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Cisplatin-induced nephrotoxicity is mediated by tumor necrosis factor-α produced by renal parenchymal cells

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Cisplatin is a chemotherapeutic agent that induces tumor necrosis factor-α (TNF-α) production in many cell types with unfortunate renal toxicity. We sought to determine the contributions of renal parenchymal cells and bone marrow-derived immune cells to the pathogenesis of cisplatin-induced renal injury *in vivo*. To do this we created chimeric mice in which the bone marrow was ablated and replaced with donor bone marrow cells from wild-type or from TNF-α knockout mice. Six weeks after reconstitution, the chimeric mice were treated with cisplatin and renal structural and functional parameters were measured. Chimeras with kidneys of wild-type animals all developed significant renal failure after 72 h of cisplatin treatment regardless of the immune cell source. Chimeras with kidneys of TNF-α knockout mice showed significantly less renal dysfunction (blood urea nitrogen, serum creatinine, and glomerular filtration rate), renal histologic injury, and serum TNF-α levels; again regardless of the immune cell source. Urinary excretion of several proinflammatory cytokines was lower in the wild-type bone marrow-knockout kidney chimera mouse than in wild-type background mice. Our results indicate that a substantial portion of circulating and urinary TNF-α is derived from nonimmune cells after cisplatin administration. We conclude that the production of TNF-α by renal parenchymal cells, rather than by bone marrow-derived infiltrating immune cells, is responsible for cisplatin-induced nephrotoxicity.

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Cisplatin is a common and effective chemotherapeutic agent for the treatment of many types of cancer. An unfortunate consequence of cisplatin treatment is renal toxicity which affects 25–35% of treated patients.[1] Studies from our laboratory[2,3] and others[4,5] have demonstrated that tumor necrosis factor-α (TNF-α) production is increased in cisplatin toxicity. We also demonstrated that TNF-α plays a pathogenic role in stimulating cytokine and chemokine expression in the kidney and in producing acute renal failure.[2] Specifically, genetic deletion of TNF-α or pharmacologic inhibition of TNF-α action reduced the expression of inflammatory cytokines and chemokines (e.g. TNF-α, monocyte chemotactic protein-1 (MCP-1), and regulated upon activation normal T-cell expressed and secreted (RANTES)) and preserved renal function after cisplatin injection. These effects of TNF-α were determined to be mediated via the TNFR2 receptor subtype.[3]

Although these studies indicated that TNF-α gene expression and protein content within the kidney increased after cisplatin administration, the source of the TNF-α which was responsible for renal injury was unclear. TNF-α is produced by a broad range of tissues and cells, including immune cells and intrinsic renal cells, such as mesangial cells,[6] glomerular[7,8] and tubular epithelial cells,[9–11] and endothelial cells.[12,13] The extent to which infiltrating immune cells and intrinsic renal cells produce TNF-α and mediate nephrotoxicity in response to cisplatin is not known. Therefore, the purpose of the present study was to determine the relative contribution of TNF-α produced by renal parenchymal cells vs leukocytes in mediating cisplatin-induced renal injury *in vivo*. We created chimeric mice in which TNF-α can be produced by either resident kidney cells or circulating immune cells. We evaluated kidney function, histology, and cytokine expression in these chimeric mice following cisplatin administration. The results indicate the local production of TNF-α by resident kidney cells is crucial in cisplatin-induced nephrotoxicity.

RESULTS

Chimeric mice were created to examine the differential role of leukocyte-derived versus kidney-derived TNF-α in the

pathogenesis of cisplatin nephrotoxicity. First, it was important to show that the bone marrow transplantation procedure resulted in a high degree of chimerism, did not alter the distribution of circulating leukocyte subtypes, and did not impact the ability of circulating immune cells to produce TNF-α (Figure 1). To determine the extent of chimerism, enhanced green fluorescent protein (EGFP) was used as a marker of the donor bone marrow. Figure 1a shows the results of flow cytometry of peripheral blood obtained from nontransplanted EGFP-expressing transgenic mice and from chimeric mice in which EGFP bone marrow was transplanted into non-EGFP expressing C57BL6 mice. The frequency of EGFP-positive cells in the two sets of mice was virtually identical, indicating almost complete replacement (>98%) of the recipient circulating leukocytes by donor cells. As studies have indicated a role for neutrophils and B and T lymphocytes in the development of acute renal failure, the effect of bone marrow transplantation on the distribution of various subtypes of peripheral blood leukocytes was determined. Two-color flow cytometry was performed on leukocytes stained for a common leukocyte antigen (CD45) and markers for CD4 and CD8 lymphocytes, B cells, macrophages, NK cells, and neutrophils. The distribution of each subtype, as a percentage of CD45-positive cells, was the same in nontransplanted and chimeric mice (Table 1). Next, intracellular cytokine staining was used to determine the ability of transplanted immune cells to produce TNF-α. Peripheral blood leukocytes were obtained from either EGFP-expressing transgenic mice or TNF-α knockout mice that had been transplanted with EGFP bone marrow 6 weeks earlier (Figure 1b). Cells were stimulated *in vitro* to produce TNF-α, fixed, permeabilized, and stained with TNF-α antibodies as described in the Materials and Methods. Flow cytometry was used to determine the levels of EGFP expression and TNF-α content. As shown in Figure 1b and c, approximately 26% of circulating EGFP-positive cells in both sets of mice stained positive for TNF-α. This result indicates that the bone marrow transplantation procedure itself did not impair TNF-α production by the donor immune cells.

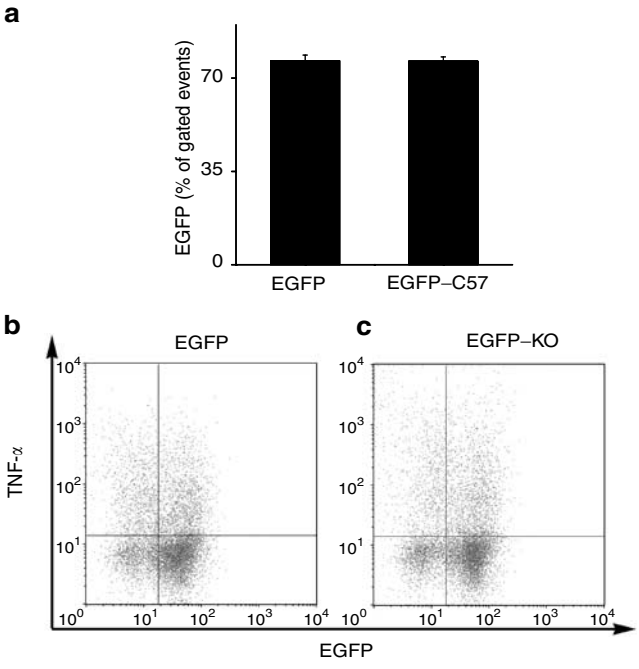

Figure 1 | Functional reconstitution of immune system by donor bone marrow. (**a**) Extent of chimerism was determined by flow cytometry of peripheral blood using EGFP as a marker of donor cells. The left-hand bar represents EGFP-positive cells in the blood of nontransplanted EGFP transgenic mice. The right-hand bar are cells from C57BL6 mice which were transplanted with bone marrow from EGFP transgenic mice (data are %±s.d. of gated events, $n=3$ in each group). (**b** and **c**) Intracellular cytokine staining for TNF-α production by peripheral blood leukocytes from a nontransplanted EGFP transgenic mouse (**b**) or a TNF-α knockout mouse transplanted with bone marrow from EGFP transgenic mice. Representative of results from three animals in each group.

Next, bone marrow chimeric mice were treated with cisplatin (20 mg/kg body weight) or saline and assessed for renal dysfunction. As shown in Figure 2, WT→WT chimeric mice developed severe renal failure between 48 and 72 h. The degree of renal dysfunction at 72 h (BUN 140±10 mg/dl, and creatinine 1.12±0.10 mg/dl) was similar to what we have observed in nontransplanted wild-type mice[2,3,14] and suggests that the bone marrow transplant procedure itself does not

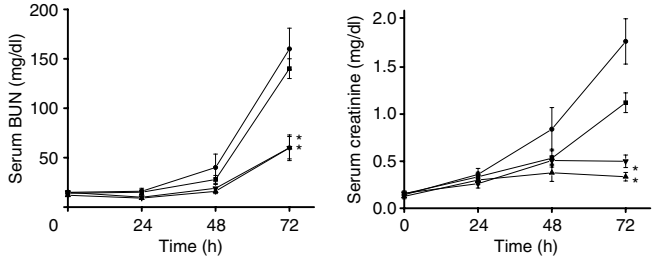

Figure 2 | Cisplatin nephrotoxicity in WT→WT (■), KO→WT (●), WT→KO (▲) and KO→KO (▼) chimeric mice. Bone marrow chimeric mice were injected with 20 mg/kg cisplatin at time = 0. Blood urea nitrogen and creatinine were measured at the indicated times. (*$P<0.05$ vs WT→WT and KO→WT $n=8$–11).

Table 1 | Distribution of circulating leukocytes in nontransplanted and chimeric mice

	CD4	CD8	B cells	Macrophages	NK cells	Neutrophils
WT (C57BL6)	10.9±1.6	10.2±2.6	51.3±9.1	0.9±0.5	1.5±0.4	12.5±7.7
WT→WT	13.0±1.4	9.8±1.4	59.3±6.5	0.6±0.2	1.0±0.3	8.1±4.1
	P=NS	*P*=NS	*P*=NS	*P*=NS	*P*=NS	*P*=NS

ANOVA, analysis of variance; NK cells, natural killer cells; NS, not significant.
All data are %±s.d. of CD45(+) cells. *N*=3 in each group. *P*-values were calculated by ANOVA.

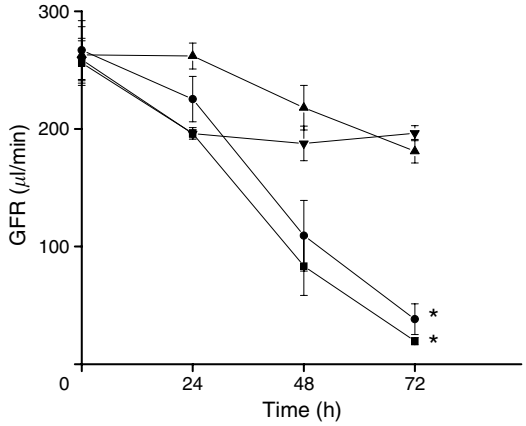

Figure 3 | GFR in cisplatin-treated chimeric mice measured using FITC-labeled inulin. WT→WT (■), KO→WT (●) chimeric mice showed significantly decreased GFR as compared with WT→KO (▲) and KO→KO (▼) mice. Data are expressed as mean ± s.e.m. ($n = 3$–4). *$P < 0.05$ vs WT→WT and KO→WT.

affect the susceptibility to cisplatin-induced acute kidney injury. Of note, KO→WT mice developed similar degrees of renal failure as the WT→WT mice. In contrast, mice with a recipient TNF-α knockout background, whether they were transplanted with wild-type (WT→KO, BUN 60 ± 11 mg/dl, and creatinine 0.33 ± 0.05 mg/dl) or TNF-α knockout bone marrow (KO→KO, BUN 60 ± 13 mg/dl, and creatinine 0.5 ± 0.07 mg/dl) had substantially less renal failure than the mice with a wild-type background ($P < 0.01$, $n = 6$–12). The results in the KO→KO group confirm our earlier report that TNF-α knockout mice are resistant to cisplatin nephrotoxicity.[2]

GFR was also determined by fluorescein isothiocyanate (FITC)-inulin clearance as a more precise measure of renal function. As shown in Figure 3, mice with a wild-type background had severely reduced GFR 72 h after cisplatin treatment (WT→WT 18 ± 4.5 μl/min, KO→WT 38 ± 13 μl/min), whereas mice with a TNF-α knockout background, regardless of the genotype of their immune cells, had well-preserved renal function (KO→KO 196 ± 7 μl/min, WT→KO 181 ± 14 μl/min, $P < 0.01$ vs WT→WT or KO→WT, $n = 3$ in each group).

The functional abnormalities in the chimeric mice were also reflected by structural changes in the kidney. Thus, 72 h after cisplatin treatment, kidneys with a wild-type background (WT→WT or KO→WT) showed severe tubular injury as evidenced by cast formation, loss of brush border membranes, sloughing of tubular epithelial cells, and dilation of tubules (Figure 4). These changes were minimal in kidneys from animals with a TNF-α knockout background (WT→KO, KO→KO). Semiquantitative assessment of histologic injury yielded tubular necrosis scores 3.1 ± 0.2 in cisplatin-treated WT→WT mice, 2.8 ± 0.2 in KO→WT mice, 1.5 ± 0.4 in WT→KO mice, and 1.1 ± 0.4 in KO→KO mice. The differences between wild-type background mice and TNF-α knockout background mice were significant ($P < 0.01$, $n = 8$–11).

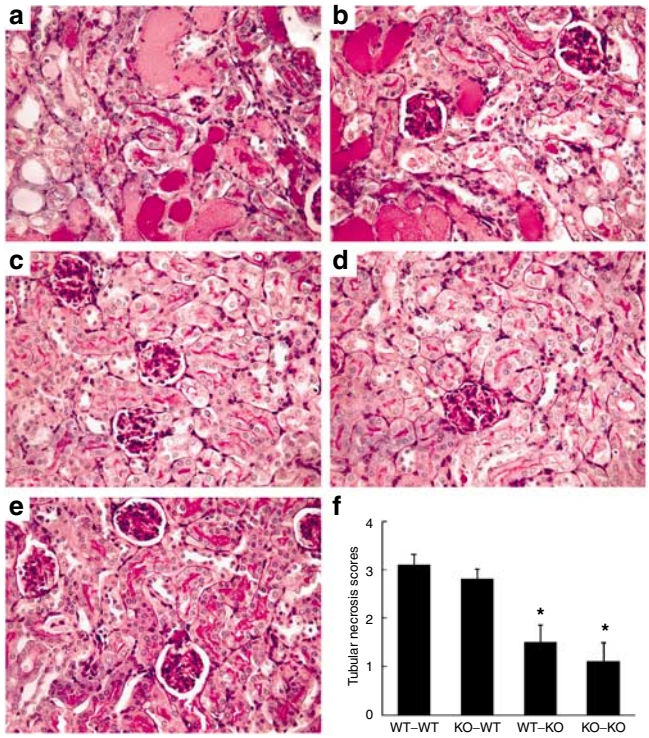

Figure 4 | Effect of cisplatin on kidney morphology in chimeric mice. Periodic acid-Schiff-stained sections of kidney removed 72 h after injection of cisplatin (**a–d**) or (**e**) saline. (**a** and **e**) WT→WT; (**b**) KO→WT; (**c**) WT→KO; (**d**). KO→KO. (**f**) Summary data of tubular necrosis scores for each group of chimeric mice. *$P < 0.01$ vs WT→WT and KO→WT, $n = 8$–11.

Leukocyte infiltration was measured using the napthol AS-D chloroacetate esterase stain. Thirty × 40 fields of esterase-stained sections were examined for quantitation of leukocytes. As shown in Figure 5, cisplatin injection produced a large increase in leukocytes within the kidney cortex in wild-type background chimeric mice (WT→WT 173 ± 63, KO→WT 107 ± 41). In contrast, TNF-knockout background mice (KO→KO 16 ± 2, WT→KO 56 ± 18) had significantly fewer infiltrating leukocytes ($P < 0.01$, $n = 6$–8). These results indicate that local production of TNF-α by resident renal cells is crucial to the recruitment of leukocytes to the kidney in cisplatin-induced nephrotoxicity.

Cisplatin administration results in the upregulation of a number of proinflammatory cytokines and chemokines in the kidney. This upregulation was blunted in TNF-α knockout mice.[2] Accordingly, we examined the expression of these cytokines in the TNF-α chimeric mice. Real-time reverse transcriptase-polymerase chain reaction (RT–PCR) was used to measure renal expression of certain inflammation-related genes (Figure 6). We previously demonstrated that Heme oxygenase 1, IL-1β, TNF-α, TNFR2, and MCP-1 are upregulated in the kidney after cisplatin injection.[2,3,14] These responses were preserved in mice with a wild-type background (WT→WT and KO→WT) but were blunted in mice which lacked the ability to produce TNF-α in their kidneys (WT→KO and KO→KO).

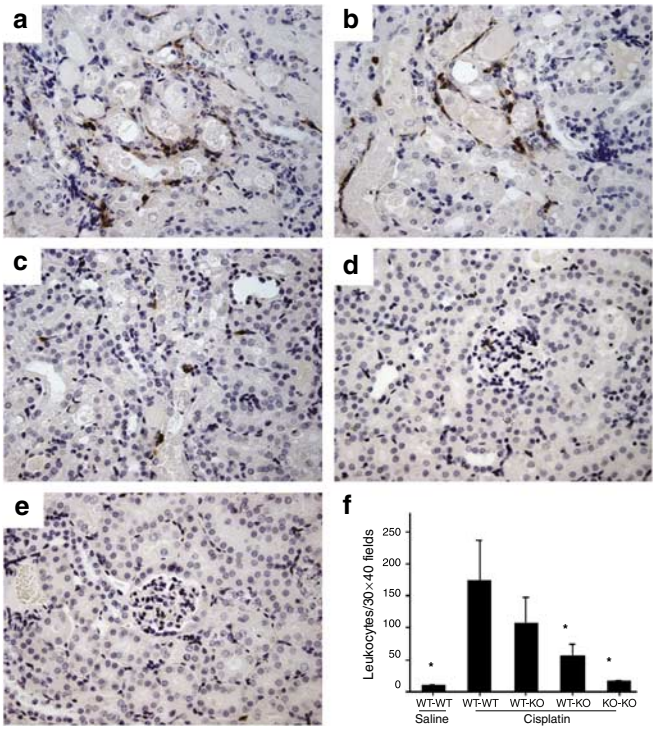

Figure 5 | TNF-α mediates leukocyte infiltration in cisplatin-induced nephrotoxicity. Sections of kidney harvested 72 h after injection of (**a–d**) cisplatin or (**e**) saline were stained for neutrophils as described in the Materials and Methods section. (**a** and **e**) WT→WT; (**b**) KO→WT; (**c**) WT→KO; (**d**) KO→KO. Neutrophils are seen as brown staining. Original magnification of all images is ×400. (**f**) Summary data of leukocyte infiltration for each group of chimeric mice Thirty ×40 fields of kidney cortex were examined from each animal. The total number of leukocytes in those 30 fields is presented. *$P<$ 0.01 vs WT→WT and KO→WT, $n=6$–8.

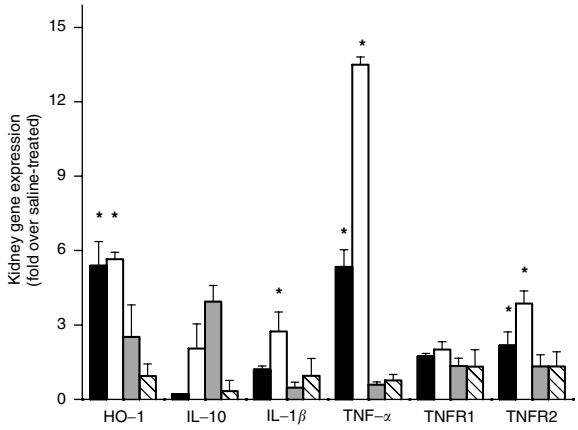

Figure 6 | Cisplatin-induced gene expression in chimeric mice. Cytokine gene expression was measured 72 h after injection of cisplatin by RT–PCR. The expression levels were normalized to the expression of actin and are expressed relative to saline-treated chimeric mice of the same genotypes. WT→WT (black) KO→WT (open), WT→KO (gray), KO→KO (hatched), *$P<0.01$ vs WT→KO and KO→KO, $n=3$.

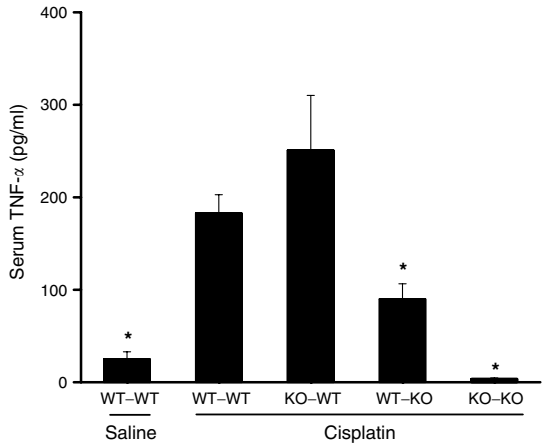

Figure 7 | Serum TNF-α 72 h after cisplatin injection in chimeric mice. The levels of TNF-α in serum were measured using an ELISA assay. *$P<0.01$ vs WT→WT and KO→WT $n=6$–8.

Cisplatin treatment increases serum TNF-α levels.[2–5,14–16] However, the source of the TNF-α is unknown. To address this issue, we measured the effect of cisplatin on serum TNF-α levels in the chimeric mice (Figure 7). Cisplatin injection increased serum TNF-α levels in WT→WT (158 ± 28 pg/ml) and KO→WT (251 ± 60 pg/ml) mice. However, TNF-α levels were lower in WT→KO (90 ± 17 pg/ml, $P<0.01$, $n=4$–5) mice and, as expected, undetectable in KO→KO mice. These results indicate that a large portion of circulating TNF-α after cisplatin injection is derived from cells of nonimmune origin.

We next measured cytokine and chemokine levels in the urine. As described in the Materials and Methods section, 16 cytokines were measured simultaneously in the urine using a multiplexed cytokine immunoassay. Upon cisplatin treatment, as shown in Figure 8, TNF-α, IP-10, IL-2, IL-6, and RANTES were detected in the urine. Of note, wild-type background mice secreted a large amount of TNF-α in urine. In contrast, there was no detectable TNF-α in the urine of mice deficient of TNF-α in their kidneys. These results suggest that resident renal cells are the major source of urinary TNF-α production. Likewise, mice with a recipient TNF-α knockout background had less urinary excretion of IL-2 and RANTES, suggesting that renal production of TNF-α is a stimulus for the renal production and excretion of these cytokines.

DISCUSSION

In the murine model of cisplatin nephrotoxicity, cisplatin increases plasma and urine levels of TNF-α and increases renal expression of TNF-α mRNA. Moreover, TNF-α was shown to play a pathogenic role in cisplatin nephrotoxicity.[2] However, the source of TNF-α which contributes to acute renal injury is not clear. In this regard, TNF-α is produced by a broad range of tissues and cells, including immune cells and intrinsic renal cells, such as mesangial cells,[6,17] glomerular[7,8] and tubular epithelial cells,[9–11] and endothelial cells.[12,13] As cisplatin nephrotoxicity is associated with the influx of bone marrow-derived inflammatory cells into the kidney,[2,3,9,14] these infiltrating leukocytes could be the source of intrarenal

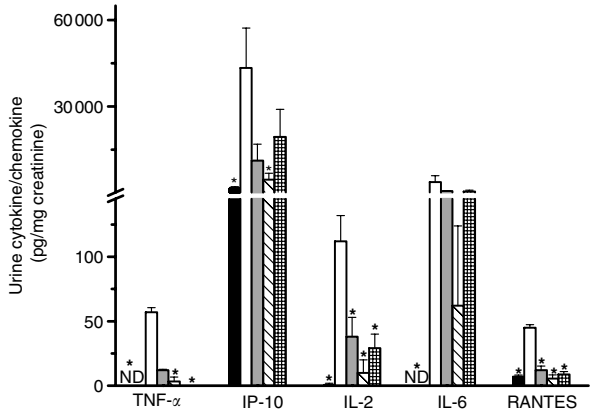

Figure 8 | Cisplatin-induced urine cytokine/chemokine profile in chimeric mice. Urine cytokine/chemokine excretion at 72 h was evaluated using a bead-based multiple cytokine assay. Levels of cytokines were normalized to creatinine. Black bars represent saline-treated WT → WT mice; the remaining bars are cisplatin-treated mice – WT → WT (open), KO → WT (gray), WT → KO (striped), KO → KO (cross-hatched). ND, not detected. *$P < 0.01$, vs cisplatin-treated WT → WT, $n = 4$–5.

and circulating TNF-α. On the other hand, we have shown that cisplatin stimulates renal epithelial cells to produce TNF-α *in vitro*[9,14] raising the possibility that renal parenchymal cells are the major source of TNF-α in cisplatin nephrotoxicity. These studies were performed to determine the role of leukocyte-derived vs kidney-derived TNF-α in the pathogenesis of cisplatin nephrotoxicity. The approach used was to create chimeric mice in which the TNF-α gene was disrupted in cells of bone marrow origin or the kidney. This approach has been used to determine the importance of leukocyte TNFR2 expression in experimental hepatitis.[18] An analogous approach has also been successfully employed to determine the site of TNF-α production in crescentic glomerulonephritis[19] and of adenosine agonist action[20] and P-selectin production[21] in ischemic acute renal failure.

The main finding of the current studies was that chimeric mice produced from wild-type recipients were equally susceptible to cisplatin nephrotoxicity regardless of whether their immune systems were from wild-type or TNF-α-deficient donors. In contrast, chimeras of TNF-α knockout recipients were resistant to nephrotoxicity regardless of the origin of their immune systems (Figures 1–3). The lack of renal failure in the WT → KO mice can not be attributed to faulty production of TNF-α by the transplanted cells because we demonstrated that transplanted cells retain their ability to produce TNF-α (Figure 1) and circulating levels of TNF-α were detectable in the WT → KO mice (Figure 7). These results indicate that, despite the fact that cisplatin nephrotoxicity is characterized by the infiltration of inflammatory cells, TNF-α produced by bone marrow-derived cells does not contribute to cisplatin nephrotoxicity. Rather, production of TNF-α by renal parenchymal cells is probably responsible for the renal failure.

We also noted that the influx of leukocytes into the kidney was reduced in WT → KO and KO → KO mice compared with KO → WT and WT → WT mice (Figure 5). This result indicates that the local production of TNF-α by resident kidney cells promotes an inflammatory response within the kidney. As both renal injury and leukocyte infiltration were reduced in mice with a TNFα-deficient background, our results do not establish if the reduction in the inflammatory infiltrate was the cause or the result of lessened kidney injury. Recent work from Rabb's group, however, demonstrated that early infiltration of the kidney by T cells is an important precedent to cisplatin injury.[22] In that study, deletion of T cells reduced cisplatin-induced renal dysfunction and also reduced kidney TNF-α content and infiltration of neutrophils and macrophages.[22] Our results indicate that the actions of T cells in cisplatin nephrotoxicity are not dependent upon the elaboration of TNF-α by those cells. Kidney expression of several chemokines and adhesion molecules is increased by cisplatin.[2] In this regard, expression of MCP-1 was increased in WT → WT mice but not in WT → KO or KO → KO mice. Likewise, cisplatin increased the urinary excretion of RANTES in WT–WT mice but not in the other chimeras. The expression of intercellular adhesion molecule-1 (ICAM1) (not shown) was modestly increased in WT → WT, WT → KO, and KO → WT mice, but not in KO → KO mice. Thus, both renal and extrarenal production of TNF-α regulates the expression of different chemotactic factors and may account for the decreases in renal leukocyte infiltration seen in the WT → KO and KO → KO mice.

Cisplatin increases both serum and urine concentrations of TNF-α.[2,4,22] Cisplatin has been shown to increase TNF-α production by renal proximal tubule cells[9] and by macrophages.[23] We found that serum levels of TNF-α were elevated to a similar extent in WT → WT and KO → WT mice but were significantly lower in WT → KO mice (Figure 6), indicating that the majority, but not all, of circulating TNF-α seen in cisplatin-treated mice has a nonimmune origin. The finding that cisplatin modestly increased serum TNF-α levels in WT → KO mice is consistent with previous reports demonstrating a cisplatin-induced increase in TNF-α production by certain immune cells.[23,24] Although a source other than the kidney can not be excluded on the basis of the present studies, we did not detect increases in TNF-α mRNA in brain, heart, liver, or skeletal muscle of cisplatin-treated mice (unpublished data). As demonstrated previously,[2] urinary excretion of TNF-α increased in cisplatin-treated WT → WT mice (Figure 8). Urinary TNFα was decreased in both the KO → WT and WT → KO mice suggesting that both immune and renal parenchymal cells contribute to urinary TNF-α. The lower TNFα excretion in KO → WT mice compared with WT → WT mice, in spite of comparable serum TNF-α levels and GFR suggests that urinary TNF-α derives mainly from intrarenal production (either by parenchymal cells or immune cells) and secretion rather than by glomerular filtration.

Although our results indicate that renal parenchymal production of TNF-α is critical to the development of nephrotoxicity, the precise site of TNF-α production within the kidney in the setting of cisplatin nephrotoxicity remains

uncertain. Several cell types within the kidney are capable of producing TNF-α,[6,7,12,13] including proximal tubule cells.[10,11] We have shown that cisplatin increases TNF-α production by proximal tubule cells *in vitro*.[9] However, our attempts to localize TNF-α production by immunofluorescence and immunohistochemistry have not been successful (unpublished results). This difficulty might reflect continuous secretion of TNF-α as it is produced. In any case, the present data do not allow us to draw any conclusions about the site of intrarenal TNF-α production.

The utility of urinary cytokine levels as early biomarkers of acute kidney injury is under investigation. In patients receiving kidney transplants, Kwon *et al.*[25] found that urinary levels of interleukin (IL)-6 and IL-8 predicted the duration of delayed allograft function. Parikh *et al.*[26] found that elevated urinary IL-18 predicted the onset of acute kidney injury in patients with respiratory distress syndrome. In a murine model of ischemic acute kidney injury, the urinary excretion of the chemokine KC increased before an elevation in the serum creatinine.[27] These studies are the first to examine the excretion of urinary cytokines in cisplatin acute kidney injury. We found that, in addition to TNF-α, cisplatin resulted in significant increases in the excretion of several cytokines and chemokines, including IL-2, IL-6, and RANTES. The data in Figure 8 are from 72 h after cisplatin injection, when renal failure is well established. Further studies at earlier time points will be required to determine the time course of cytokine excretion after cisplatin treatment and its possible predictive value in humans. Also, excretion of IL-2 and RANTES was greatest in WT→WT mice and considerably reduced in the other chimeras, suggesting a role for TNF-α in their renal excretion. On the other hand, urinary IL-6 was markedly elevated in all of the chimeras, even the KO→KO mice that sustained relatively little injury. Accordingly, urinary IL-6 may be a nonspecific response to cisplatin administration, *per se*, or is sufficiently sensitive to be increased by the less severe injury in the KO→KO mice. Further studies will be needed to distinguish between these possibilities.

In summary, we have demonstrated that the production of TNF-α by bone marrow-derived immune cells is not critical for the development of cisplatin nephrotoxicity. Rather, local production of TNF-α by renal parenchymal cells likely promotes the elaboration of chemokines and an influx of inflammatory cells resulting in functional and structural damage. In addition, the urinary excretion of several proinflammatory cytokines and chemokines is increased in cisplatin-treated animals. Further studies will be needed to determine the precise cellular source of TNF-α production within the kidney and the possible role of urinary cytokine levels as biomarkers of cisplatin nephrotoxicity.

MATERIALS AND METHODS

Creation of chimeric mice

Chimeric mice were created using C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, USA), mice expressing EGFP under control of the actin promoter (C57BL/6-TgN(ACTbEGFP)10sb, also from the Jackson Laboratories) and mice with a deletion of the TNF-α gene (B&K Universal, Ltd, East Yorkshire, UK) as either bone marrow donors or recipients. Donor mice were killed with sodium pentobarbital and the femurs were removed and flushed with RPMI medium containing 10% fetal calf serum to obtain bone marrow cells. Unfractionated bone marrow cells were washed and resuspended in phosphate-buffered saline at a concentration of 20 million cells/ml. Recipient C57/EGFP mice and TNF-α knockout mice (aged 6–7 weeks) were lethally irradiated using a Gammacell irradiator (two doses of 600 rads, 4 h apart). 8 h after the irradiation, 10 million donor bone marrow cells were injected into the lateral tail vein of recipients. Four sets of chimeric mice were created: WT→WT (wild-type donor and recipient; a control for the transplantation procedure); WT→KO (wild-type donor and TNF knockout recipient; these mice only express TNF-α in cells of bone marrow origin); KO→WT (TNF-α knockout donor and wild-type recipient; these mice express TNF-α normally in all tissues except those of bone marrow origin); KO→KO (TNF-α knockout donor and recipient; these mice do not express TNF-α in any tissue and serve as an additional control to the transplantation process). Mice were maintained in specific pathogen-free conditions before and after the bone marrow transplantation.

Drug administration

Cisplatin (Sigma-Aldrich, St Louis, MI, USA) was dissolved in saline at a concentration of 1 mg/ml and filtered through a 0.2 μm syringe filter. Mice were given a single intraperitoneal injection of either vehicle (saline) or cisplatin (20 mg/kg body weight). This dose of cisplatin produces severe renal failure in mice.[2,28] Blood was collected every 24 h by tail vein bleeding for measurement of urea nitrogen, creatinine, and TNF-α. Urine was harvested by bladder massage. Animals were killed at different time intervals, and kidney tissue was processed for histology and RNA isolation.

Renal function

Renal function was assessed by measurements of blood urea nitrogen (BUN; VITROS DT60II Chemistry slides, Ortho-clinical Diagnostics, Rochester, NY, USA) and serum creatinine (DZ072B, Diazyme Labs, San Diego, CA, USA). GFR in conscious mice was measured using a single bolus injection of FITC-labeled inulin (FITC-inulin).[29] Briefly, 5% FITC-inulin was dissolved in 0.9% NaCl and dialyzed. Dialyzed FITC-inulin (3.75 μl/g BW) was injected via tail vein. Blood samples were collected via saphenous vein at 3, 7, 10, 15, 35, 55, and 75 min post-injection. A serum sample of 10 μl was buffered with 40 μl HEPES (500 mM, pH 7.4) and fluorescence was determined using a Fluoroscan Ascent FL with 485 nm excitation, and 540 nm emission. Serum fluorescence data were fit to a two-component exponential decay curve using nonlinear regression (Origin 6.1, Microcal Software Inc., Northhampton, MA, USA). GFR was calculated using the following equation: $GFR = I/(A_1{*}T_1 + A_2{*}T_2)$ where I is the amount of FITC-inulin delivered by the bolus injection; A_1 and A_2 are the *y*-intercept values of the two decay rates, and t_1 and t_2 are the decay constants for the distribution and elimination phases, respectively.

Intracellular cytokine staining of TNF-α in peripheral blood

The BD Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA, USA) was used for TNF-α intracellular staining. Briefly, 200 μl whole blood was collected via saphenous vein and diluted with 200 μl Iscove's modified Dulbecco's medium. Next, phorbol 12-myristate

13-acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml, Sigma) were added to stimulate TNF-α production followed by a protein transport inhibitor (BD GolgiPlug, BD Biosciences). After incubation for 4 h in 5% CO_2 at 37°C, red blood cells were lysed by adding 2 ml BD PharmLyse solution, BD Biosciences. Leukocytes were centrifuged and resuspended in 100 μl of staining buffer. BD Cytofix/Cytoperm solution (500 μl) was added to fix and permeabilize the cells followed by addition of a PE-conjugated anti-mouse TNF-α antibody (1:100 dilution, BD Biosciences). After a 30-min incubation with the labeled antibody, cells were washed with BD Perm/Wash solution, centrifuged, and resuspended in 500 μl phosphate-buffered saline containing 1% fetal bovine serum and analyzed by flow cytometry.

Quantitation of mRNA by real-time RT–PCR

Real-time RT–PCR was performed in an Applied Biosystems Inc. 7900 Sequence Detection System (Foster City, CA, USA). Total RNA (1.5 μg) was reverse-transcribed in a reaction volume of 20 μl using Omniscript RT kit and random primers. The product was diluted to a volume of 150 μl and either 2 μl (actin) or 10 μl (all others) aliquots were used as templates for amplification using the SYBR Green PCR amplification reagent (Applied Biosystems) and gene-specific primers. The primer sets used were mouse IL-1β (forward: CTCCATGAGCTTTGTACAAGG; reverse: TGCTGATGTACCAGTT GGGG), TNF-α (forward: GCATGATCCGCGACGTGGAA; reverse: AGATCCATGCCGTTGGCCAG); TNFR1 (forward: CCGGGCCA CCTGGTCCG; reverse: CAAGTAGGTTCCTTTGTG); TNFR2 (forward: GTCGCGCTGGTCTTCGAACTG; reverse: GGTATACATGC TTGCCTCACAGTC. Heme oxygenase-1 (forward: AGCATGCCCC AGGATTTG; reverse: AGCTCAATGTTGAGCAGGA). IL-10 (forward: ATG CCT GGC TCA GCA CTG; reverse: GTC CTG CAT TAA GGA GTC G). MCP-1 (foward: ATGCAGGTCCCTGTCATG; reverse: GCTTGAGGTGGTTGTGGA). The amount of DNA was normalized to the β-actin signal amplified in a separate reaction (forward primer: CATGGATGACGATATCGCT; reverse: CATGAGG TAGTCTGTCAGGT).

Cytokine/Chemokine quantitation

The levels of TNF-α in serum were quantitated using an ELISA assay (Quantikine Mouse TNF-α kit; R&D Systems Inc., Minneapolis, MN, USA). Serum (50 μl) was used for the TNF-α assay. Urine cytokines and chemokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p70), IL-17, IFN-γ, IP-10, G-CSF, TNF-α, MIP-1α, MCP-1, and RANTES) were measured using a bead-based multiplexed cytokine analysis kit (Linco Research Inc., St Charles, MO, USA) using a Luminex-100 system (Luminex Corp. Austin, TX, USA). Assays were run in duplicate according to the manufacturers' protocol and data were collected and analyzed using MasterPlex QT 2.5 software (MiraiBio, Alameda, CA, USA). Amounts of cytokines were normalized to the creatinine concentration in the urine.

Histology and immunohistochemistry

Kidney tissue was fixed in buffered formalin for 24 h and then embedded in paraffin wax. Five-micrometer sections were stained with periodic acid-Schiff or naphthol AS-D chloroacetate esterase (kit no. 91A; Sigma-Aldrich). The esterase stain identifies infiltrating neutrophils and monocytes. Thirty ×40 fields of esterase-stained sections were examined for quantitation of leukocytes. Tubular injury was assessed in periodic acid-Schiff-stained sections using a semiquantitative scale[30] in which the percentage of cortical tubules showing epithelial necrosis was assigned a score: 0 = normal; 1 = <10%; 2 = 10–25%; 3 = 26–75%; 4 = >75%. The individual scoring the slides was blinded to the treatment and strain of the animal. Immunohistochemistry for neutrophils was performed by incubating dewaxed sections with a rat anti-murine neutrophil primary antibody (MCA 771 GA, SeroTec, Raleigh, NC, USA) followed by a biotinylated anti-rat IgG secondary antibody. Signal was developed using the avidin/biotin complex peroxidase method (Pierce, Rockford, IL, USA).

Statistical methods

All assays were performed in duplicate. The data are reported as mean ± s.e.m. Statistical significance was assessed by unpaired, two-tailed Student *t*-tests for single comparisons or analysis of variance for multiple comparisons.

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