed COX-2 expression and PGE2 production in neutrophils *in vivo* and *in vitro*. PGE2 mediates IL-17-induced IFNγ production, and administration of PGE2 receptor EP4 agonist exacerbated neutrophil infiltration. Our study also demonstrated that netrin-1 blocks PGE2 production but not its activity. Moreover, netrin-1 regulates COX-2 expression through inhibition of IkBα degradation. Therefore, we conclude that netrin-1 administration represents a novel therapeutic option for the treatment of ischemia reperfusion injury of the kidney and other inflammatory diseases.

## Materials and Methods

### Renal ischemia reperfusion

C57BL/6J mice (8-9 weeks of age, The Jackson Lab, Bar Harbor, ME) were anesthetized with sodium pentobarbital (50 mg/kg body weight, intra-peritoneally) and placed on a heating pad to maintain body temperature at 37° C. Both renal pedicles were identified through dorsal incisions and clamped for 26 minutes. Reperfusion was confirmed visually upon release of the clamps. As a control, sham-operated animals were subjected to the same surgical procedure except the renal pedicles were not clamped. Surgical wounds were closed and mice were given 1 ml of warm saline, intraperitoneally. The mice were kept in a warm incubator until they regained consciousness. Some animals received recombinant netrin-1 (5 µg/animal) or vehicle (0.1% BSA) and/or PGE2 receptor EP4 selective agonist ONO-AE1-329 (gift from ONO-Pharmaceuticals, Japan) (0.1 mg/kg body weight) 1 hr before renal pedicle clamping. Netrin-1 and vehicle were administered intravenously and EP4 agonist was administered subcutaneously. Urine and kidney tissue were collected 24 hrs after reperfusion and processed for ELISA and RNA isolation. The Institutional Animal Care and Use Committee of the Georgia Health Sciences University approved all of the protocols and procedures for using animals (approval number BR10-10-369).

### Isolation of neutrophils and cell culture

Neutrophils were isolated from spleen using anti-Ly6G microbead kit (Cat # 130-092-332, Milteny Biotech, Inc., Auburn, CA). Cells were counted using a hemocytometer and plated in RPMI 1640 medium containing 10% FBS. Cells were treated with different agents 2hr after plating and were harvested 48 hr after treatment. The supernatants were used for cytokine and PGE2 assays, and the pellet was used for RNA isolation.

### Experiments in RAW264.7 macrophage cell line

RAW264.7 cells (ATCC) were grown to confluence using advanced RPMI medium containing 5% serum in a 6-well plate. On the day of treatment, serum-free advanced RPMI medium was added and cells were treated with 10 ng/ml of IFNγ or 10 ng/ml of LPS with 250 ng/ml of netrin-1 or vehicle (0.1% BSA). Supernatant and RNA were isolated twenty four hours after treatment for quantification of PGE2, chemokines and inflammatory gene expression. For immunofluorescence staining for NFκB, cells were grown in a chamber slide. At confluence, cells were treated with vehicle, LPS, LPS+netrin-1 or netrin-1 for 1hr and then fixed and permeabilized. NFkB localization was determined using rabbit anti-p65 primary antibody (Cell Signaling Technology, CA) (1:280 dilution) and goat anti-rabbit secondary antibody conjugated with FITC.

A COX-2 promoter luciferase construct (US Biological) was transfected into RAW264.7 cells using Lipofectamine 2000 according to the manufacturer's instruction (Life Technologies) in a 6-well plate. 24hr after transfection, cells were treated with LPS with/ without netrin-1. Cells were harvested at 48hr after LPS addition and luciferase activity was determined and expressed as fold increase over vehicle-treated control.

### Flow Cytometry

To confirm the infiltrated immune cells that express COX-2 in the kidney after reperfusion, mice were perfused with 20 ml of saline to remove intravascular leukocytes. Kidneys were minced into fragments of 1 mm$^3$ and digested with 2 mg/ml collagenase I and 100 U/ml DNase I. The digested kidneys were then passed through 100- and 40-μm mesh, sequentially. Red blood cells in the resulting renal cells were lysed using red blood cell lysis buffer (Sigma). Renal immune cells were stained using the following fluorochrome-labeled antibodies: Anti-F4/80, Gr-1, CD4 (ebiosciences), and ant-rabbit-FITC for COX-2 (Abcam). Fc receptors on leukocytes were blocked before staining with rat anti-FcR (CD16/CD32) (BD biosciences). COX-2 staining was performed using BD cytofix/cytoperm kit (BD Biosciences). After staining renal leukocytes for surface markers, the cells were treated with fixation and permeabilization buffer containing paraformaldehyde and saponin. The cells were then washed with permeablization buffer containing saponin and stained for COX-2. Flow cytometry was performed on FACSCalibur and analyzed using Cyflogic V.1.2.1 software.

### Western blot analysis

Protein extraction from RAW264.7 cells and Western blot analysis were carried out as described before [41;42]. The membrane was probed with rabbit anti-COX-2 (Abcam, Cambridge, MA) and rabbit anti-IkBα antibodies (1:1000 dilution) (Cell Signaling

Technology, Danvers, MA). Proteins were detected using enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Inc.). Protein loading was normalized to GAPDH expression using anti-mouse GAPDH antibody (Abcam Cambridge, MA).

## Quantification of mRNA by real-time RT-PCR

Real-time RT-PCR was performed in an Applied Biosystems Inc. 7700 Sequence Detection System (Foster City, California, USA). 1.5 μg total RNA was reverse transcribed in a reaction volume of 20 μl using Omniscript RT kit and random primers. The product was diluted to a volume of 150 μl, and 5 μl aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen) and gene-specific primers. The primer sets used were: mouse TNFα (forward: GCATGATCCGCGACGTGGAA; reverse: AGATCCATGCCGTTG GCCAG), MCP-1 (forward: ATGCAGGTCCCTGTCATG; reverse: GCTTGAGGTGGTTGTGGA), ICAM-1 (forward: AGATCACATTCACGGTGCTG; reverse: CTTCAGAGGCAGGAAACAGG), COX-1 (forward: GAATGCCACCTTCATCCGAGAAG; reverse: GCTCACATTGGAGAAGGACTCC), COX-2 (forward: GCGACATACTCAAGCAGGAGCA; reverse: AGTGGTAACCGCTCAGGTGTTG) and IL-6 (forward : GATGCTACCAAACTGGATATAATC; reverse : GGTCCTTAGCCACTCCTTCTGTG ). The amount of DNA was normalized to the ß-actin signal amplified in a separate reaction (forward primer: AGAGGGAAATCGTGCGTGAC; reverse: CAATAGTGATGACCTGGCCGT).

## Renal function

Renal function was assessed by measurements of serum creatinine (cat no: DZ072B, Diazyme Labs, USA).

## Cytokine and chemokine measurements

Cytokines and chemokines in plasma were measured using ELISA array kit from eBioscience.

## Prostaglandin and thromboxane B2 quantification

PGE2 and thromboxane were quantified in mouse kidney, urine and cell culture supernatant using EIA kit (Cayman Chemicals, Ann Arbor, Michigan).

## Histology and immunostaining

Kidney tissue was fixed in buffered 10% formalin for 12 hours and then embedded in paraffin wax. For assessment of injury, 5 μM sections were stained with periodic acid-Schiff (PAS) followed by hematoxylin. Tubular injury was assessed in PAS-stained sections using a semiquantitative scale [43] in which the percentage of cortical tubules showing epithelial cell necrosis, brush-border loss, cast formation, and apoptotic bodies in the cortex was assigned a score: 0 = normal; 1 = <10%; 2 = 10–25%; 3 = 26–75%; 4 = >75%. 10 fields of 40X magnification were examined and averaged. The individual scoring of the slides was blinded to the genotype of the animal. To quantify leukocyte infiltration, sections were stained with rat anti-mouse neutrophil antibody (Abcam, Cambridge, MA) (1:200 dilution)

followed by goat anti-rat biotin conjugate. Color was developed after incubation with ABC reagent (Vector Lab). Stained sections were photographed and five 40X fields of neutrophils were examined for quantification of leukocytes. To determine COX-2 expression, sections were stained with rabbit anti-COX-2 antibody (eBiosciences) (1:100 dilution) followed by goat anti-rabbit biotin conjugate. Color was developed after incubation with ABC reagent (Vector Lab). To determine COX-2-expressing interstitial cells, sections were double-stained with rabbit anti-COX-2 antibody (eBiosciences) (1:100 dilution) and rat anti-neutrophil antibody (Monoclonal antibody NIMP-R14 raised against murine Ly-6G and Ly-6C) (Abcam, Cambridge, MA) (1:200 dilution) followed by goat anti-rabbit FITC conjugate and goat anti-rat Texas red conjugate. Fluoresecently labeled slides were mounted in aqueous mounting medium containing DAPI nuclear stain (Molecular Probes). Stained sections were photographed using an Olympus inverted microscope with color CCD camera.

### TACS TdT *in situ* Apoptosis Detection

To identify apoptotic cells, tissue sections were stained using TACS TdT *in situ* Apoptosis Detection kit (R&D Systems, Inc.) according to the manufacturer's instruction. Briefly, tissue sections were deparaffinized, hydrated, and washed with PBS. Sections were digested with proteinase K for 15 minutes at 24° C. Slides were then washed and endogenous peroxidase activity was quenched with 3% $H_2O_2$ in methanol. Slides were washed and incubated with TdT labeling reaction mix at 37° C for 1 hour and then with streptavidin-HRP. Color was developed using TACS blue label substrate solution. Slides were washed, counterstained, and mounted with Permount. Sections were photographed, and labeled cells counted and quantified.

### Statistical methods

All assays were performed in duplicate. The data are reported as mean ± SEM. Statistical significance was assessed by an unpaired, two tailed Student *t*-test for single comparison or ANOVA for multiple comparisons.

## Acknowledgements

## Reference List

1. Kinsey GR, Li L, Okusa MD. Inflammation in acute kidney injury. Nephron Exp.Nephrol. 2008; 109:e102–e107. [PubMed: 18802372]

2. Li L, Huang L, Sung Ss, Lobo PI, Brown MG, Gregg RK, Engelhard VH, Okusa MD. NKT Cell Activation Mediates Neutrophil IFN.{gamma} Production and Renal Ischemia-Reperfusion Injury. J Immunol. 2007; 178:5899–5911. [PubMed: 17442974]

3. Day YJ, Huang L, Ye H, Li L, Linden J, Okusa MD. Renal ischemia-reperfusion injury and adenosine 2A receptor-mediated tissue protection: the role of CD4+ T cells and IFN-gamma. J Immunol. 2006; 176:3108–3114. [PubMed: 16493070]

4. Burne MJ, Daniels F, El Ghandour A, Mauiyyedi S, Colvin RB, O'Donnell MP, Rabb H. Identification of the CD4(+) T cell as a major pathogenic factor in ischemic acute renal failure. J Clin Invest. 2001; 108:1283–1290. [PubMed: 11696572]

5. Rabb H, Mendiola CC, Saba SR, Dietz JR, Smith CW, Bonventre JV, Ramirez G. Antibodies to ICAM-1 protect kidneys in severe ischemic reperfusion injury. Biochem.Biophys.Res.Commun. 1995; 211:67–73. [PubMed: 7779111]

6. Bonventre JV, Zuk A. Ischemic acute renal failure: an inflammatory disease? Kidney Int. 2004; 66:480–485. [PubMed: 15253693]

7. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, Akira S, van der PT, Weening JJ, Florquin S. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. J Clin.Invest. 2005; 115:2894–2903. [PubMed: 16167081]

8. Jo SK, Sung SA, Cho WY, Go KJ, Kim HK. Macrophages contribute to the initiation of ischaemic acute renal failure in rats. Nephrol.Dial.Transplant. 2006; 21:1231–1239. [PubMed: 16410269]

9. Tadagavadi RK, Wang W, Ramesh G. Netrin.1 regulates Th1/Th2/Th17 cytokine production and inflammation through UNC5B receptor and protects kidney against ischemia-reperfusion injury. J Immunol. 2010; 185:3750–3758. [PubMed: 20693423]

10. Wang W, Reeves WB, Ramesh G. Netrin-1 and kidney injury. I. Netrin-1 protects against ischemia-reperfusion injury of the kidney. Am J Physiol Renal Physiol. 2008; 294:F739–F747. [PubMed: 18216145]

11. Grenz A, Dalton JH, Bauerle JD, Badulak A, Ridyard D, Gandjeva A, Aherne CM, Brodsky KS, Kim JH, Tuder RM, Eltzschig HK. Partial Netrin-1 Deficiency Aggravates Acute Kidney Injury. PLoS ONE. 2011; 6:e14812. [PubMed: 21625583]

12. Jayakumar C, Mohamed R, Ranganathan PV, Ramesh G. Intracellular Kinases Mediate Increased Translation and Secretion of Netrin-1 from Renal Tubular Epithelial Cells. PLoS ONE. 2011; 6:e26776. [PubMed: 22046354]

13. Ly NP, Komatsuzaki K, Fraser IP, Tseng AA, Prodhan P, Moore KJ, Kinane TB. Netrin-1 inhibits leukocyte migration in vitro and in vivo. Proc Natl Acad Sci USA. 2005; 102:14729–14734. [PubMed: 16203981]

14. Rosenberger P, Schwab JM, Mirakaj V, Masekowsky E, Mager A, Morote-Garcia JC, Unertl K, Eltzschig HK. Hypoxia-inducible factor-dependent induction of netrin-1 dampens inflammation caused by hypoxia. Nat Immunol. 2009; 10:195–202. [PubMed: 19122655]

15. Mirakaj V, Thix CA, Laucher S, Mielke C, Morote-Garcia JC, Schmit MA, Henes J, Unertl KE, K+¦hler D, Rosenberger P. Netrin.1 Dampens Pulmonary Inflammation during Acute Lung Injury. Am.J.Respir.Crit.Care Med. 2010; 181:815–824. [PubMed: 20075388]

16. Mirakaj V, Gatidou D, P+¦tzsch C, K+¦nig K, Rosenberger P. Netrin-1 Signaling Dampens Inflammatory Peritonitis. J Immunol. 2011; 186:549–555. [PubMed: 21098235]

17. Samuelsson B. Arachidonic acid metabolism: role in inflammation. Z.Rheumatol. 1991; 50(Suppl 1):3–6. [PubMed: 1907059]

18. Seibert K, Masferrer JL. Role of inducible cyclooxygenase (COX-2) in inflammation. Receptor. 1994; 4:17–23. [PubMed: 8038702]

19. Gilroy DW, Colville-Nash PR. New insights into the role of COX 2 in inflammation. Journal of Molecular Medicine. 2000; 78:121–129. [PubMed: 10868474]

20. FitzGerald GA. COX-2 and beyond: approaches to prostaglandin inhibition in human disease. Nat Rev Drug Discov. 2003; 2:879–890. [PubMed: 14668809]

21. Knight S, Johns EJ. Effect of COX inhibitors and NO on renal hemodynamics following ischemia.reperfusion injury in normotensive and hypertensive rats. American Journal of Physiology - Renal Physiology. 2005; 289:F1072–F1077. [PubMed: 15956774]

22. Do+é-Ögowska B, B+éogowski W, Doma+äski L. Dynamics of thromboxane level changes during early phase of allograft reperfusion. Clinical Transplantation. 2009; 23:716–722. [PubMed: 19298388]

23. Garvin PJ, Niehoff ML, Robinson SM, Heisler T, Salinas-Madrigal L, Contis J, Solomon H. Evaluation of the thromboxane A2 synthetase inhibitor OKY-046 in a warm ischemia-reperfusion rat model. Transplantation. 1996; 61:1429–1434. [PubMed: 8633364]

24. Hamilton TA, Rigsbee JE, Scott WA, Adams DO. gamma-Interferon enhances the secretion of arachidonic acid metabolites from murine peritoneal macrophages stimulated with phorbol diesters. J Immunol. 1985; 134:2631–2636. [PubMed: 3919102]

25. Shi H, Yokoyama A, Kohno N, Hirasawa Y, Kondo K, Sakai K, Hiwada K. Effect of thromboxane A2 inhibitors on allergic pulmonary inflammation in mice. European Respiratory Journal. 1998; 11:624–629. [PubMed: 9596113]

26. Yao C, Sakata D, Esaki Y, Li Y, Matsuoka T, Kuroiwa K, Sugimoto Y, Narumiya S. Prostaglandin E2-EP4 signaling promotes immune inflammation through Th1 cell differentiation and Th17 cell expansion. Nat Med. 2009; 15:633–640. [PubMed: 19465928]

27. Li L, Huang L, Vergis AL, Ye H, Bajwa A, Narayan V, Strieter RM, Rosin DL, Okusa MD. IL-17 produced by neutrophils regulates IFN-gamma-mediated neutrophil migration in mouse kidney ischemia-reperfusion injury. J Clin.Invest. 2010; 120:331–342. [PubMed: 20038794]

28. Li L, Huang L, Sung Ss, Vergis AL, Rosin DL, Rose CE Jr. Lobo PI, Okusa MD. The chemokine receptors CCR2 and CX3CR1 mediate monocyte//macrophage trafficking in kidney ischemia.reperfusion injury. Kidney Int. 2008; 74:1526–1537. [PubMed: 18843253]

29. Li L, Okusa MD. Macrophages, Dendritic Cells, and Kidney Ischemia-Reperfusion Injury. Seminars in Nephrology. 2010; 30:268–277. [PubMed: 20620671]

30. Lemos HP, Grespan R, Vieira SM, Cunha TM, Verri WA, Fernandes KSS, Souto FO, McInnes IB, Ferreira SH, Liew FY, Cunha FQ. Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFN+¦ production. PNAS. 2009; 106:5954–5959. [PubMed: 19289819]

31. Villanueva S, C+¬spedes C, Gonz+ílez AA, Vio CP, Velarde V. Effect of ischemic acute renal damage on the expression of COX-2 and oxidative stress-related elements in rat kidney. American Journal of Physiology - Renal Physiology. 2007; 292:F1364–F1371. [PubMed: 17244895]

32. Schneider R, Meusel M, Renker S, Bauer C, Holzinger H, Roeder M, Wanner C, Gekle M, Sauvant C. Low-dose indomethacin after ischemic acute kidney injury prevents downregulation of Oat1/3 and improves renal outcome. American Journal of Physiology - Renal Physiology. 2009; 297:F1614–F1621. [PubMed: 19794109]

33. Feitoza CQ, Goncalves GM, Semedo P, Cenedeze MA, Pinheiro HS, Beraldo FC, dos Santos OF, Teixeira VP, dos Reis MA, Mazzali M, Pacheco-Silva A, Camara NO. Inhibition of COX 1 and 2 prior to renal ischemia/reperfusion injury decreases the development of fibrosis. Mol Med. 2008; 14:724–730. [PubMed: 18769637]

34. Matsuyama M, Yoshimura R, Hase T, Kawahito Y, Sano H, Nakatani T. Study of cyclooxygenase-2 in renal ischemia-reperfusion injury. Transplantation Proceedings. 2005; 37:370–372. [PubMed: 15808647]

35. Profita M, Sala A, Bonanno A, Riccobono L, Ferraro M, La Grutta S, Albano GD, Montalbano AM, Gjomarkaj M. Chronic obstructive pulmonary disease and neutrophil infiltration: role of cigarette smoke and cyclooxygenase products. Am J Physiol Lung Cell Mol Physiol. 2010; 298:L261–L269. [PubMed: 19897740]

36. Aso H, Ito S, Mori A, Morioka M, Suganuma N, Kondo M, Imaizumi K, Hasegawa Y. Prostaglandin E2 enhances interleukin-8 production via EP4 receptor in human pulmonary microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol. 2012; 302:L266–L273. [PubMed: 22080750]

37. Simon LS. Role and regulation of cyclooxygenase-2 during inflammation. Am J Med. 1999; 106:37S–42S. [PubMed: 10390126]

38. Lukiw WJ, Ottlecz A, Lambrou G, Grueninger M, Finley J, Thompson HW, Bazan NG. Coordinate Activation of HIF-1 and NF-+¦B DNA Binding and COX-2 and VEGF Expression in Retinal Cells by Hypoxia. Investigative Ophthalmology & Visual Science. 2003; 44:4163–4170. [PubMed: 14507857]

39. Jung YJ, Isaacs JS, Lee S, Trepel J, Neckers L. IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. FASEB J. 2003; 17:2115–2117. [PubMed: 12958148]

40. Oliver K, Taylor C, Cummins E. Hypoxia. Regulation of NFkappaB signalling during inflammation: the role of hydroxylases. Arthritis Research & Therapy. 2009; 11:215. [PubMed: 19291263]

41. Wang W, Brian RW, Ramesh G. Netrin-1 and kidney injury. I. Netrin-1 protects against ischemia-reperfusion injury of the kidney. Am J Physiol Renal Physiol. 2008; 294:F739–F747. [PubMed: 18216145]

42. Ramesh G, Reeves WB. p38 MAP kinase inhibition ameliorates cisplatin nephrotoxicity in mice. Am J Physiol Renal Physiol. 2005; 289:F166–F174. [PubMed: 15701814]
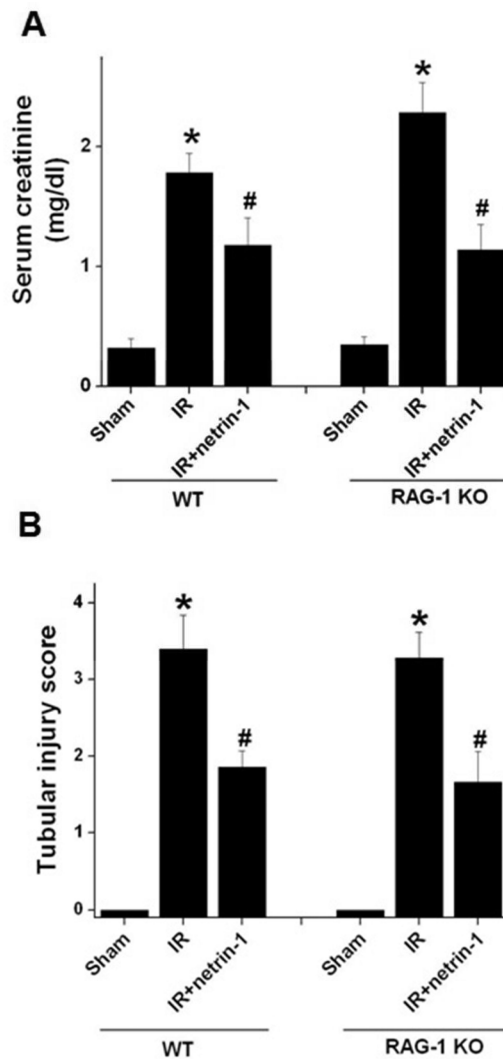
43. Ramesh G, Reeves WB. p38 MAP kinase inhibition ameliorates cisplatin nephrotoxicity in mice. Am J Physiol Renal Physiol. 2005; 289:F166–F174. [PubMed: 15701814]

**Figure 1.**
Netrin-1 administration protects kidney from ischemia reperfusion-induced dysfunction. A. Serum creatinine was measured at 24hr after reperfusion in wild-type (WT) and RAG-1 knockout mice subjected to sham operation or ischemia followed by reperfusion (IR) with vehicle or netrin-1 administration. Values are mean ± SEM. *, $p < 0.01$ vs. all other groups. #, $p<0.05$ vs. IR; n = 6-8 for each group. B. Tubular necrosis was quantified as described in Materials and Methods. Netrin-1 treatment significantly suppressed ischemia reperfusion induced tubular necrosis in both WT and RAG-1 knockout mice. Values are mean ± SEM. *, $p < 0.001$ vs. all other groups. #, $p<0.05$ vs. IR; n = 4-6 for each group.
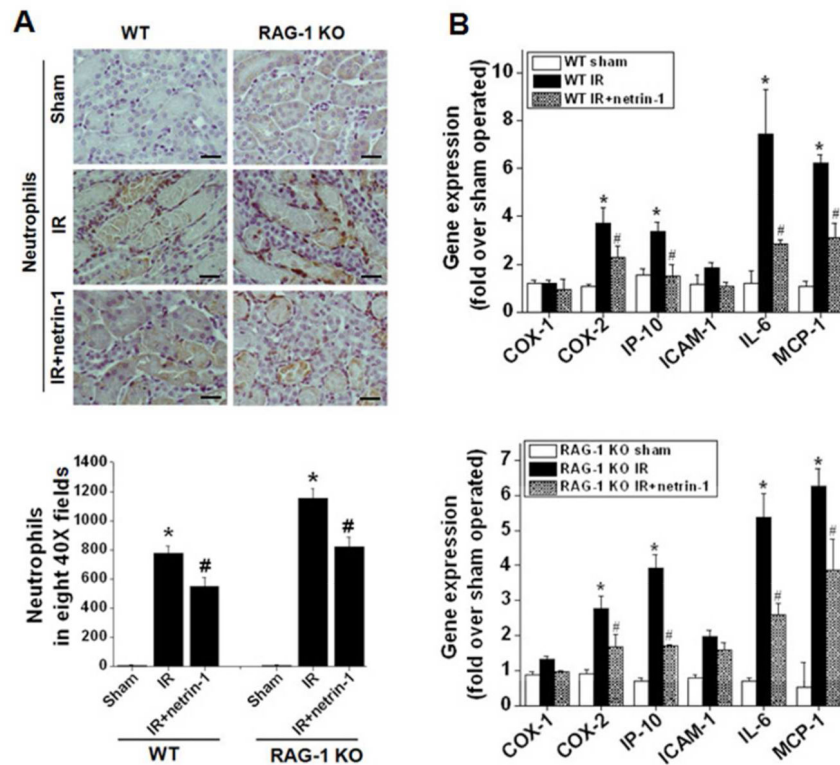
**Figure 2.**

Netrin-1 administration suppressed inflammation in kidney in response to ischemia reperfusion (IR). Neutrophil infiltration was quantified after staining kidney sections as described in Materials and Methods. A. Top: Neutrophil staining is absent in sham-operated kidney, but is increased after IR. Netrin-1 administration suppressed neutrophil staining. Scale Bar: 50μM. A. Bottom: Quantification of neutrophil staining in kidney. *, $p<0.001$ vs. all other groups. #, $p<0.05$ vs. IR. Values are mean ± SEM. n=4. B. Inflammatory mediator expression in kidney analyzed by real-time RT-PCR. Top: WT mice subjected to sham, IR with/without netrin-1 administration. Bottom: RAG-1 knockout mice subjected to sham, IR with/without netrin-1 administration. *, $p<0.05$ vs. sham. #, $p<0.05$ vs. IR. Values are mean ± SEM. n=4.
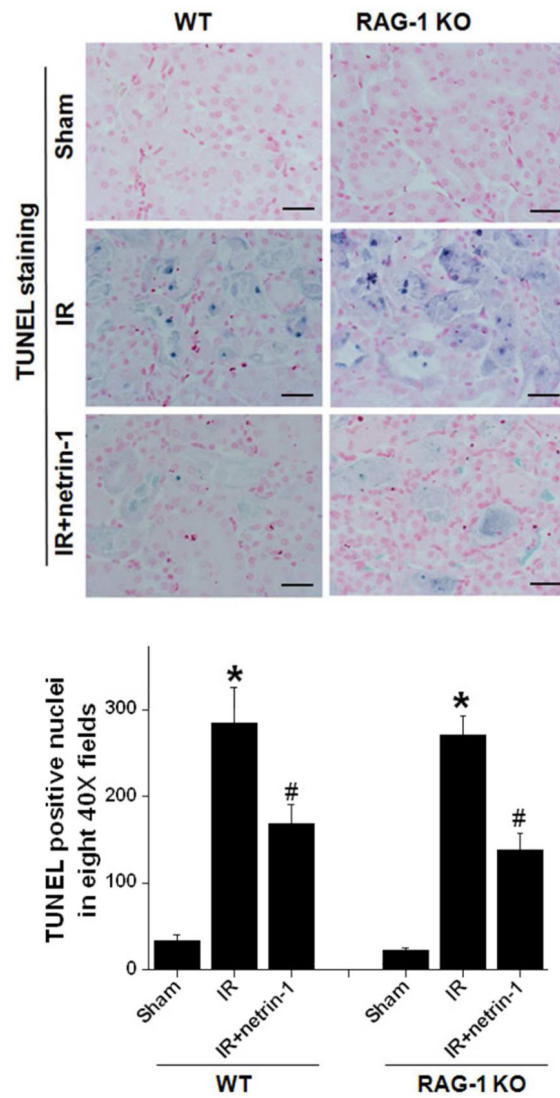
**Figure 3.**
Effect of netrin-1 administration in ischemia reperfusion-induced apoptosis. Apoptotic cells were identified using TUNEL staining (top panel). Sham.operated wild-type (WT) and RAG-1 knockout mouse kidney did not show any apoptotic cells. Twenty.four hours after reperfusion, the number of apoptotic cells (blue staining) was increased dramatically in WT as well as RAG-1 knockout mice but was decreased with administration of netrin-1. Bottom Panel. Quantification of apoptotic cells. Apoptotic-positive cells were counted in eight random 40X fields. *, $p<0.001$ vs. sham-operated. #, $p<0.001$ vs. IR. n=4-6. Scale Bar: 50μM.
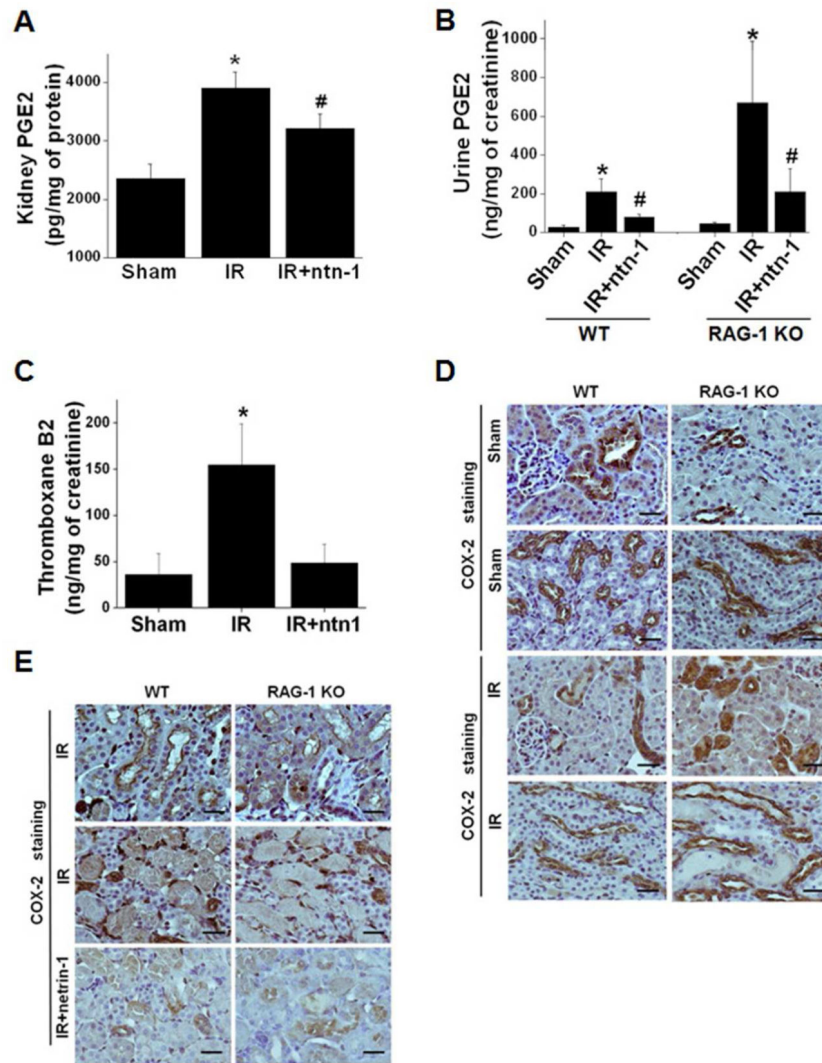
**Figure. 4.**

Netrin-1 administration suppressed COX-2 expression and PGE2 production in both wild.type (WT) and RAG-1 knockout mouse kidney. A. Quantification of kidney PGE2 by EIA kit in WT mice. Ischemia reperfusion (IR)-induced increase in PGE2 was suppressed by netrin-1. *, $p<0.001$ vs. sham. #, $p<0.05$ vs. IR. Values are means ± SEM. n=4-6. B. Quantification of urine PGE2 by EIA. Ischemia reperfusion-induced increase in the excretion of urine PGE2 was reduced significantly by netrin-1 administration. *, $p<0.001$ vs. sham. #, $p<0.05$ vs. IR. Values are mean ± SEM. n=4-6. C. Quantification of urine thromboxane B2 by EIA. Ischemia reperfusion-induced increase in the excretion of urine thromboxane B2 was reduced significantly by netrin-1 administration. *, $p<0.001$ vs. all other groups. Values are mean ± SEM. n=4-6. D and E. Immunohistochemical localization of COX-2 in WT and RAG-1 knockout mice after different treatments as described in Materials and Methods. COX-2 staining was seen in distal tubules and associated thick ascending limb, and collecting ducts from sham-operated kidney; ischemia reperfusion did not alter staining in distal tubule and thick ascending limp and collecting ducts (D) but

induced COX-2 expression in infiltrating cells, which was suppressed by administration of netrin-1 in both WT and RAG-1 knockout mice (E). Scale Bar: 50μM.
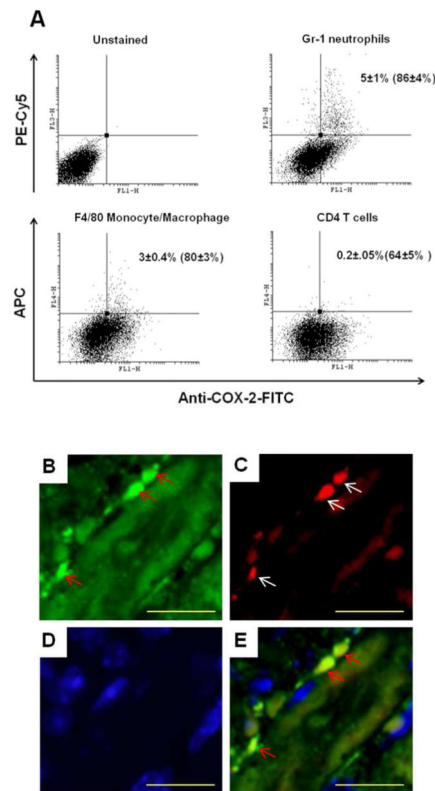
**Figure 5.**
Flow cytometry analysis of COX-2 expressing immune cells in the kidney. A. Flow cytometry analysis was carried out as described in Materials and Methods. Percentage of COX-2 postive cells shown on each histogram (outside bracket) and percentage of COX-2 positive Gr-1 positive neutrophils, F4/80 positive monocyte/macrophage and CD4 positive T cells were shown inside the bracket on each histogram. B-E. Co-localization of COX-2 and neutrophils in kidney. Immunofluorescence staining of COX-2 and neutrophils in WT mice subjected to IR was carried out as described in Materials and Methods. COX-2 (panel B, green) and neutrophil (panel C, red) staining was seen in the interstitium. Panel D is DAPI nuclear staining. Overlay of panel B, C and D is shown in E. COX-1 and neutrophils were co-localized (yellow staining, arrow). Scale Bar: 50μM.
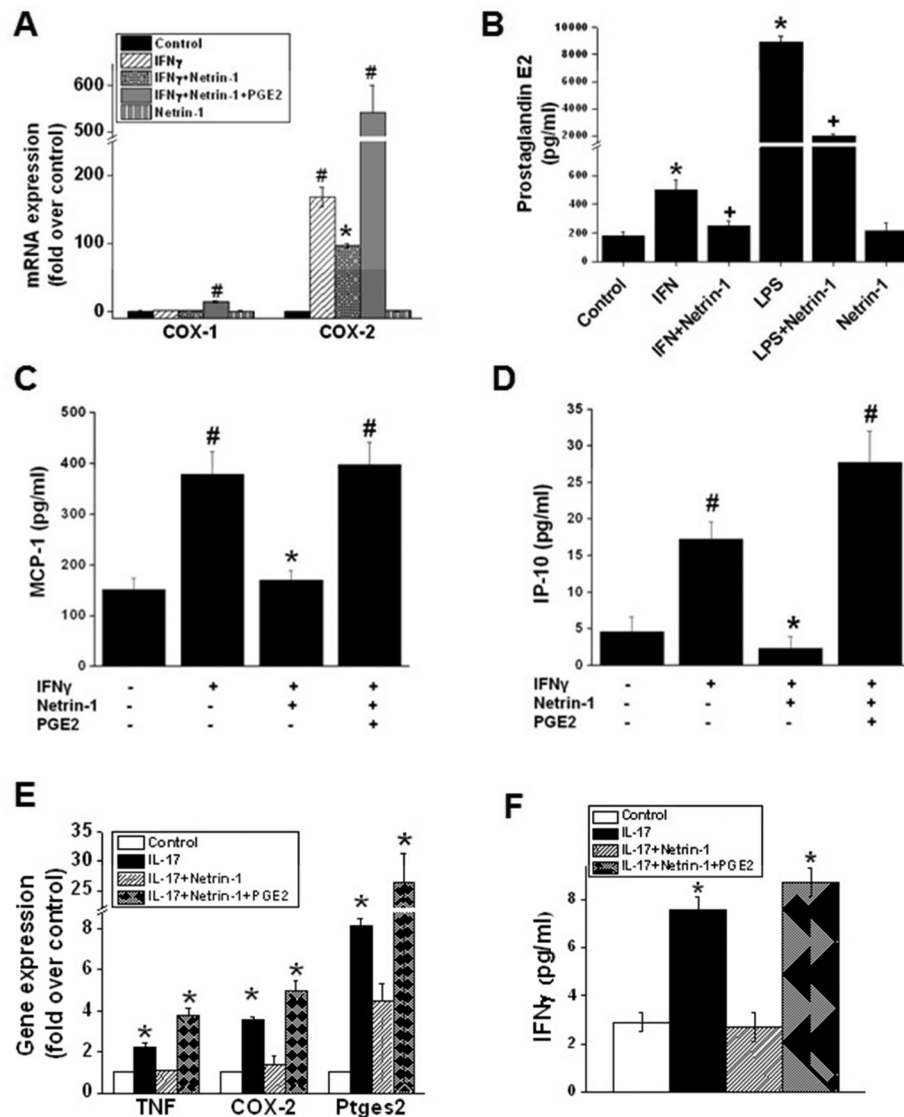
**Figure 6.**
Regulation of COX-2 expression and PGE2 production by netrin-1 in macrophages, neutrophils and T cells. A. IFNγ-induced COX-2 expression was suppressed by addition of netrin-1 in macrophages. Netrin-1-mediated suppression of COX-2 expression was abolished by addition of PGE2. #, $p < 0.001$ vs. all other groups. *$p < 0.001$ vs. IFNγ-treated. B. IFNγ and LPS-induced increase in PGE2 production in macrophages was suppressed with netrin-1 treatment. *, $p < 0.005$ vs. control. +, $p < 0.001$ vs. IFNγ and LPS-treated groups. C and D. IFNγ-induced production of chemokines MCP-1 and IP-10 was inhibited in netrin-1 treated macrophages, which was abolished with PGE2 addition. #, $p < 0.001$ vs. all other groups. *, $p < 0.001$ vs. IFNγ-treated. E and F. IL-17-induced COX-2 expression and inflammatory cytokine production is suppressed by netrin-1 in neutrophils *in vitro*. E. IL-17 induced the expression of TNFα, COX-2, and prostaglandin E synthase 2 (ptges2) in neutrophils, which was suppressed by addition of netrin-1. Addition of PGE2 along with netrin-1 abolished the suppressive effect of netrin-1. *, $p < 0.001$ vs. all other groups. F.

IL-17-induced IFNγ production in neutrophils is suppressed by netrin-1, which was abolished by addition of PGE2. *, $p<0.001$ vs. all other groups. Values are mean ± SEM. n=4-6.
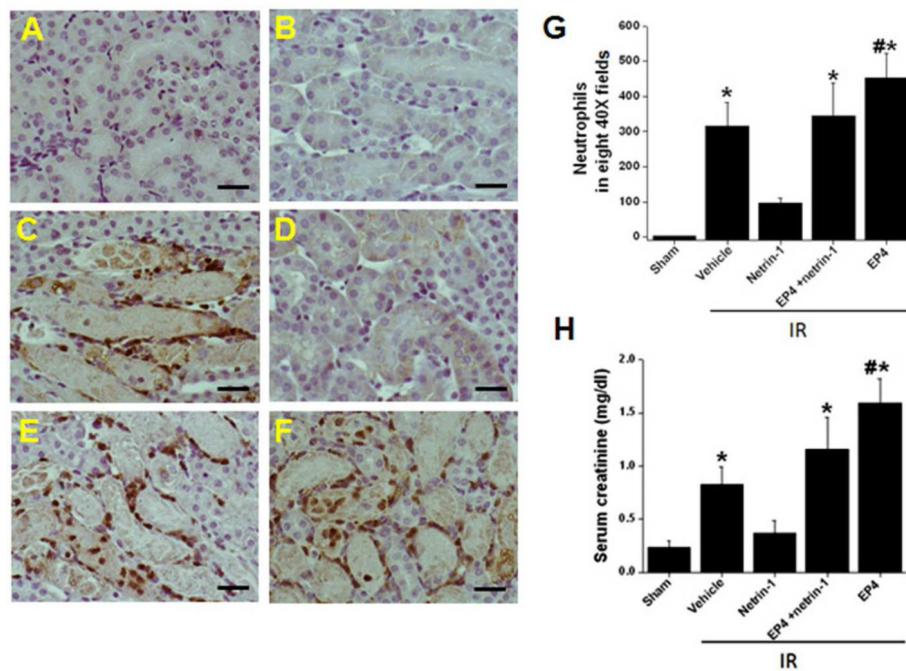
**Figure 7.**
EP4 agonist increases neutrophil infiltration and exacerbates ischemia reperfusion injury of the kidney. Neutrophil infiltration was quantified after staining the kidney section as described in Materials and Methods. Neutrophil staining was absent in antibody control (A) and sham.operated kidney (B), which is increased after IR (C). Netrin-1 administration suppressed neutrophil infiltration (D). Administration of EP4 agonist abolished netrin-1-mediated suppression of neutrophil infiltration (E). Moreover, administration of EP4 agonist alone exacerbated ischemia reperfusion-induced neutrophil infiltration (F). Quantification of neutrophil infiltration into kidney after different treatments (G). *, $p<0.001$ vs. sham and netrin-1 treated groups. #, $p<0.05$ vs. vehicle treated group. Scale Bar: 50μM. H. Kidney function measured by serum creatinine levels. IR-induced kidney dysfunction was suppressed by netrin-1; administration of EP4 agonist exacerbated kidney dysfunction. *, $p<0.001$ vs. sham and netrin-1 treated groups. #, $p<0.05$ vs. vehicle treated group. Values are mean ± SEM. n=4-6.
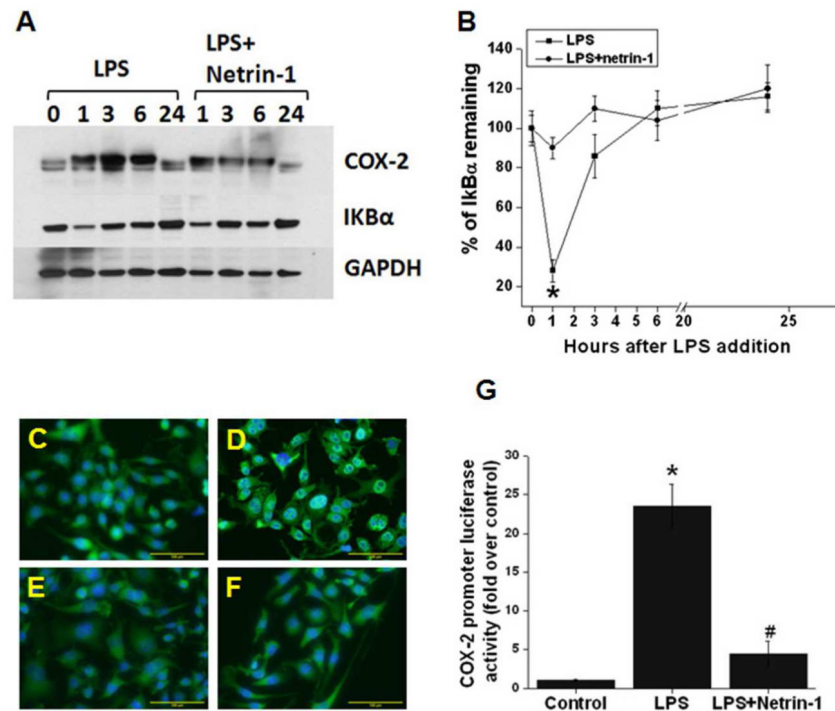
**Figure 8.**

Regulation of NFκB activation by netrin-1 in macrophage cell line (RAW264.7). A. LPS-induced increase in COX-2 expression and IκBα degradation is inhibited by netrin-1. B. Densitometric quantification of IκBα degradation in macrophage at different times after LPS addition. LPS induced IκBα degradation transiently and significantly at 1hr, which was completely inhibited by netrin-1 treatment. C.F: LPS-induced NFκB (p65) translocation into the nucleus was determined by immunofluorescence staining. C. Untreated macrophages showing cytoplasmic localization of p65 (Green). D. 1hr after addition of LPS p65 localization is shifted to the nucleus, indicating the activation state of NFκB. E. Addition of netrin-1 along with LPS suppressed NFκB translocation as determined by presence of cytoplasmic localization. F. Treatment of macrophages with netrin-1 alone did not alter cytoplasmic localization of NFκB seen in untreated control. Nucleus was stained with DAPI (blue). G. LPS-induced increase COX-2 promoter activity was suppressed by addition of netrin-1 in macrophage cell line. *, $p < 0.001$ vs. control. #, $p < 0.001$ vs. LPS-treated cells. Scale bar: 100μM.