Tim-1 promotes cisplatin nephrotoxicity

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CISPLATIN AND OTHER PLATINUM derivatives are front-line chemotherapeutic agents in the treatment of solid tumors, including ovarian, head and neck, and testicular germ cell tumors (1, 31). However, a major dose-limiting effect of cisplatin is its propensity to cause drug-induced AKI. Cisplatin’s nephrotoxic effects have been modelled in vitro and in vivo. In vitro, cisplatin induces DNA damage (11, 27), mitochondrial dysfunction (29), formation of reactive oxygen species (16), caspase activation (9), and necrotic and apoptotic cell death (19). In addition to these direct effects in vitro, in vivo studies showed significant injury to the renal microvasculature and a key role for leukocytes, including T cells, in cisplatin-induced nephrotoxicity. In particular, experimental in vivo studies defined a pathogenic role for both CD4+ and CD8+ T cell subsets in cisplatin nephrotoxicity (13).

The T cell immunoglobulin mucin (Tim) family consists of eight genes in mice (Tim1–8) and three in humans (TIM-1, -3, and -4) (4). Tim family members are cell surface glycoproteins sharing common motifs, including an IgV domain, a mucin-like domain, a transmembrane domain, and intracellular tail. Tim-1, expressed by activated T cells (4), has the capacity to act as a co-stimulatory signal and may further activate T cells in a TCR-independent manner (15). In injured kidneys, Tim-1 is expressed by tubular epithelial cells after ischemic or toxic injury (5, 8) and is known as kidney injury molecule-1 (Kim-1). The Kim-1 ectodomain is stable in urine, can be detected in the urine of humans with acute kidney injury, and is a potential biomarker (5), and functionally, Kim-1 allows tubular cells to phagocytose apoptotic debris via recognizing phosphatididyserine (7).

Given that Tim-1 can activate T cells, the current studies were conducted to test the hypothesis that inhibiting Tim-1 would attenuate cisplatin-induced acute kidney injury. To achieve this, we used an antagonistic anti-Tim-1 antibody, RMT1–10, in studies involving wild-type (WT) C57BL/6 mice and in Rag1−/− mice that lack adaptive immunity. This RMT1–10 antibody does not block the binding of apoptotic cells to T cells (10), or affect the more recently described Tim1–/−/LMIR5 interaction that assists neutrophil recruitment (35), so there is no current evidence that this antibody affects tubular cell Kim-1 function. Our results showed that anti-Tim-1 treatment reduces functional and histological renal damage after cisplatin injection, provided the mice have an intact adaptive immune system, with significant alterations in T cell activation and cytokine production. This resulted in decreased renal leukocyte recruitment, proinflammatory cytokine and chemokine mRNA expression, NF-κB activation, and reduced tubular cell apoptosis.

MATERIALS AND METHODS

Experimental design. Male mice were used for experiments. C57BL/6 mice were from Monash University Animal Services (Melbourne, Australia) and Rag1−/− mice (C57BL/6 background) were bred at the Monash Medical Centre Specific Pathogen Free Facility. Studies were approved by Monash University’s Animal Ethics Committee in accordance with the Australian National Health and Medical Research Council guidelines for animal experimentation. For experiments, mice were 8–10 wk old (25–30 g). Cisplatin (Sigma, St. Louis, MO) was freshly prepared in saline (1 mg/ml) and injected intraperitoneally at 20 mg/kg (for C57BL/6 mice) and 20 or 30 mg/kg (for Rag1−/− mice) 24 h before cisplatin injection. The doses of cisplatin used in these experiments did not result in any mortality at either the day 1 or 3 experimental timepoint.

An antagonistic anti-mouse Tim-1 monoclonal antibody (RMT1–10; rat IgG2a, 0.25 mg), specific for the IgV domain of Tim-1 and described previously (34), or nonimmune rat IgG (prepared by protein G purification of rat serum) was injected intraperitoneally 3 h before and 24 h after cisplatin injection. Mice were humanely killed on day...
cisplatin and assessed at 1 day after cisplatin injection. Antibodies for flow cytometry were APC/Cy7-anti-CD4, APC-anti-CD8, FITC-anti-CD44, FITC-anti-CD25, FITC-anti-IL-17A, and PE-anti-IFN-γ (all BD Biosciences, North Ryde, Australia) and an anti-mouse PE-Cy7 staining set (eBioscience). T cell activation was assessed by analysis of CD44 expression on splenic CD4+ cells (12). T cell apoptosis was assessed by FITC-labeled annexin-V staining (Roche Diagnostics) with propidium iodide (1 μg/ml; Calbiochem, San Diego, CA), as described previously (21). Intracellular cytokine staining was performed on cells 1 day after cisplatin injection. Splenocytes were cultured with 50 ng/ml PMA and 750 ng/ml ionomycin for 4 h and then 10 μg/ml brefeldin A (all Sigma). Cells were incubated with anti-CD4 and anti-CD8 antibodies, fixed, and permeabilized and then stained with anti-cytokine antibodies. For all markers, isotype-matched irrelevant monoclonal antibodies were used as controls.

RESULTS

Anti-Tim-1 antibodies attenuate injury in cisplatin nephrotoxicity. One day after cisplatin injection, kidneys from control antibody (rat IgG)-treated mice showed some tubular dilation and a loss of brush border in the outer medulla (Fig. 1A). By day 3, rat IgG-treated mice had developed severe tubular injury with cast formation, sloughing of tubular epithelial cells, tubular dilation, and significant injury with necrosis (Fig. 1, C and E). The anti-Tim-1 antibody (RMT1–10) protected kidneys from cisplatin nephrotoxicity. On day 1, renal morphology in anti-Tim-1 antibody-treated mice was normal, with preserved brush-border membranes and no tubular epithelial cell loss (Fig. 1B). By day 3, although renal histology had become abnormal, less histological damage was present compared with cisplatin-injected, rat IgG-treated mice (Fig. 1D). Formal semiquantitative injury scoring showed a trend toward a reduced necrosis score in anti-Tim-1 antibody-treated mice on day 1 (rat IgG 1.0 ± 0.3, anti-Tim-1 0.3 ± 0.3) and by day 3 (rat IgG 3.5 ± 0.2, anti-Tim-1 1.2 ± 0.3), mice were significantly protected (Fig. 1E). Anti-Tim-1 antibodies protected mice from functional injury, as the cisplatin-induced rise in serum urea at day 3 (rat IgG 50.3 ± 6.7, anti-Tim-1 30.1 ± 3.2 mmol/l) was significantly less in anti-Tim-1 antibody-treated mice than in rat IgG-injected mice (Fig. 1F).

Anti-Tim-1 antibodies limit renal leukocyte accumulation in cisplatin nephrotoxicity. Interstitial infiltrates of CD4+ cells (Fig. 2A) and CD8+ cells (Fig. 2B) peaked on day 1. These infiltrates were markedly diminished in mice given anti-Tim-1 antibody (CD4+: rat IgG 93 ± 14, anti-Tim-1 18 ± 5; CD8: rat IgG 58 ± 4 and anti-Tim-1 25 ± 4 c/10 hpf). When overall numbers had declined by day 3, differences between groups were not significant. Tubulointerstitial neutrophil (Fig. 2C) and macrophage (Fig. 2D) infiltrates followed a different time course, with the greatest increase in cisplatin-injected, rat IgG-treated mice at day 3. In anti-Tim-1 antibody-treated mice, neutrophil and macrophage numbers were not significantly reduced at day 1 (neutrophils: rat IgG 7 ± 3, anti-Tim-1 4 ± 2; macrophages: rat IgG 27 ± 7, anti-Tim-1 14 ± 6 c/10 hpf), but at day 3, the time of peak infiltrate in control-treated mice, renal neutrophil and macrophage numbers were reduced in anti-Tim-1 antibody-treated mice (neutrophils: rat IgG 56 ± 8
and anti-Tim-1 16 ± 3, macrophages; rIgG 52 ± 2, anti-Tim-1 37 ± 4 c/10 hpf).

Anti-Tim-1 antibodies inhibit cisplatin-induced NF-κB activation and apoptosis. Intrarenal NF-κB activation is a feature of cisplatin nephrotoxicity (30). There was a progressive increase in tubular cells positive for phosho NF-κB p65, a marker of NF-κB activation, in cisplatin-injected, rat IgG-treated mice. Anti-Tim-1 antibodies resulted in fewer cells expressing phosho NF-κB p65, at both time points (day 1: rat IgG 1.2 ± 0.1, anti-Tim-1 0.2 ± 0.2 c/10 hpf; day 3: rat IgG 29.9 ± 4.9, anti-Tim-1 13.1 ± 3.9 c/10 hpf; Fig. 3, A–C).

Apoptosis represents a key event after cisplatin injection, correlating with the degree of injury (17). Cells expressing cleaved caspase-3, a marker of apoptosis, were not present at day 1 but by day 3 were present in cisplatin-injected, rat IgG-treated mice, but significantly reduced in mice treated with anti-Tim-1 antibodies (rat IgG 15.3 ± 3.1, anti-Tim-1 5.4 ± 2.2 c/10 hpf; Fig. 3, D–F).

**Tim-1 and Tim-4 expression in the kidney after cisplatin treatment.** Tim-1 (also known as Kim-1) indicates renal injury. By day 1, Tim-1 was present in the proximal tubules in the apical region of outer medulla (Fig. 4, A and B). On day 3, Tim-1 immunostaining was present in the apical part of many of the proximal tubular cells with more diffuse cytoplasmic staining in the outer medulla (Fig. 4, C and D). Intrarenal Tim-1 mRNA expression after cisplatin injection was increased on day 1 and more so by day 3, but Tim-1 mRNA was reduced in mice given anti-Tim-1 antibodies (rat IgG 108 ± 38, anti-Tim-1 38 ± 7-fold increase over baseline). The renal expression of Tim-4, a natural ligand for Tim-1, present on macrophages and dendritic cells (3, 18) was assessed. Tim-4 immunostaining was decreased in anti-Tim-1 antibody-treated mice (rat IgG 17 ± 2, anti-Tim-1 9 ± 3 c/10 hpf) on day 3 (Fig. 5, A–C). The relative decrease in Tim-4 was similar to that observed in macrophage numbers in Fig. 2D.

*Inhibiting Tim-1 attenuates cisplatin-induced interstitial inflammatory mediators.* The expression of several cytokines and chemokines is increased in kidneys in cisplatin nephrotoxicity and contributes to renal injury (23–25). Intrarenal mRNA expression of TNF, a cytokine with a pathogenic role in cisplatin nephrotoxicity (24), was decreased (rat IgG 5.8 ± 1.2, anti-Tim-1 2.1 ± 0.5-fold increase over baseline; Fig. 6A). This reduction within the kidney was not due to changes in serum TNF levels after anti-Tim-1 treatment. Serum TNF on day 1 was not detected in any mouse (limit of ELISA’s detection).

**Fig. 1. Effects of anti-T cell immunoglobulin mucin 1 (Tim-1) antibodies in cisplatin nephrotoxicity on kidney morphology and renal function.** A: 1 day after cisplatin injection, kidneys from rat IgG-injected mice show some tubular dilation (black arrows) in the outer medulla and loss of the tubular brush border (white arrow), but anti-Tim-1-treated kidneys (B) show near normal morphology with well-preserved brush borders and no loss of tubular epithelial cells. C: by day 3, mice receiving cisplatin and rat IgG show substantial tubular epithelial cell loss, tubular dilation, and cast formation (arrowhead). D: kidneys from anti-Tim-1 antibodies-treated mice were less affected (×200 magnification). E: semiquantitative scoring of tubular necrosis in the outer medulla confirmed protection in anti-Tim-1-treated mice. F: anti-Tim-1-treated mice developed a lesser rise in serum urea. Dotted lines represent mean values from saline-injected mice not receiving cisplatin. *P < 0.05 vs. rat IgG on day 3. †P < 0.05 vs. rat IgG and anti-Tim-1 antibodies on day 1; means ± SE, unpaired t-test.

**Fig. 2. Tim-1 mediates interstitial leukocyte accumulation in the outer medulla after cisplatin injection.** Numbers of CD4+ (A) and CD8+ (B) T cells peaked at day 1 and were reduced in anti-Tim-1 antibody-treated mice compared with rat IgG-treated mice (c/10 hpf, cells per 10 high-power fields). Neutrophil (C) and macrophage (D) infiltrates were higher at day 3 but compared with rat IgG-injected mice, anti-Tim-1-treated mice had reduced neutrophils and macrophages. Dotted lines represent mean values from saline-injected mice without cisplatin. *P < 0.05 and **P < 0.01 vs. cell numbers in rat IgG-treated mice on the same day. †P < 0.05 vs. cell numbers in rat IgG- and anti-Tim-1 antibody-treated mice on day 1, and ¶P < 0.05 for CD4+ cells vs. both groups of mice on day 3; means ± SE, unpaired t-test.
detection: 20 pg/ml). On day 3, serum TNF levels were increased to a similar degree in both groups (rat IgG 88.5 ± 0.4 vs. anti-Tim-1 77.6 ± 0.3 pg/ml). Intrarenal expression of other proinflammatory cytokines, IL-1β (rat IgG 10.5 ± 2.1, anti-Tim-1 4.8 ± 1.4), IFN-γ (rat IgG 1.8 ± 0.6, anti-Tim-1 0.6 ± 0.2), and IL-6 (rat IgG 1.1 ± 0.5, anti-Tim-1 0.6 ± 0.2), was inhibited by anti-Tim-1 antibody treatment (Fig. 6, B–D), but the protective cytokine IL-10 was not reduced (rat IgG 2.1 ± 0.6, anti-Tim-1 3.9 ± 2.1; Fig. 6E). Anti-Tim-1 antibodies limited the increase in several chemokines, including, in particular, neutrophil and T cell chemotactantants (Fig. 7, A and B). In addition, the increase in mRNA for ICAM-1, an adhesion molecule important in leukocyte recruitment, was attenuated by anti-Tim-1 antibodies (rat IgG 4.3 ± 0.7, anti-Tim-1 2.5 ± 0.5; Fig. 7C).

T cell activation and IFN-γ production are reduced by anti-Tim-1 antibodies. Activation, apoptosis, and cytokine production on splenic CD4+ and CD8+ T cells 24 h after cisplatin with or without anti-Tim-1 antibody treatment were examined by flow cytometric analysis (Fig. 8). CD4+ and CD8+ T cell activation (CD44) (CD4: rat IgG 7.9 ± 0.2, anti-Tim-1 4.8 ± 0.2; CD8: rat IgG 5.1 ± 0.4, anti-Tim-1 2.3 ± 0.1%) as well as CD4+ and CD8+ cell apoptosis (CD4: rat IgG 15.3 ± 1.4, anti-Tim-1 10.4 ± 0.6; CD8: rat IgG 2.5 ± 0.2

Fig. 3. Anti-Tim-1 antibodies limit NF-κB activation and apoptosis in cisplatin-induced acute kidney injury. Immunohistochemical detection of nuclear phospho NF-κB p65 and apoptotic cells in the outer medulla. On day 3 renal tubular cells showing nuclear staining for phospho NF-κB p65 (arrows) in rat IgG-treated mice (A) were increased compared with those in mice given anti-Tim-1 antibodies (B), confirmed by counting of phospho NF-κB p65+ cells on days 1 and 3 (C). On day 3, there were more cleaved caspase-3+ cells (arrows) in rat IgG-treated mice (D) compared with anti-Tim-1-treated mice (E, F). *P < 0.05 vs. those cell numbers in rat IgG- and anti-Tim-1 antibody-treated mice on day 1; means ± SE, unpaired t-test.

Fig. 4. Kidney injury molecule-1 (Kim-1) in kidneys in rat IgG- and anti-Tim-1 antibody-treated mice after cisplatin injection. A–B: on day 1, Kim-1+ tubules are present in the proximal tubules in the apical region of outer medulla in both groups. C–D: on day 3, Kim-1 is detected in many tubules in the injured outer medulla, localized to the apical side of the epithelium with some diffuse cytoplasmic staining. Anti-Tim-1 antibody-treated mice showed fewer Kim-1+ tubules than rat IgG-injected mice (high power ×400). E: Kim-1 renal mRNA expression in anti-Tim-1 antibody-treated mice was reduced compared with that in rat IgG-treated mice. *P < 0.05 vs. rat IgG-treated mice on day 3. †P < 0.05 vs. rat IgG-treated mice on day 1; means ± SE, unpaired t-test.

Fig. 5. Immunohistochemical staining of Tim-4 expression in kidneys in rat IgG- and anti-Tim-1 antibody-treated mice after cisplatin injection. A: interstitial Tim-4-positive cells in the outer medulla of rat IgG-treated mice were increased on day 3 compared with those in anti-Tim-1-treated mice (B–C). High power ×400. *P < 0.05 vs. rat IgG-treated mice; means ± SE, unpaired t-test.
and anti-Tim-1 1.7 ± 0.3%) were diminished in anti-Tim-1 antibody-treated mice. Anti-Tim-1 treatment reduced the frequency of IFN-γ producing CD4+ and CD8+ T cells (CD4: rat IgG 4.1 ± 0.3, anti-Tim-1 2.8 ± 0.5; CD8: rat IgG 22.4 ± 3.2, anti-Tim-1 9.1 ± 2.1%), but proportions of IL-17A-producing cells were unchanged (CD4: rIgG 1.6 ± 0.2, anti-Tim-1 1.7 ± 0.1; CD8: rat IgG 1.1 ± 0.2, anti-Tim-1 1.6 ± 0.2%). There were no differences in proportions of regulatory cells (CD25+foxp3+CD4+) between the two groups (rat IgG 2.3 ± 0.2 vs. anti-Tim-1 2.1 ± 0.1% of CD4+ cells).

**Renal injury in Rag1−/− mice is not modified by anti-Tim-1 antibodies.** Tim-1 had significant effects on T cells that are important in this model of injury (13). At the dose of cisplatin (20 mg/kg) used in these studies, we confirmed that injury was substantially dependent on adaptive immunity by injecting (20 mg/kg) used in these studies, we confirmed that injury was substantially dependent on adaptive immunity by injecting anti-Tim-1 antibodies. Anti-Tim-1 antibodies affect early immune responses, including activation, apoptosis, and cytokine production on CD4+ and CD8+ T-cells, and anti-Tim-1 antibodies ameliorate the cisplatin-induced upregulation of proinflammatory cytokines, chemokines, and ICAM-1 expression, resulting in decreased infiltration of leukocytes into kidney, with less NF-κB activation and less apoptosis, and the protection from cisplatin nephrotoxicity is mediated predominantly through cells of the adaptive immune system, most likely T cells.

T cells, probably acting innately, can direct inflammatory injury in cisplatin nephrotoxicity and other forms of acute injury (Fig. 9).
kidney injury (2, 13, 22), evidence supported by the current studies. More importantly, when considering the role of Tim-1, the current studies showed that anti-Tim antibodies could modify the T cell inflammatory phenotype at an early stage. Recent evidence suggested that Tim-1 can bypass the TCR to further activate T cells (15), and it is likely that this effect is important in the current studies. No protection was afforded by anti-Tim-1 antibodies in \( \text{Rag1}^{-/-} \) mice, showing that Tim-1's effects on CD4\(^{+}\) cells (and potentially CD8\(^{+}\) cells) promote cisplatin nephrotoxicity.

Different anti-Tim-1 antibodies have different effects on Tim-1. A high-affinity anti-Tim-1 antibody (3B3) enhances the expansion, activation, and survival of T cells with increased IFN-\(\gamma\) and IL-17A production, whereas the low-affinity antibody used in the current studies (RMT1–10) has inhibitory effects on reducing T cell activation are played out in the reduced downstream inflammatory events seen within the kidney itself. T cells are well-known to activate macrophages, and, in addition to the current studies, evidence from Tim-1 inhibition in hepatic ischemia-reperfusion injury suggests that T cell-dependent macrophage activation is interrupted by anti-Tim-1 antibodies (33). These studies also show less apoptosis and hepatocellular damage after RMT1–10 administration (33). It is possible that T cell Tim-1/macrophiage Tim-4 interactions within the kidney contribute to cisplatin nephrotoxicity, as Tim-1 can affect macrophage activation (6). In the interstitium, there were fewer Tim-4\(^{+}\) cells, consistent with the diminished macrophage influx after Tim-1 inhibition.

There are other potential mechanisms by which Tim-1 may participate in cisplatin nephrotoxicity, most of which, in the context of our studies, can be effectively discounted given the nature of the anti-Tim-1 RMT1–10 antibody and our use of \( \text{Rag1}^{-/-} \) mice. Intrarenal Kim-1 plays a central role in the removal of dead cells and other debris from the tubular lumen with Kim-1-expressing epithelial cells having highly phagocytic properties in vivo (14). It is likely that Tim-1 acts locally, either on damaged tubular cells or on infiltrating leukocytes, as a phosphatidylserine receptor to mediate clearance of apoptotic cells. However, in the current studies, the effects of Tim-1 inhibition are unlikely to be directly related to local Tim-1 (Kim-1) effects, as \( \text{Rag1}^{-/-} \) mice were not protected by anti-Tim-1 antibodies, and the RMT1–10 antibody does not inhibit Tim-1 binding to apoptotic cells (10). Tim-1-dependent NKT cell activation is also mediated through binding of apoptotic cells (10). While it is impossible to exclude effects on B cells (as we used \( \text{Rag1}^{-/-} \) mice) and Tim-1 is present in some B cells at low levels (28), studies using nude mice, CD4\(^{+}\)/CD8\(^{+}\) cells, and Tim-1 is present in some B cells at low levels (28), studies using nude mice, CD4\(^{-/-}\) cell killers.

![Fig. 8. Anti-Tim-1 antibodies limit early CD4\(^{+}\) and CD8\(^{+}\) T cell responses.](http://ajprenal.physiology.org/)

One day after injection of cisplatin, splenic CD4(A) and CD8(B) T cell activation, apoptosis, and cytokine production were assessed by flow cytometric analysis of CD44 expression, annexin-V binding, and intracellular IFN-\(\gamma\) and IL-17A production. CD4\(^{+}\) and CD8\(^{+}\) cell activation, apoptosis, and IFN-\(\gamma\) production were reduced by anti-Tim-1 antibodies. *\( P < 0.05\), **\( P < 0.01\), and ***\( P < 0.001\) vs. rat IgG-treated mice; means ± SE, unpaired t-test.

![Fig. 9. Effects of anti-Tim-1 antibodies on histological evaluation (A and B: photomicrographs in the outer medulla; C: scoring of injury) and serum urea (D) in \( \text{Rag1}^{-/-} \) mice 3 days after acute kidney injury induced by 30 mg/kg of cisplatin. Both serum urea and histological injury were similar in the 2 groups. High power ×400.](http://ajprenal.physiology.org/)

Dotted lines represent mean values from saline-treated mice without cisplatin. Data are means ± SE.
and CD8−/− mice, showed a key role for T cells in cisplatin nephrotoxicity (2, 22).

In conclusion, in cisplatin nephrotoxicity, Tim-1 signaling helps activate T cells, which mediate acute kidney injury. Inhibiting Tim-1 ameliorates acute kidney injury by inhibiting T cell activation, thereby inhibiting leukocyte recruitment and inflammatory injury. The current studies define a new mechanism by which Tim-1 signaling affects T cell-directed proinflammatory cascades in acute kidney injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


