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Early and late suppression of Toll-like receptor 4 signaling induces distinct changes in a nephrotoxicant-induced mouse model of acute kidney injury

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Abstract: **Background**: Many essential genes in different signaling pathways are significantly expressed in a stress- and time-dependent manner after acute kidney injury (AKI). Despite their traditional role in driving inflammation, TLR activation is increasingly recognized to promote renal epithelial repair by inducing the secretion of specific interleukins and growth factors. **Objectives**: This study explored the effects of TLR4 inhibition at early and late time points of cisplatin-mediated AKI. **Methods**: A mouse kidney injury model was developed using a single dose of cisplatin (10 mg/kg) and treated with the TLR4 inhibitor TAK-242 at either the initial or a later time point after cisplatin induction. Kidney tissues were analyzed for histopathological and gene expression changes by HE staining and qRT-PCR, respectively. Renal functional analysis was performed by estimating serum creatinine levels in the control and all treated groups. **Results**: Elevated biochemical markers and serum creatinine levels correlated with histological findings of tubular damage in all groups. Compared with the late intervention group, we observed prominent tubular cell injury in the cisplatin AKI model and the TAK242-early groups. Cisplatin significantly increased TLR4 expression in the AKI model, which was abrogated in both TAK242-treated AKI groups. Compared to the TAK242-early group, the late intervention group showed upregulation of *Sod1* ($p \leq 0.05$), *Hsp25* ($p \leq 0.05$), *Nf-κb*, *Fzd8* and *c-Met* genes and downregulation of *Il22* ($p \leq 0.001$), *Lcn2*, and *hsp70* gene expression. Other genes, such as *Hgf1*, *Wnt4* and *Lc3 II*, showed no notable difference between the two intervention groups. **Conclusion**: The TLR4 pathway may be involved in immediate cell survival mechanisms after injury, as massive necrosis and elevated serum creatinine were observed following early inhibition of the TLR4 pathway, whereas late inhibition preserved tissue architecture and function. Understanding the optimal timing of TLR inhibition to achieve significant tissue protection can help underscore the role of this pathway in AKI repair and its associated pathological processes.

Keywords: Acute kidney injury; Gene expression; Renal repair; Toll-like receptors; TLR4 inhibition

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INTRODUCTION

Acute kidney injury (AKI) is a clinical state manifested by a rapid deterioration in kidney functional capacity (Kellum *et al*., 2021). Critically ill AKI patients show a 50–70% mortality rate. On the other hand, surviving patients represent progressive renal complications that ultimately lead to chronic form and end-stage renal failure (Kumar *et al*., 2014). In response to local injury, kidney epithelial and endothelial cells are damaged, leading to loss of the brush border typical of the proximal tubular epithelium, sloughing of tubular epithelial cells into the lumen, infiltration of inflammatory cells, and the appearance of Tamm-Horsfall protein-rich casts in the lumen and urine (Li *et al*., 2024).

Although kidneys have limited regenerative capacity, there is growing consensus that the surviving resident cells within the injured renal tubular epithelium may repair tubular damage in rodent and human kidneys to some extent (Stamellou *et al.*, 2021). The cellular mechanisms of this repair process involve dedifferentiation of surviving tubular epithelial cells, migration, proliferation and finally redifferentiation to replace damaged cells (Andrianova *et al*., 2019) In this context, Up-regulated expression of several cell signaling molecules, including cell cycle-associated kinases, cell surface receptors, cytokines signaling pathways and re-expression of some early renal developmental genes, has been observed in response to renal damage which reflects intricate interplay among several signaling molecules (Zhou *et al*., 2016; Siew *et al*., 2011; Chen *et al*., 2026). Similarly, it has been observed that during tubular cell damage, several inflammatory cell populations assemble and interact with renal tubular cells to initiate repair. However, prolonged inflammation leads to greater damage and eventually results in renal fibrosis (Huang *et al*., 2026).

Toll-like receptors (TLRs) are members of the pattern-recognition receptor (PRR) family and play significant roles in innate immunity. These receptors sense pathogen-associated molecular patterns (PAMPs) expressed on the surfaces of foreign antigens, as well as endogenous damage-associated molecular patterns (DAMPs) released

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by injured cells (Chen *et al.*, 2025). Abundantly expressed on the surface of leukocytes and kidney epithelial cells, TLRs play a key role in the secretion of proinflammatory cytokines (Zhang *et al.*, 2023). While TLR4 activation has traditionally been linked to pro-inflammatory pathways that can further damage during AKI, its potential role in renal repair has also been suggested (Valles *et al.*, 2023). Beyond its inflammatory role, TLR4 signaling can also mediate beneficial effects by maintaining extracellular matrix homeostasis, promoting tissue regeneration, and modulating macrophage polarization towards a reparative (i.e., M2) phenotype (Montero and De Andrés, 2009; El-Zayat *et al*., 2019).

Further to this, studies indicate that while blocking TLRs during renal injury prevents severe tubular necrosis and inflammation, suppressing interleukin production during the healing phase may impair kidney regeneration (Habib, 2021). TLR activation has been reported to be involved in renal epithelial cell repair by facilitating the clearance of cellular debris and initiating tissue repair programs. In this regard, Sallustio *et al*. showed that TLR2 on renal progenitor cells accelerates tubular repair via the release of inhibin A and decorin, whereas TLR2 blockade completely negates this regenerative effect (Sallustio *et al*., 2017). Additionally, TLR4-mediated release of IL-22 from leukocytes activates the ERK1/2 and Jak/STAT3 pathways, thereby accelerating re-epithelialization in injured tubular cells *in-vivo* (Kulkarni *et al*., 2014). Collectively, these observations indicate that the two stress-response programs in renal cells, namely renal inflammation and regeneration, may be linked through TLRs.

For a better prognosis and detailed understanding of AKI pathology, there is a need to identify more molecular events and biomarkers. Therefore, given the dual temporal roles of TLRs in renal injury and repair, further examination of these pathways would be of great interest. In this regard, to elucidate the role of TLR4 in AKI, the small synthetic molecule TAK-242 (Resatorvid), which targets TLR4 signaling, offers an opportunity to explore the molecular responses that contribute to kidney damage and repair mechanisms. The present study aimed to establish an animal model of AKI as a tool for biomarker-based evaluation of the damage-repair process. Histology and gene expression of various cell signaling events were analyzed at different injury time points. An improved mechanistic understanding of these pathways can further elucidate disease pathophysiology and may inform the search for potential therapeutic avenues for kidney diseases.

MATERIALS AND METHODS

Animals

Male C57BL/6 inbred mice weighing 20-30 g were acquired from the animal facility of Dow University of Health Sciences. The international guidelines for the care and experimental use of laboratory animals were followed for all animal procedures. The animals were provided with standard rodent chow and tap water.

Cisplatin-induced acute kidney injury (AKI) model and treatment with TAK242

Mice were assigned to different groups (n=3 per group). Control, cisplatin-treated and cisplatin + TAK242 (Resatorvid) groups. Cisplatin (Sigma) was freshly prepared by dissolving 1mg of the drug in 1 mL of sterile saline. A single intraperitoneal dose of 10 mg/kg was administered to mice in the test groups, while mice in the control group received an identical volume of sterile saline. The Cisplatin + TAK242 group was further divided into two subgroups: the Cisplatin + TAK-242 (early) subgroup was treated with TAK-242 (3 mg/kg) 1 h prior to and 24 h after cisplatin administration, while the Cisplatin + TAK-242 (late) subgroup was treated with TAK-242 on the 4th day of cisplatin administration. TAK-242 (Tocris) was dissolved in 2% DMSO solution and administered via the intraperitoneal route.

To distinguish the inflammatory trigger of cisplatin from preexisting baseline inflammation, a prior dose was administered to inhibit baseline TLR4 signaling and after 24 hrs, TLR4 signaling was inhibited to assess these inhibitory effects on cisplatin-mediated toxicity in the early phase of injury.

Sample collection

All animals were terminally anesthetized by volatile inhalation of terminal anesthesia and sacrificed after 7days of cisplatin injection. Mice in the cisplatin-treated group were also sacrificed 5 and 24 h after cisplatin injection (Fig.1A). Blood samples were collected via heart puncture into sterile microcentrifuge tubes. Serum was isolated as supernatant following centrifugation (Eppendorf 5430R, Germany) at1,700 x g for 10 minutes at 4°C and preserved at -80°C until analysis. After careful excision, the kidneys were cleaned of adherent adipose tissue and cut along the median longitudinal axis. Some tissue pieces were immediately immersed in formalin fixative for overnight incubation at RT, while others were washed with cold PBS and cut into smaller pieces for immediate storage at -80°C (Thermo Scientific 88700V, USA) until processed for gene expression analysis.

Analysis of renal function

As a measure of renal function, a quantitative colorimetric assay (DICT-500 , BioAssay Systems, USA) was used to determine serum creatinine (SCr) levels. According to the manufacturer's instructions, 30 µL of the standard (2 mg/dL) and 30 µL of the serum samples were placed into

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separate wells of a 96-well plate. The working reagent (200 µL) was added to each well. The optical density of the developed color was measured at 510 nm (BioTek EPOCH 2) at 0 min (OD0) and 5 min (OD5). Serum concentration was calculated using the manufacturer's formula (Habib *et al.*, 2024).

Renal histology and damage scoring

Kidney specimens were dehydrated by immersion in an ascending series of 70%, 90% and 100% alcohol, followed by xylene, all at room temperature. Tissues were embedded in paraffin using a mold and paraffin embedding system. Tissues were sliced into 5 µm-thick sections (KD-BM III, Kedee, China) and stained with Harri's hematoxylin and eosin staining kit (Carl-ROTH GmbH, Germany) according to the manufacturer's protocol. Histopathological grading of different parameters was performed using a semi-quantitative scale: normal (-) = 0; mild (+) = <25%; moderate (++) = 25-50% and severe (+++) >50% of the affected area (Chen *et al*., 2013).

Gene expression analysis by real-time PCR (qRT-PCR)

Total RNA was extracted from frozen mouse kidney tissues (50-60 mg) using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's protocol. The Integrity and purity of RNA samples were checked by 1% agarose gel electrophoresis. On a SYBR™ Safe (Invitrogen) stained gel, 8µg RNA samples were loaded and visualized under UV light. Photographs were taken using the ChemiDoc MP imaging system (BioRad gel documentation system). The quantity of RNA was measured using a NanoDrop (Thermo Fisher), and cDNA was synthesized from 0.5 µg RNA using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions.

qRT-PCR amplification was performed using a fast reaction method on a Quant Studio™ 7 Flex Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Reactions were run in triplicate using BrightGreen 2× qPCR Master Mix Low ROX (Applied Biological Materials, Canada). Primer BLAST (www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to design primers online (Table 1). β-Actin was set as the internal control. The 2^-ΔΔCt method was employed to analyze relative gene expression. The ΔCT values of control and test samples were obtained by subtracting each sample's corresponding housekeeping gene (internal control) CT values from the target gene values. Then, ΔΔCT was calculated by subtracting the ΔCT of the control from that of the test sample. Finally, the resulting fold change in gene expression was calculated using the standard formula 2^-ΔΔCt.

Statistical analysis

Means ± standard error of the mean (SEM) are used to express data. Differences among groups were analyzed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. The threshold for statistical significance was set at $p \leq 0.05$ using SPSS software (version 25).

RESULTS

Renal functional analysis

A notable increase ($p \leq 0.05$) in SCr levels was observed after 7 days of cisplatin injection. However, no increase was observed at 5 and 24 hr after cisplatin administration. The group treated with an early dose of TAK242 showed a prominent increase in SCr levels compared to the normal and cisplatin 7 days group ($p \leq 0.01$), while the group treated with a late dose of TAK242 showed SCr levels lower than the cisplatin 7 days model group, although they remained increased compared to the control group (Fig.1B).

Histopathological analysis

Kidney sections from normal control mice exhibited normal renal tissue structures. Renal tubular epithelial cells with well-arranged brush borders were observed in the normal and 5h cisplatin groups, and there were no obvious pathological changes in the glomerular or renal interstitial tissues. Mild tubular casts with disturbed intraluminal brush borders can be seen after 24 h of cisplatin injection, reflecting the initiation of tubular injury; however, abundant epithelial cell necrosis was not evident. Renal damage was noticeable after 7 days of drug administration in AKI group. Loss of brush borders, sloughing of epithelial cells, and resulting intratubular casts and tubular dilations were evident and became even more pronounced in the injury group treated with an early dose of TAK242, a TLR4 inhibitor. Massive epithelial cell necrosis, loss of brush border and dilated and disorganized tubules were observed in mice treated with an early dose of TAK242, while mice treated with a later dose of TAK242 showed preserved gross tubular structures. However, some intratubular debris with damaged brush borders was evident. The ECM of renal tissues showed infiltration of RBCs, which may indicate damage to the glomerular basement membrane (Fig. 2). A comparison of the histological parameters is shown in table 2.

Gene expression analysis

The cisplatin 7th-day group showed a significantly increased expression of Tlr4 mRNA ($p \leq 0.01$), which was suppressed in both TAK242 groups ($p \leq 0.01$). *Nfκb* expression was not significantly elevated in any group compared with the control. A massive increase in *Lcn2* expression ($p \leq 0.001$) was observed in the cisplatin 7th day group, which was significantly reduced ($p \leq 0.001$) in both TAK242 groups.

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Table 1: Details of qRT-PCR primers.

Gene primers	Primer sequence (5′–3′)	Annealing temperature (°C)	Product size (bp)	Accession number
β-Actin (F)	CCTAGGCACCAGGGTGTGAT	60°C	179	NM_007393.5
β-Actin (R)	GCCACACGCAGCTCATTGTA			
TLR4 (F)	CTCAGCAAAGTCCCTGATGACA	60°C	149	NM_021297.3
TLR (R)	TCAATTGTTTCAATTTCACACCTG			
NFκb2 (F)	CCTTCTAAAGGCTGGTGCTGA	60°C	158	NM_001177369.1
NFκb2 (R)	TGCAAGTGAGGTCAAGAGGG			
LCN2 (F)	TCTGTCCCCACCGACCAA	60°C	116	NM_008491.1
LCN2(R)	GGAAAGATGGAGTGGCAGACA			
SOD1 (F)	CGATGAAAGCGGTGTGCG	60°C	179	NM_011434.2
SOD1 (R)	TGCACTGGTACAGCCTTGTGT			
IL22 (F)	CTTGTGCGATCTCTGATGGCT	60°C	218	NM_016971.2
IL22 (R)	TTATCTGCAAGGCTGGCCTCC			
Fzd8 (F)	CCGCAGACTTCATCCAAGGT	60°C	141	NM_008058.2
Fzd8 (R)	AAGGCATCTGCCTGGTCATT			
WNT4 (F)	GGTGGTGACACAAGGGACC	60°C	186	NM_009523.2
WNT4 (R)	CCATAGGCGATGTTGTCCGA			
HGF1 (F)	ACTGAAGCTTGCTCGACCTG	60°C	117	NM_001289458.1
HGF1 (R)	GTGTAGCCCCAGCCGTAAAT			
c-Met (F)	ACGCAGAAGTTCACCACCAAGTC	60°C	180	NM_008591.3
c-Met (R)	CATCACTTCGTACAAGGCGTC			
LC3-II (F)	GGGGCAGGCGGGTGATTA	60°C	209	NM_001364358.1
LC3-II (R)	CACTTCGGAGATGGGAGTGG			
HSP70 (F)	TGCACGTGGGCTTTATCTTC	60°C	114	NM_010479.2
HSP70 (R)	ACAAATCACATCAGCGGGGC			
HSP25 (F)	CCCGGAAATACACGCTCCCT	60°C	128	NM_013560.2
HSP25 (R)	AATGGTGATCTCCGCTGACTG			

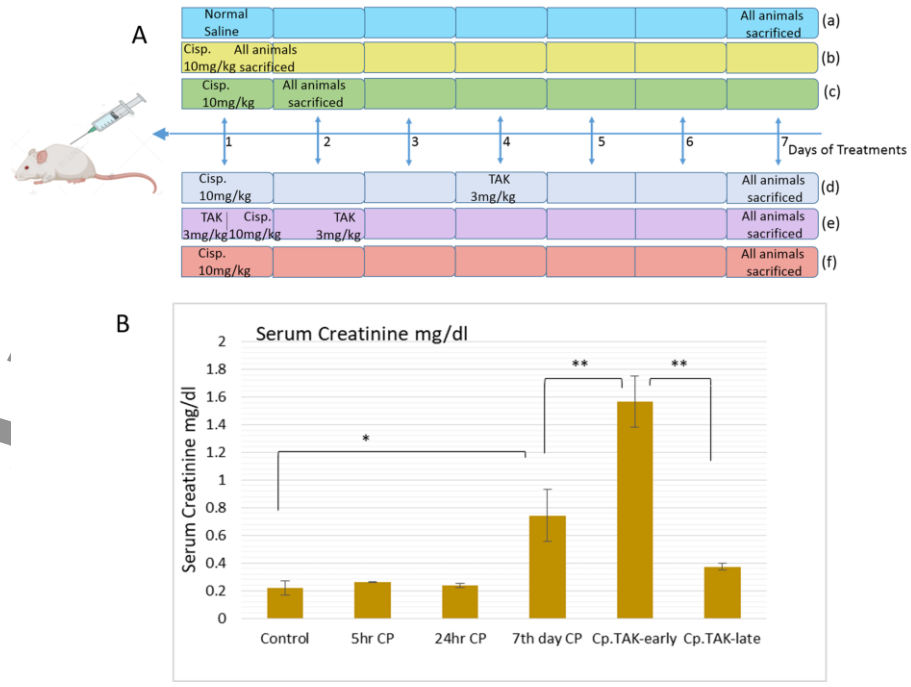


Fig. 1: Effect of cisplatin and TAK242 in different treatment groups of mice. (A) Diagram depicting *in-vivo* experimental design and time course of different treatment groups; (B) Graphical representation of renal functional analysis via estimation of serum creatinine. The normal control group was injected with saline solution. Results are expressed as means ± SEM (n = 3). *P-value* ≤0.05 was considered statistically significant.

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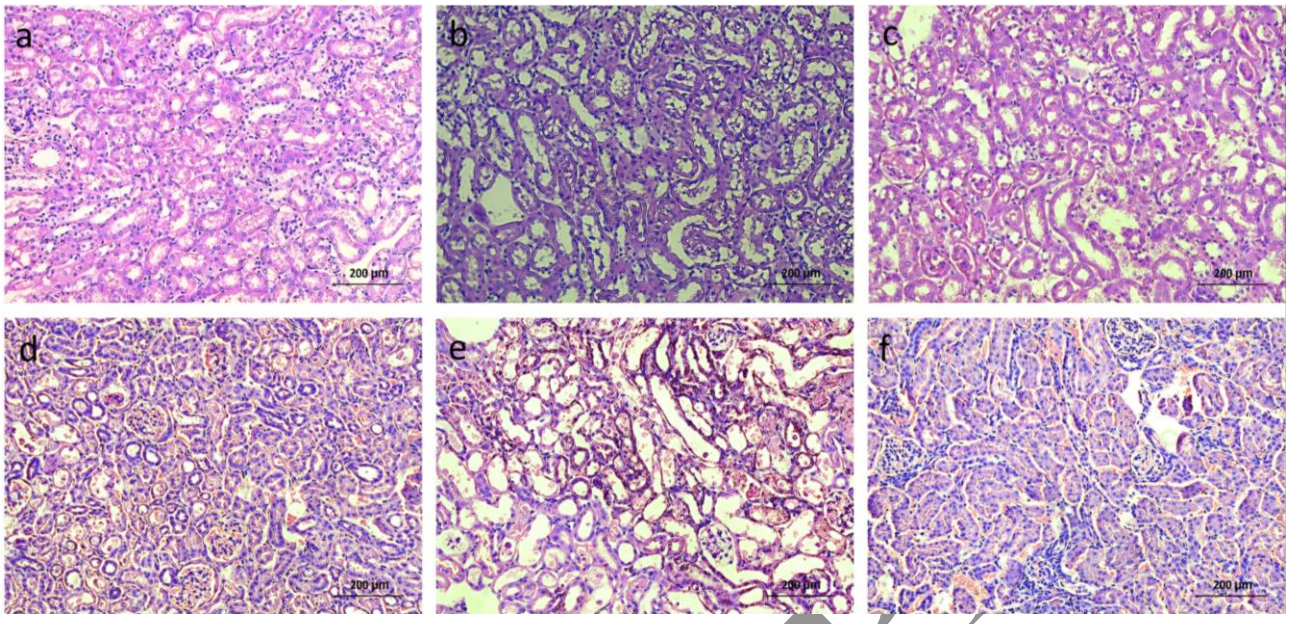


Fig. 2: Renal histopathological changes in different experimental groups. (a) Normal view of renal tissue in control group (saline treated); (b) 10 mg/kg cisplatin 5hrs group showing almost preserve tissue architecture; (c) 10 mg/kg cisplatin 24hrs group shows minimal damage with intraluminal debris in some tubules; (d) 10 mg/kg cisplatin 7days group shows intraluminal cast, loss of brush border and tubular necrosis; (e) 10 mg/kg cisplatin group with early treatment of TAK242 shows massive destruction of brush borders with intraluminal cast and; (f) 10 mg/kg cisplatin group with late treatment of TAK242 shows minimal tubular damage (HE; 20X).

Table 2: Comparison of renal histological parameters among different groups.

	Groups					
Histological parameters	Normal saline	Cisplatin 5 hrs	Cisplatin 24 hrs	Cisplatin 7days	Cisp+TAK (early)	Cisp+TAK (late)
Luminal cast	-	-	+	+++	+++	+
Tubular necrosis / apoptosis	-	-	+	++	+++	+
Loss of brush border	-	-	+	+++	+++	++
Tubular dilations			-	++	+++	-

Note: normal (-) = 0; mild (+) = <25%; moderate (++) = 25-50% and severe (+++) >50%

However, compared with the control, its expression remained increased in these groups. After 24 h of cisplatin administration, *Wnt4* expression was elevated, which became significant ($p \leq 0.01$) on the 7th day of injury. Both TAK242 groups exhibited decreased *Wnt4* expression compared with the cisplatin 7th-day group, whereas Wnt4 remained elevated compared with the control. All cisplatin groups (treated or untreated) showed improved expression ($p \leq 0.001$) of *Fzd8*.

Among all, only the cisplatin 7th day group showed increased expression of HGF ($p \leq 0.01$) with downregulation of its receptor (*c-Met*) compared with control group. Both TAK242 groups also showed a non-significant decrease in c-Met receptor expression compared with the control. In the late intervention group, a non-significant upregulation was observed compared with the TAK242-early group. *Il22* expression was increased ($p \leq 0.001$) in both cisplatin 7th day and TAK242-early groups, while it remained almost normal in TAK242-late group.

Hsp25 and *Hsp70* were upregulated in cisplatin 7th day group ($p \leq 0.001$). They were downregulated in both TAK242 groups. However, compared with control, *Hsp25* expression remained non-significantly increased in these groups. Both TAK groups reduced *Hsp25* relative to 7-day cisplatin, but TAK242-late retained substantially higher *Hsp25* than TAK242-early. All cisplatin groups (treated or untreated) showed downregulation of *Lc3II* compared to the control. After 5 h of cisplatin administration, *Sod1* expression increased ($p \leq 0.05$), which was reduced to normal ($p \leq 0.05$) on the 7th day of injury and was further downregulated in the TAK242-early group. Higher expression ($p \leq 0.05$) of *Sod1* was observed in the TAK242-late group than in the TAK242-early group (Figs. 3A, 3B and 3C).

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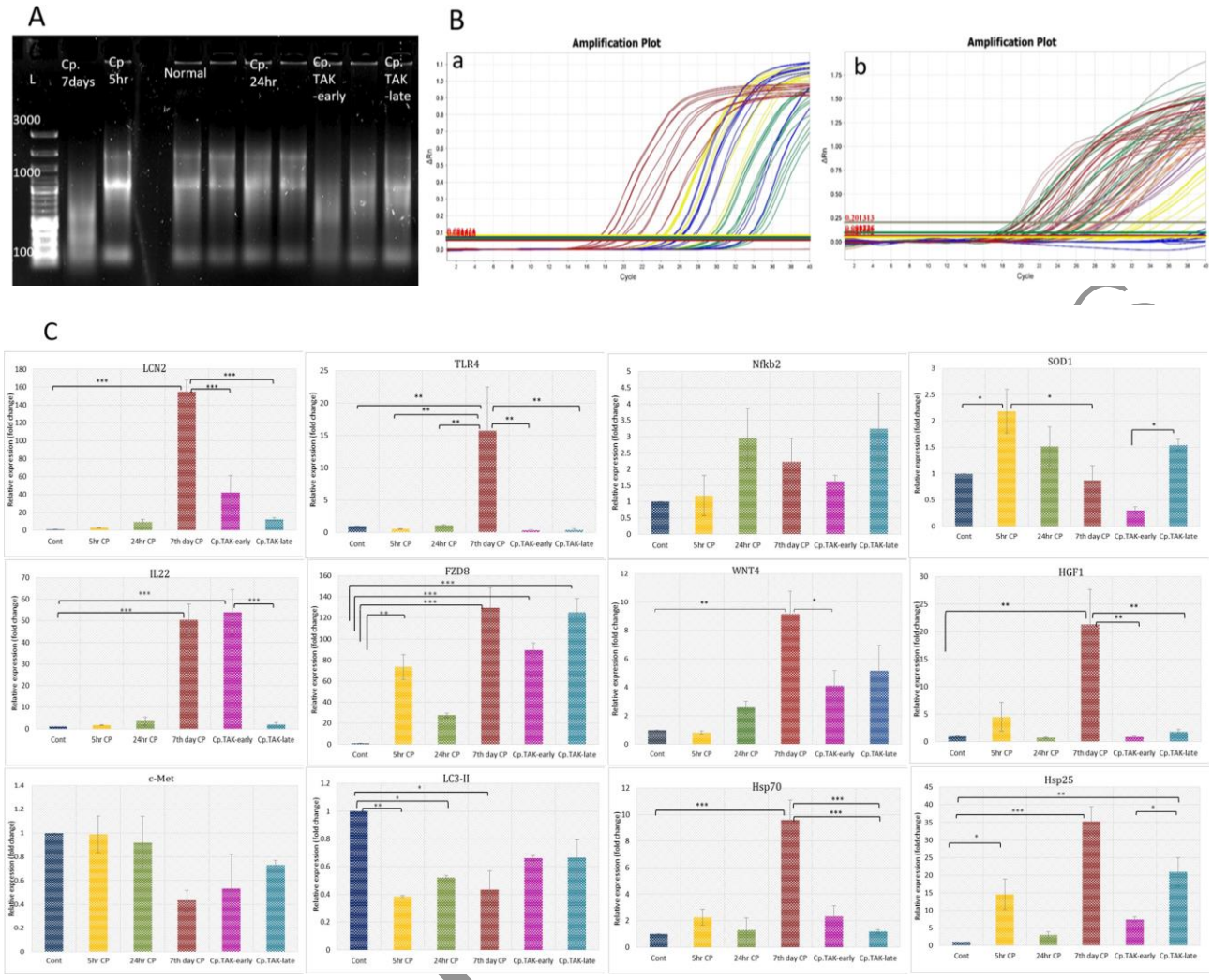


Fig. 3 Gene expression analysis by qRT-PCR in murine kidney tissues in response to different treatments. (A) Agarose gel electrophoresis of all isolated RNAs shows distinct rRNA bands with no DNA contamination; (B) SYBR Green-based qRT-PCR amplification curves (a-b) track DNA amplification of all samples; (C) Graphical representation of *LCN2, TLR4, Nf-κb, SOD-1, IL22, FZD8, WNT4, HGF1, c-Met, LC3-II, HSP70 and HSP 25* genes expression as relative fold change in different treatment groups, with *β-Actin* used as the internal control (fold-change is relative to the untreated control, which was assigned a value of 1). Results are expressed as means ± SEM (n = 3). *P-value* ≤0.05was considered statistically significant.

DISCUSSION

Understanding how different molecular events communicate to contribute to the post-injury pathophysiology of acute kidney injury remains a major area of investigation. Studies suggest that Toll-like receptors (TLRs) are significantly expressed during acute kidney injury and play a notable role in supporting tissue inflammation (Zhang *et al.*, 2023). Despite this, TLR activation leading to cytokine release highlights their role in the survival of renal epithelial cells after injury (Habib, 2021). In this study, the effects of TLR4 inhibition were evaluated and compared at two time points (Cp.TAK-early and Cp.TAK-late) in a cisplatin-induced mouse model of acute kidney injury (AKI). Drug-induced nephrotoxicity is provoked by transport-mediated (organic cation transporter 2) accumulation of drugs within proximal tubular cells (Yao *et al.*, 2007). At the cellular level, cisplatin-DNA binding transmits signals to the cytoplasm for the execution phase of apoptosis. However, because cisplatin can react with cytoplasmic proteins, protein damage may initiate necrosis (Tchounwou et al., 2021).

Treatment with the TLR4 inhibitor (TAK242, a cyclohexene derivative) at two different time points of renal injury showed significant differences in injury prognosis based on renal function, histology, and Lcn2 (a sensitive molecular marker of AKI) expression (Viau *et al.*, 2010). Although early TAK242 treatment in the AKI

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model resulted in extensive tissue destruction, it showed reduced Lcn2 expression compared to the cisplatin 7th-day group, consistent with its negative correlation with TLR4 expression. *Lcn2* has been shown to be a key mediator of EGF receptor signaling, promoting renal epithelial proliferation after injury (Urbschat *et al.,* 2020). In parallel, the decreased *Lcn2* expression may result from the notable loss of tubular cells. Consistent with previous findings (Valles *et al.,* 2023), the present observations validated that cisplatin-mediated AKI upregulates Tlr4 expression (up to 15-fold), which was significantly reduced in both other TAK242-treated AKI groups (Cp.TAK-early and Cp.TAK-late). Interestingly, inhibition of *Tlr4* did not appear to be linked to reduced *Nf-κb* expression in Cp.TAK-late group, suggesting the involvement of other additional cellular pathways or TLRs in the induction of inflammation following injury. There is no significant difference in NF-kB expression between the early and late TAK242 groups. It is worth noting that NF-κB's role in tissue injury is complex. In addition to its inflammatory role, transient *Nf-κb* activation in the acute phase of injury may act as an anti-apoptotic survival mechanism that promotes repair (Liu D *et al.,* 2022). Additionally, it has been demonstrated that *Tlr4* Deletion in ischemic AKI mice did not prevent neutrophil and macrophage accumulation following injury (Dagher *et al.,* 2016). Therefore, alternative activation of other TLRs, such as TLR2, when TLR4 is dysfunctional, is one possible explanation for this cytokine response (Yiu *et al.,* 2014). Data suggest that *NF-κB-p65* overexpression can increase the levels of antioxidant enzymes, such as SOD-1, in human kidney-2 (HK-2) cells, thereby helping reduce oxidative stress (Rojo *et al.,* 2004). In this study, increased cytoplasmic superoxide dismutase (*Sod1*) expression in Cp.TAK-late group might be a possible consequence of improved *Nf-κb* expression on the 7th day of cisplatin and Cp.TAK-early groups depict increased oxidative stress, which may be a cause of massive tissue destruction in these groups.

Compared with the control group, the massive necrosis and increased serum creatinine levels following early inhibition of TLR4 indicate immediate involvement of this pathway in cell survival mechanisms after injury, whereas comparatively late treatment with TAK242 resulted in more preserved tissue architecture and function. This observation raises the question of the optimal timing of TLR inhibition, as untimely inhibition may cause greater harm. Available data do not support the direct toxicity of TAK-242 alone, while research indicates that, as a cell-protective mechanism and to combat reactive oxygen species production, TLR4 signaling can trigger the upregulation of HIF-1α expression in HK-2 renal tubular epithelial cells; inhibiting this pathway abolished the protective effect (Liu XJ *et al*., 2023). Therefore, increased sensitivity of renal tubular cells to initial cisplatin administration in a suppressed TLR4 microenvironment can be suggested here. Further studies with a larger sample size could validate these findings.

Emerging evidence indicates that in specific situations, such as cisplatin-induced nephropathy, IL-22 acts as a cofactor that worsens AKI (Ma *et al.,* 2022). It induces DNA damage response (DDR) and promotes cell death in PTCs. However, it is also pertinent to mention that TLR4 has been shown to protect against AKI by promoting tubular regeneration via IL22 secretion through the TLR4–IL-22 pathway (Kulkarni *et al.,* 2014). As functional IL-22 receptors are predominantly expressed on renal proximal tubular epithelial cells (Weidenbusch *et al.,* 2015), the present study suggests that receptor malfunction due to damaged tubules can activate feedback mechanisms that, in turn, increase IL-22 release by immune cells, as observed on the 7th day with cisplatin and Cp TAK-early groups. This reflects that IL-22 is a "double-edged sword" in the kidney, acting as a tissue-protective mediator in some contexts and a pathogenic factor in others. Further investigation into the exclusive expression of the IL22 receptor in renal cells during AKI can elaborate on this notion. FZD8 is a receptor for Wnt ligands and activates both canonical (Wnt/β-catenin) and non-canonical signaling pathways, which are essential for normal epithelial function and kidney development (Yang *et al.,* 2017). Interestingly, the *Fzd8* receptor showed significantly increased expression in all AKI groups compared with control, suggesting its involvement in multiple cell-survival pathways. This receptor binds to multiple Wnt ligands (Ko *et al.,* 2022). The present study highlights the immediate role of this receptor in AKI, which appears independent of TLR4 signaling, as its expression remains elevated after TAK242 treatment.

Additionally, it has been reported that FZD8 promotes the expression of Vimentin and Snail, which are critical for the EMT process (Yang *et al.,* 2017). This justifies its sustained signaling in promoting tissue remodeling and fibrosis. However, because FZD8 plays a dual role in kidney injury by promoting both renal epithelial cell proliferation and fibrosis, a long-term in vivo study can confirm the ultimate fate of its activation.

WNT4 is known as a marker of renal injury and repair processes. It promotes cell proliferation and tubulogenesis (He *et al.,* 2018). Present observations highlight the indirect inhibitory effect of TLR4 antagonism on TLR4 expression, as TLR4 activation involves β-catenin phosphorylation, which can increase WNT4 expression in a positive feedback loop (Liu *et al.,* 2020). Furthermore, it can be postulated here that the activation of immune and renal cells via overexpression of different TLRs on their surface results in the release of a variety of cytokines, including IL-6, IL-1β, IFN-β, IL-8, IL10 and TNFα (El-Zayat *et al.,* 2019), which may positively influence and

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engage in cross-talk with other developmental pathways, such as Wnt/β-catenin and HGF/c-Met pathways. This cross-talk is necessary for a quick repair response (Yang *et al*., 2020; Matsumoto and Nakamura, 2001); therefore, if interrupted prematurely in the early stages of injury, it may result in more pronounced histological damage. A slight decrease in c-Met expression after 7 days of injury (all treated and untreated groups) may be due to the loss of tubular cells (Zhou *et al.*, 2013).

In tissue injury, autophagy is a pro-survival or controlled cell death process and in contrast to necrosis, it typically acts as a cleanup mechanism (Streeter *et al.*, 2016). Decreased expression of autophagy marker *Lc3-II* in all cisplatin treated groups indicates the involvement of necrosis in cisplatin-mediated tissue destruction. Heat shock proteins, HSP25 and HSP70 are molecular chaperones and components of stress response in renal proximal tubular cells. These proteins attenuate apoptosis by inhibiting reactive oxygen species (ROS) production (Tran *et al.*, 2025) and help stabilize the actin cytoskeleton, thereby reducing damage, potentially by lessening the extent of injury (Chebotareva *et al.*, 2017). Elevated expression after 5 h of injury indicates their prompt involvement in cell survival mechanisms, which remain elevated in all other groups; however, both groups treated with TAK242 showed decreased HSP expression compared to the cisplatin 7th-day group. These observations align with existing knowledge indicating that HSP-mediated protective mechanisms are inhibited through abrogation of the TLR4 pathway (Liu *et al.*, 2023). It is suggested that inhibiting these proteins in the early stages of injury (within 24–72 h) can exacerbate cellular damage.

In summary, TLRs play critical and often context-dependent roles in renal injury. These findings underscore the significance of TLR4 signaling in the pathophysiology of AKI and suggest that early inhibition of this pathway during injury may disrupt protective mechanisms of cell survival, resulting in exacerbated tissue damage. Compared with immediate blocking of this signaling pathway, a better outcome can be expected if it is halted later in the injury process. However, as the present *in-vivo* findings rely primarily on gene expression data lacking translational-level validation, discerning direct from indirect effects remains challenging, as multiple factors can influence *in-vivo* observations. *In vitro observations of* temporal treatment of renal epithelial cells in a controlled, isolated system can provide time-dependent cellular responses in a specific context.

Additionally, owing to off-target binding, chemically mediated receptor inhibition may result in unintended activation or inhibition of cellular receptors, yielding complex effects. Therefore, conditional gene knockout (cKO) experiments that target specific time points of injury can precisely elucidate the role of the gene. Since the present study focused exclusively on the impact of TAK-242 in an induced AKI model, it lacks baseline data on its effects in healthy kidney tissue. Future investigations that incorporate a TAK-242-only healthy control group may help establish its renal safety profile. Long-term observations in the AKI model are necessary to validate the potential favorable effect of TLR4 inhibition on long-term outcomes.

CONCLUSION

Later, following a toxic insult, the immediate peak requirement for extensive cell-protective activities may have subsided. In the early phases of acute kidney injury, TLR4 can help promote renal cell survival by activating genes that protect against necrosis, such as antioxidant proteins, while in the days after an injury, its inhibition may become therapeutic by reducing cell death. Given these dual-faceted roles, the timing of TLR4 modulation should be carefully considered to elucidate its complex signaling pathways and to develop targeted therapies for AKI.

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None.

Authors' contributions
RH: Conceptualized the study, performed part of the experimental work, analyzed data and wrote and edited the final manuscript; YH: Contributed to manuscript writing and performed experimental work. All authors have read and approved the published version of the manuscript. The authors declare that they did not use artificial intelligence.

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Data availability statement
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval
All experiments involving animals were approved by the institutional ethical review board for animal research and ethics at Dow University of Health Sciences (animal IRB# AR.IRB-30/DUHS/Approval/2024/57). This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict interests
The authors declare no conflict of interest.

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