IL-1 Up-Regulates Osteopontin Expression in Experimental Crescentic Glomerulonephritis in the Rat

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Osteopontin (OPN) is a macrophage chemotactic and adhesion molecule that acts to promote macrophage infiltration in rat anti-glomerular basement membrane (GBM) glomerulonephritis. The present study investigated the role of interleukin-1 (IL-1) in the up-regulation of renal OPN expression in this disease model. Accelerated anti-GBM glomerulonephritis was induced in groups of six rats. Animals were treated by a constant infusion of the IL-1 receptor antagonist or saline (control) over days -1 to 14 (induction phase) or days 7 to 21 (established disease). In normal rat kidney, OPN was expressed in a few tubules (<5%) and absent from glomeruli. During the development of rat anti-GBM disease (days 7 to 21), there was substantial up-regulation of OPN mRNA and protein expression in glomeruli (>5 cells per glomerular cross-section) and tubular epithelial cells (50–75% OPN-positive). Up-regulation of OPN expression was associated with macrophage accumulation within the kidney, severe proteinuria, loss of renal function, and severe histological damage including glomerular crescentic formation and tubulointerstitial fibrosis. In contrast, IL-1 receptor antagonist treatment of either the induction phase of disease or established disease significantly reduced OPN mRNA and protein expression in glomeruli ($\downarrow$ 75–85%, $P < 0.001$) and tubules ($\downarrow$ 45–60%, $P < 0.001$). The reduction in OPN expression was associated with significant inhibition of macrophage accumulation and progressive renal injury. *In vitro*, the addition of IL-1 to the normal rat tubular epithelial cell line NRK52E up-regulated OPN mRNA and protein levels, an effect that was dose-dependent and inhibited by the addition of IL-1 receptor antagonist, thus demonstrating that IL-1 can act directly to up-regulate renal OPN expression. In con-

clusion, this study provides *in vivo* and *in vitro* evidence that IL-1 up-regulates OPN expression in experimental kidney disease and support for the argument that inhibition of OPN expression is one mechanism by which IL-1 receptor antagonist treatment suppresses macrophage-mediated renal injury. (Am J Pathol 1999, 154:833–841)

Osteopontin (OPN) is a highly acidic glycoprotein that contains an adhesive arginine-glycine-aspartic acid sequence.¹ OPN functions as a cell adhesion and migration molecule which can bind to a number of ligands including the α v β 3 integrin (vitronectin receptor), CD44, collagen type I, and fibronectin.^{1–3} A wide range of cell types including osteoclasts, some epithelia, macrophages, T cells, smooth muscle cells, and some tumors has been shown to express OPN in a constitutive or inducible fashion.^{2–9} The adhesive functions of OPN are thought to be involved in diverse biological activities such as bone absorption, tumor metastasis, and inhibition of renal stone formation.^{2,9–11} A functional role for OPN in monocyte infiltration at sites of inflammation has recently been established. OPN, which binds avidly to macrophages, induces prominent monocyte infiltration when injected subcutaneously in mice.¹² In addition, macrophage accumulation induced by intradermal injection of the chemoattractant *N*-formyl-met-leu-phe in rats is inhibited by administration of a neutralizing anti-OPN antibody.¹³

Macrophage infiltration is thought to play an important role in mediating renal injury in both immune and non-immune forms of kidney disease.¹⁴ A clear association between up-regulation of OPN expression and macrophage infiltration has been described in a wide range of experimental models of glomerular and interstitial nephritis.^{15–22} A functional role for OPN in promoting macrophage-mediated renal injury has recently been demonstrated in rat crescentic anti-glomerular basement

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membrane (GBM) glomerulonephritis.²³ Administration of a neutralizing anti-OPN antibody during the induction phase of the disease (days 0 to 7) significantly inhibited glomerular and interstitial macrophage and T cell infiltration and the associated glomerular injury (proteinuria), loss of renal function, and histological damage including glomerular crescent formation and tubulointerstitial lesions. Furthermore, delaying administration of the anti-OPN antibody until disease was established caused a partial reversal of renal injury and damage.²³

Having established the functional importance of OPN in promoting macrophage-mediated renal injury, the next issue is to identify the factors that up-regulate OPN expression within the kidney. We postulate that the cytokine interleukin-1 (IL-1) may be an important inducer of renal OPN expression, based on two observations. First, IL-1 has been shown to up-regulate OPN gene expression in chondrocytes and osteoblasts.^{24,25} Second, blocking IL-1 activity inhibits macrophage infiltration and renal injury in rat anti-GBM disease.^{26,27} Therefore, the current study examined whether IL-1 is an important inducer of OPN expression in experimental crescentic glomerulonephritis.

Materials and Methods

Experimental Glomerulonephritis

This study used archival material from two previous studies.^{26,27} Accelerated autologous anti-GBM glomerulonephritis was induced in inbred male Sprague-Dawley rats (150–200 g) by subcutaneous immunization with 5 mg normal rabbit IgG in Freund's complete adjuvant, followed 5 days later (termed day 0) by an intravenous injection of 10 ml/kg rabbit anti-rat GBM serum (12.5 mg IgG/ml). In the first experiment, disease was induced in two groups of six rats which were treated from day –1 until being killed at day 14 (induction phase of disease) by a constant infusion of either human recombinant IL-1 receptor antagonist (IL-1ra) (Amgen, Boulder, CO) or saline by means of an Alzet 2002 miniosmotic pump implanted under the skin of the back. In the second experiment, disease was induced in three groups of six rats each. One group was killed on day 7 with no treatment. The other two groups were treated with IL-1ra or saline starting on day 7 and maintained until the animals were killed on day 21 (established disease). One group of six normal rats was also studied.

Immunohistochemistry

Immunohistochemical staining for OPN protein and macrophage accumulation was performed on formalin-fixed, paraffin-embedded sections using a microwave antigen retrieval method.^{22,23,28} Sections were dewaxed and treated with 10 minutes of microwave oven heating in 400 ml of 0.01 mol/L sodium citrate, pH 6.0, at 2450 MHz and 800W. After preincubation with 10% fetal calf serum (FCS) and 10% normal goat serum in PBS for 20 minutes, sections were drained and then labeled with mouse

mAbs to rat OPN (MPIIB10, obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA)^{29,30} or rat macrophages (ED1)^{31,32} for 60 minutes, washed 3 times in phosphate-buffered saline (PBS) and endogenous peroxidase inactivated by incubation in 0.3% H₂O₂ in methanol. Sections then were washed in PBS, incubated with peroxidase-conjugated goat anti-mouse IgG, washed in PBS, incubated with mouse peroxidase antiperoxidase complexes, and developed with 3,3-diaminobenzidine to produce a brown color.

Double immunostaining was used to detect OPN and macrophages within the same section. After staining with the ED1 mAb was completed, as described above, sections were given a second round of microwave oven heating to block antibody cross-reactivity and enhance detection of OPN. Following precubation as above, sections were incubated with the MPIIB10 mAb, then incubated sequentially with alkaline phosphatase-conjugated goat anti-mouse IgG and mouse alkaline phosphatase anti-alkaline phosphatase complexes and developed with Fast Blue BB Salt (Ajax Chemicals, Melbourne, Australia). No staining was seen in negative control sections using the 73.5 IgG1 (anti-human CD45R) irrelevant isotype control mAb. All peroxidase- and alkaline phosphatase-conjugated antibodies and complexes were purchased from Dakopatts (Glostrup, Denmark).

Probes

A 1-kb cRNA probe was generated from the rat smooth muscle osteopontin cDNA clone 2B7.⁸ Sense and antisense cRNA probes were labeled with either [³⁵S] or digoxigenin (DIG)-UTP using a RNA polymerase kit (Boehringer Mannheim, Mannheim, Germany). A 358-bp cRNA antisense riboprobe for rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also DIG-labeled. Probe labeling was determined by liquid scintillation counting (³⁵S-labeled probes) or by dot blotting (DIG-labeled probes).

In Situ Hybridization

In situ hybridization was performed on 4- μ m paraffin sections of formalin-fixed tissues using a radioactive method as described previously.³³ Tissue sections were hybridized with 300,000 cpm/section of sense or anti-sense OPN cRNA probe at 55°C. After washing, the hybridized probe was detected by emulsion photography. Only low levels of background hybridization were seen using the sense probe (0 to 2 grains/cell).

Quantitation of Immunohistochemistry and in Situ Hybridization Staining

Positively stained cells were quantitated in tissue sections as previously described.²² Briefly, the number of cells labeled with the antisense OPN cRNA probe (defined as >5 grains per cell) or the different mAbs were counted under high power in at least 50 glomerular

cross-sections (gcs) per animal. The number of tubules labeled with the antisense OPN cRNA probe or the different mAbs were scored under high power in at least 1000 cortical tubules. All scoring was performed on coded slides. Data are expressed as the mean $\pm$ SE for groups of six animals.

Cell Culture

A normal rat kidney epithelial-derived cell line, NRK52E, was obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's minimal essential medium (DMEM) containing 2% fetal calf serum (FCS). Cells were grown to confluence in 125-cm² plastic tissue culture flasks, the medium was changed to serum-free, and the cells were then cultured with or without recombinant mouse IL-1 α (1, 10, or 20 ng/ml) for up to 5 days. Preincubation of cells with IL-1ra (20 μ g/ml) for 30 minutes was used specifically to block IL-1 stimulation of cultured cells.

Northern Blot Analysis

Northern blotting was performed as previously described.^{22,34} Briefly, total cellular RNA from cultured NRK52E cells was extracted using the RNAzol reagent (Gibco BRL, Gaithersburg, MD) and 20- μ g samples denatured with glyoxal and dimethylsulfoxide, size-fractionated on 1.2% agarose gels, and capillary-blotted onto positively charged nylon membranes (Boehringer Mannheim). Membranes were hybridized overnight at 68°C with DIG-labeled cRNA probes in DIG Easy Hyb solution (Boehringer Mannheim). Following hybridization, membranes were washed finally in 0.1 $\times$ SSC/0.1% sodium dodecyl sulfate (SDS) at 68°C. Bound probes were detected using sheep anti-DIG antibody (Fab) conjugated with alkaline phosphatase and development with CPD-Star enhanced chemiluminescence (Boehringer Mannheim). Chemiluminescence emissions were captured on Kodak XAR film and densitometry analysis performed using the Gel-Pro Analyzer program (Media Cybernetics, Silver Spring, MD).

Western Blot Analysis

NRK52E cells were grown in 125-cm² flasks with or without IL-1 and analyzed by Western blotting as previously described.³⁵ Cells were washed in PBS and then lysed in 1 ml of 1% Nonidet P-40, 25 mmol/L Tris-HCl, 150 mmol/L NaCl, 10 mmol/L EDTA, pH 8.0, containing a 1:50 dilution of a protease inhibitor cocktail (P2714, Sigma-Aldrich Co., Castle Hill, Australia) for 30 minutes on ice. Samples were centrifuged at 14,000 g for 5 minutes to pellet cell debris. Samples (20 μ g) were mixed with SDS polyacrylamide gel electrophoresis sample buffer, boiled for 5 minutes, electrophoresed on a 10% SDS polyacrylamide gel, and electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham International, Buckinghamshire, UK). The membrane was blocked in PBS containing 5% skimmed milk powder, 1% FCS, and 0.02% Tween 20

and then incubated for 1 hour with 5 μ g/ml of MPIIB10 mAb diluted in the above buffer. After washing, the membrane was incubated with a 1:20,000 dilution of peroxidase-conjugated goat anti-mouse IgG in PBS containing 1% normal goat serum and 1% FCS. The blot was then developed using the ECL detection kit (Amersham) to produce a chemiluminescence signal which was captured on X-ray film.

Results

OPN Expression in Normal Rat Kidney

In situ hybridization and immunostaining identified OPN mRNA and protein expression by some glomerular parietal epithelial cells in normal rat kidney (Figure 1A). In addition, constitutive OPN expression was seen in the thick ascending limbs of the loop of Henle, accounting for less than 5% ($3.3 \pm 1.8\%$) of cortical tubules (Figures 1A, 2A, and 2B).

IL-1ra Treatment Suppresses OPN Expression in the Induction Phase of Rat Crescentic Glomerulonephritis (Experiment 1)

There was marked up-regulation of OPN mRNA and protein expression on day 14 of rat anti-GBM glomerulonephritis in saline-treated rats. In glomeruli, both podocytes and infiltrating macrophages (shown by double immunostaining) expressed OPN, whereas crescents showed strong OPN mRNA and protein staining (Figures 1B, 2A, 2B, and 3A). There was also a striking increase in tubular OPN expression ($50.6 \pm 3.2\%$ OPN-positive cortical tubules), most particularly in proximal tubular epithelial cells, which was associated with focal infiltration of ED1-positive macrophages and tubular damage (Figures 1B, 2B, and 3A).

IL-1ra treatment significantly inhibited glomerular and tubular up-regulation of OPN expression on day 14 of anti-GBM disease (Figures 1C, 2A, 2B, and 3B). This was associated with reduced macrophage accumulation within the glomeruli (10 ± 2.1 versus 4.8 ± 1.9 cells/gcs in saline and IL-1ra treated rats, respectively; $P < 0.01$) and in the cortical tubulointerstitium (170 ± 41 versus 29 ± 2.5 cells/mm²; $P < 0.01$). As previously described,²⁶ IL-1ra treatment suppressed proteinuria (332 ± 80 versus 260 ± 62 mg/24hrs; $P < 0.05$), prevented a decline in creatinine clearance (0.66 ± 0.1 versus 0.96 ± 0.25 ml/min; $P < 0.01$), and reduced histological injury, including glomerular crescent formation ($13 \pm 11.2\%$ versus $1.2 \pm 1.6\%$, $P < 0.01$).

IL-1ra Treatment Suppresses OPN Expression in Established Rat Crescentic Glomerulonephritis (Experiment 2)

The ability of IL-1 to up-regulate OPN expression during the progressive phase of established renal injury was examined in Experiment 2. Crescentic disease was es-

Figure 1. *In situ* hybridization showing OPN mRNA expression in rat anti-GBM disease. **A:** Normal rat kidney showing no OPN mRNA within the glomerular tuft, but OPN expression (black grains) is seen in some parietal epithelial cells and in the thick ascending limbs of Henle. **B:** Marked up-regulation of OPN mRNA within a glomerular crescent (*) and in many cortical tubules on day 14 of saline-treated anti-GBM disease (Experiment 1), which is substantially inhibited by IL-1ra treatment (**C**). **D:** Day 7 of untreated anti-GBM disease, clearly showing up-regulation of glomerular and tubular OPN mRNA. **E:** There is a further increase in renal OPN mRNA expression after saline treatment over days 7 to 21 (Experiment 2), with strong OPN mRNA expression seen within areas of severe tissue damage such as glomerular crescentic formation (*) and tubular atrophy and fibrosis. In contrast, IL-1ra treatment of established anti-GBM disease over days 7 to 21 completely abrogated the increase in OPN expression within glomeruli and tubules (**F**). Original magnification, $\times 250$.

Figure 2. Quantitation of OPN mRNA and protein expression in rat anti-GBM disease. The number of glomerular cells and tubules expressing OPN mRNA or protein was scored in stained tissue sections from saline-treated (closed bars) or IL-1ra-treated (hatched bars) anti-GBM disease or normal rats (open bars). A-B: Experiment 1 with IL-1ra or saline treatment over days 0 to 14 of anti-GBM disease. C-D: Experiment 2 with IL-1ra or saline treatment over days 7 to 21 of anti-GBM disease. One group of animals received no treatment before being killed on day 7 (dotted bars). The number of glomerular cells expressing OPN mRNA or protein (A and C) and the percent of cortical tubules expressing OPN mRNA or protein (B and D) were scored. Data are mean $\pm$ SE for six animals. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by ANOVA.

established by day 7 after anti-GBM serum administration and was associated with significant up-regulation of glomerular and tubular OPN expression (Figures 1D, 2C, and 2D). Saline treatment from days 7 to 21 saw a further increase in glomerular and tubular OPN mRNA and protein expression (Figures 1E, 2C, 2D, and 3C) in association with a further decline in creatinine clearance and a progressive increase in proteinuria as previously described.²⁷ Double immunohistochemistry showed a tight association between OPN expression and macrophage accumulation in areas of severe tissue damage, such as glomerular crescent formation and tubulointerstitial lesions (Figure 3C).

IL-1ra treatment of established anti-GBM disease from days 7 to 21 caused a significant reduction in glomerular and tubular OPN mRNA and protein expression (Figures 1F, 2C, 2D, and 3D). Indeed, the number of glomerular OPN-positive cells was reduced to levels below that seen on day 7 of disease, before the induction of IL-1ra treatment (Figure 2C). This was associated with recovery of

normal renal function, a minor reduction in proteinuria, and prevention of further histological damage.²⁷

IL-1 Up-Regulates OPN Expression by Renal Tubular Epithelial Cells in Vitro

To investigate whether IL-1 can act directly to up-regulate OPN expression in the development of rat anti-GBM glomerulonephritis, we examined the effect of adding IL-1 to the level of OPN mRNA expression in the normal rat epithelial cell line NRK52E. This cell line was chosen because tubular epithelial cells are the major site of OPN production within the injured kidney. Northern blot analysis showed that the NRK52E cell line constitutively expresses low levels of OPN mRNA (Figure 4). Within 6 hours of IL-1 α stimulation, there was an increase in OPN mRNA levels that peaked after 24 hours (threefold induction). The IL-1-induced up-regulation of OPN mRNA levels was dose-dependent and was maintained for a 5-day

Figure 3. Double immunohistochemistry staining showing OPN protein expression in rat anti-GBM disease. Antibody staining of OPN (blue) and macrophages (brown) is shown in anti-GBM glomerulonephritis. **A:** Day 14 of saline treated anti-GBM disease showing OPN expression and macrophage accumulation within a glomerulus with crescent formation (*). In addition, there is strong focal OPN expression in an area of tubulointerstitial injury, including tubulitis (arrowheads). **B:** Day 14 of IL-1ra-treated anti-GBM disease showing a marked inhibition of glomerular and tubular OPN expression and macrophage accumulation. **C:** Saline treatment over days 7 to 21 of anti-GBM disease showing marked up-regulation of OPN expression by glomerular and tubular epithelial cells, with colocalization of numerous macrophages within the glomerular tuft, in a crescent (*), and in tubulointerstitial lesions, including tubulitis (arrowheads). **D:** IL-1ra treatment over days 7 to 21 substantially reduces glomerular and tubulointerstitial OPN expression in association with a marked inhibition of macrophage accumulation and histological damage. Sections were counterstained with PAS. Original magnification, $\times 250$.

Figure 4. Interleukin-1 up-regulates OPN mRNA expression by cultured rat kidney epithelial cells. **A:** NRK52E cells were stimulated with various concentrations of murine IL-1 α for 6 or 24 hours. Total cellular RNA was analyzed by Northern blotting using OPN and GAPDH probes. **B:** The normalized OPN to GAPDH mRNA ratio is shown. Data are mean $\pm$ SE. *, $P < 0.05$; **, $P < 0.01$ versus the unstimulated control by *t*-test with Welch's correction.

culture period (Figures 4 and 5). Preincubation of cells with an excess of the IL-1ra completely blocked IL-1 up-regulation of OPN mRNA expression, demonstrating specificity of the effect. Western blotting showed that NRK52E cells constitutively express OPN protein of approximately 68 kd, consistent with previous studies of NRK52E cells and rat mesangial cells.^{36,37} IL-1 increased OPN protein levels within NRK52E cells in a dose-dependent fashion and this was completely inhibited by preincubation of cells with the IL-1ra (Figure 6).

Discussion

This study has shown that IL-1 is an important factor in up-regulating OPN gene expression in experimental crescentic glomerulonephritis. The ability of IL-1ra treatment to inhibit the induction of OPN expression and to down-regulate OPN expression in established anti-GBM disease clearly demonstrates a functional role for IL-1 in the up-regulation of OPN in experimental crescentic glomerulonephritis. Indeed, the inhibition of renal macrophage infiltration seen with IL-1ra treatment is entirely

Figure 5. Interleukin-1 causes sustained up-regulation of OPN mRNA by cultured rat kidney epithelial cells. **A:** NRK52E cells were stimulated with various concentrations of murine IL-1 α for 3 or 5 days in the presence or absence of 50 μ g/ml human IL-1ra. Total cellular RNA was analyzed by Northern blotting using OPN and GAPDH probes. **B:** The normalized OPN to GAPDH mRNA ratio is shown. Data are mean $\pm$ SE. *, $P < 0.05$; **, $P < 0.01$ versus the unstimulated control by *t*-test with Welch's correction.

consistent with the ability of anti-OPN antibody treatment to suppress macrophage infiltration and subsequent renal injury in this model.^{23,26,27} Furthermore, the ability of IL-1 to increase OPN mRNA levels rapidly in cultured tubular epithelial cells demonstrates that IL-1 acts directly to up-regulate OPN expression in renal cells.

This is the first demonstration that IL-1 can up-regulate OPN expression in kidney cells. The ability of IL-1 to up-regulate OPN expression in renal epithelial cells is consistent with previous studies reporting that IL-1 increases OPN mRNA expression by cultured chondrocytes and osteoblasts.^{24,25} However, it should be noted that regulation of OPN gene expression varies greatly between different cell types. For example, IL-1 failed to increase OPN gene expression in cultured rat mesangial

Figure 6. IL-1 up-regulates OPN protein expression by cultured rat kidney epithelial cells. NRK52E cells were cultured with various concentrations of murine IL-1 α for 3 days in the presence or absence of 50 μ g/ml IL-1ra. Twenty micrograms of cell lysates were analyzed by Western blotting using the MPIIB10 anti-OPN mAb. A predominant band of approximately 68 kd was seen.

cells.³⁷ In addition, vitamin D can induce or suppress OPN mRNA levels in osteoblasts depending on their state of differentiation.³⁸ Furthermore, platelet-derived growth factor induces OPN expression in vascular smooth muscle cells and marrow stromal cells, but not in NRK52E tubular epithelial cells.^{36,39,40}

It was interesting that the increase in renal OPN mRNA and protein production seen in rat crescentic glomerulonephritis was very similar to the pattern of up-regulation of renal IL-1 β expression described previously in this disease model,⁴¹ suggesting that up-regulation of OPN expression within the injured kidney may be due to local IL-1 production. In particular, strong IL-1 β and OPN mRNA and protein expression were seen in glomerular and tubular epithelial cells, including glomerular crescents and areas of tubulointerstitial injury. Whereas double staining showed that some glomerular macrophages expressed OPN, consistent with *in vitro* studies,⁶ podocytes were the major cell type expressing OPN in the glomerulus.²² Therefore, the suppression of glomerular OPN expression by IL-1ra treatment is largely attributed to inhibition of podocyte OPN production, with a lesser effect due to the reduction in macrophage infiltration and macrophage OPN expression.

It was intriguing that the rapid induction of OPN mRNA expression seen following the addition of IL-1 to cultured tubular epithelial cells was sustained for 5 days, in contrast with the 2 days required to detect TGF- β -induced up-regulation of OPN mRNA in this cell line.³⁶ Since IL-1 can induce TGF- β synthesis by tubular epithelial cells, it may be the case that the sustained IL-1 up-regulation of OPN expression by the cultured tubular epithelial cells reflects a secondary effect of IL-1-induced production of TGF- β . This possibility warrants further investigation.

IL-1 plays a crucial role in inducing renal macrophage infiltration.⁴² Indeed, IL-1 induces a broad range of chemokines and leukocyte adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1, which, acting in concert, facilitate the extravasation of blood leukocytes and their subsequent focal accumulation within the kidney. Blocking the action of just one IL-1-inducible adhesion or chemotactic molecule can inhibit leukocyte infiltration in experimental kidney disease,^{43,44} thereby demonstrating the interdependence of these molecules. This is illustrated by the ability of anti-OPN antibody treatment to suppress leukocyte infiltration and subsequent renal injury in rat anti-GBM glomerulonephritis without affecting up-regulation of ICAM-1 expression.²³ This implies that ICAM-1 is necessary for leukocyte-endothelial interactions but that renal OPN expression is required for migration and localization of macrophages and T cells within the kidney, resulting in local tissue damage.

In summary, this study provides *in vivo* and *in vitro* evidence that IL-1 up-regulates OPN expression in experimental kidney disease and argues that inhibition of OPN expression is one of the mechanisms by which IL-1ra treatment suppresses macrophage-mediated renal injury in experimental crescentic glomerulonephritis.

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