Am J Physiol Renal Physiol 314: F356–F366, 2018.
First published October 25, 2017; doi:10.1152/ajprenal.00040.2017.

RESEARCH ARTICLE | *Inflammation and Inflammatory Mediators in Kidney Disease*

IL-33 deficiency slows cancer growth but does not protect against cisplatin-induced AKI in mice with cancer

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[1]*Division of Renal Diseases and Hypertension, University of Colorado at Denver, Aurora, Colorado;* [2]*Program of Applied Translational Research, Department of Medicine, Yale University, New Haven, Connecticut; and* [3]*University of Zagreb School of Medicine and Dubrava University Hospital, Zagreb, Croatia*

Submitted 23 January 2017; accepted in final form 18 October 2017

Ravichandran K, Holditch S, Brown CN, Wang Q, Ozkok A, Weiser-Evans MC, Nemenoff R, Miyazaki M, Thiessen-Philbrook H, Parikh CR, Ljubanovic D, Edelstein CL. IL-33 deficiency slows cancer growth but does not protect against cisplatin-induced AKI in mice with cancer. *Am J Physiol Renal Physiol* 314: F356–F366, 2018. First published October 25, 2017; doi:10.1152/ajprenal.00040.2017.—The effect of IL-33 deficiency on acute kidney injury (AKI) and cancer growth in a 4-wk model of cisplatin-induced AKI in mice with cancer was determined. Mice were injected subcutaneously with murine lung cancer cells. Ten days later, cisplatin (10 mg·kg^{-1}·wk^{-1}) was administered weekly for 4 wk. The increase in kidney IL-33 preceded the AKI and tubular injury, suggesting that IL-33 may play a causative role. However, the increase in serum creatinine, blood urea nitrogen, serum neutrophil gelatinase-associated lipoprotein, acute tubular necrosis, and apoptosis scores in the kidney in cisplatin-induced AKI was the same in wild-type and IL-33-deficient mice. There was an increase in kidney expression of pro-inflammatory cytokines CXCL1 and TNF-α, known mediators of cisplatin-induced AKI, in IL-33-deficient mice. Surprisingly, tumor weight, tumor volume, and tumor growth were significantly decreased in IL-33-deficient mice, and the effect of cisplatin on tumors was enhanced in IL-33-deficient mice. As serum IL-33 was increased in cisplatin-induced AKI in mice, it was determined whether serum IL-33 is an early biomarker of AKI in patients undergoing cardiac surgery. Immediate postoperative serum IL-33 concentrations were higher in matched AKI cases compared with non-AKI controls. In conclusion, even though the cancer grows slower in IL-33-deficient mice, the data that IL-33 deficiency does not protect against AKI in a clinically relevant model suggest that IL-33 inhibition may not be useful to attenuate AKI in patients with cancer. However, serum IL-33 may serve as a biomarker of AKI.

cisplatin; kidney injury; IL-33

INTRODUCTION

Cisplatin is one of the major chemotherapeutic agents used in patients with lung cancer. Cisplatin is also used in chemotherapy of testicular cancer, ovarian cancer, bladder cancer, neuroblastoma, and osteogenic sarcoma. The development of acute kidney injury (AKI) is the major limiting factor to the use of cisplatin in cancer patients. Thus it is important to develop an animal model of cisplatin-induced AKI that resembles the human situation to test potential therapies. Most previous in vivo studies of cisplatin-induced AKI have been in models of acute (3 days), high-dose (20–40 mg/kg) cisplatin administration that leads to mortality in non-tumor-bearing mice (31). Interventions that have been tested in cisplatin-induced AKI, e.g., caspase or TNF-α inhibition, have been tested in non-tumor-bearing mice. These interventions have the potential to improve the AKI but may decrease apoptosis of cancer cells, resulting in increased growth of the cancer and impairment of the therapeutic effect of cisplatin. Thus it is important to determine the effect of therapeutic interventions on both the AKI and the cancer. In this regard, we have recently demonstrated that CD4 T-cell deficiency or depletion, which protects against AKI in the acute high-dose cisplatin-induced AKI model, did not protect against AKI in the chronic 4-wk model of cisplatin-induced AKI and resulted in larger tumors (36). The present study to further investigate the role of IL-33, a damage-associated molecular pattern (DAMP) that is released from dying cells, was performed in a chronic clinically relevant model of cisplatin-induced AKI in mice with cancer that received four weekly pulses of cisplatin.

Our published data in the acute high-dose cisplatin model of AKI demonstrate that IL-33-mediated cisplatin-induced AKI is dependent on CD4 T-cell-mediated production of CXCL1 (1). IL-33 also plays a role in other kidney diseases (45). It has been shown that IL-33 inhibition decreases lupus disease and lupus nephritis in lupus-prone mice (16) and that IL-33 deficiency decreases kidney injury and interstitial fibrosis in a unilateral urinary obstruction (UUO) model (3). The role of IL-33 in a clinically relevant chronic 4-wk model of cisplatin-induced AKI in mice with cancer is not known. Thus the first aim of the study was to determine the effect of IL-33 deficiency on the functional and histological changes of AKI in a chronic model of cisplatin-induced AKI in mice with cancer.

IL-33, in addition to mediating kidney disease, may also play a role in cancer progression. There is suppressed breast cancer progression and metastasis in mice with deficiency of the IL-33 receptor, ST2 (11). IL-33 causes colorectal cancer and intestinal polyposis by creating a microenvironment favorable to tumorigenesis (26). IL-33 signaling contributes to the

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pathogenesis of myeloproliferative neoplasms (23). Together, these studies suggest that the IL-33/ST2 axis may initiate cancer development and promote metastasis. Although serum IL-33 may serve as a marker of non-small cell lung cancer progression (44) and IL-33 is increased in lung tumors in mice (15), the effect of IL-33 deficiency on lung cancer is not known. Also, the effect of IL-33 deficiency on the therapeutic effect of cisplatin is not known. A therapy that ameliorates AKI but lessens the therapeutic effect of cisplatin would be problematic. Thus a further aim of the study was to determine the effect of IL-33 deficiency on lung cancer growth and the therapeutic effect of cisplatin on the cancer in mice.

METHODS

Cisplatin-induced AKI in mice with cancer. For all the mouse studies, 8- to 10-wk-old male C57BL/6 mice weighing 20–25 g were used. IL-33$^{-/-}$ mice were generated by Taconic Biosciences (Hudson, NY) on a C57BL/6 genetic background as described (20) and kindly shared through collaboration by Merck Research Laboratories. Utilizing the PCR-based strategy as described (20), animal genotyping was confirmed using PCR to discriminate between wild-type (WT) and mutant alleles (WT amplicon 280 bp, using PCR primers 5′-ggcattaacactaagactactcagcctcag-3′ and 5′-gcgtatgtttggtttggtgcga-3′; mutant amplicon 450 bp, using PCR primers 5′-ggcattaacactaagactactcagcctcag-3′ and 5′-cggggaaatcttggagttggaatact-3′). Of 58 mice genotyped, 16 were WT, 28 were heterozygous knockout (+/−) and 14 were homozygous knockout (−/−). On IL-33 ELISA of kidney homogenates, IL-33 was present in WT kidneys and increased with cisplatin (Fig. 1*A*). In both +/− and −/− kidneys IL-33 was absent (Fig. 1*A*). In addition, both +/− and −/− mice had decreased tumor weight at 4 wk compared with WT. Tumor weight (grams) was 1.1 in WT, 0.55 in +/− mice ($P < 0.01$ vs. WT), and 0.7 in −/− mice ($P < 0.05$ vs. WT) ($n = 6$ per group). Thus as both +/− and −/− kidneys had undetectable IL-33 by ELISA and lower tumor weights compared with +/+, the +/− and −/− mice were studied as a single group and referred to as "IL-33-deficient" mice. Age-, weight-, and sex-matched C57BL/6 mice were used as controls.

Mice homozygous for the CXCR2 mutation in the C57BL/6 strain were obtained by breeding and genotyping heterozygous mice purchased from the Jackson Laboratory as described (2, 13). Age, sex and weight matched littermates were used as controls.

All experiments were conducted with adherence to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The animal protocol was approved by the Animal Care and Use Committee of the University of Colorado at Denver. Mice were maintained on a standard diet, and water was freely available. Mice were housed five per cage under a 12:12-h light-dark schedule for at least 1 wk before cisplatin administration.

CMT167 (lung carcinoma cells; 10^5/40 μl of 10% growth factor reduced Matrigel matrix PBS) were injected with a 30-gage needle into the flank as previously described (42, 43). The cells form a primary tumor in the flank. The tumors were allowed to engraft for 10 days before cisplatin was administered.

After 10 days, cisplatin (10 mg/kg) was injected weekly for 4 wk. Six hours before cisplatin administration, food and water were withheld. Cisplatin [cis-diamminedichloro-platinum (II); Aldrich, Milwaukee, WI] was freshly prepared the day of administration in sterile normal saline at a concentration of 1 mg/ml. Mice were given 10 mg/kg of body weight of cisplatin or vehicle (saline) intraperitoneally, after which the mice again had free access to food and water. At 1, 2, 3, and 4 wk the tumor size was quantitated using digital calipers (42, 43). After 4 wk of cisplatin, the tumors and kidneys were removed and blood was drawn for blood urea nitrogen (BUN) and serum creatinine. Serum creatinine and BUN were measured using a VetAce autoanalyzer (Alfa Wassermann, West Caldwell, NJ).

Three-day high-dose model of cisplatin-induced AKI. Male C57BL/6 mice, 8- to 10-wk-old weighing 20–25 g, were used. We have described this model of cisplatin-induced AKI in detail elsewhere (7). Mice were fed by a standard diet, and water was freely available. Mice were housed five per cage under a 12:12-h light-dark schedule for at least 1 wk before cisplatin administration. Six hours before cisplatin administration, food and water were withheld. Cisplatin was freshly prepared on the day of administration in sterile normal saline at a concentration of 1 mg/ml. Mice were given 25 mg/kg body weight of cisplatin or vehicle (saline) intraperitoneally, after which the mice again had free access to food and water. Mice were euthanized on *day 3* after cisplatin injection when renal dysfunction is severe.

In the 3-day high-dose model experiments, serum creatinine concentration was analyzed with HPLC tandem mass spectrometry (Applied Biosystems 3200 Qtrap). Creatinine and [2H3] creatinine were detected in multiple reaction monitoring mode, monitoring the transitions of the *m/z* from 114 to 44.2 and from 117 to 47.2, respectively.

Histological examination. Paraformaldehyde (4%)-fixed and paraffin-embedded kidneys were sectioned at 4 μm and stained with periodic acid-Schiff (PAS) by standard methods. All histological examinations were performed by a blinded renal pathologist. Histological changes due to acute tubular necrosis (ATN score) were evaluated in the outer stripe of the outer medulla on PAS-stained tissue and were quantified by counting the percent of tubules that displayed cell necrosis, loss of brush border, cast formation, and tubule dilatation as follows: 0 = none, 1 = <10%, 2 = 10–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >75%. At least 10 fields (×250) were reviewed for each slide.

Morphologic criteria were used to count apoptotic cells on PAS-stained tissue by the pathologist experienced in the evaluation of renal apoptosis. Morphologic characteristics included cellular rounding and shrinkage, nuclear chromatin compaction, and formation of apoptotic

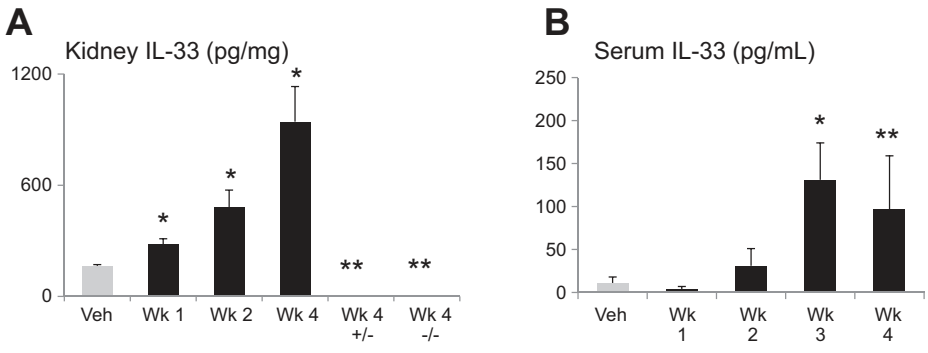

Fig. 1. Kidney and serum IL-33 increases in cisplatin-induced acute kidney injury (AKI) in WT mice. *A*: kidney IL-33 as measured by ELISA increased at *week 1* (Wk 1), *week 2* (Wk 2), and *week 4* (Wk 4) of cisplatin-induced AKI in WT mice; $n = 6$ per group. Kidney IL-33 at *week 4* was undetectable in the kidney of both heterozygous (+/−) and homozygous IL-33 knockout (−/−) mice. *$P < 0.01$ vs. vehicle (Veh); **$P < 0.05$ vs. Veh, $P < 0.001$ vs. Wks 1, 2 and 4; $n = 12$ per group. *B*: serum IL-33 in mice is increased at *weeks 3* and *4* of cisplatin administration. *$P < 0.01$ vs. Veh, **$P < 0.05$ vs. Veh; $n = 8$ per group.

bodies (9). Apoptotic tubular cells were quantitatively assessed per 10 high-power field (×400) in the outer stripe of the outer medulla.

Immunoblotting. Whole kidney extracts were immunoblotted as previously described in detail (5). Immunoblot analyses were performed with the following antibodies: *1*) a rabbit polyclonal antibody against granzyme B (no. 4275, Cell Signaling Technology, Beverly, MA); *2*) a rabbit polyclonal antibody against cleaved caspase-3 (Asp175) (no. 9661, Cell Signaling Technology); *3*) a LC3 antibody (no. 2775, Cell Signaling Technology) that detects endogenous levels of total LC3 protein (LC3 initially yields a cytosolic form, LC3-I, recognized as a 16 kDa protein; during autophagy, LC3-I is converted to LC3-II recognized as a 14 kDa protein); *4*) a β-actin rabbit mAb that detects endogenous levels of total β-actin protein (no. 4970, Cell Signaling Technology).

Measurement of cytokines. A multiplex sandwich immunoassay was used to measure the 10 following inflammatory cytokines: IL-1, IL-2, IL-4, IL-5, IL-6, CXCL1 (also known as IL-8 in humans and KC in mice), IL-10, IL-13, IFN-γ, and TNF-α using a multiarray electrochemiluminescence panel (Meso Scale Discovery, MULTI-SPOT Assay System, V-plex Proinflammatory Panel-1 for mice, catalog no: K15048D-1, Rockville, MD).

Both the mouse IL-33 ELISA (catalog no. M3300) and the CXCL1/KC ELISA (catalog no. MKC 00B) kits were obtained from R and D Systems, Minneapolis, MN. ELISA was performed according to the manufacturer's instructions.

Ki67 staining. Optimal cutting temperature compound-embedded tumors were sectioned at 7 μM and stored at −80°C. Frozen sections were brought to room temperature, fixed with neutral buffered 4% paraformaldehyde in PHS, pH 7.4 for 10 min. After washing three times, tissues were permeabilized and blocked in 5% BSA, 0.1% Triton X-100 for 30 min. Tissues were washed three times again in PBS, then incubated overnight in a humidifier at 4°C at a dilution of 1–100 with primary antibody, K_i-67 (D3B5) rabbit mAb (Alexa Fluor 647 Conjugate, 12075S, Cell Signaling Technology). After three washes again with PBS, slides were counter stained for DAPI and mounted in Antifade Mounting Medium with DAPI (H-1200, Vector Laboratories), imaged by Zeiss LSM780 spectral, and analyzed using Zen2011 Blue software and FIJI ImageJ image processing. Integrated pixel density was calculated per whole image subtracted from three unique background pixel integrated density values.

Serum IL-33 in human AKI. A nested case-control study was performed to determine whether serum IL-33 is a biomarker of AKI in humans. Perioperative plasma samples were obtained from patients enrolled in the well-described TRIBE-AKI cohort of high-risk patients undergoing cardiac surgery (33). Thirty-five cases of Acute Kidney Injury Network stage 2 AKI or higher within the first 4 postoperative days were matched to 70 controls with no AKI on preoperative GFR, age, sex, site, and year of entry into the cohort. Blood samples were collected preoperatively and daily postoperatively. The *day 1* (0–6 h) sample was collected within 6 h after surgery and *day 2* and *3* samples were collected ~48 and 72 h after surgery. *Day 1* (0–6 h) is a time point before there is an increase in serum creatinine in the AKI cases (33). Serum IL-33 was measured by ELISA (R and D systems).

Statistical analysis. In the mouse studies, nonnormally distributed data were analyzed by the nonparametric unpaired Mann-Whitney test. Multiple group comparisons were performed using analysis of variance (ANOVA) with posttest according to Newman-Keuls. A P value of <0.05 was considered statistically significant. Values are expressed as means ± SE.

In the human studies, serum IL-33 values were log transformed. Mixed regression models were used to compare postoperative serum IL-33 values after adjusting for preoperative levels and accounting for matching between cases and controls. Analyses were performed with SAS software, version 9.3 (SAS Institute, Cary, NC).

RESULTS

IL-33 in the kidney increases at week 1, 2, and 4 after cisplatin administration. IL-33 in kidney homogenates as measured by ELISA was increased at *weeks 1*, *2*, and *4* after cisplatin administration in WT mice (Fig. 1*A*). IL-33 in kidney homogenates was absent in both heterozygous (+/−) and homozygous (−/−) IL-33 knockout mice (Fig. 1*A*).

In the 4-wk model of cisplatin-induced AKI that was used in the present study, BUN and serum creatinine increase at 2 wk (36). In the present study, kidney IL-33 was increased at *week 1* before the increase in BUN and serum creatinine, suggesting that IL-33 may play a causative role in the increase in BUN and serum creatinine.

Serum IL-33. Serum IL-33, as measured by ELISA, was significantly increased at *weeks 3* and *4* (Fig. 1*B*). The increase in kidney IL-33 at *week 1* occurred before the increase in serum IL-33 at *weeks 3* and *4*, suggesting that the increase in IL-33 in kidney was not a result of the increase in IL-33 in the blood. IL-33 was undetectable in the urine by ELISA, suggesting that necrotic tubules were not the source of IL-33. Whether the origin of IL-33 in the serum is from necrotic endothelial cells in the kidney or from other organ systems merits further study.

IL-33 deficiency is not sufficient to prevent cisplatin-induced AKI in the 4-wk model. BUN and serum creatinine were significantly increased at *week 4* after cisplatin in both WT and IL-33-deficient mice (Fig. 2, *A* and *B*). Serum neutrophil gelatinase-associated lipoprotein (NGAL) is an early diagnostic biomarker of cisplatin-induced AKI (27). Serum NGAL was significantly increased at *week 4* after cisplatin in both WT and IL-33-deficient mice (Fig. 2*C*).

IL-33 deficiency was not sufficient to prevent ATN or tubular apoptosis. ATN and tubular apoptosis scores were the same in both WT and IL-33-deficient mice with cisplatin-induced AKI (Fig. 2, *D* and *E*).

AKI from tumor lysis syndrome, tumor infiltration of kidney, or thrombotic microangiopathy are clinical complications in patients with cancer (38). Thus it is possible that these factors may have contributed to the lack of protection in IL-33-deficient mice that had smaller tumors and a potentiated effect of cisplatin. Thus we determined whether IL-33 deficiency protected against AKI in the same 4-wk model of low-dose cisplatin used in the present study, but in mice without cancer. BUN and serum creatinine in cisplatin-induced AKI were the same in both WT and IL-33-deficient mice without cancer, indicating that the cancer was likely not contributing to the lack of protection in IL-33-deficient mice (Fig. 2, *F* and *G*).

We have previously demonstrated that soluble ST2 (sST2), a fusion protein that neutralizes IL-33 activity by acting as a decoy receptor, protects against cisplatin-induced AKI in the 3-day high-dose model (1). It was determined whether IL-33-deficient mice were protected against cisplatin-induced AKI in the 3-day high-dose (25 mg/kg) model. BUN (mg/dl) 3 days after one injection of cisplatin 25 mg/kg ip was 35 ± 2 in vehicle-treated WT mice, 44 ± 3 in vehicle-treated IL-33-deficient mice, 79 ± 5 in cisplatin-treated wild-type mice ($P <$ 0.01 vs. vehicle treated, $n = 4$ per group) and 31 ± 0.4 in cisplatin-treated IL-33-deficient mice ($P < 0.01$ vs. cisplatin-treated WT mice, $n = 4$ per group). Serum creatinine (mg/dl)

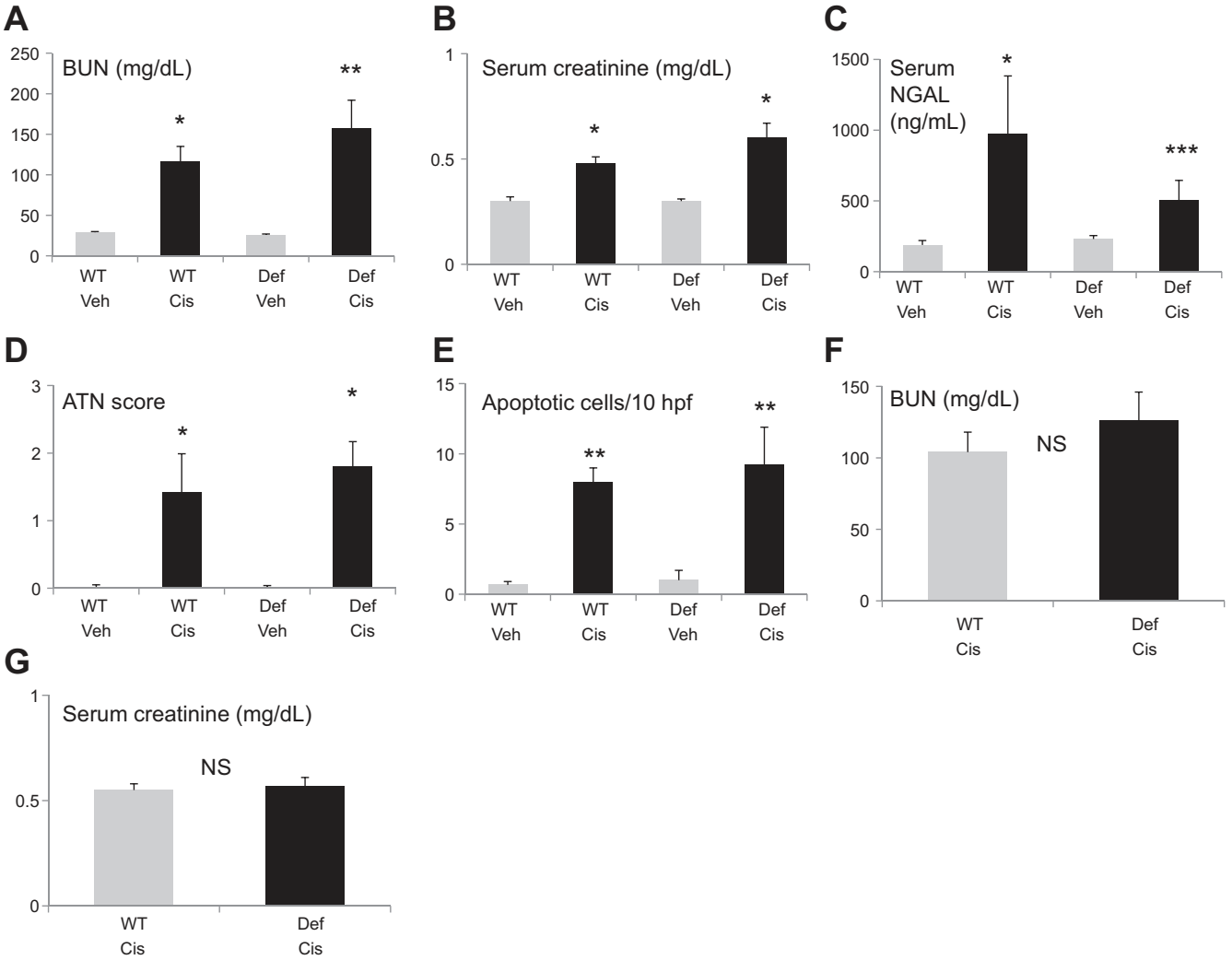

Fig. 2. IL-33 deficiency does not protect against cisplatin-induced AKI in mice with cancer (*A–E*) In mice with cancer, blood urea nitrogen (BUN), serum creatinine, serum neutrophil gelatinase-associated lipoprotein (NGAL), acute tubular necrosis (ATN) score, and apoptosis score were significantly increased by cisplatin (Cis) in both wild-type (WT) and IL-33-deficient (Def) mice. $*P < 0.05$ vs. vehicle (Veh), $**P < 0.01$ vs. Veh, $***P < 0.001$ vs. Veh; $n = 9$ for WT Veh, $n = 8$ for WT Cis, $n = 13$ for IL-33 Def Veh, $n = 14$ for IL-33 Def Cis. *F–G*: in mice without cancer, BUN and serum creatinine in cisplatin-induced AKI (Cis) were not different between WT and IL-33 Def mice; $n = 6$ per group. NS, not significant.

3 days after one injection of cisplatin 25 mg/kg ip was 0.54 ± 0.01 in vehicle-treated WT mice, 0.48 ± 0.1 in vehicle-treated IL-33-deficient mice, 1.2 ± 0.03 in cisplatin-treated WT mice ($P < 0.01$ vs. vehicle treated, $n = 4$ per group) and 0.76 ± 0.02 in cisplatin-treated IL-33-deficient mice ($P < 0.01$ vs. cisplatin-treated WT mice, $n = 4$ per group). Thus IL-33-deficient mice were functionally protected against cisplatin-induced AKI in the 3-day model.

Kidney histology. Representative histology pictures are shown in Fig. 3. In vehicle-treated mice there were clear brush borders and absence of tubular necrosis and apoptosis (Fig. 3*A*). In cisplatin-induced AKI in both WT (Fig. 3*B*) and IL-33-deficient mice (Fig. 3*C*) there was loss of brush borders, tubular necrosis and casts in dilated tubules, and tubular cell apoptosis.

IL-33 deficiency is not sufficient to prevent the increase in CXCL1 and TNF-α. IL-33 is known to bind the ST2 receptor on target inflammatory cells and stimulate production of cytokines (4, 19). The cytokines IL-1, IL-2, IL-4, IL-5, IL-6, CXCL1 (also known as IL-8 in humans and KC in mice), IL-10, IL-13, IFN-γ, and TNF-α were measured in WT kidneys and IL-33-deficient kidneys treated with vehicle or cisplatin (Table 1). Of the 10 cytokines measured, the increase in IL-1 in WT mice was blunted in IL-33-deficient mice. There was an increase in IL-6, IL-10, IL-12, CXCL1, and TNF-α in both cisplatin-treated WT and cisplatin-treated IL-33-deficient mouse kidneys (Table 1).

Tumor size and weight was significantly decreased in IL-33-deficient mice. Tumor weight in IL-33-deficient mice was nearly half that of WT mice (Table 2). Tumor weight in IL-33-deficient mice treated with cisplatin was significantly less than WT mice treated with cisplatin (Table 2). Tumor volume in IL-33-deficient mice was significantly lower than WT mice at *weeks 2*, *3*, and *4*. In WT mice, cisplatin resulted in a significant decrease in tumor volume at *weeks 3* and *4*. In IL-33-deficient mice, cisplatin completely prevented the growth of tumors from *week 1*. Representative cross sections of flank tumors are demonstrated in Fig. 4*A*.

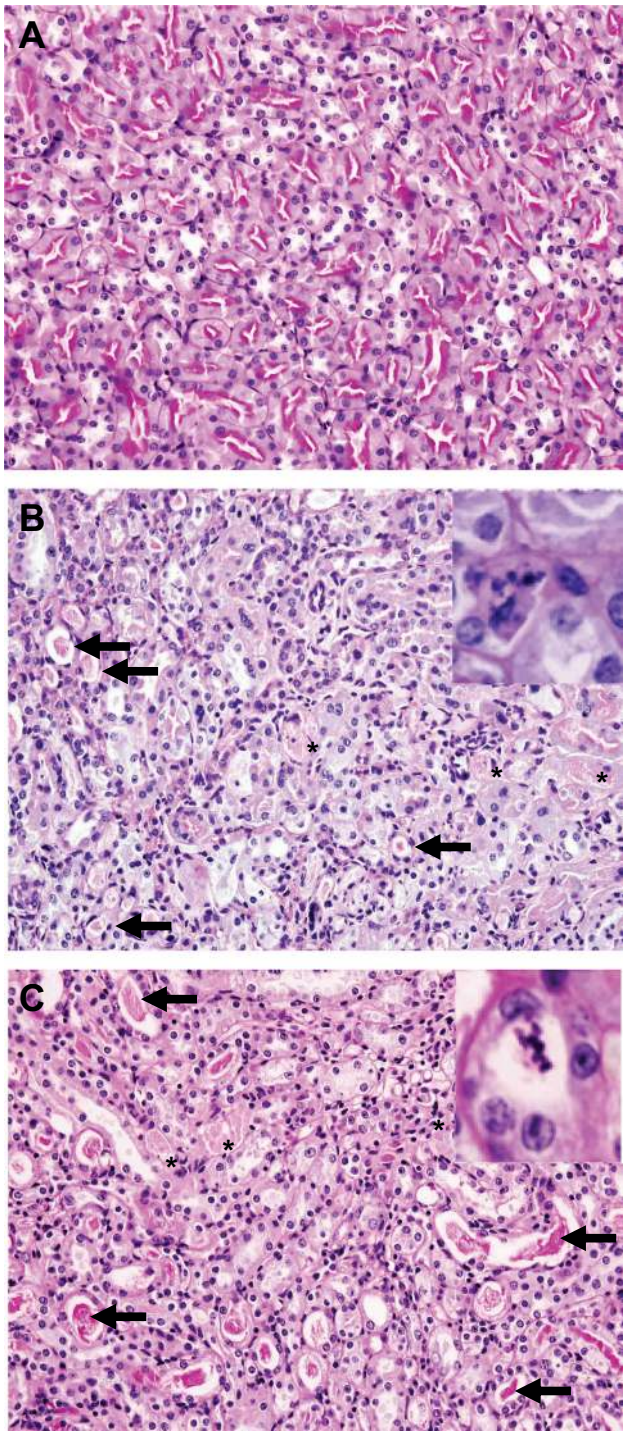

Fig. 3. Representative kidney histology. *A*: in vehicle-treated mice there were clear brush borders and absence of tubular necrosis and apoptosis. *B*: in cisplatin-induced AKI in WT mice there was loss of brush borders, tubular necrosis (asterisks), and casts in dilated tubules (arrows). Insert shows a tubular cell nucleus that is condensed and pyknotic, indicating tubular cell apoptosis. *C*: in cisplatin-induced AKI in IL-33-deficient mice there was the same amount of tubular necrosis and apoptosis. There were loss of brush borders, tubular necrosis (asterisks), and casts in dilated tubules (arrows). Insert shows a tubular cell nucleus that is condensed and pyknotic indicating tubular cell apoptosis. Magnification: ×400; *inset* magnification: ×1,000.

Cleaved caspase-3, LC3 II, and granzyme B in tumors. There was no significant difference in cleaved caspase-3, a marker of apoptosis, in tumors in vehicle-treated WT vs. IL-33-deficient mice (Fig. 4*B*). Cleaved caspase-3 was increased by cisplatin in tumors in WT but not in IL-33-deficient mice. There was no significant difference in LC3-II, a marker of autophagy, in tumors in vehicle-treated WT vs. IL-33-deficient mice (Fig. 4*C*). LC3-II was increased by cisplatin in tumors in both WT and IL-33-deficient mice. IL-33 deficiency or cisplatin had no effect on granzyme B, a marker of cytotoxic lymphocyte (CL)-mediated killing of target cells in tumors (Fig. 4*D*).

CXCL1. CXCL1, also known as IL-8 or KC, is a potent pro-inflammatory cytokine. We have shown that IL-33 stimulates CD4 T cells to make CXCL1 in the acute 3-day model of cisplatin-induced AKI (1). CXCL1 signals via the CXCR2 receptor in mice. Thus the effect of CXCR2 knockout on cisplatin-induced AKI and tumor growth in the chronic 4-wk model of cisplatin-induced AKI was determined. CXCL1 increased at *week 1* of cisplatin-induced AKI (Fig. 5*A*). However, $CXCR2^{-/-}$ mice were not protected against cisplatin-induced AKI in the 4-wk model. BUN, serum creatinine, and serum NGAL were significantly increased in both WT and $CXCR2^{-/-}$ mice (Fig. 5, *B–D*). Next the effect of CXCR2 knockout on tumor growth was determined. Tumor weight and volume was not different between WT and $CXCR2^{-/-}$ mice (Table 3). Cisplatin resulted in a similar decrease in tumor weight and volume in both WT and $CXCR2^{-/-}$ mice (Table 3). Although tumor volume at *weeks 3* and *4* was less in CXCR2 vs. WT mice, the decrease was not statistically significant (Table 3).

Proliferation in tumors. The effect of cisplatin and IL-33 deficiency on cell proliferation as determined by Ki67 staining in tumors was determined. There was an increase in Ki67 staining in tumors with cisplatin compared with vehicle in both WT and IL-33-deficient mice that was not statistically significant (Fig. 6). There was an increase in Ki67 in tumors in vehicle-treated IL-33-deficient mice compared with vehicle-treated WT mice that did not reach statistical significance (Fig. 6). There was an increase in Ki67 in tumors in cisplatin-treated IL-33-deficient mice compared with cisplatin-treated WT mice that was not statistically significant (Fig. 6). These data demonstrate that neither cisplatin nor IL-33 deficiency have a significant effect on cell proliferation as determined by Ki67 staining in tumors.

Serum IL-33 is increased in human AKI. As IL-33 is a DAMP released by damaged cells including endothelial cells (45), and endothelial cell damage and death in the nephron is an early feature of AKI (28). It was determined whether IL-33 in the serum is a marker of AKI. Serum IL-33 was increased in mice at *weeks 3* and *4* of cisplatin-induced AKI (Fig. 1*B*). In humans, using a mixed model accounting for the matching in the data, immediate postoperative serum IL-33 concentrations were higher in matched AKI cases compared with non-AKI controls. Median postoperative serum IL-33 was increased on *day 1* in AKI cases vs. controls (Table 4). The delta mean increase in serum IL-33 from baseline was increased in cases vs. controls on *day 1* (Table 5).

Table 1. *Proinflammatory cytokines*

	WT Veh, $n = 4$	WT Cis, $n = 5$	IL-33 Def Veh, $n = 5$	IL-33 Def Cis, $n = 5$
IL-1, pg/g	74 ± 24	521 ± 141*	46 ± 8	95 ± 30
IL-2, pg/g	72 ± 15	102 ± 19	58 ± 15	113 ± 36
IL-4, pg/g	48 ± 10	34 ± 6	38 ± 8	34 ± 18
IL-5, pg/g	0.01 ± 0.002	0.06 ± 0.04	0.01 ± 0.003	0.02 ± 0.003
IL-6, pg/g	728 ± 210	1,991 ± 318*	562 ± 134	2,113 ± 295**
IL-10, pg/g	87 ± 27	255 ± 21*	73 ± 13	410 ± 67**
IL-12, pg/g	918 ± 277	2,102 ± 254*	899 ± 269	2,686 ± 390**
CXCL1, pg/g	117 ± 47	3,797 ± 368*	184 ± 29	5,954 ± 1,042**
TNF-α, pg/g	55 ± 16	331 ± 62*	54 ± 11	266 ± 21**
IFN-γ, pg/g	4.2 ± 1.5	5.4 ± 0.09	3.1 ± 0.04	4 ± 1.3

WT, wild-type; Veh, vehicle; Cis, cisplatin-induced acute kidney injury; Def, deficient. *$P < 0.05$ vs. WT Veh; **$P < 0.05$ vs. IL-33 Def Veh.

DISCUSSION

Cisplatin and other platinum derivatives are the most widely used chemotherapeutic agents to treat solid tumors including ovarian, head and neck, and testicular germ cell tumors. A known complication of cisplatin administration is AKI (14). Up to 20% of AKI in hospitalized patients is caused by antineoplastic agents of which cisplatin is the most widely used for solid tumors (31). The development of AKI in the hospital is an independent risk factor for mortality and results in an increased odds-ratio of death (14, 40). The nephrotoxic effect of cisplatin is cumulative and dose dependent and often necessitates dose reduction or withdrawal. Recurrent episodes of AKI may result in chronic kidney disease. Despite this toxicity, cisplatin remains one of the most commonly used chemotherapy drugs because of its therapeutic efficacy. Therefore, an understanding of the pathogenesis of cisplatin-induced AKI, especially in a clinically relevant mouse model with cancer, is important for the development of adjunct therapies to prevent AKI, to lessen the need for dose decrease or drug withdrawal, and to lessen patient mortality and morbidity.

IL-33 is a recently discovered member of the IL-1 family of cytokines. IL-33 was originally thought to be a "leaderless" protein activated by caspase-1 in the inflammasome (39). However, in 2009 simultaneous papers demonstrated that IL-33 is not a substrate for caspase-1 and that IL-33 can be cleaved by caspase-3 from its active form to a less active form (22, 41). IL-33 is a DAMP that is released as an early response to tissue injury (29, 30, 32). Release of DAMPs from necrotic cells promote inflammation and can activate macrophages, dendritic cells, and other critical cells of the innate immune system (25). Once released from necrotic cells, full-length (active) IL-33 specifically binds the ST2 receptor that is present on epithelial cells and inflammatory cells, e.g., regulatory T cells (Treg), group 2 innate lymphoid cells (ILC2s), myeloid cells, cytotoxic NK cells, Th2 cells, Th1 cells, and CD8 T cells (4, 19, 21). IL-33 increases secretion of pro-inflammatory cytokines from inflammatory cells (19). The pro-inflammatory cytokines TNF-α (35) and CXCL1 (1) play a role in causing cisplatin-induced AKI. As IL-33 can be released from necrotic tubular cells and causes inflammation, we examined its role in AKI.

The role of IL-33 in mediating kidney diseases is becoming increasingly recognized (45). Lupus-prone model MRL/lpr mice treated mice with anti-mouse IL-33 antibody develop reduced proteinuria and reduced serum anti-dsDNA levels (16). IL-33 inhibition increased Tregs and reduced pro-inflammatory cytokines like IL-1β, IL-6, and IL-17 levels in MRL/lpr mice (16). In a mouse UUO model, deficiency of IL-33 reduced UUO-induced renal fibrosis and increased repair after AKI (3). IL-33 signaling contributes to renal fibrosis following ischemic AKI (18). In this study, administration of soluble ST2, a decoy receptor that neutralizes IL-33 activity, resulted in less severe renal dysfunction and fibrosis associated with less inflammatory cell infiltration in the kidney. However, IL-33 can also have a protective effect. Short-term treatment with IL-33 ameliorated adriamycin-induced glomerulosclerosis (37). In this study, IL-33-mediated expansion of type 2 innate lymphoid cells protected from progressive glomerulosclerosis. Thus IL-33 may have a damaging or protective effect depending on the type of kidney disease. Our earlier study in the acute 3-day model of cisplatin-induced AKI demonstrated that IL-33 exacerbates AKI likely because of stimulating CD4 T cells to make CXCL1 (1). Thus the first aim of the present study was to determine the mechanistic role of IL-33 in a clinically relevant chronic 4-wk model of cisplatin-induced AKI in mice with cancer. However, despite studies showing that IL-33 is a mediator of acute and chronic kidney diseases, the present study conclusively demonstrates that IL-33-deficient mice were not functionally or histologically protected against cisplatin-induced AKI.

Table 2. *Tumors were smaller and tumor growth was less in IL-33-deficient vs. WT mice*

	WT Veh, $n = 12$	WT Cis, $n = 13$	IL-33 Def Veh, $n = 9$	IL-33 Def Cis, $n = 8$
Tumor weight, g	0.9 ± 0.07	0.4 ± 0.1*	0.5 ± 0.06	0.2 ± 0.02**
Tumor volume *week 1*, mm^3	9.5 ± 4	1.4 ± 0.5	5 ± 1.8	0.6 ± 0.2
Tumor volume *week 2*, mm^3	55 ± 11	2.7 ± 0.6	30 ± 15^{+++}	2 ± 0.5
Tumor volume *week 3*, mm^3	151 ± 30^{+}	29 ± 14^{++}	43 ± 13^{+++}	4 ± 1.8
Tumor volume *week 4*, mm^3	122 ± 30^{+}	15 ± 9^{++}	33 ± 7^{+++}	4 ± 2.4

Cisplatin completely prevents tumor growth in IL-33-deficient mice. *$P < 0.001$ vs. WT Veh; **$P < 0.05$ vs. IL-33 Def Veh, $P < 0.05$ vs. WT Cis; +$P <$ 0.01 vs. *week 1*, $P < 0.05$ vs. *week 2*; ++$P < 0.01$ vs. *week 1*, $P < 0.05$ vs. *week 2*, $P < 0.01$ vs. WT Veh; +++$P < 0.05$ vs. *week 1*, $P < 0.01$ vs. WT Veh, $P < 0.01$ vs. IL-33 Def Cis.

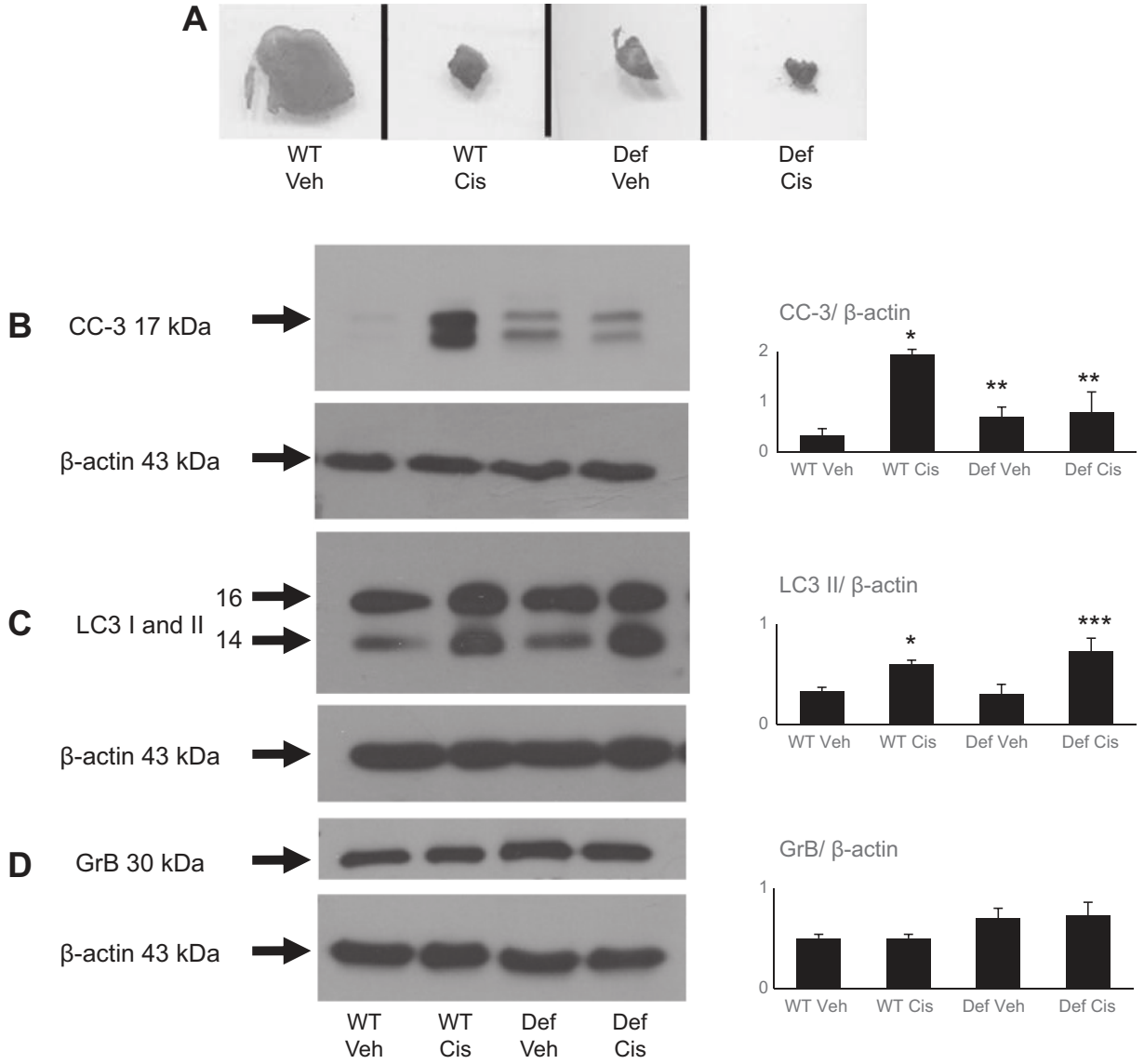

Fig. 4. Cleaved caspase-3, LC3 II, and granzyme B in tumors. *A*: representative cross sections of flank tumors from wild-type mice treated with vehicle (WT Veh), wild-type mice treated with cisplatin (WT Cis), IL-33-deficient mice treated with vehicle (Def Veh), and IL-33-deficient mice treated with cisplatin (Def Cis). Tumor size was smaller in IL-33 Def vs. WT mice. Tumor size was smallest in IL-33 Def Cis. *B* and *C*: in WT mice, cisplatin resulted in an increase in cleaved caspase-3 (CC3) and LC3 II. In IL-33 Def mice, cisplatin resulted in an increase in LC3 II but not cleaved caspase-3. *D*: granzyme B (GrB) was not different between the groups. Representative immunoblots of at least four separate experiments. $*P < 0.01$ vs. WT Veh, $**P =$ NS vs. Def Veh, $***P < 0.01$ vs. Def Veh. B-actin was assayed as a loading control.

Next, we attempted to understand why neutralization of IL-33 with injection of soluble ST2 was protective against AKI in the 3-day model (1), whereas IL-33 deficiency was not protective against AKI in the 4-wk model. In the 3-day model, we demonstrated that IL-33 promotes AKI through CD4 T-cell-mediated production of CXCL1 (1). However, in the 4-wk model, IL-33 deficiency, CD4 T-cell knockout (36), or CXCR2 knockout was not protective, suggesting that CD4 T-cell-mediated production of CXCL1 is not a mediator of the AKI. In the 3-day model, there is a large increase in CD4 T cells on *day 1* (1), whereas in the 4-wk model the increase in CD4 T cells has not been detailed. TNF-α is a known mediator of AKI in the 3-day model (35). In the 4-wk model, we demonstrate that there was an increase in TNF-α in IL-33-deficient mice. However, whether TNF-α is a mediator of AKI in the 4-wk model is not known. The ST2 receptor is present on inflammatory cells other than CD4 T cells e.g., Treg cells, group 2 innate lymphoid cells (ILC2s), cytotoxic NK cells, Th2 cells, Th1 cells and CD8 T cells (21) (4, 19). Thus, there may be a different immune response in the 4-wk model vs. the 3-day model. Also, lack of protection against AKI in the 4-wk as opposed to the 3-day model may be related to the presence of interstitial fibrosis in the 4-wk model (36).

We determined the effect of IL-33 deficiency on a panel of pro- and anti-inflammatory cytokines. There was an increase in the pro-inflammatory cytokine IL-1β in WT but not in IL-33-deficient mice. However, we have previously demonstrated that IL-1β inhibition is not protective against cisplatin-induced AKI (6), suggesting that IL-1β is not involved in the lack of protection. IL-6 was increased in both WT and IL-33-deficient

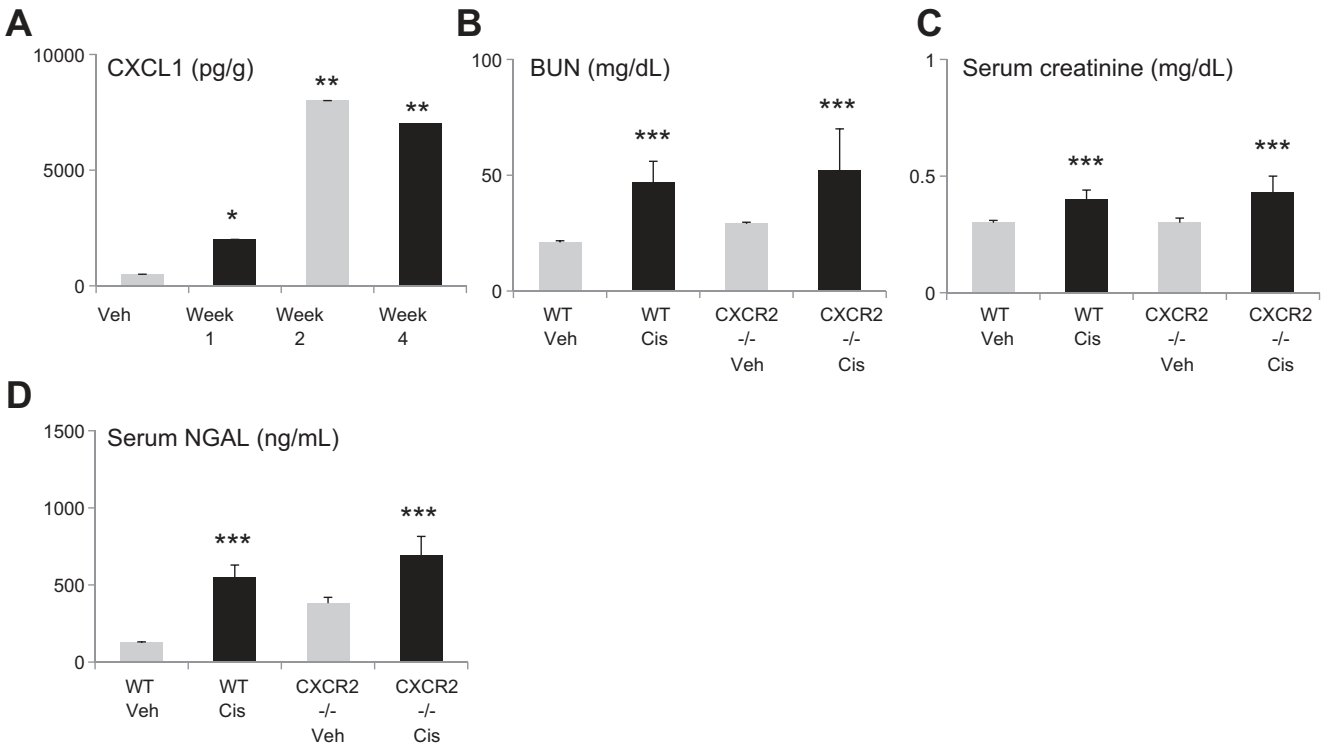

Fig. 5. CXCR2$^{-/-}$ does not protect against cisplatin-induced AKI in mice with cancer. *A*: CXCL1 as measured by ELISA was increased at *weeks 1*, *2*, and *4* after cisplatin administration in WT mice (WY). *B–D*: in mice with cancer, BUN, serum creatinine, and serum NGAL were significantly increased by cisplatin (Cis) in both WT and CXCR2$^{-/-}$ mice. *$P < 0.01$ vs. vehicle (Veh), **$P < 0.001$ vs. Veh, ***$P < 0.05$ vs. Veh; $n = 6$ per group.

mice with AKI. However, we have also demonstrated that IL-6 inhibition is not protective against cisplatin-induced AKI (6), suggesting that the persistent increase in IL-6- in IL-33-deficient mice is not the cause of the lack of protection in IL-33-deficient mice.

AKI is a complication arising from the clinical treatment of cancer. Rapid destruction of tumor cells causes an increase in nucleoside release and turnover resulting in increased synthesis of uric acid and uric acid crystal deposition in the kidney causing AKI (tumor lysis syndrome). Also, uric acid at concentrations that do not cause intrarenal crystal deposition may worsen renal injury in a mouse model of cisplatin-induced AKI (38). Thus we considered that the treatment of the cancer may have masked the protective effect of IL-33 deficiency. However, in the same 4-wk model of low-dose cisplatin-induced AKI in mice without cancer, there was still no protective effect of IL-33 deficiency, demonstrating that the lack of protection in IL-33-deficient mice is independent of the presence of cancer.

Next, the reason for smaller tumors in the IL-33-deficient mice was considered. Cleaved caspase-3, a marker of apoptosis, was measured in the tumors. If the smaller tumors in IL-33-deficient mice were due to increased apoptosis, then we would have expected more cleaved caspase-3 in IL-33-deficient tumors. However, this was not the case. If the improved therapeutic effect of cisplatin in IL-33-deficient tumors was due to increased apoptosis, we would have expected to see increased cleaved caspase-3 in IL-33-deficient mice treated with cisplatin. However, cleaved caspase-3 was decreased in IL-33-deficient mice with cisplatin treatment, suggesting that alterations in apoptosis did not explain the heightened therapeutic effect of cisplatin in IL-33-deficient mice. Next, LC3 II, a marker of autophagy, was determined in the tumors. The emerging hypothesis is that in established tumors, autophagy confers a survival advantage to tumor cells (8). LC3 II was increased comparably in the tumors in both WT and IL-33-deficient mice, suggesting that a lack of autophagy was not the cause of smaller tumors in IL-33-deficient mice. Granzyme B

Table 3. *Tumor weight, tumor growth, and response to cisplatin was the same in CXCR2$^{-/-}$ vs. WT mice*

Experiment 1	WT Veh, $n = 8$	WT Cis, $n = 6$	CXCR2$^{-/-}$ Veh, $n = 7$	CXCR2$^{-/-}$ Cis, $n = 6$
Tumor weight, g	0.9 ± 0.09	0.2 ± 0.04*	0.7 ± 0.2	0.3 ± 1**
Tumor volume *week 1*, mm^3	46 ± 10	14 ± 6	81 ± 14	13 ± 6
Tumor volume *week 2*, mm^3	152 ± 25^{+}	66 ± 20^{++}	137 ± 36	43 ± 13^{++++}
Tumor volume *week 3*, mm^3	404 ± 139^{+}	84 ± 32^{++}	184 ± 37^{+++}	76 ± 29^{++++}
Tumor volume *week 4*, mm^3	444 ± 266^{+}	81 ± 14^{++}	274 ± 62^{+++}	112 ± 62

NS, not significant. *$P < 0.001$ vs. WT Veh; **$P < 0.05$ vs. CXCR2$^{-/-}$ Veh, NS vs. WT Cis; +$P < 0.05$ vs. *week 1*; ++$P < 0.05$ vs. *week 1*, $P < 0.05$ vs. WT Veh; +++$P < 0.05$ vs. *week 1*, NS vs. WT Veh; ++++$P < 0.05$ vs. *week 1*, $P < 0.05$ vs. CXCR2$^{-/-}$ Veh.

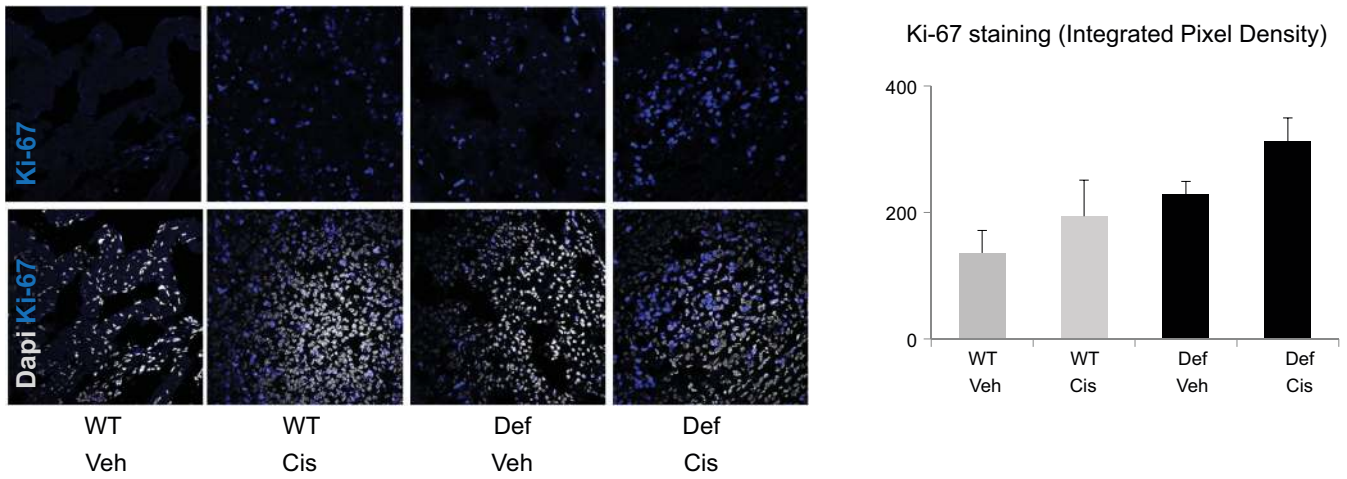

Fig. 6. Neither cisplatin nor IL-33 deficiency have a significant effect on cell proliferation in tumors. Cell proliferation was determined by Ki67 staining in tumors. The increase in Ki67 staining in tumors with cisplatin (Cis) compared with vehicle (Veh) in both wild-type (WT) and IL-33-deficient mice (Def) was not statistically significant. The increase in Ki67 in tumors in vehicle-treated IL-33-deficient mice compared with vehicle-treated WT mice did not reach statistical significance. The increase in Ki67 in tumors in cisplatin-treated IL-33-deficient mice compared with cisplatin-treated WT mice was not statistically significant. Data represents the mean of 5 images/animal, 4 animals/treatment group.

is known to be produced by CD8 T cells and is cytotoxic to tumors. Cisplatin or IL-33-deficient had no effect on granzyme B, suggesting that an increase in granzyme B in IL-33-deficient tumors is not the cause of the smaller tumors. Thus, what may be the reason for smaller tumors in IL-33-deficient mice? Tumor development results in downregulation of IL-33 in tumor cells themselves that increases immunogenicity and promotes type 1 antitumor immune responses through CD8 T cells and NK cells (21). However, upregulation of IL-33 in the tumor stroma and serum results in immune suppression via Treg and myeloid-derived suppressor cell. WT mouse cancer cells were injected into the IL-33-deficient mice. Thus it is likely that smaller tumors were due to a lack of IL-33 in the surrounding tumor macroenvironment rather than the tumor itself.

The IL-33, CD4 T-cell, CXCL1 axis was further considered in this model. CXCL1 is a pro-inflammatory chemokine produced by CD4 T cells. Our previous study in the acute 3-day model demonstrated that IL-33-mediated cisplatin-induced AKI is dependent on CD4 T-cell-mediated production of CXCL1, concluding that inhibition of IL-33 or CXCL1 has therapeutic potential in cisplatin-induced AKI (1). To complete our study of the IL-33, CD4 T-cell, CXCL1 axis in the 4-wk model of cisplatin-induced AKI in mice with cancer, we determined whether inhibition of CXCR2, the receptor for CXCL1, protected against AKI. CXCR2 knockout was not protective against AKI or cancer. The lack of protection in IL-33-deficient, CD4 T-cell knockout or CXCR2 knockout mice suggests that the IL-33, CD4 T-cell, CXCR2 axis is not important in mediating AKI in a chronic 4-wk model in mice with cancer.

DAMPs are endogenous molecules released by damaged cells, including endothelial cells (45). IL-33, a DAMP, has been shown to be present in blood vessels in the kidney in mice (1). As endothelial cell damage and death in the nephron is an early feature of AKI (28), it is logical to consider that IL-33 may be increased in the serum in AKI. Serum IL-33 was increased in mice at *weeks 3* and *4* of cisplatin-induced AKI (Fig. 1*B*). Serum IL-33 is a known biomarker for a variety of disorders such as heart failure, non-small-cell lung cancer, pulmonary inflammatory diseases, and systemic lupus erythematosus activity (46). Elevated serum IL-33 may serve as a biomarker for cardiovascular events in stable renal transplant recipients (24). Serum IL-33 reflects disease activity in patients with ulcerative colitis (34), rheumatoid arthritis (12), and ankylosing spondylitis (17). Serum IL-33 is increased in patients with vasculitis compared with control patients (10). The present study is the first demonstration that serum IL-33 is increased in both mice and patients with AKI.

In summary, in a clinically relevant model of cisplatin-induced AKI in mice with cancer, there was a mild but significant degree of kidney dysfunction and tubular injury, and cisplatin significantly slowed the growth of tumors. Unlike our previous study in the acute high-dose model of cisplatin-induced AKI in which IL-33 inhibition or CXCR2 knockout was protective against AKI and lethality (1), IL-33-deficient or

Table 4. *Median pre- and postoperative levels of serum IL-33 (pg/ml) in patients undergoing cardiac surgery*

Time Point	AKI Cases, $n = 35$	Controls, $n = 70$	P Value
Preoperative	155 (0, 516)	58 (0, 641)	0.87
Day 1	331 (71, 1, 922)	72 (0, 759)	0.048
Day 2	315 (34, 2, 731)	158 (0, 905)	0.08
Day 3	413 (12, 1, 391)	135 (0, 1, 170)	0.38

Reported values are median (25th percentile, 75th percentile). AKI, acute kidney injury.

Table 5. *Mean pre- and postoperative levels of serum IL-33 (pg/ml) in patients undergoing cardiac surgery*

Time Point		AKI Cases	Controls	P Value
Preoperative	Actual	1,082 (2, 174)	1,160 (2, 214)	0.87
Day 1	Delta from pre	355 (926)	12 (759)	0.05
Day 2	Delta from pre	456 (1, 252)	111 (1, 069)	0.29
Day 3	Delta from pre	282 (1, 402)	222 (1, 190)	0.67

Reported values are means ± SD.

CXCR2 knockout mice were not protected against AKI in a 4-wk model of low-dose cisplatin in mice with cancer. The present study in a clinically relevant 4-wk low-dose cisplatin model of AKI in mice with cancer highlights the fact that interventions thought to protect against cisplatin-induced AKI in the acute high-dose model in mice without cancer, may make the cancer grow slower but may not prevent AKI. In conclusion, the lack of a protective effect of CD4 T-cell deficiency or depletion in our previous study (36) and IL-33 deficiency or CXCR2 knockout in the present study, suggest that blockade of the IL-33, CD4 T-cell, CXCR2 axis may not be beneficial in reducing AKI and have variable effects on cancer growth. However, the increase in serum IL-33 in mice and humans with AKI suggests that further studies are warranted to clarify the role of serum IL-33 as an early biomarker of AKI.

GRANTS

This work was supported by Veterans Affairs Merit Award 1I01BX001737-01A1 (to C. Edelstein); National Institutes of Health Grants CA-162226, CA-198619, and CA-058187 (to R. Nemenoff); HL-121877 and HL-123616 (to M. Weiser-Evans); and RO1 HL-85757 (to C. Parikh).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.R., S.H., C.N.B., Q.W., A.O., M.C.W.-E., R.A.N., H.T.-P., C.R.P., and C.L.E. conceived and designed research; K.R., S.H., C.N.B., A.O., M.M., D.L., and C.L.E. performed experiments; K.R., S.H., C.N.B., H.T.-P., and C.L.E. analyzed data; K.R., S.H., C.N.B., H.T.-P., and C.R.P. interpreted results of experiments; K.R., H.T.-P., C.R.P., and C.L.E. prepared figures; K.R., S.H., C.N.B., M.C.W.-E., R.A.N., and C.L.E. edited and revised manuscript; K.R., S.H., C.N.B., M.C.W.-E., R.A.N., D.L., and C.L.E. approved final version of manuscript; C.L.E. drafted manuscript.

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