# 1. TITLE PAGE

Cisplatin toxicity is mediated by direct binding to Toll-like Receptor 4 through a mechanism that is distinct from metal allergens

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# 2. RUNNING TITLE PAGE

Running Title: Cisplatin activates TLR4 by Direct Binding

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## (Non-Standard) Abbreviations

CaM Calmodulin

CD14 Cluster of Differentiation 14
CIT Cisplatin-Induced Toxicity

DAMP Damage-Associated Molecular Pattern

DMSO Dimethyl Sulfoxide

ELISA Enzyme-Linked Immunosorbent Assay

HA Hemagglutinin

HEK Human Embryonic Kidney

hrs Hours

hTLR4 Human Toll-Like Receptor 4

MD-2 Myeloid Differentiation Factor 2

MST Microscale Thermophoresis

mTLR4 Mouse Toll-Like Receptor 4

MTT 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide

NF-κB Nuclear Factor Kappa B

LPS Lipopolysaccharide

PAMP Pathogen-Associated Molecular Pattern

PBS Phosphate Buffered Saline PRR Pattern Recognition Receptor

TLR4 Toll-Like Receptor 4

# 3. ABSTRACT

Cisplatin is an effective chemotherapeutic agent, yet its use is limited by several adverse drug reactions, known as cisplatin-induced toxicities (CITs). We recently demonstrated that cisplatin could elicit pro-inflammatory responses associated with CITs through Toll-like Receptor 4 (TLR4). TLR4 is best recognized for binding bacterial lipopolysaccharide (LPS) via its coreceptor, MD-2. TLR4 is also proposed to directly bind transition metals, such as nickel. Little is known about the nature of the cisplatin-TLR4 interaction. Here, we show that soluble TLR4 was capable of blocking cisplatin-induced, but not LPS-induced TLR4 activation. Cisplatin and nickel, but not LPS, were able to directly bind soluble TLR4 in a microscale thermophoresis binding assay. Interestingly, TLR4 histidine variants that abolish nickel binding, reduced, but did not eliminate, cisplatin-induced TLR4 activation. This was corroborated by binding data that showed cisplatin, but not nickel, could directly bind mouse TLR4 that lacks these histidine residues. Altogether, our findings suggest that TLR4 can directly bind cisplatin in a manner that is enhanced by, but not dependent on, histidine residues that facilitate binding to transition metals.

## 4. SIGNIFICANCE STATEMENT

This work describes how the xenobiotic cisplatin interacts with Toll-like receptor 4 (TLR4) to initiate pro-inflammatory signaling that underlie cisplatin toxicities, which are severe adverse outcome in cisplatin treatment. Here, we provide a mechanistic bridge between cisplatin extracellular interactions with TLR4 and previous observations that genetic and chemical inhibition of TLR4 mitigates cisplatin-induced toxicity.

# **5. INTRODUCTION**

Cisplatin is the oldest and most potent of the platinum-based chemotherapeutics available (Rosenberg & VanCamp, 1970; Rosenberg *et al*, 1965). It is used to treat a variety of solid-state cancers – ranging from head-and-neck to ovarian and testicular (Nagasawa *et al*, 2021; Muzaffar *et al*, 2021; Low *et al*, 2012; Bookman, 2016; Einhorn, 2002; de Vries *et al*, 2020). Consisting of two amine ligands and two chloride ions bound to a platinum core, cisplatin mediates its anti-tumour effects by intercalating into the DNA of replicating cells. Cisplatin binding to DNA inhibits strand separation and DNA damage repair, leading to the build-up of DNA damage that eventually leads to cell death (Dasari & Tchounwou, 2014). Treatment regimens that include cisplatin can have 5-year overall survival rates of up to 90% making it integral to cancer therapy.

Despite its effectiveness, cisplatin use has been limited by the discovery of cisplatin-induced toxicities (CITs) (Tsang *et al*, 2009; El-Awady *et al*, 2011; Shahid *et al*, 2018). The development of CITs such as nephrotoxicity, peripheral neurotoxicity, and ototoxicity, appears to be dependent on both the dosages administered and the age of patients. Children appear to be a highly susceptible population, particularly to cisplatin-induced hearing loss (Kamalakar *et al*, 1977; Romano *et al*, 2020; Ruggiero *et al*, 2021). Newer platinum-based drugs were explicitly designed to reduce toxicities and overcome chemotherapy resistances (Kelland, 2007). Unfortunately, most modifications made to the underlying structure of cisplatin come at the cost of its therapeutic efficacy - leaving cisplatin the primary choice for treatment and underscoring the need to develop mitigations for its toxicities. Studies of the underlying mechanisms of CITs have revealed both unique features, such as anatomical location, and shared features amongst CITs. Inflammation has proven to be a critical aspect of CITs (Domingo *et al*, 2022). The inhibition of direct and indirect mediators of inflammation has proven to be effective in mitigating CITs in *in-vitro* and *in-vivo* pre-clinical studies. (So *et al*, 2007; Babolmorad *et al*, 2021; Kim *et al*, 2011; Kaur *et al*, 2011; Rybak *et al*, 1999, 2009; Domingo *et al*, 2022).

Models of CIT where pro-inflammatory signalling systems, such as the Toll-like Receptor 4 (TLR4) pathway, have been removed or otherwise disabled have also exhibited resistance to cisplatin toxicity (Babolmorad et al., 2021; Gao et al., 2020; Tsuruya et al., 2003; Li et al., 2019; Park et al., 2014; Ramesh & Reeves, 2003; H. S. So et al., 2008; Woller et al., 2015; B. Zhang et al., 2008; Q. Zhang et al., 2022; Y. Zhang et al., 2014; Zhou et al., 2018, 2020).

TLR4 is a membrane-bound pattern-recognition receptor (PRR) typically responsible for recognizing and mounting pro-inflammatory innate immune responses to both pathogen-associated and damage-associated molecular patterns, referred to as PAMPs and DAMPs (Kawai & Akira, 2006). TLR4 exists in complexes with co-receptors which can help dictate its specificity. The best characterized agonist of TLR4 is bacterial lipopolysaccharide (LPS), which is bound to TLR4 in conjunction with the co-receptor, MD-2. The current understanding of TLR4 activation suggests that TLR4 itself contains features that allow for the binding of various potential agonists – with co-receptors providing support to complete the TLR4 dimerization necessary for downstream signalling (Kawasaki & Kawai, 2014). Agonists interact with TLR4 through its extracellular/ectodomain. The ectodomain region contains unique amino acid residue combinations that in human TLR4 confers a greater affinity for hexa-acetylated LPS versus penta-acetylated LPS compared to that of murine TLR4 (Hajjar et al, 2002). Certain single nucleotide polymorphisms can also confer LPS-specific TLR4 hypoactivation (Richard et al, 2021). Similarly, histidines 456 and 458 in the TLR4 ectodomain were found to mediate the interaction of TLR4 with Group 9 and Group 10 transition metals, leading to contact allergen hypersensitivities (Schmidt et al, 2010; Raghavan et al, 2012; Rachmawati et al, 2013; Oblak et al, 2015).

Given that platinum, the core of cisplatin, is a Group 10 transition metal as well, we hypothesized that TLR4 may be helping mediate toxicities like CITs by binding cisplatin (and platinum agents) directly and triggering hypersensitivity responses. We recently showed that genetic inhibition of Tlr4 reduced cisplatin toxicity *in vitro* and protected hair cells from cisplatin-induced death in zebrafish (Babolmorad *et al*, 2021).

We further showed that TLR4 activation by cisplatin was not dependent on MD-2 and could be suppressed by TLR4 inhibitors. In this new study, we provide evidence that CITs are in part mediated by direct interactions between cisplatin and TLR4. We show that this interaction is aided, but not dependent on, key metal-binding residues in TLR4.

# **6. MATERIALS AND METHODS**

#### **Cell Culture and Treatments**

Human Embryonic Kidney HEK293-Null2 cells (Cat #hkb-null2) and HEK293-hTLR4 cells (Cat #hkb-htlr4) were obtained from Invivogen and are isogenic reporter cell lines, where HEK293hTLR4 are stably transfected with human TLR4 and MD-2. Cells were grown in DMEM supplemented with FBS (10%), penicillin-streptomycin (100μg/mL), and Normocin (100μg/mL) at 37°C and 5% CO<sub>2</sub>. For experiments with soluble recombinant TLR4, cells were grown in 24-well plates at a density of 5.0 x 10<sup>4</sup> cells/well for 24 hrs. Either soluble mTLR4 (Biotechne R&D Systems, Cat #9149-TR-050) or hTLR4 (Biotechne R&D Systems, Cat #1478-TR-050), were added 24 hrs post-seeding for 1 hr prior to agonist treatments. Recombinant proteins were resuspended in PBS, diluted for use in culture media as described. For experiments with TLR4 histidine variants, cells were seeded in 6-well plates at 2.5 x 10<sup>5</sup> cells/well. Cells were transfected 24 hrs post-seeding, where appropriate. LPS (Invitrogen, L23351), nickel chloride hexahydrate (Sigma, 654507), platinum (II) chloride (Sigma, 520632), platinum (IV) chloride (Sigma, 379840), and/or cisplatin (Teva, 02402188) treatments were made 48 hrs post-seeding, or 24hrs posttransfection. Supernatant collection, ELISAs, and cell viability analyses were performed 48 hr postagonist treatment. The concentrations used were based on dose-response experiments of each agonist conducted for a prior publication (Babolmorad et al, 2021). Metal agonist treatment concentrations were also specifically chosen to ensure they retained solubility in media.

#### TLR4 Histidine-456 and Histidine-458 Multi-Site Directed Mutagenesis

TLR4 histidine residues 456 and 458 were replaced with alanine and leucine, respectively, by site-directed mutagenesis according to the manufacturer protocols ((Agilent, Cat# 200514/200515) The mutagenic primers were: 5'-TACCTTGACATTTCTGCTACTCTCACCAGAGTTGCTTTCAATGGC-3' and 5'-GCCATTGAAAGCAACTCTGGTGAGAGTAGCAGAAATGTCAAGGTA-3'. TLR4 mutations were confirmed by Sanger sequencing using primers: 5'-TTGGGACAACCAGCCTAAG-3' and 5'-GAGAGGTCCAGGAAGGTCAA-3'

#### **Immunoblotting**

Transfected cells were collected and lysed using 400μL of Pierce RIPA Lysis and Extraction Buffer (ThermoScientific, Cat#89900), containing Pierce protease inhibitors (ThermoScientific, Cat#A32953). To lyse cells, cells were kept on ice and scraped after 15 minutes of exposure to lysis buffer. Samples were heated at 80°C for 10 min. and protein separated by SDS-PAGE prior to transfer to nitrocellulose membrane. Membranes were probed overnight with mouse anti-HA (1:2500) (Santa Cruz, 12CA5 sc-57592) or mouse anti-GAPDH (1:5000) (Invitrogen, MA5-15738) and then probed for 1 hr with goat anti-mouse secondary antibody (1:5000) (LiCor, IRDye 800CW). Probed membranes were imaged on a LiCor Odyssey and the immunoblotting procedure was performed according to their recommendations.

## **Cell Transfections**

HEK293-Null2 cells were transfected with either an empty vector control, human *TLR4* expression clone (kindly provided by Dr. A. Hajjar, Cleveland clinic), or *TLR4* variant expression clone. To assess the impact of histidine variants on TLR4-mediated immune responses to LPS, HEK293-Null2 cells were also co-transfected with a human MD-2 expression clone (OriGene, RC204686). JetPRIME (Polyplus, CA89129-924) reagent was used for all transfections in accordance with manufacturer specifications.

#### Enzyme-Linked Immunosorbent Assay (ELISA) and Cell Viability Assays

IL-8 secretion was used as a measure of TLR4 activation in HEK-null2 and HEK-hTLR4 cells and was quantified through ELISAs (88-8088, Invitrogen) as recommended by the provider (Invivogen). Secreted cytokines were collected 48 hrs post-agonist treatment and quantified by ELISA following the manufacturer protocols. Protein secretion was normalized to cell viability to account for the differing toxicities of agonists.

Briefly, 96-well plates were coated with anti-IL-8 antibodies and used to capture IL-8 present in sample supernatants. A secondary anti-IL-8 antibodies conjugated with biotin was used to immobilize streptavidin-horse radish peroxidase (HRP) enzymes that oxidized TMB substrate to produce a colorimetric signal. The signal was quantified using a SpectraMAX i3x plate reader (Molecular Devices) and IL-8 concentrations were derived from manufacturer-provided standard curves.

Cell viability was measured using MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (ACROS, 158990010) for the purpose of normalizing ELISA data. MTT was added to cells post-treatment at 1mg/mL and incubated for 4 hrs. The absorbance of solubilized formazan was measured at 590 nm on a SpectraMAX i3x plate reader (Molecular Devices).

#### Microscale Thermophoresis (MST)

The NanoTemper Microscale Thermophoresis Monolith system was used to measure normalized fluorescence changes associated with protein-ligand binding. Soluble recombinant TLR4 (human and mouse) and TLR4 agonists were prepared, separately, in PBS containing 2% DMSO, and 0.5% Tween-20 except for MST experiments involving nickel. Nickel MST experiments were performed with reagents resuspended in 2% DMSO, 0.1% Pluronic F-127 PBS buffer. Serial dilutions of agonists were mixed 1:1 with solutions of hTLR4 (100μg/mL) or mTLR4 (100μg/mL). Binding assays were performed using NanoTemper NT.LabelFree instrument (NanoTemper Technologies). All experiments were carried out at

room temperature in hydrophobic capillaries with 20% light-emitting diode power (fluorescence lamp intensity) and 40% microscale thermophoresis power (infrared laser intensity). Microscale thermophoresis data were analyzed by Monolith Affinity Analysis v.2.2.6 software. Binding affinities and associated statistics reported are estimates from GraphPad Prism.

#### **Data Analyses and Statistical Analyses**

For soluble TLR4 experiments, residual TLR4 activity was calculated by normalizing the data from soluble TLR4-treated cells to their respective control cells that lacked soluble TLR4. For investigating the dose-response to soluble hTLR4, data fitted to non-linear best fit curves following four parameters with a variable slope. 2-way ANOVA with Bonferroni multiple comparison tests were used to calculate the statistical significance of the effects of soluble TLR4 pre-treatment. To determine the percentage of IL-8 secretion retained or lost due to the mutation of histidine residues, data was normalized to the amount of IL-8 secretion triggered by the wild-type hTLR4. For experiments involving variant hTLR4 constructs, actual individual data from each experiment are plotted as boxes (25<sup>th</sup> and 75<sup>th</sup> percentile borders; median central band) with Tukey whiskers.

Statistical analyses were performed through 2-way ANOVA (for the LPS experiments) or 1-way ANOVA (for all remaining experiments) with Bonferroni multiple testing correction. For all thermophoresis experiments, data fitted to non-linear best-fit curves follow three parameters with a standardized slope. EC<sub>50</sub> parameters derived from the curves are reported as apparent K<sub>d</sub> values. All statistical analyses were performed using GraphPad Prism 7.2.

## 7. RESULTS

#### Soluble recombinant TLR4 can reduce cisplatin-induced TLR4 activation in vitro

Platinum (II) ions, platinum (IV) ions, and cisplatin have all been shown to elicit TLR4-dependent proinflammatory cytokine secretion (Babolmorad *et al*, 2021). While these interactions appeared to be independent of MD-2, their molecular nature remains to be elucidated. Metal allergens, such as nickel, can elicit similar inflammatory signalling via direct activation of TLR4 (Raghavan *et al*, 2012; Schmidt *et al*, 2010; Oblak *et al*, 2015; Rachmawati *et al*, 2013). Notably, this can be blocked by soluble TLR4. Accordingly, we sought to determine whether soluble forms of TLR4 could block cisplatin activation of TLR4.

HEK293 cells stably expressing human TLR4 (hTLR4), MD-2 and CD14 (HEK293-hTLR4), were pre-treated for 1 hr with 0.1 nM soluble hTLR4 or mouse TLR4 (mTLR4), and then treated with either no agonist, 1 ng/mL LPS, 200 μM nickel or 25 μM cisplatin for 48 hrs prior to measuring cell viability and pro-inflammatory cytokine (IL-8) secretion as described in the Methods section. Residual TLR4 activity was calculated as the percentage of normalized IL-8 secretion the absence of soluble TLR4 treatment. Differences in residual TLR4 activity were assessed by 2-way ANOVA with Bonferroni multiple testing correction.

LPS activation of TLR4 could not be blocked by either soluble hTLR4 (Fig. 1A) or mTLR4 (Fig. 1B). This was expected since LPS binding to TLR4 requires MD-2. By contrast, soluble hTLR4, but not soluble mTLR4, reduced pro-inflammatory response to nickel by 25% (Fig.1A, B). This was expected because mTLR4 lack the critical histidine residues found in hTLR4 necessary for binding Group 10 metal allergens (Schmidt *et al*, 2010). Notably, both soluble mTLR4 and hTLR4 could inhibit TLR4 activation by cisplatin in this assay by approximately 50%. These data support a model where cisplatin-binding to TLR4 does not require co-receptors.

#### Metal allergens and cisplatin can directly bind hTLR4 without additional cellular components

Our experiments with soluble recombinant TLR4 indicate that TLR4 co-receptors are not required to block nickel and cisplatin-induced TLR4 signalling but they do not confirm a direct interaction between these ligands and TLR4. To rule out the possibility that soluble TLR4 was binding (and blocking)

endogenous TLR4, or DAMPs released in response to cellular toxicity, we attempted to detect direct binding events using recombinant protein via microscale thermophoresis (MST).

To validate this system, soluble recombinant forms of hTLR4 were exposed to increasing concentrations of LPS without MD-2. The range of LPS concentrations chosen (0.009-2.5 $\mu$ M) was based on estimated binding affinities associated with TLR4:MD-2 complexes (Viriyakosol et al., 2001). No detectable binding was observed between hTLR4 and LPS in this assay (Fig. 2A) as evidenced by the lack of curve fit to a 3-parameter agonist binding model. By contrast, nickel has been demonstrated to be a TLR4-dependent metal contact allergen, so we reasoned it could be a positive control for our MST studies, though we are not aware of any methods that have shown direct binding of nickel to TLR4. In line with previous *in-vitro* reports, our MST data suggested that hTLR4 bound nickel within the tested range of 0.49-500 $\mu$ M. The curve fit to a 3-paramater agonist binding model was  $R^2 = 0.695$ , suggesting that the estimated (apparent) binding affinity (K<sub>d</sub>) of 2.72  $\mu$ M (95% confidence interval 0.736-7.71  $\mu$ M) supports a direct binding model of nickel to human TLR4.

To examine the specificity of our MST assay for detecting soluble TLR4 binding events, we tested calcium chloride as a negative control for metal binding to TLR4. Calcium is a Group 2 metal, and we are not aware of any reported calcium interactions with TLR4, so we did not expect to observe binding interactions between TLR4 and calcium. Unlike nickel, hTLR4 displayed little, to no capacity, to bind calcium within the concentration range of 0.49-500mM in our assay (Fig. 2C), with a curve fit  $R^2 = 0.3$  to the agonist binding model. This contrasts with previous reports showing that calcium binding to calmodulin (CaM) is robustly detected by MST (Wienken *et al.*, 2010; Seeger *et al.*, 2017).

We next tested cisplatin in the MST assay within the concentration range of  $0.73\text{-}1665\mu\text{M}$  and detected clear binding of cisplatin to hTLR4, with a strong curve fit of  $R^2 = 0.928$  to the agonist binding model (Fig. 2D). The apparent  $K_d$  of this interaction was  $50.57~\mu\text{M}$  (95% confidence interval of 38.43-

66.76μM). Pt (II) and Pt (IV) solubility were incompatible with the MST buffer system and this precluded our ability to test their binding to hTLR4.

## TLR4 activation by platinum and cisplatin is enhanced by known metal-binding residues

To further understand the interaction between hTLR4 and cisplatin, we used site-directed mutagenesis to replace histidines 456 and 458 (located within the extracellular region of hTLR4 that is part of soluble hTLR4) with alanine and leucine, respectively. These histidines have been reported to be critical mediators of nickel-TLR4 binding (Schmidt et al, 2010; Raghavan et al, 2012). HA epitope-tagged hTLR4 variant constructs were transiently expressed in HEK293-Null2 cells (which do not express endogenous hTLR4, MD-2 or CD14) prior to treatment with LPS, nickel, platinum (II), platinum (IV) or cisplatin. Transiently expressed hTLR4 levels were assessed by immunoblotting, which revealed that hTLR4 WT and hTLR4 H456A-H458L levels were comparable, indicating these variants do not induce gross destabilization of hTLR4 (Fig. 3A). Further supportive of this conclusion, no difference in IL-8 secretion was observed between WT and variant TLR4 when stimulated with 1 ng/mL LPS in the presence of MD-2 (Fig. 3B). As expected, the hTLR4 H456A-H458L variant was impaired in IL-8 secretion in response to 200 µM nickel compared to wild-type hTLR4 (Fig. 3C). These data indicate that while not impacting global TLR4 structure, the histidine variants do impair metal activation of hTLR4. Interestingly, the histidine variant TLR4 had differential effects on responses to platinum (II), platinum (IV) and cisplatin. Similar to the nickel phenotype, the replacement of His456/458 completely abrogated IL-8 secretion in response to 100 μM platinum (II) (Fig. 3D). By contrast, His456/458 replacement only partially reduced IL-8 secretion induced by 100 µM platinum (IV) (Fig. 3E) and 25 µM cisplatin (F). Here, variant TLR4 activation was reduced 5-fold in response to platinum (IV) and 2.5-fold in response to cisplatin while retaining significant IL-8 secretion compared to the negative control (EV). Taken together, the data suggests that the direct interactions between cisplatin and hTLR4 occur, in part, due to the metalbinding properties intrinsic to hTLR4.

#### Cisplatin and nickel use distinct mechanisms to bind TLR4

Previous investigations have identified critical differences in how mTLR4 and hTLR4 interact with potential agonists. For example, mTLR4 does not contain histidines at conserved positions 456 and H458. Our blocking experiments with soluble mouse TLR4 and the differential impact of His456 and His458 mutation on TLR4 activation by nickel and cisplatin hinted at differences in the ways these agonists interact with TLR4. To further explore this concept, we investigated nickel and cisplatin binding to soluble mouse TLR4 in the MST assay. Unlike our experiments with human TLR4, we were unable to detect binding of nickel to mouse TLR4 within the same range of concentrations (0.49-500 μM) by MST as no curve could be fit to the agonist binding model (Fig. 4A).

By contrast, mouse TLR4 was able to bind cisplatin within a similar range of tested concentrations (0.29-1665  $\mu$ M) as indicated by a curve fit of R<sup>2</sup> = 0.72 to the agonist binding model. The interaction of cisplatin and mouse TLR4 in the MST assay occurred with a lower apparent affinity  $K_d$  = 104.9  $\mu$ M (95% confidence interval of 52.24-205.5  $\mu$ M) compared to hTLR4. To determine if these affinities were appreciably different we normalized the MST data to "Fraction bound" and compared the curve fit parameters (Fig. S1). These analyses showed that affinity of cisplatin significantly increased by ~2.5-fold for hTLR4 compared to mTLR4 (P < .0002).

## **8. DISCUSSION**

The connection between TLR4 and the development and severity of CITs has been a key area of investigation. TLR4 has been linked molecularly to CITs; TLR4 activation can induce the production of reactive oxygen and nitrogen species, apoptosis, and pro-inflammatory signalling pathways, e.g., NF-κB. Yet, the molecular details of this linkage were unclear.

Initial models suggested that DNA damage generated by cisplatin could elicit the release of DAMPs that could then be subsequently detected through PRRs such as TLRs (Miller *et al*, 2010; Manohar & Leung, 2018). The relevance of TLR4-specific DAMPs to CIT development remains inconclusive, however. Cisplatin has minimal effects on TLR4-specific DAMP expression (Zhang *et al*, 2008) and in our previous work, we established that DAMP signalling can be differentiated from cisplatin-induced TLR4 activation, since TLR4 activation in response to HMGB1 depended on the MD-2 co-receptor, in contrast to cisplatin (Babolmorad *et al*, 2021). Alternatively, others have posited that cisplatin-induced activity was dependent on synergies with other TLR4 ligands, such as LPS (Oh *et al*, 2011).

This work uncovers new molecular insights on the interaction between cisplatin and TLR4, supporting a role for direct activation of TLR4 by cisplatin. Our use of microscale thermophoresis with recombinant soluble TLR4 protein removes confounding factors normally associated with working with cell-based assays. Using MST, we have shown that TLR4 can bind nickel, as implied in the literature, and also cisplatin, without the need for any additional cellular factors, *e.g.*, MD-2. Nevertheless, while these findings indicate that cisplatin is sufficient to bind and activate TLR4, it does not rule out the involvement of other TLR4 agonists, *e.g.*, LPS or DAMPs, that may contribute to TLR4 activation during CITs *in vivo*.

Our studies also identified intriguing distinctions in how mTLR4 and hTLR4 bind cisplatin and other metal agonists that requires further characterization. Our work confirmed that histidines 456 and 458 of human TLR4 are critical for activation by nickel but their contribution to activation by cisplatin was less definitive, since replacement of these residues retained considerable activity. It should be noted that while we interpreted the activation of hTLR4 H456A-H458L by LPS as an indication that these variants did not

create global perturbations in TLR4 structure, we cannot rule out the fact that MD-2 may have stabilized general perturbations induced by histidine replacement. Nevertheless, one explanation for the residual activation of His variant TLR4 by cisplatin, as well as the direct binding of cisplatin to mTLR4 is that additional residues that are conserved in human and mouse TLR4 contribute to cisplatin binding. One candidate is His431, a residue known to contribute to metal-induced hypersensitivities (Schmidt *et al.* 2010).

Our evidence of direct mTLR4-cisplatin binding justifies follow-up studies to evaluate the role of H431 in cisplatin binding to mTLR4 and hTLR4. H431 (but not H456/458) also exists on the zebrafish *tlr4bb* gene. We previously showed that zebrafish neuromasts, structurally analogous to human ear hair cells, are susceptible to cisplatin-induced toxicity in a *tlr4*-dependent manner (Babolmorad *et al*, 2021).

Future work should seek to better characterize the chain of events that follow from direct TLR4 binding. Metal agonists are known to induce TLR4 homodimerization and elicit TLR4 downstream signalling and activation differently, with some only capable of facilitating homodimerization without activation compared to others (Raghavan *et al*, 2012). Cisplatin, platinum ions, and perhaps other platinum-based agonists may thus interface with TLR4 to induce homodimerization and activation to different degrees.

In summary, our data provides evidence for direct TLR4-cisplatin binding interactions that are enhanced by, but are not dependent on, TLR4-metal-binding interactions. This is reinforced by our prior work wherein we showed that both mTLR4 and hTLR4 were mediators of cisplatin-induced ototoxicity that can be chemically targeted to mitigate cisplatin toxicity. Moreover, our findings argue for distinct mechanisms of TLR4 activation by cisplatin, nickel and LPS (Figure 5). This raises the attractive opportunity of selectively inhibiting TLR4 activation rather than ablating its function entirely. A precision treatment to reduce cisplatin-induced toxicities, while preserving TLR4 bacterial detection, would be an ideal otoprotectant to optimize the safety of cisplatin treatment.

#### 9. ACKNOWLEDGEMENTS

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# 10. AUTHOR CONTRIBUTIONS

Participated in research design: Domingo I, Groenendyk J, Michalak M, Bhavsar A

Conducted experiments: Domingo I.

Contributed new reagents or analytic tools: Michalak M, Bhavsar A

Performed data analysis: Domingo I, Groenendyk J, Michalak M, Bhavsar A

Wrote or contributed to the writing of the manuscript: Domingo I, Groenendyk J, Michalak M, Bhavsar A

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12. FOOTNOTES

**Conflicts of Interest** 

The authors declare that they have no conflict of interest.

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## 13. FIGURE LEGENDS

FIGURE 1. Soluble recombinant TLR4, TLR4(s), can inhibit TLR4-activated pro-inflammatory IL-8-secretion to distinct agonists in HEK293 hTLR4 cells. (A) IL-8 secretion of HEK293-hTLR4 cells pre-treated with 0.1 nM soluble recombinant human TLR4 and subsequently treated with either 1 ng/mL LPS, 200 μM Ni<sup>2+</sup>, or 25 μM cisplatin. Data are shown as the percentage of TLR4 activity in the absence of soluble hTLR4. Nil representative of cells treated without agonists (*n* = 4 independent biological replicates for all conditions). (B) IL-8 secretion of HEK293-hTLR4 cells pre-treated with 0.1 nM soluble recombinant mouse TLR4 and subsequently treated with either 1 ng/mL LPS, 200 μM Ni<sup>2+</sup>, or 25 μM cisplatin. Data are shown as the percentage of TLR4 activity in the absence of soluble mTLR4 (*n* = 4 for all conditions). Nil representative of cells treated without agonists
Data Information: For all panels, all individual data points (technical replicates) from each experiment are plotted with a central bar for the mean and whiskers denoting SD (standard deviation). Statistical analyses were performed through 2-way ANOVA and Bonferroni multiple testing correction.

\*\*\*\*\*, *P* < 0.0001; ns, not significant.

FIGURE 2. Human TLR4 can directly bind nickel and cisplatin. Microscale thermophoresis analysis showing normalized fluorescence of hTLR4 plotted against the indicated concentrations of (A) LPS (*n* = 5 independent replicates), (B) Ni<sup>2+</sup> (*n* = 3 independent replicates), (C) Ca<sup>2+</sup> (*n* = 6 independent replicates) or (D) cisplatin (*n* = 3-6 independent replicates), respectively.

Data Information: For all panels, data fitted to non-linear best-fit curves follow three parameters with a standardized slope. (A) Apparent K<sub>d</sub> unavailable/undetectable. (B) Apparent K<sub>d</sub> = 2.72 μM, R<sup>2</sup> = 0.695.

(C) Apparent K<sub>d</sub> unavailable/undetectable. (D) Apparent K<sub>d</sub> = 50.57 μM, R<sup>2</sup> = 0.927.

FIGURE 3. Histidine 456 & 458 mutations (H456A-H458L) reduce, but do not abrogate TLR4 activation by cisplatin. (A) HEK293-Null2 cells were transfected with either empty vector (EV), hTLR4, or hTLR4 mutant constructs and immunoblotting of lysate was used to analyze relative TLR4 protein levels. Images from different parts of the same gel are shown. IL-8 secretion from HEK293-Null2 cells transfected with either nothing (control), empty vector (EV), hTLR4, or hTLR4 mutant constructs and subsequently treated with (B) 1 ng/mL LPS, (C) 200  $\mu$ M Ni<sup>2+</sup>, (D) 100  $\mu$ M platinum (II) [Pt(II)], (E) 100  $\mu$ M platinum (IV) [Pt(IV)], or (F) 25  $\mu$ M cisplatin displayed as a percentage of the response elicited by wild-type hTLR4 (n=4 independent biological replicates for experiments performed with LPS, Ni<sup>2+</sup>, and Pt(II); n=3, independent biological replicates for experiments involving Pt(IV) and cisplatin). Cells treated with LPS were also co-transfected with an MD-2 construct.

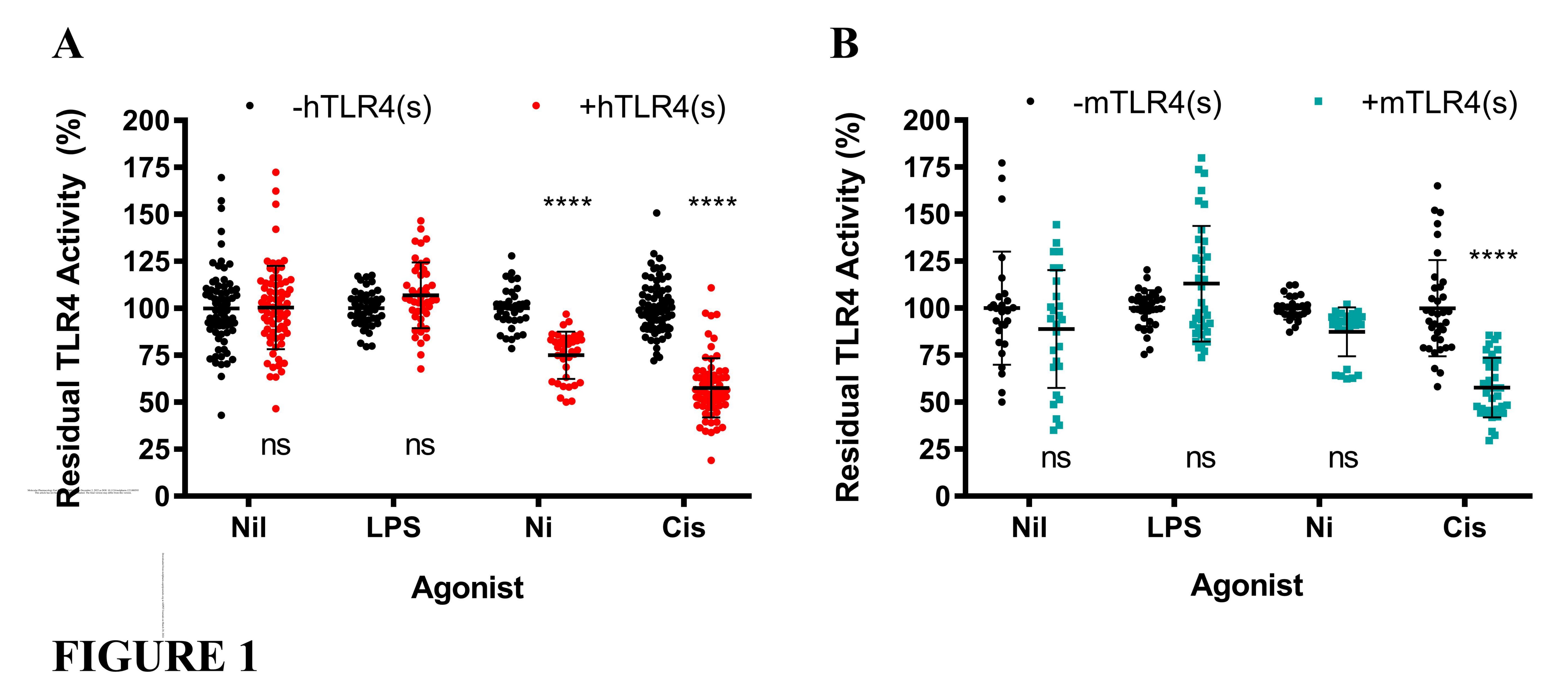
Data Information: For panels B-F, individual data (technical replicates) from each experiment are plotted with the mean and SD (standard deviation) displayed. Statistical analyses were performed through 1-way ANOVA (for all remaining experiments) with Bonferroni multiple testing correction. \*, P < 0.05; \*\*P < 0.01; \*\*\*\*, P < 0.001; \*\*\*\*, P < 0.0001; n.s., non-significant.


FIGURE 4. Mouse TLR4 directly binds cisplatin but not nickel. Microscale thermophoresis analysis showing normalized fluorescence of mTLR4 plotted against the indicated concentrations of (A)  $Ni^{2+}$  (n = 3 independent replicates) or (B) cisplatin (n = 3-4 independent replicates).


Data Information: (A) Apparent  $K_d$  unavailable/undetectable. (B) Apparent  $K_d = 104.9 \mu M$ ;  $R^2 = 0.721$ .

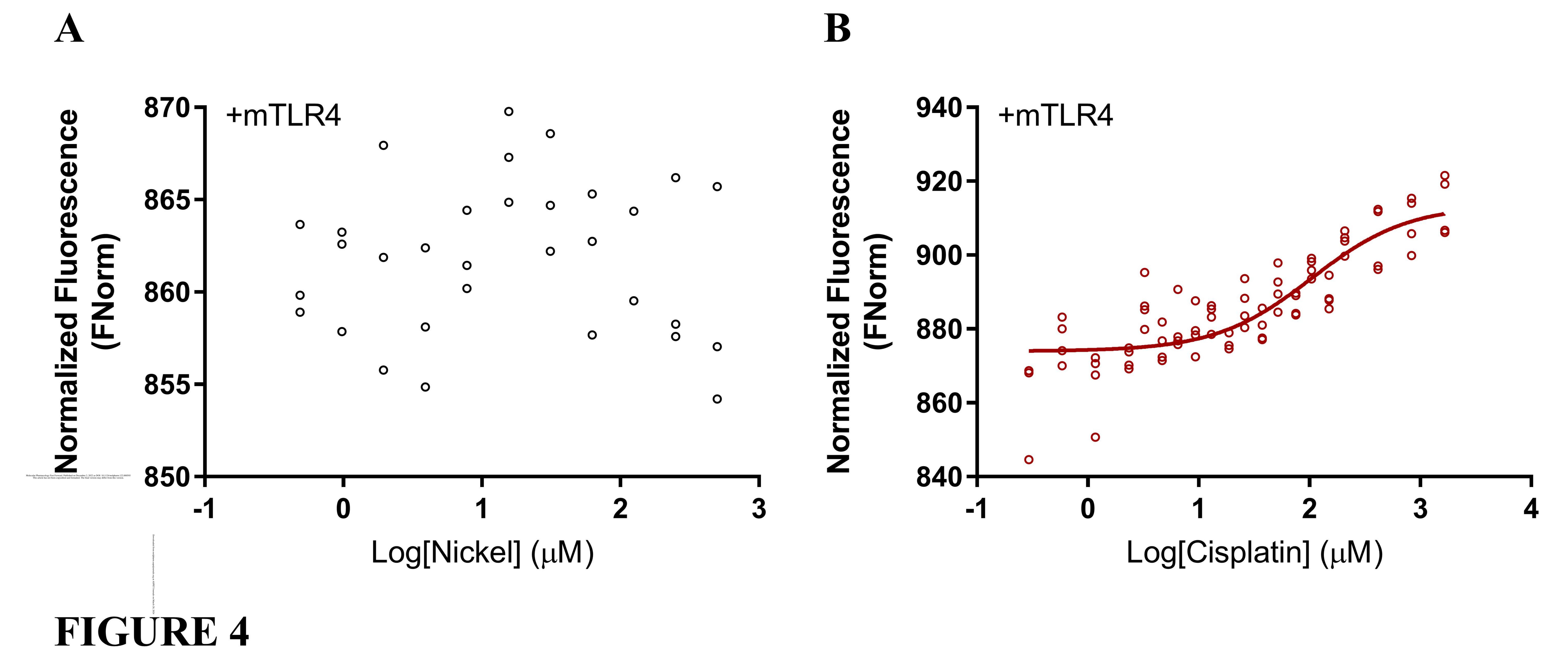
FIGURE 5. TLR4 is activated by LPS, nickel and cisplatin by distinct mechanisms. (Left panel) LPS requires MD-2 for TLR4 activation and cannot directly bind soluble TLR4 species. (Middle panel) Nickel can directly bind human TLR4 and this requires His456/His458. Soluble hTLR4 can block nickel activation of TLR4 (denoted by black line). Nickel cannot bind to mouse TLR4, which lacks these histidine residues. (Right panel) Cisplatin can directly bind both human and mouse TLR4 and this is not strictly dependent on His456/His458.

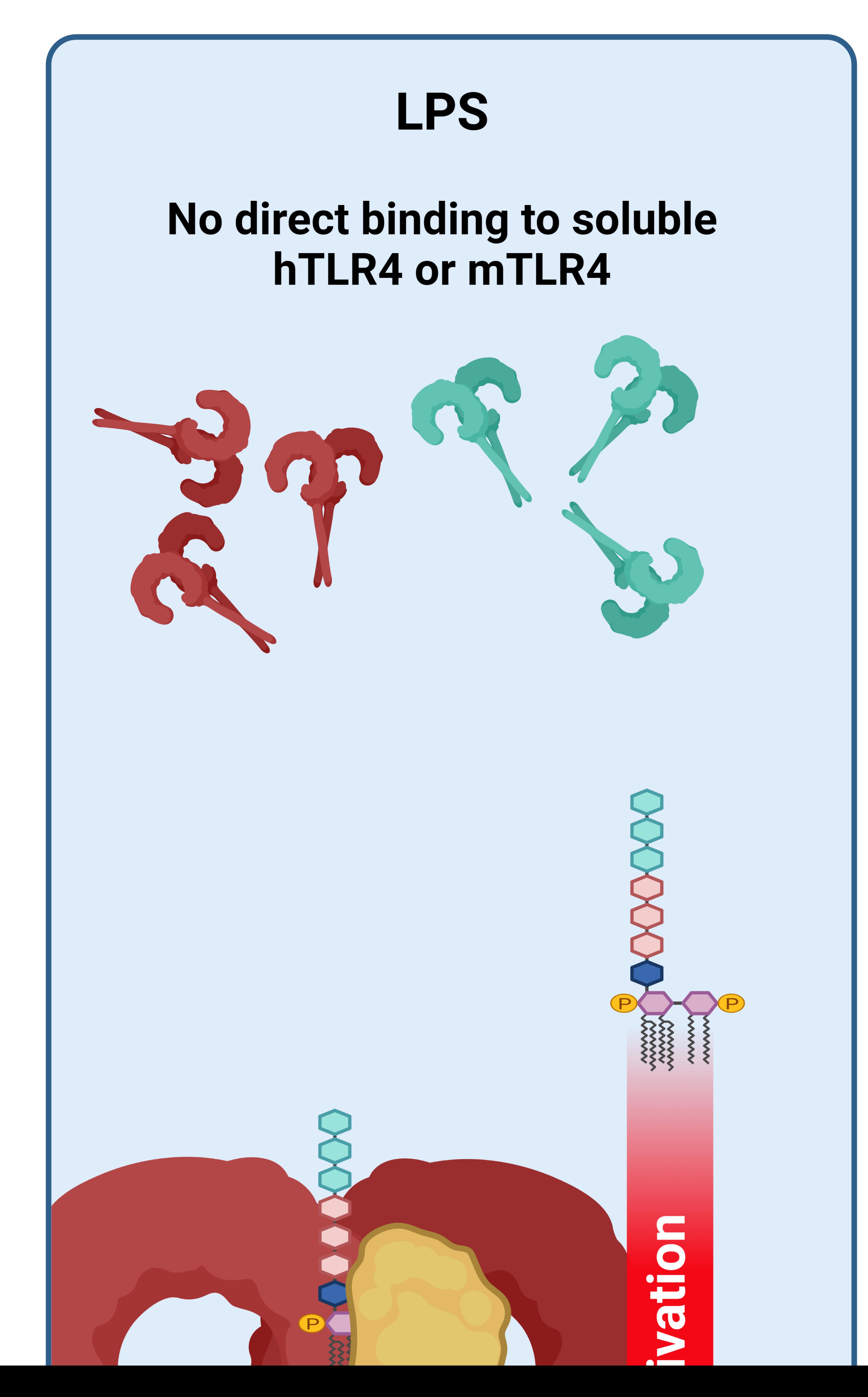
Cisplatin has higher affinity for soluble hTLR4, which is more effective at blocking cisplatin activation of TLR4, compared to soluble mTLR4 (denoted by solid black vs. grey dashed lines). The additional TLR4 residues that contribute to cisplatin binding remain to be elucidated (denoted by ?). Figure was created with BioRender.com.

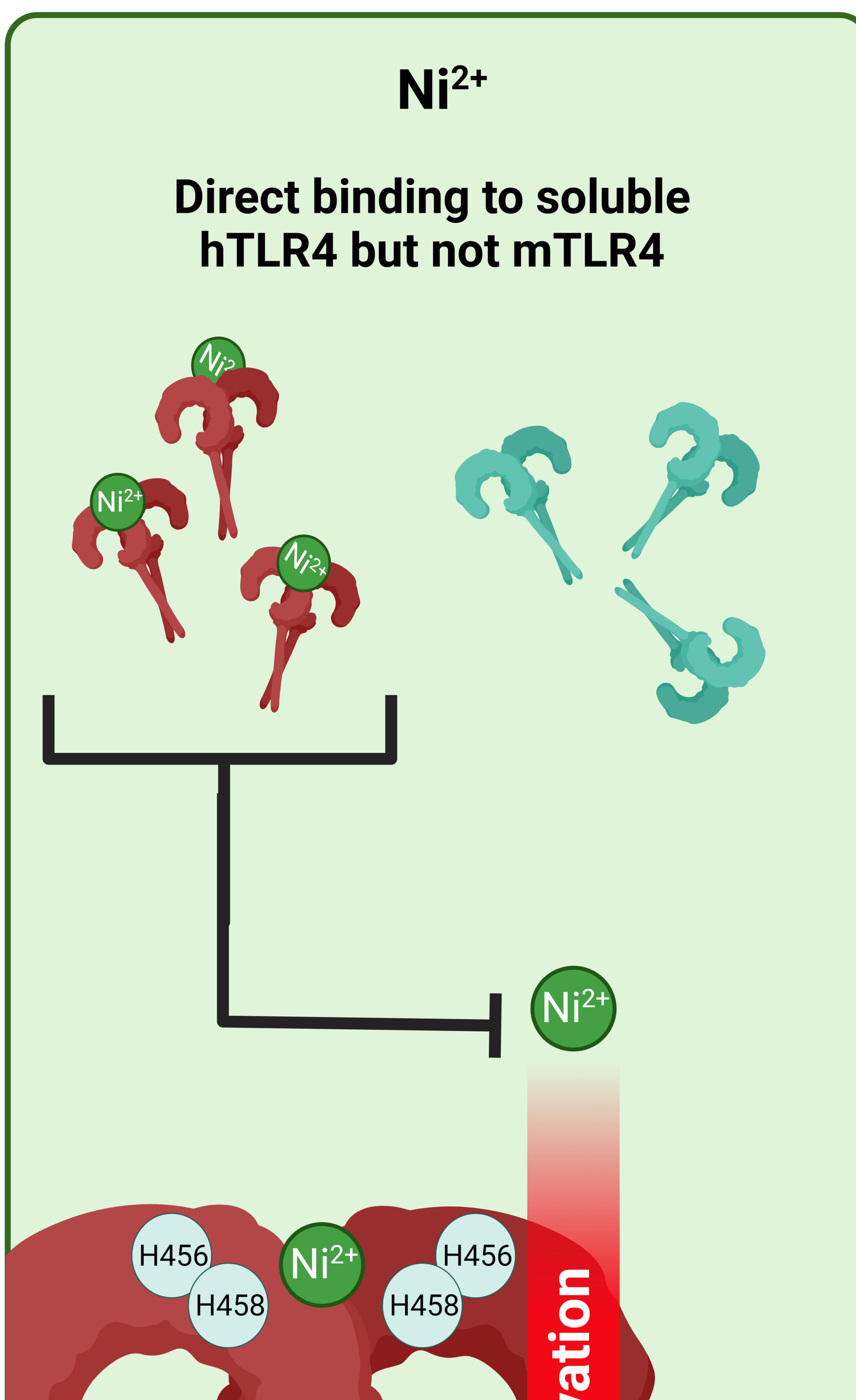


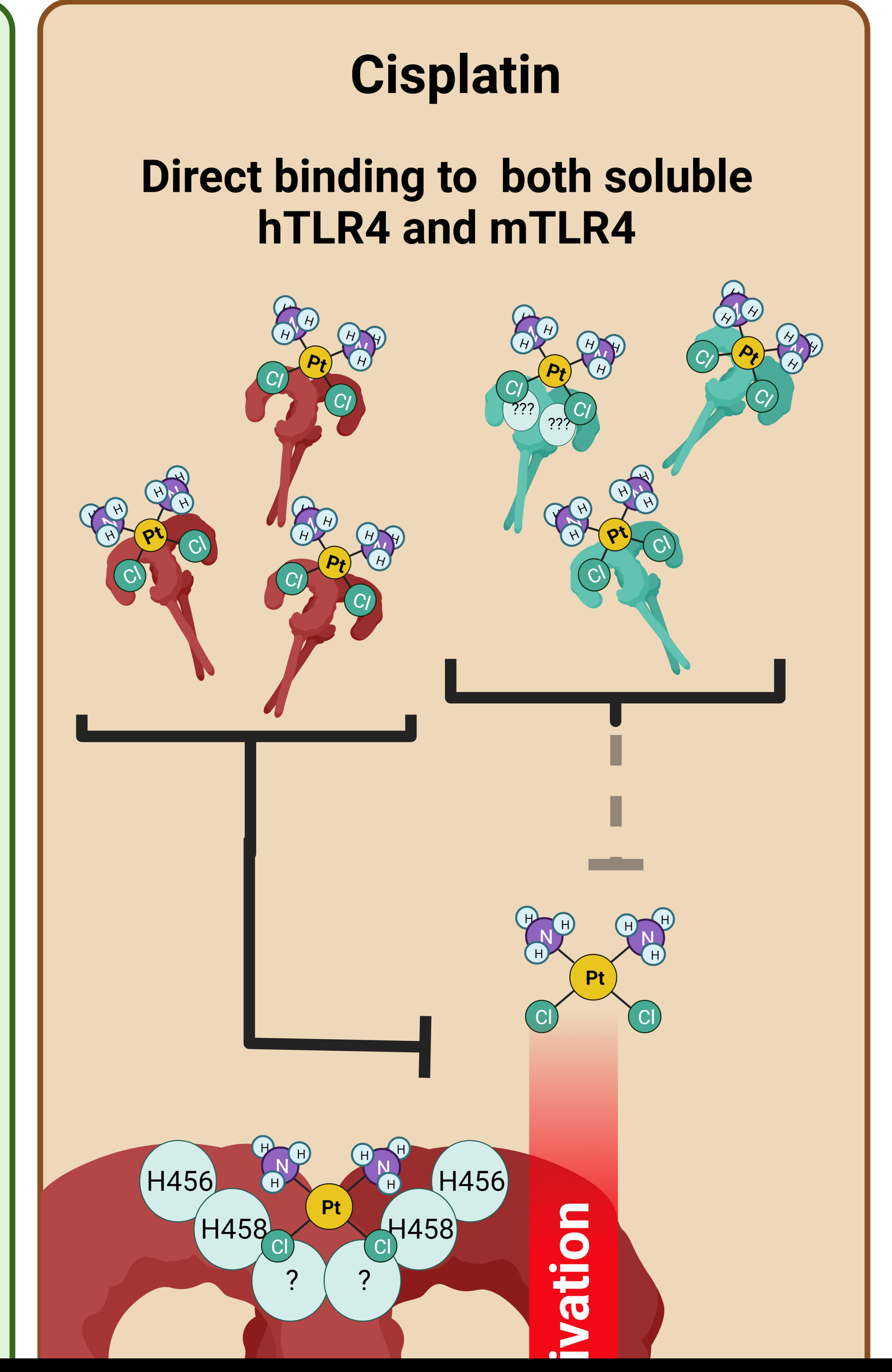










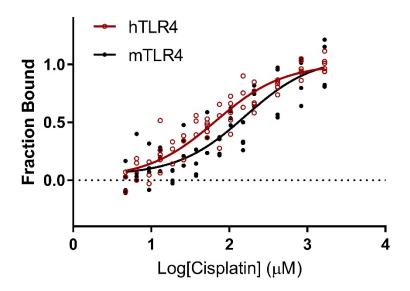


# **Molecular Pharmacology**

# Cisplatin toxicity is mediated by direct binding to Toll-like Receptor 4 through a mechanism that is distinct from metal allergens

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**SUPPLEMENTAL FIGURE 1. Cisplatin binds to hTLR4 with higher affinity than mTLR4.** Depicted is a direct comparison of cisplatin binding curves to hTLR4 and mTLR4 over the same cisplatin concentration ranges. Microscale thermophoresis data was normalized by determining the fraction of ligand bound (also accounts for differences in number of tryptophan residues in TLR4 species).

Data Information: Data were fit to a non-linear regression log(agonist) 3-parameter model. hTLR4 Apparent  $K_d$  = 66.55  $\mu$ M, (95% Confidence Interval of 44.29-99.46  $\mu$ M);  $R^2$  = 0.90. mTLR4 Apparent  $K_d$  = 161  $\mu$ M (95% Confidence Interval of 87.85-300.9  $\mu$ M);  $R^2$  = 0.783. Comparison of the derived  $K_d$  parameter using the Extra Sum of Squares F-Test provides a P < 0.0002, indicating that the hTLR4 and mTLR4 binding data cannot be described by the same curve.