

# The Combination of G-CSF/AMD3100 Mobilizes Bone Marrow-Derived Stem Cells to Rescue Mice from Cisplatin-Induced Acute Kidney Injury

Zhi Chen

Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology

Xiang Ren

Shanxi Bethune Hospital

Ruimin Ren

Shanxi Bethune Hospital

Yonghong Wang

Shanxi Bethune Hospital

Jiwen Shang (✉ [sjw139@126.com](mailto:sjw139@126.com))

Shanxi Bethune Hospital

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## Research

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# Abstract

**Background:** Several studies have confirmed that mobilizing bone marrow-derived stem cells (BMSCs) ameliorates renal function loss following cisplatin-induced acute kidney injury (AKI). The aim of this study was to explore whether the combination of G-CSF/AMD3100 exerts beneficial effects with respect to renal function recovery in a mouse model of cisplatin-induced nephrotoxicity.

**Methods:** C57BL/6J mice received intraperitoneal injections of G-CSF (200 µg/kg/d) for 5 consecutive days. On the day of the last injection, the mice received a single subcutaneous dose of AMD3100 (5 mg/kg) 1 hour before cisplatin 20 mg/kg injection. 96 hours after cisplatin injection, the mice were euthanized, blood and tissue samples were collected to assess renal function and tissue damage. Cell mobilization was assessed by flow cytometry.

**Results:** Mice pretreated with G-CSF/AMD3100 exhibited longer survival and significantly lower serum creatinine and BUN levels than mice treated with only G-CSF or saline, exhibited attenuated tissue injury and cell death and enhanced tissue repair and cell regeneration. C57BL/6J mice pretreated with G-CSF/AMD3100 exhibited higher numbers of stem cells in peripheral blood than mice treated with only G-CSF or saline. Furthermore, G-CSF/AMD3100 administration prevented increases in the expression of proinflammatory factors, such as IL-6 and TNF-α, and increased the expression of the anti-inflammatory factor IL-10.

**Conclusions:** These results suggest that G-CSF/AMD3100 mobilizes bone marrow cells to improve renal function and prevent cisplatin-induced renal tubular injury and that the combination of G-CSF/AMD3100 is superior to G-CSF alone for preventing AKI. This combination may represent a new therapeutic option for the treatment of AKI and warrants further investigation.

## Background

The two most common causes of acute kidney injury (AKI) are ischemia/reperfusion (I/R) injury and nephrotoxic agent exposure<sup>1</sup>. Cisplatin is a chemotherapeutic drug widely administered to treat various solid tumors. Nephrotoxicity is a severe side effect of cisplatin administration and often results in acute renal disease<sup>2</sup>. The rate of cisplatin-induced renal damage is 25% to 35%, and the curative effects and nephrotoxicity of cisplatin are dose-dependent. Cisplatin-induced toxic nephropathy is typically characterized by tubular necrosis and apoptosis and greatly limits the use of this agent in clinical settings. Hence, novel therapies for cisplatin-induced AKI that do not compromise its anti-tumor effects are needed.

Stem cell therapy is of great interest as a treatment for AKI. Numerous studies have demonstrated that stem cells can repair damaged tubular cells and attenuate cisplatin-induced AKI<sup>3,4</sup>. Bone marrow-derived stem cells (BMSCs) can transdifferentiate into endothelial and epithelial cells<sup>5</sup>. Several studies have demonstrated that mobilizing BMSCs to treat AKI in animal models facilitates significant

improvements in renal function and enables the repair of renal tissue structural damage<sup>6,7</sup>. Granulocyte colony-stimulating factor (G-CSF) mobilizes BMSCs to sites of renal injury, where these cells transdifferentiate into renal stem cells<sup>6,8</sup>. The CXCR4 antagonist plerixafor (AMD3100) exerts beneficial effects against I/R-induced AKI<sup>9</sup> and myocardial infarction<sup>10</sup>. However, several studies have reported that continuous AMD3100 administration accelerates renal functional decline and exerts adverse effects on renal tissue repair<sup>11,12</sup>. Theiss et al. demonstrated that high concentrations of AMD3100 mobilize BMSCs, whereas low concentrations of AMD3100 do not mobilize BMSCs and do not improve survival in the setting of myocardial infarction<sup>13</sup>. However, Zuk et al. demonstrated that a single dose of 5 mg/kg AMD3100 was less effective at mobilizing BMSCs than a single dose of 1 mg/kg AMD3100 in a rat model of renal I/R<sup>14</sup>. Other studies have demonstrated that the combination of G-CSF and AMD3100 increases BMSC mobilization compared with G-CSF alone<sup>15,16</sup>. Therefore, the usefulness and optimal dosage of AMD3100 remain controversial. In addition, few studies have examined the combination of G-CSF/AMD3100 as a therapy for cisplatin-induced AKI.

Based on the above findings, we speculated that combination therapy with G-CSF/AMD3100 might be more effective for recruiting BMSCs into renal tissue to repair and ameliorate cisplatin-induced AKI than either therapy alone. In addition, the mechanisms underlying the renoprotective effects of BMSCs require further elucidation. In the present study, we first demonstrated that G-CSF/AMD3100 mobilizes BMSCs into the peripheral blood and rescues mice from cisplatin-induced AKI. Second, we demonstrated that mobilization of BMSCs by G-CSF/AMD3100 decreases tubular cell apoptosis and promotes proliferation. The protective effects of this combination are associated with reductions in the levels of putative biomarkers of renal injury and inflammation.

## Materials And Methods

### Ethics statement

All experiments were performed using C57BL/6J mice and were conducted according to the Huazhong University of Science and Technology Guide for the Care and Use of Laboratory Animals. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology, Wuhan, China.

### Animals

Experiments were performed with 7- to 8-week-old male C57BL/6 mice (weighing 20-25 g) purchased from Hua Fukang Company (Beijing, China). The animals were housed according to institutional animal research guidelines and were maintained under constant temperature, a 12-hour light/dark cycle, and 50±5% humidity with standard mouse chow and water *ad libitum*.

### Cisplatin-induced acute kidney injury (AKI) model and treatment protocol

The experimental design is shown in Figure 1. AKI was induced in C57BL/6J mice via intraperitoneal injection of 20 mg/kg body weight cisplatin (Sigma Chemical Co.) which was dissolved in saline. Mice were randomly allocated to the following groups: saline alone (0.9% NaCl) (control) (n=12), Cis alone (cisplatin, 20 mg/kg) (n=12), Cis+G-CSF (200 µg/kg/d intraperitoneally; Peprotech, Rocky Hill, NJ) (n=12), and Cis+G-CSF/AMD3100 (5mg/kg subcutaneously; Sigma-Aldrich, St. Louis, MO) (n=12). G-CSF treatment was administered over five consecutive days, and AMD3100 was administered 60 min before AKI induction. At 96 hours after the last cisplatin injection, the mice were euthanized. Blood and tissue samples were collected to assess renal function and tissue damage.

### **Ablation of BM stem cells in mice**

To confirm BM (bone marrow) stem cell mobilization following G-CSF/AMD3100 administration, mice were irradiated with 2 separate doses of 4.5 Gy of whole-body  $\gamma$ -radiation at a 2-hour interval for bone marrow ablation (BMA). Mice were randomly assigned to the following groups: irradiation + saline group, irradiation + G-CSF/AMD3100 group, non-irradiation + saline group, and non-irradiation + G-CSF/AMD3100 group. Beginning 1 day after irradiation, G-CSF/AMD3100 was administered as described in the above-mentioned treatment protocol. Control mice that were not subjected to irradiation were injected with an equivalent dose of saline (n=6 per group). The mice were euthanized at 96 hours after treatment, and peripheral blood samples were collected for subsequent flow cytometry (FCM) analyses.

### **Peripheral blood flow cytometric analysis**

Mice were anesthetized with chloralhydrat, and peripheral blood samples were collected for FCM analyses. Red blood cells were lysed using Lysing Buffer (Sigma). The remaining cells were labeled with APC-labeled rat anti-mouse CD34, CD133 and CD44 antibodies and PE-labeled rat anti-mouse CXCL4 and c-kit antibodies (all from BD Pharmingen).

### **Measurement of blood urea nitrogen and serum creatinine levels**

Serum samples were obtained from mice 4 d after cisplatin injection. Blood urea nitrogen (BUN) and creatinine levels were subsequently measured using an autoanalyzer (Hitachi 7150 Auto-analyzer; Hitachi, Tokyo, Japan).

### **Tissue processing and histopathological scoring**

After the mice were euthanized, kidney specimens were fixed immediately in 10% buffered formalin for 24 hours at room temperature and then embedded in paraffin, and some kidney specimens were embedded in OCT compound for freezing, then were rapidly frozen in liquid nitrogen. Kidney tissues were cut into 3µm thick sections and were stained with PAS staining for immunohistochemical analysis. Tubular injury was diagnosed based on the presence of tubular epithelial necrosis, cast formation, tubular dilatation and brush border loss. Renal injury severity was scored in a blinded fashion as described in a previous study<sup>17</sup> based on the percentage of tubule lesions in ten randomly selected, non-overlapping fields (magnification, 200x) as follows: 0, 0%; 1, ≤10%; 2, 11-25%; 3, 26-45%; 4, 46-75%; and 5, 76-100%.

## Immunofluorescence and immunohistochemical (IHC) studies

Mice were euthanized, and their kidneys were perfused with precooled PBS via the left ventricle. Specimens were embedded in paraffin and were cut into 3µm thick sections and processed for immunostaining. IHC labeling was performed to identify Ki-67-positive cells, which were counted by a blinded investigator in 20 randomly selected cortical and outer medullary (OSOM) fields at a magnification of 400×. In addition, kidney tissue sections were subjected to immunofluorescence staining for BrdU using mouse anti-BrdU monoclonal antibodies (Roche) and Dylight 594-conjugated secondary antibodies (Amyjet) to evaluate tubular epithelial cell proliferation. The number of BrdU-positive cells was determined by counting the numbers of positive nuclei in 20 randomly selected, non-overlapping cortical and OSOM fields at a magnification of 400×.

## Apoptosis assay

Apoptosis was evaluated using a terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay kit (Roche, Indianapolis, IN, USA). Briefly, C57BL/6J mouse kidney sections were deparaffinized, rehydrated, digested with proteinase K and labeled with a TUNEL reaction mixture for 60 minutes at 37°C. The TUNEL-positive cells corresponding to apoptotic tubular epithelial cells were counted in 20 randomly selected cortical fields at high-power magnification (x400). All tissue sections were viewed and labeled by a blinded examiner

## Western blot analysis

A 50µg quantity of total renal cell lysate was separated by SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore; Billerica, MA, USA). Western blotting was performed as described in our previous study<sup>18</sup>. The following primary antibodies were used: PCNA (Ruiyng, China), Bcl-2 (Santa Cruz Biotechnology), and Bax (Santa Cruz Biotechnology). Anti-mouse or goat HRP-conjugated secondary antibodies (Amyjet) were used to detect protein using an ECL Assay Kit (Bipecc Biopharma). β-Tubulin or β-actin was used as an internal control. Band intensity was quantified using ImageJ software (1.44 P).

## Quantitative RT-PCR

Total RNA was extracted from tissues with TRIzol reagent (TaKaRa) according to the manufacturer's instructions. RNA reverse transcription was conducted using a PrimeScript™ RT Reagent Kit (TaKaRa). PCR enzymes and master mixes (DBI Bioscience) were used for real-time PCR, along with primers specific for mouse GAPDH, TNF-α, IL-6, IL-10, Kim1, and Ngal. Relative expression levels were normalized to GAPDH and calculated using the  $2^{-\Delta\Delta Ct}$  method. The primer sequences were as follows:

IL-6 forward (F): 5'-TCCAGTTGCCTTCTTGGGAC-3'

reverse (R): 5'-TGCACAACCTCTTTTCTCATTTCCAC-3'

IL-10 forward (F): 5'-ATCAGCAGGGGCCAGTAC-3'

reverse (R): 5'-AAGGCTTGGCAACCCAAGT-3'

Kim1 forward (F): 5'-TACCTGGAGTAATCACACTGAAGCA-3'

reverse (R): 5'-TTCAATCTTAGAGACACGGAAGGC-3'

Ngal forward (F): 5'-GGCAGCTTTACGATGTACAGCA-3'

reverse (R): 5' -TCTGATCCAGTAGCGACAGCC -3'

TNF- $\alpha$  forward (F): 5'-TCACAAAACCTTGAGAGTCGTGGTG-3'

reverse (R): 5'-AAAGTGGCTCTACGTTATATTCTGCC-3'

GAPDH forward (F): 5'-GCCAGCCTCGTCTCATAGACA-3'

reverse (R): 5'-AGAGAAGGCAGCCCTGGTAAC-3'

### **Statistical analysis**

Results are presented as the mean  $\pm$  SEM. For normally distributed data, differences within groups were assessed by ANOVA, and differences between groups were assessed using Tukey's post hoc test. Student's *t*-test was performed to analyze differences between two groups. For data that were not normally distributed, differences within groups were evaluated using the Kruskal–Wallis Htest, and differences between groups were evaluated using the Mann–Whitney *U*-test. All tests were two-tailed, and a P-value  $<0.05$  was considered significant.

## **Results**

### **Pretreatment with the combination of G-CSF/AMD3100 improves survival in cisplatin-treated mice**

BMSCs can repair injured renal tubules, and G-CSF can mobilize BMSCs into the peripheral blood. Therefore, we examined the ability of pretreatment with G-CSF/AMD3100 to prolong survival in cisplatin-treated mice. As shown in Figure 2A, compared with saline pretreatment, G-CSF and G-CSF/AMD3100 pretreatment improve survival rate in cisplatin-treated mice. Only 16.7% of mice survived up to 10 days following cisplatin treatment, whereas 58.3% of mice pretreated with G-CSF/AMD3100 survived up to 10 days after cisplatin injection ( $P<0.01$ ). Furthermore, G-CSF/AMD3100 group had better survival rate than the G-CSF alone group ( $P<0.05$ ). These results indicate that G-CSF/AMD3100 protects against cisplatin-induced renal toxicity.

### **The combination of G-CSF/AMD3100 ameliorates cisplatin-induced renal functional deterioration**

Cisplatin causes renal tubular damage and induces acute renal failure. We measured serum BUN and creatinine levels to assess the severity of renal dysfunction in each group. As shown in Figure 2B and C, after 4 days of cisplatin treatment, serum creatinine and BUN levels were significantly increased in saline-

treated mice ( $P < 0.05$  vs. untreated with cisplatin). By contrast, mice that received G-CSF or G-CSF/AMD3100 injections exhibited significantly lower serum creatinine and BUN levels than mice that were treated with saline. Moreover, mice pretreated with G-CSF/AMD3100 exhibited lower BUN and creatinine levels than mice in G-CSF-treated ( $P < 0.05$ ). These results suggest that G-CSF and G-CSF/AMD3100 pretreatment improve renal function in cisplatin-treated mice and that the combination of G-CSF/AMD3100 improves renal function more effectively than G-CSF alone.

### **The combination of G-CSF/AMD3100 ameliorates renal tubule lesions**

To evaluate the effects of G-CSF/AMD3100-induced BMSC mobilization on cisplatin-induced renal impairment, we subjected the kidneys of cisplatin-treated mice to Periodic Acid-Schiff stain (PAS) staining (Figure 3A-H). Histological scoring was based on the typical microscopic features of acute tubular damage, including extensive tubular necrosis, cast formation, tubular dilatation, and brush border loss. Cisplatin-treated mice exhibited more severe tissue injury than G-CSF and G-CSF/AMD3100-treated mice, and G-CSF/AMD3100-treated mice exhibited significantly lower histopathological scores than G-CSF-treated mice ( $P < 0.05$ ; Figure 3I). These results suggest that G-CSF/AMD3100 pretreatment protects against cisplatin-induced renal damage.

### **The combination of G-CSF/AMD3100 mobilizes BMSCs**

CD34<sup>+</sup> and CD133<sup>+</sup> cells are widely recognized as hematopoietic stem cell (HSC) and endothelial progenitor cell (EPC) biomarkers, respectively. In this study, BMSCs in peripheral blood samples from C57BL/6 male mice were analyzed by flow cytometry. The CD34<sup>+</sup>, CD133<sup>+</sup> staining results were depicted in Figure 4. As expected, G-CSF ( $*P < 0.05$  vs. cisplatin) and G-CSF/AMD3100 ( $**P < 0.01$  vs. cisplatin) treatment enhanced circulating CXCR4<sup>+</sup> cell mobilization. In addition, the G-CSF/AMD3100 group exhibited a much larger CXCR4<sup>+</sup> cell population than the G-CSF treated group ( $P < 0.01$ ) (Figure 4A). The CXCR4<sup>+</sup>CD34<sup>+</sup> and CXCR4<sup>+</sup>CD133<sup>+</sup> cell populations were also harvested and identified by double-staining (Figure 4B,C). G-CSF/AMD3100 treatment increased the numbers of CXCR4<sup>+</sup>CD34<sup>+</sup> and CXCR4<sup>+</sup>CD133<sup>+</sup> cells in the peripheral blood by 1.8-fold and 1.9-fold, respectively, compared to G-CSF treatment. The percentages of CXCR4<sup>+</sup>CD34<sup>+</sup> and CXCR4<sup>+</sup>CD133<sup>+</sup> were significantly increased in the G-CSF/AMD3100-treated and G-CSF-treated groups compared with the cisplatin-treated group. We also measured the numbers of CXCR4<sup>+</sup>CD44<sup>+</sup> and c-Kit<sup>+</sup> peripheral blood mononuclear cells (Supplemental Figure 1). G-CSF/AMD3100 treatment increased the numbers of CXCR4<sup>+</sup>CD44<sup>+</sup> and c-Kit<sup>+</sup> cells in the peripheral blood compared with G-CSF or saline treatment. These findings suggest that G-CSF/AMD3100 effectively mobilizes stem cells into the peripheral blood and facilitates tubule repair and regeneration.

### **Bone marrow ablation prevents BMSC mobilization by the combination of G-CSF/AMD3100**

To further determine whether G-CSF/AMD3100 administration mobilizes BMSCs into the peripheral blood, we performed bone marrow ablation (BMA) via irradiation to prevent BMSC development and mobilization. As shown in Supplemental Figure 2, when the bone marrow was irradiated, the percentages

of CXCR4<sup>+</sup>CD34<sup>+</sup> and CXCR4<sup>+</sup>CD133<sup>+</sup> cells were not different between the irradiation + saline group and irradiation + G-CSF/AMD3100 groups ( $P>0.05$ ); however, these percentages were significantly higher in the non-irradiation + G-CSF/AMD3100 group than in the non-irradiation + saline and irradiation + G-CSF/AMD3100 groups ( $P<0.01$ ). These results further proved that BMSCs were mobilized by G-CSF/AMD3100.

### **The combination of G-CSF/AMD3100 enhances tubular epithelial cell regeneration**

To determine whether G-CSF/AMD3100 pretreatment facilitates tubular epithelial cell proliferation and regeneration, we utilized Western blotting to determine whether the combination of G-CSF/AMD3100 significantly increases PCNA protein expression compared with cisplatin treatment ( $P<0.05$ ). G-CSF/AMD3100-treated mice exhibited higher levels of PCNA protein expression than G-CSF-treated mice (Figure 5A and B). We also assessed the effects of G-CSF/AMD3100 treatment on tubular cell proliferation and regeneration by counting the numbers of BrdU<sup>+</sup> cells in each group. G-CSF-treated and G-CSF/AMD3100-treated mice exhibited higher numbers of BrdU<sup>+</sup> cells than saline-treated mice, and G-CSF/AMD3100-treated mice exhibited significantly higher numbers of BrdU<sup>+</sup> cells than G-CSF-treated mice ( $69.1\pm 8.3$  cells/HPF,  $36.1\pm 5.5$  cells/HPF in G-CSF and G-CSF/AMD3100-treated mice, respectively,  $P<0.05$ ) (Figure 5C-G). Similar results were observed for Ki-67 expression. Low numbers of Ki-67-positive cells were detected in the renal tissues of control and cisplatin-treated mice on day 4 of treatment (control group,  $32.4\pm 4.4$ ; cisplatin group,  $45.9\pm 5.7$  cells/HPF) (Figure 5H and I). G-CSF/AMD3100 pretreatment resulted in a 4-fold increase in tubular cell proliferation compared with saline treatment ( $P<0.01$ ) and a 1.5-fold increase in cell proliferation compared with G-CSF treatment (Figure 5H-L). These results suggest that G-CSF/AMD3100 pretreatment promoted tubular epithelial cell regeneration to a greater extent than G-CSF pretreatment.

### **The combination of G-CSF/AMD3100 promotes the protective effect of BMSCs against cisplatin-induced injury**

To determine the relationship between cisplatin nephrotoxicity and apoptosis, we performed Western blotting to quantify Bcl-2 and Bax protein expression. Bcl-2 expression was notably lower in the cisplatin-treated group than in the other three groups and was significantly lower in the G-CSF-treated group than in the G-CSF/AMD3100-treated group ( $P<0.05$ , Figure 6A and C). By contrast, Bax expression was increased in the cisplatin-treated group; however, G-CSF and G-CSF/AMD3100 administration decreased Bax expression, and a significant difference in Bax expression was noted between G-CSF-treated mice and G-CSF/AMD3100-treated mice based on band intensities ( $P<0.05$ , Figure 6A and B). Furthermore, to evaluate renal tubular epithelial cell (RTEC) damage, we examined the percentages of apoptotic RTECs via TUNEL assay. As expected, both G-CSF-treated mice and G-CSF/AMD3100-treated mice exhibited significantly decreased proportions of TUNEL<sup>+</sup> cells compared with cisplatin-treated mice. Interestingly, G-CSF/AMD3100-treated mice exhibited significantly fewer TUNEL<sup>+</sup> cells than G-CSF-treated mice (Figure 6D-G). To confirm that the combination of G-CSF/AMD3100 attenuates renal epithelial injury, we measured the levels of kidney injury molecule-1 (Kim-1) and neutrophil gelatinase-associated lipocalin

(Ngal) by qRT-PCR. Kim1 and Ngal mRNA expression levels were 2-fold and 1.9-fold lower, respectively, in G-CSF/AMD3100-treated mice than in cisplatin-treated mice. G-CSF/AMD3100 treatment also decreased Kim1 and Ngal mRNA expression to a greater extent than G-CSF administration alone, although these differences were not statistically significant ( $P=0.065$ ,  $P=0.058$ , respectively) (Supplemental figure 3). These results indicate that G-CSF/AMD3100 administration prevents cisplatin-induced RTEC apoptosis.

### **G-CSF/AMD3100-induced BMSC mobilization impact the immune response**

Recent studies have demonstrated that MSC administration protects against I/R injury by significantly downregulating the expression of pro-inflammatory cytokines such as IL-1b, TNF- $\alpha$ , IFN- $\gamma$ <sup>19,20</sup>. We examined the expression of the pro-inflammatory cytokine IL-6, which increases abruptly in the setting of cisplatin-induced nephrotoxicity. G-CSF/AMD3100 treatment significantly decreased IL-6 mRNA expression ( $P<0.01$ ) to a greater extent than G-CSF treatment alone ( $P<0.05$ ) (Figure 7A). We observed similar changes in TNF- $\alpha$  levels, although there was no significant difference in TNF- $\alpha$  levels between G-CSF-treated mice and G-CSF/AMD3100-treated mice ( $P=0.065$ ) (Figure 7B). However, cisplatin-treated mice exhibited much higher mRNA expression levels of the anti-inflammatory cytokine IL-10 than control mice. Moreover, IL-10 mRNA expression levels were increased in G-CSF/AMD3100-treated mice compared with cisplatin-treated mice ( $P<0.01$ ) (Figure 7C). These data indicate that G-CSF/AMD3100 pretreatment ameliorates cisplatin-induced renal injury.

## **Discussion**

Stem cells can facilitate renal repair and regeneration after AKI. Recent studies have focused specifically on BMSCs mobilization to sites of renal injury to facilitate tissue repair and regeneration<sup>21-23</sup>. However, the repair efficiency of BMSCs is limited due to the low survival and migration efficiency of BMSCs; only a small proportion of intravenously injected BMSCs migrate successfully to sites of renal tissue damage<sup>24</sup>. Thus, the viability of this therapy in clinical settings is limited. In the present study, we explored the effects of the combination of G-CSF/AMD3100 in a mouse model of cisplatin-induced AKI and observed that G-CSF/AMD3100 pretreatment exerted marked renoprotective effects against cisplatin-induced nephrotoxicity.

G-CSF is considered the gold standard for mobilizing hematopoietic and mesenchymal lineage cells from the BMSC population into the circulation and promoting the proliferation and differentiation of mobilized BMSCs<sup>25,26</sup>. AMD3100 has been approved by the United States Food and Drug Administration and the EU for hematopoietic stem cell mobilization in lymphoma and multiple myeloma patients who have failed hematopoietic stem cell-mobilization therapy with G-CSF alone<sup>27,28</sup>. In the present study, G-CSF/AMD3100-treated mice exhibited higher numbers of BMSCs in the peripheral blood than G-CSF-treated mice. These findings are consistent with those of previous studies demonstrating that the combination of G-CSF/AMD3100 mobilized BMSCs more effectively than G-CSF alone<sup>29-31</sup>. The role of the CXCR4/SDF-1 pathway in stem cell mobilization and progenitor cell trafficking has recently been studied, revealing a vital role of this chemokine receptor in regulating stem cell fate. CXCR4 is expressed

on the surface of MSCs, and SDF-1 (stromal cell-derived factor-1 $\alpha$ ) is expressed on the surface of bone marrow stromal cells<sup>32,33</sup>. To increase BMSC mobilization into the peripheral blood, we used AMD3100 to disrupt the interactions between cell receptors and their ligands in specific microenvironments (Supplemental figure 4). We observed that G-CSF/AMD3100-treated mice exhibited a higher proportion of CXCR4<sup>+</sup> cells in the peripheral blood than mice in another groups, consistent with a previous study that demonstrated that combining G-CSF with AMD3100 dramatically improved HSC mobilization into the peripheral blood<sup>15</sup>. Several studies have demonstrated that acute AMD3100 administration enhances tissue repair<sup>10,34</sup>, whereas continuous AMD3100 administration has adverse effects on tissue regeneration<sup>13,14</sup>. AMD3100 exerts rapid cell-mobilization effects that peak within 1–3 hours of administration in mice<sup>31,35</sup>. Therefore, we elected to administer ADM3100 subcutaneously 1 hour before cisplatin injection. The combination of G-CSF/AMD3100 synergistically enhanced BMSC mobilization into the peripheral circulation.

Previous studies have demonstrated that G-CSF significantly ameliorates ischemia/reperfusion- and cisplatin-induced renal injury<sup>22,36</sup>. Wu et al.<sup>37</sup> demonstrated that subcutaneous administration of 6mg/kg AMD3100 prevents ischemic acute kidney injury. In the present study, we observed that the combination of G-CSF/AMD3100 ameliorated cisplatin-induced nephrotoxicity than G-CSF, findings supported by our analyses of plasma creatinine and BUN levels and renal tissue morphology. The mechanism underlying stem cell-based therapy involves the transdifferentiation of stem cells into renal cells and subsequent release of factors that increase cell survival and proliferation, exert anti-inflammatory and anti-apoptotic effects and modulate the immune response<sup>38</sup>. Zuket al.<sup>14</sup> reported that AMD3100 administration ameliorates I/R-induced kidney injury characterized by leukocyte infiltration and pro-inflammatory chemokine/cytokine expression. In the present study, we failed to elucidate the mechanism by which BMSCs transdifferentiate into RTECs. However, we found that pretreatment of cisplatin-treated mice with G-CSF/AMD3100 or G-CSF could contribute to promote renal repair by promoting cell proliferation and decreasing apoptosis, as indicated by the increased protein expression levels of several cell proliferation-related markers, such as PCNA, Ki-67 and BrdU. In addition, significantly decreased protein expression levels of Bax and notably enhanced protein expression levels of Bcl-2 were observed in cisplatin-treated mice pretreated with G-CSF/AMD3100 or G-CSF. Furthermore, these effects were greater in mice pretreated with G-CSF/AMD3100 than in those pretreated with G-CSF alone. TUNEL assays of DNA fragmentation further confirmed the positive effects of G-CSF/AMD3100 on BMSC mobilization. Taken together, our findings are consistent with those of previous studies demonstrating that mesenchymal stem cells treatment could alleviate the cisplatin-induced renal injury and I/R-induced renal failure, at least in part, by attenuating apoptosis and tubular injury and by promoting tubular regeneration<sup>14,39-41</sup>.

Numerous studies have demonstrated that BMSCs exert potent immunomodulatory effects *in vitro* and *in vivo*<sup>42-44</sup>. In the current study, we observed the levels of IL-10 mRNA expression were high in cisplatin-treated animals pretreated with G-CSF/AMD3100 compared with animals pretreated with G-CSF alone. Milwidet al.<sup>43</sup> reported that BMSC-secreted IL-10 contributed to the attenuation of severe cisplatin-induced AKI. Moreover, the levels of the pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$  are increased in

renal tubular cells during cisplatin-induced AKI<sup>45, 46</sup>. Our results for IL-6 and TNF- $\alpha$  mRNA expression are consistent with these findings. We observed significantly lower levels of IL-6 and TNF- $\alpha$  following G-CSF/AMD3100 or G-CSF pretreatment, consistent with the results of Overath et al.<sup>47</sup>, who observed that adipose-derived MSCs pretreated by exposure to hypoxia significantly decreased pro-inflammatory cytokine levels and significantly attenuated cisplatin-induced renal injury in mice.

## Conclusions

Some reagents can prevent kidney damage caused by toxic molecules. Our findings provide some evidences for the potential of combination of G-CSF/AMD3100 as a therapy protocol for cisplatin-induced AKI. This combination maybe enhance the migration and homing of BMSCs to sites of renal tissue damage to produce beneficial effects.

## Abbreviations

BMSCs: Bone marrow-derived stem cells; AKI: Acute kidney injury; I/R: Ischemia/reperfusion; G-CSF: Granulocyte colony-stimulating factor; BMA: Bone marrow ablation; FCM: Flow cytometry; BUN: Blood urea nitrogen; IHC: Immunohistochemical; TUNEL: Transferase dUTP nick end-labeling; PVDF: Polyvinylidene difluoride; PAS: Periodic Acid-Schiff stain; HSC: Hematopoietic stem cell; EPC: Endothelial progenitor cell; RTEC: Renal tubular epithelial cell

## Declarations

### Ethics approval and consent to participate

All experiments were performed using C57BL/6J mice and were conducted according to the Huazhong University of Science and Technology Guide for the Care and Use of Laboratory Animals. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology, Wuhan, China.

### Consent for publication

Not applicable.

### Availability of data and materials

All data generated or analyzed during this study are included in this article.

### Competing interests

The authors declare no conflicts of interest.

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### Authors' contributions

ZC, XR and RR performed the in vitro tests and data analysis and prepared the manuscript. XR and YW performed animal experiments; ZC and XR performed the statistical analysis. JS and ZC designed and modified the manuscript. The authors read and approved the final manuscript.

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Not applicable.

### Author details

<sup>1</sup>Department of Urology, Shanxi Bethune Hospital, Taiyuan 030032, China. <sup>2</sup>Department of Neurosurgery, Shanxi Bethune Hospital, Taiyuan 030032, China.

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## Figures

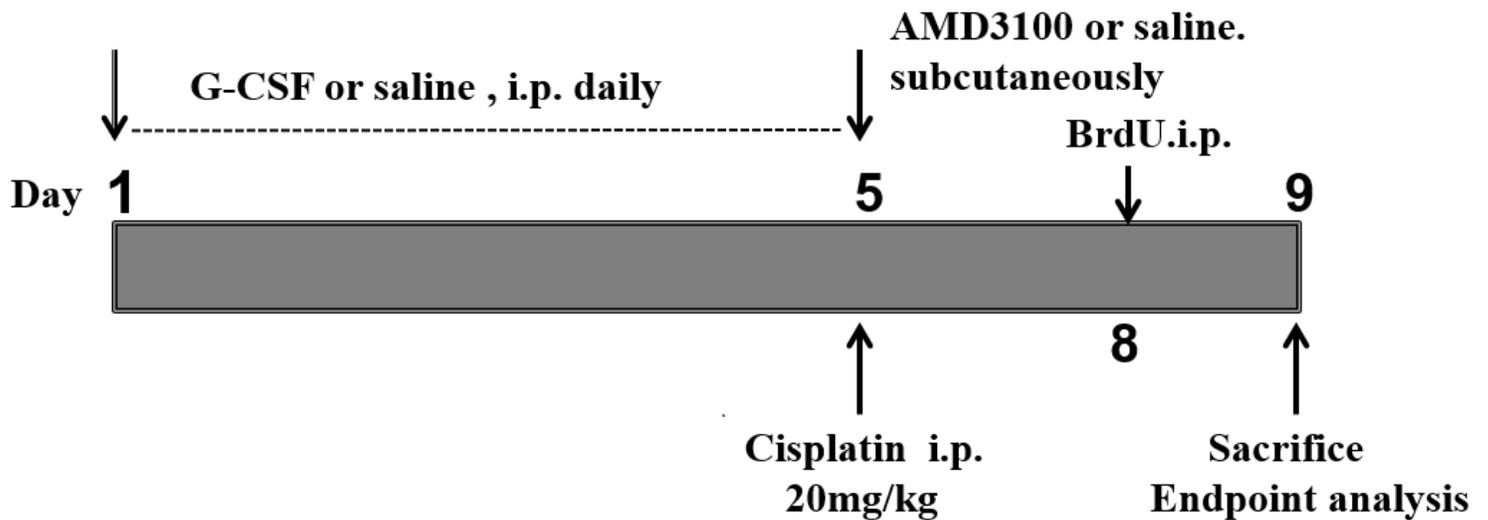
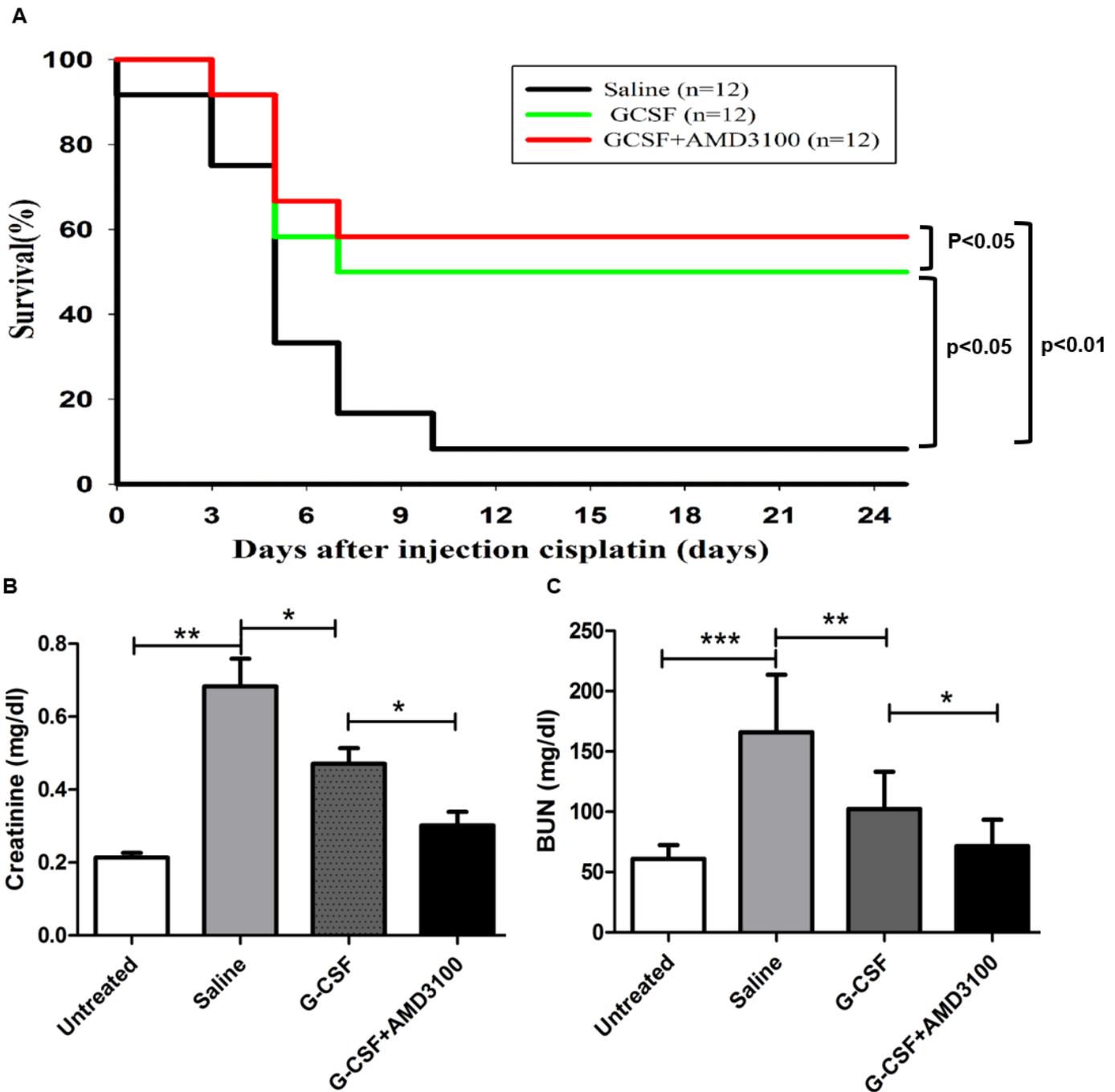


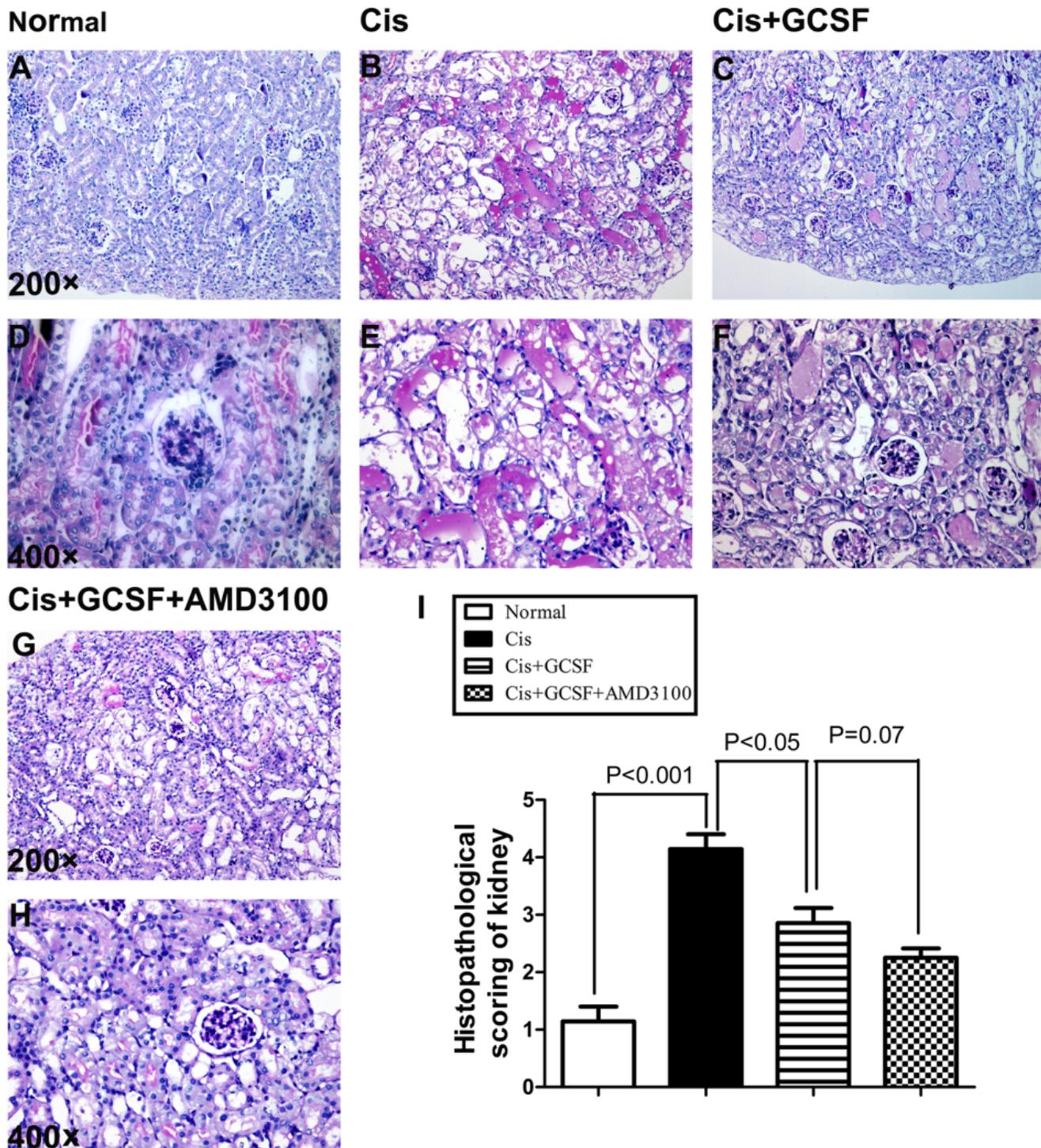
Figure 1

Timeline of the experimental design of this study. 7 to 8 week-old male C57BL/6 mice were divided into four groups. All mice received G-CSF (200 µg/kg/d) for 5 consecutive days, and received subcutaneously a signal AMD3100 (5mg/kg) 1 hour before an intraperitoneal injection of cisplatin 20mg/kg, injections of 5-bromo-2-deoxyuridine (BrdU) (100mg/kg/day) for 2 consecutive days from the indicated time.



**Figure 2**

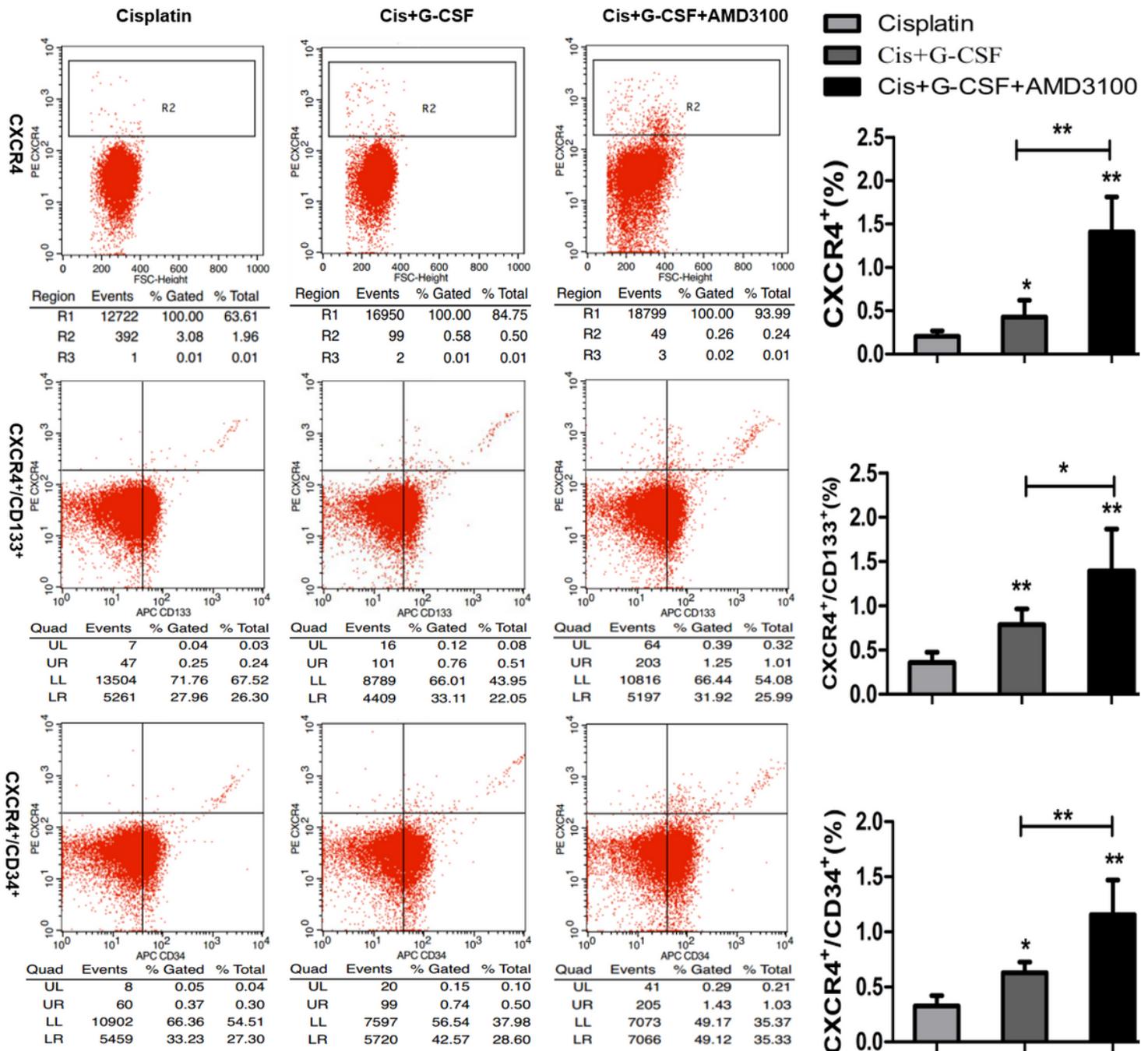
Survival rates and blood of serum creatinine and urea nitrogen (BUN) levels of cisplatin-administered mice that were pretreated with or without G-CSF and/or AMD3100. (A) C57BL/6 mice received an injection of of G-CSF (200  $\mu\text{g}/\text{kg}$  per d), G-CSF (200  $\mu\text{g}/\text{kg}$  per d)/AMD3100 (5mg/kg), or saline (as control) into the intraperitoneal before treated with cisplatin. Mice were observed to determine survival rate (n =12, per group). (B) Serum creatinine and (C) Serum BUN levels of the mice 4 d after cisplatin injection are shown. "Untreated" means mice without treatment with cytokine and cisplatin. Results are expressed as mean $\pm$ SD, \*P <0.05, \*\*P <0.01 and \*\*\* P <0.001.



**Figure 3**

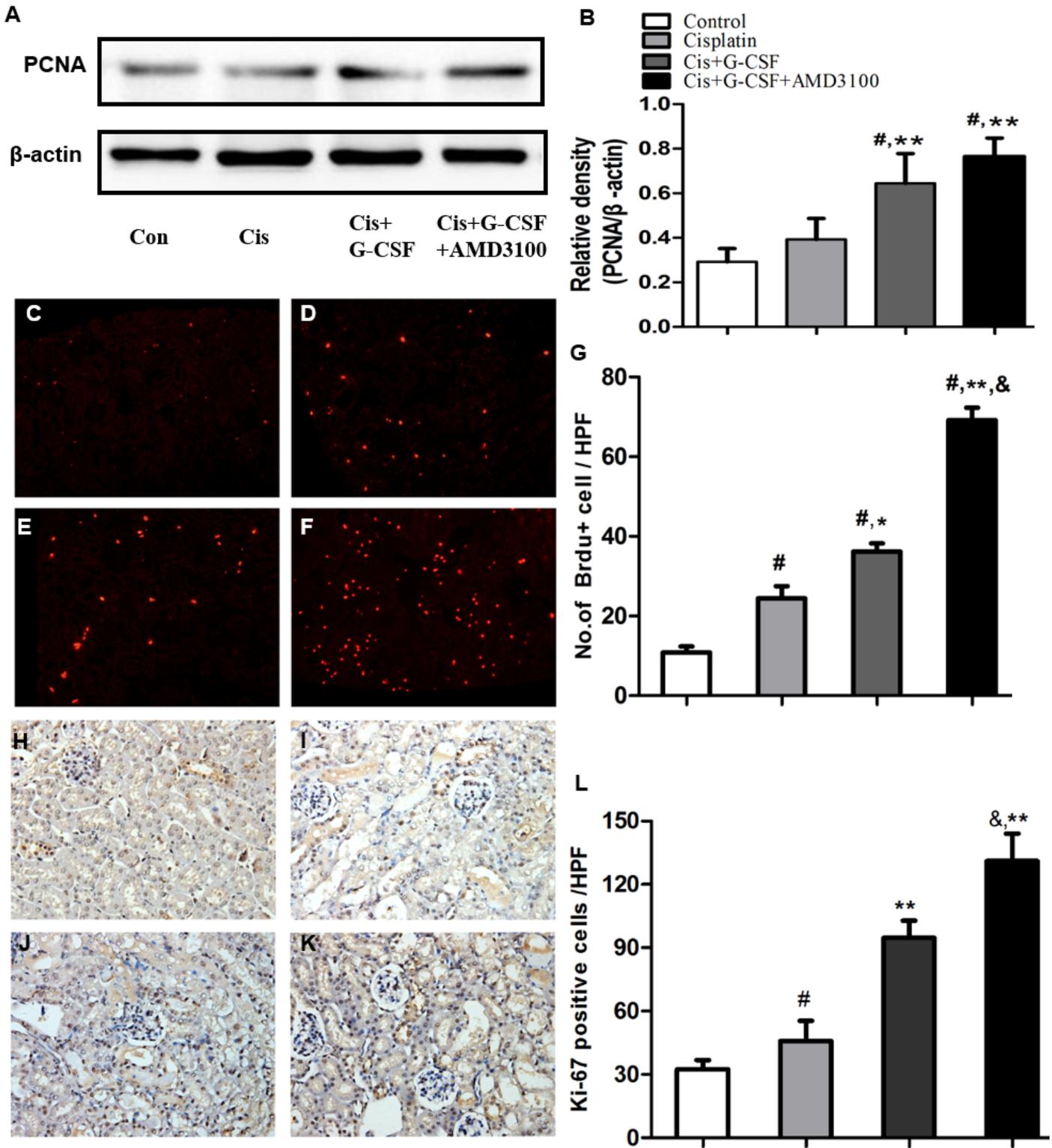
Histologic analyses of kidneys of cisplatin-treated mice. C57BL/6 mice received an injection of G-CSF (200  $\mu$ g/kg per d), G-CSF (200  $\mu$ g/kg per d)/AMD3100 (5mg/kg), or saline (as a control of cytokines) into the intraperitoneal space. The day after the last injection of cytokines, single intraperitoneal injections of cisplatin (20 mg/kg body wt) were given to these mice. Morphologic changes in PAS staining are shown. The figures indicate renal tubules of saline-pretreated mice (A-D), Cisplatin-treated mice (B-E), G-CSF

pretreated + Cisplatin-treated mice (C̄F), and G-CSF/AMD3100 pretreated+Cisplatin-treated mice (ḠH). Representative data are shown for three independent experiments. Magnification, ×200 in ĀB̄C and G, ×400 in D, E, F and F. Histopathological scoring of cisplatin-induced renal injury (I). Tubular injury was defined as tubular necrosis, cast formation, loss of brush border in renal tubules and tubular dilatation. Data are presented as the means ± SD.



**Figure 4**

Effects of G-CSF/AMD3100 on mobilization of stem cells. Four days after cisplatin injection, the peripheral blood of the mice was obtained and analyzed by a flow cytometer. (A) CXCR4+ cells, (B) CXCR4+CD34+ cells, (C) CXCR4+CD133+ cells. Data represent the mean ± SD (n = 6 per group). \*p < 0.05, \*\*p < 0.01, and vs. the cisplatin group, and the indicated test group.



**Figure 5**

Effects of G-CSF/AMD3100 on renal tubular regeneration. (A and B) western blot analyses of PCNA protein. G-CSF/AMD3100 pretreatment showed a significantly increase in PCNA expression. (C-F) immunofluorescent staining tested the expression of BrdU in renal tubular, and red fluorescence represents the nucleus of BrdU+ cells.(G) The number of BrdU+ cells/ HPF. The figures indicate BrdU+ cells in renal tubules of saline-treated mice (control) (C), Cisplatin treated mice (D), Cisplatin+G-CSF

pretreated mice (E), and Cisplatin+G-CSF/AMD3100 pretreated mice (F). (H-K) immunohistochemical staining tested the expression of ki-67 in renal tubular (L) The number of ki-67+ cells/ HPF. The figures indicate ki-67+ cells in renal tubules of saline-treated mice (control) (H), Cisplatin treated mice (I), Cisplatin +G-CSF pretreated mice (J), and Cisplatin+G-CSF/AMD3100-pretreated mice (K). Data represent the mean  $\pm$  SD (n = 6 per group). \*p < 0.05, \*\*p < 0.01, and compared with the cisplatin group; # p < 0.05 compared with control group; & p < 0.05 compared with G-CSF group. Original magnification  $\times$ 400.

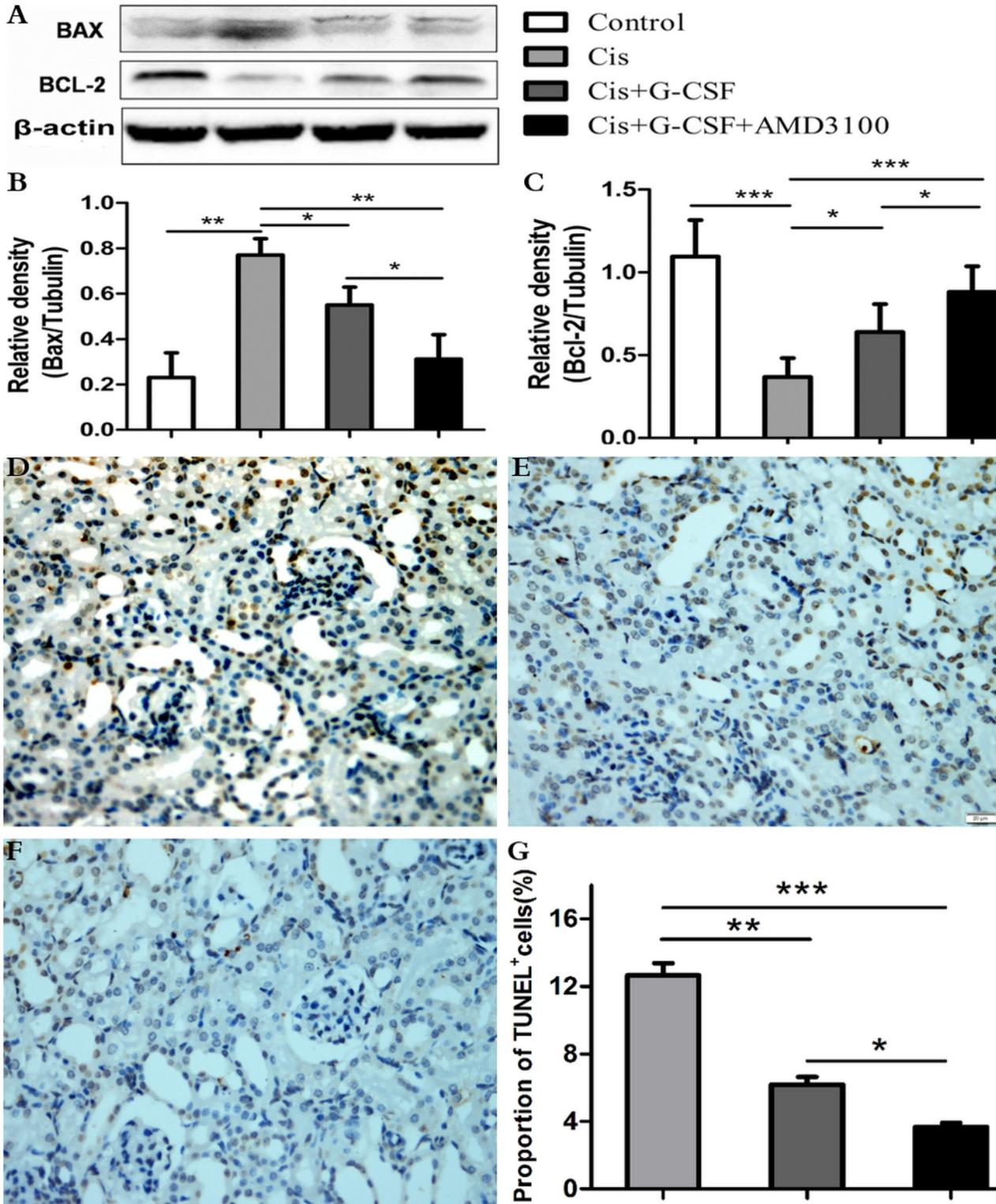


Figure 6

Effects of G-CSF/AMD3100 on renal tubular cells apoptosis. (A -C) western blot analyses of BCL-2 and BAX protein. G-CSF/AMD3100 pretreatment showed a significantly increase in BCL-2 expression, but decrease in BAX expression. (D-G) Representative micographs showing the TUNEL immuno- staining among different groups. (D) Cisplatin treated mice; (E) Cisplatin +G-CSF–pretreated mice; (F) Cisplatin+G-CSF/AMD3100–pretreated mice. (G) Quantitative analysis for the number of TUNEL staining positive cells among different groups. Data represent the mean  $\pm$  SD (n = 6 per group). \*p <0.05, \*\*p < 0.01, \*\*\* P<0.001. Original magnification  $\times$ 400.

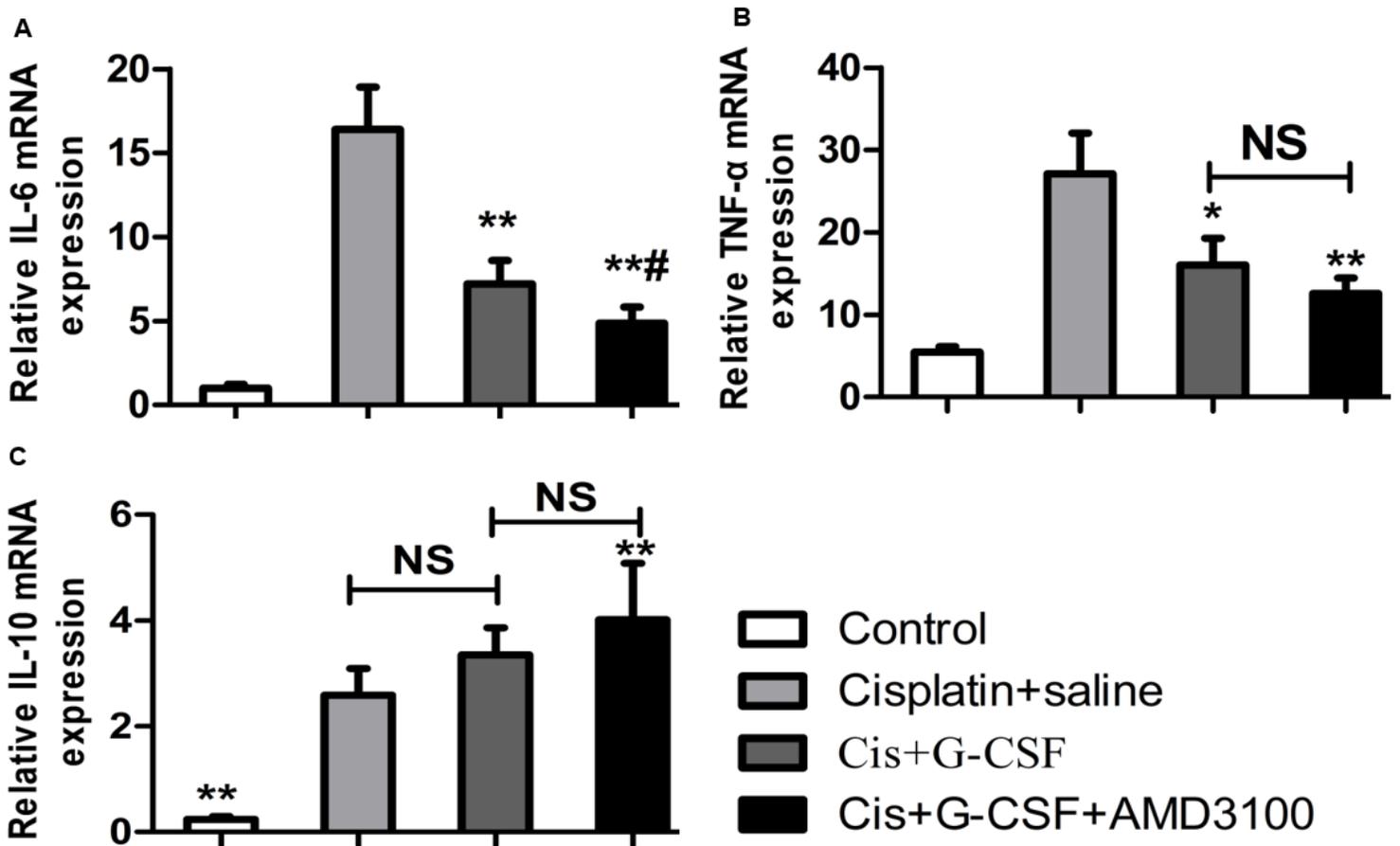


Figure 7

Effects of G-CSF/AMD3100 on the inflammatory cytokines mRNA expression. Total RNA was extracted from renal tissue in the saline-, cisplatin+saline-, G-CSF- and G-CSF/AMD3100 treated mice, 4 days after AKI and analyzed for IL-6(A), TNF- $\alpha$  (B), IL-10(C) mRNA expression by real-time RT-PCR analysis. Results are expressed as the means  $\pm$  SD (n=6), \*P<0.05, \*\*P<0.01, vs. the cisplatin group and the indicated test group.

## Supplementary Files

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