GM-CSF Promotes Macrophage Alternative Activation after Renal Ischemia/Reperfusion Injury

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ABSTRACT
After kidney ischemia/reperfusion (I/R) injury, monocytes home to the kidney and differentiate into activated macrophages. Whereas proinflammatory macrophages contribute to the initial kidney damage, an alternatively activated phenotype can promote normal renal repair. The microenvironment of the kidney during the repair phase mediates the transition of macrophage activation from a proinflammatory to a reparative phenotype. In this study, we show that macrophages isolated from murine kidneys during the tubular repair phase after I/R exhibit an alternative activation gene profile that differs from the canonical alternative activation induced by IL-4–stimulated STAT6 signaling. This unique activation profile can be reproduced in vitro by stimulation of bone marrow-derived macrophages with conditioned media from serum-starved mouse proximal tubule cells. Secreted tubular factors were found to activate macrophage STAT3 and STAT5 but not STAT6, leading to induction of the unique alternative activation pattern. Using STAT3-deficient bone marrow-derived macrophages and pharmacologic inhibition of STAT5, we found that tubular cell-mediated macrophage alternative activation is regulated by STAT5 activation. Both in vitro and after renal I/R, tubular cells expressed GM-CSF, a known STAT5 activator, and this pathway was required for in vitro alternative activation of macrophages by tubular cells. Furthermore, administration of a neutralizing antibody against GM-CSF after renal I/R attenuated kidney macrophage alternative activation and suppressed tubular proliferation. Taken together, these data show that tubular cells can instruct macrophage activation by secreting GM-CSF, leading to a unique macrophage reparative phenotype that supports tubular proliferation after sterile ischemic injury.


Macrophages are key participants in regulating the inflammatory response to infections and sterile injuries. Macrophages are activated in response to pathogens and endogenous injury stimuli. The inflammation that ensues acts to eliminate pathogens/toxins, restore tissue homeostasis, and promote tissue repair. Broadly defined, macrophage activation has been divided into two major subtypes: classically activated proinflammatory M1 macrophages and alternatively activated anti-inflammatory M2 macrophages. Classically activated M1 macrophages are induced by IFN-γ and activation of Toll-like receptors and promote antimicrobial responses downstream of STAT1 and NF-κB signaling, respectively. Alternatively activated macrophages were first defined as IL-4/IL-13–induced macrophages seen in parasitic infections but have subsequently been detected in multiple types of infectious and sterile tissue injuries. M2 macrophages have been typically subcategorized into M2a wound-healing macrophages induced by IL-4/IL-13 activation of the IL-4Rα–STAT6 signaling pathway and regulatory M2b/c macrophages, which are activated by immune complexes and IL-10 or TGF-β, respectively.¹

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These categories have largely been defined in vitro with individual stimuli. In vivo, macrophage differentiation and activation of effector functions during the inflammatory response are dictated by a dynamic and complex microenvironment. Investigation of in vivo macrophages in a wide variety of disease models associated with inflammation, both infectious and sterile, has shown a diverse spectrum of macrophage phenotypes that are temporally and functionally dynamic. Identification of the in vivo signaling pathways that govern macrophage activation and regulate macrophage effector functions may, therefore, provide therapeutic targets to promote inflammation resolution and tissue repair.

In sterile ischemic kidney injury models, macrophages seem to play roles in all phases of the injury process, including the initial injury, subsequent repair, and late fibrosis. Within 24 hours after ischemia/reperfusion (I/R) injury, monocytes are recruited to the kidney, where they differentiate into macrophages. Proinflammatory macrophages predominate during the early injury phase, during which tubular apoptosis is prominent. During the tubular repair phase, when tubular cells are proliferating and repopulating the denuded basement membrane, kidney macrophages begin to express markers of alternative activation. Macrophage depletion studies suggest that the functional phenotypes of the macrophages correlate with each phase. Our previous in vivo studies using fluorescently tagged ex vivo IFN-γ-primed macrophages suggested that signals within the injured kidney were sufficient to induce this proinflammatory to anti-inflammatory phenotypic switch in infiltrating macrophages. Both in vivo studies using IL-4Rα null mice and in vitro coculture studies suggest that the primary signal for alternative macrophage activation in the injured kidney uses an IL-4Rα-independent pathway.

In this study, we show that the pattern of in vivo alternative macrophage activation after I/R injury is distinct from that seen after in vitro IL-4 stimulation but consistent with the pattern of activation seen after macrophage exposure to tubular cell conditioned media (CM). To determine the mechanism by which tubular cells can promote macrophage alternative activation, we defined the signaling pathways activated in macrophages by secreted tubular factors. In vitro studies with primary bone marrow-derived macrophages (BMMs) and CM from serum-starved mouse proximal tubule (MPT) cells showed that tubular cell-secreted factors activate JAK–STAT (STAT3 and STAT5) pathways in macrophages. Using BMMs from LysM-Cre;Stat3fl/fl mice as well as pharmacologic inhibition of STAT5, we found that tubule cell-mediated macrophage alternative activation is regulated by STAT5 activation. GM-CSF, a well known STAT5 activator, is upregulated in renal proximal tubule cells after I/R injury. We found that GM-CSF is secreted by MPT cells and required to induce alternative activation in BMMs. Functional blockade of GM-CSF with a neutralizing antibody both in vitro and in vivo attenuates tubular-mediated macrophage alternative activation, resulting in decreased tubular cell proliferation during the repair phase after kidney injury.

RESULTS

Tubular-Mediated Alternative Activation Is IL-4–STAT6 Independent

We have found that proinflammatory macrophages cocultured with MPT cells or primary tubular epithelial cells show increased expression of the classic alternative activation genes arginase-1 (Arg1) and Cd206 (mannose receptor), which is independent of IL-4Ra–STAT6 signaling (Supplemental Figure 1). To determine if tubular cell-induced alternative activation is distinct from IL-4–induced alternative activation, naïve BMMs were stimulated with IL-4 or CM from serum-starved MPT cells. The gene expression profiles of IL-4– compared with MPT CM-treated macrophages show both overlapping (Arg1 and Cd206) (Figure 1A) and divergent gene expression. Msr1 is induced by MPT CM but not IL-4 (Figure 1B), whereas Dectin-1, Fizz1, Ym1, and Igf-1 are specific to IL-4–induced macrophage alternative activation (Figure 1C). To determine the relevance of these distinct alternative activation profiles for in vivo macrophage activation, macrophages were flow-sorted from control kidneys and ischemically injured kidneys on day 5 after reperfusion (the time at which we detected increased Arg1 and Cd206 expression) and analyzed for alternative activation gene expression. Consistent with the tubular cell activation profile, Msr1 expression is upregulated in macrophages at day 5 after I/R injury compared with macrophages from uninjured kidneys, whereas the expression of downstream IL-4 gene targets (Dectin-1, Fizz1, and Ym1) is not induced (Figure 1D). Of note, Igf-1 expression is upregulated on day 5 after I/R, suggesting that either there is selective IL-4 signaling to induce Igf-1 but not other IL-4 targets or a third pathway (nontubular and non–IL-4) may be responsible for Igf-1 expression.

Tubular Factors That Induce Macrophage Alternative Activation Are Basolaterally Secreted

Proximal tubular cells are polarized epithelia, with the apical surface facing the tubule lumen exposed to the glomerular filtrate and the basolateral surface attached to the basement membrane facing the interstitium. After I/R injury, macrophages traffic to the interstitium of the kidney and line areas of tubular damage adjacent to the tubular basement membrane. To model in vivo polarity and determine whether the polarity of secreted tubular factors would be physiologically relevant in activating macrophages in the interstitium, MPT cells were grown to confluency on 0.4-μm Transwell inserts, and CM were collected from either the apical- or basolateral-facing chambers (Figure 2A). The basolateral MPT CM induced significantly higher expression of Arg1 and Cd206 than the apical CM (Figure 2, B and C), consistent with the model that tubular cell-secreted factors can signal to macrophages in vivo.

Tubular-Secreted Factors Activate JAK–STAT Signaling Pathways

The JAK–STAT pathway is a major signaling pathway that regulates macrophage activation. To determine
Whether a JAK–STAT signaling pathway other than IL-4Rα–STAT6 is involved in tubular cell-mediated macrophage activation, STAT signaling pathways were analyzed in naïve BMMs at various time points after exposure to MPT CM. Macrophage STAT3 and STAT5 were phosphorylated early (within 5 [STAT3] to 30 [STAT5] minutes after treatment with tubular CM, whereas STAT6 was not activated by CM. Compared with IFN-γ-induced macrophages, STAT1 was not significantly activated. mRNA expression of the macrophage alternative activation markers Arg1, Cd206, and Msr1 was induced at 6–12 hours after treatment with tubular CM, whereas the expression of Igf-1 was not induced (Figure 3B).

A pan-JAK inhibitor, JAK inhibitor I, was used to determine whether JAK–STAT signaling is required for the induction of tubular cell-mediated macrophage alternative activation. JAK inhibitor I efficiently inhibited MPT CM-induced STAT3 and STAT5 activation in BMMs (Figure 4, A and B). This JAK–STAT inhibition led to almost complete inhibition of ARG1 protein expression (Figure 4, A and B) and Arg1, Cd206, and Msr1 mRNA expression (Figure 4C).

**Tubule Cell-Mediated Macrophage Activation Requires STAT5 but Not STAT3 Activation**

STAT3-dependent induction of alternative activation has been described through IL-10–16 and more recently, MyD88-dependent autocrine production of IL-6, IL-10, and CSF-3 in mycobacteria-infected macrophages.17 To determine whether STAT3 is required for tubular-mediated alternative activation in macrophages, a transgenic mouse model with myeloid-specific knockout of STAT3 was generated. LysM-Cre transgenic mice with myeloid-specific expression of Cre recombinase were mated with Stat3fl/fl mice, and there was no detectable STAT3 expression (Figure 5, A and B). However, elimination of STAT3 activation did not affect CM-stimulated STAT5 activation or induction of ARG1 protein expression (Figure 5, A and B) or Arg1, Cd206, and Msr1 mRNA expression (Figure 5C).

To determine whether STAT5 is required for tubular cell-mediated macrophage activation, N-(4-Oxo-4H-chromen-3-yl)methylene)nicotinohydrazide, a nonpeptidic small molecule...

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**Figure 1.** Macrophages activated in vitro by secreted tubular factors and in vivo by the microenvironment of the postischemic kidney exhibit an alternative activation gene profile that differs from IL-4-activated macrophages. (A–C) Naïve BMMs were treated with vehicle media (white bars), tubular CM (black bars), or IL-4 (20 ng/ml; gray bars). Cell lysates were collected at 12 hours after treatment for RNA. mRNA expression by quantitative PCR is shown relative to Hprt (n=3). (D) CD45+F4/80+CD11c+ kidney macrophages were isolated from uninjured kidneys and postischemic kidneys 5 days after I/R injury. mRNA expression by quantitative PCR is shown relative to Hprt (n=3 for day 0 and n=5 for day 5). Data are shown as means±SEMs. Exp, expression; Veh, vehicle. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

**Figure 2.** Alternative activation is induced by basolaterally secreted tubular cell factors. (A) MPT cells (0.356×10⁶/4.2 cm²) were plated on 0.4-μm Transwell inserts and allowed to grow to confluence (transepithelial resistance of 61.4±9.2 μOhm·cm²) over a 4-day period. The cells were washed, and media were changed to serum-free media with equal volumes (2 ml) within the insert (apical) and below within the cell culturewell (basolateral) as shown. CM were collected 48 hours later. Naïve BMMs were then treated with vehicle media, apical CM, or basolateral CM. Cell lysates were collected at (B) 12 hours for mRNA expression (shown relative to Hprt and (C) 24 hours for protein analysis (densitometry shown normalized to β-actin; n=3). Data are shown as means±SEMs. ACTB, β-actin; Basolat, basolateral; Exp, expression; veh, vehicle. *P<0.05; **P<0.01 (vehicle, apical, and basolateral).
STAT5 inhibitor that targets the SH2 domain of STAT5 was used. The STAT5 inhibitor reduced MPT CM-induced STAT5 activation by approximately 70% (P = 0.02) and significantly decreased Cd206, with a concomitant reduction in ARG1 protein and Arg1 and Msr1 mRNA expression that did not reach statistical significance (Figure 6). These data suggest that STAT5 signaling rather than STAT3 is required for tubular cell-mediated macrophage alternative activation.

GM-CSF Expression Is Upregulated in the Kidney after I/R Injury and Precedes STAT5 Activation

GM-CSF (or CSF2) is known to be a regulator of monocyte/macrophage differentiation and mediates its actions through the activation of JAK2 and STAT5. We found that GM-CSF expression in the whole kidney is increased within 24 hours after ischemic injury and peaks on day 3, preceding detectable STAT5 activation and protein expression of the alternative activation markers ARG1 and CD206 seen on days 3–5 after I/R injury (Figure 7, A and B). To determine whether ischemic injury specifically induces tubular cell GM-CSF expression, proximal tubule cells were cell sorted by flow cytometry from kidneys 3 days after I/R injury. Expression of Csf2 is significantly upregulated in proximal tubule cells from the postischemic kidney compared with proximal tubule cells from uninjured kidneys (Figure 7C, Supplemental Figure 2 shows sorting specificity for proximal tubule cells). Dual immunostaining for phosphorylated STAT5 and the macrophage marker F4/80 revealed that 11.8% ± 1.6% of F4/80+ cells in the outer medulla were positive for phosphorylated STAT5 on day 5 after I/R (Figure 7D).

GM-CSF Secreted by Renal Tubular Cells Is Required to Induce Alternative Activation in Macrophages

The observation that GM-CSF expression and macrophage alternative activation are sequential in vivo led us to hypothesize that GM-CSF secreted by tubular cells mediates STAT5-dependent macrophage alternative activation. Assessment of naïve macrophage activation in response to recombinant GM-CSF revealed that expression of Arg1, Cd206, and Msr1 was detectably induced at concentrations between 300 and 500 pg/ml, whereas maximal activation of Arg1 and Msr1 required much higher concentrations (Figure 8A). Interestingly, the concentration of GM-CSF in basolateral MPT CM was 374 ± 54.6 pg/ml, whereas GM-CSF levels in the apical MPT CM, which failed to induce alternative activation, were below the induction threshold (185.4 ± 25.4 pg/ml). To determine if tubular cell–secreted GM-CSF is required for in vitro macrophage alternative activation, naïve BMMs were treated with MPT CM with or without the addition of a GM-CSF–neutralizing antibody. These experiments revealed that the neutralizing antibody to GM-CSF prevented STAT5 activation after either GM-CSF or MPT CM stimulation (Figure 8, B and C, quantified in Figure 8D). Furthermore, the neutralizing antibody effectively inhibited Arg1, Cd206, and Msr1 expression in response to MPT CM, showing that GM-CSF is the tubule-derived signal for STAT5 activation and required for tubule cell–induced alternative activation (Figure 8E).

Comparison of Arg1, Cd206, and Msr1 expression in response to macrophage stimulation with either MPT CM or GM-CSF revealed that Arg1 and Msr1 were induced to a greater degree by MPT CM than GM-CSF at concentrations comparable with those in MPT CM (compare the columns for 300 and 500 pg/ml in Figure 8A with MPT CM in Figure 8E). In contrast, Cd206 expression seems to be maximally stimulated by GM-CSF in this dose range. These findings suggest that MPT CM contains factors in addition to GM-CSF that can induce Arg1 and Msr1 expression. Macrophage colony-stimulating factor (M-CSF) is known to promote M2 macrophage activation, expressed in proximal tubular cells after tubular...
Cd206-mediated STAT5 phosphorylation and that GM-CSF is both necessary and sufficient for tubule cell-induced macrophage alternative activation. Here, we sought to investigate the mechanism by which tubular cells induce macrophage alternative activation.

Because JAK–STAT pathways are critical in regulating macrophage activation, we first determined whether a JAK–STAT pathway other than IL-4R signaling is involved in tubular cell-mediated macrophage alternative activation. Using mRNA expression profiling of macrophages isolated from injured kidneys, comparisons of IL-4– and tubular CM-induced macrophages, STAT3-deficient BMMs, and STAT5 inhibition, we identified GM-CSF as a tubule-derived STAT5 activator mediating a unique macrophage alternative activation phenotype that differs from the canonical Th2 IL-4–induced alternative activation state.

**DISCUSSION**

The signals that instruct macrophages to alter their gene expression profile and their functional repertoire to promote tubular repair after renal I/R injury are unknown. Our previous studies showed that macrophages transition from a proinflammatory state during the tubular apoptotic injury phase to a reparative state during the tubular proliferative phase. We found that tubular cells induce macrophage alternative activation in vitro through an IL-4Rα–independent pathway.

Here, we sought to investigate the mechanism by which tubular cells can promote macrophage alternative activation.
GM-CSF was first described for its ability to generate granulocyte and macrophage populations from bone marrow precursor cells. It has become evident that GM-CSF also plays important roles in the activation and function of more mature myeloid cells in the setting of infection and inflammation. Macrophages differentiated in vitro in the presence of GM-CSF or M-CSF have basal cytokine expression profiles and responsiveness to LPS similar to M1 and M2 macrophages, respectively. However, recent characterization of global gene expression profiles of GM-CSF– and M-CSF–derived macrophages suggests that the M1 and M2 categorizations of these cells may be limited, especially when comparing murine and human macrophage gene expression. Furthermore, because the M1/M2 designation of GM-CSF– and M-CSF–differentiated BMMs is primarily on the basis of in vitro macrophages stimulated with LPS, it is unclear whether this designation holds true in the setting of sterile inflammation and in vivo in the presence of additional stimuli. The complexity of GM-CSF biology is evident, because it is implicated in inflammatory conditions, such as rheumatoid arthritis and crescentic GN, but also, as an inducer of M2 markers in microglia/macrophages surrounding glioblastoma tumor cells. Nonhematopoietic cells, such as vascular smooth muscle cells and colon epithelial cells, have been reported to be important sources of GM-CSF, mediating atherogenic monocyte activation and promoting colonic epithelial cell proliferation in colitis, respectively. In addition, GM-CSF has been associated to Th2 immune responses in allergic lung inflammation. Thus, macrophage activation by GM-CSF and M-CSF treatment seems to be highly context dependent.

In our model of mouse renal I/R injury, an increase in GM-CSF expression within the kidney was detectable within 24 hours after I/R and peaked on day 3, at which time proximal tubule cell expression of GM-CSF significantly increased. STAT5 activation and expression of alternative activation markers in macrophages

Figure 5. Alternative activation of macrophages induced by secreted tubular cell factors is independent of STAT3. Naive BMMs from wild-type and LysM-Cre;Stat3<sup>fl/fl</sup> mice were cultured with MPT CM. (A) Cell lysates were harvested at 30 minutes and 24 hours for protein. (B) Densitometry of three separate experiments as in B normalized to β-actin loading control. (C) Cells stimulated as above were harvested 12 hours after stimulation for RNA, and expression is shown relative to Hprt (n=3). Data are shown as mean±SEM. ACTB, β-actin; Exp, expression; Veh, vehicle. *P<0.05; **P<0.01; ****P<0.0001.

Figure 6. Macrophage alternative activation by secreted tubular factors correlates with STAT5 activation. Naive BMMs were pretreated for 30 minutes with vehicle, 1.0 μM JAK inhibitor I, or 100 μM STAT5 inhibitor and then cultured in normal media and vehicle or MPT CM with or without the same inhibitor. (A) Cell lysates were harvested at 30 minutes and 24 hours for Western blot analysis. (B) Densitometry of Western blot analysis from four separate experiments as in A shown relative to vehicle and normalized to β-actin loading control. (C) mRNA expression 12 hours after MPT CM treatment with vehicle or STAT5 inhibitor. Expression shown relative to Hprt (n=4). Data are shown as mean±SEM. ACTB, β-actin; Exp, expression; Inh, inhibitor. *P<0.05; **P<0.01.
follow during the tubular repair phase (3–5 days after I/R). General depletion of macrophages during this tubular repair phase results in decreased reparative tubular proliferation, suggesting that the induction of alternative activation genes in macrophages during this time is critical for macrophage-mediated tubular repair.9,10 These studies used in vivo GM-CSF blockade after renal I/R to assess the importance of this pathway in macrophage activation and tubule repair. One time daily injection of the GM-CSF–neutralizing antibody achieved only a partial reduction in STAT5 activation in the injured kidney at the one time point assessed (day 5), but the expression of all three alternative activation markers shown to require GM-CSF–STAT5 signaling in vitro were significantly reduced in macrophages isolated from these kidneys. Furthermore, the proliferation of tubular cells that is known to underlie effective kidney repair was significantly reduced after GM-CSF blockade.

Although the above observations provide strong support for the importance of tubule-cell-secreted GM-CSF in promoting alternative macrophage activation after kidney injury, it is interesting to note that GFR, as estimated by serum creatinine, was not reduced in the mice exposed to the GM-CSF–neutralizing antibody. It remains to be determined whether this is because we achieved only a partial blockade of GM-CSF signaling or because GM-CSF–STAT5-independent factors, such as tubule-secreted M-CSF24–26 and/or macrophage-secreted IGF-1, can provide sufficient reparative signals to partially compensate for the loss of GM-CSF signaling. Serum creatinine can return to baseline levels, even when there is significant induction of late tubular fibrosis, suggesting that hyperfiltration can underlie normalization of creatinine and mask failure of normal repair. Although this study has identified an important pathway mediating the alternative activation of kidney macrophages after I/R injury, the modest ability of the GM-CSF–neutralizing antibody to prevent STAT5 activation in vivo will likely limit its use for studying these late outcomes.

Our data show that the full expression of alternative activation after tubule injury requires factors other than GM-CSF and are consistent with previous reports of M-CSF as an activator of the reparative macrophage phenotype.24–26 GM-CSF and M-CSF are both secreted by tubular cells and seem to serve as coinducers for maximizing macrophage expression of protective factors, such as arginase-1. In contrast, secreted tubular factors do not induce macrophage IGF-1 expression, although IGF-1 expression is upregulated in kidney macrophages at day 5 after I/R (Figure 1D). IGF-1 is a well known inducer of tubule cell proliferation,41 and the mechanism behind its macrophage expression after kidney injury clearly deserves additional study. Identification of

Figure 7. Macrophage STAT5 activation follows an increase in whole-kidney GM-CSF expression and correlates with alternative activation. (A and B) Whole-kidney lysates of uninjured kidneys (day 0) and kidneys 1, 3, and 5 days after I/R injury immunoblotted for pSTAT5, GM-CSF, CD206, and ARG1; expression was normalized to loading control β-actin. Densitometry in B is shown relative to baseline expression at day 0 (n=4 per group). Data are shown as means±SEMs. *P<0.05; ****P<0.0001 versus day 0. (C) Real-time quantitative PCR for Csf2 using mRNA isolated from proximal tubule cells from kidneys 3 days after I/R. Expression is shown relative to proximal tubule cells from uninjured sham kidneys normalized to Hprt (n=4/group). ***P<0.001. (D) Representative images of F4/80 (red) and pSTAT5 (green) immunofluorescence at day 5 after renal I/R. Original magnification, ×400. ACTB, β-actin; DAPI, 4′,6-diamidino-2-phenylindole.
these signals within the microenvironment of the postischemic kidney will further enhance our understanding of how reparative macrophages are activated.

Taken together, our data show that GM-CSF secreted by tubular epithelial cells after I/R injury mediates a novel STAT5-dependent macrophage activation profile that promotes tubular proliferation during kidney repair. Future studies using conditional knockout of the GM-CSF–STAT5 pathway will be needed to fully address the functional significance of this unique mode of alternative macrophage activation in the process of normal tubule repair as well as maladaptive fibrosis after kidney injury.

CONCISE METHODS

Animals

C57BL/6 (National Cancer Institute/National Institutes of Health), Stat6−/− (The Jackson Laboratory), Lyso-M-Cre18 (The Jackson Laboratory), and Stat3−/− (gift from Xin-Yuan Fu) mice were used for primary bone marrow-derived macrophage cultures. All animal protocols were approved by the Yale University Institutional Animal Care and Use Committee.

Reagents and Antibodies

JAK inhibitor I (420097) and STAT5 inhibitor (573108) from Calbiochem were purchased from EMD Millipore. Rabbit antibody anti-pSTAT1-Y701 (9171), anti-pSTAT3-Y705 (9145), antitotal STAT3 (9132), anti-pSTAT5-Y694 (9359), and anti-pSTAT6-Y641 (9361) were obtained from Cell Signaling Technology. Rabbit anti-CD206 antibody (ab64693) was purchased from Abcam, Inc. Goat antibody anti-ARG1 (sc-18354) and mouse antibody anti-β-actin (sc-69879) are from Santa Cruz Biotechnology. Recombinant mouse GM-CSF and M-CSF were obtained from R&D Systems. Anti-mouse F4/80 (BM8), anti-mouse GM-CSF antibody for Western blot (clone MP1–22E9, 14–7331), anti-mouse GM-CSF functional-grade antibody for neutralization (clone MP1–22E9, 16–7331), and rat isotype control antibody (rat IgG2a, k, 16–4321) were obtained from eBioscience.

In Vitro Cell Culture and CM Protocol

BMMs were isolated and cultured in the presence of L929 supernatant as per the protocol of Cui et al.43 and as previously described.10 In all in vitro BMM experiments, after 7 days of differentiation in the presence of L929, M-CSF, and GM-CSF normalized to loading control β-actin is shown relative to vehicle-treated controls) and (E) 12 hours for RNA. mRNA expression is shown relative to Hprt (n=3 for all experiments). Data are shown as means±SEMs. ACTB, β-actin; Exp, expression; Veh, vehicle. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

Figure 8. GM-CSF secreted by tubular cells promotes alternative activation of macrophages. (A) BMMs were treated with recombinant mouse GM-CSF at the indicated concentrations for 12 hours. Cell lysates harvested for RNA isolation and mRNA expression are shown relative to Hprt. **P<0.01; ***P<0.001 versus 0 pg/ml. (B) Five micrograms per milliliter neutralizing (Neut) GM-CSF antibody or isotype (Iso) antibody was added to culture media containing 300 pg/ml GM-CSF (GM) and incubated for 3 hours at 37°C before treating naive BMMs. Cell lysates of treated BMMs were harvested at 30 minutes for Western blot analysis of pSTAT5. (C–E) Five micrograms per milliliter Neut GM-CSF antibody or Iso antibody was added to MPT CM and incubated for 3 hours at 37°C before treating naive BMMs. Cell lysates of BMMs harvested at (C) 30 minutes for Western blot analysis of pSTAT5 (densitometry in D normalized to loading control β-actin is shown relative to vehicle-treated controls) and (E) 12 hours for RNA. mRNA expression is shown relative to Hprt (n=3 for all experiments). Data are shown as means±SEMs. ACTB, β-actin; Exp, expression; Veh, vehicle. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.
collected after 48 hours, centrifuged to remove debris, and filtered with a 0.2-μm filter. All BMM and MPT CM experiments were performed with 10% FBS containing MEM-a media and either serum-free DMEM:F12 media or serum-free MPT CM at a 1:1 ratio.

Collection of apical and basolateral CM was performed by plating 0.356×10^6 MPT cells on 0.4-μm transparent PET membrane transwell inserts for a six-well plate (353090; Corning). Cells were cultured for 4 days in DMEM:F12 media with 10% FBS with antibiotics/antimycotic (Invitrogen). After 4 days, the cells were washed with PBS three times, and the medium was changed to serum-free DMEM:F12 for 48 hours (2 ml in well: basolateral CM; 2 ml in insert: apical CM). Cell density at the end of the 48-hour serum starvation period was approximately 3.5×10^6 MPT cells on a 4.2-cm² surface of the transwell insert for the six-well plate. Separate experiments were performed using the 12-mm, 0.4-μm PET Snapwell Insert (9.5×10^4 MPT cells plated on a 1.12-cm² growth area and grown to confluence in 4 days) for Ussing chamber transepithelial potential measurements. Transepithelial resistance of the MPT cells at this cell density was 61.4±9.2 mOhm/cm², consistent with polarized proximal tubule cells.

MPT cells deemed to be free of mycoplasma contamination were used to collect CM. Mycoplasma testing was performed with the LookOut Mycoplasma PCR Detection Kit (Sigma-Aldrich). MPT CM were also tested for endotoxin using the ToxinSensor Gel Clot Endotoxin Assay Kit (L00351; Genscript). MPT CM endotoxin level was tested to be <0.25 endotoxin units (EU) /ml (1 EU/ml is approximately 0.1 ng/ml). Of note, MEM-a media containing 10% FBS used

Figure 9. GM-CSF regulates the expression of a subset of kidney macrophage alternative activation genes and promotes tubular proliferation. (A) Mice were subjected to 24 minutes of bilateral I/R and then injected with isotype antibody or neutralizing GM-CSF antibody daily starting on day 1.5 after I/R. All mice were euthanized on day 5 (2 hours after the final antibody injection). (B) Serum creatinine was measured on day 1 (before initiation of antibody treatment) and day 5 after I/R. (C) Whole-kidney lysates of day 5 postischemic kidneys from three mice receiving isotype control and three mice receiving neutralizing antibody immunoblotted for pSTAT5. (D) Densitometry of pSTAT5 relative to β-actin for all mice as shown in C. (E and G) Real-time quantitative PCR for the indicated genes using mRNA isolated from CD45⁺F4/80⁺CD11c⁻ kidney macrophages; expression is shown relative to Hprt. (F) Percentage of total gated kidney cells analyzed by FACS that are CD45⁺F4/80⁺CD11c⁻. (H–J) Representative images of Ki67 immunostaining of kidney sections 5 days after I/R injury from mice that received either (H) isotype antibody or (I) GM-CSF–neutralizing antibody. (J) Quantification of Ki67-positive tubular cells in outer medulla (n=4 for isotype and n=5 for GM-CSF neutralizing antibody). Data are shown as means±SEMs. ACTB, β-actin; Exp, expression; ISO, isotype. Original magnification, ×400. *P<0.05; **P<0.001.
as the vehicle control media tested positive for endotoxin levels > 0.25 EU/ml (industry standard for FBS is < 10 EU/ml).

Renal I/R Model
Male 8- to 10-week-old C57BL/6 mice (National Institutes of Health/National Cancer Institute) were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg) and then subjected to renal I/R using a modified approach to that previously described. Briefly, both the right and left renal pedicles were isolated through a midline abdominal incision and clamped for 24 minutes using a nontraumatic microaneurysm clip (Fine Science Tools). Mice were kept at 37°C using a warming pad, and reperfusion of both kidneys was confirmed after clamp release. Mice were given 1 ml normal saline intraperitoneally to prevent dehydration. Mice were euthanized on days 0, 1, 3, and 5 after clamp release. Mice were given 1 ml normal saline intraperitoneally daily starting 36 hours after I/R injury. Mice were euthanized on day 5; tissue and blood were collected for analysis. Serum creatinine was measured by HPLC (Yale Mouse Metabolic Phenotyping Center).

Isolation of Kidney Proximal Tubule Cells and Macrophages.
Kidney macrophage isolation at day 5 of I/R injury was performed with kidney digestion as previously described. After blocking nonspecific Fc binding with anti-mouse CD16/32 (clone 93; eBioscience), kidney single-cell suspension was stained with anti–CD45-PE (30F11; BD Biosciences), anti-F4/80 FITC (BM8; eBioscience), and anti–CD11c-PE-Cy7 (HL3; BD Bioscience). Using unstained and isotype controls to identify autofluorescent kidney cell populations, CD45+ F4/80+ CD11c+ cells were first selected, and then, the CD11c+ population of the CD45+ F4/80+ cells was gated for isolation on the Sony SY3200. For isolation of kidney proximal tubule cells, kidney single-cell suspensions were stained with Fluorescein-labeled Lotus Tetragonolobus Lectin (1:200; Vector Laboratories). Total RNA was extracted from CD45+ F4/80+CD11c+ or Lotus lectin-positive cells and reverse transcribed for real-time PCR analysis of gene expression as described below.

Immunostaining
Kidneys were perfused with PBS, fixed with 10% neutral buffered formalin, and processed for histology (hematoxylin and eosin; paraffin blocks) or immunostaining (5-μm cryostat sections). After antigen retrieval with BD Retriever A, sections were permeabilized in methanol for 10 minutes at −20°C. The sections were then blocked at room temperature using PBS containing 0.1% BSA and 10% donkey serum. The following primary antibodies were used: anti-F4/80 (clone BM8; eBioscience and anti-pSTAT5 (9359; Cell Signaling Technology). Ki67 immunohistochemistry staining was performed by Yale Research histology. Quantification of cells expressing the specified marker was performed in a blinded fashion by counting positive cells per total cells (identified as 4′,6-diamidino-2-phenylindole+ nuclei for immunofluorescence staining) in 10 randomly chosen ×400 fields from the outer medulla. Images were taken at ×400 using a Nikon microscope system.

Western Blot
Lysates of mouse kidney were prepared by homogenization in 0.5 ml RIPA buffer containing SDS (Teknova) with Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). Cultured cells were also lysed directly in RIPA buffer containing SDS, supplemented with a protease and phosphatase inhibitor cocktail. Equal amounts of protein were loaded, and electrophoresis was performed in 10% polyacrylamide separating gel/5% stacking gel or precast Mini-Protean TGX 4%–15% gradient gel (Bio-Rad). Proteins were transferred to PVDF membrane and blocked with 5% milk in Tris-buffered saline with Tween 20 for 1 hour. Membranes were incubated overnight at 4°C. Blots were then washed and incubated with secondary antibody for 1 hour at room temperature. After washing, the second antibody was visualized by chemiluminescence reagents (PerkinElmer).

ELISA
GM-CSF in MPT CM was measured by sandwich ELISA using anti-mouse GM-CSF antibody (554404; BD Pharmingen) as a capture antibody and biotin anti-mouse GM-CSF antibody (554407; BD Pharmingen) as a detection antibody, with recombinant mouse GM-CSF (R&D) as the standard.

RNA Isolation, RT-PCR, and Quantitative Real-Time PCR
RNA was extracted with an RNeasy Mini Kit (Qiagen) and reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). Gene expression analysis was determined by quantitative real-time PCR (Taq Universal Sybr Green Supermix; Bio-Rad) and normalized to Hprt. Data are expressed using the comparative threshold cycle (dCt) method, and mRNA ratios are given by 2−dCt or fold difference (dD) relative to a control. Primers used include previously published primers for Arg1, Cd206, Hprt, Igf-1, Msr1, Tgfb1, and Ym1 as well as Cis forward: TCGGGAATCTGCGTGCATCT, reverse: GGTTGTCTGTCTCGAATACGG; Csfl forward: GCTGTGATCCCGGTGGTCAG; Csfl reverse: GCTGGAGAAGTCTCATGG; Csf2 forward: TGGTCTACAGGGCTCTCAGCA; reverse: CCGTGAACCTGGCTCTGABA; Dectin1 forward: GAAATCTGTGCTTGGTGTT; reverse: TAGGTGAGGATCCTTGGGAG; and Fizz1 forward: AGGAAACTTCTTGGCACAATCCA, reverse: CTGGTATCCTCCTCCTTCA.

Statistical Analyses
All results are expressed as means ± SEMs. Comparisons were tested with unpaired t tests and one- or two-way ANOVAS with post-test Bonferroni or Tukey multiple comparison analyses where appropriate using Prism 6.0 (GraphPad Software, Inc.). Data are presented as means ± SEMs. P ≤ 0.05 was considered to be statistically significant.

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DISCLOSURES
None.

REFERENCES


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