Ischemia-reperfusion induces G-CSF gene expression by renal medullary thick ascending limb cells in vivo

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Zhang, Ying, Vanessa K. Woodward, John M. Shelton, James A. Richardson, Xin J. Zhou, Daniel Link, Mariusz L. Kielar, D. Rohan Jeyarajah, and Christopher Y. Lu. Ischemia-reperfusion induces G-CSF gene expression by renal medullary thick ascending limb cells in vivo and in vitro. Am J Physiol Renal Physiol 286: F1193–F1201, 2004. First published January 20, 2004; 10.1152/ajprenal.00379.2002.—Ischemic acute renal failure involves not only the kidney but also extrarenal organs such as the bone marrow that produces inflammatory cells. By ELISA and RNase protection assays, we now show that renal ischemia-reperfusion increases serum concentrations of granulocyte macrophage colony-stimulating factor (G-CSF) protein and increases both G-CSF mRNA and protein in the ischemic kidney. In situ hybridization localized the increased G-CSF mRNA to tubule cells, including medullary thick ascending limb cells (mTAL), in the outer medulla. We also show that mTAL produce G-CSF protein and increase G-CSF mRNA after stimulation by reactive oxygen species in vitro. The production of G-CSF by the kidney after ischemia-reperfusion provides a means of communication from the injured kidney to the bone marrow. This supports the known inflammatory response to ischemia.

Acute renal failure; ischemia; granulocyte colony-stimulating factor; reactive oxygen species; medullary thick ascending limb tubules

ISCHEMIC ACUTE RENAL FAILURE (ARF) of native kidneys remains a major clinical problem with high mortality (for reviews, see Refs. 29, 44, 47). Recent data suggest that the response to such renal injury involves not only the kidney but also extrarenal organs (reviewed in Ref. 15) such as the lung that produces hepatocyte growth factor (21, 23), the liver (15, 19), and the bone marrow. The bone marrow produces inflammatory cells, including macrophages (5) and neutrophils (see review in Ref. 18), which infiltrate the kidney and exacerbate ischemic injury. The bone marrow may also produce stem cells that help repair renal injury (13, 20).

If the kidney produced granulocyte colony-stimulating factor (G-CSF) after renal ischemia, this molecule would stimulate the marrow to produce neutrophils and macrophages (12, 54), as well as any stem cells, that migrate into the kidney. Furthermore, G-CSF activates endothelium (1); this might facilitate the inflammatory response to ischemia. It also regulates T cell functions (46, 53); this may be important given the role of T cells in ischemic renal injury (36).

Given the above potential importance of G-CSF in ischemic ARF, we assessed this molecule during this disease. We now report that ischemic ARF increases peripheral serum concentrations of G-CSF, as well as renal G-CSF mRNA and protein. Furthermore, renal tubular cells [including medullary thick ascending limb cells (mTAL)] contain G-CSF mRNA in vivo by in situ hybridization; mTAL in vitro produce G-CSF protein and increase G-CSF mRNA after exposure to reactive oxygen species (ROS) that are generated during the reperfusion phase of ischemic ARF (28).

MATERIALS AND METHODS

Animals. Six-week-old male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME). They were used according to the guidelines from the National Institutes of Health and the University of Texas Southwestern Medical Center.

Cell lines. mTAL cells were kindly provided by Dr. F. N. Ziyadeh (51). Cells were grown in DMEM, supplemented with 10% FCS, 2 mM glutamine, and 1% penicillin/streptomycin, and incubated at 37°C and 5% CO2. RAW macrophages were maintained as previously described (7).

ROS. mTAL cells were grown to confluence in tissue culture plates. Monolayers were treated with 5 mM hypoxanthine (HX) and 0.01 U/ml xanthine oxidase (XO; both from Sigma, St. Louis, MO). After incubation at 37°C and 5% CO2 for 2–6 h, total RNA was isolated for RNase protection assays. In some experiments, XO was heated at 90°C for 1 h; this inactivates the enzyme but not any endotoxin contamination (49). The heated XO/HX did not increase G-CSF mRNA; this indicates that no significant endotoxin was present.

Renal ischemia-reperfusion. Mice were anesthetized with inhaled isoflurane, and rectal temperatures were maintained at 37°C (TR-100 Temperature Controller; Fine Science Tools). Ischemia was induced by clamping the left renal pedicle for 18–28 min with a bulldog clamp (catalog no. 2715326V; ASSI). After 2–5 h of reperfusion, both kidneys were harvested for RNase protection assay. The nonischemic right kidney was the control. In some experiments, the right kidney was removed immediately after removal of the clamp on the left renal pedicle. Blood urea nitrogen (BUN) was measured 24 h after reperfusion using an automated analyzer (Reflotron; Roche Diagnostics, Indianapolis, IN).

RNase protection assays. Total RNA was isolated from the whole mouse kidney using an Rneasy Midi Kit (catalog no. 75144; Qiagen). RNase protection assays were performed using a RPA kit (catalog no. 556134; Pharmingen). The template is mCK-4 (catalog no. 556145; Pharmingen). Total RNA was used for each determination. The protected samples were run on a precast gel (8 M urea-6% acrylamide-bis-acrylamide 19:1). The intensity of the specific bands was quantitated using a Molecular Dynamics Storm 840 PhosphorImager, normalized to L32 gene expression.

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We clamped the left renal pedicle for 24 min and then either removed the right kidney (L ischemia, no Rt nephrectomy) or did not remove the right kidney (L ischemia, no Rt nephrectomy). At 24 h we measured the blood urea nitrogen (BUN) in these mice and control mice. There was no significant difference between the BUN of control and “L ischemia, no Rt nephrectomy” (P < 0.01 by t-test); n = 3 animals/group.

Each kidney was suspended in 2 ml lysis buffer containing Triton X-100, pepstatin A, leupeptin, and aprotinin, homogenized, and centrifuged. ELISA was performed on the supernatants. This technique was previously used to measure tissue cytokines (40).

In situ hybridization. Transcardial perfusion was performed with heparinized diethylpyrocarbonate (DEPC)-saline, followed by chilled 4% formaldehyde (freshly prepared from paraformaldehyde)/DEPC-PBS, pH 7.4. The kidneys were fixed for 16 h with rocking at 4°C, transferred to DEPC-saline, dehydrated, paraffin embedded, sectioned at 5 μm on microscope slides previously treated with Vectabond (Vector Laboratories, Burlingame, CA), and stored desiccated at 4°C. Hybridization, darkroom photographic work, and photomicrography were performed as previously detailed (43).

Histology. Kidneys were fixed in 10% formalin, embedded in paraffin, sectioned, and stained in hematoxylin and eosin.

Construction of the sense and antisense riboprobes for G-CSF. Previously reported primers for murine G-CSF (45) amplified a 514-bp fragment from a cDNA library obtained from a kidney that had 24 min of ischemia and 2 h of reperfusion. The cDNA was made using the ThermoScript RT-PCR System (catalog no. 11146–024; Invitrogen Life Technologies) and oligo(dT)20 primers. PCR was performed using 35 cycles at 94°C for 2 min, 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min. The resulting fragment was initially subcloned into the pDrive Cloning Vector (catalog no. 231222; Qiagen PCR Cloning Kit Ligation Protocol). The plasmid containing the G-CSF insert was transferred into Qiagen EZ Competent Cells (catalog no. 231222) and sequenced to confirm its identity. The BamHI–SalI G-CSF fragment, containing the G-CSF insert, was then subcloned into pBluescript KS+ (catalog no. 231122; Stratagene), which is the preferred vector for our Molecular Pathology Core. To construct the antisense riboprobe, the plasmid was linearized with both enzymes SalI and ClaI to ensure a clean cut. The linearized plasmid was purified by degrading the enzymes with 2.5 μl 10% SDS, 5 μl 0.5 M EDTA, and 2 μl proteinase K at 50°C for 30 min. The linearized plasmid was further purified by water-saturated phenol/chloroform-isoamyl alcohol (50:1) extraction, precipitated by 100% cold ethanol/sodium acetate. Antisense G-CSF was labeled with S35 by using a MAXIScript T7/T3 kit from Ambion (catalog no. 1308–1326) and the T7 enzyme. The radiolabeled fragment was purified using Quick Spin Columns (TE) for radiolabeled DNA purification (catalog no. 1523023; Roche Diagnostics).

To construct the sense riboprobe, the plasmid was linearized with both enzymes BamHI and PstI and exposed to the T3 enzyme; labeling of the sense riboprobe was done with the same procedure described above.

Immunohistology. Immunostaining was performed as previously described (42). To localize mTAL cells, we used a rabbit antibody to mouse Tamm-Horsfall protein that was a gift from Dr. J. Hoyer (11).
To localize proximal tubule cells, we used tetramethylrhodamine isothiocyanate-coupled Lotus tetragonolobus agglutinin.

**Effects of G-CSF on renal function in vivo.** Recombinant murine G-CSF (4 μg; catalog no. 414-CS/CF; R&D Systems) was dissolved in 0.5 ml of 0.1% human serum albumin (RIA grade, catalog no. 7888; Sigma) and given intraperitoneally. Control animals received albumin only. Absolute neutrophil counts in peripheral blood were performed by Antech Diagnostics (Irving, TX).

**RESULTS**

*mTAL tubular cells express G-CSF after ischemia-reperfusion injury in vivo.* Our first experiments demonstrated that G-CSF protein appears in the peripheral circulation in mice with ischemic ARF. Figure 1A shows that G-CSF protein is increased in peripheral blood 4 h after left kidneys are made ischemic by clamping the left renal pedicle for 18–24 min. Figure 1B shows that G-CSF is present at 2 h and remains elevated for over 24 h.

In the above experiments, the right kidney was not removed. As discussed below, there was no increase in the BUN; Fig. 3. Increased leukocytes 24 h after renal ischemia. A: low-power photomicrograph at 24 h after renal ischemia. G, glomerulus. B: high-power photomicrograph of the outer medulla. T, some of the injured tubules. Arrows show some of the peritubular inflammatory cells.

Fig. 4. Ischemia-reperfusion increases the renal abundance of G-CSF mRNA in vivo. The left renal pedicles were clamped for 24 min. After either 2 or 5 h of reperfusion, the total RNA from the right (nonischemic) and left (ischemic) kidneys was harvested. Top: RNase protection gels of two experiments; bottom: densitometry. The y-axis is the ratio of the mRNA densities for G-CSF-L32 (“housekeeping” gene). Filled bars, mRNA from the ischemic left kidney; hatched bars, mRNA from nonischemic right kidney. mRNA is from the right and left kidneys of the same mice (1–8).

Fig. 5. Renal ischemia-reperfusion increases renal G-CSF protein in vivo. After reperfusion (5 h), the left (ischemic) and right (nonischemic) kidneys were perfused with normal saline and homogenized, and their content of G-CSF protein was determined by ELISA. See MATERIALS AND METHODS; n = 6 for each group. *P < 0.001 for the difference between the ischemic and nonischemic kidneys.

To localize proximal tubule cells, we used tetramethylrhodamine isothiocyanate-coupled Lotus tetragonolobus agglutinin.
Fig. 6. In situ hybridization shows increased G-CSF mRNA in the outer medulla after ischemia-reperfusion. The left renal pedicle was clamped for 24 min. After 2 h of reperfusion, that kidney (A and C) and the right nonischemic kidney (B) were harvested for in situ hybridization for G-CSF mRNA. In these darkfield photomicrographs, the bright dots represent anti-sense (A and B) and sense (C) hybridization. O, outer stripe of the outer medulla; the box adjacent to O delineates the area of the outer stripe that is shown in greater detail in Fig. 7. I, inner stripe of the outer medulla; the box adjacent to I delineates the area of inner stripe that is shown in greater detail in Fig. 8. Bar = 100 μm.

Fig. 7. In the outer stripe of the outer medulla, increased G-CSF mRNA is expressed by ischemic S3 tubules and medullary thick ascending limb (mTAL). High-power views of the outer stripe of the outer medulla (the region marked by box adjacent to O in Fig. 6). The ischemic kidney is shown in A, C, E, and F; the nonischemic kidney is shown in B and D. Darkfield is shown in A and B; brightfield is shown in C and D. E shows mTAL stained with anti-Tamm-Horsfall-protein; F shows proximal tubules stained with tetramethylrhodamine isothiocyanate (TRITC)-coupled Lotus tetragonolobus. Bar = 100 μm. White arrow in A shows one of many ischemic tubules expressing more G-CSF (more white dots) than nonischemic tubule indicated in B. White arrow in E shows one of many mTAL; white arrow in F shows one of many proximal tubules.
therefore, the increased serum G-CSF was not the result of decreased renal clearance. At 24 h after clamping of the left renal pedicle, the unmanipulated right kidney maintained the same BUN as control mice (Fig. 2). The functional significance of clamping the left kidney was demonstrated by performing a right nephrectomy in one group of mice. That group of mice had a BUN of 161 ± 5 mg/dl compared with control mice, which had a BUN of 26 ± 2 mg/dl.

The amount of damage inflicted by the ischemia-reperfusion at 24 h is shown in Fig. 3. Note the damaged tubules in the outer medulla and the peritubular leukocytes. The large number of leukocytes at 24 h (Fig. 3) contrasts with the undetectable number of leukocytes at 2 h after ischemia (see Figs. 7C and 8C).

We found that a source of G-CSF in peripheral blood is the ischemic kidney by measurement of both renal G-CSF protein and mRNA. G-CSF mRNA was measured using RNase protection assays. The right nonischemic kidney was compared with the left ischemic kidney in eight different mice. The results are shown in Fig. 4. After 2 and 5 h of reperfusion, the G-CSF mRNA was increased in the ischemic kidney. We also measured the amount of G-CSF protein in the left ischemic and right control kidneys. After 5 h of reperfusion, the ischemic left kidney contains more than two times the G-CSF protein as the nonischemic right kidney (Fig. 5).

We localized the structures in the ischemic kidney that were producing G-CSF mRNA in Figs. 6–8. After 24 min of ischemia and 2 h of reperfusion, there was increased in situ hybridization of antisense G-CSF mRNA in the outer medulla of an ischemic left kidney (Fig. 6A) compared with the outer medulla of the contralateral nonischemic right kidney (Fig. 6B). Although ischemia slightly decreased G-CSF expression in the cortex (compare Fig. 6, A and B), the major effect was the increased expression in the outer medulla because both G-CSF mRNA (Fig. 4) and protein (Fig. 5) were increased for the entire kidney. Note the low control sense G-CSF staining of the ischemic kidney (Fig. 6C).

Figure 7 shows high-power views of the outer stripe of the outer medulla of ischemic vs. nonischemic kidneys. A comparison of Fig. 7, A and B, shows more G-CSF mRNA in most, if not all, tubules of the ischemic outer stripe of the outer

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**Fig. 8.** In the inner stripe of the outer medulla, increased G-CSF mRNA is expressed by ischemic mTAL. High-power views of the inner stripe of the outer medulla (the region marked by box adjacent to I in Fig. 6). The ischemic kidney is shown in A, C, E, and F; the nonischemic kidney is shown in B and D. Darkfield views are shown in A and B; brightfield views are in C and D. E shows mTAL stained with anti-Tamm-Horsfall-protein; F shows the absence of proximal tubules after staining with TRITC-coupled Lotus tetragonolobus. Bar = 100 μm. White arrow in A shows one of many ischemic tubules expressing more G-CSF (more white dots) than nonischemic tubule indicated in B. White arrow in E shows one of many mTAL.
medulla. A comparison of hematoxylin- and eosin-stained sections (Fig. 7, C and D) shows tubular injury in the ischemic outer stripe but few, if any, leukocytes. This last point is important because G-CSF was previously thought to be produced only by leukocytes (17, 30, 41). The absence of detectable leukocytes, together with the large number of renal tubules expressing G-CSF in Fig. 7A, indicates that the G-CSF is being produced by ischemic renal tubule cells. Furthermore, the absence of leukocytes at 2 h of reperfusion (Fig. 7C) contrasts with the large number of leukocytes in the outer medulla at 24 h of reperfusion (Fig. 3). Figure 7, E and F, shows the results of immunostaining with markers specific for either mTAL or proximal tubules. As expected, both mTAL and proximal tubules (S3 segments) are present in the outer stripe. Because Fig. 7A shows increased G-CSF in all tubules, both mTAL and S3 proximal tubules must express G-CSF after ischemia.

Figure 8 is similar to Fig. 7 except that the inner stripe is studied at high power. Figure 7, A and B, shows that more G-CSF mRNA is expressed by most, if not all, tubules in the ischemic inner stripe. Figure 7C shows injury but no detectable leukocytes in the ischemic inner stripe. Figure 7, E and F, shows that mTAL, but not proximal tubules, are present in the inner stripe. Because Fig. 7A shows increased G-CSF in all ischemic tubules, mTAL must be one type of tubule expressing this G-CSF.

mTAL express G-CSF after exposure to ROS in vitro. To better understand the regulation of G-CSF gene expression after ischemia, we chose to study mTAL cells in vitro for the following reasons. As shown above, mTAL are one type of tubule that expresses this gene in vivo after ischemia-reperfusion. Furthermore, although the production of G-CSF has not previously been examined, these cells do produce other regulatory molecules after ischemia-reperfusion injury in vivo and in vitro (2, 38, 39). We chose to stimulate these cells with ROS because ROS appear during the reperfusion phase of ischemic ARF (28). Furthermore, ROS, generated by the action of XO on HX, has previously been used to stimulate intracellular signaling pathways in mTAL (6).

ELISA shows that mTAL exposed to ROS release G-CSF protein in the supernatant (Fig. 9). This increased G-CSF protein results from increased mRNA in mTAL exposed to ROS for 2 or 5 h (Fig. 10). In these last two experiments, heat-inactivated XO is used as a control. Treatment at 90°C inactivates the XO enzymatic activity but not endotoxin. The fact that heated XO did not stimulate G-CSF mRNA expression thus indicates that contaminating endotoxin is not responsible for this expression. This control is necessary because endotoxin sometimes contaminates enzyme preparations and endotoxin does activate G-CSF gene expression (30).

Macrophages do not express G-CSF after stimulation by ROS in vitro. Figures 7 and 8 show that tubules are the major cells expressing G-CSF after ischemia in vivo, and in vitro studies (Figs. 9 and 10) show that mTAL produce G-CSF in response to ROS that are generated during the reperfusion phase of ischemic injury. Previously, G-CSF production has been studied in the setting of infection, not ischemia-reperfusion, and the major G-CSF-producing cell was a macrophage responding to endotoxin or cytokines (17, 30, 31, 41). Although small numbers of macrophages are present in the interstitium of the normal kidney (for example, see Ref. 35), the ability of macrophages to produce G-CSF in response to
ROS, in contrast to endotoxin, has not previously been examined. Therefore, we addressed this issue in Fig. 11. Unlike mTAL, macrophages did not activate their G-CSF gene in response to ROS in vitro. Note the positive control: macrophages did respond to endotoxin as previously reported.

G-CSF itself does not injure the kidney. To determine whether G-CSF itself injured the kidney, we injected 4 μg recombinant murine G-CSF and measured BUN and absolute neutrophil counts in peripheral blood 24 h later. Consistent with previous reports, the G-CSF caused neutrophilia (32). However, we found that it did not cause an increase in BUN (Fig. 12).

**DISCUSSION**

Our data indicate that renal ischemia-reperfusion injury increased peripheral serum concentrations of G-CSF (Fig. 1), increased renal G-CSF mRNA (Fig. 4) and protein (Fig. 5) in vivo, increased G-CSF mRNA in tubular cells (including mTAL) of the outer medulla in vivo (Figs. 6–8), and increased production of G-CSF protein and mRNA in mTAL in vitro (Figs. 9 and 10). We attempted two techniques to demonstrate renal G-CSF protein in vivo. We were unable to demonstrate G-CSF in the ischemic kidney by immunohistochemistry; to our knowledge, there are no reports of immunohistochemistry being used successfully to demonstrate G-CSF in any tissue in vivo. However, we were able to demonstrate increased G-CSF protein in the ischemic kidney by ELISA on renal homogenates (Fig. 5).

This study makes two important points. **Point 1** is that we show that renal tubule cells make G-CSF in response to renal ischemia. This point is supported by the following observations: in situ hybridization shows that most cells in the ischemic outer medulla at 2 h reperfusion have increased G-CSF mRNA (Figs. 7A and 8A). The mRNA is associated with renal tubules. Furthermore, Figs. 7C and 8C show no detectable leukocytes at 2 h after reperfusion by hematoxylin/eosin staining. [Note the absence of leukocytes at 2 h of reperfusion (Figs. 7C and 8C) contrasts with the large number of leukocytes present in the outer medulla at 24 h of reperfusion (Fig. 3)]. This is an important point. Because previous studies of G-CSF production were performed in the setting of infection and...
showed production by macrophages stimulated by endotoxin or cytokines (17, 30, 41), it is important to exclude leukocytes as major producers of G-CSF. Because there are undetectable leukocytes, and G-CSF mRNA is associated with renal tubules, we conclude that the G-CSF gene is activated in renal tubules after ischemia-reperfusion in vivo. This conclusion is further supported by the inability of macrophages to produce G-CSF in response to ROS in vitro (see below).

Point two is that mTAL produce G-CSF in response to ROS in vitro. Such ROS are produced during the reperfusion phase of ischemic ARF (28). We chose to study mTAL in vitro because they are a major cell type expressing G-CSF in vivo. Figures 7 and 8 show that most, if not all, tubules in the outer and inner stripes of the outer medulla produce G-CSF mRNA. Because immunohistology shows that mTAL are a major population of both the inner and outer stripes, they must produce G-CSF. Furthermore, we chose to study mTAL in vitro because mTAL are known to survive moderate ischemia and produce other regulatory molecules (2, 38, 39). We found that mTAL increase G-CSF mRNA and produce G-CSF protein in response to ROS in vitro (Figs. 9 and 10). In contrast to mTAL, ROS does not activate the G-CSF gene in macrophages in vitro (Fig. 11). This last experiment buttresses our point that renal tubules, not macrophages, produce G-CSF in vivo.

This production of G-CSF by the kidney after ischemic injury is expected to stimulate the bone marrow to produce both neutrophils and macrophages (12, 54). These cells are in the kidney by 24 h after ischemia (9, 16, 50). Therapies that prevent their entry in the kidney ameliorate ischemic injury in some studies. Such therapies include transgenic knockout (14) or antisense inactivation (4, 37) of endothelial adhesion molecules, antibodies against chemokines (26, 39), and pharmacological inhibition of chemotactic leukotrienes (31, 31). The relative contribution of neutrophils and macrophages to the renal inflammatory infiltrate is controversial because these cells may be difficult to differentiate by antibodies and histological dyes (52).

However, not all studies that decrease inflammation after ischemic ARF ameliorate injury (for example, see Ref. 24). How may one reconcile these seemingly contradictory studies? One approach may be to divide ischemic ARF into phases (3, 27) including an "initiation phase," when ischemia-reperfusion initiates epithelial and vascular cell injury, and an "extension phase," when maladaptive responses exacerbate the initial injury. The inflammatory response to ischemia may occur during the extension phase. However, if the injury during the initiation phase is severe, it cannot be further exacerbated by inflammation. In other words, an irreversibly damaged kidney resulting from a severe initial insult cannot be further damaged by inflammation, and therapy to decrease inflammation will have no effect. Only if the initial injury is moderate will inflammation exacerbate the injury; only under such circumstances will therapy to decrease inflammation ameliorate injury.

Irrespective of whether the inflammatory response to ischemia exacerbates injury, it may be important for renal transplantation. In that setting, the leukocytes may be from the recipient, and their entry in the renal allograft may contribute to activation of the immune response and the resulting rejection (see reviews in Refs. 8 and 22). G-CSF is only one factor in the pathogenesis of ischemic ARF. Injections of G-CSF are used to treat neutropenic patients with cancer or transplant patients with cytomegalovirus infections. Although transient renal failure results rarely (10, 25, 48), such injections are usually safe (34). This suggests that G-CSF alone is not sufficient to injure the kidney. This conclusion is supported by our observation that injections of recombinant G-CSF do not injure the kidney (see Fig. 12 and Ref. 17).

As a final point, G-CSF stimulates the mobilization of stem cells from the bone marrow (for example, see Ref. 33). Such stem cells may help repair the kidney after ischemic injury (13, 20).

In conclusion, our data are consistent with the hypothesis that the response to ischemic renal injury involves not only the kidney but also extrarenal organs that influence events in the kidney (reviewed in Ref. 15). One of these organs is the bone marrow that produces inflammatory cells that exacerbate injury. We show in this report that G-CSF is produced by mTAL in the injured kidney in response to ROS. This G-CSF may be a signal from the injured kidney that mobilizes inflammatory cells, and possibly stem cells, from the bone marrow.

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