Selective nanoparticle-mediated targeting of renal tubular Toll-like receptor 9 attenuates ischemic acute kidney injury

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We developed an innovative therapy for ischemic acute kidney injury with discerning kidney-targeted delivery of a selective Toll-like receptor 9 (TLR9) antagonist in mice subjected to renal ischemia reperfusion injury. Our previous studies showed that mice deficient in renal proximal tubular TLR9 were protected against renal ischemia reperfusion injury demonstrating a critical role for renal proximal tubular TLR9 in generating ischemic acute kidney injury. Herein, we used 300-400 nm polymer-based mesoscale nanoparticles that localize to the renal tubules after intravenous injection. Mice were subjected to sham surgery or 30 minutes renal ischemia and reperfusion injury after receiving mesoscale nanoparticles encapsulated with a selective TLR9 antagonist (unmethylated CpG oligonucleotide ODN2088) or mesoscale nanoparticles encapsulating a negative control oligonucleotide. Mice treated with the encapsulated TLR9 antagonist either six hours before renal ischemia, at the time of reperfusion or 1.5 hours after reperfusion were protected against ischemic acute kidney injury. The ODN2088-encapsulated nanoparticles attenuated renal tubular necrosis, inflammation, decreased proinflammatory cytokine synthesis. neutrophil and macrophage infiltration and apoptosis, decreased DNA fragmentation and caspase 3/8 activation when compared to the negative control nanoparticle treated mice. Taken together, our studies further suggest that renal proximal tubular TLR9 activation exacerbates ischemic acute kidney injury by promoting renal tubular inflammation, apoptosis and necrosis after ischemia reperfusion. Thus, our studies suggest a potential promising therapy for ischemic acute kidney injury with selective kidney tubular targeting of TLR9 using mesoscale nanoparticle-based drug delivery.

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Translational Statement

Acute kidney injury (AKI) due to ischemia and reperfusion is a frequent clinical problem with high morbidity and mortality. Here, we developed an innovative therapy for ischemic AKI with kidney-targeted delivery of a selective Toll-like receptor 9 (TLR9) antagonist in mice subjected to renal ischemia and reperfusion injury. We show that kidney-targeted delivery of a TLR9 antagonist encapsulated in a mesoscale nanoparticle (MNP) that allows approximately 30-fold kidney selective drug delivery protects against ischemic AKI by reducing renal tubular necrosis, inflammation, and apoptosis. Our studies suggest a potential promising therapy for ischemic AKI with selective kidney tubular targeting of TLR9 using MNP-based drug delivery.

Acute kidney injury (AKI) has been a major clinical problem with health care costs of more than $10 billion per year in the United States.[1] Unfortunately, there is no effective preventive measure or therapy for AKI.[2,3] Renal ischemia reperfusion (IR) injury is a major leading cause of AKI as patients undergoing cardiac, vascular, or liver transplant surgical procedures have an approximately 50%–80% chance of developing AKI.[4,5] Renal IR results in rapid proximal tubular necrosis, and it is becoming increasingly clear that necrotic renal cells after IR release several damage-associated molecular pattern ligands that orchestrate additional renal cell death.[6,7] Indeed, subsequent renal tubular apoptosis with rapid upregulation of proinflammatory cytokines and chemokines that causes influx of inflammatory leukocytes into the renal parenchyma potentiates ischemic AKI.[8–10]

Toll-like receptors (TLRs) are pattern recognition receptors that regulate innate as well as adaptive immunity.[11,12]

Both cell surface and intracellular TLRs (13 identified for mice and 11 for humans) play important roles in protecting against microbial invasion.[11–13] Numerous endogenous damage-associated molecular pattern ligands released after cell death activate TLRs including histones, high mobility group box 1, heat shock proteins, and mitochondrial DNA.

TLR9 is a cytosolic receptor for unmethylated cytosine-phosphate-guanosine deoxyribonucleic acid found in microbial DNA and DNA viruses.[14,15] In addition, TLR9 also recognizes endogenous mitochondrial DNA products released from injured cells to trigger MyD88-dependent gene transcription leading to inflammation and apoptosis.[14,16–18] We recently demonstrated that mice lacking renal proximal tubular TLR9 were protected against ischemic AKI with reduced renal tubular necrosis, inflammation, and apoptosis when compared with wild-type mice subjected to renal IR. Consistent with these findings, a selective TLR9 agonist ODN1668 exacerbated renal IR injury in wild-type but not in renal proximal tubular TLR9 null mice.[19] TLR9-mediated renal tubular inflammation and injury after renal IR is mediated by NFκB activation as well as caspase 3 and 8 activation.

Based on our previous findings, a selective TLR9 antagonist (ODN2088) would be a potential drug therapy to treat ischemic AKI. The limitation of this approach is that a systemic TLR9 antagonist administered will target every cell type in the body. As TLR9 is expressed in almost every organ system and because TLR9 activation produces diverse effects depending on cell types and organs studied,[15] systemic administration of the TLR9 antagonist may have limited therapeutic potential. For example, TLR9 activation induces inflammation in hepatic IR and plays a role in septic AKI.[16,17,20,21] In contrast, TLR9 induces cytoprotective signaling in immune, cardiac, and neuronal cells.[22–24] Although renal proximal tubular TLR9 may exacerbate renal IR injury by inducing apoptosis and inflammation, other cell types in the kidney may benefit from TLR9 signaling.

To circumnavigate these limitations, we used a novel approach to deliver the selective TLR9 antagonist ODN2088 to renal tubular cells to protect against ischemic AKI. We recently demonstrated that mesoscale nanoparticles (MNPs), which are polyethylene glycol (PEG)-coated poly(lactic-co-glycolic acid) polymer particles with diameters of approximately 300–400 nm, localize into renal tubule cells preferentially over other organs, with greater than 26-fold selectivity.[25] We packaged the selective TLR9 antagonist ODN2088 into this MNP and tested the hypothesis that selective renal tubular delivery of the TLR9 antagonist would attenuate ischemic AKI in mice by reducing renal tubular necrosis, inflammation, and apoptosis.

RESULTS

Generation of MNPs containing ODN2088 and confirmation of renal tubular MNP delivery

Lyophilized MNPs containing ODN2088 exhibited a mean diameter of 311.7 ± 12.1 nm and a polydispersity index of 0.316 ± 0.048. These particles encapsulated 89 ng ODN2088/1 mg MNP. Lyophilized MNPs containing control ODN exhibited a mean diameter of 311.6 ± 5.0 nm and a polydispersity index of 0.208 ± 0.032. These particles encapsulated 56.7 ng control ODN/1 mg MNP.

To confirm renal tubular delivery of MNPs, kidney sections of mice injected with MNPs or with saline vehicle control 6 hours before renal IR injury were stained with anti-PEG antibody. The renal proximal tubular distribution pattern for the MNPs was confirmed by colocalization of PEG staining with phytohemagglutinin (PHA) lectin staining in 75 mg/kg MNP-injected mice (Figure 1a, representative of 3 experiments). As described previously, we again show that there was almost no MNP localization to other tubular segments, endothelial cells, or mesangial cells in the glomeruli.[25,26]

We also confirmed that administration of MNPs does not result in systemic distribution. Figure 1b shows representative *in vivo* near-infrared fluorescence images of the kidney, liver, spleen, lung, and heart (top), and average fluorescence intensity quantified (bottom) after injection of 75 mg/kg Cy5-loaded MNPs given i.v. The images show approximately 30-fold kidney selectivity over other organs including the lung, liver, spleen, and heart (N = 3). Furthermore, administration in mice that were subjected to 30-minute renal IR also demonstrated >30-fold kidney selectivity (N = 3).

Selective tubular delivery of ODN2088-encapsulated MNPs protects against ischemic AKI in mice

Plasma creatinine and blood urea nitrogen (BUN) values were similar between mice injected with MNPs encapsulated with control ODN (control MNPs) and ODN2088-encapsulated MNPs (MNP-ODN2088) subjected to sham operation (Figure 2). Mice treated with control MNPs and subjected to renal IR had significantly higher plasma creatinine and BUN as well as kidney neutrophil gelatinase-associated lipocalin (NGAL) mRNA (N = 4–5) compared with sham-operated mice (N = 4). We show here that mice treated with 37.5 or 75 mg/kg MNP-ODN2088 6 hours before renal ischemia were protected against ischemic AKI compared with control MNP-treated mice as demonstrated by reduced plasma BUN and creatinine as well as kidney NGAL mRNA expression (N = 6–7). Furthermore, mice treated with 75 mg/kg MNP-ODN2088 at the time of reperfusion or 1.5 hours after reperfusion were also significantly protected against ischemic AKI compared with control MNP-treated mice (N = 6, Figure 2). In contrast, naked ODN2088 given i.v. 6 hours before renal ischemia failed to protect against ischemic AKI in mice (N = 5–6).

MNP-ODN2088 reduces renal tubular necrosis after ischemic AKI

Figure 3a shows representative kidney hematoxylin and eosin images of control MNP-injected or MNP-ODN2088-injected mice subjected to sham surgery or 30-minute renal IR and 24-hour reperfusion (original magnification ×200, N = 6–7). Control MNP-injected mice subjected to renal IR showed

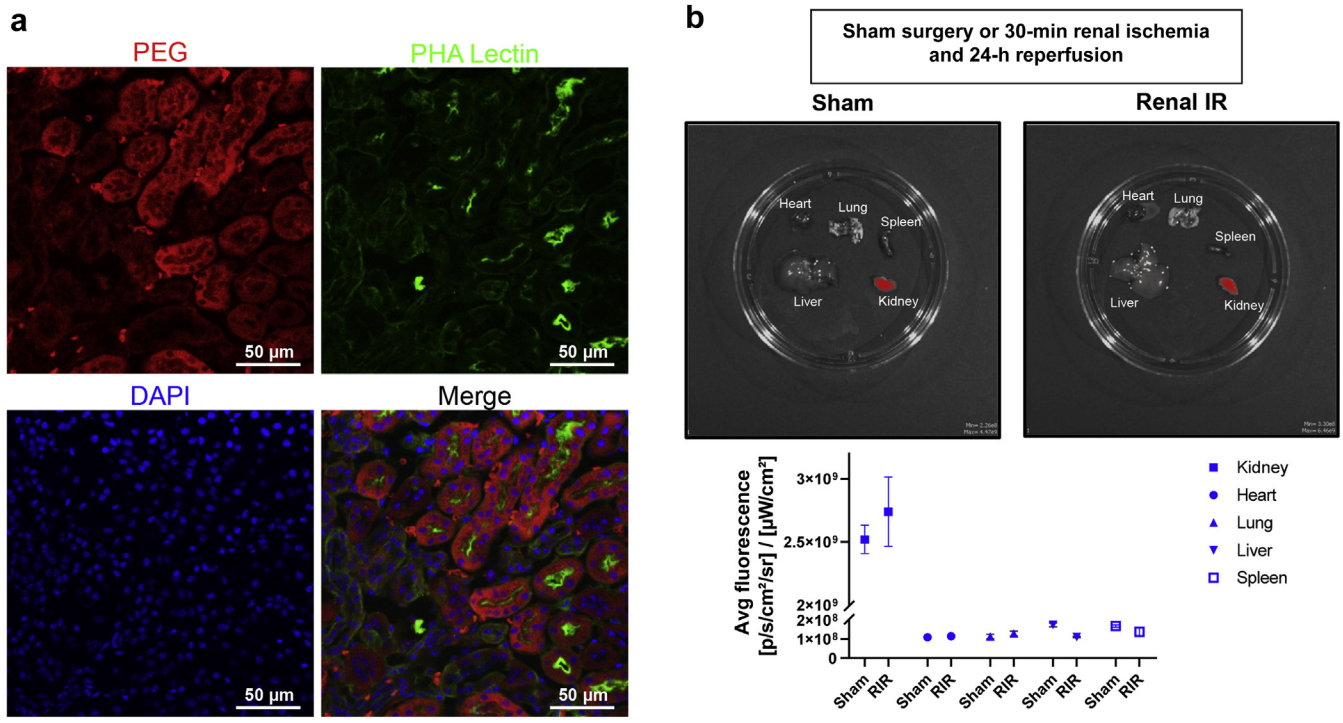

Figure 1 | Confirmation of kidney selective and renal tubule cell mesoscale nanoparticle (MNP) delivery. (**a**) Mouse kidney sections stained with anti–polyethylene glycol (PEG) antibody to detect MNP localization and phytohemagglutinin (PHA) lectin to mark renal proximal tubule cells and counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. Mice were injected with ODN2088-encapsulated MNPs or with vehicle control 6 hours before renal ischemia reperfusion (RIR) injury. Renal proximal tubular distribution pattern for PEG was confirmed by colocalization of PEG antibody staining and PHA lectin staining in MNP-injected mice (representative of 3 experiments). Mice injected with vehicle control showed no PEG staining (data not shown). There was almost no PEG localization in other tubular segments, endothelial cells, or mesangial cells in the glomeruli. (**b**) (Top) *In vivo* near-infrared fluorescence images of the kidney, liver, lung, heart, and spleen of mice injected with 75 mg/kg i.v. Cy5 mimic 3,3′-diethylthiadicarbocyanine iodide–loaded MNPs 6 hours before sham surgery or 30-minute renal ischemia and 24-hour reperfusion (RIR). (Bottom) Quantification of average fluorescence efficiency per square centimeter in each organ demonstrating approximately 30-fold selective delivery of Cy5 MNPs to the kidneys compared with other organs in both sham-operated mice and mice subjected to RIR injury (N = 3). Data are background subtracted and represent mean ±SEM. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

severe tubular necrosis and proteinaceous casts as well as increased tubular dilatation and congestion (Figure 3a). MNP-ODN2088 treatment 6 hours before renal ischemia, at the time of reperfusion, or 1.5 hours after reperfusion led to decreased renal tubular necrosis, congestion, and cast formation compared with control MNP-injected mice subjected to renal IR. Kidneys from MNP-ODN2088-injected mice had significantly reduced renal tubular injury scores compared with control MNP-injected mice after IR (Figure 3b). In contrast, naked ODN2088 given i.v. 6 hours before renal ischemia failed to reduce kidney necrosis score in mice subjected to renal IR (N = 5–6).

MNP-ODN2088 attenuates kidney apoptosis in mice after ischemic AKI

Figure 4a shows representative terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL)–stained images indicative of renal apoptosis, and Figure 4b shows counts of TUNEL-positive kidney cells from control MNP-injected and MNP-ODN2088-injected mice subjected to sham surgery (N = 4) or to renal IR (N = 6, original magnification ×200). Many TUNEL-positive (fragmented DNA) cells were detected, suggestive of renal tubular apoptosis in the kidneys from control MNP-injected mice subjected to renal IR injury. TUNEL-positive kidney cell counts were significantly reduced in MNP-ODN2088-injected mice given 6 hours before renal ischemia, at the time of reperfusion, or 1.5 hours after reperfusion. In contrast, naked ODN2088 given i.v. failed to reduce kidney apoptosis in mice subjected to renal IR (N = 5–6). We also detected caspase 3 and 8 activation by measuring cleaved caspases 3 and 8 in the kidney lysates from sham-operated mice and mice subjected to renal IR injury (Figure 4c). Caspase 3 and 8 cleavages were increased in control MNP-injected mice subjected to renal IR and were significantly reduced in MNP-ODN2088-injected mice 6 hours before renal ischemia. To show specific inhibition of the TLR9 signaling pathway in tubular cells treated with ODN2088-encapsulated MNPs, freshly isolated mouse proximal tubule cells were treated with a selective murine TLR9 ligand 5 μM ODN1668 for 3 days with or without pretreatment of 1 μM ODN2088-encapsulated MNPs (N = 3). We show that MNPs encapsulating ODN2088 completely prevented ODN1688-mediated induction of caspase 3 and 8 fragmentation (Figure 4d).

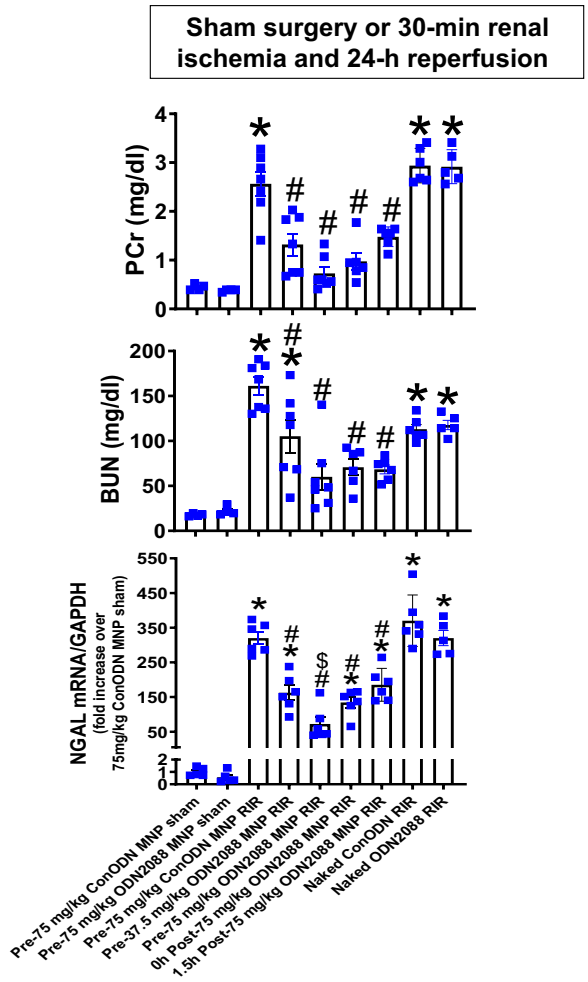

Figure 2 | Mesoscale nanoparticle (MNP)–ODN2088-mediated protection against ischemic acute kidney injury (AKI) in mice. Mice were injected with control MNP-ODN or with ODN2088 encapsulated in MNPs (MNP-ODN2088) and subjected to sham surgery (N = 4) or to 30-minute renal ischemia and 24-hour reperfusion (ischemia reperfusion, N = 6–7). Some mice were injected with MNP-ODN2088 6 hours before renal ischemia. A separate cohort of mice were injected with MNP-ODN2088 at the time of reperfusion or 1.5 hours after reperfusion. Another cohort of mice were injected with 5 mg/kg naked ODN2088 i.v. 6 hours before renal ischemia (N = 5–6). Plasma blood urea nitrogen (BUN) and creatinine as well as kidney neutrophil gelatinase-associated lipocalin (NGAL) mRNA were measured. For statistical analysis, the 1-way analysis of variance plus Tukey's *post hoc* multiple comparison test was used to detect significant changes. $^{*}P < 0.05$ versus control MNP-injected mice subjected to sham surgery. $^{\#}P < 0.05$ versus control ODN mice subjected to renal ischemia reperfusion (RIR). Error bars represent 1 SEM. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

MNP-ODN2088 reduces kidney neutrophil infiltration after ischemic AKI

Figure 5a shows representative immunohistochemistry images, and Figure 5b shows counts of infiltrating kidney neutrophils in the kidneys of control MNP-injected mice and MNP-ODN2088-injected mice subjected to sham surgery (N = 4) or renal IR (N = 6, original magnification ×200). Kidney neutrophil infiltration increased significantly in control MNP-injected mice subjected to renal IR. MNP-ODN2088 treatment either 6 hours before renal ischemia, at the time of reperfusion, or 1.5 hours after reperfusion significantly attenuated kidney neutrophil infiltration after renal IR compared with MNP-ODN2088-injected mice. In contrast, naked ODN2088 given i.v. 6 hours before renal ischemia failed to neutrophil infiltration after renal IR in mice (N = 5–6).

MNP-ODN2088 reduces kidney macrophage infiltration after ischemic AKI

Figure 5c shows representative immunohistochemistry images, and Figure 5d shows counts of infiltrating kidney macrophages in the kidneys of control MNP-injected mice and MNP-ODN2088-injected mice subjected to sham surgery (N = 3) or renal IR (N = 4, original magnification ×200). Kidney macrophage infiltration increased significantly in control MNP-injected mice subjected to renal IR. MNP-ODN2088 treatment 6 hours before renal ischemia significantly attenuated kidney macrophage infiltration after renal IR compared with MNP-ODN2088-injected mice.

MNP-ODN2088 downregulates proinflammatory chemokine and cytokine induction after ischemic AKI

Figure 6 shows fold increases in proinflammatory mRNAs normalized to glyceraldehyde-3-phosphate dehydrogenase for each indicated mRNA in the kidneys of control MNP-injected mice and MNP-ODN2088-injected mice subjected to sham surgery (N = 4–5) or renal IR (N = 6). Ischemic AKI increased all proinflammatory genes measured in control MNP-injected mice. Consistent with the renal protective role of TLR9 antagonism via reduction of neutrophil and macrophage attracting chemokines, we show that macrophage inflammatory protein-2 and monocyte chemoattractive protein-1 expression was significantly attenuated in mice injected with MNP-ODN2088 6 hours before renal ischemia, at the time of reperfusion, or 1.5 hours after reperfusion. Moreover, interleukin-6, keratinocyte-derived cytokine, and tumor necrosis factor-α induction was attenuated in MNP-ODN2088-injected mice. In contrast, naked ODN2088 given i.v. 6 hours before renal ischemia failed to attenuate proinflammatory cytokines in the kidney of mice subjected to renal IR injury (N = 5–6).

DISCUSSION

In this study, we found that MNP technology allows selective renal tubular delivery of a potent TLR9 antagonist ligand oligonucleotide ODN2088 to protect against ischemic AKI. The MNP-loaded ODN2088 reduced renal tubular necrosis, noted by decreased plasma BUN and creatinine as well as kidney NGAL mRNA induction compared with control MNP-injected mice. Increasing clinical significance further, MNP-ODN2088 given at the time of reperfusion or 1.5 hours after reperfusion also protected against renal IR injury.

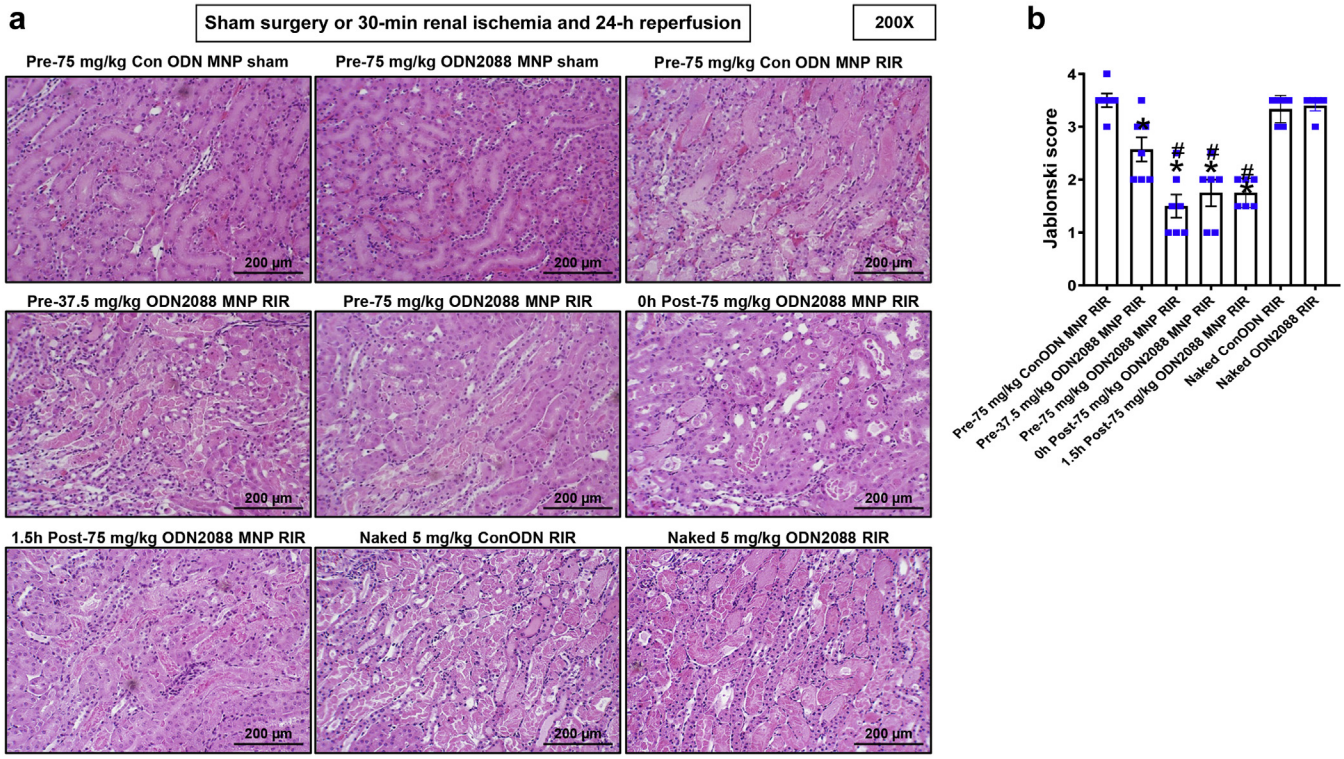

Figure 3 | Mesoscale nanoparticle (MNP)–ODN2088-mediated reduction of renal tubular necrosis after ischemic acute kidney injury (AKI). Representative hematoxylin and eosin images (from 4 to 7 experiments) of kidneys from mice injected with control MNPs or with MNP-ODN2088 and subjected to sham surgery (N = 4) or to 30-minute renal ischemia and 24-hour reperfusion (N = 6–7, original magnification ×200). Some mice were injected with MNP-ODN2088 6 hours before renal ischemia. A separate cohort of mice were injected with MNP-ODN2088 at the time of reperfusion or 1.5 hours after reperfusion. Another cohort of mice were injected with 5 mg/kg naked ODN2088 i.v. 6 hours before renal ischemia (N = 5–6). Renal injury scores assessing the degree of renal tubular necrosis are also shown here (scale: 0–4) 24 hours after renal ischemia reperfusion (RIR). *$P < 0.05$ versus control MNP-injected mice subjected to RIR injury. For statistical analysis, the Mann-Whitney nonparametric test was used to detect significant changes. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

After IR, necrotic renal cells release mitochondrial DNA that can target intracellular renal tubular TLR9.[20] We previously demonstrated that renal proximal tubular TLR9 activation exacerbates ischemic AKI by promoting renal tubular epithelial apoptosis and inflammation. Mice deficient in renal proximal tubular TLR9 were protected against ischemic AKI with reduced plasma creatinine and kidney NGAL expression as well as renal tubular apoptosis and necrosis.[19] Furthermore, a selective TLR9 agonist exacerbated renal tubular injury in wild-type mice but not in mice deficient in renal tubular TLR9 subjected to renal IR injury. These findings suggest a potential therapeutic role for a specific TLR9 antagonist in protecting against ischemic AKI. However, systemic administration of a selective TLR9 antagonist may not be effective as a treatment for ischemic AKI. This is because TLR9 activation produces complex and perhaps divergent effects depending on cell types and organ systemic affected[22,23,27] and renal IR injury results in systemic inflammatory response and extra-renal organ dysfunction.[28,29] Renal tubular epithelial TLR9 promotes cell injury and death, whereas TLR9 signaling in other cell types may promote cytoprotective effects. Indeed, TLR9 activation protects against cerebral as well as cardiac IR injury via activation of phosphatidylinositol-3′-kinase/protein kinase B (also known as AKT) signaling.[22,23] In addition, TLR9 activation may mediate cardiac and neuronal protection by modulating energy metabolism.[27] Furthermore, in cisplatin-induced AKI, TLR9 may promote tissue protection by promoting accumulation of beneficial regulatory T cells.[30] Consistent with these findings, global TLR9-deficient mice were not protected against ischemic AKI again demonstrating the complex and differential effects of TLR9 activation in different organs and cell types.[19,31] In agreement with the global TLR9 knockout mice studies, we demonstrated here that systemic TLR9 blockade with 5 mg/kg naked ODN2088 (15 nucleotides in length) failed to protect against ischemic AKI in mice. It is possible that global TLR9 knockout mice or mice injected with naked ODN2088 systemically i.v. were not protected against ischemic AKI, as they may lack the tissue protective regulatory T cells modulated by TLR9 signaling.

Previous studies demonstrated that i.v. injection of oligonucleotides (approximately 16–21 nucleotides) displays broad distribution into most organ systems (liver, kidney, bone marrow, adipocytes, and lymph nodes) except the central

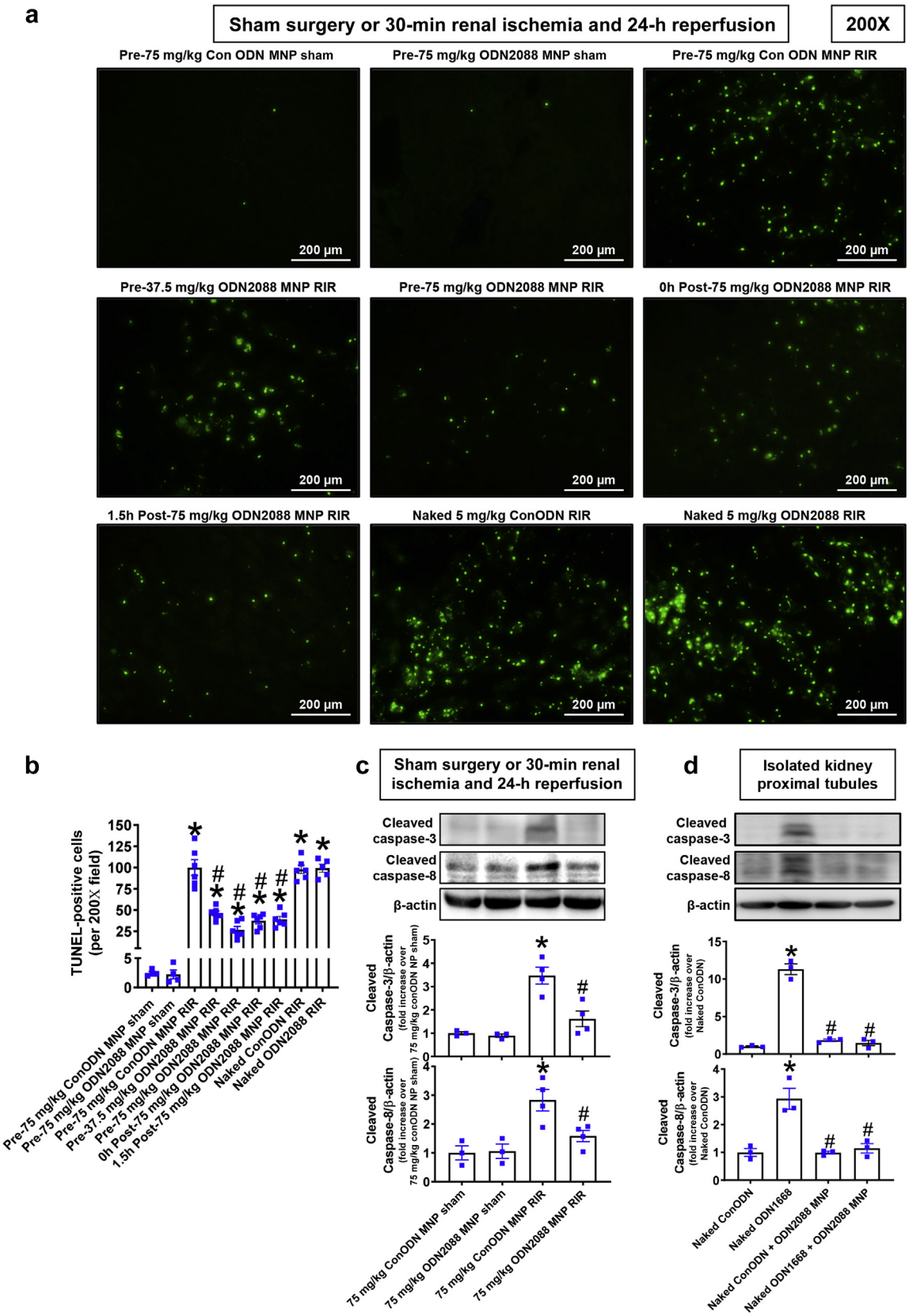

Figure 4 | Mesoscale nanoparticle (MNP)–ODN2088 attenuates kidney apoptosis after ischemic acute kidney injury (AKI). (**a**) Representative images of terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) staining indicative of renal tubular apoptosis and counts of TUNEL-positive kidney cells (**b**) in kidneys of control MNP-injected and MNP-ODN2088–injected mice subjected to sham surgery (N = 4) or 30-minute renal ischemia and 24-hour reperfusion (N = 6, original magnification ×200). Some mice (continued)

nervous system, due to the blood-brain barrier.[32] Therefore, broad biodistribution of the naked TLR9 antagonist oligonucleotide again demonstrates the need for kidney selective delivery to effect renal protection. It is exciting and clinically relevant that MNPs encapsulating 3.26 or 6.5 μg/kg ODN2088 provided protection against ischemic AKI and attenuated renal tubular necrosis, inflammation, and apoptosis.

The highest dose of MNPs encapsulating ODN2088 (75 mg/kg) used here is higher than doses administered (25–50 mg/kg) to characterize the kidney selective delivery of MNPs in previous studies.[25,26] However, the imaging studies still resulted in similar approximately 30-fold kidney-specific delivery of MNPs compared with other organs studied (Figure 1b). Furthermore, it was unknown until this study whether kidneys subjected to IR would still allow similar kidney selective delivery of MNPs given i.v., because renal tubular necrosis and inflammation have severe effects on kidney function. Our studies found that MNPs delivered i.v. continued to demonstrate kidney selective delivery, even in mice that were subjected to ischemic AKI, further adding clinical relevance to these studies.

There is no effective therapy or preventive measure for clinical AKI. Many promising potential therapies based on preclinical research failed to be effective clinically.[33,34] Lack of effective pharmacological therapy is clearly due to the complex nature of clinical AKI in patients with multiple comorbidities in comparison with simplified laboratory models of AKI.[35,36] An additional factor that may preclude effective clinical therapy for AKI is that systemic administration of drugs to treat clinical AKI is problematic for several pharmacologic reasons. Drugs given systemically (oral or intravenous) normally target every organ system and multiple cell types. Concomitantly, experimental therapeutics that produce protective effects in renal epithelial cells may produce unwanted detrimental effects in other cell types (e.g., liver, heart) as discussed above. Another reason for the failure of systemic therapies is the pharmacokinetics/biodistribution with respect to the nephron. Often, an insufficient amount of drug reaches the renal tubular cells when drugs are given in nontoxic doses. Therefore, one way to circumvent the confounding effects of multiple-organ targeting of drugs and less-than-effective dosing problems is to devise a strategy to selectively deliver or concentrate drugs in the kidney by renal-specific delivery methods.

The recently developed MNP drug delivery system may hold a promise in targeting renal cells specifically and provide potential therapy for clinical AKI. Recent studies demonstrated that the relatively large (approximately 300–400 nm) polymer-based MNPs preferentially localized to the kidney when compared with other organs by approximately 26- to 94-fold.[25] MNPs injected via the i.v. route preferentially localized to renal tubular cells and remained for approximately 7 days. These findings are exciting, as renal tubular cells are the major site of injury during and after ischemic AKI.[19,37] Moreover, MNP treatment does not have any detrimental effect on renal or hepatic function, inflammation, or hematological problems as MNP toxicity was tracked for up to 30 days in preclinical studies.[25,26,38]

Specific renal tubular localization of MNPs appears to be dependent on the large size (approximately 300–400 nm) and hydrophilic PEG surface chemistry of the MNP.[25] PEGylation also appears to prevent macrophages scavenging the MNPs.[39,40] The clinically relevant major components of the MNP (poly(lactic-co-glycolic acid) and PEG) are US Food and Drug Administration approved.[41] Our previous studies, using both immunofluorescence and immunohistochemistry, showing renal tubular specific delivery of MNPs,[25] were confirmed in this study. It appears that MNP localization is greater in renal proximal tubules when compared with distal tubules and this is significant as renal proximal tubules are the most susceptible cell types for injury and death due to hypoxia after renal IR.[19,25,26,33,38,42] Furthermore, i.v. injected MNPs appear not to localize in endothelial cells and renal glomeruli based on immunofluorescence and immunohistochemistry studies.

Another exciting aspect of our study is that MNP-ODN2088 delivered at the time of reperfusion as well as 1.5 hours after reperfusion were as protective as MNP-ODN2088 given 6 hours before renal ischemia. Because MNPs take approximately 3–6 hours to maximally localize to the kidney,[26] these findings show that MNP-ODN reaching renal tubular cells 3–7.5 hours after ischemic injury can be effective and equally protective as pretreatment raising clinical significance greatly as not all cases of ischemic AKI can be anticipated in advance.

Based on our findings, MNP-ODN2088 protects against ischemic AKI by reducing renal tubular necrosis and apoptosis after IR. In particular, MNP-ODN2088 significantly attenuated renal tubular apoptosis in mice subjected to severe

Figure 4 | (continued) were injected with MNP-ODN2088 6 hours before renal ischemia. A separate cohort of mice were injected with MNP-ODN2088 at the time of reperfusion or 1.5 hours after reperfusion. Another cohort of mice were injected with 5 mg/kg naked ODN2088 i.v. 6 hours before renal ischemia (N = 5–6). (**c**) We detected caspase 3 and 8 activation by measuring cleaved caspases 3 and 8 in kidney lysates from sham-operated mice and mice subjected to renal ischemia reperfusion (RIR) injury after control MNP or MNP-ODN2088 treatment. $^{*}P < 0.05$ versus control MNP-injected mice subjected to sham surgery. $^{\#}P < 0.05$ versus control ODN mice subjected to RIR. Error bars represent 1 SEM. (**d**) We detected caspase 3 and 8 activation in primary cultures of mouse renal proximal tubule cells treated with control ODN or with 5 μM ODN1668 (a selective mouse TLR9 activating ligand). Some proximal tubule cells were pretreated with MNPs encapsulating control ODN or MNPs encapsulating 1 μM ODN2088. $^{*}P < 0.05$ versus control ODN-treated mouse proximal tubule cells. $^{\#}P < 0.05$ versus ODN1668-treated mouse proximal tubule cells. Error bars represent 1 SEM. TLR9, Toll-like receptor 9. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

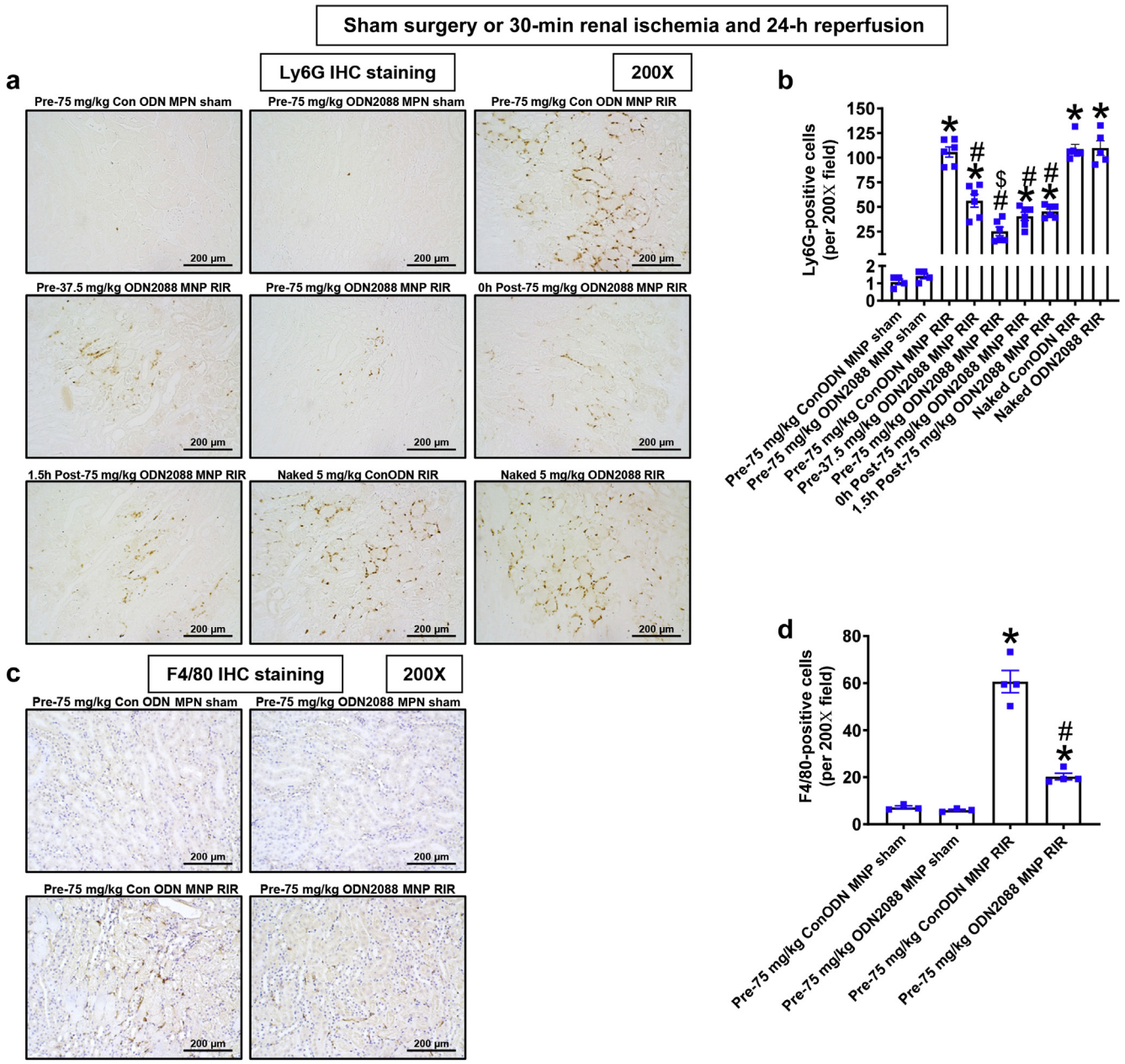

Figure 5 | Mesoscale nanoparticle (MNP)–ODN2088 decreases kidney neutrophil infiltration after ischemic acute kidney injury (AKI). Representative images of immunohistochemistry (IHC) for neutrophils (**a**) and macrophages (**c**) (dark brown) and counts of infiltrating kidney neutrophils (**b**) and macrophages (**d**) in kidneys of control MNP-injected and MNP-ODN2088–injected mice subjected to sham surgery (N = 4) or 30-minute renal ischemia and 24-hour reperfusion (N = 6, original magnification ×200). Some mice were injected with MNP-ODN2088 6 hours before renal ischemia. A separate cohort of mice were injected with MNP-ODN2088 at the time of reperfusion or 1.5 hours after reperfusion. Another cohort of mice were injected with 5 mg/kg naked ODN2088 i.v. 6 hours before renal ischemia (N = 5–6). $^{*}P < 0.05$ versus control MNP-injected mice subjected to sham surgery. $^{\#}P < 0.05$ versus control ODN mice subjected to renal ischemia reperfusion (RIR). Error bars represent 1 SEM. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

ischemic AKI (reduced TUNEL staining and reduced caspase 3 and 8 activation) compared with control MNP-treated mice. Moreover, MNPs encapsulating ODN2088 attenuated TLR9 agonist–mediated activation of caspases 3 and 8 in cultured mouse proximal tubule cells. Renal IR injury causes renal proximal tubular TLR9 activation to induce kidney apoptosis via caspase activation.[19,43] In addition to regulating necrosis and apoptosis, MNP-ODN2088 significantly attenuated kidney proinflammatory cytokine expression compared with control MNP-treated mice. Consistent with reduced proinflammatory cytokines and chemokines, MNP-ODN2088-treated mice had reduced neutrophil infiltration after IR. Taken together, our studies suggest that selective blockade of renal tubular TLR9 effectively attenuates apoptosis and inflammatory pathways that occur during and after renal IR injury.

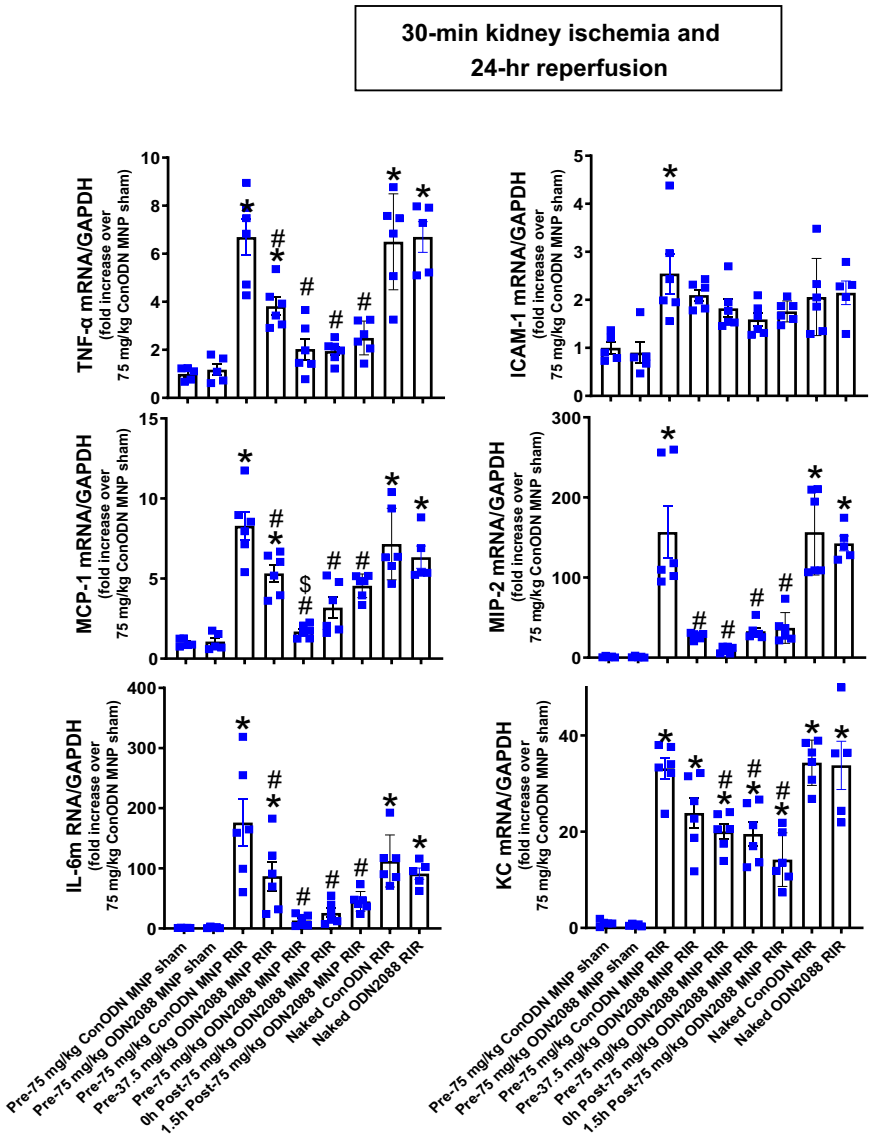

Figure 6 | Mesoscale nanoparticle (MNP)–ODN2088 attenuates kidney proinflammatory chemokine/cytokine induction and neutrophil infiltration after ischemic acute kidney injury (AKI). With quantitative real-time polymerase chain reaction (RT-PCR), we measured the expression of proinflammatory cytokine and chemokine mRNAs in the kidney (keratinocyte-derived cytokine [KC], monocyte chemoattractive protein-1 [MCP-1], macrophage inflammatory protein-2 [MIP-2], tumor necrosis factor-α [TNF-α], interleukin-6 [IL-6], and intercellular adhesion molecule-1 [ICAM-1]) 24 hours after sham surgery or 30-minute renal ischemia. Some mice were injected with MNP-ODN2088 6 hours before renal ischemia. A separate cohort of mice was injected with MNP-ODN2088 at the time of reperfusion or 1.5 hours after reperfusion. Another cohort of mice was injected with 5 mg/kg naked ODN2088 i.v. 6 hours before renal ischemia. Fold increases in proinflammatory mRNAs normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from quantitative RT-PCR reactions for each indicated mRNA (N = 4–6) are shown. $^{*}P < 0.05$ versus control MNP-injected sham-operated mice. $^{\#}P < 0.05$ versus control ODN-injected mice subjected to renal ischemia reperfusion (RIR) injury. Error bars represent 1 SEM.

It is becoming increasingly clear that necrotic cell death is a highly regulated process. Necroptosis is one form of the programmed necrosis and is mediated by members of receptor-interacting protein kinases 1 and 3.[44] Caspase 8 can protect against necroptosis by cleaving receptor-interacting protein kinases 1 and 3 and attenuating necroptosis.[45] Here we show that MNPs encapsulating ODN2088 attenuated caspase 8 activation. These findings imply that in this mouse model of ischemic AKI, caspase 8 is involved in regulating apoptosis rather than necroptosis because both necrosis and apoptosis are significantly attenuated by MNP-ODN2088.

In a prior work, we reviewed the relationship between the size of nanoparticles and their organ targeting.[25,26] We determined that nanoparticles less than 10 nm are rapidly cleared from the body and those between 10 and 250 nm are phagocytosed by the reticuloendothelial system and are taken up in the liver or spleen. Microparticles (>1000 nm) deposit mostly in the lungs. It is highly intriguing that MNPs with the sizes of approximately 350–400 nm accumulated in the

Table 1 | Primers used in quantitative reverse transcription polymerase chain reactions to amplify mouse cDNAs based on published GenBank sequences, and annealing temperatures used for each primer

Primers	Sequence (sense/antisense)	Annealing temperature (°C)
Mouse TNF-α	5′-TACTGAACTTCGGGGTGATTGGTCC-3′ 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	65
Mouse MCP-1	5′-ACCTGCTGCTACTCATTCAC-3′ 5′-TTGAGGTGGTTGTGGAAAAG-3′	60
Mouse MIP-2	5′-CCAAGGGTTGACTTCAAGAAC-3′ 5′-AGCGAGGCACATCAGGTACG-3′	60
Mouse KC	5′-CAATGAGCTGCGCTGTCAGTG-3′ 5′-CTTGGGGACACCTTTTAGCATC-3′	60
Mouse IL-6	5′-CCGGAGAGGAGACTTCACAG-3′ 5′-GGAAATTGGGGTAGGAAGGA-3′	62
Mouse ICAM-1	5′-TGTTTCCTGCCTCTGAAGC-3′ 5′-CTTCGTTTGTGATCCTCCG-3′	60
Mouse NGAL	5′-CACCACGGACTACAACCAGTTCGC-3′ 5′-TCAGTTGTCAATGCATTGGTCGGTG-3′	66
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-CACCACCCTGTTGCTGTAGCC-3′	65

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1; IL-6, interleukin-6; KC, keratinocyte-derived cytokine; MCP-1, monocyte chemoattractive protein-1; MIP-2, macrophage inflammatory protein-2; NGAL, neutrophil gelatinase-associated lipocalin; TNF-α, tumor necrosis factor-α.

kidney with >30-fold selectivity.[25,26] It remains to be determined in future mechanistic studies exactly how MNPs with sizes ranging from 300 to 400 nm injected i.v. can reach renal proximal tubules.[25,38] Indeed, the size of MNPs used here precludes glomerular filtration (approximately 10 nm cutoff), and we believe that they are transcytosed across the peritubular capillary endothelium.[25,38] Histologically, we showed that MNPs localize predominantly in the basolateral region of proximal tubule epithelial cells.[25,26] We hypothesize that the MNPs are transcytosed across the endothelial cells (<500 nm) of the peritubular capillaries and are subsequently released into the tubulointerstitium between the capillary and epithelial cells of the renal tubules. The MNPs would likely then be endocytosed by epithelial cells of the tubule. Because of the pressure gradient in this segment of the nephron (50–10 mm Hg), we hypothesize that MNPs are endocytosed by peritubular endothelial cells rather than glomerular endothelial cells.

In summary, we demonstrate in this study a novel and innovative method to treat ischemic AKI using MNPs. Our study suggests that kidney-targeted MNP-mediated selective drug delivery is an exciting method to treat AKI with improved therapeutic specificity and potentially reduced systemic toxicity by allowing lower drug dosage.

METHODS

Generation of MNPs incorporating the selective TLR9 antagonist ODN2088

MNPs encapsulating the selective TLR9 antagonist ODN2088 or control ODN were formulated similarly to previously described methods with minor modifications.[25,26] Briefly, poly(lactic-co-glycolic acid) of molecular weight 38–54 kDa (Sigma, St. Louis, MO) was conjugated to 5 kDa carboxylic acid–terminated PEG (Nanocs, NY) (poly[lactic-co-glycolic acid]-PEG) before particle formulation. The conjugated copolymer (100 mg) was dissolved in 2 ml acetonitrile. Then, 50 μg of oligonucleotide (ODN2088 or control ODN; InvivoGen, CA) dissolved in water was added to the copolymer solution and bath sonicated for 2 minutes. The resultant emulsion was added to a solution of purified water (4 ml) and Pluronic F-68 (75 μl; Fisher Scientific, NH) and centrifuged at 5400 × *g* for 15 minutes. The nanoparticle pellet was washed with 10 ml purified water and centrifuged under the same specifications. The resultant pellet was resuspended in a 2% sucrose solution and lyophilized for storage at −20 °C.

The hydrodynamic diameter and polydispersity index of the MNPs were characterized via dynamic light scattering (Malvern, UK) in a 10 mg/ml phosphate-buffered saline suspension. To quantify oligonucleotide loading into the particles, approximately 10 mg of lyophilized particle powder was dissolved in 200 μl acetonitrile and shaken at room temperature for 30 minutes. To this solution we added 300 μl Tris-EDTA buffer (Fisher Scientific) before centrifugation at 31,000 × *g* for 30 minutes. The supernatant containing liberated oligonucleotide was used for quantification via the Quant-iT RiboGreen RNA Assay Kit (Fisher Scientific) according to the manufacturer's instructions.

Renal IR injury in mice

After Columbia University IACUC approval, 20–25 g male C57BL/6 mice (Jackson Labs, ME) were anesthetized with pentobarbital i.p. (Sigma: 50 mg/kg body weight or to effect). Some mice received i.v. MNPs encapsulating control ODN or MNPs encapsulating the TLR9 antagonist ODN2088 (37.5 or 75 mg/kg MNP that delivers 3.26 or 6.5 μg/kg ODN2088) 6 hours before renal ischemia. Mice were then subjected to right nephrectomy and 30-minute left renal ischemia as described.[46,47] Sham-operated animals underwent anesthesia followed by laparotomy, right nephrectomy, bowel manipulations, and wound closure without renal ischemia. Body temperature was sustained at approximately 37 °C during surgery as well as during recovery from anesthesia. A separate cohort of mice received i.v. 75 mg/kg MNP encapsulating control ODN or MNP encapsulating the TLR9 antagonist ODN2088 at the time of reperfusion or 1.5 hours after reperfusion. Finally, some mice received naked ODN2088 6 hours before renal ischemia i.v. to determine whether systemic TLR9 blockade protects against ischemic AKI. For pain management, all mice received 0.5–1 mg/kg s.c. buprenorphine SR before surgery.

Detection of renal injury after IR

Twenty-four hours after renal IR or sham surgery, we measured plasma BUN and creatinine using an enzymatic creatinine reagent kit (Thermo Fisher Scientific, MA). We also performed quantitative real-time polymerase chain reaction for kidney NGAL mRNA from mice subjected to sham surgery or to renal IR injury.[48]

Histological detection of kidney injury

Twenty-four hours after renal IR injury or sham surgery, kidney hematoxylin and eosin sections were assessed using a grading scale of kidney necrotic IR injury to the proximal tubules (0–4, Renal Injury Score), as outlined by Jablonski *et al.*[49] The renal pathologist was blinded to the experimental conditions. Deidentified slides were hematoxylin and eosin–stained coronal cross-sections of bivalved whole kidney showing full-thickness cortex and medulla. The cortical and medullary parenchyma was evaluated in its entirety in all

the microscopic fields covering the entire slide to generate the Jablonski score.

Detection of kidney apoptosis

Twenty-four hours after renal IR or sham surgery, TUNEL staining detected fragmented DNA as described.[50] Apoptotic TUNEL-positive cells were quantified in 5–7 randomly chosen ×200 microscope images fields in the corticomedullary junction and results were expressed as apoptotic cells counted per ×200 field. In addition, kidney caspase 3 and 8 immunoblotting were performed as described previously.[51] Primary antibodies for mouse caspase 3 and 8 were from Cell Signaling Technology (Danvers, MA).

Detection of kidney neutrophil and macrophage infiltration

We performed kidney immunohistochemistry using rat anti-mouse Ly6G monoclonal antibody or with anti-mouse F4/80 (Thermo Fisher Scientific, Inc., Pittsburgh, PA) as described.[52,53] Primary IgG_{2a} antibody (MCA1212; AbD Serotec, Raleigh, NC) was used as a negative isotype control. Quantification of kidney infiltrating neutrophils or macrophages was performed using 5–7 randomly chosen ×200 microscope image fields and results were expressed as neutrophils counted per ×200 field.

Quantitative real-time polymerase chain reaction for proinflammatory cytokine and chemokine mRNA expression

Renal inflammation after IR was also assessed by measuring proinflammatory mRNAs including interleukin-6, intercellular adhesion molecule-1, monocyte chemoattractive protein-1, keratinocyte-derived cytokine, macrophage inflammatory protein-2, and tumor necrosis factor-α with quantitative real-time polymerase chain reaction as described previously with primers listed in Table 1.[52,54] To confirm equal RNA loading, glyceraldehyde-3-phosphate dehydrogenase mRNA expression was also measured.

PEG immunohistochemistry

We performed florescent immunohistochemistry for PEG to detect renal proximal tubular localization of MNPs administered as the surface of MNPs are composed of PEGylated poly(lactic-co-glycolic acid). Kidneys from mice treated with 75 mg/kg MNP encapsulating ODN2088 6 hours before renal IR injury were fixed with 4% paraformaldehyde, dehydrated with 30% sucrose, frozen in optimal cutting temperature compound (Tissue-Tek, Torrance, CA), and cryosectioned (5 μm). Kidney sections were incubated with anti-PEG antibody (ab94764; Abcam, MA) specific to the PEG backbone plus PHA lectin antibody (proximal tubule specific marker; Molecular Probes, Eugene, OR). After washes, kidney slides were counterstained with 4′,6-diamidino-2-phenylindole to visualize cell nuclei, mounted with Vectashield (Vector, Burlingame, CA) mounting media, and imaged with a fluorescent microscope (Olympus IX81, Melville, NY).

In vivo imaging to test kidney selective delivery of MNPs

Mice were injected with fluorescent 75 mg/kg i.v. Cy5 mimic 3,3′-diethylthiadicarbocyanine iodide MNP 6 hours before either sham surgery or renal IR as described above. Twenty-four hours later, the heart, lungs, liver, spleen, and kidney were harvested and fluorescently imaged using an IVIS Spectrum Preclinical In Vivo Imaging System (Perkin Elmer, Waltham, MA) using 640/680 nm excitation/emission filters. Living Image Software v4.3 (Perkin Elmer) quantified average fluorescence efficiency per square centimeter in each organ of interest.

Mouse proximal tubule cell culture and TLR9 ligand treatments

Mouse kidney proximal tubules were isolated with Percoll density gradient separation as described previously.[55] Confluent cells were treated with control oligonucleotides (ODN) or with selective 5 μM TLR9 agonist ligand ODN1668 (InvivoGen) for 3 days as described.[56] Some cells were pretreated with MNPs encapsulating ODN2088 (87 ng ODN2088/mg MNPs) 30 minutes before control ODN or ODN1668 treatment. We then performed caspase 3 and 8 immunoblotting as described.[51]

Statistical analysis

Data were analyzed with Student's *t*-test, 1-way analysis of variance plus Tukey's *post hoc* multiple comparison test or Mann-Whitney nonparametric *U* test to analyze renal injury scores. All data are expressed throughout the text as means ± SEM.

DISCLOSURE

DAH is a cofounder and officer with equity interest in LipidSense, Inc. and Goldilocks Therapeutics, Inc. and is a member of the scientific advisory board of Concarlo Holdings, LLC. RMW is a scientific advisor with equity interest in Goldilocks Therapeutics, Inc. EAJ is a cofounder and chief medical officer with equity interest in Goldilocks Therapeutics Inc.

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