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# Distinct Macrophage Phenotypes Contribute to Kidney Injury and Repair

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## ABSTRACT

The ischemically injured kidney undergoes tubular cell necrosis and apoptosis, accompanied by an interstitial inflammatory cell infiltrate. In this study, we show that iNos-positive proinflammatory (M1) macrophages are recruited into the kidney in the first 48 hours after ischemia/reperfusion injury, whereas arginase 1– and mannose receptor–positive, noninflammatory (M2) macrophages predominate at later time points. Furthermore, depletion of macrophages before ischemia/reperfusion diminishes kidney injury, whereas depletion at 3 to 5 days after injury slows tubular cell proliferation and repair. Infusion of Ifnγ-stimulated, bone marrow–derived macrophages into macrophage-depleted mice at the time of kidney reperfusion restored injury to the level seen without macrophage depletion, suggesting that proinflammatory macrophages worsen kidney damage. In contrast, the appearance of macrophages with the M2 phenotype correlated with the proliferative phase of kidney repair. *In vitro* studies showed that IFNγ-stimulated, proinflammatory macrophages begin to express markers of M2 macrophages when cocultured with renal tubular cells. Moreover, IL-4–stimulated macrophages with an M2 phenotype, but not IFNγ-stimulated proinflammatory macrophages, promoted renal tubular cell proliferation. Finally, tracking fluorescently labeled, IFNγ-stimulated macrophages that were injected after injury showed that inflammatory macrophages can switch to an M2 phenotype in the kidney at the onset of kidney repair. Taken together, these studies show that macrophages undergo a switch from a proinflammatory to a trophic phenotype that supports the transition from tubule injury to tubule repair.

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Ischemic injury of the kidney in humans results in tubular cell death and the clinical syndrome of acute tubular necrosis. In otherwise healthy patients, tubular injury peaks histologically 2 to 3 days after injury and is followed by a repair phase in which proliferation of surviving tubular cells promotes tubule regeneration. Examination of postmortem specimens has shown that ischemic tubular injury also leads to the accumulation of mononuclear inflammatory cells in the vasa recta of the outer medulla.[1] Animal models of ischemia/reperfusion (I/R), where the inflammatory re-

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sponse can be more carefully studied, show that ischemic injury is accompanied by the rapid influx of polymorphonuclear leukocytes, lymphocytes, and macrophages into the interstitium.[2–4] These cells are believed to home to the injured kidney in response to inflammatory cytokines such as monocyte chemoattractant protein-1, stromal cell-derived factor-1, IL-6 and IL-8/keratinocyte chemoattractant produced locally by cells such as the resident dendritic cell and injured tubular and endothelial cells.[5,6]

In the early phase of renal I/R injury, leukocyte populations seem to be detrimental because they promote apoptotic cell death by releasing reactive oxygen species and activating caspases. In agreement with this proposed negative role of inflammatory cells during acute kidney injury (AKI), blocking the initial inflammatory response by inhibition of cytokine action, preventing inflammatory cell homing, or depleting the inflammatory cells themselves seem to decrease the degree of morphologic and functional injury in models of renal I/R.[7–10]

One of the major cell types that accumulates around tubules in the rodent kidney after I/R is the mononuclear phagocyte/macrophage.[11] Several groups have shown that systemic depletion of phagocytes, including monocytes and macrophages, with liposomal clodronate before I/R decreases morphologic kidney damage and attenuates the increase in blood urea nitrogen (BUN) and creatinine.[11,12] These observations are consistent with the idea that macrophage infiltration augments the inflammatory response and promotes tubular injury. Recent studies in other forms of immune-mediated renal injury raise the possibility that it is the effector phenotype of the recruited macrophages rather than their presence alone that determines the extent of renal parenchymal injury.[13,14]

Macrophages exhibit a range of phenotypes, a phenomenon that has been described as macrophage polarization or heterogeneity.[15–17] The "classically" activated or F4/80$^+$Cd11c$^-$MR$^-$ (M1) macrophage is induced by exposure to IFN$\gamma$ (Ifn$\gamma$), LPS, TNF$\alpha$, or GM-CSF and expresses proinflammatory cytokines such as IL-1$\beta$, TNF$\alpha$, and IL-6. These M1 macrophages help confer resistance to infection and can be distinguished by their expression of high levels of inducible nitric oxide synthase 2 (iNos), IL-12, IL-23, and Ly-6C. In contrast, exposure of macrophages to IL-4 or IL-13 inhibits expression of these proinflammatory markers and instead activates expression of high levels of arginase-1, mannose receptor (MR), and IL-10. These "alternatively" activated or F4/80$^+$Cd11c$^-$MR$^+$ (M2) macrophages are believed to modulate the inflammatory response and to promote tissue repair.[13,18,19] This M2 classification has been further subdivided into M2a–c based on the expression of additional cytokines such as Ccl1, Ccl16, and IL-1.[16,19]

To study the possible role of macrophage heterogeneity in the process of transitioning from the early stages of tubular injury, necrosis, and apoptosis to the later stages of proliferation and repair, we combined marker analysis with functional studies in a mouse model of kidney I/R. We found that macrophages expressed proinflammatory markers during the initial period of reperfusion injury, whereas macrophages resembling the alternatively activated or trophic phenotype predominated during the recovery and repair phase. *In vitro* studies showed that renal epithelial cells promote the coexpression of markers characteristic of classic and alternative activation in both uninduced or Ifn$\gamma$-activated macrophages and that IL-4–induced alternatively activated macrophages stimulated epithelial cell proliferation. Depletion of macrophages before I/R reduced injury, whereas depletion during the phase of M2 predominance diminished tubular cell proliferation and delayed tubule repair. The phenotypic switching of macrophages therefore reflects a functional change in the transition from initial tubular cell loss to eventual repair/recovery.

## RESULTS

### Phagocyte Depletion before Renal I/R Reduces Tubular Injury

Mice subjected to a period of renal ischemia sufficient to induce tubular cell death exhibit a well-described loss of glomerular filtration accompanied by a rapid increase in BUN (Supplemental Figure 1A). Immunostaining with an antibody specific for the mouse macrophage marker F4/80 showed an influx of mononuclear phagocytes beginning 1 day after I/R injury. Macrophages accumulated in all areas of the injured kidney, frequently surrounding the injured tubules in the outer medulla (Supplemental Figure 1B). As previously reported, injection of liposomal clodronate significantly decreased both circulating monocytes and tissue macrophages, as well as CD11c$^+$ dendritic cells and neutrophils (Supplemental Figure 1, C and D).[11,12] When mice were treated with liposomal clodronate to deplete myeloid phagocytes before I/R, the peak BUN level was markedly diminished (Supplemental Figure 1E). These data support previously published studies showing that the initial influx of macrophages and neutrophils after I/R worsens the tubular injury.[11,12]

### Alternative Macrophage Activation during the Repair Phase of Ischemic Renal Injury

After tubule injury, the process of repair begins with a marked increase in tubular cell proliferation that peaks on day 3 and slowly declines over the ensuing week (Supplemental Figure 2, A and B). Immunostaining showed that the majority of dividing cells were tubular cells, with approximately 88% of the bromodeoxyuridine (BrdU)-positive proliferating cells expressing the proximal tubule marker megalin and 1% expressing the thick ascending limb marker Tamm-Horsfall protein. The remaining 10% were unclassifiable (Supplemental Figure 2C and data not shown).

Immunostaining with F4/80 showed that the number of F4/80$^+$ mononuclear phagocytes increases progressively after reperfusion and remained high in the renal interstitium during the period of tubular cell proliferation and recovery (Figure 1A, quantified in B). In contrast, staining for Cd11c-expressing cells showed that the number of renal dendritic cells did not

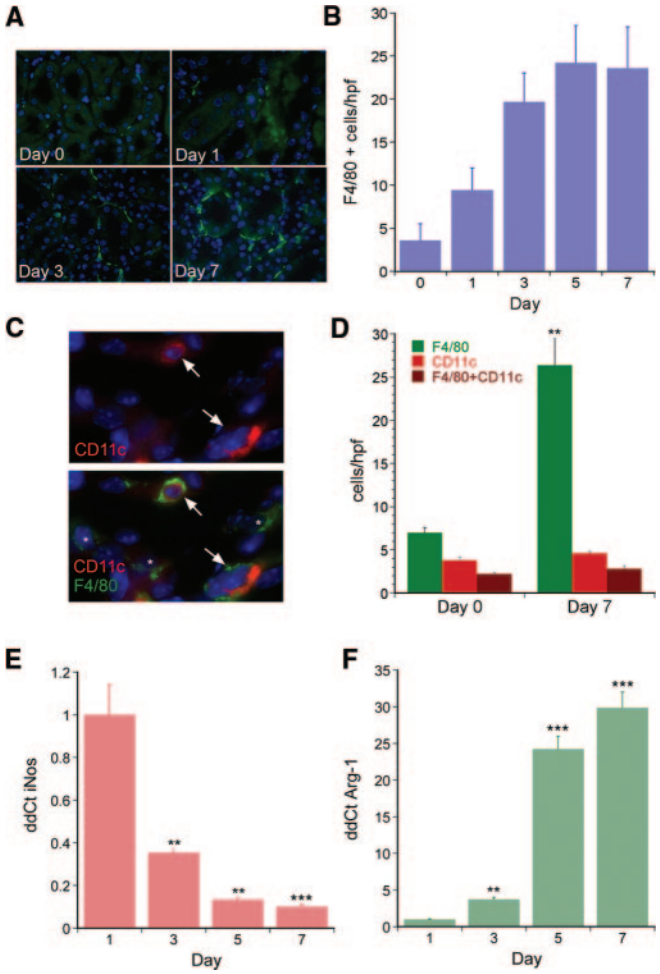


**Figure 1.** Macrophages switch phenotypes during kidney injury and repair. (A and B) Immunostaining for F4/80 (green) shows a progressive increase in macrophages surrounding the injured tubules from days 1 to 7. $n = 4$ to 8 mice/group, $P < 0.001$ for days 3 to 7 *versus* day 1. (C) Immunostaining for Cd11c and F4/80 shows that a small percentage of F4/80$^+$ cells coexpress the dendritic cell marker Cd11c (arrows), whereas other F4/80$^+$ cells do not express Cd11c (asterisks). (D) Quantification of F4/80$^+$Cd11c$^+$ cells in control mice and on day 7 after injury. $^{**}P < 0.01$ *versus* day 0. (E and F) qPCR for iNos (E) and Arg-1 (F) from F4/80$^+$ cells isolated from kidneys at indicated times after I/R. Values reported as ddCt relative to day 1. $^{**}P < 0.01$, $^{***}P < 0.001$.

change over this time period (Figure 1C, quantified in D). The high numbers of macrophages present during the proliferative phase raised the possibility that macrophages might also assist in kidney repair.

To determine whether the macrophages present at the time of injury differ phenotypically or functionally from those present during recovery, F4/80$^+$ cells were isolated by FACS-sorting from the kidney at multiple time points after I/R injury, and the expression of iNos (a marker of proinflammatory macrophages) and Arginase-1 (Arg1, expressed by alternatively activated macrophages) was quantitatively assessed using real-time PCR. At 24 hours after injury, F4/80$^+$ cells expressed high levels of iNos and little Arg1 (Figure 1, E and F), suggesting that they were inflammatory macrophages. Over the ensuing 6 days, the expression of iNos in F4/80$^+$ cells progressively decreased, whereas Arg1 markedly increased. This observation suggests that F4/80$^+$ cells present during the repair phase have adopted a phenotype more similar to that of alternatively activated macrophages.

To provide more detailed evidence that this change in marker expression reflects a shift from proinflammatory to alternatively activated macrophage phenotypes, we induced these activation states *in vitro* by stimulating cultured mononuclear phagocytes with defined cytokines and compared them to macrophages obtained from the kidney 1 and 7 days after injury. For *in vitro* cell profiling, mononuclear phagocytes were harvested from mouse bone marrow and cultured for 7 days (BMMs), followed by stimulation with either Ifnγ or IL-4 for 24 hours. As previously reported, stimulation with Ifnγ resulted in expression of iNos and the p40 subunit of IL-12 and IL-23, consistent with a proinflammatory phenotype, whereas treatment with IL-4 induced expression of Arg1 and the MR, which define the alternatively activated phenotype (Figures 2, A and B, and 3D). Macrophages were isolated from the kidney at days 1 and 7 after I/R injury using the surface expression of F4/80, Cd11c, and MR. Macrophages isolated on day 1 were predominately F4/80$^+$Cd11c$^-$MR$^-$ and exhibited high expression of iNos and the p40 subunit of IL-12/23, whereas macrophages isolated on day 7 were predominately F4/80$^+$Cd11c$^-$MR$^+$ and expressed high levels of Arg1 (Figure 2C).

Together, these results suggest that the macrophage phenotypes in the I/R-injured kidney change from a proinflammatory expression profile in the acute phase of injury to an alternatively activated phenotype during the repair phase.

## Macrophages Exhibit Signal-Specific Expression Plasticity

To address the possibility that kidney macrophages alter their expression profile in the days after I/R injury in response to cues in the microenvironment, we labeled BMMs with PKH26, stimulated them for 24 hours with Ifnγ to induce their differentiation into iNos-positive, proinflammatory macrophages (Figure 3A), and followed their marker expression after intravenous injection into host mice. Intravenous injection of labeled M1 macrophages at the time of injury followed by harvest and examination of the kidney 24 hours later showed that the majority of the PKH26-labeled cells that had homed to the injured kidney retained iNos expression (quantified in Figure 3C). In contrast, the majority of PKH26-labeled M1 cells that were injected 3 days after injury downregulated iNos expression and instead activated MR expression by day 5 (Figure 3B, quantified in C). These results support the concept that macrophages can alter their expression profile in response to the signals received in their local microenvironment.

Because the proinflammatory macrophages enter the in-

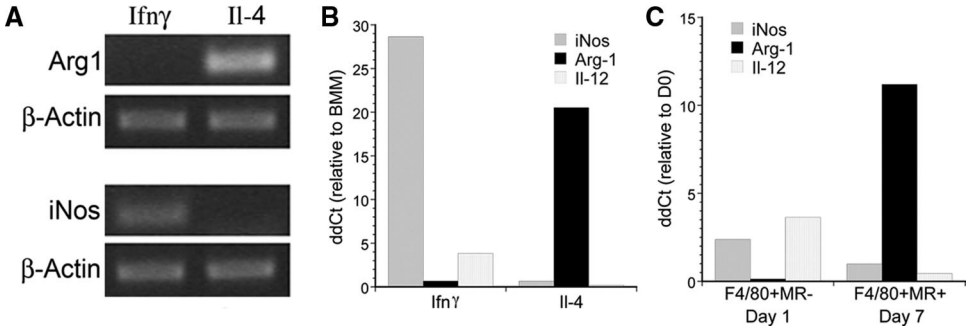


**Figure 2.** Macrophages induced *in vitro* and activated *in vivo* demonstrate similar expression patterns. (A) PCR of mRNA isolated from BMMs treated for 24 hours with either Ifnγ or IL-4. Ifnγ induces expression of the M1 marker iNos, whereas IL-4 induces expression of the M2 marker Arg-1. (B) RNA isolated from BMMs stimulated for 24 hours with either Ifnγ or IL-4 was used for real-time PCR of message levels for iNos, Arginase-1, or IL-12 relative to their expression in uninduced BMMs. A representative experiment is shown (from an *n* of 3). (C) $F4/80^+Cd11c^-$ macrophages were isolated from kidneys at baseline (D0) and compared with $F4/80^+Cd11c^-MR^-$ macrophages 24 hours after I/R injury and $F4/80^+Cd11c^-MR^+$ macrophages 7 days after I/R injury. A representative real-time PCR for iNos, Arg-1, and IL-12 relative to their expression in D0 cells is shown (from an *n* of 3).

jured kidney and surround the damaged tubules, we examined the possibility that tubular cells can induce the expression of an alternatively activated macrophage phenotype. Mouse proximal tubule cells (Boston University mouse proximal tubule cells [BU-MPT][20]) were cocultured in either direct contact (data not shown) or via Transwell with naive BMMs. Immunostaining with antibodies to the MR showed that coculture with MPT cells activated MR expression to a level that was indistinguishable from that obtained after stimulation with IL-4 (Figure 3, D and E). Consistent with the *in vivo* findings, a substantial percentage of BMMs that were first stimulated with Ifnγ to activate iNos expression followed by MPT coculture showed upregulation of MR expression (Figure 3, D and E).

These results suggest that proximal tubule cells secrete a

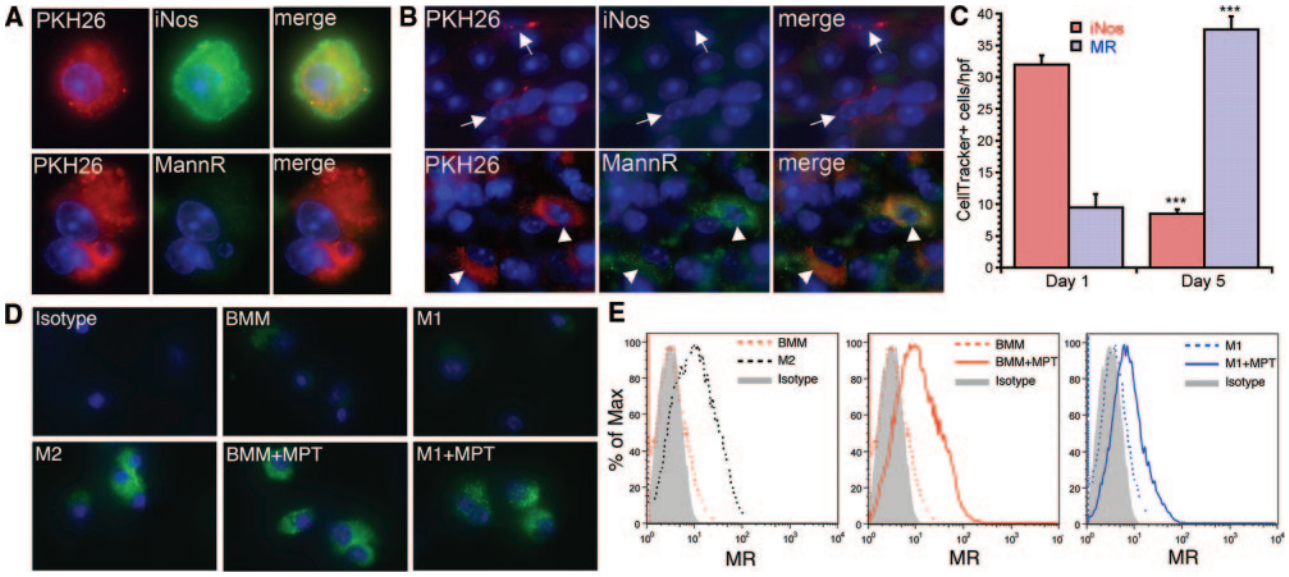


**Figure 3.** Macrophages demonstrate plasticity *in vitro* and *in vivo*. (A) BMMs were stimulated with Ifnγ to induce iNos expression and labeled with the cytosolic tracker dye PKH26. Original magnification: ×1000. (B) PKH26 labeled M1 macrophages were injected intravenously at 3 days after I/R, followed on day 5 by immunofluorescence analysis of PKH26, iNos, and MR. Multiple $PKH26^+MR^+$ cells were detected in the injured kidneys on day 5 (arrowheads), whereas most $PKH26^+$ cells had lost iNos expression (arrows). Original magnification: ×400. (C) Quantification of $PKH26^+iNos^+$ and $PKH26^+MR^+$ macrophages injected either on day 0 and analyzed on day 1 after I/R or injected on day 3 and analyzed on day 5 after I/R. $n = 4$ separate mice/group, $^{***}P < 0.001$ *versus* day 1. (D) BMM were stimulated for 48 hours with vehicle (BMM), Ifnγ (M1), IL-4 (M2), or vehicle followed by MPT coculture (BMM+MPT) or Ifnγ followed by MPT coculture (M1+MPT) and then immunostained for MR (green) or isotype control. Stimulation with IL-4 or coculture with MPT results in increased expression of MR (bottom three panels). Original magnification: ×400. (E) FACS analysis of MR expression by naive BMM *versus* IL-4–induced BMMs (M2), naive BMMs *versus* naive BMMs cocultured with MPT cells, and Ifnγ-activated BMMs (M1) *versus* M1 cocultured with MPT cells.

factor or factors that can induce a noninflammatory macrophage phenotype. To examine this response more carefully, BMMs were first cultured for 48 hours in the presence of Ifnγ to induce a proinflammatory phenotype, followed by culture with either vehicle, Ifnγ, IL-4, or Transwell coculture with MPT cells and examination of mRNA expression of macrophage proteins. These experiments confirmed that switching from Ifnγ stimulation to either IL-4 stimulation or MPT coculture leads to marked upregulation of the alternative activation markers Arg1 and MR (Figure 4A). However, analysis of other macrophage-expressed proteins showed that MPT coculture induces a hybrid macrophage phenotype with elements of both proinflammatory and alternative activation. Specifically, MPT coculture failed to induce expression of either Ym1 or Igf1, both of which have been shown to be expressed during alternative activation by IL-4,[17,21,22] whereas expression of the proinflammatory cytokine IL-1β and the macrophage scavenger receptor 1 (Msr1) were highly upregulated after MPT–macrophage coculture but not after treatment with IL-4 (Figure 4B).

In light of these findings, we examined the role of IL-4 signaling in the macrophage response to renal tubular cell–se-

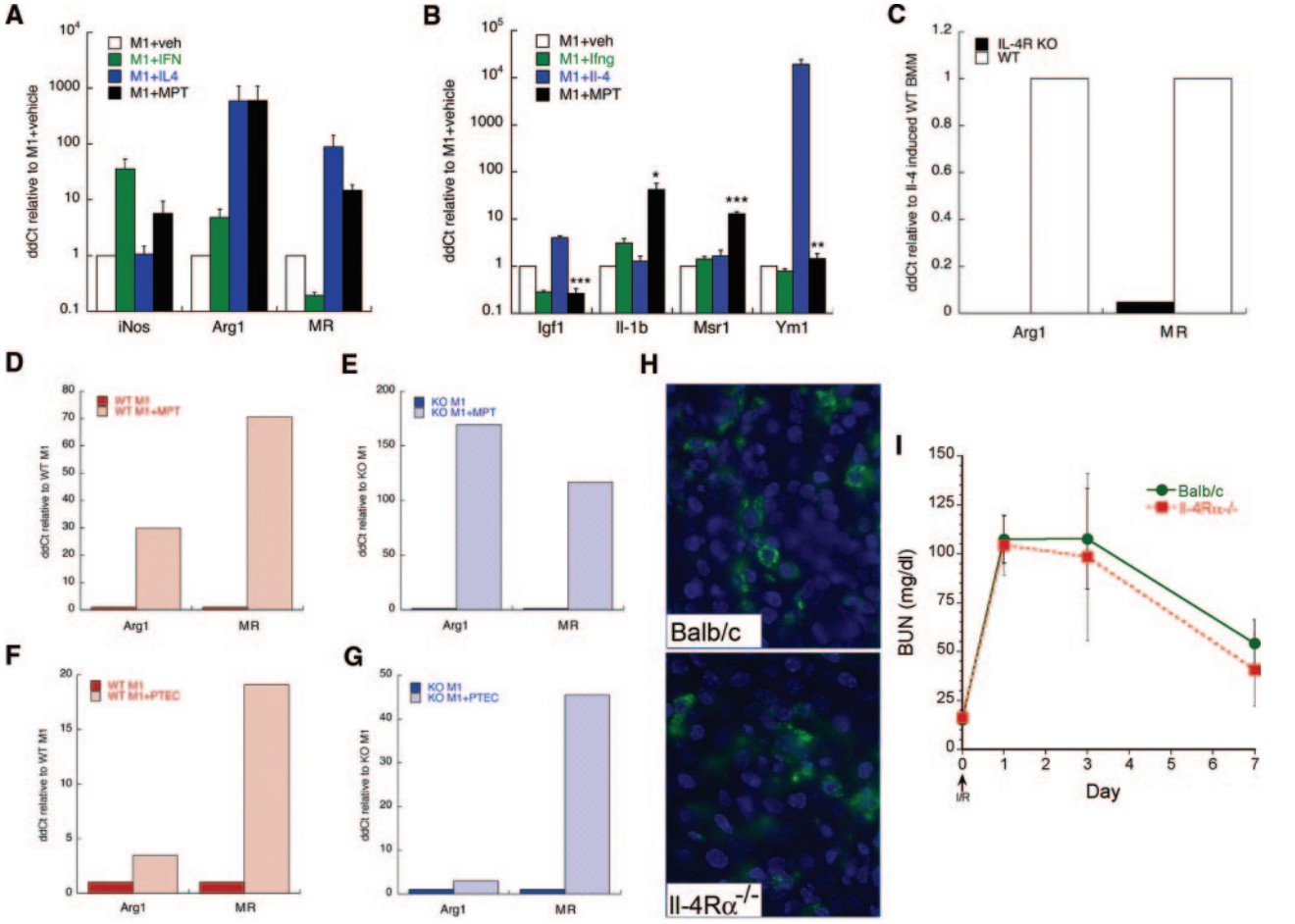


**Figure 4.** MPT coculture induces a hybrid activation state in macrophages that is IL-4 independent. BMMs were stimulated with Ifnγ for 48 hours (M1), followed by culture for 48 hours in the presence of Ifnγ, vehicle, IL-4, or MPT coculture, followed by RNA harvest and quantitative RT-PCR, normalized to Hprt expression. (A) Expression of iNos, Arg1, and MR after coculture of M1-induced cells with MPT was not statistically different from that seen after stimulation with IL-4. (B) Ym1 and Igf1 expression are significantly lower in M1+MPT compared with M1+IL-4, whereas IL-1β and Msr1 mRNA expression in M1+MPT are significantly upregulated. Expression is presented relative to M1-activated BMMs treated with vehicle. $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for M1+IL-4 *versus* M1+MPT. (C) Quantitative RT-PCR analysis of Arg1 and MR expression in IL-4rα$^{tm1Sz}$ BMMsw (IL-4R KO) induced with IL-4 for 24 hours relative to that in IL-4–stimulated strain-matched Balb/c wild-type BMMs. (D–G) IL-4R KO BMMs or WT BMMs stimulated with Ifnγ for 24 hours (M1) were cocultured with (D and E) MPT cells (M1+MPT) or (F and G) primary tubular epithelial cells isolated from wild-type Balb/c kidneys (M1+PTEC) for 48 hours. ddCt expression shown relative to M1-induced KO and WT BMMs, respectively. A representative experiment is shown for each condition (from an *n* of 3). (H and I) Wild-type (Balb/c) or IL-4Rα–null mice were subjected to unilateral ischemia/reperfusion and contralateral nephrectomy. Sections from the ischemically injured kidney were stained for MR on day 7 after injury (H), and samples were obtained for BUN on the indicated days (I). The infiltration of MR-positive cells and the course of BUN after I/R were indistinguishable in the two groups of mice. $n = 11$ mice/group.

creted factors. Macrophages were obtained from mice that lack the $\alpha$ subunit of the IL-4 receptor and are thus unable to activate Stat6 in response to IL-4 or IL-13.[17,23,24] As predicted, stimulation of these IL-4r$\alpha$–null macrophages with IL-4 fails to induce expression of either Arg1 or MR compared with macrophages obtained from wild-type mice (Figure 4C). In contrast, coculture of the IL-4r$\alpha$–null macrophages with MPT cells results in marked upregulation of both Arg1 and MR to levels that are indistinguishable from that seen with wild-type macrophages (Figure 4, D and E). In a series of parallel experiments, freshly isolated primary tubular epithelial cells (PTECs) were used for coculture rather than the immortalized MPT cells. PTEC coculture was also found to induce expression of Arg1 and MR in both wild-type and IL-4r$\alpha$–null macrophages, albeit to a lesser degree than that seen with MPT coculture (Figure 4, F and G). Finally, IL-4r$\alpha$–null mice exhibited a similar degree of macrophage accumulation in the ischemically injured kidney as that seen in wild-type mice, with indistinguishable changes in BUN during injury and recovery (Figure 4, H and I). Cumulatively, these experiments suggest that macrophages can exhibit great plasticity in their expression profile depending on the signals received and that tubular cells secrete a factor or factors that are capable of regulating that expression profile in an IL-4 receptor–independent fashion.

### Alternatively Activated Macrophages Promote Tubule Cell Proliferation

The finding that the predominant F4/80-expressing macrophage phenotype transitions from proinflammatory to alternatively activated as the injured kidney progresses from cell necrosis/apoptosis to proliferation and repair led us to examine the role of classically and alternatively activated macrophages in mediating kidney injury. To determine whether Ifn$\gamma$- and IL-4–induced macrophages exert different effects after kidney injury, circulating and tissue myeloid phagocytes, including monocytes and resident macrophages, were partially depleted by treatment with liposomal clodronate, followed by I/R and intravenous infusion of $1 \times 10^6$ macrophages stimulated with either Ifn$\gamma$ or IL-4 *in vitro*. Pilot studies in which the infused macrophages were labeled with a membrane tag before infusion showed that $7.5 \pm 3.1$ Ifn$\gamma$-stimulated cells/hpf and $5.3 \pm 2.2$ IL-4–stimulated cells/hpf were found in proximity to the injured tubules 24 hours after infusion. To determine the functional effects of these cells, animals were treated with either liposomal vehicle (LV) or liposomal encapsulated clodronate (LC) for 2 days, followed by unilateral I/R and contralateral nephrectomy to induce AKI. Infusion of Ifn$\gamma$-stimulated cells in macrophage-depleted, I/R-injured animals resulted in an increase in BUN 24 hours after injury that was indistinguishable from LV-treated, I/R-injured control animals and that was significantly worse than that seen in I/R-injured animals with macrophage depletion alone (Figure 5A). Consistent with the results of Wang *et al.*,[13] infusion of IL-4–stimulated macrophages in macrophage-depleted I/R-injured mice did not increase BUN above that seen with macrophage depletion alone. Examination of renal histology (Figure 5B) and tubular injury scoring (Figure 5C) 24 hours after I/R injury and M1 or M2 cell infusion confirmed that tubule damage was more severe in the LC+M1-treated animals compared with LC alone or LC+M2 macrophage infusion. These data suggest that proinflammatory macrophages, but not alternatively activated cells, promote increased tubular damage after AKI.

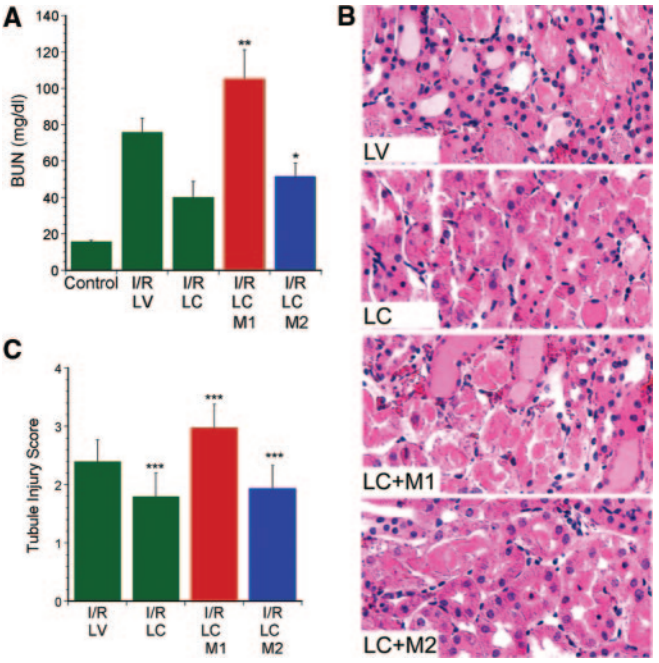


**Figure 5.** Proinflammatory macrophages promote tubule injury. Mice treated for 2 days with liposomal vehicle (LV) or liposomal clodronate (LC) were subjected to unilateral I/R and contralateral nephrectomy, followed by infusion of $1 \times 10^6$ M1 or M2 macrophages immediately after I/R. (A) BUN values are shown 24 hours after I/R ± macrophage infusion. $n = 4$ to 8 animals/group, $^{**}P < 0.01$ *versus* I/R+LC alone, $P$ = not significant *versus* I/R+LV alone. $^{*}P < 0.05$ *versus* I/R+LC+M1, $P$ = not significant *versus* I/R+LC alone. (B) Histology of mice treated as in A shows increased tubular injury in the LV control and LC+M1 group compared with LC or LC+M2. Original magnification: ×400. (C) Blinded scoring of tubular injury shows increased injury in the mice receiving M1 macrophages ($n = 4$ mice/group, $^{***}P < 0.001$ LC *versus* LV, $P < 0.001$ LC+M1 *versus* LC, $P < 0.001$ LC+M2 *versus* LC+M1, $P$ = not significant *versus* LC+M2 *versus* LC).

The presence of proinflammatory macrophages in injured organs such as the kidney has been shown to increase reactive oxygen species generation and induce apoptotic death of sublethally injured tubular cells. However, the observation that the number of M2 macrophages is increasing at the time when cell proliferation is occurring in the injured kidney led us to examine the possibility that M2 macrophages might promote proliferation of renal epithelial cells rather than cell death. To partially mimic the *in vivo* setting of macrophage interaction with injured tubular cells, MPT cells were subjected to ATP depletion via culture in glucose-free media containing 2-de-

oxyglucose before macrophage coculture. MPT cells underwent 24 hours of ATP depletion in the bottom chamber of a Transwell culture dish, and surviving cells were washed and placed in glucose-containing media with 2% FBS. Macrophages that had been previously activated with Ifnγ or IL-4 were added to the top chamber and cocultured for 3 days. The number of MPT cells present in the bottom well was determined after 24 to 72 hours of coculture. Control MPT cells cultured in the absence of macrophages began to recover and divide by 24 to 48 hours after ATP depletion, with a rapid increase in cell number by 72 hours after ATP depletion (Figure 6A). Coculture with IL-4–stimulated macrophages resulted in a further increase in cell numbers at both 48 and 72 hours after ATP depletion, whereas Ifnγ-stimulated macrophage coculture was indistinguishable from control.

To determine whether alternatively activated macrophages are important for epithelial proliferation *in vivo* during the repair phase after AKI, mice were subjected to unilateral I/R and contralateral nephrectomy followed by injection of LC or LV at 48 and 72 hours after reperfusion. In this manner, the initial proinflammatory macrophage influx and tubule injury are unaltered, whereas the later increase in alternatively activated cells on days 3 to 5 was significantly diminished (Figure 6B). In agreement with the *in vitro* results, examination of cell proliferation on day 5 after injury showed that there was a 45% reduction in the number of Ki-67$^+$ cells in the group with late macrophage depletion (Figure 6C, quantified in D). BUN values showed that the initial injury was indistinguishable in the two groups; however, mice in which macrophages were depleted beginning on day 3 after injury exhibited less improvement in glomerular filtration at 5 and 7 days after injury (Figure 6E). Histologic examination of the kidneys on day 5 showed the persistence of tubule injury in both LV- and LC-treated animals (Figure 6F). Animals receiving LC exhibited more tubules in which the surviving epithelial cells were highly simplified with persistent luminal casts (Figure 6F, green arrows), whereas those receiving LV had more regenerating tubules with greater numbers of epithelial cells/luminal cross section (Figure 6F, yellow arrows). It is of interest to note that, although the Ki-67 staining and tubule histology suggest a substantial diminution of tubule regeneration in mice depleted of macrophages during the repair phase, the improvement in BUN is only modestly impaired in these animals. This suggests that, as in humans with AKI, the improvement in GFR after acute tubular necrosis in the rodent is likely to represent a combination of tubule repair and hyperfiltration through less

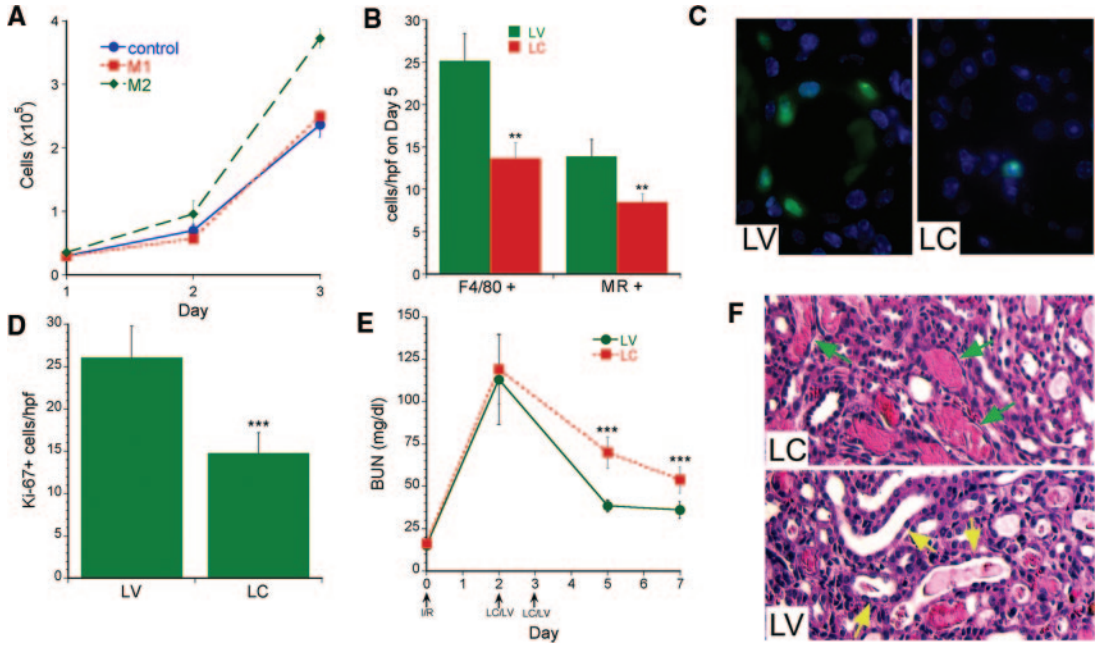


**Figure 6.** Alternatively activated macrophages promote tubule proliferation and kidney repair. (A) Transwell coculture of ATP-depleted MPT cells with M2 macrophages results in increased cell numbers compared with coculture with M1 macrophages or MPT cells alone ($n = 3$, $P < 0.01$ for M2 *versus* M1 and M2 *versus* control; $P$ = not significant for M1 *versus* control). (B) Mice were subjected to unilateral I/R and contralateral nephrectomy followed 48 and 72 hours later by injection of LV or LC. Quantitation of F4/80 and/or MR expressing cells/400× field in the outer medulla on day 5 after injury. $n = 3$, $^{**}P < 0.01$ *versus* LV. (C and D) Proliferating epithelial cells on day 5 after injury were detected by Ki-67 immunostaining (C, 1000×) and quantified/400× field in the outer medulla (D, $n = 3$, $^{***}P < 0.001$). (E) BUN determination shows less improvement in GFR at 5 and 7 days after injury in the LC-treated group ($^{***}P < 0.001$ *versus* LV at days 5 and 7). (F) Histology on day 5 after I/R of kidneys from mice treated on days 2 and 3 with LC or LV shows that the majority of injured tubules in LV-treated animals are undergoing repair with increasing numbers of cells/tubule (yellow arrows). In contrast, many tubules from LC-treated mice continue to have small numbers of highly simplified cells lining the basement membrane (green arrows). Original magnification: ×400.

injured nephrons. In agreement with this possibility, we found that there is a significant component of tubule atrophy and interstitial fibrosis in normal mice 2 weeks after I/R injury, despite return of the BUN to baseline (data not shown).

## DISCUSSION

These studies showed a biphasic effect of mononuclear phagocytes in the injured kidney. The early influx of macrophages seem to promote a proinflammatory milieu that may serve to remove damaged cells and promote the apoptosis of some sublethally injured cells, thus worsening the initial level of tubule injury and augmenting the decrement of glomerular filtration. However, in response to input received from the injured kidney, there is a subsequent switch to an alternatively activated macrophage phenotype, leading to suppression of the inflammatory response and promotion of a proliferative repair phase. This phenotypic switch seems to be partially independent of the IL-4 and IL-13 Stat6 signaling pathway, because macrophages with markers of alternative activation are induced in IL-4r$\alpha$–null macrophages cocultured with renal tubular cells. Thus, it is likely that multiple signals derived from tubular cells, endothelial cells, interstitial cells, and other inflammatory cells are sensed by the macrophages that have entered the injured kidney, resulting in a highly complex array of potential effector responses. Mechanisms by which macrophages are polarized to a trophic phenotype and promote tubular repair after I/R injury have yet to be fully elucidated. Tubular epithelial secreted Csf1 has been implicated as a potential signal that mediates repair after I/R, although macrophages only partially contribute to Csf1-dependent tubular repair.[25] In addition, recent evidence suggested that macrophage-derived Wnt7b plays a major role in stimulating repair and regeneration of renal epithelial cells after ischemic injury.[26] Ultimately, the resolution of this cycle would be predicted to require an additional set of signals leading to the efflux and/or apoptosis of the alternatively activated macrophages to allow full epithelial redifferentiation. The identity of each of these signals and how they cross-talk to define the exact expression profile of the macrophages is currently unknown, but this information will be an important advance in our understanding of how injury and repair are regulated under normal and pathologic conditions.

## CONCISE METHODS

### Animal Models

C57BL/6 male mice, 8 to 12 weeks old, were obtained from Charles River (Wilmington, MA). IL-4r$\alpha$ knockout (KO; BALBcJ/Il-4r$\alpha^{tm1Sz}$) and BALB/cJ mice were obtained from Jackson Laboratory (Bar Harbor, ME). Male 8- to 10-week-old mice were anesthetized and subjected to renal I/R using a modified approach to that previously described.[27] Briefly, the left renal pedicle was clamped for 30 minutes using a nontraumatic micro-aneurysm clip (Harvard Apparatus), whereas the right renal pedicle was ligated with two sutures followed by nephrectomy. Mice were kept at 37°C using a warming pad, and reperfusion of the left kidney was confirmed after clamp release. Mice were given 1 ml of normal saline intraperitoneally to prevent dehydration. Blood for BUN was obtained before I/R and at the indicated times after reperfusion. All experiments were repeated on at least two separate occasions. For detection of proliferation, mice were injected with BrdU (1 mg/g intraperitoneally, BD Bioscience) 3 hours before death, or immunostaining was performed for Ki-67. All animal protocols were approved by the Yale University Institutional Animal Care and Use Committee.

### Mononuclear Phagocyte Depletion with LC

LC and LV were generous gifts of Dr. Van Roojien.[28] Three hundred microliters of LC or LV was injected intraperitoneally on 2 successive days either before I/R or 48 hours after I/R. In some experiments, mice received an intravenous injection of $1 \times 10^6$ Ifn$\gamma$ or IL-4 activated macrophages given via the retroorbital sinus immediately after I/R injury. The depletion of circulating monocytes was determined using a Hemavet 950 automated cell counter (DREW Scientific, Oxford, CT), and the depletion of tissue macrophages was determined by quantifying the number of F4/80$^+$ cells/400$\times$ field (hpf) in the nephrectomized kidney.

### Immunostaining

Kidneys were perfusion fixed with 4% paraformaldehyde and processed for histology (hematoxylin and eosin, paraffin blocks) or immunostaining (4-$\mu$m cryosections). The sections were blocked at room temperature using saline containing 0.1% BSA and 10% goat serum. The following primary antibodies were used: anti-BrdU (Sigma), anti-TammHorsfall protein (Chemicon), anti-Megalin (a kind gift by Dr. Dan Biemesderfer), anti-F4/80 (clone BM8, eBioscience), anti-NOS2 (clone C-11, Santa Cruz Biotechnology), anti-Ki-67 (clone SP6, NeoMarkers), and anti-MR (clone MR5D3, Cell Sciences). Quantification of cells expressing the specified marker was performed in a blinded fashion by counting positive cells/total cells (identified as 4′,6-diamidino-2-phenylindole$^+$ nuclei) in 10 randomly chosen 400$\times$ fields from the outer medulla. At least two sections per kidney from at least four kidneys were counted for each experiment. Images were taken at 200, 400, or 1000$\times$ using a Nikon microscopy system.

### Isolation of F4/80-Expressing Cells from Injured Kidneys for Real-Time PCR

Kidneys were harvested at each time point, minced, and homogenized, followed by incubation with 0.1% collagenase (Worthington) and 20 $\mu$g/ml DNase I (Qiagen) for 30 minutes at 37°C. Mononuclear cells were obtained by density separation using Lympholyte M (Cedarlane), and the F4/80$^+$ cell fraction was enriched using MACS (Miltenyi Biotec). Briefly, macrophages were labeled with biotin-conjugated rat anti-mouse F4/80 and purified using sepharose-coated nanobeads (Stem Cell Technology) according to manufacturer's protocol. To select M1 and M2 cells, the kidneys were minced and digested as above, followed by staining with anti-F4/80-FITC (BM8, eBioscience), anti-Cd11c-PE (HL3, BD Biosciences), anti-CD45-PerCP (30F11, BD Biosciences), and anti-CD206 (MR)-Alexa Fluor 647 (MR5D3, AbD Serotec), after incubating for 10 minutes at 4°C in

the presence of anti-CD16/32 (2.4G2, BD Biosciences) antibody to block Fc receptors. Stained cells were sorted by FACS Aria and analyzed with LSR II (BD Biosciences). All sorted macrophage subsets were >98% pure by postsort analyses. Total RNA was extracted from enriched M1 and M2 and reverse transcribed for real-time PCR analysis of gene expression.

### Analysis of mRNA

RNA was extracted with a RNeasy Mini kit (Qiagen) and reverse transcribed. Gene expression analysis was determined by quantitative real-time PCR using an iCylcer iQ (Bio-Rad) and normalized to $\beta$-actin or hypoxanthine guanine phosphoribosyl transferase. The following primers were used: Arg1 Fw: CAGAAGAATGGAAGAGTCAG, Rev: CAGATATGCAGGGAGTCACC; MR Fw: CAAGGAAGGTTGGCATTTGT Rev: CCTTTCAGTCCTTTGCAAGC; iNos Fw: CCAAGCCCTCACCTACTTCC, Rev: CTCTGAGGGCTGACACAAGG; Il-12p40 Fw: GGTGTAACCAGAAtk;1AGGTGCG, Rev: AAGGTGTCATGATGAACTTAGG; Igf1 Fw: TGGATGCTCTTCAGTTCGTG Rev: GTCTTGGGCATGTCAGTGTG; Il-1$\beta$ Fw: TGTGAAATGCCACCTTTTGA Rev: TGTCCTCATCCTGGAAGGTC; Msr1 Fw: AAAGGGAGAGAAGGGGAGTG Rev: GCATGACACAGGAACCAATG; Ym1 Fw: CTTCCACAGGAGCAGGAATC Rev: GCTCCATGGTCCTTCCAGTA; $\beta$-actin Fw: TGGAATCCTGTGGCATCCATGAAAC, Rev: TAAAACGCAGCTCAGTAACAGTCCG; HPRT Fw: CAGTACAGCCCCAAAATGGT Rev: CAAGGGCATATCCAACAACA. Data are expressed using the comparative threshold cycle (dCt) method, and mRNA ratios are given by $2^{-\mathrm{dCT}}$.

### Isolation and *In Vitro* Polarization of Mononuclear Phagocytes

BMMs were isolated and cultured as per the protocol of Cui *et al.*[29] Mouse bone marrow cells were flushed from the femurs of 8- to 12-week-old mice and passed through a 40-$\mu$m cell strainer (BD Falcon), and red blood cells were lysed in ACK buffer (0.15 M $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA). Remaining cells were incubated in MEM$\alpha$ medium (Life Technologies) containing 10% FBS (Sigma), 10% L929 fibroblast cell supernatant (generously provided by Dr. Agnes Vignery), 1% glutamine (Life Technologies), 1% MEM vitamin (Life Technologies), and 1% penicillin/streptomycin (Life Technologies). After 18 hours, nonadherent cells (containing the majority of BMMs) were transferred to a new dish and incubated in 30% L929 supernatant for an additional 7 days. The resulting BMMs were judged to be >98% pure based on F4/80 staining. For *in vitro* polarization, BMMs were stimulated with either 100 U/ml murine recombinant Ifn$\gamma$ (Roche) or 20 ng/ml murine recombinant IL-4 (Sigma) for 24 to 48 hours. To track injected M1 or M2 cells, the cells were labeled using the PKH26 Red Fluorescence Cell Linker Kit (Sigma) according to the manufacturer's instructions.

### Isolation of Primary Tubular Epithelial Cells

Isolation of mouse primary tubular epithelial cells was performed under a modified protocol described by Schafer *et al.*[30] Briefly, kidneys were harvested after cardiac perfusion with 0.025% collagenase (Worthington) in M199 Hank's solution (Lonza). Renal cortex was isolated, minced, and incubated at 37°C in the collagenase solution while 5% $CO_2$ balanced with room air gas was injected into the solution for 40 minutes. Enzyme-containing solution was removed. Tissue and cells were resuspended in renal epithelial growth medium (Lonza) containing 2.5% FBS and penicillin/streptomycin and passed through a 40-$\mu$m cell strainer. Cells were plated onto 0.4-$\mu$m transwell inserts (BD Falcon) and grown to confluence before coculture experiments.

### *In Vitro* Coculture Assays

Mouse proximal tubule cells (BU-MPTs) were grown to a monolayer on a 0.4-$\mu$m Transwell insert (Falcon) and cocultured with $5 \times 10^5$ uninduced BMMs or BMMs induced with Ifn$\gamma$ or IL-4, respectively) for 48 hours. The macrophages were harvested for RNA isolation and real-time PCR, FACS analysis, or immunostaining. For FACS analysis, the cells were stained with rat anti-mouse CD206 (MR conjugated to Alexa Fluor 647, Serotec) for 20 minutes on ice after Fc block with rat anti-mouse CD16/32 (clone 93, eBioscience). Cell analysis was performed using a FACSCalibur flow cytometer (BD Pharmingen). Dead cells were distinguished by 7-amino-actinomycin D staining, and all FACS data were analyzed using Cell Quest and FlowJo software.

In some experiments, MPT cells were subjected to ATP depletion using a modification of our previous procedure.[31] Cells were incubated in glucose-free D-MEM medium (Life Technologies) containing 2% FBS and 10 mM 2-deoxyglucose for various times in a humidified 5% $CO_2$ incubator at 37°C. A time course showed that approximately 60% of the MPT cells had detached and died after 24 hours of ATP depletion, and this time point was used for subsequent coculture experiments. MPT cells ($1 \times 10^5$) plated in six-well tissue culture plates (Corning) were ATP-depleted as above and changed to glucose-containing MEM$\alpha$ + 2% FBS. Control, Ifn$\gamma$-stimulated, or IL-4–stimulated BMMs were seeded on a 0.4-$\mu$m Transwell insert (Falcon) and cocultured with ATP-depleted MPT cells for 1 to 3 days. Numbers of MPT cells were determined at each time point by trypsinizing and counting the cells.

### Statistical Analysis

All scoring was performed in a blinded fashion as per the methodology of Shih *et al.*[32] Briefly, hematoxylin and eosin-stained sections were scored for the percentage of tubules in the outer medulla showing tubular atrophy, tubular dilation, epithelial desquamation, apical blebbing, or brush border loss in 10 randomly chosen, nonoverlapping fields per section. The degree of tubular injury was designated as 0 if <5% of tubules in the field were designated as damaged; 1 for 6 to 25%; 2 for 26 to 50%; 3 for 51 to 75%; and 4 if damage involved >75% of the observed tubules. Statistical comparisons were made using unpaired or paired *t* tests where appropriate, and data are presented as mean ± SEM.

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## DISCLOSURES

None.

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See related editorial, "Macrophages in Kidney Repair and Regeneration," on pages 199–201.