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Netrin-1 Regulates Th1/Th2/Th17 Cytokine Production and Inflammation through UNC5B Receptor and Protects Kidney against Ischemia–Reperfusion Injury

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Overwhelming evidence suggests that ischemia–reperfusion injury of the kidney is an inflammatory disease mediated by innate and adaptive immune systems. The neuronal guidance molecule netrin-1 was shown to modulate inflammatory responses. Given that ischemic kidney is particularly prone to reperfusion-elicited inflammation, we sought to determine the function of netrin-1 and its receptor UNC5B in ischemia–reperfusion-induced inflammation. Renal ischemia–reperfusion caused a rapid decrease in serum netrin-1 levels. Administration of recombinant netrin-1 before or after renal ischemia–reperfusion reduced kidney injury, apoptosis, monocyte and neutrophil infiltration, and cytokine (IL-6, IL-1β, and TNF-α) and chemokine (MCP-1, macrophage-derived cytokine, monokine-induced IFN-γ, keratinocyte-derived chemokine, and chemokine with 6 cysteines) production. Analysis for different netrin-1 receptors on leukocytes showed very high expression of UNC5B, UNC5D, neogenin, or deleted in colorectal cancer. Expression of UNC5A was low. Neutralization of UNC5B receptor reduced netrin-1–mediated protection against renal ischemia–reperfusion injury, and it increased monocyte and neutrophil infiltration, as well as serum and renal cytokine and chemokine production, with increased kidney injury and renal tubular cell apoptosis. Finally, investigation into netrin-1’s effect on CD4 T cell stimulation showed suppression of Th1/Th2/Th17 cytokine (IL-2, IL-6, IL-10, IL-13, IL-17, IFN-γ, IL-4, and TNF-α) production in vitro. Our studies demonstrate that netrin-1 acting through UNC5B receptor reduces renal ischemia–reperfusion injury and its associated renal inflammation. The Journal of Immunology, 2010, 185: 3750–3758.
expressed in kidneys, and its level is downregulated in serum after renal IRI. Administration of netrin-1 to mice before or after renal IRI protected kidneys and suppressed leukocyte infiltration. This protective function of netrin-1 against renal IRI was mediated through UNC5B receptor.

Materials and Methods

Mice and surgical procedure
Male C57BL/6J mice, 8–9 wk of age (The Jackson Laboratory, Bar Harbor, ME), were used to induce renal IRI, as described earlier (20, 21). Briefly, mice anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.) were placed on a heating pad maintained at 37°C and subjected to bilateral renal pedicle clamping through dorsal incisions for 26 min. Reperfusion was confirmed visually upon release of the clamps. As control, sham-operation was subjected to the same surgical procedure except for renal pedicle clamping. Surgical wounds were closed, and mice were given 1 ml warm saline i.p. The mice were kept in a warm incubator until they gained consciousness. Experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine.

Netrin-1 quantification by ELISA

Netrin-1 levels in plasma were determined using an ELISA kit (USCN Life Science, Wuhan, China), according to the manufacturer’s instructions. The minimal detection limit is 7.8 pg/ml. All measurements were made in duplicate.

Isolation of mononuclear leukocytes

Mononuclear leukocytes were isolated from peripheral blood using Lymphoprep solution (AXIS-SHIELD, Oslo, Norway) for determining netrin-1 receptor expression. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), and the expression of different netrin-1 receptor mRNA was determined using an SYBR green PCR amplification kit (Qiagen, Valencia, CA).

Flow cytometry

To quantify infiltration of leukocytes into kidneys after reperfusion, mice were perfused with 20 ml saline to remove intravascular leukocytes (15, 22). Kidneys were minced into fragments of 1 mm and digested with 2 mg/ml collagenase D and 100 U/ml DNase I at 37°C. The digested kidneys were passed through 100- and 40-μm mesh, sequentially. RBCs in the resulting renal cells were lysed using RBC lysis buffer (Sigma–Aldrich, St. Louis, MO). Mice blood leukocytes were used to determine UNC5B expression. Renal cells and blood leukocytes were stained using the following fluorochrome-labeled Abs: anti-CD45, UNC5B (AXXORA, San Diego, CA), CD11c, F4/80, CD11b, Gr-1, Ly-6G (BioLegend, San Diego, CA), 7/4 (AbD Serotec, Raleigh, NC) CD4, CD8, B220, NK1.1, CD3, and PDCA-1. FcRs on leukocytes were blocked before staining with rat anti-FcR from 2.4G2 hybridoma supernatant (22). 2.4G2 hybridoma supernatant was kindly provided by Dr. Robert Bonneau (The Penn State College of Medicine). Unless otherwise indicated, the Abs used for flow cytometry were obtained from eBioscience (San Diego, CA) or BD Biosciences (San Jose, CA). Intracellular cytokine staining for IFN-γ was performed using a BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA). After staining renal cells, the cells were treated with fixation and permeabilization buffer containing paraformaldehyde and saponin. The cells were then washed with permeabilization buffer containing saponin and stained for intracellular IFN-γ. We used normal mouse IgG conjugated with FITC as a control for UNC5B receptor staining. Flow cytometry was performed on a FACS Calibur and analyzed using CellQuest (BD Pharmingen) or WinMDI 2.8 software.

Administration of UNC5B blocking Ab and recombinant netrin-1

To determine the effect of netrin-1 in renal IRI, netrin-1 (5 μg/mouse, R&D Systems, Minneapolis, MN) was injected i.v. 2 h before or after renal pedicle clamping or sham surgery. To investigate the role of UNC5B receptor in netrin-1 attenuation of renal IRI, mice were subjected to four treatments: UNC5B blocking Ab (800 μg/kg body weight, R&D Systems); UNC5B blocking Ab + netrin-1; netrin-1; or isotype control Ab (goat IgG). UNC5B Ab or isotype control was injected i.p. 18 h before renal pedicle clamping. The dose of UNC5B Ab and netrin-1 was chosen based on previous studies (20, 23–25). Plasma and kidneys obtained from mice at different time intervals after reperfusion were used to determine the extent of inflammation and kidney injury for different treatments.

Renal function

Renal function was determined by measuring creatinine (DZ072B; Diazyme Labs, Poway, CA).

Histology

Kidney tissue was fixed in buffered 10% formalin for 12 h and then embedded in paraffin wax. For assessment of injury, 5-μm kidney sections were stained with periodic acid-Schiff. Acute tubular necrosis was determined in the outer stripe of the outer medulla and cortex using a semi-quantitative score in which the percentage of tubules showing epithelial necrosis, brush-border loss, and cast formation was assigned a score: 0 = normal, 1 = <10%, 2 = 11–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >76% (26, 27).

TACS TiT in situ apoptosis detection

To identify apoptotic cells, tissue sections were stained using a TACS TiT in situ Apoptosis Detection kit (R&D Systems), according to the manufacturer’s instructions. Briefly, tissue sections were deparaffinized, hydrated, and washed with PBS. Sections were digested with protease K for 15 min at 24°C. After washing the slides, the endogenous peroxidase activity was quenched with 3% H2O2 in methanol and incubated with TiT labeling reaction mixture at 37°C for 1 h. The color was developed by treating with TACS blue label substrate solution. Slides were washed, counterstained, and mounted with Permount solution.

Cytokine and chemokine measurement

Cytokines and chemokines in plasma were measured using an ELISA array kit from SABiosciences (Frederick, MD) and ELISA kit from eBioscience.

Quantitation of mRNA by real-time RT-PCR

Real-time RT-PCR was performed in an Applied Biosystems 7700 Sequence Detection System (Foster City, CA). 1.5 μg total RNA was reverse transcribed in a reaction volume of 20 μl using an Omniscript RT kit and random primers. The product was diluted to a volume of 150 μl, and 6-μl aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Qiagen) and gene-specific primers. UNCSA, UNC5C, and neogenin primers were designed using Primer 3 software, and specificity of the primers was confirmed using Basic Local Alignment Search Tool of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov). All other primers used were described previously (20, 27, 28). The primer sets used were netrin-1 forward: 5′-AAGGCTTACACCCACCGGAAG-3′, reverse: 5′-GGCCCAACAGATTCTTGACGCT-3′; netrin-1 forward: 5′-ATACTTAACAGAGATACACGACGAC-3′, reverse: 5′-CAATAGTGATGACCTGGCCGT-3′; UNC5A, forward: 5′-GGATTTGAGGACGGTGAGCAAGCC-3′, reverse: 5′-TTTCTAATGCTCTTCTACTCTC-3′; DCC, forward: 5′-CTCTTCAGAGATTTGAGGAGGAC-3′, reverse: 5′-AGAGAAGTTGTCACACTATGAG-3′; neutogenin, forward: 5′-CGCTAATCTTGTGATTCTTCT-3′, reverse: 5′-GATGATGTTAACCTAATGCTTGGC-3′; mouse TNF-α, forward: 5′-GCATGATCAGGGACGTTGGAAGA-3′, reverse: 5′-GATCAGTACGGTTCAGAGACCAG-3′; IFN-γ, forward: 5′-CTCGAACAACAGGCGAAAAGG-3′, reverse: 5′-AACCCGAAATCGAGGGACT-3′; IL-6, forward: 5′-GGAGGCTCGACCAACCTGATGATC-3′, reverse: 5′-GCTTCTTGACCC-TCCTTTCTGTG-3′; IL-1β, forward: 5′-CTCTACGAGCTTGCTGCAACG-3′, reverse: 5′-TGCTGATGACCTAGGTTGGGG-3′; IL-4, forward: 5′-CAACGAAGAACACCCAGACGAG-3′, reverse: 5′-GAGATTGCGTACTTACCTGAC-3′; and ICAM-1, forward: 5′-AGATCAGTACGGTTCAGAGACCAG-3′, reverse: 5′-CTCTACGAGCTTGCTGCAACG-3′.

Isolation and stimulation of CD4 T cells and quantification of cytokines

CD4 T cells were isolated from mice (C57BL/6J) spleens using a Dynabeads FlowComp Mouse CD4+D25+ Treg isolation kit (Invitrogen). Briefly, spleens perfused with isolation buffer (PBS with 0.1% BSA and 2
mM EDTA) were passed through 40-μm mesh. RBCs were lysed using RBC lysis buffer (Sigma-Aldrich). Spleen cells suspended at a concentration of 1 × 10^8 cells/ml were mixed with 200 μl Ab mixture containing rat anti-mouse CD45R (B220), CD11b (Mac-1), Ter-119, CD16/32, and CD8 Abs. After incubating the spleen cells for 20 min, non-CD4 T cells were removed by adding depletion beads. Bead-free CD4 T cells were treated with rat anti-mouse CD25 Ab, followed by magnetic beads to remove CD4+D25- T regulatory cells. CD4 T cell purity was determined by flow cytometry using fluorochrome-conjugated CD45, CD4, and CD25 Abs. Purity of CD4+CD25- T cells was >90%. CD4 T cell viability was >98%, as determined by a trypan blue dye exclusion test. CD4 T cells were plated at a concentration of 2 × 10^5 cells/ml in a CD3 Ab-coated 96-well plate or control plate with or without netrin-1. Culture supernatant was harvested at 48 h after plating and assayed for Th1/Th2/Th17 cytokines using ELISA array from SABiosciences. To determine the effect of netrin-1 on CD4 T cell viability, the cells were incubated with MTT (Cell proliferation assay kit; Promega, Madison, WI) for 2 h, and absorbance was recorded at 490 nm. The percentage of viable CD4 T cells was calculated by comparing with untreated cells in a control plate.

Statistical analysis
Results are presented as mean ± SEM from five to eight animals or replicate per condition. Graphpad Instat 3 software was used for statistical analysis. We performed statistical analysis using an unpaired, two-tailed t test for single comparisons and ANOVA for multiple comparisons; p < 0.05 was considered significant.

Results
Netrin-1 is downregulated in circulation after ischemia–reperfusion of the kidney
Our earlier studies showed a decrease in netrin-1 expression on vascular endothelial cells as early as 3 h after renal IRI (20). However, netrin-1 concentration in circulation subsequent to renal IRI is not known. Therefore, we investigated the level of plasma netrin-1 at different time intervals after renal IRI. Netrin-1 in normal mouse plasma was detectable (106 ± 14 pg/ml), and the level was significantly downregulated within 2 h after reperfusion (p < 0.005; Fig. 1A). Downregulation was observed even at 6 and 24 h after reperfusion, consistent with our earlier observation that endothelial cells exhibit decreased expression of netrin-1 after renal IRI (20).

Administration of netrin-1 before or after renal ischemia protects kidneys against IRI
To determine whether netrin-1 modulates renal IRI, mice were administered vehicle or netrin-1 2 h before or after bilateral renal IRI, and renal dysfunction was determined by measuring serum creatinine (Fig. 1B, 1D). As shown in Fig. 1B and 1D, netrin-1 administration resulted in a significant reduction in renal dysfunction at 24 and 48 h after reperfusion compared with vehicle-treated mice.
animals (p < 0.05). These data suggest that netrin-1 can protect kidneys even after the initiation of ischemic injury. Protection of kidneys was associated with a reduction in the infiltration of monocytes and neutrophils (Fig. 1C), suggesting that netrin-1–mediated suppression of leukocyte infiltration may be responsible for the protection of kidneys against renal ischemia–reperfusion.

Netrin-1 receptors, but not netrin-1, are expressed in leukocytes

Our earlier studies (20) and current findings suggest that netrin-1 may mediate its protective functions in renal IRI, in part by suppressing inflammation and infiltration of leukocytes. Earlier studies showed expression of netrin-1 receptor UNC5B but not DCC on granulocytes, monocytes, and lymphocytes (28). However, data on netrin-1 and other netrin-1 receptors’ (UNC5A, UNC5C, UNC5D, and neogenin) expression in leukocytes remain unclear.

Therefore, we examined netrin-1 and netrin-1 receptor expression in different subsets of leukocytes with the notion that netrin-1 acting on leukocytes mediates anti-inflammatory functions. As shown in Fig. 2A, only UNC5B mRNA was expressed significantly, whereas UNC5A was detectable at a lesser level. Expression of UNC5C, UNC5D, neogenin, or DCC was undetectable. To determine the UNC5B expression on leukocytes, blood cells were stained for UNC5B and different populations of leukocytes. As shown in Fig. 2B, with the exception of NK cells, all leukocyte populations in mice showed expression of UNC5B receptor. There was no significant difference in the intensity of UNC5B receptor expression among different leukocyte populations, with the exception of dendritic cells, as calculated from mean channel fluorescence.

**UNC5B neutralization Ab inhibits netrin-1–mediated attenuation of renal IRI**

To determine whether UNC5B mediates netrin-1 protective functions against renal IRI in vivo, four groups of mice were subjected to bilateral renal pedicle clamping and were followed for 72 h after reperfusion. UNC5B Ab or isotype-matched Ab was administered i.p. 18 h before clamping of renal pedicles. Netrin-1 or vehicle was administered i.v. 2 h before renal pedicle clamping. As shown in Fig. 3, netrin-1–treated mice showed improved renal function compared with mice treated with UNC5B Ab or isotype control Ab, as determined by measuring serum creatinine. Mice administered netrin-1 plus UNC5B Ab showed increased renal injury, suggesting that the protective effects of netrin-1 are mediated through UNC5B receptor.

Improved renal function with netrin-1 administration was associated with better preservation of renal morphology compared with vehicle-treated kidneys (Fig. 4I, IV). Administration of UNC5B Ab abolished netrin-1–mediated preservation of kidney morphology by increasing necrosis and cast formation in the renal tubules. In addition, administration of netrin-1–inhibited ischemia–reperfusion-induced apoptosis of tubular epithelial cells (Fig. 4III), which was inhibited in response to UNC5B Ab treatment (Supplemental Figs. 1, 2).
Netrin-1 attenuates renal infiltration of leukocytes through UNC5B receptor in vivo

Our earlier studies (20) and present findings suggest that administration of netrin-1 reduces inflammation in renal IRI, but the role of UNC5B in netrin-1–mediated attenuation of leukocyte infiltration is not known in vivo. To determine whether UNC5B receptor mediates suppression of ischemia–reperfusion-induced renal leukocyte infiltration, kidneys isolated from wild-type (WT) mice subjected to renal IRI and treated with netrin-1 and/or UNC5B Ab were analyzed for different subsets of leukocytes at 6 and 24 h after reperfusion. As shown in Fig. 5, renal IRI in vehicle-treated mice was characterized by a drastic increase in the infiltration of monocytes and neutrophils into kidneys (Fig. 5) compared with sham-operated mice. This increase in renal monocytes and neutrophils was significantly reduced in netrin-1–treated mice, suggesting that netrin-1 attenuates renal infiltration of leukocytes. However, administration of UNC5B Ab prevented netrin-1 suppression of monocyte and neutrophil infiltration into kidneys, suggesting that netrin-1 attenuates leukocyte infiltration through UNC5B receptor. UNC5B Ab inhibition of netrin-1’s protective effects on kidneys was also observed at 24 h after reperfusion (data not shown). Mice treated with UNC5B Ab alone that were subjected to IRI also showed increased infiltration of leukocytes compared with sham-operated mice (data not shown). However, except for a minimal increase in B cells, the number of renal CD4 and CD8 T cells, NK cells, CD11c+ B220+ B cells, 7/4+Ly-6G+ neutrophils, 7/4+Ly-6G− monocytes, CD11b+PDCA-1+ plasmacytoid dendritic cells, CD11c+F4/80− macrophages, and F4/80−CD11c+ dendritic cells in mice subjected to renal IRI was similar to sham-operated mice. These results suggest that netrin-1 inhibits infiltration of leukocytes into kidney by acting through UNC5B receptor.

Studies showed that IFN-γ produced from neutrophils mediates IRI of the kidney (4, 15). Having found that netrin-1 acting through UNC5B receptor inhibits infiltration of monocytes and neutrophils into kidney in renal IRI, we investigated IFN-γ production in them in response to netrin-1 and/or UNC5B Ab administration. Renal cells obtained at 6 h after reperfusion were stained for IFN-γ in response to netrin-1 and analyzed by flow cytometry. We

FIGURE 4. Neutralization of UNC5B inhibits netrin-1 protection of renal morphology and apoptosis. Mice injected with UNC5B Ab, isotype control Ab, netrin-1 + UNC5B Ab, or netrin-1 + isotype control Ab were subjected to renal pedicle clamping or sham surgery. Renal morphology was assessed by periodic acid-Schiff staining. I. Netrin-1 administration reduced ischemia–reperfusion-induced necrosis and cast formation in tubules (C) compared with vehicle-treated (B) mice at 6 h of reperfusion. II. Administration of UNC5B Ab in netrin-1–treated mice increased renal tubular necrosis and cast formation. A, Sham-operated mice kidneys had no visible impact on renal morphology. II. Necrosis was quantified as described in Materials and Methods (n = 4–6). III. Quantification of apoptosis by TUNEL staining (n = 4). *p < 0.001, versus all other groups.

Netrin-1 attenuates renal infiltration of leukocytes through UNC5B receptor in vivo

Our earlier studies (20) and present findings suggest that administration of netrin-1 reduces inflammation in renal IRI, but the role of UNC5B in netrin-1–mediated attenuation of leukocyte infiltration is not known in vivo. To determine whether UNC5B receptor mediates suppression of ischemia–reperfusion-induced renal leukocyte infiltration, kidneys isolated from wild-type (WT) mice subjected to renal IRI and treated with netrin-1 and/or UNC5B Ab were analyzed for different subsets of leukocytes at 6 and 24 h after reperfusion. As shown in Fig. 5, renal IRI in vehicle-treated mice

FIGURE 5. Netrin-1 injection reduces infiltration of monocytes and neutrophils into kidneys. Mice administered isotype control Ab (vehicle), UNC5B Ab, netrin-1, or netrin-1 + UNC5B Ab were subjected to renal pedicle clamping or sham surgery. A and B, Kidneys obtained at 6 h after reperfusion were analyzed by flow cytometry, gating on CD45+ cells for CD4+ and CD8+ T cells, CD3+ “NK1.1” NK cells, CD11c+B220+ B cells, 7/4+Ly-6G+ neutrophils, 7/4+Ly-6G− monocytes, CD11b+PDCA-1+ plasmacytoid dendritic cells, CD11c+F4/80− macrophages, and F4/80−CD11c+ dendritic cells (n = 4). *p < 0.001, versus sham surgery; **p < 0.05, versus vehicle-treated group.
found more neutrophils positive for IFN-γ compared with kidneys of sham-operated mice (Fig. 6). However, the expression level of IFN-γ in neutrophils of different treatment groups was similar to sham-operated control mice (Fig. 6C, 6D).

Our finding is consistent with an earlier report that infiltration of neutrophils positive for IFN-γ, rather than the expression level of IFN-γ in neutrophils, is increased in renal IRI (4).

Netrin-1 reduction of cytokine and chemokine production is mediated through UNC5B receptor in vivo

Cytokines and chemokines play an important role in the activation and migration of leukocytes into injured organ and the exacerbation of tissue injury (3, 12). Having determined that netrin-1 attenuates renal leukocyte infiltration through UNC5B receptor, we investigated the effect of UNC5B receptor neutralization on netrin-1–mediated reduction of chemokine and cytokine production in serum and kidneys after renal IRI. Serum and kidneys obtained from mice treated with netrin-1 and/or UNC5B Ab and subjected to sham or bilateral renal pedicle clamping were analyzed for different cytokines and chemokines by ELISA and real time RT-PCR, respectively. Ischemia–reperfusion significantly increased the levels of cytokines (IL-6 and IL-10) and chemokines (MCP-1, monokine-induced IFN-γ, eotaxin, macrophage-derived cytokine, keratinocyte-derived chemokine, and chemokine with 6 cysteines) in the circulation at 6 h (Fig. 7A) and 24 h (data not shown) after reperfusion in vehicle-treated mice compared with sham-operated mice. Netrin-1 administration significantly reduced the levels of different chemokines and cytokines in the circulation. Netrin-1 suppression of cytokine and chemokine production in renal IRI was significantly reduced in UNC5B Ab-treated mice. Similarly, the expression of different cytokines and chemokines (IL-1β, TNF-α, IL-6, IL-10, MCP-1, and ICAM-1) in kidneys was increased at 6 h (Fig. 7B) and 24 h (data not shown) after reperfusion in mice subjected to IRI compared with sham-operated mice. Administration of netrin-1 suppressed the expression of IL-6 and MCP-1 at 6 and 24 h after reperfusion and the expression of ICAM-1, IL-1β, and TNF-α at 24 h after reperfusion (data not shown). Mice treated with UNC5B Ab before netrin-1 administration exhibited significantly decreased netrin-1–mediated reduction of renal cytokine and chemokine expression in renal IRI, suggesting that netrin-1 attenuates cytokine and chemokine production through UNC5B receptor in renal IRI. Administration of UNC5B Ab alone had no impact on the reperfusion-induced increase in serum and renal cytokine and chemokine production. These results suggest that netrin-1 attenuation of cytokine and chemokine production is mediated through UNC5B receptor.

Netrin-1 regulates production of Th1/Th2/Th17 cytokines from CD4+CD25− T cells

The mechanism through which netrin-1 inhibits infiltration of leukocytes into kidneys is unknown. It is possible that netrin-1 acting
directly on leukocytes suppresses cytokine and chemokine production. Given that CD4 T cells produce cytokines and chemokines and regulate neutrophil infiltration in renal IRI (4), we investigated the effect of netrin-1 on CD4 T cell cytokine production. CD4 T cells depleted of regulatory T cells (Fig. 8A) were stimulated with CD3 Ab in the presence or absence of netrin-1. As shown in Fig. 8B and 8C, the levels of different cytokines produced by CD4 T cells in the absence of CD3 Ab were very low and were comparable to those produced by cells treated with netrin-1 alone. However, in the presence of CD3 Ab, CD4 T cells showed a dramatic and significant increase in Th1/Th2/Th17 cytokines (IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, IFN-γ, and TNF-α) compared with nontreated CD4 T cells (p < 0.0001). The production of IL-5, IL-12, IL-23, and TGF-β cytokines was low and comparable between different groups. Addition of netrin-1 to CD3 Ab-treated CD4 T cells significantly reduced the production of different cytokines (IL-2, IL-4, IL-6, IL-10, IL-13, IFN-γ, and TNF-α) compared with vehicle-treated CD4 T cells (p < 0.0001). The inhibition of cytokine production by netrin-1 was dose dependent. Minimal effect was noticed with 100 ng/ml of netrin-1, and maximal inhibition was observed with 500 ng/ml. It is possible that the observed netrin-1 suppression of cytokine production resulted from the induction of cell death in CD4 T cells. Therefore, we investigated CD4 T cell viability in response to netrin-1 treatment by MTT assay. Rather than cell death, we noticed a moderate increase in CD4 T cell proliferation in response to netrin-1 and CD3 Ab treatment compared with nontreated CD4 T cells (p < 0.001; Fig. 9). These findings suggest that by acting directly on leukocytes, netrin-1 can suppress cytokine production and, thereby, it can attenuate renal IRI.

Discussion

Inflammation plays a very critical role in the progression and exacerbation of ischemic injury to acute kidney injury. Concurrent with the induction of soluble mediators of inflammation, which promote tissue injury, kidneys and other organs were shown to produce a variety of factors in response to ischemic stress (3, 8, 12, 20, 29, 30). Although the function of these stress-induced proteins is not well understood, it was suggested that some of them are cytoprotective. These secreted proteins were reported to reduce ongoing cell injury and/or facilitate renal remodeling after tubular injury. Netrin-1 is one of the cytoprotective proteins expressed in normal kidneys, and it increases in response to injury (20). UNCSB is a receptor of netrin-1 expressed in different organs and tissues, including on lymphocytes, monocytes, and neutrophils (28). Recent findings
and then analyzed for their viability by MTT assay. Netrin-1 had no impact on CD4 T cell viability \((n = 5), p < 0.05, \text{versus control plate.}\)

in vivo and in vitro indicate that netrin-1 regulates migration of leukocytes through UNC5B receptor (28), thereby it may control inflammation. In our previous studies, we showed the expression pattern of netrin-1 and its receptors in renal IRI and netrin-1-mediated amelioration of kidney injury (20, 21, 31), but the receptor through which netrin-1 attenuates renal IRI is unknown. Likewise, the role of UNCSB in renal IRI is not known. In this study, we investigated the effect of netrin-1 neutralization on netrin-1 suppression of inflammation and chemokine and cytokine production in renal IRI.

We noticed a drastic decrease in circulating netrin-1 levels in response to renal IRI. Administration of netrin-1, before or after renal ischemia–reperfusion, attenuated renal dysfunction. Leukocytes showed very high expression of UNCSB receptor, and neutralization of UNCSB receptor inhibited the protective effects of netrin-1 in renal IRI. We also determined that netrin-1, acting through UNCSB receptor, inhibits the infiltration of monocytes and neutrophils, as well as the production of serum and renal chemokines and cytokines. Finally, using CD4 T cells, we showed that netrin-1 inhibits Th1/Th2/Th17 cytokine production in vitro.

Our previous study showed a decrease in renal netrin-1 mRNA levels but an increase in protein levels between 3 and 24 h after reperfusion (20). Under normal physiological conditions, netrin-1 is expressed in peritubular capillaries in the interstitium of the kidneys (20). In response to ischemia–reperfusion, netrin-1 expression decreases in the peritubular capillaries, but it starts to appear in tubular epithelial cells. In the current study, we investigated the effect of renal IRI on circulating netrin-1 levels. Netrin-1 was downregulated rapidly in serum within 2 h after reperfusion. These findings are consistent with our previous findings (20) and studies by Ly et al. (28), suggesting that downregulation of netrin-1 in the circulation and renal endothelial cells may predispose kidneys to exacerbated inflammation and injury. The mechanism by which netrin-1 is decreased rapidly in response to ischemia–reperfusion is not clear. Earlier studies indicated that inflammatory cytokines, such as TNF-α and IFN-γ, can suppress endothelial expression of netrin-1 (28). In our studies, expression of inflammatory chemokines and cytokines increased immediately after renal IRI, suggesting a possible effect of these proinflammatory mediators on netrin-1 levels in serum.

Receptors for netrin-1 include DCC, neogenin, UNCSA–D, and adenosine receptor 2b (18). Kidneys were reported to show significant expression of UNCSB, UNCSA, and neogenin receptors, whereas other netrin-1 receptor expression is negligible (20, 31). Earlier studies showed expression of UNCSB but not DCC on leukocytes (28). Consistent with their observation, only UNCSB receptor expression was noticed in mononuclear leukocytes; other netrin-1 receptor (UNCSA, UNCSD, DCC, and neogenin) expression was undetectable. Very low expression of UNCSA was also observed in leukocytes. Netrin-1 is bifunctional in that it attracts some axons and repels others. The chemorepulsive ability of netrin-1 likely contributes to the inhibition of leukocytes to chemotactic stimuli (18, 32). Studies showed that the interaction of netrin-1 with UNCSB receptor results in potent inhibition of the migration of myeloid cells toward chemotactic stimuli in vivo and in vitro (23, 28). Inflammatory cytokines and chemokines in circulation and kidneys were increased after renal IRI (12, 15, 20, 33). Netrin-1 was shown to attenuate pulmonary inflammation, as well as chemokine and cytokine production in acute lung injury (23). Consistent with these observations, our findings indicate an important function of netrin-1 in attenuation of cytokine and chemokine production and infiltration of monocytes and neutrophils into kidney in renal IRI, most likely acting through UNCSB receptor on leukocytes.

Infiltration of neutrophils is one of the hallmarks of tissue injury. Neutrophils were shown to accentuate renal IRI (4, 15, 34). Cytokines, such as IL-17 and IFN-γ, produced by neutrophils are known to mediate renal IRI (4, 15). We noticed a marked decrease in neutrophil infiltration in response to netrin-1 treatment and attenuation of netrin-1–mediated neutrophil infiltration upon UNCSB receptor neutralization. These neutrophils showed IFN-γ expression as determined by intracellular flow cytometry. Neutrophils are known to contain small stores of IFN-γ that are released upon stimulation by degranulation (4). In this regard, an earlier study showed amelioration of renal IRI subsequent to IFN-γ neutralization (15). Although the infiltration of neutrophils positive for IFN-γ was greater subsequent to ischemic injury, the IFN-γ staining in neutrophils was similar in sham-operated and IRI kidneys. This observation is consistent with a previous study in renal IRI (4) and suggests possible release of IFN-γ from kidney-infiltrated neutrophils in renal IRI.

The response of the innate immune system to pathogens or tissue injury is very rapid. Thus, we see a rapid influx of monocytes and neutrophils into kidney subsequent to renal IRI. In contrast to the innate immune system, the activation of adaptive immune cells takes longer. However, Ag-independent activation of the adaptive immune system is possible (35). For example, a hypoxic condition augments cytokine production in CD4 T cells (36). CD4 T cell cytokines, such as IL-6, IL-17, TNF-α, and IFN-γ, contribute to the pathogenesis of IRI of kidney, liver, lung, and intestine (15, 37–40). The CD4 T cell cytokines are also known to regulate the expression of other soluble mediators of inflammation and the function of leukocytes, including monocytes and neutrophils. Because CD4 T cells show high expression of UNCSB receptor and are known to produce inflammatory cytokines after ischemia–reperfusion (41, 42), we investigated the effects of netrin-1 on CD4 T cells. Interestingly, netrin-1 dramatically inhibited Th1/Th2/Th17 cytokine production, without affecting CD4 T cell viability. However, the signaling pathway through which netrin-1 suppresses cytokine production is not clear. Netrin-1 is known to activate adenylyl cyclase/cAMP pathways (43, 44). cAMP/protein kinase A-mediated activation of CREB protein suppresses cytokine production from immune cells (45, 46). Therefore, it is possible that netrin-1–mediated activation of adenylyl cyclase/cAMP pathways may inhibit production of proinflammatory mediators of inflammation. Taken together, these results are consistent with the view that netrin-1 inhibits cytokine and chemokine production and protects kidney against IRI.

Although our present study provided evidence of netrin-1 attenuation of cytokine production from CD4 T cells, the direct effect of netrin-1 on neutrophil and monocyte inflammatory functions is

**FIGURE 9.** Effect of netrin-1 on CD4⁺CD25⁺ T cell viability. CD4 T cells were incubated for 48 h in the presence or absence of netrin-1 and then analyzed for their viability by MTT assay. Netrin-1 had no impact on CD4 T cell viability \((n = 5), p < 0.05, \text{versus control plate.}\)
not known. Because UNCSCb receptor is highly expressed on mono-
cytes and neutrophils, and earlier studies suggest that netrin-1 mediates suppression of neutrophil migration in vitro (28), it is possible that netrin-1 acting on UNCSCb receptor regulates neutrophil and monocyte chemotaxis, as well as chemokine and cytokine production. Also, it is possible that the decrease in infiltration of monocytes and neutrophils in response to netrin-1 treatment is due to a decrease in renal injury and suppression of chemokine re-
ceptor expression rather than to inhibition of neutrophil functions.

In conclusion, we determined the role of UNCSCb receptor of netrin-1 in renal IRI and examined the effect of netrin-1 on leukocyte migration. Our studies suggest that netrin-1 mediates sup-
pression of inflammatory chemokine and cytokine production and attenuates kidney injury through UNCSCb receptor. The protective function of netrin-1 might be linked to inhibition of production of pro-
flammatory mediators by leukocytes. Further studies on the netrin-1–
directed effects on neutrophils and monocytes are warranted. Elucidation of these mechanisms may serve as a powerful tool for using netrin-1 to treat ischemic diseases of the kidney and other organs.

Disclosures
The authors have no financial conflicts of interest.

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