Investigating and characterizing the direct binding interactions between chemotherapeutic cisplatin and pattern recognition receptor, Toll-like Receptor 4

by

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ABSTRACT

Cisplatin is one of the most effective chemotherapeutic agents available. Unfortunately, adverse drug reactions - cisplatin-induced toxicities - often limit the use of cisplatin. These toxicities develop in at least half of those treated, are diverse, and are especially problematic for pediatric patients. Research into the development of these toxicities has repeatedly implicated the innate immune receptor, TLR4 (Toll-Like Receptor 4), in the process. TLR4 usually helps promote immune response to bacterial pathogens, but it is also known to do the same towards metal allergens, like nickel. Because cisplatin-induced toxicities are associated with TLR4-dependent immune responses and cisplatin has a metal (platinum) core, I explored the possibility of direct binding interactions between TLR4 and cisplatin.

I first probed for direct binding interactions (cisplatin:TLR4 interactions) by pre-treating HEK293-hTLR4 cells with soluble mouse (mTLR4) or human TLR4 (hTLR4). I then examined the effect on TLR4 activation by several agonists. Here, I found that both mTLR4 and hTLR4 can impede endogenous TLR4 activation in response to cisplatin. Through microscale thermophoresis, I acquired evidence for direct binding interactions between hTLR4, nickel, and cisplatin. To my knowledge, this is the first explicitly direct demonstration of TLR4-metal allergen binding. To ascertain whether known metal-binding TLR4 residues, H456 and H458, were responsible for cisplatin binding and pro-inflammatory IL-8 secretion in response to platinum ions, I created and tested the responsiveness of mutant hTLR4 constructs. In doing so, I discovered that these residues only enhance an underlying capacity for TLR4s to bind and respond to cisplatin. Returning to microscale thermophoresis, I determined that mTLR4 could also bind cisplatin directly, despite being incapable of binding nickel.

Ultimately, I not only provide evidence for direct binding events between hTLR4 and cisplatin but also characterize some of the features involved in this process. As a result, I raise the possibility that there are additional, conserved, residues specific and sufficient for binding cisplatin and platinum ions.

PREFACE

This thesis contains original work that I, Ivan Kristell Domingo, completed in full or made substantial contributions to. The protocol for Microscale Thermophoresis (MST) outlined in Chapter 2 was optimized with the aid of Jody Groenendyk from the Michalak Lab.

This thesis will also reference original and collaborator data from co-authored publications. Figures 5 and 6 (and associated texts) in particular are derived from the publication:

"Babolmorad G, Latif A, Domingo IK, et al. Toll-like receptor 4 is activated by platinum and contributes to cisplatin-induced ototoxicity. *EMBO Rep*. 2021;22(5):e51280. doi:10.15252/embr.202051280"

I was responsible for designing and completing the experiments involving the use of siRNA. I worked with fellow graduate student, Asna Latif, to design and complete the experiments quantifying IL-8 secretion in response to different agonists. Cole Delyea was responsible for the design and completion of experiments measuring NF-κB activation. The manuscript and remainder of the data in the cited publication was completed by PhD student, Ghazal Babolmorad. Zebrafish experiments that will be subsequently alluded to were completed by Niall Pollock of the Ted Allison Lab.

The Appendix also contains data published in collaboration:

"Meijer AJM, Diepstraten FA, Langer T, et al. TCERG1L allelic variation is associated with cisplatin-induced hearing loss in childhood cancer, a PanCareLIFE study. *npj Precision Oncology*. 2021;5(1):64. doi:10.1038/s41698-021-00178-z"

I was only responsible for the design and execution of the functional assays noted. Dr. Meijjer and her colleagues were responsible for the genomic studies.

ACKNOWLEDGEMENTS

Thank you to everyone who has put up with me for the past 5 years.

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LIST OF ABBREVIATIONS

ALR	AIM (Absent-In Melanoma)-2 Like Receptor
AP1	Activator Protein 1
API5	Apoptosis Inhibitor 5
APR	Acute Phase Reactants / Acute Phase Proteins
ATF	Activating Transcription Factor
Bcl	B-Cell Lymphoma
BLITZ	Biolayer Interferometry
CaM	Calmodulin
CD	Cluster of Differentiation
CFP	Cyan Fluorescent Protein
CIH	Cisplatin-Induced Hepatotoxicity
CIC	Cisplatin-Induced Cardiotoxicity
CIN	Cisplatin-Induced Nephrotoxicity
CIO	Cisplatin-Induced Ototoxicity
CIPN	Cisplatin-Induced Peripheral Neurotoxicity
CLR	C-Type Lectin Receptor
CREB	cAMP (Cyclic Adenosine Monophosphate) Response Element Binding
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRP	C-Reactive Protein
Cryo-EM	Cryogenic Electron Microscopy
Ctr1	Copper Transporter 1
Ctr2	Copper Transporter 2
DAMP	Damage Associated Molecular Pattern
DMEM	Dulbecco Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
ELISA	Enzyme Linked Immunosorbent Assay
EV	Empty Vector
FRET	Fluorescent Resonance Energy Transfer
GWAS	Genome Wide Association Study
HEK293	Human Embryonic Kidney 293 Cells
HIV	Human Immunodeficiency Virus
HMGB1	High Mobility Group Box 1
HSP	Heat Shock Protein
HUVEC	Human Umbilical Vein Endothelial Cells
IBD	Inflammatory Bowel Disease
IFN	Interferon
IKK	IκB (I Kappa B) Kinase
IL	Interleukin
IRAK	Interleukin 1 Receptor Associated Kinase
KC	Keratinocyte-Derived Chemokine
LBP	LPS Binding Protein
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
Ly96	Lymphocyte Antigen 96
MAF	Musculoaponeurotic Fibrosarcoma

MAPK	Mitogen Activated Protein Kinase
MD-2	Myeloid Differentiation Factor 2
MST	Microscale Thermophoresis
MTT	3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide
MyD88	Myeloid Differentiation Factor 88
NF-κB	Nuclear Factor Kappa B
NLR	NOD (Nucleotide Oligomerization Domain)-Like Receptor
OCT2	Organic Cation Transporter 2
ORF	Open Reading Frame
PAUF	Pancreatic Adenocarcinoma Upregulated Factor
pCMV	Plasmid Cytomegalovirus
PRR	Pattern Recognition Receptor
PAMP	Pathogen Associated Molecular Pattern
RIPA	Radioimmunoprecipitation Assay
RLR	RIG (Retinoic Acid-Inducible Gene)-Like Receptor
RPS3	Ribosomal Protein S3
SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
siRNA	Silencing RNA
SLE	Systemic Lupus Erythematosus
SNP	Single-Nucleotide Polymorphism
SPR	Surface Plasmon Resonance
TAB	TGF-β Activated Kinase Binding Protein
TAK	TGF-β Activated Kinase 1
TAK242	Resatorvid
TBK	TANK (TRAF-Family-Member Associated NFκB Activator) Binding Kinase
TBS	Tris (Trisaminomethane)-Buffered Saline
TCERG1L	Transcription Elongation Regulator 1-Like Protein
TGF-β	Transforming Growth Factor Beta
TIR	Toll/Interleukin-1 Receptor
TIRAP	TIR-Domain-Containing Adaptor Protein
TMB	3,3',5'5-tetramethylbenzidine
TNFα	Tumour Necrosis Factor α
TNFR	Tumour Necrosis Factor Receptor
TLR	Toll-Like Receptor
hTLR4	Human Toll-Like Receptor 4
mTLR4	Mouse Toll-Like Receptor 4
zTLR4	Zebrafish Toll-Like Receptor 4
TRAF	Tumour-Necrosis-Factor Receptor Associated Factor
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-Domain-Containing Adaptor-Inducing Interferon β
TWEEN	Polysorbate 20
YFP	Yellow Fluorescent Protein

CHAPTER 1 - INTRODUCTION

SECTION 1 – CISPLATIN & CISPLATIN-INDUCED TOXICITIES

1.1.1 - Cisplatin

Cisplatin is one of the most powerful chemotherapeutic agents available. Cisplatin, formally known as *cis*-diamminedicholoroplatinum (II), has been in use for decades and continues to be used to treat a wide variety of solid-state cancers[1,2] – ranging from head-and-neck[3] and lung,[4] to ovarian[5] and testicular.[6] It was the first metal chemotherapeutic agent ever developed and the first platinum-based chemotherapeutic agent approved by the US Food and Drug Administration (FDA).[7] It has been used to treat malignancies independently and as part of treatment regimens. It is currently used as an adjuvant chemotherapeutic agent for use following surgical intervention or used in conjunction with radiation therapy.[8,9]

Consisting of just a platinum (II) ion bound to two amide and two chloride groups, cisplatin owes its antineoplastic activity primarily to its ability to limit safe cell division (Figure 1A).[1] Being a relatively simple and extremely small molecule, cisplatin enters cells through both passive diffusion and active transport mechanisms. Once inside, cisplatin changes into its reactive form – replacing its chloride groups with hydroxides through either mono- or di-aquation and intercalates into the DNA of reproducing cells by binding onto nucleophilic purine bases (Figure 1B).[10] While both guanine and adenine are apt targets for electrophilic attack, evidence suggests that guanine is particularly more susceptible. In doing so, it can form both inter-strand and intra-strand adducts and crosslinks that, once accumulated, ultimately reduces DNA access and replication, and inhibits DNA repair. This results cell cycle arrest which culminates in multiple mechanisms of cell death, such as necrosis and apoptosis.[11]

In the clinic, cisplatin is administered through IV in sterile saline solution.[8,10] As such, cisplatin often exists systemically within patients undergoing active treatment. While portions of cisplatin inevitably reach their target tumour cells, significant amounts also remain in the bloodstream; up to 65-95% of the initial dose administered.[12] Such systemic cisplatin does not undergo aquation due to the relative abundance of chloride ions in circulation.[13] As a result, systemic cisplatin is left to either be bound and deactivated by plasma proteins[14] and secreted out of the body in urine,[15,16] or retained for prolonged periods of time, if not indefinitely, in particular organs.[17] This retention, ephemeral or lasting, subsequently leads to the one major drawback of cisplatin, and platinum-based antineoplastic use as a whole: toxicity.

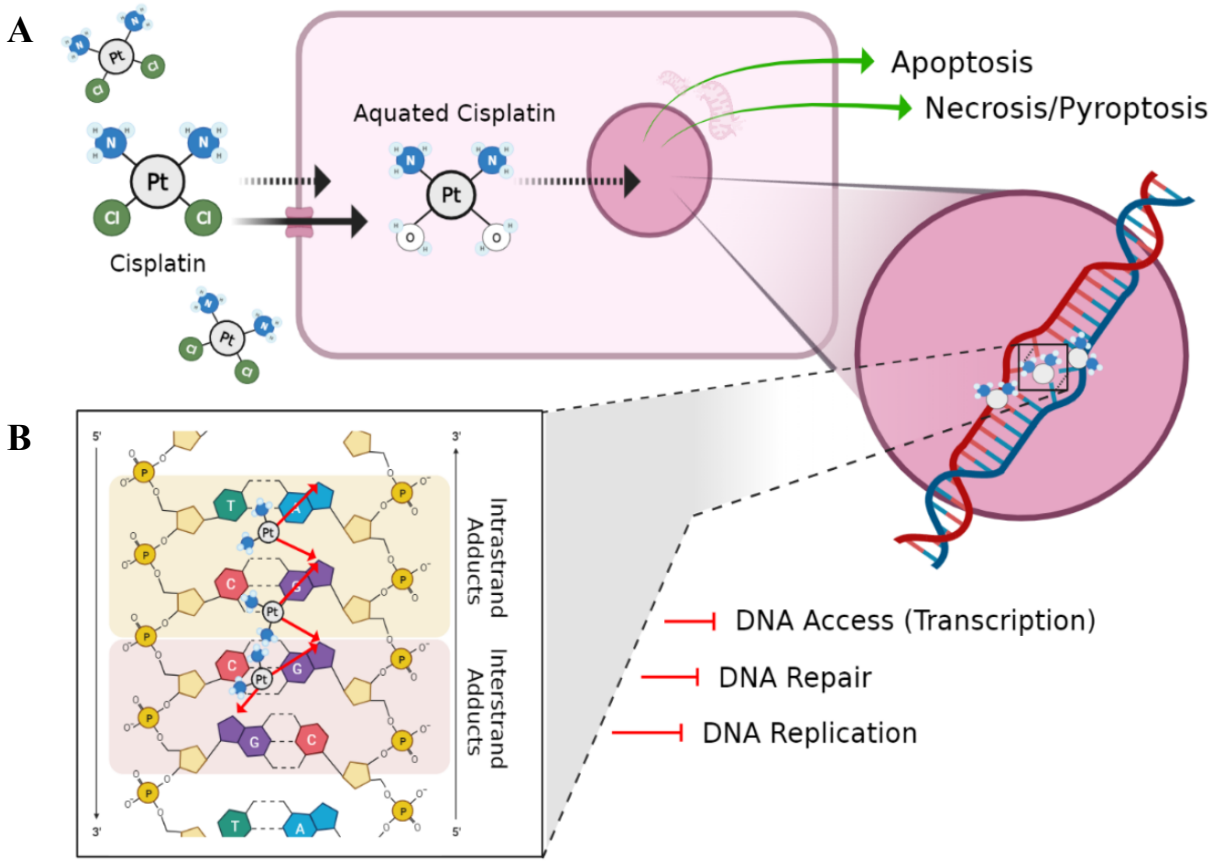


FIGURE 1. Cisplatin anticancer action is largely associated with its active, aquated, state that intercalates into DNA and causes eventual cell death. (A) Structural depiction of cisplatin as described. (B) Summary of cisplatin antineoplastic activity mechanism. Cisplatin primarily enters cells through passive diffusion and active transport. Active transport is facilitated by a wide variety of membrane transport proteins, such as copper transporters, Copper Transporter 1 & 2 (Ctr1 & Ctr2) as well as organic cation transporters, like OCT2.[18] Once inside the cell, cisplatin undergoes rapid changes, some of which are mediated by dedicated metabolic systems, such as the glutathione metabolic system.[19] The most important change that cisplatin undergoes for the function of cancer treatment is aquation – in which its labile chloride groups are 'replaced' by water either singly or as a pair, creating mono- or di-aquated cisplatin respectively. While the original form of cisplatin is itself capable of ultimately entering the nucleus and creating both intra-strand and inter-strand DNA crosslinks, the aquated forms, mono-aquated especially, have been shown to be several magnitudes more reactive.[20] Following the production and accumulation of these adducts,[21] there is an established marked reduction in DNA access for protein synthesis, repair, and replication.[22] These effects eventually lead to cancer cell death, with cells possessing innate DNA repair deficiencies being the most sensitive to the formation of cisplatin-DNA lesions.[23–26] Cisplatin-induced DNA damage is also not limited to genomic DNA; cisplatin can also produce similar structures in mitochondrial DNA.[27]

1.1.2 – Cisplatin-Induced Toxicities

Cisplatin-induced toxicities (CITs) come in various forms, but most are situated in regions of the body that either are intrinsically sensitive or are exposed to cisplatin longer than clinically intended. While there are rarer cases of cisplatin-induced hepatotoxicity (CIH)[28,29] and cisplatin-induced cardiotoxicity (CIC),[30] the three most common CITs fit the aforementioned description and are cisplatin-induced nephrotoxicity (CIN),[31–33] cisplatin-induced ototoxicity (CIO),[34–36] and cisplatin-induced peripheral neuropathy/neurotoxicity (CIPN).[37,38] It is important to note that while cisplatin does have intrinsically harmful properties as discussed, such properties are most effective, if not predominantly effective, on cells undergoing active replication. As such, CITs are believed to be linked to additional mechanisms beyond that of cisplatin-mediated cell death through DNA damage.

Cisplatin-induced nephrotoxicity, for example, is particularly linked to the physiological role of kidneys in the filtration of toxins; the rate of exposure to cisplatin is particularly higher in the kidneys than other locations in the body as it mediates the active excretion of the chemical.[39] CIN displays clinically in diverse ways, ranging from reversable or acute kidney tissue injury, to complete renal failure.[40] Luckily, besides the reduction of cisplatin dose used in treatments, CIN can be mitigated if not completely reversed through medical intervention. The standard and sufficient operating procedure at the moment consists of hydration, supplemented with or without mannitol.[41]

Cisplatin-induced ototoxicity, on the other hand, is believed to develop due to the anatomical limitations on filtration imposed by the structure of the inner ear. Evidence suggests that the inner ear, specifically the Organ Corti, can accumulate systemically administered cisplatin functionally indefinitely - being a relative ‘dead end’ within the body.[42,43] Following drug accumulation, there is a substantial loss in the hair cells that mediate the sensorineural reception of specific sound frequencies (starting from 8kHz+ to lower frequencies with increased severity).[44,45] While CIO can manifest differently in terms of severity and complications between patients for various reasons including age of treatment and dose of treatment, the worst-case-scenario is permanent bilateral hearing loss.[46] Unlike CIN, CIO is specifically insidious given that cisplatin treatment is used extensively in the treatment of malignancies in children and can significantly impair learning, speech, and psychosocial development.[47,48]

Moreover, CIO, unlike CIN, is fundamentally irreversible; limiting cisplatin use at the cost of efficacy is the only clinically-approved option currently available.[49]

Cisplatin-induced neuropathy/neurotoxicity further differs from the former as more of the underlying mechanisms have yet to be elucidated. Currently, data suggests that peripheral neurons are more sensitive cisplatin due to their higher expression of, and reliance on, various active ion transport proteins, such as Ctr1, and ionic balance that could mediate cisplatin influx.[50] In murine models, the CIPN phenomenon connotes to increased sensitivity to pain and extreme temperatures.[51,52] In humans, the onset of CIPN can vary wildly from patient to patient as the prior CITs (and most CITs in general).[53] Like CIO, there are several strategies under review, but no preventative or treatment measures have passed the threshold for clinical use.[54]

Despite all the differences that may exist between CITs, however, there are quite a few similarities that appear to harken to the underlying molecular mechanisms that may be driving or contributing to the onset of disease. One such commonality considered a hallmark of CITs is inflammation – including those associated with the innate immune pattern-recognition receptor, Toll-Like Receptor 4.

SECTION 2 – INNATE IMMUNITY, PATTERN-RECOGNTION RECEPTORS, TOLL-LIKE RECEPTORS & SIGNALLING

1.2.1 – Innate Immunity & Pattern-Recognition Receptors (Overview)

Innate immunity is the branch of the immune system that is responsible for mediating rapid but non-specific responses to toxins and foreign agents, or 'antigens'.[55] It is considered the 'first line of defense' against immune insult and is dependent on both anatomical defenses, such as skin, as well as cellular and chemical processes.[56] One cellular process critical for innate immunity is the activation of pro-inflammatory downstream signalling systems through pattern-recognition receptors (PRRs).[56]

Pattern-recognition receptors bind pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) and trigger downstream signalling mechanisms that culminate in the production of pro-inflammatory factors, such as cytokines and chemokines.[57,58] These serve to propagate and enhance the state of inflammation by recruiting and assisting in the activation of dedicated innate and adaptive immune cells with more direct and comprehensive anti-pathogenic capabilities, such as phagocytosis. There are five types of PRRs, based on protein homology: the Toll-like Receptors (TLRs), the Nucleotide Oligomerization Domain (NOD)-like Receptors (NLRs), the Retinoic Acid-Inducible Gene (RIG)-like Receptors (RLRs), the C-Type Lectin Receptors (CLRs), and the Absent-in-Melanoma (AIM)-2-like Receptors (ALRs). The member receptors of these families are each responsible for the recognition of a specific PAMP or subset of PAMPs.[59] Of these, the TLRs appear to be the most involved in the development of cisplatin-induced toxicities.

1.2.2 – Toll-Like Receptors & Toll-Like Receptor 4 (Overview)

TLRs share the same general structure - consisting of 3 fundamental regions: the leucine-rich repeat (LRR) ectodomain, (2) the transmembrane domain and (3) the Toll/Interleukin-1 (IL-1) Receptor (TIR) cytoplasmic domain. Topologically, this makes TLRs Type I transmembrane proteins. TLR transmembrane domains extend through the lipid bilayers that they localize to and have their N-terminus domains (ectodomains) exposed directly to the extracellular space or endosomal cavity (Figure 2A).[60,61] Contained within their ectodomains are the residues and other structural components that define their target specificity and facilitate direct binding interactions.

13 TLRs have been identified in mammals, TLR1-TLR13, with additional TLRs present in other species (such as birds, frogs, and teleosts), each with differing agonists or sets of agonists (Figure 2B).[62,63] Humans express functional TLR1-TLR10. Mice, on the other hand, can express the full gamut of TLRs but insertion mutations have rendered their TLR10 defective.[62] Of course, the expression of different TLRs can differ not just between species but individuals and cell types as well, with dedicated immune cells expressing TLRs like TLR4 the most. Nevertheless, TLRs can be considered functionally ubiquitous overall; TLRs are not strictly restricted to dedicated innate or adaptive immune cells. The expression of TLRs occurs in all tissues, somatic or germ-line,[64] and their purpose remains the same: detect PAMPs and/or DAMPs and moderate inflammatory responses. With respect to cisplatin-induced inflammatory responses and cisplatin-induced toxicities, the two most relevant TLRs are TLR4 and TLR2, though there are reports of TLR9 involvement as well.[65] Given that this study pertains to the characterization of interactions between cisplatin and TLR4, I will be putting a particular focus on TLR4 and will use it as context to outline the remaining core concepts associated with TLRs.

TLR4 is also a particularly interesting TLR and overall frame of reference. It was one of the first TLRs discovered,[66] and as a function of that, it has become one of the most well-characterized, if not the most well-characterized. It shares traits with distinct subsets of TLRs beyond the structural and archetypical for PRRs and, in a way, possesses traits distinct from most other TLRs. Research has, for example, shown that TLR4 is the one of the few TLRs that could be considered both an extracellular and intracellular TLR. Canonically, TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 encompass the extracellular, plasma membrane-bound, TLRs while TLR3, TLR7-9 and TLR11-13 comprise the intracellular TLRs, targeted towards endosomes and other intracellular compartments.[62] Studies, however, have shown that endosomal functional TLR4 exist and subcellular localization drives changes to signalling dynamics (Figure 2B).[67,68] TLR4 is, however, not especially noteworthy when it comes to the process of ligand binding and signal transduction initiation. Whether they are localized to the plasma membrane or endosomes, all TLRs consequently undergo hetero- or homo-dimerization upon binding their target.[61] This binding can depend on a myriad of intrinsic and extrinsic factors based on the target ligand, such as the availability of certain residues or coreceptors.[62] It is the completion of this step that facilitates signal transduction through the recruitment of different adaptor proteins.[69]

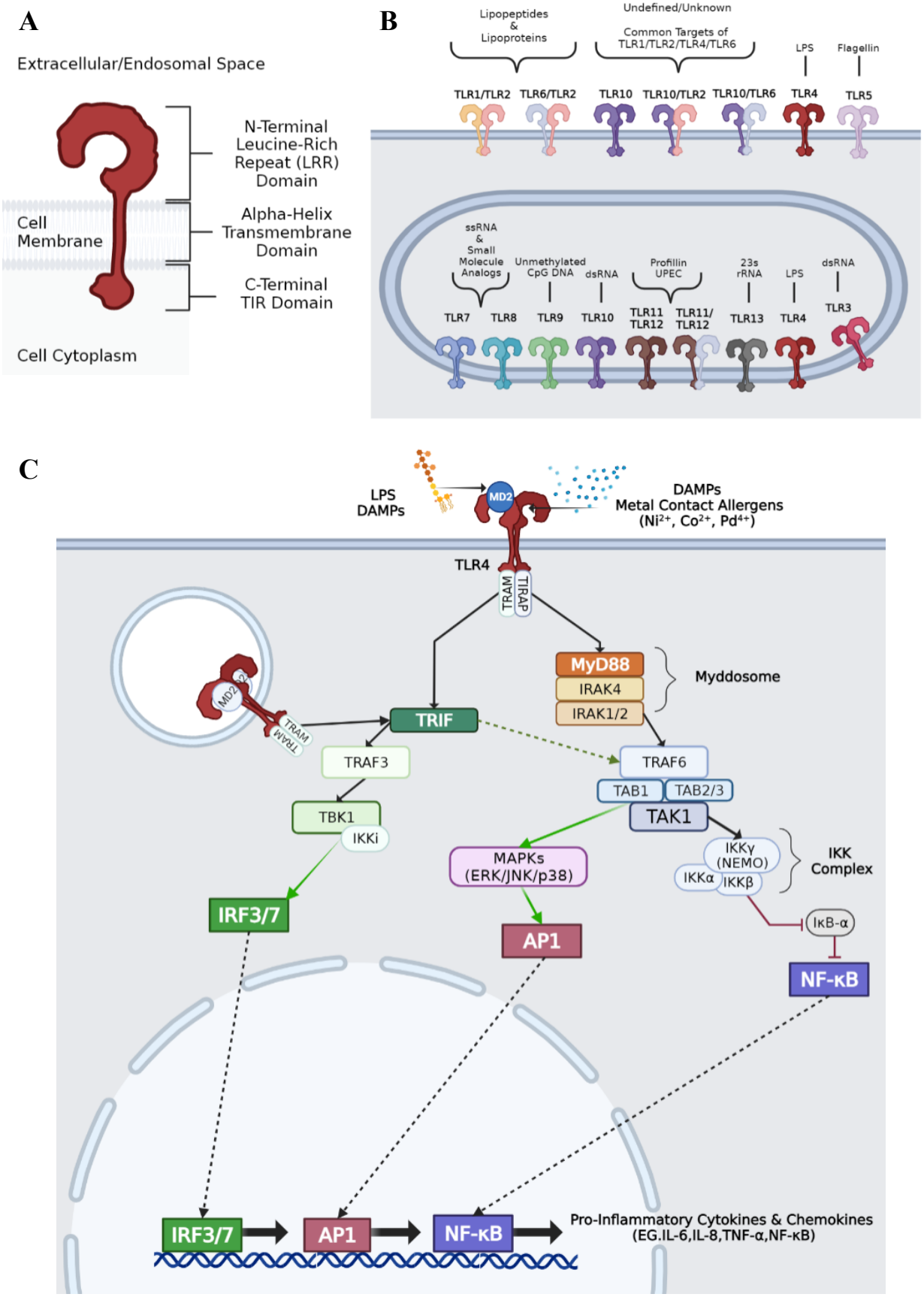
TLR4 is, as mentioned, no exception to this rule; TLR4 must dimerize after binding its chief ligand, LPS (lipopolysaccharide), to initiate downstream signalling.[70] Dimers formed by TLR4 are conventionally homodimeric, but extremely rare cases of pro-inflammatory heterodimers (such as TLR4/TLR2 and TLR4/TLR6) in response to atypical LPS and Amyloid-β fibrils have also been reported recently.[71,72] The PAMP, LPS, is an essential component of Gram-negative, bacterial, outer cell membranes and conventionally can only be bound by TLR4 with the assistance of three coreceptors: (1) MD-2 (Myeloid Differentiation Factor 2), (2) LBP (LPS Binding Protein), and (3) CD14 (Cluster of Differentiation 14).[73] In zebrafish, Ly96 (Lymphocyte Antigen 96) serves as core of their version of TLR4-LPS binding interactions as the ortholog of MD-2 (Figure 2C).[74] TLR4-coreceptor complexes have also been shown to bind and dimerize in response to a multitude of DAMPs.[58,75] For example, direct interactions between TLR4 and HMGB1 (High Mobility Group Box 1) have been demonstrated and appear to be dependent on the presence of MD-2.[76,77] DAMPs capable of binding TLR4 independent of all known coreceptors thus far have also been reported, though the amount of evidence available for each does differ and some remain controversial. These include but are not limited to: (1) Heat Shock Protein B8 (HSP22),[78] (2) Pancreatic Adenocarcinoma Upregulated Factor (PAUF),[79] (3) Apoptosis Inhibitor 5 (API5),[80] and (4) Ribosomal Protein S3 (RPS3).[81] In these instances, the potential for direct binding has either been evaluated indirectly through assays of downstream effectors, or directly through high-throughput biomolecular assays – such as SPR (Surface Plasmon Resonance) or BLITZ (Biolayer Interferometry). Whatever the target, or targets, may be, the successful dimerization of TLRs and subsequent signal transduction and recruitment of specific adaptor proteins results in at least one of three possible signalling pathways: (1) the MyD88 (Myeloid Differentiation Factor 88)-Dependent signalling pathway, (2) the MyD88-Independent/TRIF (TIR-Domain-Containing-Adaptor-Inducing Interferon β Protein) signalling pathway, and (3) the MAPK-associated signalling pathway (Figure 2C).[62] TLR4 is once again unique in this respect, as it is capable of triggering all three signalling cascades as opposed to one combination or the other. The intricacies of these mechanism are outlined further in Figure 2C, but briefly, TLR4 is able to mediate both MyD88-Dependent and MyD88-Independent signalling pathways in conjunction with MAPK signalling through its access to both intermediary adapter proteins, TIRAP (TIR-Domain-Containing Adaptor Protein) and the auxiliary TRAM (TRIF-Related Adaptor Molecule).

TIRAP, also known as MAL (MyD88-Adaptor-Like Protein), uniquely mediates both TLR4 and TLR2 access to MyD88-Dependent signalling, while TRAM uniquely allows TLR4 to recruit the TRIF necessary for MyD88-Independent signalling. TLRs outside of TLR3 and TLR4 are restricted in direct access to MyD88-Dependent and MAPK signalling pathways, while TLR3 is conversely limited in direct access to MyD88-Independent/TRIF and MAPK signalling.

Together, these signalling systems, lead to the activation of transcription factor regulators directly responsible for controlling pro-inflammatory responses, such as IRF3/5/7 (Interferon Regulator 3/5/7), AP-1 (Activator Protein 1), and NF-κB (Nuclear Factor Kappa B) (Figure 2C).[62] IRFs 3/5/7 facilitate inflammation as the primary mediator/s of Type I Interferon expression.[82] Type I Interferons such as Interferon Alpha (IFNα) and Beta (IFNβ) are typically associated with anti-viral immune responses but are also known to influence the development of innate and adaptive immune cells and the persistence of autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus (SLE).[83] API-1 exist as either homodimeric or heterodimeric complexes composed of Jun-family proteins, Fos-family proteins, ATF/CREB (Activating Transcription Factor/cAMP Response Element Binding) family proteins, and MAF (Musculoaponeurotic Fibrosarcoma) family proteins. Depending on the specific subunits involved, different genes can be expressed but as a whole, the genes upregulated encode for pro-inflammatory cytokines and chemokines.[84–86] NF-κB similarly mediates the expression of pro-inflammatory cytokines and chemokines,[87,88] but it also serves as the 'master regulator' for a multitude of critical cell processes. NF-κB is known to regulate the expression of genes that drastically affect, if not dictate, cell homeostasis, cell cycles, and cell maturation and differentiation – all of which can themselves affect the course and outcomes of inflammation downstream.[89–91]

The pro-inflammatory cytokines and chemokines alluded to thus far, refer mostly to:
(1) the pro-inflammatory interleukins (IL), IL-1β and IL-6, (2) the CXC-motif chemokines, CXCL1/KC (Keratinocyte-derived Chemokine) and CXCL8/IL-8, and (3) Tumour-Necrosis Factor α (Tumour Necrosis Factor α).[92] Collectively, these factors sustain and propagate the state of inflammation through autocrine and endocrine communication.

The secretion of pro-inflammatory cytokines, for instance, can drive positive feedback loops that induce the expression of additional pro-inflammatory effectors in cells that have yet to be directly activated by PAMPs or DAMPs. At the same time, chemokines are capable of attracting dedicated immune cells to sites of damage or infection. Extended exposure to IL-6 and TNF-α can be especially dangerous due to their pleiotropic effects. IL-6 receptors are capable of not only activating dedicated immune cells that can then produce their own pro-inflammatory cytokines, but modifying vasculature to facilitate immune cell chemotaxis, and promoting deleterious reactive oxygen species (ROS) production.[93,94] TNF-α, on the other hand, can elicit cell death through apoptosis or necroptosis when bound by TNF-α Receptor 1 (TNFR1), or additional NF-κB activation when bound by TNF-α Receptor 2 (TNFR2).[95,96] Cytokine receptors themselves can trigger downstream signalling pathways that result in the expression of additional cytokines as well as other pro-inflammatory elements - such as acute phase proteins, or reactants (APRs). These can be divided into two subgroups, positive and negative.[97] Positive APRs increase in response to inflammation to promote or regulate the process; prolonged exposure to some, such as CRP (C-Reactive Protein), can itself contribute to pathogenesis and inflammation.[98]


A
Extracellular/Endosomal Space
N-Terminal
Leucine-Rich
Repeat (LRR)
Domain
Alpha-Helix
Transmembrane
Domain
C-Terminal
TIR Domain
Cell
Membrane
Cell Cytoplasm
B
Lipopeptides
&
Lipoproteins
Undefined/Unknown
Common Targets of
TLR1/TLR2/TLR4/TLR6
LPS
Flagellin
TLR1/TLR2
TLR6/TLR2
TLR10
TLR10/TLR2
TLR10/TLR6
TLR4
TLR5
ssRNA
&
Small
Molecule
Analogs
Unmethylated
CpG DNA
dsRNA
Profillin
UPEC
23s
rRNA
LPS
dsRNA
TLR7
TLR8
TLR9
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TLR11
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TLR13
TLR4
TLR3
C
LPS
DAMPs
MD2
DAMPs
Metal Contact Allergens
(Ni2+, Co2+, Pd4+)
TLR4
TRAM
TIRAP
MyD88
IRAK4
IRAK1/2
Myddosome
TRIF
TRAF3
TRAF6
TAB1
TAB2/3
TAK1
TBK1
IKKi
MAPKs
(ERK/JNK/p38)
IKKγ
(NEMO)
IKKα
IKKβ
IKK
Complex
IRF3/7
AP1
IκB-α
NF-κB
IRF3/7
AP1
NF-κB
Pro-Inflammatory Cytokines & Chemokines
(EG.IL-6,IL-8,TNF-α,NF-κB)

FIGURE 2. Toll-Like Receptors, like TLR4, mediate pro-inflammatory responses to various PAMPs, DAMPs, and chemicals through three distinct signalling pathways.
(A) Simplified structure of TLRs as described. (B) Summary figure of known mammalian TLRs, their locations throughout a cell, and their principal ligands. (C) Overview and summary of the primary TLR signalling mechanisms as exemplified by TLR4. TLR4, upon binding its target ligand either independent or with the aid of coreceptors, undergoes dimerization. The combination of intracellular TIR domains allows for the recruitment of adaptor proteins, TIRAP or TRAM. TIRAP subsequently recruits MyD88 through its own TIR domain interactions. This results in the formation of a 'Myddosome' – a complex comprised of the former in conjunction with IRAK4 and IRAK1/2 (interchangeably). This results in the activation of IRAK1, which subsequently recruits and activates TRAF6. TAK1 is then activated by TRAF6, and consequently activates the IKK complex. The IKK complex phosphorylates and targets IκB-α for degradation – allowing NF-κB to enter its active state and translocate to the nucleus. Simultaneously, or conversely in the event of endosomal TLR4 activation, TRAM mediates that recruitment and activation of TRAF3, which leads to the eventual activation of TBK1 and IKKi which can then directly mediate the activation of IRF3. Activated IRF3, like NF-κB, translocates to the nucleus. In both of these instances, the MAPK signalling systems can be concomitantly activated through the TRAF6-TAK1 activated complex; TRIF can access this branch in-directly through the recruitment of other key proteins as connoted by the dashed lines. The activation of the various MAPKs leads to a multiplicity of effects, the most pertinent to immunity being the activation of AP-1. AP-1 follows its fellow transcription factors to the nucleus to assist in the production of pro-inflammatory factors as described.

1.2.3 – Toll-Like Receptor 4 In Cisplatin-Induced Toxicities

As noted earlier, TLR4 is an emerging topic of interest in CIT research. It is one of seven genes implicated in the development of CIPN by transcriptomic analyses.[99] The upregulation of TLR4 occurs in both the kidneys and the cochlea in parallel with the onset of disease.[100–102] TLR4 upregulation and activation attributed to other causes, like septic shock, can even have synergistic effects – worsening the development of cisplatin-induced toxicities.[100,103]

Meanwhile, the deliberate inhibition, or lack of expression, of TLR4 downstream signalling cascade components, such as the MyD88-dependent and MAPK signalling pathways, corresponds to reduced cell death associated with CITs such as CIN and CIO. For example, pharmacological targeting of TAK1, the intermediary between the MyD88-dependent and MAPK signalling pathways, diminishes CIN-associated renal damage.[104] TLR4-deficient (TLR4-/-) and TLR4-signalling deficient mice (C3H/HeJ) experience protection from CIN based on histology that aligns with reduced pro-inflammatory cytokine secretion and MAPK signalling mediator activation.[105,106] TLR4-deficient mice are also subject to reduced neutrophil influx. Interestingly, the primary drivers of CIN appear to be renal TLR4-expressing cells though; wild-type mice transplanted with TLR4-deficient bone marrow still experienced CIN albeit to a lesser extent.[105] In CIO, blocking these signalling branches increased cell viability and reduced NF-κB activation and pro-inflammatory cytokine secretion. In *in-vitro* and zebrafish models of CIO, commercially available TLR4 signalling inhibitor TAK242 increased the survival of fish neuromasts (hair cell orthologs) and fish following cisplatin treatment.[107] The absence of expressed TLR4 itself can also bestow a modicum of protection against CITs. CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) knockout of TLR4 in HEI-OC1 murine inner ear cells abrogates pro-inflammatory responses emblematic of CIO.[107] TLR4 deficiencies also safeguard mice from CIO – translating to retained hearing.[100] Antagonistic binding of TLR4 and the downstream mediator, IRAK1, can effectively reduce the signs and symptoms of chemotherapy-associated neuropathies overall.[108–112] Mice with MyD88 and TRIF deficiencies have increased resistance to cisplatin-induced pain hypersensitivity (mechanical allodynia), a presentation of CIPN.[113,114]

TLR4-dependent downstream effectors - pro-inflammatory cytokines and chemokines - have been shown to influence CIT outcomes further. The most commonly studied pro-inflammatory cytokines and chemokines associated with CITs are IL-1β, IL-6, IL-8, and TNF-α. Disease severity tends to be directly proportional to their secretion. Using exogenous pro-inflammatory cytokines, So et al. (2007) demonstrated that downstream effectors associated with CITs could independently account for up to $1/5^{th}$ of the cell viability loss associated with CIO.[115] Their sequestration of pro-inflammatory cytokines and chemokines with blocking antibodies also reduced cell death following cisplatin treatment. Anti-TNF-α antibodies were the most effective and used in follow-up studies.[116] Faubel et al. (2007) reported similar findings in the context of CIN, except the group found that IL-6 signalling inhibition alone was insufficient for rescue.[117] Pro-inflammatory cytokines, induced or exogenously provided, have also been shown to promote pain and thermal hypersensitivities *in-vivo* - symptoms of chemotherapy-induced peripheral neuropathies like CIPN.[118,119]

SECTION 3 – PROJECT RATIONALE & HYPOTHESIS

1.3.1 – Project Rationale

Given that such a strong correlation exists between the expression of TLR4 and the sensitivity to, and severity of, CITs, possible direct interactions between TLR4 and cisplatin itself warrant further investigation. TLR4 is not limited to binding LPS or DAMPs; as alluded to in Figure 2C, TLR4 has also been shown to activate in response to contact metal allergens – specifically, Group 10 metal allergens. This was first reported on by Schmidt et al. (2010), who were able to demonstrate that nickel-induced pro-inflammatory cytokine secretions (IL-8 and TNF-α) were completely dependent on TLR4 expression and downstream signalling components in HUVEC and HEK293 models.[120] To be brief, HEK293 cells, transiently or stably expressing TLR4 were able to elicit pro-inflammatory IL-8 in response to nickel while wild type HEK293, which express minimal to no components of the TLR4 surface complex, proved incapable. Using MyD88-targeted siRNA and blocking anti-IRAK1 antibodies, they were able to determine that TLR4 downstream signalling itself was necessary for nickel-induced IL-8 secretion. Most importantly, they were able to abrogate nickel-induced pro-inflammatory responses through the selective mutation of two key residues: (1) Histidine 456 and (2) Histidine 458 – without impeding functionality in total. These residues were isolated based on differences between murine TLR4 responsiveness to nickel insult compared to human TLR4 (hTLR4) and sequence analysis of human TLR4 (hTLR4) and murine TLR4 (mTLR4). It was also informed by previously uncovered qualities of TLR4 that outline discrete binding regions in TLR4 ectodomains with highly variable amino acid sequences (summarized in Figure 3). In short, previous studies by the Miller group had identified a hypervariable region with an additionally 'interspecies hypervariable' region centrally located within the hTLR4 ectodomain with residue differences that heavily dictated the specificity of hTLR4 to differing forms of LPS (and only LPS).[121] Studies following up promptly broadened the range of potential metal contact allergens from just nickel to both cobalt and palladium, among others.[122–124] Platinum is a Group 10 metal ion and sits at the core of cisplatin and is the heart of an entire class of platinum-based chemotherapeutic agents (with their own degrees of toxicity). [107107]

In a nutshell, there is thus evidence that: (1) TLR4 can activate in response to metal ions such as nickel, (2) TLR4 activation by metal allergens is direct and mediated by two key histidine residues and (3) platinum ions (cisplatin core metals) can activate TLR4.

1.3.2 – Hypothesis & Project Objectives

Bearing these in mind, I hypothesized that TLR4 may be directly interacting with cisplatin based on the same principles that allow it to directly bind and activate in response to other Group 10 metal allergens. In other words, in this work I will detail my assessment of three primary hypotheses grounded on precedents established in the literature:

(1) Soluble hTLR4 will block pro-inflammatory responses to cisplatin and nickel by endogenous hTLR4, but not those associated with LPS.
(2) Soluble hTLR4 will bind cisplatin directly without cellular factors
(3) Mutations of H456 and H458 will abrogate TLR4-dependent platinum and cisplatin-induced proinflammatory cytokine secretion, but will have no impact on LPS-induced TLR4 activation.

In doing this, I endeavored to also evaluate prior findings in the field through both replication and novel methods. I examined whether nickel could be bound by hTLR4 in the absence of other confounding cellular factors. I also investigated potential discrepancies in expectations by evaluating mTLR4 binding properties in contrast with that of hTLR4 within the context of MST.

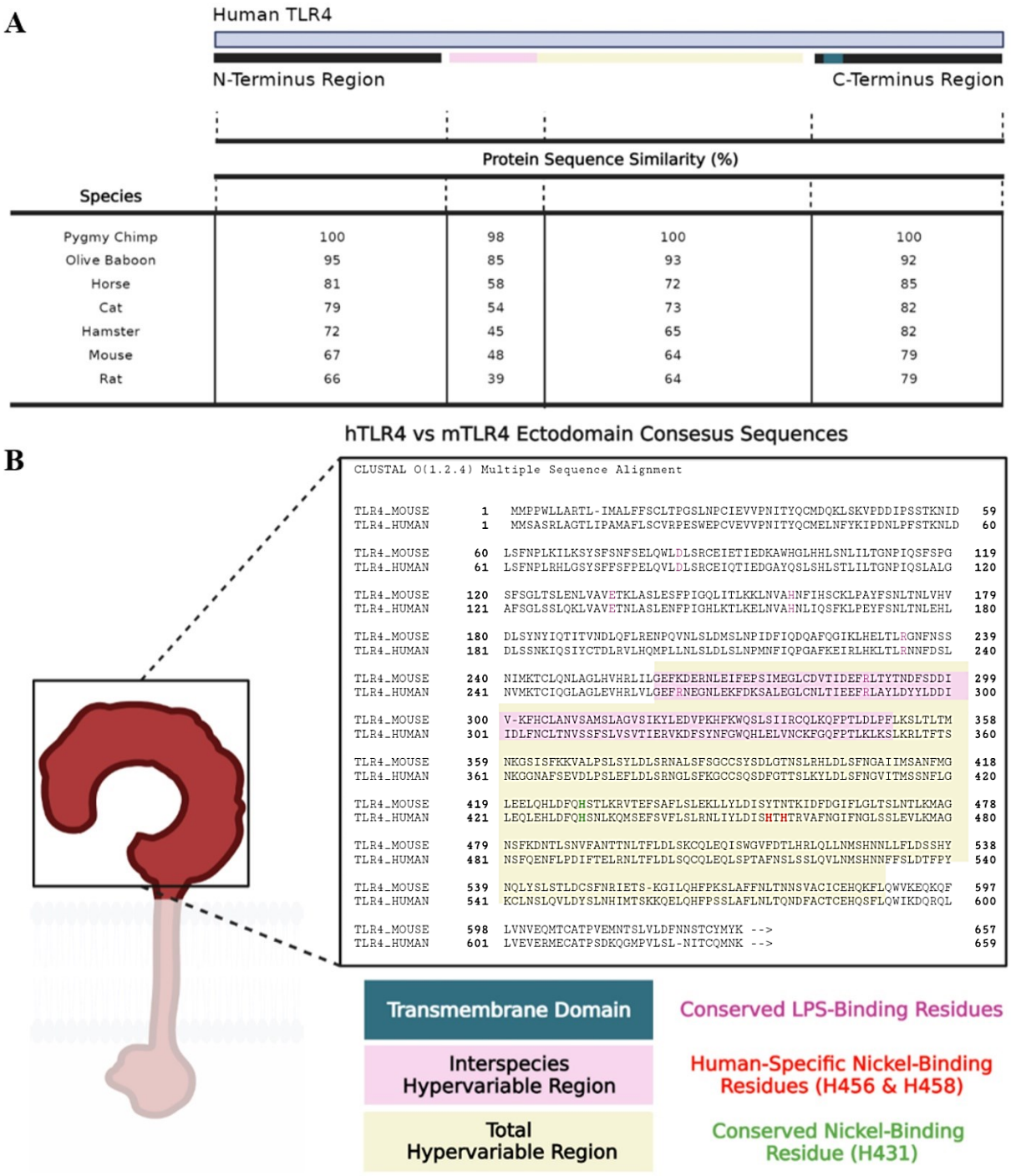

FIGURE 3. TLR4 contains a hypervariable region which itself contains an 'interspecies' hypervariable region; regions are associated with and define binding to differing ligands – either variants of LPS or metal allergens. (A) Adapted figure from Hajjar et al. detailing the sequence similarities between human TLR4 and the TLR4 of recent and distant relatives and identification of the most differentiated regions.[121] (B) Breakdown of the human TLR4 ectodomain sequence. Conserved and human-specific residues critical for binding LPS and metal allergens are regionally distinct. Figure designed with use of the UniProt protein alignment tool.

CHAPTER 2 - METHODS

2.1 – Tissue Culture & General Treatment Procedures

Human Embryonic Kidney HEK293-Null2 cells (Cat #hkb-null2), HEK293-hTLR4 cells (Cat #hkb-htlr4), and HEK293-isohTLR4 cells (Cat #293-htlr4a) were obtained from Invivogen and are isogenic reporter cell lines. HEK293-hTLR4 are stably transfected with human *TLR4* and *MD-2* while HEK293-isohTLR4 are stably transfected with only human *TLR4*. HEK293-Null2 cells are only stably transfected with an inducible alkaline phosphatase controlled by NF-κB promoter elements. Cells were grown in DMEM supplemented with 10% FBS, penicillin-streptomycin (100µg/mL), and 100µg/mL Normocin at 37ºC and 5% CO2. HeLa cells (ATCC CCL-2) were grown under similar conditions.

Experiments exploring the efficacy of soluble recombinant TLR4, mTLR4 (Biotechne, R&D Systems, Cat #9149-TR-050) or hTLR4 (Biotechne R&D Systems, Cat #1478-TR-050), in blocking endogenous TLR4 activity were performed with cells grown in 24-well plates at a density of 5.0×10^4 cells/well for 24HRs. Seeded cells were pre-treated with the recombinant proteins for 1HR prior to the application of agonist treatments. Recombinant proteins were resuspended in PBS, diluted for use in culture media as described. Experiments investigating the effects of histidine mutations were performed in 6-well plates with a cell density of 2.5×10^5 cells/well. Any transfections conducted were done 24HRs after seeding. Treatments with LPS (eBioscience, 00-4976-93), nickel chloride hexahydrate (Sigma, 654507), platinum (II) chloride (Sigma, 520632), platinum (IV) chloride (Sigma, 379840), and/or cisplatin (Teva, 02402188) followed 48HRs post-seeding, or 24HRs post-transfection, with fresh media. Supernatant collection, ELISAs, and cell viability analyses were performed 48HRs post-agonist treatment.

For siRNA-related experiments, HeLa cells were grown at 1.5×10^5 cells/well. Cells were transfected with siRNA 24HRs post-seeding and treated with agonists 24HRs post-transfection. Cells were exposed to agonists for 72HRs prior to supernatant collection, ELISAs and cell viability analyses.

2.2 – Histidine Multi-Site Directed Mutagenesis

The QuikChange Multi Site-Directed Mutagenesis Kit (Agilent, Cat# 200514/200515) was used to substitute the *Tlr4* histidine 456 and 458 residues with alanine and leucine respectively. The amino acid residues, alanine, and leucine were chosen for being chemically distinct from histidine residues. Both are smaller and less polar than histidine residues. Substitutions with chemically distinct residues are more likely to change the functionality of a protein as chemically distinct residues would be less likely to possess whatever key properties were associated with and afforded by the original. The alanine and leucine mutations were also the easiest to produce following the constraints imposed by kit primer design protocols. Manufacturer protocols were followed; controls for all phases were provided in kit and used. Primers used for the process were designed through the Primer3 program and are as follows:

5’-TACCTTGACATTTCTGCTACTCTCACCAGAGTTGCTTTCAATGGC-3’

5’-GCCATTGAAAGCAACTCTGGTGAGAGTAGCAGAAATGTCAAGGTA-3’

Generated clones with successful and specific mutations were isolated and validated through Sanger Sequencing. An excerpt of validation of mutation can be seen in below, in Figure 4. Constructs mutated were amplified prior to sequencing. The portions of *Tlr4* sequenced were compared with those from the original, in pDisplay-hTLR4 construct provided by Dr. Adeline Hajjar. Primers used for sequencing were also designed using Primer3 and were as follows:

5’- TTGGGACAACCAGCCTAAG-3’

5’-GAGAGGTCCAGGAAGGTCAA-3’

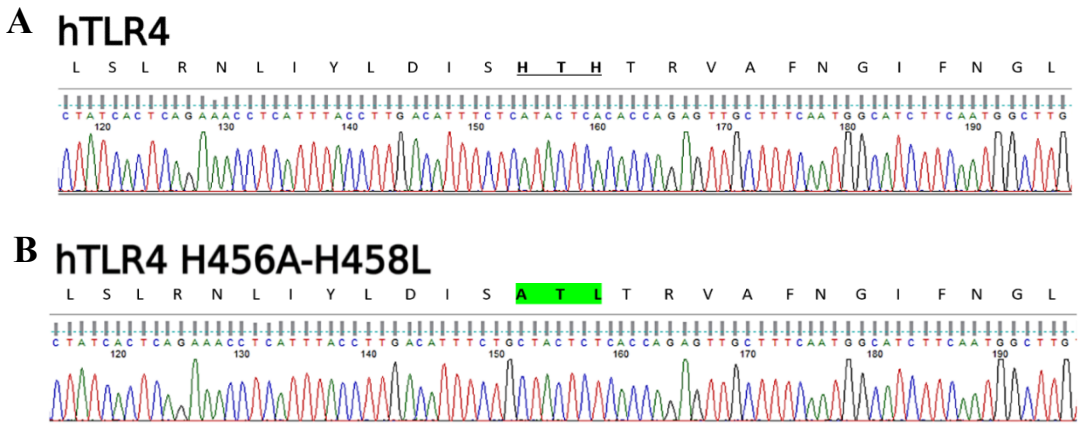

FIGURE 4. Sanger sequencing validation of hTLR4 double histidine mutation – H456 to A456 (H456A) & H458 to L458 (H458L). (A) Excerpt of the Sanger sequencing chromatogram for control (non-mutagenized) hTLR4 ORFs focused on the region associated with metal-binding histidines. Associated amino acids positioned above. (B) Excerpt of the Sanger sequencing chromatogram for mutagenized hTLR4 ORFs. Mutagenesis was performed according to the Agilent QuikChange Multi-Site Directed Mutagenesis kit.

2.3 – Transient Cell Transfection

HEK293-Null2 cells were transfected with either an empty vector control, a human *Tlr4* expression clone (kindly provided by Dr. Hajjar, of the Cleveland Clinic, as noted previously), or a mutated *Tlr4* expression clone (as described above). To assess the impact of histidine mutations 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) reagents were used for all transfections in tandem with 0.5µg of DNA in accordance with manufacturer specifications.

2.4 – siRNA Gene Knockdown

siRNA gene knockdown was completed using the dsiRNA TriFECTa kit (Integrated Technologies). HeLa cells were either transfected with 5nM of non-targeting/negative control siRNA or *hTLR4* siRNA (hs.Ri.TLR4.13) in RNAiMAX transfection reagent (Thermofisher). The negative control siRNA provided in the kit is not scrambled but is reported (advertised) to not recognize sequences in human, mouse, or rat transcriptomes. Three commercial, proprietary, pre-designed *hTLR4* siRNA sequences came with the kit (hs.Ri.TLR4.13.1, hs.Ri.TLR4.13.2, and hs.Ri.TLR4.13.3) and, were pooled for transfections.

2.5 – Immunoblotting

Transfected cells were lysed with 400µL of Pierce RIPA Lysis and Extraction Buffer (ThermoScientific, Cat #89900), containing Pierce protease inhibitors (ThermoScientific, Cat #A32953). Specifically, cells were exposed to lysis buffer and kept on ice for 15 minutes prior to cell scraping. Lysates were then spun at 16,000 x *g* and the resulting supernatants collected. These were then prepared for SDS-PAGE - mixed with 5x Laemmli Buffer and heated at 80ºC for 10 minutes. Proteins were separated using 4% Stacking 10% Resolving bis-acrylamide SDS-PAGE gels prepared in 1.0mm molds. Gel electrophoresis was induced through 200V applied for 50 minutes. Separated proteins were then transferred from the gels onto nitrocellulose membranes with the use of a Trans-Blot Turbo Transfer System (BioRad). Transfer was completed with singular mini-gels, exposed to 1.3A for 9 minutes, to accommodate for the mixed molecular weight of proteins present. Following transfer, nitrocellulose membranes were rinsed with water and subsequently dried for 1HR and rehydrated with TBS for 2-5 minutes prior to blocking with LiCor TBS Blocking Buffer (at 4ºC with agitation) overnight.

Membranes were then cut and immersed into LiCor TBS Blocking Buffer supplemented with 0.2% TWEEN and different primary antibodies: mouse anti-HA (Santa Cruz, 12CA5 sc-57592) or mouse anti-GAPDH (Invitrogen, MA5-15738). Primary antibody treatment continued overnight as well. The following day, membranes were washed with TBST 3x and then immersed in LiCor TBS Blocking Buffer supplemented with 0.2% TWEEN and goat anti-mouse secondary antibodies (LiCor, IRDye 800CW) for 1 hour. Membranes were washed again 3-5x prior to final visualization using a LiCor Odyssey CLX system. Visualization conditions were based on system recommendations.

2.6 – Cell Viability (Cell Survival/Count) Assays

Cell viability in response to treatment was estimated roughly – based on cell survival/count 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 prepared at a concentration of 5mg/mL and was added to cells post-treatment diluted to a final concentration of 1mg/mL. Cells provided with MTT were placed back into incubation at 37ºC and 5% CO2 to grow in the dark for another 4HRs.

Following incubation, the leftover supernatant and excess MTT reagent in wells were aspirated and replaced with DMSO (Sigma, D109) and kept at room temperature, shaking in absence of light, for 20 min. The absorbance at 590nm was measured upon completion using a SpectraMAX i3x plate reader (Molecular Devices); data collected was subsequently consolidated using the SoftMax Pro 7 program (Molecular Devices).

2.7 – Enzyme-Linked Immunosorbent Assays (ELISAs)

IL-8 ELISAs (88-8088, Invitrogen) were used to assess TLR4 activation IL-8 is a pro-inflammatory cytokine characterized in literature as a response related to both Group 10 metal ion hypersensitivities and cisplatin-induced toxicities. It is also a method of measuring the TLR4 activation in HEK-Null2 and HEK293-hTLR4 cells specifically recommended by provider (Invivogen).

ELISAs, or enzyme-linked immunosorbent assays, are assays that take advantage of antibodies specific for the protein of interest. For the experiments performed, 96-well plates were coated with anti-IL-8 antibodies specifically. These antibodies capture any IL-8 present in the supernatant they are exposed to. Secondary anti-IL-8 antibodies with conjugated biotin are then bound to the still-captured IL-8. The biotin subsequently serves as a binding interface for streptavidin-horse radish peroxidase (HRP) proteins. These proteins, as enzymes, oxidize TMB (3,3',5,5'-tetramethylbenzidine) provided into a deep blue substance. It is this change in coloration that can be measured quantitatively to indirectly quantify the amount of target protein initially captured. The color change is directly proportional to the amount of IL-8 first bound.

ELISAs were performed following manufacturer protocols; supernatants used for ELISAs were collected 48HRs post-treatment of cells as described prior. Color change was measured with a SpectraMAX i3x plate reader (Molecular Devices) following the manufacturer protocols. Protein secretion was normalized with cell viability (cell count) assays to account for the differing toxicities of agonists. ELISAs were performed so that there could be three or more technical replicates per condition and independent biological replicate. All data points (technical replicates from each biological replicate) are shown on figures.

2.8 – Microscale Thermophoresis (MST)

The NanoTemper Microscale Thermophoresis Monolith system was used to measure normalized fluorescence changes associated with protein-ligand binding. Soluble recombinant TLR4 and TLR4 agonists were prepared, separately, in 2% DMSO 0.5% TWEEN PBS buffer.

Serial dilutions of the prepared agonists were mixed 1:1 with solutions of prepared hTLR4 (100μg/mL) or mTLR4 (100μg/mL). Samples were then loaded into NanoTemper NT.LabelFree capillaries and loaded into the NanoTemper NT.LabelFree instrument (NanoTemper Technologies) to assay binding at room temperature. Capillaries used were hydrophobic and assays were completed using 20% light-emitting diode power (fluorescence lamp intensity) and 40% microscale thermophoresis power (infrared laser intensity).

Data was collected using the Monolith Control software (NanoTemper) and subsequently analyzed using the Monolith Affinity Analysis software (NanoTemper).

2.9 – Data Analyses & Statistical Analyses

All statistical analyses were performed using GraphPad Prism 7.2.

To determine the residual TLR4 activity of soluble TLR4-pretreated cells, their IL-8 secretions in response to the agonists were normalized to those of their respective control cells not pretreated with soluble TLR4. To characterize the dose-response to soluble hTLR4, the data was fit to non-linear best fit curves with four parameters and a variable slope. 2-way ANOVAs with Bonferroni multiple comparison tests were used to calculate statistical significance. To evaluate the importance of H456 and H458 in cisplatin-induced pro-inflammatory cytokine secretion, the IL-8 secretions of cells transfected with mutant TLR4 were normalized to the IL-8 secretions of cells transfected with wild-type TLR4 and treated with the same agonist. The resulting data is shown as boxes (25^{th} and 75^{th} percentile borders; median central band) with Tukey whiskers. Statistical analyses were performed through 1-way ANOVAs with Bonferroni multiple comparison testing correction, except for the LPS experiments which required 2-way ANOVAs.

For the thermophoresis experiments, the data obtained was fit to non-linear best-fit curves with three parameters and a standardized slope. Dose-response curve models were chosen to ensure that consistent comparisons could be made between the linear response ranges of different agonists.

CHAPTER 3 - RESULTS

3.1 – Cisplatin Induces TLR4 Activity In An LPS-Unrepsonsive System

To help probe the relationship between cisplatin treatment and TLR4 activation, I first wanted to determine the role of MD-2 in cisplatin-induced TLR4 activation. I used HeLa cells to do this as early reports indicated that HeLa cells do not express endogenous MD-2 despite their expression of TLR4.[125]

In line with these reports, HeLa cells proved incapable of mounting a proinflammatory cytokine response to LPS, even when supplied at extreme concentrations. Interestingly, I found that HeLa cells could secrete IL-8 in response to cisplatin (Figure 5A). Without the ability to use LPS as a TLR4-specific positive control for possible subsequent experiments, and to more importnatly ensure that cisplatin-induced IL-8 secretion was specifically dependent on the presence of TLR4, I used *TLR4*-targeting siRNA. siRNA, or short interfering RNA, are small RNA sequences that can be used by cells to perform "RNA interference". 24HRs post-seeding, I transfected cells with either 5nM of non-targeting siRNA or *TLR4*-targeting siRNA. 24 HRs post-transfection, cells were either left untreated or treated with 30μM cisplatin. 72HRs post-treatment, supernatants were collected for IL-8 ELISAs; MTT cell viability (cell count) assays were performed for normalization. RNA interference results in mRNA, complementary to the siRNA used, to be degraded – preventing mRNA translation and target protein expression.[126]

Ultimately, the capacity for cisplatin to induce IL-8 secretion appeared dependent on TLR4 expression, as siRNA inhibition of TLR4 expression proved sufficient to reduce IL-8 responses up to 75% (Figure 5B).

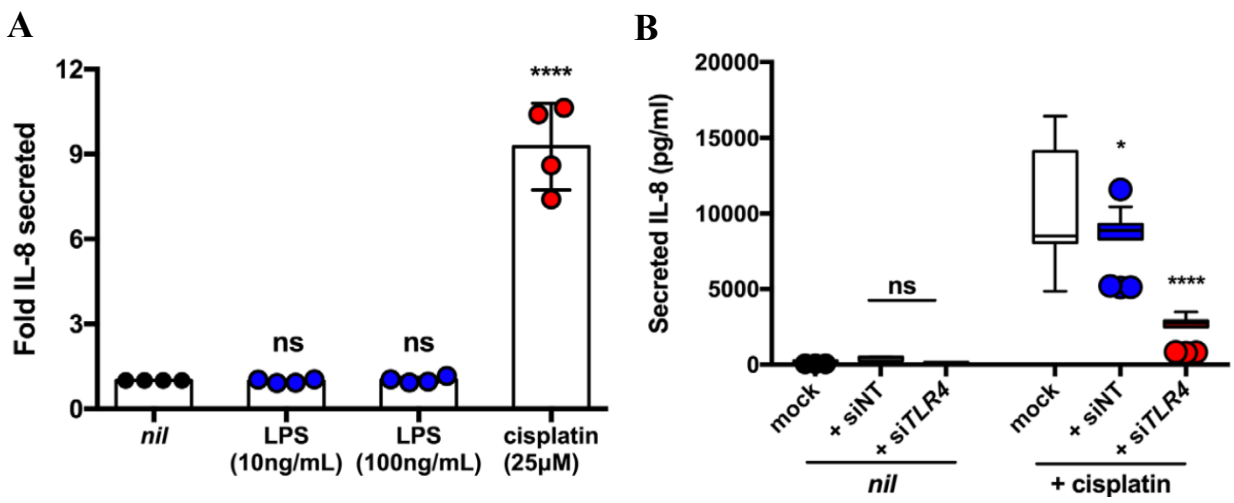

FIGURE 5. Cisplatin-induced TLR4-dependent pro-inflammatory response does not require MD-2 expression; siRNA-mediated inhibition of TLR4 expression prevents cisplatin-induced pro-inflammatory responses. (A) IL-8 secretion following treatment with listed agonists relative to *nil* (untreated) HeLa cells (n = 4 independent biological replicates). (B) Secreted IL-8 of HeLa cells either mock transfected or transfected with the described corresponding siRNA. (n = 3 independent biological replicates).

Data Information: Bar data for (A) represents mean values; error bars represent standard deviation. Three technical replicates were performed for each independent experiment and each data point is plotted in (B). Boxes contain the data points within the constraints of 25th and 75th percentile borders. Data points that exist outside of these borders and the associated Tukey whiskers are also shown. Central band represents median. Statistical analyses were determined in comparison to *nil* treatments using either one-way (A) or two-way (B) ANOVA. *, $P < 0.05$; ****, $P < 0.0001$; n.s., not significant (Dunnett's Test). Figure panels are derived from a co-authored publication.

3.2 – Platinum Ions & Cisplatin Elicit Pro-Inflammatory Cytokine Secretion Dependent on TLR4, Independent of MD-2

In addition to studying cisplatin-induced TLR4 activation through HeLa cells, I contributed to the characterization of TLR4-dependent pro-inflammatory responses to platinum ions and cisplatin. In work performed by alumnus Cole Delyea, nickel, and platinum ions (II) and (IV) induced NF-κB activation in HEK293 hTLR4 cells in a dose-dependent manner (Figure 6A). HEK293-Null2 cells and HEK293-hTLR4 cells were stimulated with either 1ng/mL LPS, 400μM Ni^{2+}, 25, 50, or 100μM Pt(II) or 25,50, or 100μM Pt(IV) for 36HRs before the extent of NF-κB activation was measured through an integrated alkaline phosphatase reporter system. The HEK293 hTLR4 cells used stably express the human TLR4 (hTLR4) complex - consisting of CD14, MD-2 and hTLR4. The upregulation appeared dependent on TLR4 expression as HEK293-Null2 cells that do not express TLR4 failed to respond to any of the agonists used. These findings fit with the work that fellow graduate student, Asna Latif, and I completed. HEK293-Null2 cells and HEK293-hTLR4 cells were similarly treated, albeit with either 50pg/mL LPS, 200μM Ni^{2+}, 100μM Pt (II), or 100μM Pt(IV) for 48HRs prior to supernatant collection, cell viability assays, and IL-8 ELISA. Cell viability was used to normalize raw data and obtain final secreted IL-8 values. Together, we demonstrated that HEK293 hTLR4 cells could secrete IL-8 in response to LPS and metal ions (Figure 6B). The HEK293-Null2 cells, in this context, failed to elicit pro-inflammatory cytokine secretion in response any of the agonists used (Figure 6B). Asna conducted follow-up experiments following the same procedure to establish the utility of our model for the study of CITs and she subsequently found that hTLR4 cells could also secrete IL-8 to cisplatin and in a dose-dependent manner (Figure 6C). Like in the previous experiments, the HEK293-Null2 cells failed to secrete IL-8 in response to cisplatin, regardless of the concentration of cisplatin used. Though platinum and cisplatin-induced TLR4 activation did not require MD-2, it was technically possible for MD-2 expression to affect the extent of TLR4 activation regardless. To address this, we transfected HEK293-isohTLR4 cells with either empty vectors (EV) or plasmids encoding human *MD-2*. HEK293-Null2 cells and HEK293-isohTLR4 cells that stably express hTLR4 but not MD-2 or CD14 were transfected with either empty vector (EV) or MD-2 prior to treatment with 1ng/mL LPS, 200μM Ni^{2+}, 100μM Pt(II), 100μM Pt(IV) or 25μM cisplatin. In doing so, we discovered that MD-2 could bolster platinum and cisplatin-induced IL-8 secretion (Figure 6D).

This finding was not particularly surprising. Prior publications revealed that MD-2 could affect the functionality of metal-induced, MD-2-independent, TLR4 homodimerization.[122,124] Nickel, for example, behaved in line with precedent for these experiments; it failed to elicit an increase in IL-8 secretion that was statistically significant in the absence of MD-2.

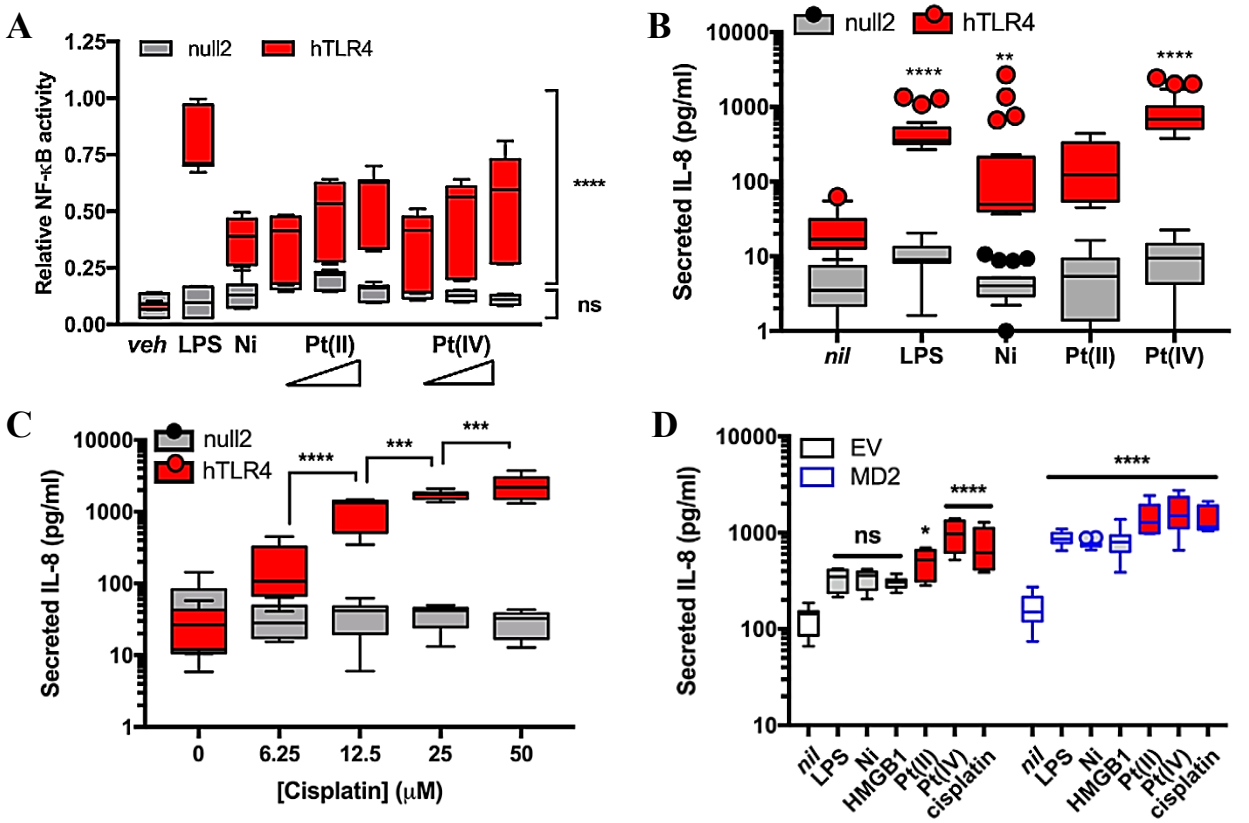

FIGURE 6. Platinum ions elicit pro-inflammatory responses in a hTLR4-dependent, dose-dependent, manner; MD-2 enhances metal and cisplatin-induced TLR4 activation. (A) NF-κB activation in response to the listed agonists relative to the response to the vehicle control for each group. This portion of data was contributed by alumni, Cole Delyea (n = 3 independent biological replicates). (B) IL-8 secretion of HEK293-Null2 and HEK293 hTLR4 cells in response to the listed agonists. (n = 4 independent biological replicates) (C) HEK293-Null2 and hTLR4 IL-8 secretion following treatment with different concentrations of cisplatin (n = 3 independent biological replicates). This data was obtained by MSc student, Asna Latif. (D) IL-8 secretion of HEK293-isohTLR4 transfected with either EV (outlined in black) or human MD-2 (outlined in blue). White boxes represent secretion associated with negative untreated control groups. Grey boxes represent secretion associated with positive controls groups. Red boxes represent secretion associated with platinum-related compounds. Experiments (n = 3-4 independent biological replicates) were completed in conjunction with Asna Latif.

Data Information: For all panels, all of the data points from each replicate are plotted. Each independent biological replicate corresponds to 3 data points. The boxes contain the data points within the 25th and 75th percentile borders. Boxes have Tukey whiskers and a central band denoting the median value. The individual data points that exist outside the Tukey whiskers are also shown. 2-way ANOVA was used to determine statistical significances in both panels. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; n.s., non-significant (Dunnett's Test). Figure panels are derived from a co-authored publication.

3.3 – Soluble Recombinant TLR4 Reduces Cisplatin-Induced Endogenous TLR4 Activation (*In-Vitro*)

Soluble recombinant TLR4 has been used in the past to probe the potential for direct binding interactions between TLR4 and agonists of TLR4 downstream signalling. Logically, if an agonist is capable of binding onto and activating TLR4 without the need of particular co-receptors, then soluble recombinant TLR4 would pose a threat to that process by acting as non-functional competitors – sequestering ligands and limiting the amount available to endogenous TLR4 on cells. The capacity for metal allergens to bind human TLR4 (hTLR4) directly, for example, was first demonstrated in part through the use of soluble recombinant TLR4; the application of soluble recombinant hTLR4 proved capable of blocking the activation of endogenous hTLR4 in response to nickel.[122] I sought to determine whether the same could be said in regard to cisplatin.

HEK293 cells stably expressing hTLR4, MD-2 and CD14 (HEK-293-hTLR4), were pre-treated with soluble hTLR4 or mouse TLR4 (mTLR4) and then treated with LPS, nickel, or cisplatin. LPS-induced TLR4 activation could not be blocked by either form of soluble TLR4 as LPS binding requires a stable connection to co-receptor/complex MD-2/CD14 (Figure 7A, B). Nickel-induced TLR4 activation, on the other hand, could be blocked – but only by soluble recombinant hTLR4 (Figure 7A, B). This was expected because mTLR4 lack the histidine residues found in hTLR4 critical for binding established Group 10 metal allergens. Interestingly, cisplatin-induced TLR4 activation could be blocked by both soluble recombinant mTLR4 and hTLR4. Soluble mTLR4 reduced cisplatin-induced IL-8 secretion within a more variable range compared to soluble hTLR4 which seemed to be able to reduce IL-8 secretion more consistently (Figure 7B) though they both could facilitate up to a 50% reduction of response on average. This is alluded to by the different ranges of residual TLR4 activity (box sizes) they are associated with. To ensure that the soluble TLR4 used was the active component to reductions in TLR4 activation observed, I evaluated the dose-response to soluble recombinant hTLR4 pre-treatment. Showcased (Figure 7C) is a direct, saturable, correlative relationship between the degree of blocking and amount of available soluble recombinant TLR4 used.

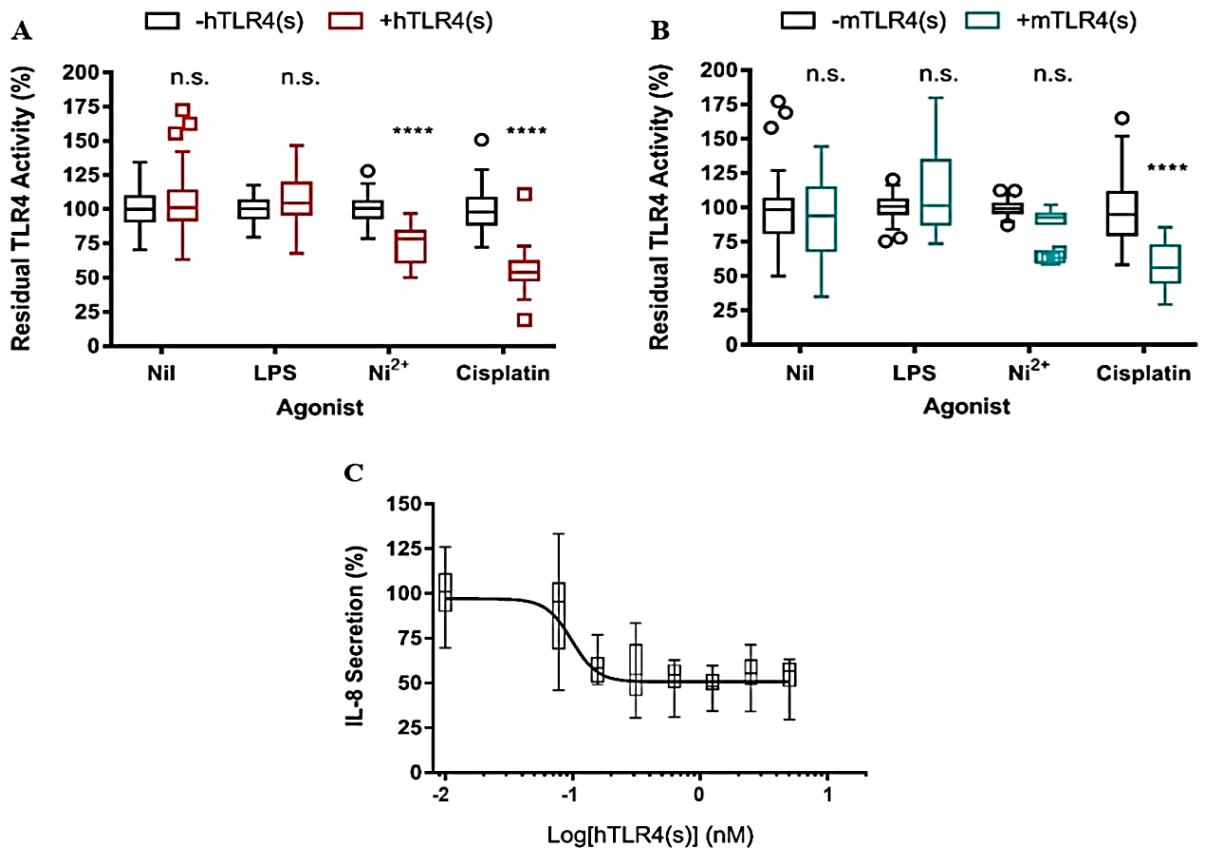

FIGURE 7. Soluble recombinant TLR4, TLR4(s), can inhibit TLR4-activated pro-inflammatory IL-8-secretion to distinct agonists in HEK293 hTLR4 cells. (A) IL-8 secretion of HEK293-hTLR4 cells pre-treated with 0.1nM soluble recombinant human TLR4 and subsequently treated with either 1ng/mL LPS, 200µM Ni^{2+}, or 25µM 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 0.1nM soluble recombinant mouse TLR4 and subsequently treated with either 1 ng/mL LPS, 200µM Ni^{2+}, or 25µM cisplatin. Data are shown as the percentage of TLR4 activity in the absence of soluble mTLR4 (n = 3 independent biological replicates for Nil & Ni^{2+} conditions, n = 4 independent biological replicates for LPS & Cisplatin conditions). Nil representative of cells treated without agonists. (C) IL-8 secretion of HEK293-hTLR4 cells pre-treated with different concentrations of soluble recombinant hTLR4 after treatment with 25µM cisplatin as a percentage of IL-8 secretion without the pre-treatment condition (n = 4 independent biological replicates).

Data Information: For all panels, actual individual data from each experiment are plotted as box (25^{th} and 75^{th} percentile borders; median central band) with Tukey whiskers. 27 total data points are shown per condition; each independent biological replicate corresponds to 9 data points. Statistical analyses were performed through 2-way ANOVA and the Bonferroni multiple testing correction. ****, $P < 0.0001$; ns, not significant. For panel C, non-linear best-fit-curve follows four parameters, variable slope;$R^2 = 0.59$. Apparent $IC_{50} = 0.09813$nM.

3.4 – Metal Allergens & Cisplatin Directly Bind hTLR4 Independent Other Factors

In an aforementioned prior publication, I showed that HeLa cells could respond to cisplatin despite their reported minimal-to-no expression of MD-2 (Figure 5A) and that their responses were dependent on the expression of TLR4 through the use of siRNA (Figure 5B).[107] This is consistent with the prior finding that provides evidence to suggest that soluble recombinant TLR4, and therefore TLR4, can bind metal targets without the need for, at least the prototypical array of, coreceptors. The *in-vitro* nature of prior works however, imposed several confounding variables. For example, reduced TLR4 activation observed could have been due to soluble TLR4 interaction with endogenous TLR4, resulting in dysfunctional signalling regardless. While the LPS data may partly discount such possibility, DAMPs could have simply been necessary for confounding interactions. Similarly, the impact of soluble TLR4 could have been due to the sequestration of DAMPs, as opposed to metal agonists. Thus, to circumvent the potential effects of these externalities, microscale thermophoresis (MST), an *in-vitro* biophysical assay for protein-ligand interaction, was used (Figure 8).

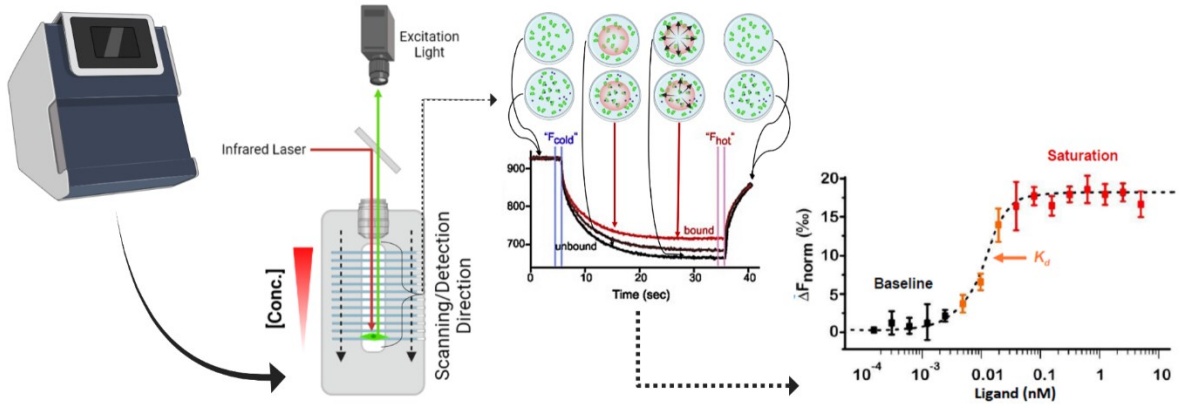

FIGURE 8. Microscale Thermophoresis detects changes in localized fluorescence to measure protein-ligand binding affinities. Samples prepared are loaded into capillaries arranged based on concentration and inserted into the system. Upon entry, samples are exposed to an excitation light and a temperature gradient via infrared laser. Binding is detected based on changes to localized fluorescence caused by thermophoresis (temperature-induced motion) over time. The thermophoretic ability of a protein is dependent on three properties: size, charge, and solvation shell. All three of these properties are altered in the event of ligand binding. Monitored changes over time and concentrations are plotted to estimate binding affinities. Figure adapted from the NanoTemper Monolith MST System user manuals.[127]

As a negative control for the system, soluble recombinant hTLR4 were exposed to increasing concentrations of LPS without MD-2 or any other cellular factors present. As expected, no binding was observed (Figure 9A).

Conversely, hTLR4 nickel-binding was implied based upon curve fitting to a non-linear, best fit 3 parameter curve, though the curve fit was only $R^2 = 0.65$. This implied binding is in line with previous reports characterizing it as a TLR4-dependent metal contact allergen (Figure 9B).

To ensure that the data acquired through MST was based on the specific detection of binding to soluble TLR4, as opposed to non-specific factors caused by the presence of metal ions in the system, calcium chloride was subsequently used. Calcium is a Group 2 metal ion that has had some of its interactions with proteins explored through MST. Calmodulin (CaM), for example, has been shown to bind calcium directly through MST. Meanwhile, there appears to be no reported cases of calcium-induced TLR4 activation or TLR4-calcium binding interactions, by MST or otherwise.[128,129] In line with this, hTLR4 displayed little to no capacity to bind calcium, even when presented with calcium at extremely high concentrations (Figure 9C), as indicated by the R^2 of 0.31 and inverse binding mode. Moreover, the binding affinity (Apparent K_d) for calcium (1629mM) was an extrapolation beyond the actual highest concentration tested (500mM) further indicating that no binding was detected over the range of concentrations used in the experiment.

In contrast, hTLR4 bound cisplatin much more explicitly. The predicted hTLR4-cisplatin binding curve fit was $R^2 = 0.927$. Surprisingly however, hTLR4 bound cisplatin with a lower apparent binding affinity compared to nickel (Figure 9D).

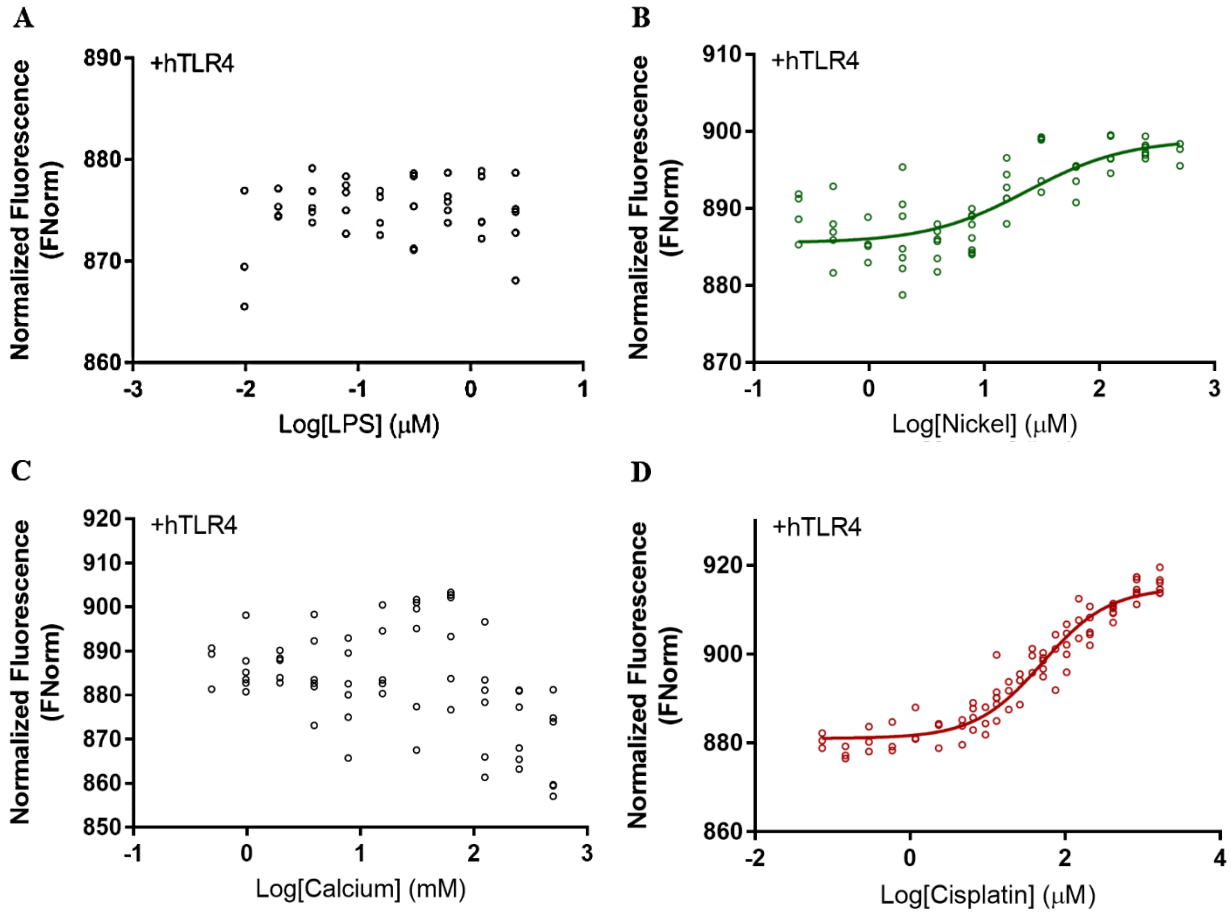

FIGURE 9. Human TLR4 can directly bind nickel and cisplatin. (A-D) Microscale thermophoresis analysis showing normalized fluorescence of hTLR4 plotted against the indicated concentrations of LPS (n = 5 independent replicates), Ni^{2+} (n = 3-6 independent replicates over entire range of concentrations), Ca^{2+} (n = 6 independent replicates) or cisplatin (n = 3-6 independent replicates over entire range of concentrations), respectively.

Data Information: For all panels, data fitted to non-linear best-fit curves follow three parameters with a standardized slope. Curves were generated on GraphPad Prism 7.2. (A) Apparent K_d undetectable. (B) Apparent K_d = 23.53μM, R^2 =0.651. (C) Apparent K_d = 1629 mM, R^2 = 0.31. (D) Apparent K_d = 50.57μM, R^2 = 0.927.

3.5 – Metal-Binding Residues Influence Platinum & Cisplatin-Induced Activation of TLR4

In an effort to study the underlying facets of TLR4 that confer the observed direct binding interactions *in-vitro*, I mutagenized TLR4 histidine 456 and 458 into the biochemically dissimilar alanine and leucine to assess the subsequent effects on metal and cisplatin-induced TLR4 activation. Histidines 456 and 458 exist within the TLR4, and soluble TLR4, ectodomains and are established elements critical for Group 10 metal binding and metal contact allergen-induced TLR4 activation. HEK293-Null2 cells (which do not express endogenous hTLR4, MD-2, and CD14) were then transfected with the HA-epitope-tagged mutagenized hTLR4 constructs and then treated with LPS, nickel, platinum (II), platinum (IV) or cisplatin. Successful transfection and protein expression was substantiated through immunoblotting (Figure 10A).

Treatment with LPS, in this instance, served two purposes. As with prior experiments, treatment with LPS served as a negative control, as the absence of H456 and H458 has had no reported effects on LPS-induced TLR4 activation. In addition, treatment with LPS served as a positive control for the capacity of mutagenized TLR4 constructs to not only be expressed to a detectable extent through immunoblot, but expressed onto the cell surface and conduct canonical functions. And as to be expected, H456A-H458L hTLR4 displayed equal capacity to respond to LPS, when provided with MD-2, as wild-type hTLR4 (Figure 10B). Nickel-induced IL-8 secretion mediated by H456A-H458L hTLR4 was equal to or less than 25% that of wild-type hTLR4, which was similarly unsurprising (Figure 10C). The Group 10 metal ion binding properties of H456 and H458 were discovered through the explicit deconstruction of nickel-induced TLR4 activation.

The mutagenesis of H456 and H458 also impaired TLR4 activation in response to platinum (II), platinum (IV), and cisplatin (Figures 10D, E, F). Furthermore, the degree of impairment caused by the mutagenesis appeared to differ between the metal agonists. Based on statistical analysis comparing to EV negative controls, the mutagenesis of known metal-binding residues resulted in the complete abrogation of nickel and platinum (II)-induced TLR4 activation but only hindered platinum (IV) and cisplatin-induced TLR4 activation. Given that the two forms of hTLR4 were expressed comparably and that mutagenesis itself did not result in the intrinsic destabilization of TLR4 or typical TLR4 functions, then the data taken altogether suggest that hTLR4 cisplatin-binding is, only partially dependent on recognized metal-binding elements specific to hTLR4.

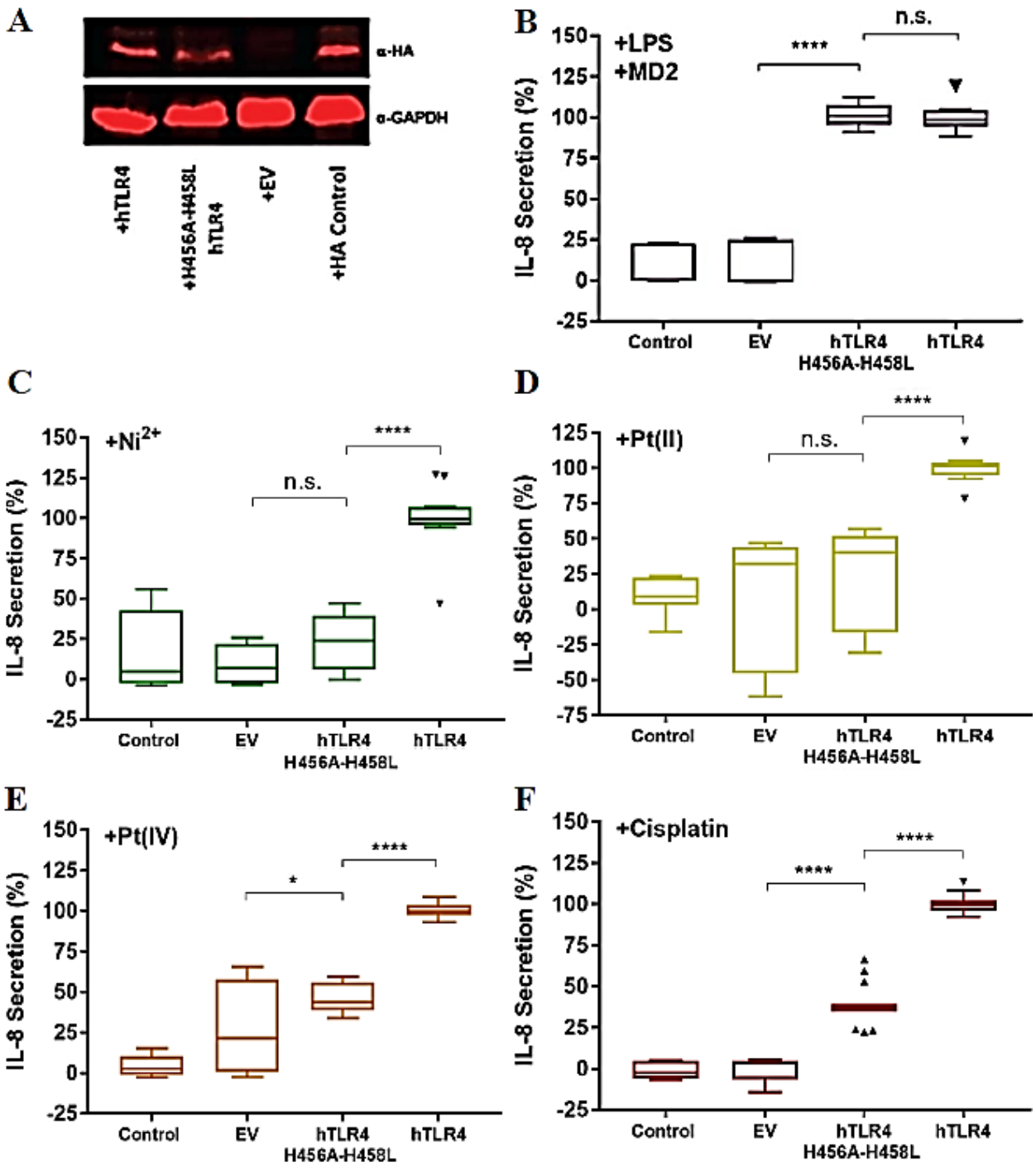
A
α-HA
α-GAPDH
+hTLR4
+H456A-H458L hTLR4
+EV
+HA Control
B
+LPS
+MD2
IL-8 Secretion (%)
n.s.
****
Control
EV
hTLR4
hTLR4 H456A-H458L
C
+Ni2+
IL-8 Secretion (%)
n.s.
****
Control
EV
hTLR4
hTLR4 H456A-H458L
D
+Pt(II)
IL-8 Secretion (%)
n.s.
****
Control
EV
hTLR4
hTLR4 H456A-H458L
E
+Pt(IV)
IL-8 Secretion (%)
*
****
Control
EV
hTLR4
hTLR4 H456A-H458L
F
+Cisplatin
IL-8 Secretion (%)
****
****
Control
EV
hTLR4
hTLR4 H456A-H458L

FIGURE 10. Histidine 456 & 458 mutations (H456A-H458L) partially inhibit TLR4 activation by platinum ions and cisplatin. (A) HEK293-Null2 cells were transfected with either empty vector (EV), hTLR4, or hTLR4 mutant constructs; immunoblotting was used to analyze relative TLR4 protein levels. (B-F) IL-8 secretion from HEK293-Null2 cells transfected with either nothing (control), empty vector (EV), hTLR4, or hTLR4 mutant constructs and subsequently treated with either 1 ng/mL LPS, 200μM Ni^{2+}, 100μM platinum (II) [Pt(II)], 100μM platinum (IV) [Pt(IV)], or 25μM cisplatin displayed as a percentage of the response elicited by wild-type hTLR4 (n = 3 independent biological replicates for experiments with Pt(IV), n = 4 independent biological replicates for experiments performed with LPS, Ni^{2+}, Pt(II) and cisplatin). Cells treated with LPS were also co-transfected with an MD-2 construct.

Data Information: For panels B-F, all individual data points from each experiment are plotted as box (25^{th} and 75^{th} percentile borders; median central band) with Tukey whiskers. Each independent biological replicate corresponds to 3 data points. 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. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; n.s., non-significant.

3.6 – TLR4 Cisplatin-Binding is Similar To, But Distinct From, TLR4 Nickel-Binding

Returning to the soluble TLR4 experiments for a moment, recall that mTLR4 also proved sufficient to inhibit up to 50% of cisplatin-induced TLR4 activation despite being incapable of inhibiting nickel-induced TLR4 activation. This hinted at the existence of unaddressed metal-binding residues or features sufficient to confer binding to cisplatin, but not nickel and other Group 10 metal ions. This premise however also happens to coincide with the previous result, as the mutagenesis of H456 and H458 proved insufficient to abrogate cisplatin-induced TLR4 activation.

To further explore, or validate, the concept of extant undiscovered residues that are sufficient to facilitate direct TLR4 cisplatin binding, I tested the direct binding capabilities of mTLR4 through MST. And as to be expected based on the previous results and the literature, no direct binding could be detected between mTLR4 and nickel ions (Figure 11A). In stark contrast is mTLR4-cisplatin binding, which could be robustly detected (Figure 11B). Interestingly, and equally important to note, is that the apparent binding affinity of mTLR4 appears lower than that of hTLR4. This may not be biologically relevant however, at least with the present data set and the potential for error. The standard error (SE) for the K_d of hTLR4-cisplatin binding is reported as the $\log IC_{50} = 0.054$, placing the accurate K_d within the 95% confidence interval of 38.43-66.76μM. The same statistic for mTLR4-cisplatin binding is 0.174, placing the accurate K_d within the 95% confidence interval of 60.77-313μM. That said, comparison of the binding curves (Bottom, Top, and $\text{Log}IC_{50}$ values) through both an Extra-Sum-Of-Squares F-Test and Student's t-test suggest statistically significant differences ($P < 0.0001$) (Figure 11C).

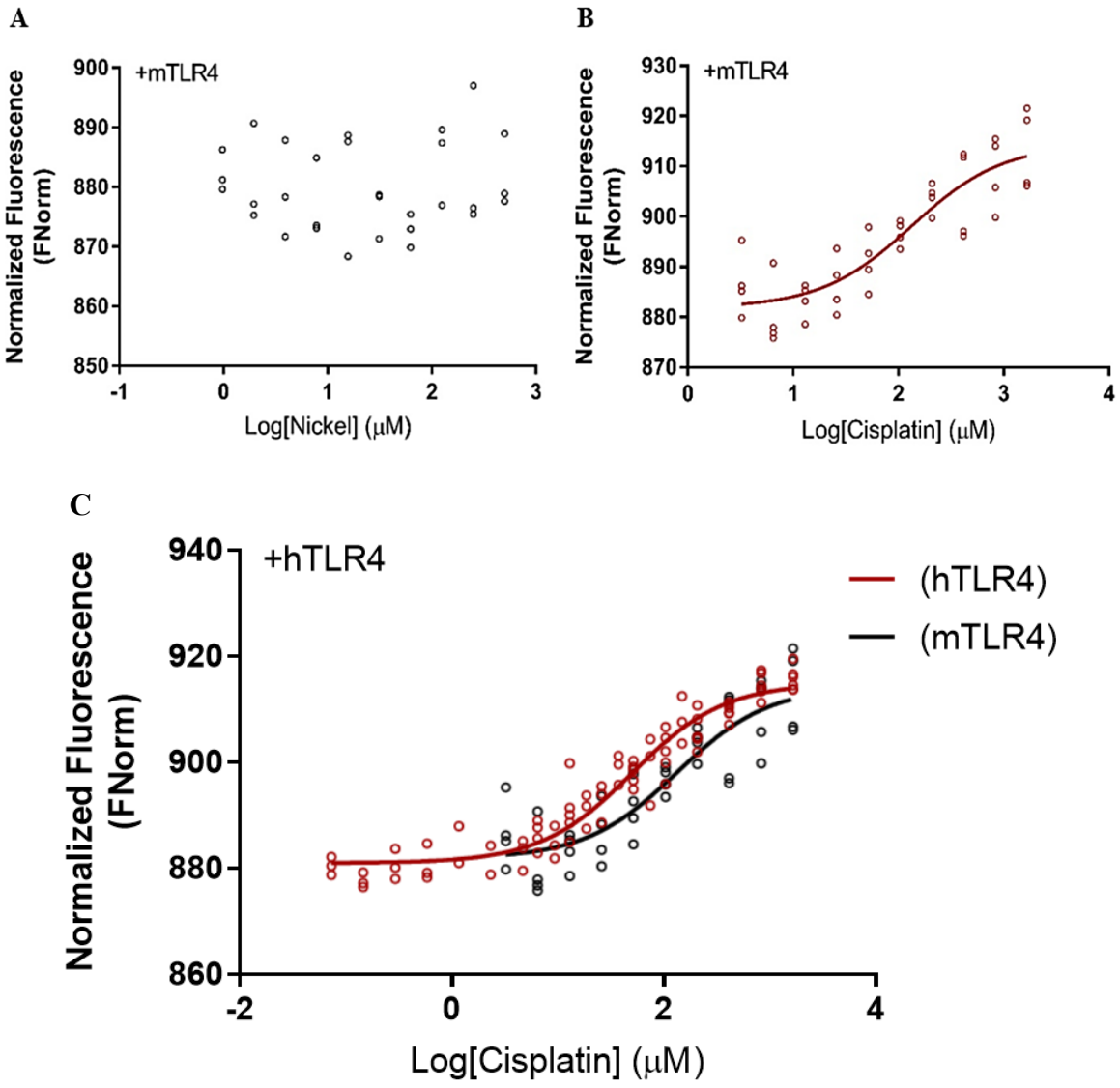

FIGURE 11. Mouse TLR4 directly binds cisplatin but not nickel. (A-B) Microscale thermophoresis analysis showing normalized fluorescence of mTLR4 plotted against the indicated concentrations of (A) Ni^{2+} (*n* = 3 independent replicates) or (B) cisplatin (*n* = 4 independent replicates), respectively. (C) Comparison of hTLR4 and mTLR4 binding to cisplatin through MST.

Data Information: (A) Apparent K_d unavailable/undetectable. (B) Apparent K_d = 133.6μM; R^2 = 0.781. (C) Curve comparisons were performed with an Extra-Sum-of-Squares (F) Test and a modified Student's t-test (with Mean = IC_{50}, SE, and N = DF (Degrees of Freedom) +1) following GraphPad Prism 7.2 protocols. For both, **** statistically significant differences were reported, P < 0.0001.

CHAPTER 4 - SUMMARY & DISCUSSION OF RESULTS

4.1 – Context

Our understanding of the relationship between TLR4 and CITs has grown over the years, but research has lacked explanatory power regardless. The common assumption is that the two are indirectly related, but this has likely only placed constraints on thorough investigations.

Two, ultimately compatible, theories have risen to prominence thus far. The first proposes that cisplatin primarily causes TLR4 upregulation. This upregulation then enhances immune responses to latent bacterial infections, leading to local persistent inflammation and damage. The second proposes that cisplatin neoplastic activity results primarily in the release of DAMPs (and TLR4 upregulation). It is then these DAMPs that trigger increased TLR4 activation. In clinical cases of cisplatin-induced toxicity, both models would likely be relevant, as DAMPs and bacterial contaminants would presumably be present, but existing literature suggests neither model is perfectly sufficient to explain the entirety of CIT development. This study may thus fill the knowledge gap, as the data alludes to another pathway through which TLR4 may influence the onset and progression of CITs yet explored.

4.2 – Cisplatin-Induced TLR4-Activation Can Occur Independently From LPS & MD-2 DAMP-Induced TLR4 Activity

The expression of MD-2 is considered the minimum prerequisite for LPS-induced TLR4 activation.[130] While sequence variations in TLR4 may affect the capacity to bind specific isoforms of LPS, the binding of LPS ligands, as a whole, always requires access to at least MD-2. For example, while the binding of 'smooth' (S) form LPS - LPS containing the O-antigen side-chain - requires the entire LBP, CD14, MD-2, TLR4 complex, the binding of 'rough' (R) form LPS by TLR4 - which does not possess the O-antigen side-chain - can bypass the need for both LBP and CD14, but not MD-2.[131] Such is why transfection of the isogenic HEK293-hTLR4 cells and the HEK293-Null2 cells co-transfected with hTLR4 with MD-2 proved sufficient to confer LPS responsiveness; the LPS used was 'rough' (R).[132] It would therefore stand to reason that if pro-inflammatory immune responses to cisplatin chemotherapy stem primarily from latent bacterial infections and DAMPs, then MD-2 would have to be a critical component in the process.

Of course, the totality of my work suggests that is not the case. Both the soluble hTLR4 blocking experiments and the histidine mutation experiments showcased robust TLR4 activation in response to platinum ions and cisplatin in the absence of MD-2, corroborating published data. The results are all also consistent with the findings of others that have explored the role of TLR4 in CITs under similar conventionally sterile conditions, like the Zhang and Ramesh groups.[105] Our reagents are also tested for endotoxin using the Pyrotell Gel Clot Formulation kit (Pyrotell, GS125-5) and have remained free from detectable contamination. The data also fits current models and conventional understanding of LPS and metal-ion binding;[120,133,134] interaction interfaces appear distinct and the fact that mutation of strictly metal-binding residues did not impair the core functionality of TLR4 in response to LPS only further indicates that it is, for one thing, possible to at least decouple the exacerbation of CITs by latent infections and MD-2-specific DAMPs from other evidenced concurrent mechanisms. HMGB1 is one such MD-2 specific DAMP that has already explicitly been dissociated from cisplatin-induced TLR4 activation. While publications have also implied the existence of LPS-specific elements to CITs through the use of *in-vivo* models such as C3H/HeJ mice, it is critical to note that the operative mutation in the TLR4 of these mice is P712H which exists in the most conserved region of the cytoplasmic domain.[135] P712H, in truth, protects against TLR4 pro-inflammatory signalling in response to various other DAMPs (such as HSPs 60 and Gp96)[136,137] and toxic agents (such as paclitaxel)[138] as structural assessments suggest that it tampers with adaptor recruitment downstream.

4.3 – TLR4 Can Bind Metal Allergens & Cisplatin Directly Dependent on Histidine Residues

Separating the influence of LPS and certain DAMPs from cisplatin-induced pro-inflammatory responses was a valuable endeavour, but the crux of this project was to determine whether the relationship between TLR4 and CITs was physically direct. By using established indirect methods, and direct methods novel in context, I provide evidence to suggest that TLR4 can bind platinum ions and cisplatin.

Soluble hTLR4 blocking experiments and hTLR4 histidine mutation experiments were indirect binding assays. Raghavan et al. (2012) used the same techniques to deduce that TLR4 was responsible for metal contact hypersensitivities and pinpoint the histidine residues required for metal-induced TLR4 activation.[122] Here, those same strategies suggest that platinum and cisplatin-induced pro-inflammatory responses also depend, in part, on the same properties that control metal contact hypersensitivities. Soluble hTLR4 blocked nickel and cisplatin-induced endogenous TLR4-dependent pro-inflammatory cytokine secretion. The mutation of H456 and H458 reduced platinum and cisplatin-induced TLR4-dependent pro-inflammatory cytokine secretion.

Indirect methods can be affected by confounding factors and side effects, however. Mitigating bacterial infections and removing LPS as a factor from experiments is standard practice; DAMP signalling is harder to address. Cisplatin and metal allergens are innately toxic *in-vitro* as cell culture consists of actively reproducing cells, meaning that they likely do trigger the release of DAMPs. To my knowledge, the evidence implicating TLR4 in the development of metal contact hypersensitivities consists primarily of observed changes to reporters downstream of TLR4 activation - IL-6, IL-8, or NF-κB - *in-vitro*. The latter of these is itself often observed indirectly through luciferase reporter assays. And while Schmidt et al. and follow-up groups did attempt to address confounding variables and narrow down alternative explanations through other approaches, the very nature of indirect *in-vitro* observation limits the ability to remove DAMPs from the equation. The blocking of endogenous hTLR4 activation with specific soluble hTLR4 could be rooted more in the sequestration of DAMPs as opposed to metal ions. The consequences of generating histidine mutations could be due to impaired human-specific DAMP recognition - not impaired metal binding. So, rather than go through the arduous task of removing every known TLR4-specific DAMP from play (and still have to contend with the existence of undiscovered TLR4-specific metal-specific DAMPs regardless), I opted to use microscale thermophoresis as a direct and immediate assay for binding. The beauty of MST, as described in the methods, is that it is an entirely cell-free procedure for detecting and quantifying protein-ligand binding events.

The MST data verifies that human TLR4 can bind metal allergens akin to nickel and definitively illustrates that direct TLR4-cisplatin binding can occur. The lack of detectable binding between hTLR4 and calcium, and mTLR4 and nickel, matches expectations based on literature and attests to assay specificity. All in all, the MST data provides a novel physical explanation for prior findings and why bacterial infections and DAMPs may not be necessary to produce the immunological hallmarks of CITs, even if they may be nevertheless involved in real pathologies.

4.4 – Implications, Limitations & Future studies

In terms of complicated implications, several of my observations do not perfectly align with the literature and expectations set by preceding experiments. In the three studies that first identified binding interactions between hTLR4 and transition metals, metal ions (nickel, cobalt, palladium) did not require MD-2 to bind TLR4 and mediate homodimerization but did require MD-2 to trigger complete TLR4 activation. My research, in contrast, suggests that nickel-induced TLR4 activation can occur independently of MD-2. There are possible explanations for this based on differences in methodology. The three precedent papers treated their control HEK293 cells with 1.5mM of their respective metal ions for 8-16HRs; I treated cells with 100-200μM of metal ions for 48HRs. The precedent papers, and our EMBO Reports publication, purport that MD-2 is likely integral to small metal ion-binding as it stabilizes metal-induced homodimerization. It is possible that higher concentrations of nickel used in the absence of MD-2 result in either inaccessible aggregates or rapid toxicity that kills cells before they can adequately begin production and secretion of markers of inflammation; 1mM of nickel is sufficient to cull almost 50% of cells within 4HRs.[139] Alternatively, this same process could reduce the number of residual nickel aggregates that could impede or affect ELISA and similar biochemical assays. Nickel aggregation is not surprising even for otherwise soluble formulations; extreme concentrations of Group 10 metal ions in combination with media containing phosphate can lead to insoluble and less reactive products. Likewise, extreme nickel concentrations could have shifted the mode of interaction. Reports indicate that HaCat keratinocytes (which express TLR4) internalize metal ions (nickel, cobalt, and chromate) exponentially higher at higher concentrations. HaCat keratinocytes treated with 1mM of radiolabelled nickel contained 35-50x the amount of radiolabelled intracellular nickel compared to 100μM treatments.[139]

This could lead to less effective nickel on the cellular surface at high concentrations as opposed to at lower concentrations, which could affect binding that could be compensated for by MD-2 stability. It also should be noted that for IL-8 ELISAs, the primary papers did not measure and consider the inherent dynamic effects of cell viability loss over the course of treatment on final readouts. Interestingly, their scale of positive responses also goes beyond kit range which can likely obscure lower but nevertheless relevant signals. Moreover, in a paper by Oblak et al. (2015), critical mutations in MD-2 proved capable of abrogating LPS-induced TLR4 activation despite being incapable of completely inhibiting metal-induced TLR4 activation.[124] This contributes further to the idea that endotoxin-induced signalling can be distinguished from metal-induced signalling and leaves open the possibility for MD-2 independent metal-induced TLR4 activation.

The extended exposure time is unlikely to have led to confounding amounts of MD-2-independent DAMPs relevant to pro-inflammatory immune responses as histidine mutations would not have been able to uniquely abrogate nickel-induced TLR4 activation completely. If the mutational effects were instead specific to MD-2-independent DAMPs, there would have been little to no significant differences between the inhibition of nickel, platinum, and cisplatin-induced activation. It is important to note in this case, that despite experiencing similar degrees of inhibited activation associated with histidine mutations, platinum ions (II) and (IV) have cytotoxic profiles distinct from each other and cisplatin. In other words, the metals likely trigger different degrees of DAMP release - and yet experience similar degrees of stunted activation due to histidine mutation regardless. Stunted activation that is also, again, distinct from the canonical LPS-mediated signalling of TLR4.

The differences between hTLR4 Apparent K_d values for nickel and cisplatin also do not seem to mesh well with the data from the other experiments. Even though hTLR4 has a higher reported binding affinity for nickel ions based on MST, soluble hTLR4 appears to sequester cisplatin more effectively (up to 50% on average as opposed to up to 30% on average versus nickel). This would also go against expectations based on amino acid residue analysis as additive residues beyond H456 and H458 unique for binding cisplatin must exist compared to nickel based on the mTLR4-hTLR4 and histidine mutation comparisons.

The most likely explanation for these inconsistencies is in the difference in accuracy between the two estimated binding curves. The best-fit curve for hTLR4-nickel binding had an $R^2 = 0.651$ while the best-fit curve for hTLR4-cisplatin binding had an $R^2 = 0.927$. This discrepancy in accuracy is itself likely rooted in differences in solubility between the two agonists, cisplatin being far more soluble within the optimized buffer for MST compared to nickel. Due to the insolubility of nickel relative to cisplatin, aggregation and adsorption events were much more common. It would not be surprising if the reduced solubility directly limited hTLR4 access and binding to nickel ions or impaired the detection of any binding-related fluorescence changes.

Otherwise, the data presented portrays a strong case for the existence of direct cisplatin-TLR4 binding through accepted metal-based binding properties. The nuances of this TLR4 mechanic have yet to be fully elucidated and could be vital for future research ventures. Though H456 and H458 appear important for cisplatin-induced TLR4 activation, the underlying residue or remaining set of residues sufficient for cisplatin-binding has yet to be determined. This property must, at the very least, exist in human and mouse TLR4 based on the soluble TLR4 data and MST. mTLR4 only has access to the conserved histidine, H431, while hTLR4 has access to H431 and the two human-specific histidines, H456 and H458.[120] This distinction could be why mTLR4 can still bind cisplatin but with an apparent affinity (133.6μM) statistically significantly lower than, hTLR4 (50.57μM). While the extent of this dissimilarity may be partially rooted in the fact that the mTLR4 cisplatin binding curve was estimated using a narrower range of cisplatin concentrations, H431 may nevertheless prove sufficient for binding platinum, but not atomically smaller Group 9 and 10 transition metal ions in the absence of additional residues.

Whatever this property may be, it may also exist in zebrafish; morpholino-mediated knockdown of the two zebrafish TLR4 receptors (zTLRs), *tlr4ba* and *tlr4bb*, confers a degree of protection from the zebrafish-equivalent of cisplatin-induced ototoxicity. Analysis of the zTLR4 sequences reveals that zTLR4bb does have a conserved H431 but does not possess H456 or H458. It does, however, contain three additional histidines nearby (H445, H450, and H461) – none of which exist in zTLR4ba (Figure 12). These distinctions could help evaluate the importance of precise histidine positions over general charge placement in the TLR4 metal-binding. Alternatively, platinum-specific residues may exist elsewhere – in the broader hypervariable region or the interspecies hypervariable region.

Chimeric mTLR4-hTLR4 proteins facilitated the discovery of H456 and H458 by allowing researchers to test the metal-binding capabilities of individual and combined TLR4 domains. Similar tests could identify undiscovered minimum regional requirements for platinum-induced TLR4 activation instead of nickel. I did manage to get the original chimeric mTLR4-hTLR4 proteins used by the original metal-binding research groups from Dr. Adeline Hajjar. Given that the overarching goal was to detect any physically direct and causative binding between TLR4 and cisplatin and that I succeeded without the chimeric proteins, I only initiated their validation and preliminary testing. Follow-up studies can incorporate their use should the granular approach of histidine comparisons fail. Biochemical analysis of the protein crystal structures may also help in this regard, as it did for theoretically validating the importance of H456 and H458 for nickel ions.

FIGURE 12. Zebrafish zTLR4bb and human hTLR4 share a conserved H431; both zTLRs have added local histidine residues but no H456 and H458. Zebrafish TLR4 (zTLR4) have recently been found to elicit responses to LPS with their version of MD-2 (Ly96) and are no longer considered true 'orphan receptors'. Zebrafish TLR4 also appear to be relevant in development of CIO-like symptoms. (A) Ectodomain sequence of zTLR4ba has little to no similarities captured within the TLR4 hypervariable regions. (B) Ectodomain sequence of zTLR4bb shares the conserved H431 with both mTLR4 and hTLR4. While missing H456 and H458, it contains far more local histidines compared to both zTLRba, mTLR4, and hTLR4. Beyond the depicted ectodomains, zTLR4ba and zTLR4bb share an approximate 38% sequence similarity with hTLR4.

Beyond addressing the internal complexities of platinum and cisplatin-TLR4 binding, additional work can provide a more accurate understanding of binding dynamics. While MST proved adequate for detecting binding as a whole, other techniques could provide valuable insight into binding rates but more so establish more accurate binding affinities. Strategies along the same line include SPR and BLITZ are far more sensitive assays for binding. Preliminary platinum (II) and platinum (IV) binding analyses through MST yielded results suggestive of success, but the work remained inconclusive. Adsorption and aggregation issues also emerged due to their impaired solubility, especially at higher concentrations, in the available optimal (lab-standard) solvent for MST. These factors were also relevant in the testing of the nickel and cisplatin, which limited the range of concentrations for testing. This in turn affected binding curve analyses; the lack of both completely defined low and high concentration response plateaus made the assumption of four-parameter sigmoidal curve fits for analyses untenable. The fluidic and more sensitive nature of SPR may alleviate this issue by allowing for flowing analytes at smaller concentration ranges. Another option could involve less-MST-specific solvents better suited for dissolving platinum (II) and platinum (IV) with additional optimization. Because SPR and BLITZ only recognize changes to reporter variables (incidental light reflection or white light interference) at a specific location, they may additionally circumvent the need for conditions that make for ideal MST, such as protein fluorescence/labelling and low-background solvents. One other reason for the reporting of Apparent K_d values, as opposed to absolute values, is that the soluble TLR4 proteins were unlabelled; detection hinged on binding effects on the intrinsic autofluorescence of ectodomain tryptophan residues which likely restricted sensitivity. For reference, the hTLR4 ectodomain contains just 3 tryptophan residues compared to the 6 in mTLR4. TLR4 immobilization in both SPR and BLITZ could help limit the use of detergents and reduce restrictions on solvent that would otherwise have confounding degrees of autofluorescence at higher concentrations. Immobilization also better mimics biological systems where TLRs are membrane-bound. Because there has been a noted distinction between metal-mediated TLR4 homodimerization and metal-mediated TLR4 activation events, follow-up studies should also include the analysis of TLR4 dimerization events and immediate activation indicators (such as TIRAP/TRAM recruitment) following exposure to platinum (II), platinum (IV) and cisplatin.

I attempted to probe this process through Fluorescence Resonance Energy Transfer (FRETs) assay using CFP-tagged and YFP-tagged TLR4, but machine limitations led to inconsistent results. This could be viable in the future with additional optimization or a switch in approach (towards Flow Cytometry), but to overcome this challenge, I have begun work with SEC (Size Exclusion Chromatography) in tandem with immunoblotting. Dimerization should facilitate a size change sufficient for protein samples to register different values on a chromatogram, elute in separate fractions, and immunoblot at differing positions. During the 65th Annual Meeting of the Canadian Society for Molecular Biosciences, I was privy to the stunning visualization of a single water molecule through Cryo-EM. It may be possible to use this technology to capture snapshots of cisplatin-TLR4 and Group 9/10 metal-TLR4 formations in the future. Regardless of the particular methods chosen for subsequent investigations, verifying the inability of soluble H456A-H458L hTLR4 to bind metal ions through those methods should be considered a priority. Soluble humanized mTLR4 (Y456H-T458H) should also be produced and tested for gained binding properties. The Schmidt group did create and use these constructs for *in-vitro* cell culture experiments but did not evaluate binding capabilities in isolation through direct binding assays.

4.5 – Additional Considerations & Inquiries

Now that there is evidence for direct binding interactions between cisplatin and TLR4, new questions about CITs can be raised beyond the mechanistic. Questions pertaining to the applicability of the work described. If, as my experiments and the literature demonstrate, it is possible to curb metal and cisplatin-induced TLR4 activation without impeding natural antibacterial responses, then TLR4 ought to be investigated as a target for alleviating CITs. In a recently published review, I underline the abundance of anti-inflammatory approaches in pre-clinical and clinical CIT research. Most remedies currently under investigation focus on the inhibition of downstream inflammatory signalling - post-transduction.[65] The discovery of the cisplatin-TLR4 direct interaction axis may open the possibility of pre-transduction inhibition. Experiments must scale up to in-*vivo* models to broach this prospect, however. While I have contributed to work that has become pertinent to zebrafish, it would be interesting to see if my findings have implications relevant to mammalian models. CRISPR/Cas9 systems have been used produced mice with specific TLR4 residue substitutions (D298G/N39671) to study potential causes of LPS hypo-responsiveness in humans.[140]

It could thus be worthwhile to scale planned mutation experiments, such as H431 deletion, through the same process to observe changes to platinum ion responses and CIT development; mice are already model organisms for CIN, CIPN and CIO. It would provide additional insight into the significance of each TLR4-related CIT model proposed in a much more realistic environment – with a fully functioning immune system available. If removing the entire collection of metal-binding residues discovered provides little rescue, then DAMPs and upregulated LPS recognition would have to be the prime TLR4-related drivers of CITs. Of course, the opposite could also hold true.

4.6 – Conclusion

In summary, in the completion of my thesis, I provide novel evidence to suggest that cisplatin can be directly bound by human TLR4. This binding can occur independent of other cellular factors and, in cell culture, is sufficient to elicit pro-inflammatory responses, like IL-8 secretion, reflective of genuine CITs. Using recognized methods, I determined that two accepted metal-binding histidine residues, H456 and H458, contribute to, but are not solely responsible for, mediating this interaction (Figure 13). The underlying residue sufficient for this interaction has yet to be identified, but the fact that murine and human (and perhaps zebrafish) TLR4 can bind and elicit responses to cisplatin suggests that the remaining factor is conserved between species. In the process of doing this research, I took advantage of microscale thermophoresis, a direct method for detecting and quantifying binding interactions, to also present novel verification of TLR4 Group 9-Group 10 transition metal binding capabilities. The importance of these observed cisplatin-TLR4 interactions to CIT development as a whole – in living organisms - compared to other pre-existing and compatible models has yet to be determined. Full characterization of binding requirements, dynamics, and immediate events following initial cisplatin binding, such as dimerization and adaptor recruitment, should be the focus of subsequent studies. Through this work, and the work I have contributed to other CIT investigators, I hope to have made a difference - no matter how small - to the collective body of knowledge available so that one day it can translate into positive material changes to patient chemotherapeutic outcomes and quality of life.

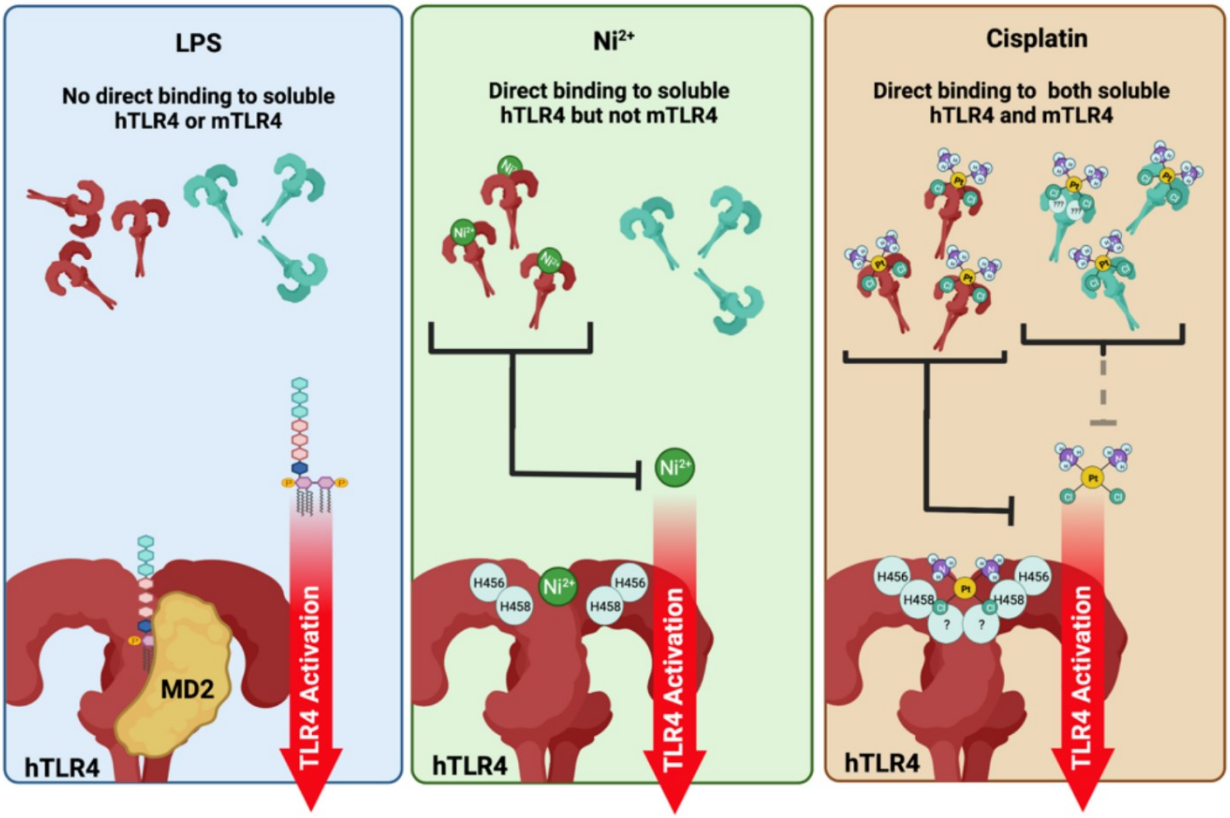

FIGURE 13. Model summarized; TLR4 is activated by LPS, nickel and cisplatin through distinct mechanisms. (Left Panel) MD-2 (and other co-receptors) mediate TLR4 binding of LPS. Soluble forms of TLR4 are insufficient to inhibit this interaction. (Middle Panel) TLR4 binds nickel independent of MD-2, but entirely dependent on H456 and H458. Soluble hTLR4, but not mTLR4, can mitigate this process. (Right Panel) TLR4 binds cisplatin; activity resultant from binding is enhanced by, but not entirely dependent on, H456 and H458. Both soluble mTLR4 and hTLR4 can pose a challenge to this interaction. There is a discrepancy in their binding affinities and protection, as signified by solid versus dashed lines. The requisite residue or residues, (?), are unknown and requires further investigation.

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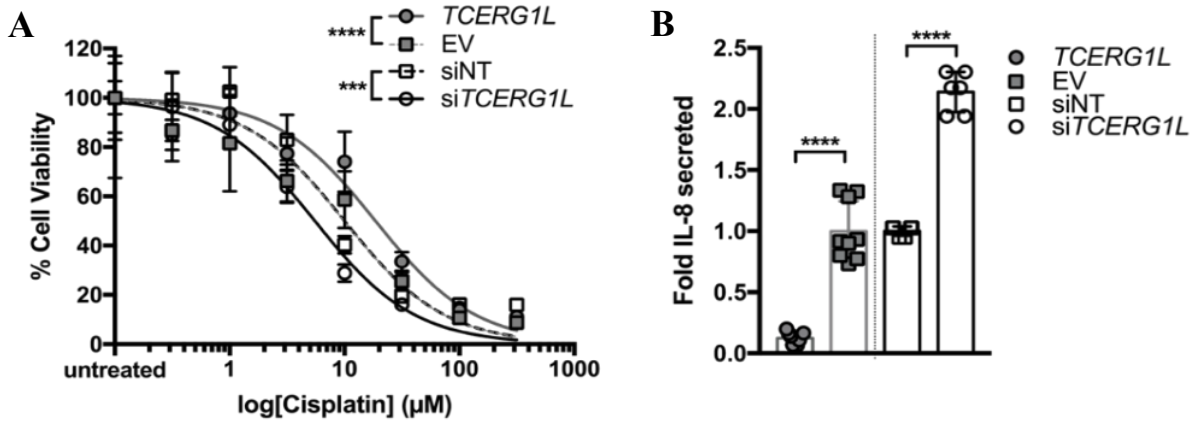
APPENDIX

Appendix 1.1 – Summary

The work detailed in this section was performed in collaboration with Dr. A.J.M. Meijer and the PanCareLife Consortium, a pan-European research group dedicated to studying the long-term effects of childhood cancer and chemotherapy on survivor quality of life. The data is part of a report published in NPJ Precision Oncology.[141]

Briefly, GWAS (Genome-Wide Association Studies) identified SNPs (single-nucleotide polymorphisms) of the *Tcerg1L* (Transcription Elongation Regulator 1-Like) gene as a barometer for susceptibility to CIO in non-cranially irradiated pediatric patients. Tcerg1L is a transcription elongation factor associated with the pathology of numerous diseases - ranging from viral infections with HIV and diabetes to various IBDs (inflammatory bowel diseases). It is a paralog of Tcerg1 and is expressed in humans and mice; in humans, expression occurs in a diverse selection of cells. These include cells typically affected by CITs, such as inner ear hair cells. Interested in identifying other possible models for CIT development, I offered to determine the functional impact of Tcerg1L on cisplatin-induced toxicity in an *in-vitro* model. Using HeLa cells transfected with either empty vector (EV), additional Tcerg1L (pCMV6-Tcerg1L), non-targeting siRNA (NT) or *Tcerg1L*-siRNA, I was able to determine the effects of Tcerg1L on cisplatin-induced toxicity and pro-inflammatory induction. Based on data, Tcerg1L overexpression is protective against cisplatin-induced toxicity while suppression accomplishes the opposite (Appendix Figure 1A). The same trend applies to cisplatin-induced pro-inflammatory IL-8 secretion. Transfection with Tcerg1L drastically impedes pro-inflammatory cytokine secretion; gene silencing roughly doubles secreted IL-8 (Appendix Figure 1B). The mechanism through which Tcerg1L modulates the effects of cisplatin remains unknown, but Tcerg1 is known to affect the course of diseases by directly regulating RNA Polymerase II and altering coupled splicing of transcripts.[142–144] Two genetic targets of its transcription regulation are mediators of cell death, Bcl-x (the Bcl-xL precursor) and the Fas/CD95 ligand.[145] Surprisingly, the literature suggests that Tcerg1L is naturally pro-apoptotic, which clashes with our findings. This difference may stem from the multiplicity of toxic effects cisplatin can produce, including direct DNA damage.



APPENDIX FIGURE 1. Tcerg1L expression has an inverse relationship with cisplatin-induced toxicity and pro-inflammatory response. (A) Cell viability of HeLa cells following treatment with a gradient of cisplatin concentrations plotted as a percentage of untreated cell viability. (B) IL-8 secretion shown in terms of relative fold-induction in response to 100μM cisplatin treatment post-transfection with either pCMV6-Tcerg1L or *TCERG1L*-targeted siRNA. For both experiments, transfection was performed 24HRs post-seeding; treatment with cisplatin followed 24HRs after transfection for 48HRs.

Data Information: (A) Cisplatin CC_{50} = 5.5μM (si*TCERG1L)*, 18.6μM (TCERG1L), 9.92μM and 10.01μM (EV, siNT, respectively). Statistics comparing cell viability curves determined through Extra Sum of Squares F Test; $n = 9$ (for siRNA) and $n = 21$ (for overexpression), from 2-3 independent experiments. (B) Bars denote mean with standard deviation. Statistics obtained through two-tailed Student *t*-tests; $n = 6$ and $n = 9$, from two independent experiments, respectively.
For all data sets ***, $P < 0.001$; ****, $P < 0.0001$.