TIM2 Gene Deletion Results in Susceptibility to Cisplatin-Induced Kidney Toxicity

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T-cell Immunoglobulin and Mucin domain 2 (TIM2) belongs to the receptor family of cell surface molecules expressed on kidney, liver, and T cells. Previous studies have revealed that TIM2-deficient mice (TIM2$^{-/-}$) are more susceptible to the Th2-mediated immune response in an airway inflammation model. Here, we investigated the phenotypic response of TIM2$^{-/-}$ mice to cisplatin-induced kidney toxicity. A lethality study in male BALB/c wild-type (TIM2$^{+/+}$) and TIM2$^{-/-}$ mice, administered with 20 mg/kg cisplatin ip, resulted in 80% mortality of TIM2$^{-/-}$ mice as compared with 30% mortality in the TIM2$^{+/+}$ group by day 5. The TIM2$^{-/-}$ mice showed approximately fivefold higher injury as estimated by blood urea nitrogen and serum creatinine at 48 h that was confirmed by significantly increased proximal tubular damage assessed histologically (H & E staining). A significantly higher expression of Th2-associated cytokines, TNF-α, IL-1β, IL-6, and TGFβ, with a significant reduction of Th1-associated cytokines, RANTES and MCP-1, by 72 h was observed in the TIM2$^{-/-}$ mice as compared with TIM2$^{+/+}$ mice. A higher baseline protein expression of caspase-3 (approximately twofold) coupled with an early onset of p53 protein activation by 48 h resulted in an increased apoptosis by 48–72 h in TIM2$^{-/-}$ compared with TIM2$^{+/+}$. In conclusion, the increased expression of the proinflammatory and proapoptotic genes, with a higher number of apoptotic cells, and a pronounced increase in injury and mortality of the TIM2-deficient mice collectively suggest a protective role of TIM2 in cisplatin-induced nephrotoxicity.

Key Words: TIM2; cisplatin; kidney; inflammation; apoptosis.

The T cell and airway phenotype regulator (Tapr) region on the human 5q33.2 chromosome and murine 11B1.1 is most frequently linked with asthma, allergy, and other immune responses. Within the confines of the Tapr region are a class of type I cell surface molecules called the T-cell Immunoglobulin domain and Mucin domain (TIM) family. They have been studied for their role in the regulation of immune functions (McIntire *et al.*, 2001, 2004). One of the first members of the family to be identified was the hepatitis A virus cellular receptor 1 (HAVCR1) in the liver, which was later found to be expressed on the apical surface of the kidney epithelial cells following injury as the kidney injury molecule-1 (Kim-1) (Ichimura *et al.*, 1998). It is also expressed as the T-cell Immunoglobulin domain (TIM1) on all activated T cells, particularly Th2-type differentiated cells (Chakravarti *et al.*, 2005; McIntire *et al.*, 2001). The TIM family is comprised of three members in humans (hTIM1, hTIM3, and hTIM4) and eight in mice (mTIM1-8). Mouse TIM1 and TIM2 share a homology of 66% with each other and 41 and 36%, respectively, with hTIM1. This has resulted in a postulation that mTIM2 evolved as a gene duplication of mTIM1 and that it also shares functional characteristics with hTIM1 because it is found only in rodents and not in primates (Rodriguez-Manzanet *et al.*, 2009). In fact, humans express higher levels of TIM1/HAVCR1 in the liver, whereas higher levels of TIM2 transcripts are found in the mouse liver (Chen *et al.*, 2005).

Thus far, studies have detected TIM2 expression on the surface of Th2 cells (Rennert *et al.*, 2006) and in splenic B cells, bile duct cells, and kidney epithelial cells (Chen *et al.*, 2005). TIM2 has been shown to be involved in inducing T-cell activation upon binding to Semaphorin 4A (found on B cells and dendritic cells), thereby exerting its effects on the immune system (Kumanogoh *et al.*, 2002). *In vitro* transfection experiments by Chen *et al.* (2005) have shown ferritin being sequestered by TIM2 to endosomes, which may be another potential ligand for this molecule. Watanabe *et al.* (2007) have shown an unknown ligand in the mouse liver that binds to TIM2 through cell-cell contact on adjacent hepatocytes that is capable of inhibiting the negative effect elicited by TIM2 on liver differentiation genes. This implies the presence of a molecule capable of suppressing the action of TIM2 in the liver and thereby potentially in inflammation following injury to the liver (Watanabe *et al.*, 2007).

Observations of the TIM2 knockout (ko) mouse phenotype in an airway inflammation model confirmed that the resting immune system was unaffected. However, disregulated CD4+ T-cell activity and over expression of Th2 cytokines

contributed to the severity of inflammation asserting the critical role TIM2 exerts in T-cell effector pathways (Rennert *et al.*, 2006). In fact, TIM2 is believed to play an important role during the effector phase of the immune response rather than the initial differentiation phase by specifically being up-regulated in Th2 cells and inducing a negative regulation in the Th2 cell response (Chakravarti *et al.*, 2005). Knickelbein *et al.* (2006) found that a transfected T-cell line with TIM2 complementary DNA exerted its inhibitory effect in the T-cell receptor cascade starting below or at the phospholipase C $\gamma1$ (PLC$\gamma1$) activation and above the NFAT/AP-1–dependent transcription factors.

With the understanding that T cells particularly CD4+ cells play an important role in cisplatin-induced acute nephrotoxicity (Liu *et al.*, 2006) and that TIM2 is a critical negative regulator of Th2 response, the objective of this study was to characterize the phenotype of TIM2 ko mice using a cisplatin-induced kidney toxicity model.

MATERIALS AND METHODS

Animals. Male wild-type (wt) and TIM2$^{-/-}$ BALB/c mice, 8–10 weeks of age weighing 25–29 g, were given standard laboratory diet and water *ad libitum* and were maintained in our central animal facility over wood chips free of any known chemical contaminants under conditions of $21 \pm 1°C$ and 50–80% relative humidity at all times in an alternating 12-h light-dark cycle. All animal maintenance and treatment protocols were in compliance with the Guide for Care and Use of Laboratory animals as adopted and promulgated by the National Institutes of Health and were approved by respective Institutional Animal Care and Use Committees.

Experimental design. TIM2-deficient mice were generated using TIM2-targeting GAL4 knock-in vector as reported previously (Rennert *et al.*, 2006) and were bred onto the BALB/c background for 10 generations. Wt littermates were used as controls in all studies. Cisplatin (Sigma, St Louis, MO) was freshly prepared at 20 mg/kg in saline and administered by a single ip injection. A lethality study was conducted in the TIM2$^{+/+}$ and TIM2$^{-/-}$ ($n = 10$ each) by injecting them with 20 mg/kg cisplatin ip, respectively, in 0.9% saline (10 ml/kg). Survival/mortality were observed and recorded twice daily for 10 days. Animals ($n = 5$/group/time point) were euthanized by an overdose of pentobarbital sodium (180 mg/kg, ip) on days 1, 2, and 3 after cisplatin administration. Control animals were injected with equal volume of vehicle (saline) ip and were euthanized on day 1. Heparinized tubes were used to collect blood from the dorsal aorta for measurement of blood urea nitrogen (BUN) and serum creatinine (SCr) as indicators of kidney function. The kidneys were perfused with PBS through the left ventricle. One kidney was diced into small fragments and flash frozen in liquid nitrogen for RNA and protein extractions. One half of the second kidney was flash frozen into OCT blocks in liquid nitrogen for cryosectioning and immunostaining. The other half of the second kidney was fixed in formalin for 16 h for paraffin sections, histology, and immunohistochemistry.

Analysis of kidney function. Serum creatinine (SCr) concentrations were measured using a Beckman Creatinine Analyzer II. BUN was measured spectrophotometrically at 340 nm using a commercially available kit (Thermo Scientific, Rockford, IL).

Immunofluorescence staining. Kidney tissues were fixed in 4% para-formaldehyde and embedded in paraffin. The tissue sections were deparaffi-nized in xylene and rehydrated in ethanol followed by antigen retrieval using Vector Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA).

The samples were then blocked with 10% normal goat serum (Vector Laboratories) for an hour at room temperature. The sections were incubated overnight at 4°C in rabbit monoclonal anti-Ki67 (1:500) (Vector Laboratories) and rabbit polyclonal anti-*Kim-1* (1:200) (raised in Bonventre laboratory) (Ichimura *et al.*, 1998). The primary antibody was detected using goat anti-rabbit Cy3-labeled secondary antibody (Jackson ImmunoResearch Laborato-ries, West Grove, PA). 4′,6-diamidino-2-phenylindole (Sigma Aldrich, St Louis, MO) was used for nuclear staining. The tissue sections were mounted using ProLong Gold Antifade Reagent (Invitrogen, Carlsbad, CA). The imaging was performed using a NIKON Eclipse 90i fluorescence microscope. The number of proliferating cells (Ki67) was counted in 10 different fields at ×400 magnification. Results are represented as the fraction of positively stained cells out of the total number of cells/field.

TUNEL assay. Apoptosis was measured in kidney tissues by TUNEL assay using the In Situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) according to manufacturer's instructions. Briefly, kidney tissues were fixed with 4% paraformaldehyde and paraffin embedded. Tissue sections were deparaffinized, permeabilized, and exposed to TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides, including fluorescein isothiocyanate- labeled 2′-deoxyuridine, 5′-triphosphate. Positive staining was identified by DNA breakage in the nucleus using NIKON Eclipse 90i fluorescence microscope. The number of apoptotic cells was counted in 10 different fields at ×400 magnification. Results are represented as the fraction of positively stained cells out of the total number of cells/field.

RNA extraction and real-time PCR. Total RNA was extracted from frozen tissue samples using TRIZOL reagent (Invitrogen) according to manufacturer's instructions. Briefly, 30 mg of tissue sample was homogenized in 1 ml of TRIZOL reagent to which 200 μl of chloroform was added and mixed well. After a brief incubation at room temperature, the samples were centrifuged at $12,000 \times g$ for 15 min. To the upper aqueous phase, 500 μl of isopropyl alcohol was added to precipitate RNA. The samples were incubated at room temperature and centrifuged at $12,000 \times g$ for 10 min. The pellet was washed in 75% ethanol, air dried, and dissolved in RNase-free water (Qiagen Sciences, Germantown, MD). Integrity of the isolated total RNA was determined by 1% agarose gel electrophoresis, and the RNA concentration was measured by ultraviolet light absorbance at 260 nm using the Nanodrop 2000C spectrophotometer (Thermo Scientific).

The isolated RNA was treated with DNase I Amp Grade (Invitrogen). The DNA-free RNA samples were then reverse transcribed according to the iScript cDNA Synthesis Kit protocol (Biorad, Hercules, CA). Real-time PCR of the tissue samples was performed with SYBR Green (Biorad) detection system using CFX96 RTPCR instrument (Biorad). Primers were designed to amplify 120–150 base pair fragment with the following cycle conditions: 95°C for 3 min; the following steps were repeated 40 times: 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Forward and reverse primer sequences for mouse-specific genes were designed using MacVector software (MacVector Inc., Cary, NC) and are listed in Table 1.

Protein extraction and Western blot analysis. Tissue samples were homogenized in 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.4, 5M sodium chloride, and 0.5M EDTA. Complete Protease Inhibitor Cocktail tablets (Roche Applied Science) were added to the lysed solution and centrifuged at $6000 \times g$ for 8 min at 4°C to remove cell debris and nuclei. Using the supernatant, protein concentrations were measured at 562 nm using the BCA Protein Assay Kit (Pierce, Rockford, IL) according to manufacturer's instructions. Immunoblotting was performed using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene fluoride membrane. The blots were blocked with 5% Blotting Grade Blocker Non Fat Dry Milk (Biorad) for an hour and incubated overnight at 4°C on a rotor with mouse monoclonal anti-p53 (1:2000) (Abcam, Cambridge, MA), mouse monoclonal anti-Hsp70 (1:5000) (Stressgen, Ann Arbor, MI), rabbit polyclonal anti-caspase-3 (1:2000) (Cell Signaling Technology, Danvers, MA), and rabbit monoclonal anti-β-Actin (1:5000) (Cell Signaling Technology).

TABLE 1
RT-PCR Primers Used for the Quantification of mRNA Expression Levels in the Study

Gene	F/R	Sequence
Caspase-3	F	5′-TGTGGCATTGAGACAGACAGTGG-3′
	R	5′-TCCAGGAATAGTAACCAGGTGCTG-3′
CD68	F	5′-TCTTTCTCCAGCTGTTCACC-3′
	R	5′-ATGATGAGAGGCAGCAAGAG-3′
GAPDH	F	5′-GAATACGGCTACAGCAACAGG-3′
	R	5′-GGTCTGGGATGGAAATTGTG-3′
Hsp70	F	5′-CACCACCTACTCGGACAACCAG-3′
	R	5′-ATCTCCTCCTTGCTCAGGCG-3′
ICAM1	F	5′-TGTTTTGCTCCCTGGAAGGC-3′
	R	5′-AGTCACTGCTGTTTGTGCTCTCC-3′
IL-1β	F	5′-ACCTGTCCTGTGTAATGAAAGACG-3′
	R	5′-TGGGTATTGCTTGGGATCC-3′
IL-6	F	5′-CAAGAGACTTCCATCCAGTTGCC-3′
	R	5′-CATTTCCACGATTTCCCAGAGAAC-3′
Kim-1	F	5′-GGAAGTAAAGGGGGTAGTGGG-3′
	R	5′-AAGCAGAAGATGGGCATTGC-3′
MCP1	F	5′-CCCAATGAGTAGGCTGGAGAGC-3′
	R	5′-TGGTTGTGGAAAAGGTAGTGGATG-3′
MIP2	F	5′-CAATGCCTGAAGACCCTGCC-3′
	R	5′-CTTTTTGACCGCCCTTGAGAG-3′
p53	F	5′-TGGAAGACAGGCAGACTTTTCG-3′
	R	5′-ATGGTAAGGATAGGTCGGCGGTTC-3′
RANTES	F	5′-GCCCTCACCATCATCCTCACTG-3′
	R	5′-TTCTTGAACCCACTTCTTCTCTGG-3′
TGFβ	F	5′-CTCCACCTGCAAGACCAT-3′
	R	5′-CTTAGTTTGGACAGGATCTGG-3′
TNFα	F	5′-AGAAGAGGCACTCCCCCAAAAG-3′
	R	5′-TTCAGTAGACAGAAGAGCGTGGTG-3′

Note. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

The blots were incubated in horseradish peroxidase–conjugated secondary antibody (GE healthcare, Buckinghamshire, UK), and antigens on the blots were revealed using enhanced chemiluminescence kit (GE healthcare) by autoradiography.

Statistical analysis. Data are expressed as average ± SE. Statistical difference ($p < 0.05$) from respective vehicle-treated control as calculated by student's t-test is represented by "$." Statistical differences between the wt and ko groups over time were determined using one-way ANOVA, followed by Tukey's multiple comparison test. "*" represents statistical difference ($p < 0.05$) between wt and ko groups at the same time point. "#" represents statistical difference within the groups (wt vs. ko) between 24 versus 48 h and 24 versus 72 h. "!" represents statistical difference within the groups (wt vs. ko) between 48 versus 72 h.

RESULTS

Higher Mortality Rate and Increased Kidney Injury in the TIM2 ko Mice as Compared with wt Mice following Cisplatin Administration

In order to determine if there were any differences in response to cisplatin-induced nephrotoxicity upon deletion of the TIM2 gene, TIM2$^{-/-}$ and wt male BALB/c mice were administered 20 mg/kg cisplatin and survival was recorded at the end of 10 days. Of the 10 mice used for the study in each group, 7 survived in the wt group, whereas only 2 survived in the TIM2$^{-/-}$ group by day 10 of the study (Fig. 1A). BUN and SCr remained unchanged at 24 h after cisplatin injection (Fig. 1B); however, approximately fivefold greater BUN (118.65 ± 22.75 vs. 26.46 ± 4.42) and sevenfold greater SCr (1.55 ± 0.48 vs. 0.2 ± 0) was observed by 48 h in the TIM2$^{-/-}$ mice as compared with the wt mice. Elevated levels of BUN and SCr persisted at 72 h in both groups. Kim-1 messenger RNA (mRNA) levels in the kidney were used as a metric to assess nephrotoxicity in the absence of availability of a quantitative mouse urinary Kim-1 assay. A significant correlation between the transcription and ectodomain shedding of Kim-1 ($r = 0.83$) following kidney tubular damage has been reported previously (Vaidya *et al.*, 2010). No differences in the baseline *Kim-1* mRNA levels were found between the wt and TIM2$^{-/-}$ mice. *Kim-1* mRNA levels increased significantly in both groups following injury ($p < 0.05$) that gradually peaked by 72 h (Fig. 1C). Histologically, at 48 and 72 h, a substantially higher number of necrotic tubules, sloughed off cells, and intratubular casts were observed in the TIM2$^{-/-}$ mice compared with the wt mice (Fig. 1D).

Increased Expression of Inflammatory Genes in TIM2 ko Mice as Compared with wt Mice

Marked increase in kidney dysfunction, particularly by 48 h in TIM2$^{-/-}$ mice, led us to further investigate the differential expression of inflammatory chemokines that may potentially aggravate cisplatin-induced nephrotoxicity. Consistent with previous reports, there was a significant elevation in all nine candidate chemokines (TNF-α, CD68, IL-1β, IL-6, RANTES, MCP-1, TGFβ, ICAM1, and MIP-2α) following cisplatin administration in both TIM2$^{-/-}$ and TIM2$^{+/+}$ mice (Fig. 2). A 15-fold up-regulation of the proinflammartory cytokine TNF-α was observed in the TIM2$^{-/-}$ as compared with close to sixfold in TIM2$^{+/+}$ mice at 24 h. Similarly, CD68 showed a marked increase of around 76-fold at 48 h and 60-fold at 72 h as compared with wt mice (approximately 45-fold at 48 and 72 h, respectively). An increased expression of Th2 cytokines like IL-1β (around 20-fold vs. 3-fold, $p < 0.05$) and IL-6 (around 280-fold vs. 70-fold, $p < 0.05$) were found in the TIM2$^{-/-}$ mice versus wt by 72 h following nephrotoxicity. However, levels of the Th1-associated cytokines RANTES and MCP-1 were lower in the TIM2$^{-/-}$ as compared with TIM2$^{+/+}$ mice. Similarly, TGFβ transcript levels initially decreased in the TIM2$^{-/-}$ mice at 24 h but gradually increased to approximately 1.3-fold and 3.7-fold at 48 and 72 h in the TIM2$^{-/-}$ mice, whereas they largely remained unchanged in the wt mice. Levels of ICAM1 and MIP-2α remained elevated throughout the time course in both the groups without any significant difference between the TIM2$^{-/-}$ and wt mice.
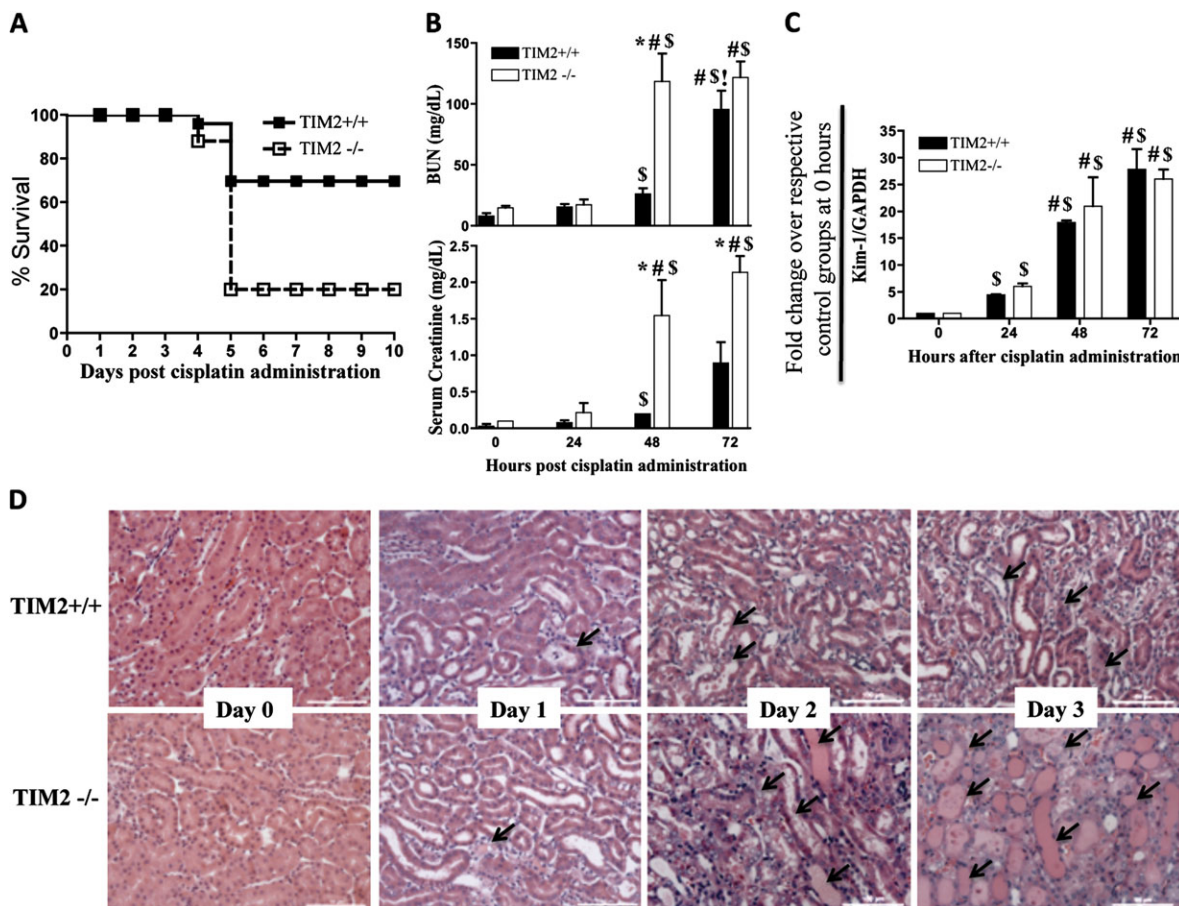
FIG. 1. Comparison of mortality, plasma BUN, serum creatinine (SCr), and renal histology over a time course after administration of cisplatin. (A) Survival curve of TIM2$^{-/-}$ and wt mice ($n = 10$/group) over a period of 10 days following administration of cisplatin (20 mg/kg, ip). (B) BUN and SCr levels were measured using plasma samples separated from blood of wt and TIM2$^{-/-}$ mice from day 0 through 3 after cisplatin administration (20 mg/kg, ip). (C) Quantitative real-time-PCR results of Kim-1 mRNA normalized to GAPDH and fold change recorded over baseline up to 72 h, following cisplatin administration. Data are the average ± SEM. *$p < 0.05$ between wt and ko groups at the same time point. #$p < 0.05$ within the groups (wt or ko) between 24 versus 48 h and 24 versus 72 h. !$p < 0.05$ within the groups (wt or ko) between 48 versus 72 h and \$$p < 0.05$ with respective vehicle-treated control groups at 0 h. ($n = 5$/group/time point). (D) Representative hematoxylin and eosin–stained kidney sections of wt and TIM2$^{-/-}$ mice during the course of study, following cisplatin administration (bar = 100 μm). Arrows indicate necrosis.
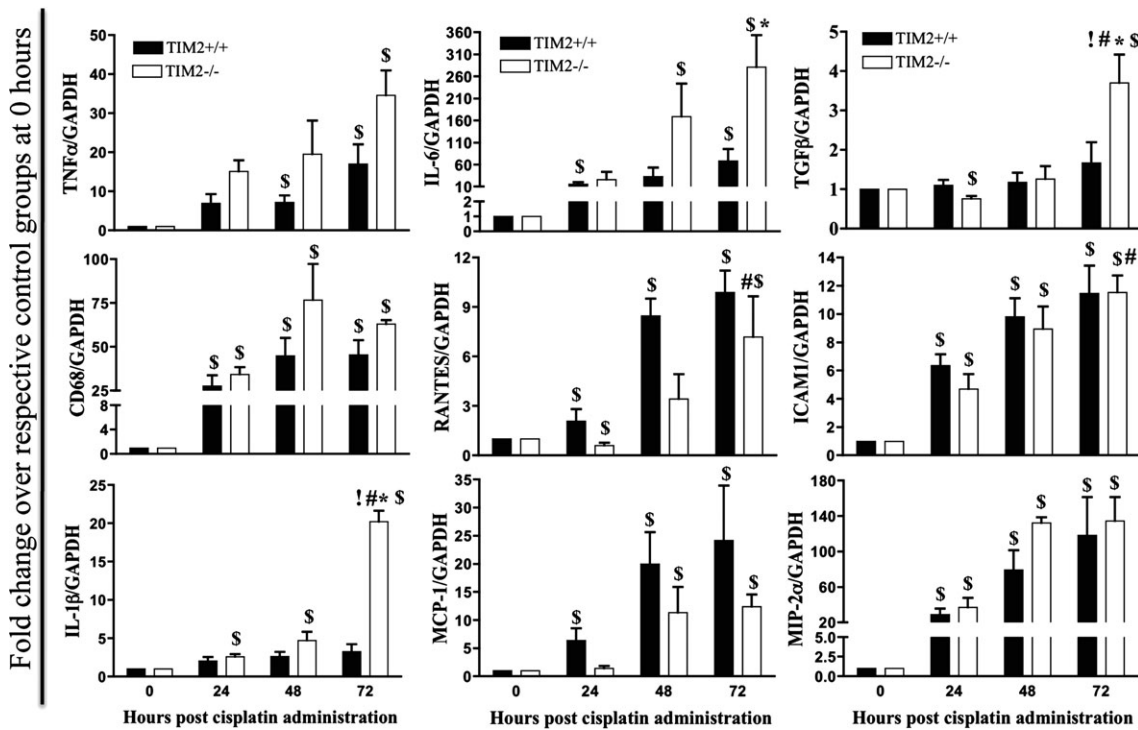
Increased Apoptosis in TIM2 ko Mice as Compared with wt Mice

The apoptotic and proliferative responses by the renal tissues following cisplatin administration were examined using TUNEL assay (Fig. 3A) and Ki67 staining (Fig. 3B), respectively. Approximately twofold increase in the TUNEL positive cells was found by 48 h along with a significant difference by 72 h in the TIM2$^{-/-}$ mice compared with wt mice (Fig. 3i). The apoptotic cells largely appeared in the outer stripe of the outer medulla (OSOM), which is consistent with higher histological damage in this region (representative figures of wt and TIM2$^{-/-}$ are shown in Figs. 3a–d and Figs. 3e–h, respectively). A significant number of cells appeared to be in a proliferative state in the OSOM region by 48 h in the TIM2$^{-/-}$ mice as compared with wt mice, shown by the representative Figs. 3j–m and Figs. 3–q stained for Ki67 in wt and TIM2$^{-/-}$,

respectively. However, by 72 h, the numbers of cells appear to return back to baseline levels in the two groups.

Increased Expression of Apoptotic Genes and Proteins in TIM2 ko Mice as Compared with wt

p53 mRNA levels increased approximately threefold in TIM2$^{-/-}$ and wt mice at 24 h following cisplatin-induced kidney toxicity (Fig. 4A). The p53 protein expression increased significantly in the TIM2$^{-/-}$ mice versus wt mice by 48 h and remained elevated at 72 h indicating an early trigger of apoptotic signal in the TIM2$^{-/-}$ mice, almost a day ahead of the protein detection in the wt mice (Figs. 4A and 4B). The caspase-3 mRNA levels remained elevated above controls in both groups with a slight increase by 48 and 72 h in TIM2 kos. Cleaved caspase-3 protein levels gradually increased up to 72 h in the wt mice. High levels of cleaved caspase-3 were found in

FIG. 2. Changes in gene expression of inflammatory cytokines and chemokines in TIM2 ko mice after cisplatin administration. Quantitative RT-PCR data of mRNA levels normalized to GAPDH and fold change calculated over baseline levels of wt and $TIM2^{-/-}$ mice for nine genes known to play a role in inflammation over a period of 72 h following cisplatin administration (20 mg/kg, ip). Data are the average ± SEM. $*p < 0.05$ between wt and ko groups at the same time point. $\#p < 0.05$ within the groups (wt or ko) between 24 versus 48 h and 24 versus 72 h. $!p < 0.05$ within the groups (wt or ko) between 48 and 72 h and $\$p < 0.05$ with respective vehicle-treated control groups at 0 h. ($n = 5$/group/time point).
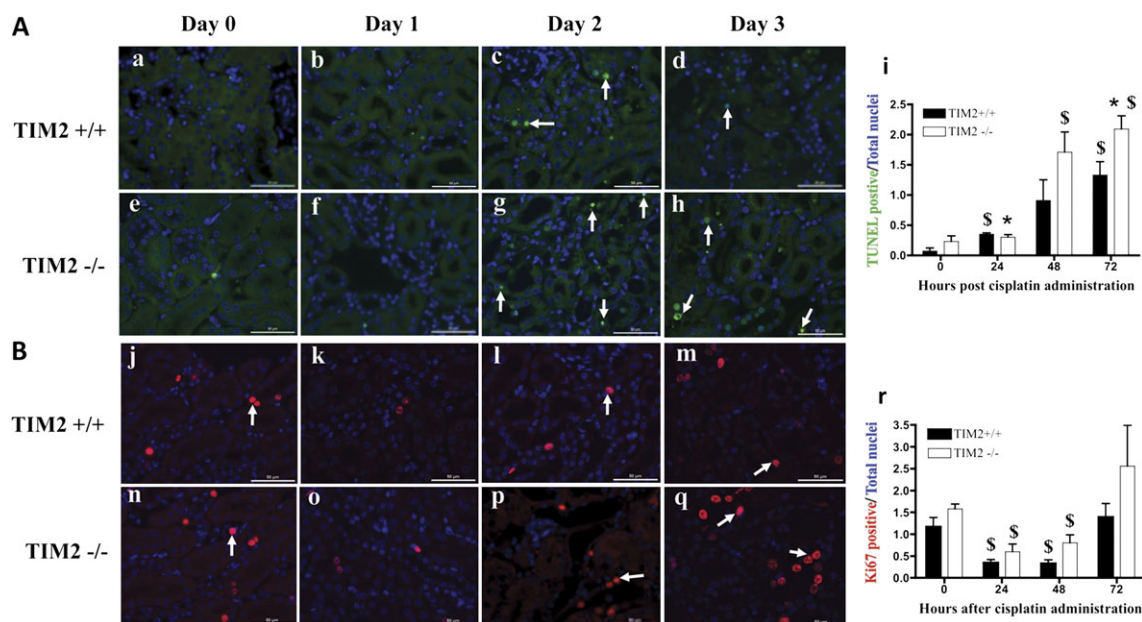
the $TIM2^{-/-}$ mice kidney at baseline, levels of which fluctuated after cisplatin administration (Fig. 4B). $TIM2^{-/-}$ mice expressed around sixfold higher levels of Hsp70 mRNA at the baseline and remained statistically significant during the course of the study. Baseline levels of Hsp70 protein were confirmed to be higher in the uninjured $TIM2^{-/-}$ mice compared with the wt mice. Similar to wt mice, Hsp70 levels increased following cisplatin injection up to 48 h in $TIM2^{-/-}$ mice as well. However, although the protein expression continued to increase by 72 h in the wt, Hsp70 protein levels decreased in the $TIM2^{-/-}$ mice by 72 h.

DISCUSSION

In an effort to understand the effect of TIM2 deficiency to kidney toxicity, we evaluated kidney injury, dysfunction, inflammation, and apoptosis following cisplatin administration in $TIM2^{-/-}$ and $TIM2^{+/+}$ mice. The $TIM2^{-/-}$ mice exhibited a significant elevation of BUN, SCr as renal dysfunctional parameters, and Kim-1 mRNA levels and proximal tubular necrosis as indicators of kidney injury resulting in higher mortality as compared with the $TIM2^{+/+}$ mice. A significant increase in inflammation characterized by increases in TNF-α, IL-1β, IL-6, and TGFβ along with greater proportion of renal

cells undergoing apoptosis because of an early onset of p53 and cleaved caspase-3 resulted in dramatic increase in cisplatin-induced nephrotoxicity in the TIM2-deficient mice as compared with wt mice.

The CD4+ Th cells play an important role in tissue injury and inflammation by phagocytic infiltration (Liu *et al.*, 2006; Singbartl *et al.*, 2005). The Th1 cellular response is associated most commonly with delayed-type hypersensitivity and intracellular pathogenic protection with the secretion of cytokines like RANTES, MIP-2α, and IFNγ, whereas Th2 subset of cells play a role in humoral and allergic responses and are chemoattracted mostly to ligands like IL-6 and TNF-α (Zhang *et al.*, 2000). Increased mRNA production of TNF-α and its receptors within resident kidney cells, triggering the production of inflammatory cytokines that cause renal injury (Pabla and Dong, 2008), has been proposed to be one of the major drivers of cisplatin nephrotoxicity. Our model of cisplatin nephrotoxicity in the wt mice elicited a similar response to previously established proinflammatory cytokine responses in the renal tissue following systemic injection of cisplatin (Ramesh and Reeves, 2002) where increased mRNA expression levels of TNF-α, IL-1β, TGFβ, RANTES, MIP-2α, MCP-1, and ICAM1 were detected. We also found significantly up-regulated levels of the Th2-associated cytokines (IL-6, IL-1β, and TNF-α) in the $TIM2^{-/-}$ by 72 h along with

FIG. 3. Effect of cisplatin on apoptosis and proliferation in TIM2 ko mice. Paraffin embedded kidney tissue sections of wt and TIM2$^{-/-}$ mice from day 0 through 3 following cisplatin administration were used to determine TUNEL- and Ki67-positive staining cells. Representative figures of TUNEL (a–d) and Ki67 (j–m) staining in renal tissues of wt mice and TUNEL (e–h) and Ki67 (n–q) staining in renal tissues of TIM2$^{-/-}$ mice are shown. The numbers of positive staining TUNEL and Ki67 nuclei are represented graphically in (i) and (r), respectively. Data are the average ± SEM. *$p < 0.05$ between wt and ko groups at the same time point and $$p < 0.05$ with respective vehicle-treated control groups at 0 h ($n = 5$/group/time point) (bar = 50 μm).

TGFβ. It was interesting to note significant down-regulated expression levels of Th1-associated cytokines (RANTES) in TIM2$^{-/-}$ mice compared with wt mice. Although MCP-1 appeared to be slightly down-regulated in the TIM2$^{-/-}$, ICAM1 transcripts measured in wt and TIM2$^{-/-}$ were almost equally elevated by 72 h and were not significantly different in expression levels. The up-regulation of MCP-1 and ICAM1 is largely governed by activation of NF-κB because of production of reactive oxygen species (ROS) that are generated as a result of cisplatin-induced kidney toxicity (Lee et al., 2006; Ramesh and Reeves, 2004). It appears that modulating or deleting TIM2 possibly enhances the Th2 while suppressing the Th1 response following cisplatin administration. Experiments using TIM2$^{-/-}$ mice to study airway inflammation (Rennert et al., 2006) found similar results with consistently up-regulated levels of Th2-associated cytokines, such as IL-4, IL-5, IL-6, and IL-10. Our study further strengthens the hypothesis that TIM2 is a critical negative regulator of Th2 T-cell responses, and the TIM2-deficient phenotype is because of disregulated CD4+ T-cell activity and the robust over expression of Th2 cytokines.

Following cisplatin administration, several studies have noted apoptosis by the intrinsic, extrinsic, and/or the endoplasmic reticulum stress–mediated pathways (Pabla and Dong, 2008) in the kidney. The extrinsic pathway is triggered from the plasma membrane where the death receptors like Fas and TNF-α receptor bind to ligands leading to the activation of caspase-8 and ultimately ending in apoptosis. Fas and caspase-8 showed no statistically significant differences between the two groups during the course of this study (data not shown) but were significantly up-regulated (around 4.3-fold and 2.2-fold, respectively, by 72 h) following injury in both groups.

The intrinsic pathway involves the activation of proapoptotic molecules like Bcl2, which puncture the mitochondrial membrane releasing apoptotic factors. A number of proapoptotic factors belonging to the Bcl2 family of proteins like PUMAα are believed to be activated downstream of the tumor suppressor protein p53 (Jiang et al., 2006). Following cisplatin administration, previous studies have noted elevated expression of cell cycle inhibitory protein p21, which is known to cause cell cycle interruption, and its induction is partly controlled by p53-dependent mechanisms (Price, Megyesi, et al., 2004). Clearly, there appears to be an overlap between apoptosis and cell repair/proliferation. In support, proliferative data from the TIM2$^{-/-}$ group appear to be significantly higher by 48 h corresponding to an increase in p53 levels around the same time point in the group. Studies have shown p53-dependent mechanisms participate in reparative functions after DNA damage (Price, Safirstein, et al., 2004). It would be interesting to investigate the expression of p21 protein as it has been strongly associated with cell survival than with cell death and could possibly aid better in understanding the course of increased injury in the mice lacking TIM2. Some researchers have proposed that the generation of ROS can lead to increased stabilization and phosphorylation of the proapoptotic p53 molecule through protein kinases that detect DNA damage,

A **Gene expression**

B **Protein expression**
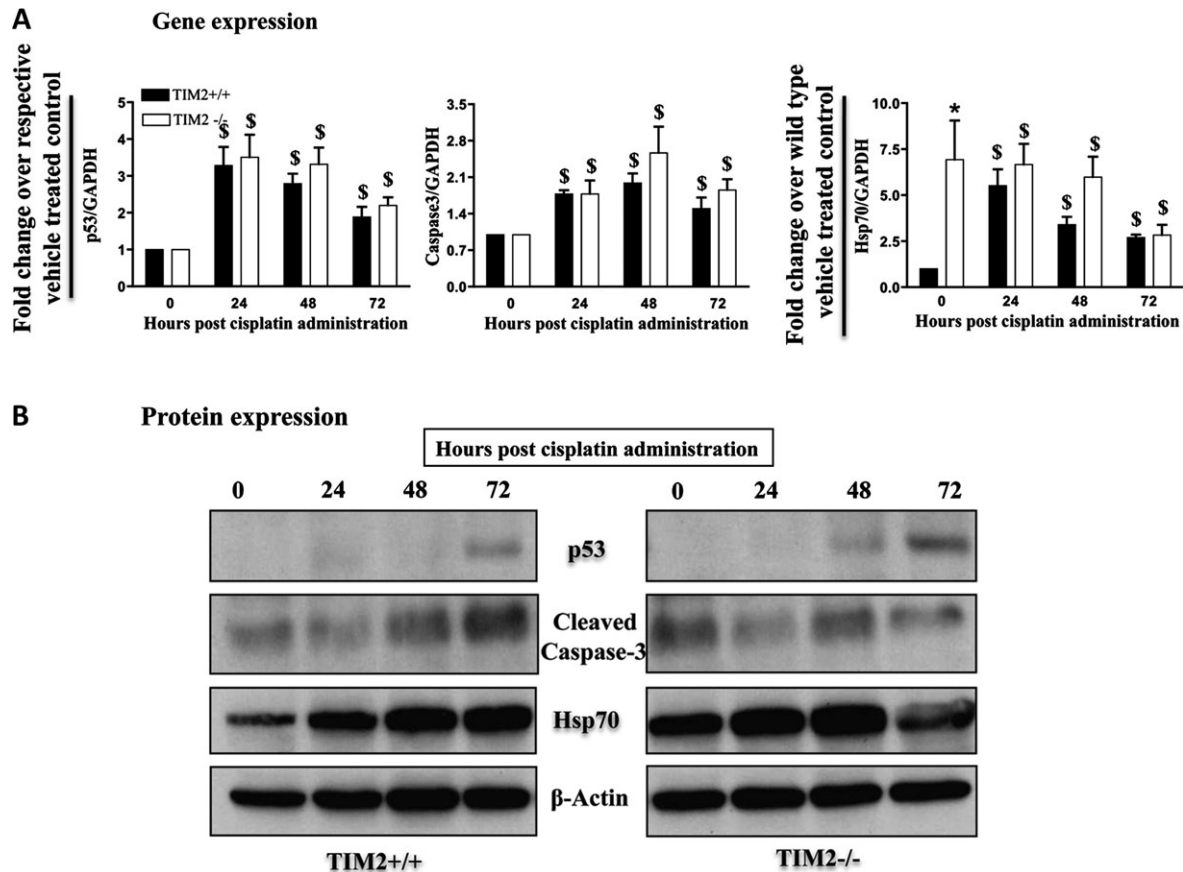
FIG. 4. Apoptotic molecular expression differences in TIM2 ko mice after cisplatin injection. (A) Graphical representation of quantitative RT-PCR data of p53, caspase-3, and Hsp70 mRNA levels normalized to GAPDH and fold change calculated over respective vehicle-treated baseline control groups. Data are the average ± SEM. *$p < 0.05$ between wt and ko groups at the same time point and \$$p < 0.05$ with respective vehicle-treated control groups at 0 h. ($n = 5$/group/time point). (B) Representative Western blots of p53, 17 kDa band of cleaved caspase-3 and Hsp70 with corresponding β-actin as loading controls. ($n = 3$ pooled samples/group/time point).

leading to the activation of caspase-3, and ultimately apoptosis in renal tubular cells (Jiang and Dong, 2008; Yano *et al.*, 2007).

The molecular chaperone Hsp70 is involved in preventing protein aggregation, refolding, and maturation and is constitutively expressed in the kidney (Yokoo and Kitamura, 1997). Hsp70 gets activated in response to cellular stress, which then averts apoptosis by preventing cleavage of the DNA repair enzyme PARP by caspase-3 (Mosser *et al.*, 1997). Thus, Hsp70 levels increase proportionally to caspase-3 levels and protect the cells from monocyte- and/or TNF-mediated cell death (Mosser *et al.*, 1997). In the wt and TIM2$^{-/-}$ mice, the Hsp70 and caspase-3 levels gradually increased as the injury progressed. By 72 h, the Hsp70 expression decreased in the TIM2$^{-/-}$ mice, whereas it continued to increase in the wt mice. Investigations conducted by Yokoo and Kitamura (1997), on the effect of IL-1β in the kidney, found that Hsp70 is down-regulated by IL-1β in glomeruli, thereby subjecting these cells to oxidant-induced apoptosis. The significant increase in IL-1β levels in our study correlates with the significant drop in Hsp70

protein levels by 72 h in the TIM2$^{-/-}$ mice, which could possibly account for the early activation of p53 by 48 h and the onset of apoptosis in these mice. A number of studies have also shown that preconditioned induction of Hsp70 is cytoprotective to the kidney and is capable of conferring resistance to stress and cytokine-induced cell death while preventing apoptotic cell death because of ischemia/reperfusion or cisplatin (Yokoo and Kitamura, 1997; Zhang *et al.*, 2008; Zhou *et al.*, 2003). The higher endogenous Hsp70 gene (c. sixfold) and protein expression that we detected in TIM2$^{-/-}$ mice may probably be in response to the elevated cleaved caspase-3 levels at baseline thereby averting apoptotic cell death in the kidney. However, further downstream mechanisms that inhibit Hsp70's protective response and the mechanisms resulting in increased expression of caspase-3 in the TIM2$^{-/-}$ mice remain to be investigated.

Taking into consideration that of the four most commonly studied TIM genes, TIM1 and TIM2 share the closest homology, one may propose that they might share some functional similarities as well. Both molecules are expressed on

Th2 cells (Umetsu *et al.*, 2005), and recently, TIM1 has been noted to act as a phosphatidyl serine receptor signaling for apoptosis and phagocytosis (Ichimura *et al.*, 2008). It is possible that TIM2 plays a similar role as TIM1 by protecting the mouse kidney upon tubular injury. Although there was a significant increase in Kim-1 mRNA levels following kidney injury, there was no increase in the TIM2 mRNA levels following cisplatin-induced toxicity in the wt mice (data not shown). Furthermore, TIM2 ko mice did not exhibit higher Kim-1 mRNA in spite of having greater proximal tubular damage (Fig. 1C). One possible explanation is that the renal injury is so severe in the TIM2 ko mice that a marked increase in Kim-1 mRNA and protein synthesis is not instigated in the dying cells of the kidney. This is consistent with previous studies using rat models of bilateral ischemia/reperfusion injury (Vaidya *et al.*, 2006), high-dose cisplatin administration (Vaidya *et al.*, 2006), or high (fatal)-dose mercuric chloride administration (Zhou *et al.*, 2008) where a saturation or lack of dose-dependent increase or marked decrease in urinary Kim-1 levels, respectively, was reported.

Study of the TIM2$^{-/-}$ phenotype upon cisplatin nephrotoxicity has provided insights into inflammatory and immune responses mediated by both the Th1 and the Th2 subsets of cells, which may help in understanding the role played by other TIMs that are expressed on Th cells. Whether deficiency of TIM1 in mice exhibits a similar susceptibility to nephrotoxicity remains to be investigated. A further study of interest would be to try and understand how expression of H-ferritin is modulated in the absence of TIM2 in the kidney. As H-ferritin primarily functions to sequester iron in order to buffer levels of ROS (Chen *et al.*, 2005), it might be proposed to affect ROS in TIM2$^{-/-}$ mice.

Thus, we report that the TIM2-deficient mice exhibit a dramatic susceptibility to kidney toxicity because of the Th2-mediated inflammatory cytokine damages with higher rate of apoptosis and necrosis, resulting in increased cell death. TIM family members, including TIM2, may play a critical role in preventing inflammation and apoptosis following kidney damage caused by nephrotoxicants.

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