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Osteopontin Expressed in Tubular Epithelial Cells Regulates NK Cell-Mediated Kidney Ischemia Reperfusion Injury

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Renal ischemia reperfusion injury (IRI) occurs after reduced renal blood flow and is a major cause of acute injury in both native and transplanted kidneys. Studies have shown diverse cell types in both the innate and the adaptive immune systems participate in kidney IRI as dendritic cells, macrophages, neutrophils, B cells, CD4+ NK+ cells, and CD4+ T cells all contribute to this form of injury. Recently, we have found that NK cells induce apoptosis in tubular epithelial cells (TECs) and also contribute to renal IRI. However, the mechanism of NK cell migration and activation during kidney IRI remains unknown. In this study, we have identified that kidney TECs express a high level of osteopontin (OPN) in vitro and in vivo. C57BL/6 OPN-deficient mice have reduced NK cell infiltration with less tissue damage compared with wild-type C57BL/6 mice after ischemia. OPN can directly activate NK cells to mediate TEC apoptotic death and can also regulate chemotaxis of NK cells to TECs. Taken together, our study’s results indicate that OPN expression by TECs is an important factor in initial inflammatory responses that involves NK cells activity in kidney IRI. Inhibiting OPN expression at an early stage of IRI may be protective and preserve kidney function after transplantation. The Journal of Immunology, 2010, 185: 967–973.

In the case of renal transplantation, injury that occurs during organ preservation and the early stages of surgery may lead to delayed graft function, which may then contribute to both early allograft loss and chronic allograft dysfunction (1). Renal ischemia reperfusion injury (IRI), which follows a period of reduced renal blood flow, is a major cause of acute renal failure from many etiologies. Indeed, severe injury can result in a very high mortality rate of 50% (2). Kidney IRI results from multiple molecular events. Although biological changes in tubular epithelial cells (TECs) play an important role in kidney injury and dysfunction, IRI is considered an Ag-independent inflammatory condition that involves multiple factors leading to tubular and endothelial dysfunction (3–5). We and others have demonstrated that TEC injury contributes to kidney dysfunction after exposure to inflammation and proinflammatory cytokines (6–8). After ischemia, oxidative stress induces cytokine and chemokine upregulation, which enhances the recruitment of inflammatory cells that are capable through the expression of cytokines, such as IFN-γ and TNF-α, of mediating both direct and indirect tissue injury (9, 10). Recent studies suggest in addition to classical involvement of innate immunity effectors, such as neutrophils, diverse cell types of the adaptive immune system can also participate in kidney IRI, including resident dendritic cells, B cells, CD4+ NK+ cells, CD3− NK.1+ cells, and CD4+ T cells that also infiltrate into the kidney shortly after injury (11–21). Interestingly, as T and B cell-deficient Rag1−/− mice develop severe IRI, innate immune responses are clearly important in this type of kidney injury (22, 23). However, this does not exclude a significant role for diverse effectors in acute kidney injury.

Our recent study indicates that NK cells can directly kill TECs, and that NK cells contribute substantially to kidney IRI (24). NK cell killing may represent an important under-recognized mechanism of kidney injury that results from diverse forms of inflammatory injury, including transplantation. In that study, we demonstrated that activation of NK cells was essential to induce apoptosis of TECs in vitro. However, it is unknown what kidney factors activate NK cells or enhance their migration to the kidney after IRI.

Osteopontin (OPN), also known as the early T cell activation gene 1, is a secreted phosphorylated gp that is expressed in macrophages, T lymphocytes, and NK cells rapidly after activation (25–27). OPN acts as a proinflammatory cytokine and induces Th1-type cell immune responses (25, 28). It costimulates TCR-mediated T cell proliferation and prevents apoptosis (29). OPN augments production of IFN-γ and IL-12 but inhibits IL-10 production (28, 29). Furthermore, OPN has been shown to regulate Th1-mediated autoimmune demyelinating diseases in the rodent experimental autoimmune encephalomyelitis model (30, 31) as well as in infectious diseases (25, 28). As OPN levels increase after renal IRI, we considered whether OPN might play a role in NK cell activation or function in IRI.

Studies have reported that kidney cells produce a high level of OPN after injury as previously reviewed (32), suggesting OPN might play an important role in kidney injury after IRI. The impact of OPN in kidney injury could be both positive and negative, namely, inhibiting apoptosis or recruiting inflammatory cells. Indeed, a previous study reported that OPN deficiency significantly inhibited...
macrophage infiltration and increased apoptosis with less tubulo-interstitial fibrosis (33). In contrast to this finding, others have found OPN deficiency elicited significantly higher serum creatinine levels and more prominent morphological injury than occurred with wild-type mice (34). As the role of OPN appears to have divergent functions, we were interested in determining the role of OPN in NK activation, chemotaxis, and kidney injury.

Materials and Methods

Animals

C57BL/6 (B6, H-2b) and Rag1−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). OPN−/− were initially developed in 129 mice and backcrossed with C57BL/6 mice for nine generations (35). All animals were maintained in the animal facility at the University of Western Ontario using approved protocols and procedures.

TEC culture

The TEC line B6 NG1.1 was developed as previously described (6). Primary TECs were obtained from C57BL/6 or OPN−/− mice kidney after digestion with collagenase (Sigma-Aldrich, St. Louis, MO) for 15 min and then centrifugation, leukocytes were collected at the interface and counted, and Biotec, Auburn, CA). Purity of NK cells was confirmed by flow cytometry (Invitrogen, Carlsbad, CA), 100 ng/ml sodium selenite, and 18 ng/ml hydrocortisone), 100 U/ml penicillin and streptomycin (100 µg/ml), glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), and β-mercaptoethanol (0.5 mM). Activated or naive NK cells were used as effector cells in a killing assay. Cell death was confirmed with 7-aminoactinomycin-D (7-AAD) staining, according to the manufacturer’s protocol. Formalin-fixed kidney sections were stained with H&E, and injury was scored in a double-blinded fashion by a single pathologist, using an arbitrary scoring system (0: no change; 1+: <25% area change; 2+: 25–50% area change; 3+: >50% area change). Criteria for kidney injury included tubular necrosis, immune cell infiltration, tubular casts, and glomerular necrosis. Kidney sections were embedded in OCT compound and snap frozen in liquid nitrogen. Cryostat sections were fixed in acetone and stained with OPN Ab (R&D Systems) using a standard protocol. All slides were stained by a strepavidin-biotin immunoperoxidase and its substrate method by a standard method.

Abs and reagents

Cells were characterized with fluorescent-conjugated mAbs that specifically recognize CD3, CD4, CD8, NK1.1, DX5 (CD49b), and NKGD2 (eBioscience, San Diego, CA). Anti-OPN and recombinant mouse OPN (rmOPN) were purchased from R&D Systems (Minneapolis, MN).

Isolation of NK cells

NK cells were purified from C57BL/6 or Rag1−/− mouse spleens using anti-CD49b MACS beads selection after depletion CD3+ T cells (Miltenyi Biotec, Auburn, CA). Purity of NK cells was confirmed by flow cytometry and >94% cells were CD3− CD49b+. To isolate infiltrating NK cells, kidneys were collected at 0, 4, 24, and 48 h after IRI (renal artery clamping, 45 min at 32˚C), then digested with collagenase (Sigma-Aldrich) to make single-cell suspensions. All kidney cells were then suspended in 38% Percoll before being laid onto 72% Percoll. After 1000 g and 30 min of centrifugation, leukocytes were collected at the interface and counted, before being stained with anti-CD45, anti-CD3, and anti-CD49b (DX5).

Cytotoxicity assay

NK cells were purified by CD49b+ selection on a MACS beads column (Miltenyi Biotec), then activated in the presence of rmOPN (0.1–5 µg/ml) in RPMI 1640, supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), glutamine (2 mM), sodium pyruvate (1 mM), HEPES (10 mM), and β-mercaptoethanol (0.5 mM). Activated or naive NK cells were used as effectors cells in a killing assay. Cell death was confirmed with 7-aminoactinomycin-D (7-AAD) staining, according to the manufacturer’s protocol (BD Biosciences, San Diego, CA). Briefly, TECs were labeled with 0.5 µM CFSE (Molecular Probes/Invitrogen, Burlington, Ontario, Canada) for 10 min and washed three times with medium. The CFSE-labeled TECs were mixed with NK cells or cultured alone for 5 h. 7-AAD staining and flow cytometry were used to determine the percentage of the apoptotic/necrotic TECs by gating on CFSE− cells.

Kidney reperfusion injury

Ischemia was induced in mice by clamping the left kidney for 45 min at 32˚C on a thermo-regulated pad. To assess function of the ischemic kidney, after the clamps were released, the right kidney was removed. Kidneys were sampled at different time points after being flushed with cold PBS. Sham controls were treated with the same operative procedure as in the injury group but kidneys were not clamped. Serum was collected for creatinine detection by a Jaffe reaction method with an automated CX5 clinical analyzer (Beckman, Fullerton, CA).

Quantitative real-time PCR

Total RNA was extracted from kidneys with a spin column, according to the manufacturer’s protocol (Qiagen, Mississauga, Ontario, Canada). cDNA pools were synthesized with the First Strand Synthesis System according to the manufacturer’s protocol (Stratagene, La Jolla, CA). Primers were designed using Primer Express, primer designing software from Applied Biosystems (Streetsville, Ontario, Canada). OPN: 5′-GCC TGT TTG CTA GTG CCT CCT C-3′ and 5′-CAC AGC ATT CTG TGG CCG AAG G-3′; IFN-γ: 5′-CAT TGA AAG CCT AGA AAG TCT GA-3′ and 5′-TAG CGA TGC AAA ATG TTG ATA TC-3′; IL-4: 5′-AGC TAG TTG TCA TCC TGC TCT TC-3′ and 5′-AGC ATG GTG CTC CAG TAC TAC-3′; and β-actin: 5′-CCA GCC TTC CTT CCT GGG TA-3′ and 5′-CTA GAA GCA TTT GGG GGT CA-3′. The gene sequences were obtained from the www.ncbi.nlm.nih.gov database. Real-time quantitative PCR was performed on standardized quantities of cDNA using the Brilliant SYBR Green QPCR Master Mix kit, and amplified DNA products were generated and detected using the Mx4000 system (Stratagene). Each PCR amplification condition was set up in triplicate. β-actin amplification was used as the endogenous control. The normalized Δ threshold cycle value and relative expression levels (2ΔΔCt) were calculated according to the manufacturer’s protocol.

Histology and immunohistochemistry

Formalin-fixed kidney sections were stained with H&E, and injury was scored in a double-blinded fashion by a single pathologist, using an arbitrary scoring system (0: no change; 1+: <25% area change; 2+: 25–50% area change; 3+: >50% area change). Criteria for kidney injury included tubular necrosis, immune cell infiltration, tubular casts, and glomerular necrosis. Kidney sections were embedded in OCT compound and snap frozen in liquid nitrogen. Cryostat sections were fixed in acetone and stained with OPN Ab (R&D Systems) using a standard protocol. All slides were stained by a strepavidin-biotin immunoperoxidase and its substrate method by a standard method.

FIGURE 1. Expression of OPN in kidney. A, Real-time PCR analysis of OPN expression. The kidneys were collected at different reperfusion time points after being perfused with PBS to remove blood from kidneys. Total RNA was extracted (Qiagen) and cDNA pools were synthesized (Stratagene). Real-time quantitative PCR was performed using the Brilliant SYBR Green QPCR Master Mix kit and the Mx3005P system (Stratagene). β-actin amplification was used as the endogenous control. The normalized Δ threshold cycle value was calculated according to the manufacturer’s protocol. The results represented average data from three mice at each time point. *p < 0.05; **p < 0.01; ***p < 0.001. B, Expression of OPN in naive and 48 h IRI kidneys was determined by immunohistochemistry (anti-OPN, R&D Systems). Similar staining was obtained from other mice in the same group. The positive staining areas are depicted by arrows in the pictures. IRI and naive kidney were compared. Magnification ×200.
Migration assays
Assays with primary lymphocytes used transwells with 5-μm pores (Costar, Corning, NY). Briefly, NK cells (1 \times 10^6) from C57BL/6 or Rag \(-/-\) mice were labeled with 0.5 μM CFSE and placed in each upper chamber. Lower chambers contained complete medium with TECs or varying doses of rmOPN (R&D Systems). Transwell plates were incubated for 2–4 h. Total migrated cells were quantified by FACS to confirm CFSE positive NK cells. Quantification of migrated NK cells was determined as: migration index = migrated cells in the presence of TECs or OPN/ migrated cells in the absence of TECs or OPN.

ELISA
OPN in TEC culture was detected using ELISA kits according to the manufacturer’s protocol (R&D Systems). B6 TECs or OPN \(-/-\) TECs were seeded in 24-well plates the day before supernatants were collected. The supernatants were spun for 1500 rpm for 5 min before ELISA analysis. The obtained OD value was compared with serial diluted standard control of OPN (R&D Systems).

Statistical analysis
Data were compared using Student t test (Statview, Nesbit, MS) for unpaired values and one-way ANOVA for multigroup differences. The p values <0.05 were considered significant. Significance levels are provided in figures.

Results
Kidneys express high levels of OPN during IRI
Our recent study demonstrated that activated, but not naive, NK cells can induce apoptosis of TECs in vitro, and activated NK cells (CD69\(+\)) were present in kidneys during IRI (24). We were interested in identifying kidney factors that promote NK cell migration and as well as activate NK cells in the kidney after IRI. In general, chemokines and cytokines present within the kidney during early injury might alter NK cell function. IFN-γ, IL-2, and IL-15 are well-documented cytokines for activation and function of NK cells. However, as lymphocytes very quickly infiltrate into the kidney within 30 min after injury (14, 18) with a peak around 4 h after IRI (14, 24), we hypothesized that pre-existing or quickly released kidney factors in the kidney must participate in the initial NK cell migration after injury. We used real-time PCR to quantify various cytokines in the kidney after IRI. The expression of IL-15 and IFN-γ in the kidney was very low, and we were not able to detect significant levels of IL-2 and IL-7 after IRI (data not shown). In contrast, we have found that mRNA and protein expression of OPN was markedly increased within the kidney shortly after IRI (Fig. 1A). Importantly, in immunohistochemistry analysis, basal expression of OPN was evident in naive kidneys and, in keeping with previous findings (32), kidney expression of OPN increased after IRI (Fig. 1B).

Kidneys collect more numbers of NK cells
We generated C57BL/6 mice as described in Materials and Methods. A, Serum creatinine in naive and IRI mice. Data represented average of 4 or 12 experiment mice in each group. B and C, Representative histology of IRI kidney. Data represented one mouse from each group. Similar data were obtained from all mice in the same group. The injury areas are depicted by arrows in the pictures. Magnification \( \times 100 \). C, Kidneys were collected for pathology scoring analysis as described in Materials and Methods. Data represented average of 12 experiment mice in each group. Criteria for kidney injury, including tubular necrosis, immune cell infiltration, cast, and glomerular necrosis were scored: 0: no change; 1+: <25% area change; 2+: 25–50% area change; 3+: 50–75% area change; 4+: >75% area change. Data were obtained using six mice from each group. *p < 0.05; **p < 0.01.

FIGURE 2. OPN \(-/-\) mice have less kidney IRI. A, Sera, collected after 1–5 d of IRI, were used for creatinine level analysis. Data represented average of 4 or 12 experiment mice in each group. B, Representative histology of IRI kidney. Data represented one mouse from each group. Similar data were obtained from all mice in the same group. The injury areas are depicted by arrows in the pictures. Magnification \( \times 100 \). C, Kidneys were collected for pathology scoring analysis as described in Materials and Methods. Data represented average of 12 experiment mice in each group. Criteria for kidney injury, including tubular necrosis, immune cell infiltration, cast, and glomerular necrosis were scored: 0: no change; 1+: <25% area change; 2+: 25–50% area change; 3+: 50–75% area change; 4+: >75% area change. Data were obtained using six mice from each group. *p < 0.05; **p < 0.01.

FIGURE 3. OPN \(-/-\) kidneys have less NK cells infiltrating after IRI. A, Kidneys were collected after 0, 4, and 24 h of IRI. After being digested by collagenase, the cells were suspended in 38% Percoll (Sigma-Aldrich) and overlaid on 72% Percoll, then centrifuged at 1000 \( \times g \) for 30 min. Leukocytes were collected at interface of Percoll and counted before being stained with anti-CD45, anti-CD3, and anti-CD49b (DX5). Average number of CD3 \(^+\) CD49b \(^+\) NK cells from each mouse before and after IRI. The results represent pooled data from 10–16 mice. B and C, Real-time PCR analysis of IFN-γ and IL-4 expression in kidney. The kidneys were collected at different reperfusion time points after being perfused with PBS to remove blood from kidneys. β-actin amplification was used as the endogenous control. The normalized β threshold cycle value was calculated. The results represented average data from four mice at each time point. D and E, Real-time PCR analysis of IFN-γ and IL-4 expression in TECs or NK cells cocultures. Primary TECs were obtained from C57BL/6 or OPN \(-/-\) mice kidneys as described in Materials and Methods. TECs alone or cocultured with NK cells were collected 24 h after and used for real-time PCR analysis. Data were pooled from three repeated experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
OPN deficiency leads to less kidney injury

Previous studies have had diverging conclusions on the role of OPN in renal IRI as OPN deficiency either inhibited kidney injury (33) or elicited more prominent morphological damage (34). As many variables alter IRI susceptibility, including background strain and condition of ischemia, we tested IRI using OPN-deficient mice on the C57BL/6 background, in a standardized model of injury controlling clamp time and kidney temperature. All mice were treated with 45 min of ischemia, followed by reperfusion injury. As shown in Fig. 2A, OPN−/− mice consistently had less injury and better function, demonstrated by lower creatinine levels at 24 and 48 h compared with wild-type C57BL/6 mice (24 h: 75 ± 40 versus 104 ± 42 μmol/l, p < 0.01 and 48 h: 43 ± 28 versus 75 ± 31 μmol/l, p < 0.01). Consistent with serum creatinine levels, an independent, double-blind pathology analysis also indicated that OPN−/− kidneys had lower scores of injury compared with wild-type C57BL/6 mice (1.1 ± 0.6 versus 1.9 ± 0.8, p < 0.05, Fig. 2B, 2C). Collectively, our data indicate that OPN clearly promotes ischemic kidney injury.

OPN−/− mice have less infiltrating NK cells after IRI

Previous studies have demonstrated that CD4+ T cells and NK cells infiltrate into the kidney shortly after IRI, typically within 30 min to a few hours (11–18). However, infiltrating leukocytes appear to return to previous basal levels by 24 h after injury. We have also found that NK cells infiltrate into the kidney shortly after IRI and peak at around 4 h (24). We analyzed NK cell infiltration in OPN−/− and C57BL/6 male mice that underwent 45 min of ischemia, followed by reperfusion. Whole kidneys were digested with collagenase V and kidney cells were separated from infiltrating leukocytes using gradient Percoll, as described in Materials and Methods. Isolated total cells were analyzed by flow cytometry. Similar to previous reports and our study in wild-type mice, NK cells (CD3−CD49b+) increased quickly after 4 h; however, significantly fewer infiltrating NK cells were found in kidneys after 4 h in OPN−/− mice compared with C57BL/6 mice (Fig. 3A, 6.3 ± 1.6 × 104 versus 3.6 ± 1.5 × 104, p < 0.01).

OPN is considered to be a proinflammatory cytokine and induces Th1-type cell immune responses (25, 28). Less NK cell migration in OPN deficient kidney may be due to Th1 and Th2 balance alternation in the kidney. To test this possibility, we first analyzed expressions of Th1 (IFN-γ) and Th2 (IL-4) cytokines by real-time PCR. Expressions of IFN-γ and IL-4 were increased in kidneys of C57BL/6 and OPN−/− mice after IRI. IFN-γ is higher in wild-type B6 kidney than OPN−/− kidney at 4 h IRI (Fig. 3B), but there is no difference of IL-4 expression between the two groups (Fig. 3C). Serum cytokine levels were collected from IRI mice and used for ELISA. We did not find significant levels of IFN-γ and IL-4 proteins in

![Figure 4](http://www.jimmunol.org/10.4049/jimmunol.1700970)

**FIGURE 4.** OPN regulates NK cell migration through an indirect effect. A. Production of OPN in TECs. The 1–4 × 10⁵ C57BL/6 or OPN−/− TECs were placed in 12-well plates, and supernatants were collected after 24 h culture without any treatment. Production of OPN by TECs was confirmed by ELISA (R&D Systems). Data were pooled from three independent experiments. B. NK cells migration to TECs. NK cells (10⁵) were purified from C57BL/6 mice by anti-CD49b MACS beads after depleting T cells, then labeled with 0.5 μM CFSE, and placed in each upper chamber of transwells with 5-μm pores (Costar). Lower chambers were seeded with TECs (2 × 10⁵) developed from C57BL/6 or OPN−/− mice for 24 h. Neutralizing anti-OPN (0.1–10 μg/ml, R&D Systems) was added 30 min before NK cells were placed on top chambers. Total migrated cells were quantified by FACS to gate on CFSE positive NK cells after 2 h. Level of migrated NK cells was determined by migration index (migration index = total migrated cells/cells in medium control well). Data were averaged from four different experiments. C. NK cell migration toward OPN assay. Various concentrations of OPN were added into the lower chamber of the transwell. The 10⁵ purified NK cells were added on the top wells. Two hours after, migration of NK cells was quantified, as described previously. Data were averaged from four different experiments. D. NK cell migration toward OPN-treated TEC assay. Various concentrations of rmOPN protein (R&D Systems) were added into the lower chamber of the transwell together with OPN−/− TEC (2 × 10⁵) for 24 h. NK cells (10⁵) were added on the top wells. Two hours after, migration of NK cells was quantified as described previously. Data were averaged from four repeated experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
NK cells migrate to OPN-producing TECs

The finding that OPN−/− kidneys have less infiltrating NK cells, compared with wild-type mice, after injury suggests a role for OPN in promoting NK cell migration. We tested the effect of OPN on NK cell migration using an in vitro transwell system. We first determined whether TECs produce soluble OPN in vitro. TECs were purified and developed as described in Materials and Methods. As ELISA results showed in Fig. 4A, TECs produce high basal levels of OPN in supernatant without stimulation, whereas, as expected, OPN−/− TECs do not produce OPN protein. We then tested the capacity of OPN−/− TECs to attract NK cells in a transwell system. Naive NK cells were purified and loaded on upper chambers without any further treatment. Lower wells were seeded with 2 × 10^5 TECs that were allowed to attach and culture for 24 h. Migration of CFSE-labeled NK cells was quantified by flow cytometry after 2 h incubation. NK cells were found to migrate toward wild-type C57BL/6 TEC, whereas much fewer NK cells were attracted by OPN−/− TEC (Fig. 4B). Interestingly, anti-OPN Ab (0.1, 1, and 10 μg/ml) consistently failed to inhibit NK cell migration rate to TECs (Fig. 4B, data not shown). These data would suggest that OPN expression by TECs was involved in NK migration but that the effect of OPN may be indirect. To further test this, we added recombinant OPN to lower wells without any TECs. NK cells did not show a significant increase of migration to rOPN protein (Fig. 4C). Furthermore, when OPN protein was added to OPN−/− TECs at lower wells, migration of NK cells was increased compared with OPN−/− TECs alone (Fig. 4D).

OPN can activate NK cells that then mediate TEC death

Our data indicate OPN does play an important albeit a possible indirect role in NK cell migration to kidney. OPN can act as a proinflammatory cytokine and induces Th1-type cell immune responses (25, 28). It costimulates TCR-mediated T cell proliferation, including promoting proinflammatory cytokine expression, upregulation of adhesion molecules, leukocyte infiltration, and activation. Various chemokines, including CC, CXC, and CX3C families, have been identified during kidney ischemia reperfusion injury (36). Hence, chemokines should have a significant effect on the migration of macrophages, neutrophils, NK cells, and T cells during kidney IRI. However, as demonstrated in our study and others, leukocytes infiltrate into the kidney as quickly as 30 min to 4 h (14, 18, 24) suggesting that factors that promote injury must be rapidly upregulated or released within the kidney. Naive kidneys express OPN as confirmed in the current study (Fig. 1) and monocyte/macrophage accumulation has been reported at OPN-expressing regions in the kidney (37–41), whereas OPN deficiency has a significant reduction in macrophage infiltration in the kidney after IRI (33). In other study models, OPN promotes accumulation of macrophages in obesity-induced adipose tissue (42) and OPN can trigger neutrophil infiltration into injured livers or into inflammation sites (43–45). Collectively, these studies suggest that OPN might be a candidate protein for modifying renal IRI by its effects on many structures in the kidney, including TECs.

Discussion

Both innate and adaptive immune systems participate in kidney IRI and the successful attenuation of IRI will need to consider DC, CD4+ T cells as well as macrophages and neutrophils. Our previous study has supported a role for NK cells in tubular cell injury in IRI, as they infiltrate into the kidney quickly after ischemia and can induce kidney TEC death and kidney injury in vitro and in vivo (24). In this study, we have identified OPN to play a key role in attracting and activating NK cells within the kidney during IRI. We have demonstrated that OPN expression by TECs and within kidneys regulates chemotactic activity of TECs for NK cell migration. Importantly and for the first time, we have demonstrated that OPN activates NK cells to enhance cytotoxic capacity to TECs in vitro. Consistent with its inflammatory functions, OPN-deficient mice had decreased injury and improved function after acute IRI. Hence, our data support a key role for OPN in promoting NK cell-mediated injury, with relevance to diverse forms of inflammatory kidney injury during transplantation that invariably results in ischemic injury.

In general, chemokines are early major mediators for inflammation, including promoting proinflammatory cytokine expression, upregulation of adhesion molecules, leukocyte infiltration, and activation. Various chemokines, including CC, CXC, and CX3C families, have been identified during kidney ischemia reperfusion injury (36). Hence, chemokines should have a significant effect on the migration of macrophages, neutrophils, NK cells, and T cells during kidney IRI. However, as demonstrated in our study and others, leukocytes infiltrate into the kidney as quickly as 30 min to 4 h (14, 18, 24) suggesting that factors that promote injury must be rapidly upregulated or released within the kidney. Naive kidneys express OPN as confirmed in the current study (Fig. 1) and monocyte/macrophage accumulation has been reported at OPN-expressing regions in the kidney (37–41), whereas OPN deficiency has a significant reduction in macrophage infiltration in the kidney after IRI (33). In other study models, OPN promotes accumulation of macrophages in obesity-induced adipose tissue (42) and OPN can trigger neutrophil infiltration into injured livers or into inflammation sites (43–45). Collectively, these studies suggest that OPN might be a candidate protein for modifying renal IRI by its effects on many structures in the kidney, including TECs.

![Image](http://www.jimmunol.org/)

FIGURE 5. OPN activates NK cells in vitro. A, Activation of NK cells by OPN. OPN (5 μg/ml, R&D Systems) was added into NK cells that were purified from C57BL/6 mice as described in Materials and Methods. After 4 d, NK cells were collected for FACS analysis with anti-CD49b, granzyme B, and anti-CD69 staining. B, OPN-activated NK cells were used as effectors. TEC lines C57BL/6 NG1.1 were labeled with CFSE and used as a target. The mixtures were cultured for 5 h before harvest and stained by 7-AAD. CFSE−/7-AAD− cells were calculated after a flow cytometry analysis. The results were averaged from triplicate wells. Similar results were repeated in a separate experiment. *p < 0.05; **p < 0.01; ***p < 0.001.
cell types. In the current study, we have found that NK cells have augmented migration to OPN-producing TECs in vitro (Fig. 4). In vivo as well, OPN-null kidneys had fewer infiltrating numbers of NK cells after injury compared with wild-type mice, also suggesting chemotactic activity of OPN (Fig. 3). However, our data indicate that OPN does not directly attract NK cells (Fig. 4C) and adding OPN protein to TECs could increase NK cell migration (Fig. 4D). Hence, our data suggest that OPN is an important regulator for chemotaxis of NK cells, but this is an indirect effect on inducing the expression of chemotactic factors, such as chemokines.

Previous studies demonstrate that OPN-deficient mice have a reduced number of NK cells in comparison with wild-type mice (46), which may result in a decrease of NK cell migration into kidney after IRI. However, a significant amount of NK cells exist in OPN−/− mice. NK cells still maintain their function in OPN−/− mice. In our study, a significant level of NK cells still migrate into kidney in OPN−/− mice after IRI (Fig. 3A). Furthermore, we used NK cells purified from OPN−/− mice in a transwell assay, and a similar migration result was seen in wild-type C57BL/6 NK cells (data not shown). Although it is not likely that reduced NK cell infiltration was due to a lower number of NK cells in OPN−/− mice, we cannot rule out this possibility.

IFN-α, IFN-γ, IL-2, IL-12, and IL-15 are well-documented cytokines involved in the activation and function of NK cells. We used quantitative PCR to assess expression of cytokines in the kidney after IRI. Although IL-15, IFN-α, and IFN-γ were expressed at very low levels, we could not detect IL-2, IL-7, or IL-12. Similar to previous observations (32), and in contrast to other cytokines, we found that kidney expression of OPN mRNA was markedly increased after IRI. Although OPN is a pleiotropic functional protein with proinflammatory Th1-like cytokine activity, it is also an antiapoptotic molecule that can promote cell survival. This is consistent with observations with increased expression of OPN in various animal and human kidney injury models (32) in which regulation of organ injury and regeneration after inflammation might be expected (33, 47–49). OPN is an important factor for tissue repair and can inhibit TEC apoptosis as well as participate in the regeneration and repair of TECs (32, 34, 47–52). The role of OPN in kidney injury is controversial. In the current study, we have demonstrated that OPN activates NK cells, which we show to be a prerequisite to induce TEC apoptotic death in vitro (Fig. 5). These data indicate that OPN can activate naive NK cells to induce apoptosis in TECs and suggest that OPN-expressing TECs may be capable of activating NK cells in situ to worsen kidney injury.

Although TECs may express OPN after IRI to prevent apoptosis and enhance tissue repair, OPN together with other chemokines and cytokines may contribute to injury by the activation of infiltrating leukocytes, including NK cells and T cells. In this scenario, inhibiting OPN expression at an early stage of injury may limit the extent of renal injury. Indeed, in our study, OPN-deficient mice experienced less acute kidney injury. This is in agreement with a previous report in which kidney function and morphology were preserved in OPN-null kidneys early in IRI with reduced macrophage infiltration and fibrosis in later stages (33). In contrast, OPN-null mice have reported to have more pronounced IRI (higher creatinine level and kidney histological abnormality) than wild-type mice, which would support a renoprotective function of OPN on TECs (34). The reason(s) for this discrepancy is unknown but may be due to the variable resistance and susceptibility of different mouse strains to IRI (53). In our model, OPN-null mice clearly had less severe kidney injury with reduced creatinine levels, less tissue damage, and fewer infiltrating NK cells. There are several possible mechanisms that may help explain the difference in results compared with previous reports (33, 34). Different genetic backgrounds may contribute to this difference in observed injury (53). This might result from pleiotropic functions of OPN as aforementioned. OPN is a proinflammatory cytokine that can enhance Th1-like immune responses (28–34). OPN also participates in tissue repair and prevents TEC apoptosis. Induction of inflammatory responses and contribution of OPN to antiapoptosis/tissue repair may vary in mice with different genetic backgrounds. Hybrid strains in particular with mixed MHC genetic backgrounds (129xC57BL/6 F2 versus 129xBalb Black Swiss) display various degrees of IRI. In this study, we used OPN-null mice on a near-complete C57BL/6 background to control for this variability between control and experimental groups to more clearly define the role of OPN in kidney injury. In addition, the amount of experimental injury is altered by organ temperature. In comparison with a previous study (33), we were able to obtain a reduced level of injury by applying a lower temperature, and this may have made differences in injury levels more apparent.

In summary, the current study demonstrates that OPN augments NK cell migration, activates NK cells, and enhances NK cell-mediated TEC apoptotic death. These effects would be expected to potentiate inflammation and injury in the kidney during ischemia and reperfusion. As OPN-deficient mice have less infiltrating NK cells and less tissue damage after IRI, inhibition of OPN expression at an early stage of injury may be beneficial in preserving kidney function during inflammation.

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Disclosures

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