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Immunological Impact of CLI-095 on Dendritic Cell Maturation and Hypoxia-re-oxygenation induced inflammatory injury

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Abstract

Immunological Impact of CLI-095 on Dendritic Cell Maturation and Hypoxia-re-oxygenation induced inflammatory injury

Introduction: Ischemia reperfusion injury (IRI) activates innate immunity through the engagement of Toll-Like Receptors (TLRs) by endogenous ligands. TLR4 expressed within the kidney is a potential mediator of innate activation and inflammation. Stimulation of TLR4 induces distinct patterns of gene expression, which not only leads to the activation of innate immunity but also to the development of acquired immunity. CLI-095, a novel synthetic small-molecule, suppresses production of multiple cytokines by inhibiting TLR4 signaling. In this study, we have determined the role of TLR4 in hypoxia and re-oxygenation injury model, which mimics IRI in vitro, and investigated the effect of CLI-095 (a specific TLR4 inhibitor) on TLR4 mediated inflammation and maturation of dendritic cells (DCs).

Hypothesis: TLR4 signaling plays an important role in activation of innate immunity, and that targeting its pathway with CLI-095 will prevent inflammation and subsequent injury caused during hypoxia re-oxygenation.

Method: Bone marrow derived dendritic cells were stimulated by hypoxia re-oxygenation with or without CLI-095. Expression of TLR4, proinflammatory cytokines, and dendritic cells maturation markers were then tested by Flow Cytometry, qRT-PCR, and ELISA.

Results: We have shown that CLI-095 is able to blocks the TLR4 signaling pathway and reduce the expression of pro inflammatory cytokines (IL6 and TNFα) in response to

hypoxia re-oxygenation. In addition, DCs that were pretreated with CLI-095 showed low expression of maturation markers in comparison to cells that were subjected to hypoxia re-oxygenation.

Conclusion: TLR4 is involved in innate immunity activation in response to IRI or hypoxia re-oxygenation and CLI-095 is able to block TLR4 signaling pathway and suppress the activation of the inflammatory response. Therefore, ameliorating TLR4 by new therapies such as CLI-095, which specifically targets TLR4, may have potential implication in reducing IRI in clinical transplantation. Since almost all immune cells including DCs express TLR4.

Keywords:

Kidney, Ischemia Reperfusion Injury, hypoxia re-oxygenation, Toll like receptors, Innate immunity, CLI-095

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List of abbreviations

7-AAD	7 Aminoactinimycin D
ABMR	Antibody-mediated rejection
APC	Antigen presenting cell
ATP	Adenosine triphosphate
Ca	Calcium
CD 80	Cluster of differentiation 80
CD 83	Cluster of differentiation 83
CD 86	Cluster of differentiation 86
cDNA	Complimentary Deoxyribonucleic Acid

DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FC	Fragment crystallisable

GMCSF	Granulocyte macrophage colony stimulating factor
HMGB1	High mobility group box 1
ICAM-1	Intracellular adhesion molecule-1
IFN-γ	Interferon gamma
IRAK1	Interleukin -1 receptor-associated kinase
IRAK4	Interleukin-1 receptor-associated kinase 4
IRI	Ischemia reperfusion injury
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase

MHC	Major histocompatibility complex
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger Ribonucleic Acid
MyD88	Myeloid differentiation primary response gene 88
Na	Sodium
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOD-Like	nucleotide-binding oligomerization domain-like receptors
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate Buffered Saline

qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
TAK-1	Transforming growth factor beta-activated kinase 1
TCR	T-cell receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TRAF	TNF receptor-associated factor

VCAM-1	Vascular cell adhesion molecule-1

Introduction

Kidney transplant

Kidney disease affects 200 million people worldwide, and on average 16 people experience kidney failure daily in Canada alone [1]. Transplant remains the best option for patients suffering from end-stage renal disease [2]. However, chronic allograft rejection and side effects of immunosuppressive drugs are still a barrier to the long-term success of kidney transplant. Many studies have linked acute kidney injury, delayed graft function, and acute and chronic rejection to the damage incurred by ischemia reperfusion injury (IRI) during and after the transplant [3].

1.1.1 IRI and transplant

IRI often occurs when transplanting a kidney and is the result of a disturbance of the blood flow. Brain death influences disturbance of blood flow and is caused by chronic hemodynamic interruptions in the cadaveric donors. The innate immune system and complement cascade in the donor are activated by these disturbances. During the harvesting operation, a brief but chronic renal ischemia is caused by clamping of the renal artery. Further ischemia damage may result from cold ischemia during storage of the allograft kidney [4]. In living donors, there is subjection of the allograft kidney transplantation into warm ischemia, where there are no condition interruptions relating to brain death. However, IRI is less severe in living donor's transplantation [5].

1.1.2 Delayed graft function and IRI

After the transplantation of the kidney, another often early condition is delayed graft function (DGF), which is occures after ischemic acute tubular necrosis as a result of

IRI [6]. The level of IRI is dependent on many factors occurring in the donor, the transplantation process, and the recipient [7]. The first post-transplant period is when the severe complication DGF occurs. Based on the severe complications relating to the renal impairment and the related costs of longer hospital periods, many studies have linked DGF occurrence and the acute and chronic allograft dysfunction [8, 9]. A two-phase injury is determined by IRI in the transplanted kidney. The initial phase, occurring immediately after transplantation, is linked to the ischemic damage, while the second phase, which happens later, is associated with IRI-linked activation of the adaptive and innate immune response. It has the capacity to influence either cell-mediated rejection or antibody-mediated rejection (ABMR) [10, 11].

The following factors affect DGF and IRI. (I) Donor factors: Donor-associated factors that increase DGF risks are donor age, results of biopsy at implantation, and brain or cardiac death [12, 13]. (II) Recipient factors: The significant recipient factors that influence the occurrence and severity of DGF and IRI are high panel-reactive antibodies, a body mass index higher than 30, the African American race, and the male gender [14]. (III) Storage preservation: The duration of cold ischemia and storage time is related to DGF. Sufficient preservation of renal allograft is essential during cold storage for prevention of DGF [15, 16].

The clinical influence of IRI on the rate of survival and kidney graft function is related to DGF, rejection of the graft, and severe graft dysfunction with a continuous interstitial fibrosis. (I) DGF: DGF is caused by IRI-linked ischemic graft damage, which affects the long-term and short-term results of the kidney graft [17]. There is a lack of a basic DGF definition, and controversy exists regarding the effect of DGF on long-term

graft survival. (II) Graft rejection: After kidney transplantation, the inflammatory response following IRI leads to a higher immunogenicity of the graft [18]. IRI may also increase the response of humoral immunity to antigens. An enabled cross relation between B and T cells also favors this increase. This results in increased rates of ABMR. Furthermore, immunogenicity of the graft is enhanced by the presentation of antigens by dendritic cells to the naive T cells, hence causing rejections of T cells [19]. (III) Chronic graft dysfunction: Continuous interstitial fibrosis of the kidney graft is caused by IRI in experimental models of kidney transplantation. In humans, in contrast, IRI is associated with the establishment of interstitial fibrosis or tubular atrophy [20, 21].

IRI

IRI is a common cause of acute kidney damage and has an impact on both short- and long-term graft survival post-transplant [22]. Ischemia refers to the restriction of blood to body tissues, which results in shortages of the oxygen and glucose necessary for cell metabolism. There are instances in which blood flow returns to affected tissues after an ischemic period, and damage that occurs during this period is referred to as reperfusion injury [23]. A decreased metabolic supply in an ischemic organ leads to severe microvascular dysfunction [24]. Interestingly, reperfusion does not reinstate normal circumstances, but instead increases the injury by activating numerous mechanisms, such as innate and adaptive immune activation and cell death programs [25].

1.2.1 Kidney IRI pathophysiology

1.2.2 Oxidative stress and mitochondrial dysfunction

The first change stimulated by ischemia is a shortage in oxygen availability, which leads to a change from aerobic to anaerobic metabolism [26]. The latter does not satisfy the demand from aerobic tissues, and intracellular ATP levels therefore decrease. In addition, intracellular acidosis increases due to increased lactic acid, which results from lactate-dependent ATP creation. These procedures result in (1) destabilization of lysosome membranes with lysosome enzyme leakage and cell structure breakdown [27]; and (2) inhibition of membrane-bound Na^+-K^+-ATPase action [28]. Eventually there is a huge intracellular release of Na^+ ions and water, along with edema [26]. The levels of intracellular Ca^{2+} also increase because it is no longer being pumped out of the cells [29] and the depletion of ATP prevents Ca^{2+} re-uptake [30]. In turn, the overproduction of calcium leads to the stimulation of calcium-dependent proteases such as calpains. Calpains stay inactive because of the acid environment, although they might harm the cells after the normalization of pH upon reperfusion [31]. Another consequence of Ca^{2+} overproduction is the production of reactive oxygen species (ROS) in the mitochondrial stage of ischemia. This leads to the opening of mitochondrial transition pores (mPTPs) after reperfusion, along with cell death and apoptosis [32, 33].

1.2.3 Inflammation

Based on observations of abnormities in kidney IRI, inflammation appears to link different types of cells and is important in the pathophysiology. An inflammatory cascade in renal IRI can result in further damages. Therefore, renal tissue is protected by the inhibition of inflammatory response as a key therapeutic tactic [34, 35]. The key inflammation mediators are chemokines, cytokines, and interleukins, which have a major

role in regulating pro-inflammatory reaction, infiltration, and activation of leukocytes, as well as the expression of adhesion molecules [36]. Renal dysfunction is highly stimulated by pro-inflammatory cytokines such as interleukin 6 (IL6) and TNFα [36, 37].

Neutrophils and leukocytes are recruited into post-ischemic tissue by inflammatory mediators, ROS, and cell adhesion molecules P-selectin and intracellular adhesion molecule-1 (ICM-1). This later results in improved leukocyte-endothelial interactions, which can increase damage to and inflammation of the endothelial cells, and interfere with blood flow[38]. Both chronic and acute renal failure have been associated with the participation of the inflammatory leukotriene pathway in IRI [39, 40]. In summary, it is evident that inflammation, leukocytes, and adhesion molecules are extremely integrated with the process of IRI. Thus, any agents capable of inhibiting neutrophils and leucocyte infiltration or suppressing inflammation could be able to reduce the effects of IRI on the kidney.

1.3 Innate and adaptive immunity

The innate immune system comprises the initial line of host defense throughout infection. It therefore plays a decisive role during early detection and consequent activation of pro-inflammatory reactions to attacking pathogens [41]. Conversely, the adaptive immune mechanism is responsible for the eradication of pathogens during the late stage of infection and during the production of immunological memory [42].

The innate immune system consists of cellular and non-cellular components that react instantly and non-particularly to microorganisms at the spot of infection. The cellular elements of innate immunity include macrophages, neutrophils, dendritic cells

(DCs), and natural killer (NK) cells [43]. The molecular elements include toll-like receptors (TLR), chemokines, complement proteins, and cytokines [44, 45]. Due to IRI, innate elements are stimulated (complement activation and TLR triggering) [46], and these activate adaptive immunity and add to the delay of graft functions. In contrast, the adaptive immune system consists of an antigen-specific immune reaction against an identified foreign antibody and leads to memory. The adaptive system is further separated into cell-mediated immunity and humoral immunity, which involve antibody-releasing B cells and cytotoxic/helper T cells, respectively.

1.3.1 Cells involved in innate immunity

After reperfusion, neutrophils adhere to the endothelium and drift into the tissue. The neutrophils respond to any unspecific injuries and discharge proteases, ROS, and pro-inflammatory cytokines, such as IL-4, IL-6, interferon-γ, and tumor necrosis factor-α [47]. Macrophages also generate pro-inflammatory cytokines and can be found in injured tissues from the early phases of IRI [48]. The NK cells serve vital roles in renal IRI, including the perforin-dependent killing of tubular cells [49]. DCs represent a necessary stage during the pathogenesis of IRI and experience an antigen-independent maturation process stimulated by damage-associated or pathogen-associated molecular proteins (DAMPs, PAMPs). In addition, DCs can symbolize a bridge between innate and adaptive immune stimulation. In renal transplantation, brain death induces oxidative stress in the deceased donor. The donor DCs are stimulated in this case and can trigger the recipient's T cells [50].

1.3.2 Kidney DCs

Kidney DCs are present in the interstitial extracellular compartment and are perfectly located to interact with other substances that are transported from the tubule lumen and directly into the peritubular capillaries [51]. These DCs also interact with endogenous molecules, exogenous invading organisms, lymphocytes, NK T cells, fibroblasts, and epithelial cells. Therefore, DCs are activated by DAMPs or PAMPs [52]. If these two molecular patterns are present, then the DCs act as key effectors and initiators of the innate immune system, which comprises NK cells, DCs, monocytes, and neutrophils. In reperfusion damage from kidney ischemia, the kidney DCs play an integral role in the antigen-independent inflammatory response after reperfusion has taken place. They also play a substantial role in the production of chemokines and cytokines, and both of these signaling proteins drive neutrophil infiltration through the processes of activating NK T cells and the signal pathway [48, 53].

At the same time, DCs can also stimulate tolerance due to the phenotypic plasticity and function of the kidney. The kidney DCs that induce tolerance are immature, insufficiently produce positive co-stimulatory signals, and inadequately enhance negative stimulatory signals. They also express reduced pro-inflammatory cytokines and have the capacity to produce immune tolerance via induced T cell energy.

1.3.3 Formation and maturation of DCs

DCs are extracted from bone-marrow hematopoietic cells. They initially transform into immature DCs and are characterized by low T cell activation potential and high endocytic activity. Immature kidney DCs continuously sample their environment for bacteria, viruses, and other pathogens. They do this via pattern-recognition receptors, one

of which is TLR4. Immature DCs use phagocytose nominal amounts of the membranes of live cells, which is also known as nibbling. When these immature cells interact with a presentable antigen, the cells turn into DCs and then travel themselves to a lymph node.

The cells also phagocytose different pathogens and extract their proteins in tiny pieces. Once these cells have matured, they present fragments at the cell surface via MHC. These cells simultaneously enhance the ability to activate T cells and upregulate co-receptors for the purpose of T cell activation, such as CD40, CD86, and CD80. Moreover, these cells also regulate chemokine receptor type 7, which is a chemotactic receptor that causes DCs to travel via the blood stream or the lymphatic system. This is where they play the role of antigen-presenting cells, which trigger helper and killer T cells. Furthermore, they activate B cells by providing them with different antigens that are extracted from a pathogen and co-stimulatory signals. These cells can also activate T cell tolerance and some C-type lectin receptors, which function as pattern-recognition receptors and regulate DCs when lymphocyte activation is appropriate instead of inducing immune tolerance [54].

Helper T cells are limited to a single antigen and are professional antigen-presenting cells like DCs. These cells can activate resting helper T cells when the corresponding antigen is provided. B cells and macrophages have the capacity to activate memory T cells, while DCs can activate both naïve T cells and memory T cells. Naïve T cells and memory T cells are some of the most potent among antigen cells. Mature DCs have the potential to activate CD8± T cells, while the formulation of memory T cells can only be completed by the interaction of CD4 helper T cells and DCs [55]. The help provided by CD4 cells subsequently triggers the matured cells and induces CD8 memory

T cells, which have the capacity to expand twice [55, 56]. This activation process requires simultaneous interaction from CD8 T cells, CD4 DCs, and T cells [56].

As previously mentioned, mature DCs are generated from white blood cells and monocytes, which can transform into DCs or macrophages. The macrophages are formed from stem cells in bone marrow, whereas monocyte DCs are produced through peripheral blood mononuclear cells (PBMCs). These monocytes can be treated with interleukin and granulocyte macrophage colony-stimulating factor, both of which result in the differentiation of immature DCs in approximately one week's time. After this treatment, tumor necrosis factor then turns the immature DCs into mature DCs [57].

1.3.4 Mechanism of IRI-induced immune system activation

There are two stages in the sequence caused by ischemia reperfusion and the resulting immune system activation. The first is the stimulation of the innate system. The detection receptors of innate immunity are mainly TLRs (both intra- and extracellular), NOD-like receptors, intracellular receptors, and retinoic acid-inducible gene 1 receptor (RIG1). TLRs are necessary to identify PAMPs or DAMPs. The TLRs trigger several kinases [IL-1-receptor-linked kinase 1 (IRAK1), IL-1-receptor-linked kinase 4 (IRAK4), TANK fastening kinase 1, inhibitor of NF-κB kinase] enlisting within the cytoplasm adaptor fragments [myeloid separation 88 (MyD88), Toll/IL receptor-containing adaptor protein, TRIF-related adaptor molecule, and TIR domain-containing adaptor-inducing interferon (TRIF)]. The kinases intensify and broadcast the signal to the transcription elements NF-κB, MAP3 kinase (MAPK3), and interferon regulatory element 3. Finally, the transcription elements program the genes controlling the inflammatory cells [9].

The second stage is the stimulation of the adaptive mechanism. In tissues influenced by inflammation, the immature DCs develop into mature cells, attach to the antigen, and drift to the lymph nodes, where they present the antigen to the T cells. The T cell activation is mediated through signals produced by the T cell receptor and the co-activation fragments. The strict relations between T and B cells might produce an alloimmune reaction. It has also been established that the renal IRI might increase the humoral immune reaction and produce an ABMR [11].

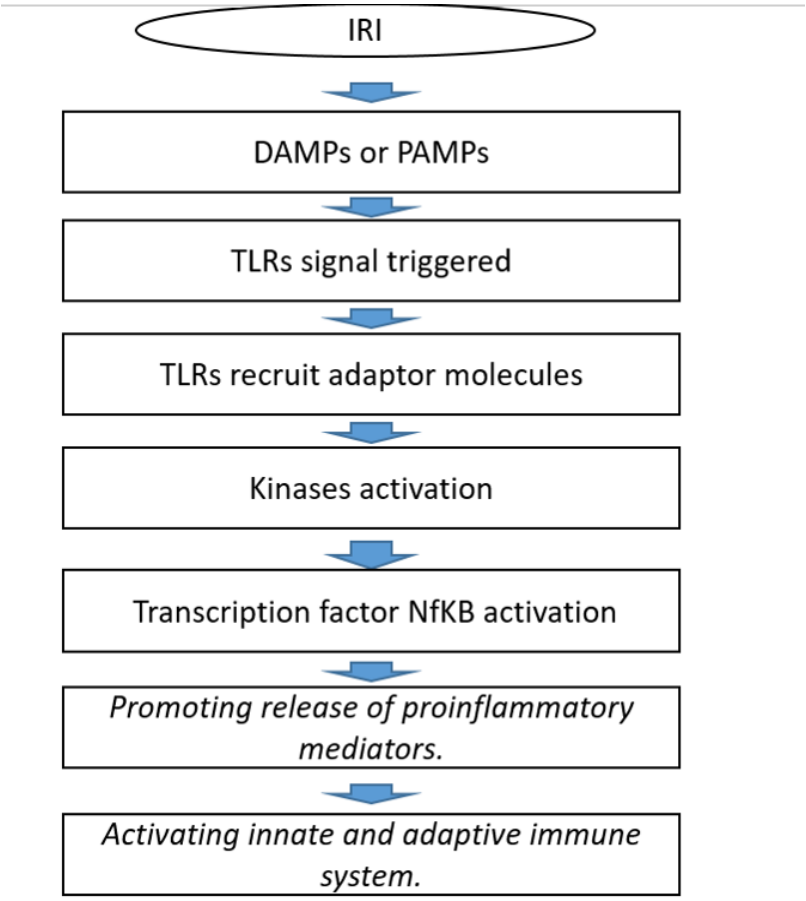

Figure 1: The mechanism of IRI-induced immune system activation

1.4 TLRs

TLRs are germline-encoded pattern-recognition receptors that detect invading pathogens in the host immune system [58]. To date, 10 TLRs have been defined in humans, while mice have 12 [58]. TLRs are types of transmembrane proteins that are expressed primarily in the immune system cells, such as macrophages, DCs, neutrophils, B cells, and NK cells [59-61]. However, TLRs can also be expressed in non-immune cells, such as epithelial and endothelial cells [61]. Signaling via TLRs depends on association with a group of cytoplasmic adaptor molecules, principally MyD88 and Toll/IL-1 receptor domains containing adaptor-inducing IFN-β (TRIF) [62]. Upon activation via MyD88, TLRs initiate downstream signals that liberate transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) to be translocated to the nucleus. This in turn initiates the transcription of pro-inflammatory cytokines, chemokines, and adhesion molecules, as well as neutrophil infiltration in graft tissue and DC maturation [60]. Moreover, mature DCs interact with T cells and promote the adaptive immune response [63]. In the context of transplantation, TLR-driven innate immune responses are critical to IRI.

1.4.1 TLRs ligands

TLR receptors recognize microbial products such as bacterial lipopolysaccharides (LPS) and CpG DNA. These are also known as PAMPs. Endogenous ligands released from damaged tissues known as DAMPs can also signal through TLRs. These include high-mobility group box 1 (HMGB-1), heat shock proteins, and necrotic nucleosome-DNA, which can be recognized by TLR2, TLR3, TLR4, and TLR9 to promote inflammation [60, 64, 65]. Among them, only the nuclear protein HMGB-1 has been

confirmed to be related to the pathogenesis of IRI [66, 67]. HMGB-1 unites the DNA and controls the transcription and modeling of chromatin. In deceased-donor kidneys, in which IRI is recurrent and severe, TLR-4 is upregulated and tubular HMGB-1 is noticeable [68]. The TLR4 receptors play a major role in IRI.

1.4.2 HMGB-1

The chromatin-linked protein HMGB-1 is released by hematopoietic cells via a lysosome-mediated pathway [69]. It is a key mediator of endotoxin shock [70] and serves in numerous immune cells to activate inflammatory reactions as a DAMP [71]. Recognized receptors for HMGB-1 include TLR2, TLR4, and RAGE (receptor for advanced glycation end products). HMGB1 can stimulate maturation of dendritic cell through upregulation of CD80, CD83, CD86, and CD11c; and stimulate creation of other pro-inflammatory cytokines within myeloid cells (IL-1, TNF-α, IL-6, IL-8), including upregulated articulation of cell adhesion particles (ICAM-1, VCAM-1) on endothelial cells.

1.4.3 TLRs' signaling pathway

The innate immune system uses TLRs to identify preserved molecules connected with pathogens, resulting in inflammatory reactions and a link to adaptive immunity [72, 73]. Upon binding to the conventional TLR ligands, all of the TLRs apart from TLR3 employ the adaptor particle MyD88 via the TIR domain and arbitrate the MyD88-dependent pathway [74]. After that, MyD88 employs the serine-threonine kinases IL-1, receptor-linked kinase4 (IRAK4), and IRAK1. IRAK4 phosphorylates IRAK1 and subsequently mediates the employment of TRAF6 by the receptor compound [75]. The IRAK1-TRAF6 compound then detaches from the receptor to interact with and stimulate

MEKK3 and TAK1, which are members of the MAP kinase kinase kinase (MAPKKK) family [76]. The activation of TAK1 and MEKK3 ultimately stimulates of NF-κB and JNK [77], which results in the transcription of chemokine genes and inflammatory cytokines such as those encoding TNFα, IL-1β, IL-6, and IL-8. Moreover, TLR3 and TLR4 recruit a MyD88-independent pathway that employs TIR domain-containing adaptor stimulating IFN-β (TRIF) to stimulate NF-kB and IRF3.

1.4.4 TLR4 and IRI

Several studies have suggested that the expression of both TLR4 and its multiple endogenous ligands is amplified in the ischemic kidney in response to the devolvement of renal IRI signaling through the TLR4/MyD88 pathway [78-80]. In one study, mice hereditarily lacking in TLR4 were protected from kidney dysfunction and histological harm. This protection was linked with a decrease in pro-inflammatory cytokine and chemokine production associated with the reduction of macrophage and neutrophil infiltration. TLR4 signaling in kidney parenchymal cells significantly contributes to kidney damage, although articulation of TLR4 on leukocytes is also clearly significant in IRI [78]. This underlines the role of TLR4 in renal ischemia reperfusion, and suggests that blocking TLR4 signaling might offer a foundation for therapeutic strategies to diagnose or protect against renal ischemic damage.

1.5 Therapeutic targeting of TLRs

Research using TLR-deficient mice has implied a role of TLRs in several pathological circumstances, and the topic of TLRs and the signals they produce is attracting huge interest. A majority of the research has been conducted using different ischemia reperfusion models, such as intestinal, renal, hepatic, cerebral, and myocardial

models. Such studies have also focused on the functionality of TLR4 to various degrees. Moreover, TLR4 is also thought to be engaged in numerous pathologies, such as cardiac diseases [81], sepsis [82], and IRI [83-85]. Therefore, TLR4 is a notable therapeutic target for the management of inflammatory diseases. In this vein, numerous small-molecule complexes that control TLR signaling were explored in a clinical study [86].

1.5.1 CLI-095

CLI-095 is a cyclohexene derivative that is also known as TAK-242. It is an antagonist for TLR4 and selectively inhibits its pathway [87]. CLI-095 was initially categorized as a suppressor of nitric oxide (NO) and cytokine generation by LPS-stimulated macrophages and in endotoxin shock in mice [88]. CLI-095 attaches to cysteine 747 in the intracellular sphere of TLR4, thus hindering both MyD88-independent and MyD88-dependent pathways stimulated by LPS [89]. Furthermore, one study demonstrated that CLI-095 suppresses LPS-induced inflammation [90]: CLI-095 restrains the production of TNF-α in LPS-activated human U-937, mouse RAW264.7, and P31/FUJ cells [90]. Moreover, CLI-095 hinders the stimulation of ligand-independent NF-κB, resulting in overexpression of TLR4. CLI-095 also suppresses the linking of Toll/interleukin-1 together with the TLR4 receptor domain, consisting of an adaptor protein. Furthermore, CLI-095 inhibits the activation of TIRAP-mediated nuclear factor and activates TRAM-mediated and interferon-sensitive components in HEK293 cells articulating MD-2, CD14, and TLR4 [91]. In addition, in one study CLI-095 management hindered IRAK1 stimulation in RAW264.7 cells. Furthermore, a study carried out on a cerebral ischemia reperfusion model established that CLI-095 considerably decreased cerebral infarction, enhanced neurological function, hindered the

phosphorylation of downstream protein kinases in the TLR4 signaling pathway, and down-regulated the articulation of inflammatory cytokines [92].

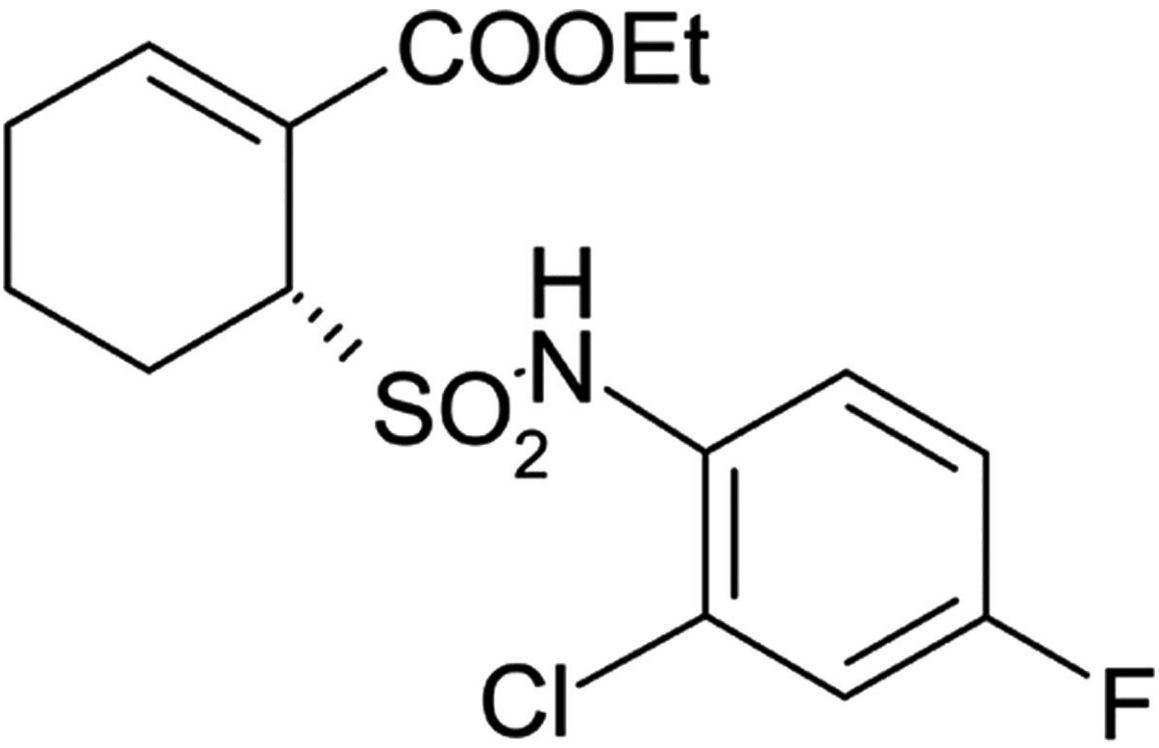

Figure 2: CLI-095 structure

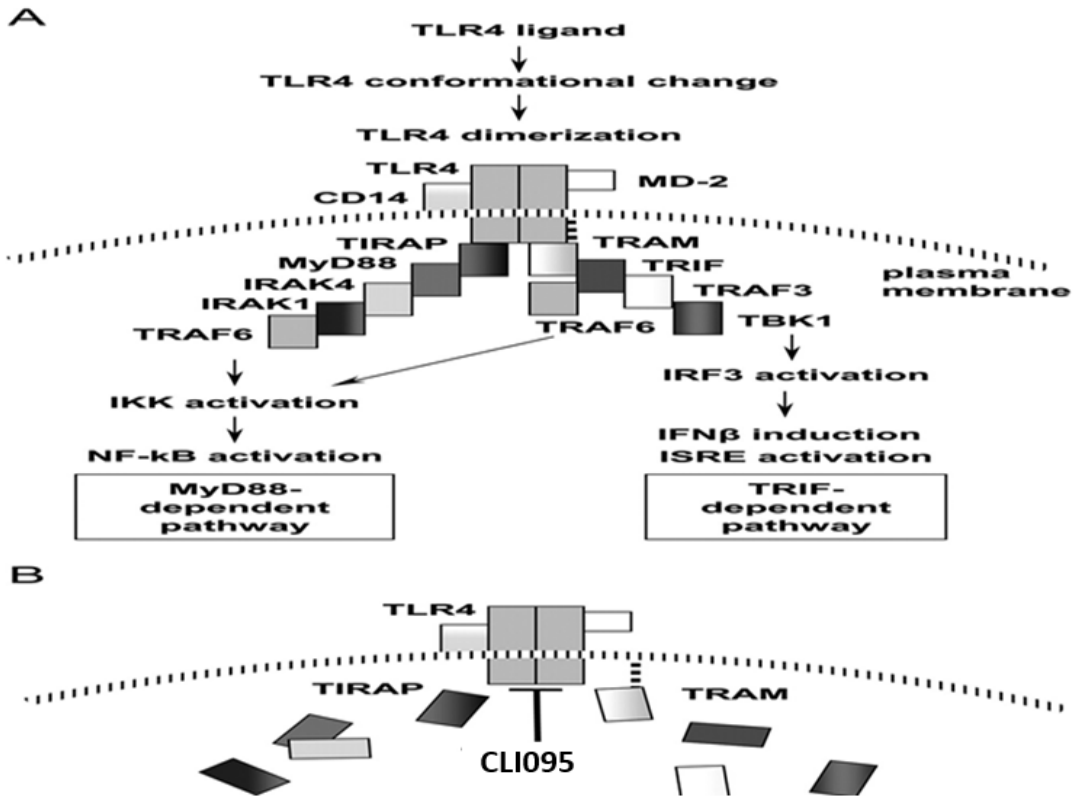

Figure 3 (A) TLR4 signaling pathway (B) CLI-095 mechanism of action on TLR4. The TLR4 pathway transduces signaling in both MyD88-dependent and TRIF-dependent pathways (MyD88 independent). CLI-095 interferes with and blocks the interaction between TLR4 and its adaptor molecules [93].

1.6 Rationale and hypothesis

1.6.1 Rationale

In one study, RAG-1-deficient mice lacking both T and B cells were not protected from IRI, suggesting that cells of the innate immune system (such as DCs) play a significant role [94]. In fact, DCs are the first responders to DAMPs released from damaged or necrotic cells after oxygen deprivation, with subsequent activation of TLR4 and TLR9 signaling. In contrast, DCs significantly infiltrate the kidney after IRI [95]. These findings suggest that it is important to determine whether CLI-095 can modulate TLR4 signaling in DCs to reduce activation of innate immune response to re-oxygenation after hypoxia.

1.6.2 Hypothesis

We hypothesize that TLR4 signaling plays an important role in the activation of innate immunity, and that targeting its pathway will prevent innate immunity activation in response to re-oxygenation after hypoxia.

2 Material and methods

2.1 Cell culture methods

Maintenance of cell lines. Dulbecco's modified Eagle medium (DMEM; Corning cellgro), supplemented with 10% fetal calf serum (FCS; Gibco) and 1.1% penicillin-streptomycin (Gibco), was used to maintain RAW 264.7 cells. The cells were kept in a humidified incubator (37^{o}C; 5% CO_2).

Cell culture. For passaging, adherent cells were washed with pre-warmed (37^{o}C) 1X PBS (Gibco), sub-cultured using 5 mL 0.25% trypsin, and stored in a 37°C incubator for 5 min. Trypsin dilution was performed by adding the appropriate cell medium, which was then pooled into 50-mL tubes. Cells were pelleted by centrifugation (1,000 rpm; 5 min) at room temperature. For washing, the cells were re-suspended in appropriate cell media and centrifuged (1,000 rpm; 5 min) at room temperature. The pelleted cells were re-suspended in the appropriate cell medium and re-plated on 12-well plates at the required density (1×10^6 cells/well).

Primary cells: isolation of bone-marrow-derived DCs. Femurs of C57BL/6 mice were removed and freed from tendons and muscles. The bones were placed in 70% ethanol for 2 min and then washed in DMEM. The bone ends were cut off, and DMEM was used to flush out the marrow. The isolated cells were centrifuged (1,000 rpm; 5 min), and then cultured in a six-well dish at a density of $1x10^6$ cells/mL in DMEM with 1% L-glutamine (Gibco), 1.1% penicillin-streptomycin, and 10% FBS. The cells were then supplemented with 20-30 ng/mL of mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF). Half of the medium was replaced by fresh medium on day 3 of

culture. On day 5, the medium was completely replaced with fresh medium. On day 6, the medium was removed, and cells were incubated with Accutase (Innovation Cell Technologies) at 25°C for 15 min. Cells were pooled into 50-mL tubes and were pelleted by centrifugation (500 rpm; 5 min) at room temperature. The pelleted cells were re-suspended in DC medium and re-plated on 12-well plates at the required density (1×10^6 cells/well).

2.2 Experiment design

2.2.1 Incubation with LPS

DCs that were plated on 12-well plates (1×10^6 cells/well) were divided into four groups of three wells each. Group 1, the normal cells, comprised untreated DCs that were incubated for 12 hrs. Group 2, the LPS cells, were treated with 50 ng/ml of LPS for 12 hrs. Group 3, the CLI-095 group, included cells treated with 100 μg/ml of CLI-095 (Invivogen) 3 hrs before the end of the incubation time (12 hrs). Group 4, the CLI-095+LPS group, comprised cells that were treated with CLI-095 for 3 hrs and then incubated with LPS for 12 hrs. At the end of the incubation time, cell-culture supernatants were collected for ELISA, and the cells were obtained for PCR and flow cytometry studies.

2.2.2 Hypoxia, re-oxygenation, and incubation

DCs that were plated on 12-well plates (1×10^6 cells/well) were divided into two groups of six wells each. Group 1, the hypoxia group, included cells that were incubated without treatment, and group 2, the CLI-095+hypoxia group, comprised cells treated with 100 μg/ml of CLI-095 before incubation. Media were replaced by DMEM (glucose- and FBS-free media); 1 ml was used for each well, and then cells were incubated for 12 hrs in

a hypoxic environment using a GENbag anaer (bioMerieux® sa, Marcy I'Etoile, France; 2% O2). After 12 hrs, the media were replaced by warm DC media, and the cells were incubated in normal conditions for 12 hrs (re-oxygenation). At the end of the incubation time, the cell-culture supernatants were collected for ELISA, and the cells were obtained for PCR and flow cytometry studies.

2.2.3 Normoxia incubation

DCs in 12 wells (1×10^6 cells/well) were divided into two groups. In group 1, the normoxia group, cells were incubated without treatment, and in group 2, the CLI-095 group, cells were treated with 100 µg/ml of CLI-095 before incubation. Cells were incubated for 24 hrs at 37^{o}C. At the end of the incubation time, the cell-culture supernatants were collected for ELISA, and the cells were obtained for PCR and flow cytometry studies.

2.2.4 Nucleic acids

Extraction of nucleic acids

The mRNA was extracted from cultured cells according to the protocol supplied with the PureLink RNA Mini Kit (Thermo Fisher Scientific). A NanoDrop ND-1000 (Thermo Fisher Scientific) was employed to determine the concentration of RNA samples.

cDNA synthesis

To synthesize cDNA, 0.5-1.0 µg of RNA and 4 µl of SuperScript VILO Master Mix (Thermo Fisher Scientific) with 20 µL of H_2O were incubated at 25^{o}C for 10 min. The reaction was carried out in a thermocycler (Eppendorf Mastercycler) with the following parameters: 25 °C for 10 min, 42°C for 1 hr, 80°C for 5 min, and holding at 25°C. The concentration of cDNA samples was determined using a NanoDrop ND-1000.

Real-time PCR

The mRNA expressions of TLR4, MyD88, IRAK4, NF-kB, and β-actin (Sigma) were determined using real-time PCR. Each cDNA sample was amplified using SYBR Green (Bioline) on a Bio-Rad CFX Connect Real-Time PCR System. Briefly, the reaction conditions comprised 2 µl of cDNA and 0.2 µM primers in supermix with a final volume of 20 µl. Each cycle comprised denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, and extension at 72°C for 10 sec. β-actin was used as an endogenous control to normalize each sample. Fold changes were calculated using the ΔΔCT method. The experiment was performed in triplicate and repeated in five independent trials. Primers were purchased from Sigma-Aldrich (ON, Canada) and are listed in Table 1.

2.2.5 Flow cytometry

Standard procedures were employed to perform flow cytometry analysis. All antibodies were purchased from GeneTex, Inc. The following antibodies were used: CD11c-FITC, MHCII-FITC, CD80-FITC, CD83-FITC, and CD86-FITC. The cells were washed, and the cell numbers were adjusted to 1-5 x 10^6 cells/mL in an ice-cold Fluorescence-Assisted Cell-Sorting (FACS) buffer, which contained PBS, 0.1% NaN_3 (Sigma), and 5-10% FBS. The cells were incubated with 2% FC blocker for 20 min, and subsequently centrifuged (1,200 rpm; 5 min; 4°C), followed by resuspension in FACS buffer. The cells were then incubated with 0.1-10 µg/mL of primary labeled-antibody in the dark for 45 min at 4°C and washed twice with FACS buffer. The cells were analyzed using a CytoFLEX Flow Cytometer (Beckman-Coulter) and treated with 7-AminoActinomycin D (7- AAD; BD Via-Probe) for 10 min to identify dead cells. They were then passed through Nitex into an FACS tube before analysis to avoid any remaining clumps.

2.2.6 ELISA

The amount of cytokine proteins secreted in the cell culture media was determined using IL-6 and TNF ELISA kits (BioLegend). To obtain culture supernatants, each cell culture (1×10^6 cells/well) was grown alone and together on 12-well plates (1 mL/well). The resulting cell-cultured media supernatant was harvested and centrifuged to remove cellular debris. Cytokines in the cell-culture supernatant were measured using ELISA kits according to the manufacturer's instructions. The cytokine protein levels were corrected for the total amount of proteins, and the results are expressed in pg/mL. The absorbance was read at 450 nm and 570 nm using an iMark Microplate Absorbance Reader (Bio-Rad), and the concentration of the samples was determined using a standard curve.

2.3 Statistical analysis

For all the experiments, statistical analysis was done using GraphPad Prism 6 and Microsoft Excel 2010 software. The results are expressed as the standard deviation (SD) of at least three technical and biological replicates. An unpaired student's t-test was used to determine the statistical significance. A two-way ANOVA followed by a post-hoc Tukey's test was employed for the analysis of parametric data. P-values ≤ 0.05 were considered statistically significant.

Table 1: List of primers

PRIMER	**Forward**	**Reverse**
TLR4	AACTCTGGATCTACCCTTTC	AAAGCTCAGTGCATTTCTAC
MyD88	TATACTGAAGGAGCTGAAGTC	TAAAGGCATCGAAAAGTTCC
IRAK4	CTGCCTTGTTTATGGCTTCTTG	AGAAGAATGTCCAGTCGTTGAG
NF-κB	CCTTCCTCAACTTCCAGAACAA	CCTTCTCTCTGTGTGTGAGTTG
B-Actin	GATGTATGAAGGCTTTGGTC	TGTGCACTTTTATTGGTCTC

3 Results

The objective of the present study is to evaluate the role of CLI-095 in reducing the TLR4-mediated innate immune response to hypoxia and re-oxygenation injury. In our *in vitro* model, we were able to induce this injury and assess its effects on DC maturation. We were also able to evaluate the effect of CLI-095 on the early events in TLR4 signaling. In addition, CLI-095 reduced the levels of DC maturation markers and pro-inflammatory cytokines in response to the injury.

3.1 CLI-095 binds to TLR4 and blocks the TLR4 signaling pathway

We tracked the TLR4 signaling pathway to define the mechanism behind the ability of CLI-095 to reduce the innate immune response. We examined the mRNA expression of two mediators of the TLR4 signal: NF-kB and IRAK4. IRAK4 is an essential activator of NF-κB, and NF-κB is a protein complex that controls DNA transcription, cytokine production, and cell survival. The cells were subjected to hypoxia and re-oxygenation, and then compared to CLI-095-treated cells, which were incubated with CLI-095 for 3 hrs and then also subjected to hypoxia and re-oxygenation. IRAK4 and NF-κB expression levels were greatly reduced in CLI-095-treated cells compared to untreated cells, indicating that CLI-095 can block the TLR4 signal.

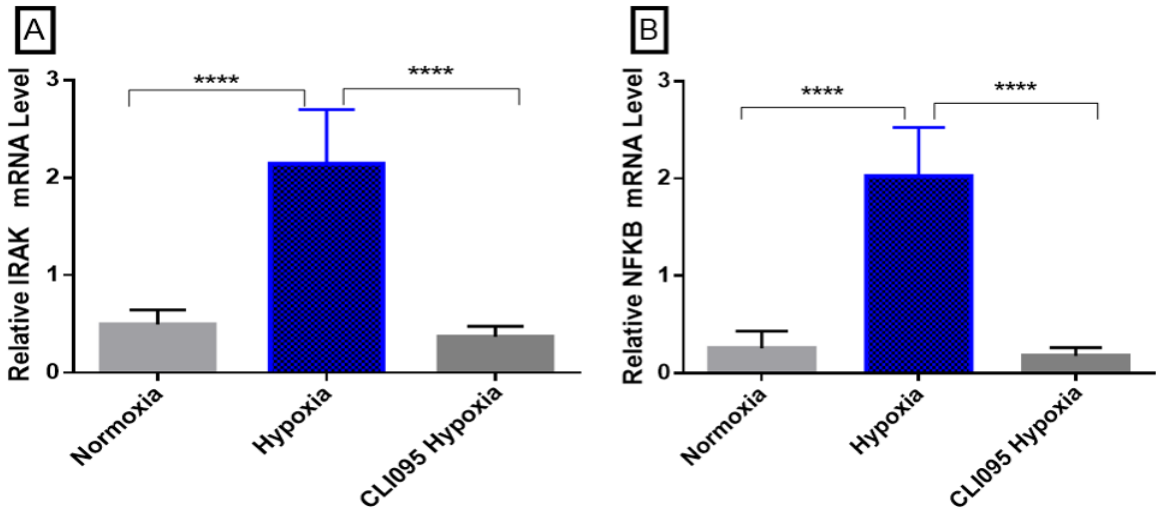

Figure 4: Expression of IRAK4 and NF-κB mRNA in DCs incubated in normal conditions (normoxia), hypoxia, and re-oxygenation (hypoxia), and cells treated with CLI-095 (100 μg/ml) for 3 hrs and then subjected to hypoxia and re-oxygenation (CLI-095 hypoxia).

DCs were subjected to hypoxia for 12 hrs and then re-oxygenated for 12 hrs. IRAK4 and NF-κB expression levels were compared to those of untreated cells (normoxia) and cells treated with CLI-095 (100 μg/ml) for 3 hrs and then subjected to hypoxia and re-oxygenation. Relative levels of NF-κB and IRAK4 mRNA were normalized to β-actin and the normal sample's mRNA. Error bars represent SD, n=3. **** $p < 0.0001$.

3.2 CLI-095 attenuated the expression of inflammatory mediators in innate immune cells

3.2.1 CLI-095's protective effects on DC pro-inflammatory cytokines in response to hypoxia re-oxygenation injury

Cells injured by hypoxia and re-oxygenation that were treated with CLI-095 showed reduced levels of pro-inflammatory cytokines (TNFα and IL6) compared to cells that were only subjected to hypoxia and re-oxygenation injury (Fig. 5).

3.2.2 CLI095's protective effects on the RAW264.7 cell line's levels of pro-inflammatory cytokines in response to LPS and hypoxia re-oxygenation injury

To determine whether CLI-095 could reduce the levels of pro-inflammatory cytokines in other innate immune cells, we demonstrated that RAW cells subjected to hypoxia re-oxygenation injury showed the same result as DC cells in the same incubation conditions (Figure 6).

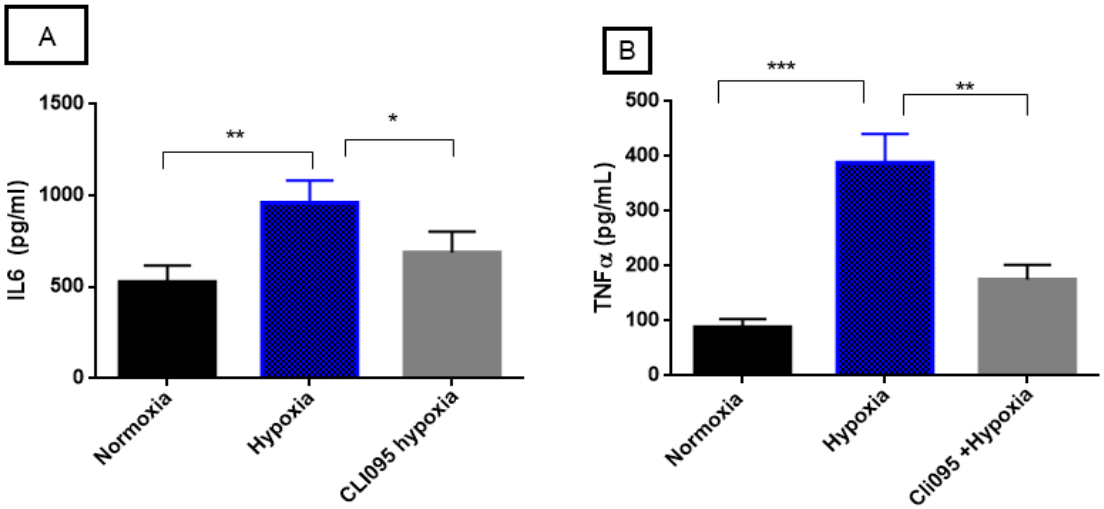

Figure 5: Cytokine production by DCs incubated in normal conditions (normoxia), hypoxia, and re-oxygenation (hypoxia), and cells that were treated with CLI-095 (100 µg/ml) for 3 hrs and then subjected to hypoxia and re-oxygenation (CLI-095+hypoxia).

DCs were subjected to hypoxia for 12 hrs and then re-oxygenated for 12 hrs. Subsequent cytokine production was compared to that of untreated cells (normoxia) and cells treated with CLI-095 (100 µg/ml) for 3 hrs and then subjected to hypoxia and re-oxygenation. The culture media were collected, and IL6 (A) and TNF-α (B) were quantified by ELISA. Results are expressed as mean pg/ml ± SE from four independent experiments. Cells treated with CLI-095 showed reductions in pro-inflammatory cytokine levels (TNFα and IL6) compared to cells that were only subjected to hypoxia and re-oxygenation. * $p < 0.05$, ** $p < 0.002$, *** $p < 0.001$, **** $p < 0.0001$.

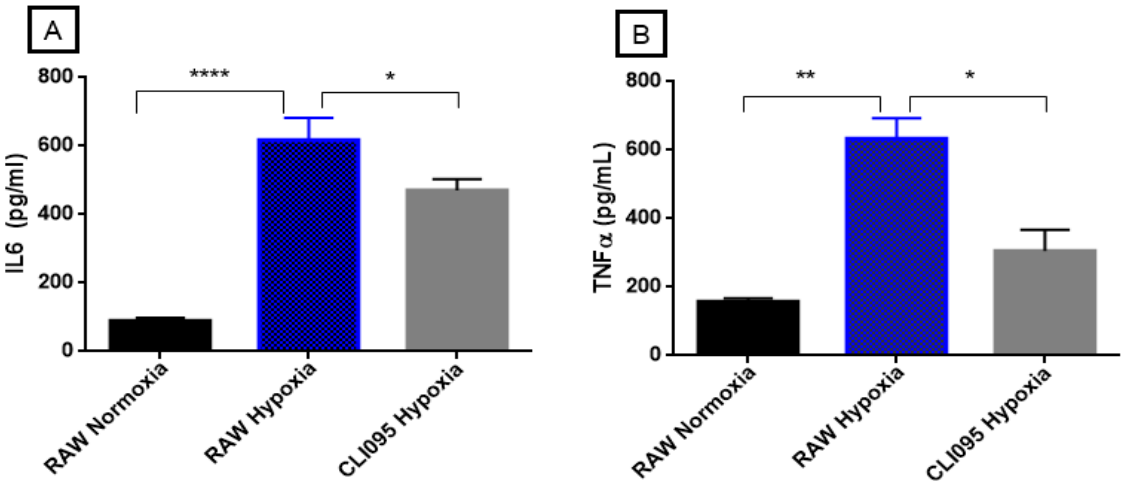

Figure 6: Cytokine production by RAW264.7 cells incubated in normal conditions (normoxia), hypoxia, and re-oxygenation (hypoxia), and cells treated with CLI095 (100 µg/ml) for 3 hrs then subjected to hypoxia re-oxygenation (CLI-095+hypoxia).

RAW cells were subjected to hypoxia for 24 hrs and then re-oxygenated for 24 hrs; subsequent cytokine production was compared to that of untreated cells (normoxia) and cells treated with CLI-095 (100 µg/ml) for 3 hrs then subjected to hypoxia re-oxygenation. The culture media were collected and IL6 (A) and TNF-α (B) were quantified by ELISA. Results are expressed as mean pg/ml ± SE from three independent experiments. Cells treated with CLI-095 showed reductions in pro-inflammatory cytokine levels (TNFα and IL6) compared to cells that were only subjected to hypoxia re-oxygenation. * $p < 0.05$, ** $p < 0.002$, *** $p < 0.001$, **** $p < 0.0001$.

3.3 DC maturation marker activation after hypoxia and re-oxygenation injury

3.3.1 Cell maturation in response to hypoxia and re-oxygenation

To assess the ability of damaged cells to induce DC maturation in response to hypoxia and re-oxygenation, DCs were subjected to hypoxia for 12 hrs, followed by re-oxygenation for 12 hrs. Afterwards, we examined DC maturation markers (MHCII, CD80, CD83, and CD86) using flow cytometry. MHCII, CD80, CD83, and CD86 increased in samples without treatment compared to non-hypoxic cells that were kept for 24 hrs in normal culture conditions (Figure 4).

3.3.2 CLI-095's protective effects on DC maturation in response to hypoxia and re-oxygenation injury

Cells treated with 100ug/ml of CLI095 3 hrs before hypoxia and re-oxygenation showed decreased levels of DC maturation markers (MHCII, CD80, CD83, and CD86) compared to untreated hypoxic cells. When compared to non-hypoxic samples, CLI095-treated cells exposed to hypoxia re-oxygenation injury showed no significant differences, indicating the potent protective effects of CLI095 against hypoxia re-oxygenation injury (Figures 5 and 6).

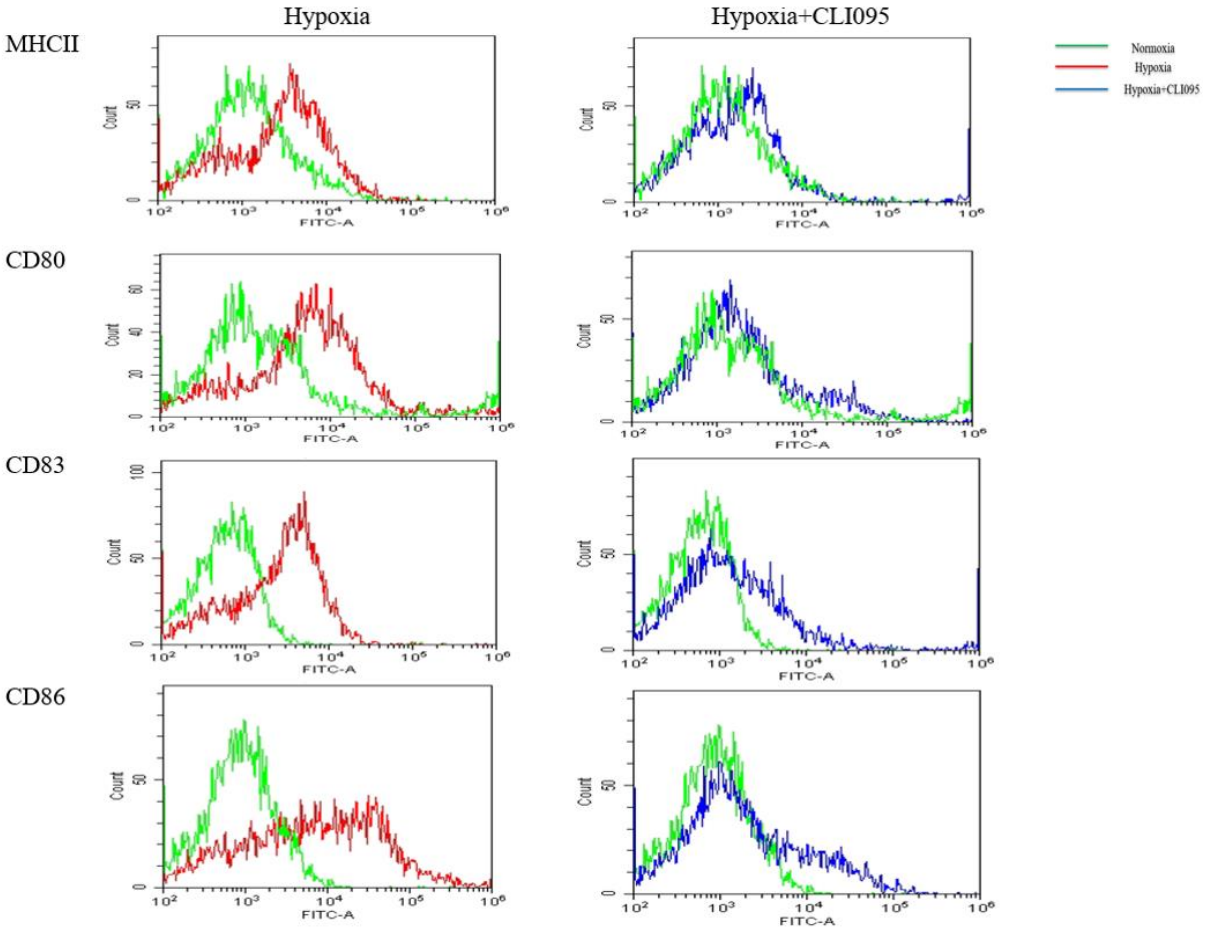

Figure 7: Analysis of cell surface maturation marker expression in DCs in response to hypoxia and re-oxygenation, and CLI-095 treatment

Histogram of MHCII, CD80, CD83, and CD86 expression in cells subjected to hypoxia and re-oxygenation, and CLI-095-treated hypoxia and re-oxygenation cells. The percentage represents hypoxia-stained cells in comparison to normoxia-stained cells. Histograms are representative of four separate experiments.

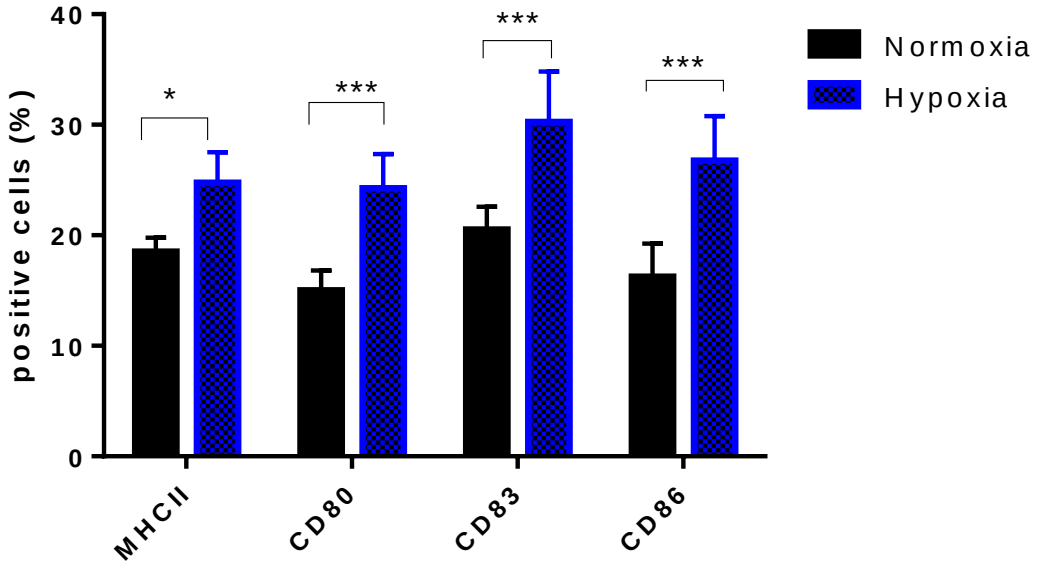

Figure 8: Analysis of cell surface maturation marker expression in DCs in response to hypoxia and re-oxygenation

Cells subjected to hypoxia and re-oxygenation in comparison to non-hypoxic cells. Flow cytometry analysis of MHCII, CD80, CD83, and CD86 showed that hypoxia and re-oxygenation significantly increased the expression levels of MHCII, CD80, CD83, and CD86. Results are representative of four different experiments. Error bars represent SD, * $p < 0.05$, *** $p < 0.001$.

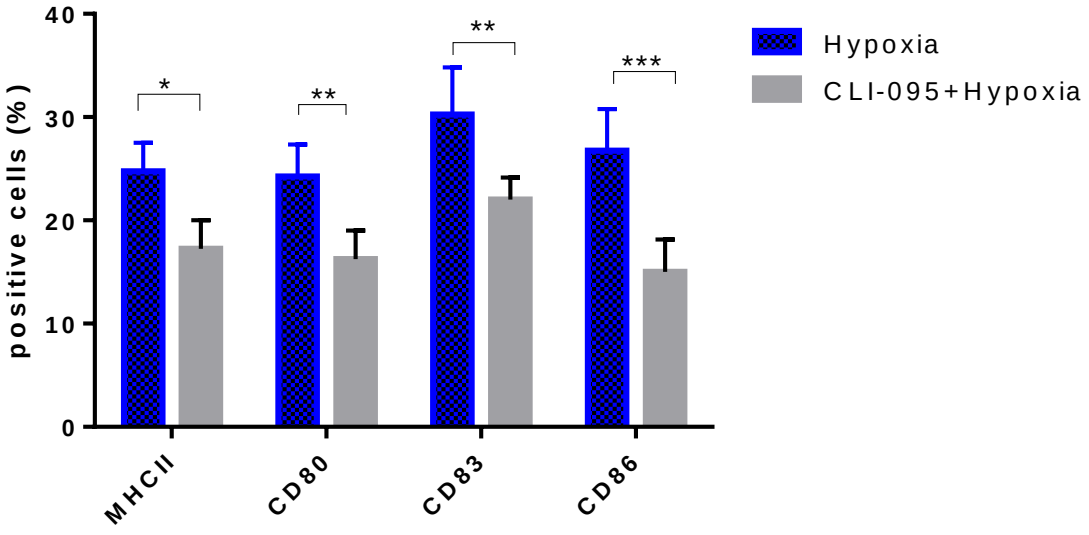

Figure 9: Analysis of cell surface maturation marker expression in DCs in response to hypoxia and re-oxygenation, and CLI-095 treatment

Cells subjected to hypoxia and re-oxygenation were compared to CLI-095-treated cells incubated with CLI-095 for 3 hrs and then subjected to hypoxia and re-oxygenation. Flow cytometry analysis of MHCII, CD80, CD83, and CD86 showed that CLI-095 treatment before hypoxia and re-oxygenation significantly reduced MHCII, CD80, and CD86 levels compared to untreated stressed cells. Results are representative of four different experiments. Error bars represent SD, * $p < 0.05$, ** $p < 0.002$, *** $p < 0.001$.

4 Discussion

IRI is an inevitable consequence of kidney transplant and is the primary cause of reduced graft survival after transplantation [22, 46]. IRI activates innate immune response, which in turn results in varying degrees of tissue damage [61]. The immune response occurs when a group of receptors known as TLRs (mainly TLR4) become activated after IRI. In microbial infections, TLRs recognize multiple molecules arises from microbes collectively called PAMPs. Similarly, molecules secreted from damaged cells (DAMPs) can also activate endogenous TLR in sterile conditions, and these molecules are also known to trigger innate immune responses and to increase the transcription of pro-inflammatory cytokines, chemokines, and adhesion molecules, as well as neutrophil infiltration in the graft. Antigenic mimicking of DAMPs helps in DC maturation. All of these processes ultimately induce IRI and subsequently cause allograft rejection [60].

Mature DCs interact with T cells to promote an adaptive immune response [63]. Several studies have demonstrated the implication of TLR4 in activating innate immunity in various tissues during IRI, including liver, heart, and kidney tissues. The results showed that *TLR4*$^{-/-}$ mice were protected from hepatic, myocardial, and renal IRI [79, 96, 97]. Therefore, TLR4 could be an important therapeutic target for the management of IRI.

DCs are important immune cells with a major role in antigen presentation and adaptive immunity activation. They have always received significant interest from researchers of IRI [98]. In facilitating their role in renal IRI, DCs are first activated, which initiates their movement into the lymph nodes and their interaction with both T and

B cells. This in turn results in an adaptive immune response [99]. The immune system has a combination of cells, tissues, and molecules to protect the body from pathogens and toxins. For this process to work efficiently, two major reactions occur: innate immunity and adaptive immunity. In the innate and adaptive immune responses to acute IRI, neutrophils, DCs, and macrophages play the largest and most important roles [95]. Studies on the role of the innate immune cells in renal IRI have shown that mice lacking both T and B cells were not protected from IRI, suggesting that cells from the innate immune system such as DCs play a significant role [94]. However, due to IRI in the kidney, DCs can be activated by resulting DAMPs. When IRI-affected organs are transplanted, activation of DCs can initiate the adaptive immune response. In the present study, we found that intraperitoneal injection of CLI-095 in the mouse IRI model did not decrease the creatinine level in the blood. Therefore, we assume that CLI-095-mediated TLR4 abrogation may have little in IRI. However, DCs and macrophages are the most common and readily available leukocytes in the kidney, where they play a fundamental role. In fact, DCs are the initial responders to DAMPs released from damaged necrotic cells after oxygen deprivation with subsequent activation of TLR4 and TLR9 signaling. In addition, DCs significantly infiltrate the kidney after IRI [95, 99].

CLI-095 is a specific and selective inhibitor of TLR4 signaling. In this study, it inhibited the production of cytokines (TNF-α and IL-6) induced by TLR4 ligands. Furthermore, in LPS-induced DCs, CLI-095 inhibited both MyD88-dependent and independent pathways [100]. A study conducted on RAW264.7 cells and HEK293 (human embryonic kidney) cells showed similar results [91]. Furthermore, CLI-095 has been shown to inhibit the activation of NF-κB in LPS-induced HEK293 cells [101].

However, the effect of CLI-095 on innate immune cells has not yet been tested in response to hypoxia and re-oxygenation. Therefore, we tested the ability of CLI-095 to inhibit the activation of innate immune cells in response to this condition.

In this study, a hypoxic culture of immature DCs induced co-stimulatory molecules, such as CD80, CD83, CD86, and MHCII (**Figure 8)**. This data suggests that hypoxia and re-oxygenation trigger DC maturation. Previous studies on hypoxic culture of DCs have shown the same result [102]. However, the protective effect of CLI-095 occurred only when we treated the cells 3 hrs before the hypoxia and re-oxygenation activation (**Figure 9**). This indicates that blocking the TLR4 signal with CLI-095 reduced the DC maturation marker. Moreover, this also suggests that DC maturation requires TLR4.

Cytokines and chemokines are pro-inflammatory mediators that are secreted when DCs are activated [103, 104]. The stimulation of DCs with hypoxia and re-oxygenation induces these cells to secrete inflammatory cytokines such as TNF-α and IL-6 [105, 106]. These are important cytokines that stimulate the immune response and are associated with many inflammatory conditions, such as trauma, sepsis, infection, and rheumatoid arthritis. In addition, TNF-α and IL-6 are involved in cell proliferation, differentiation, and apoptosis. Upregulation of TNF-α and IL-6 levels in DCs indicates that the TLR4 signal is activated and the cells are ready to initiate the immune response. Our results showed that DCs that were treated with CLI-095 and subjected to hypoxia and re-oxygenation secreted less TNF-α and IL-6 than cells that were stimulated without treatment (**Figure 5**). This suggests that CLI-095 has suppressive effects on the production of pro-inflammatory mediators. Our data also showed that the inhibitory

effects of CLI-095 on the production of TNF-α and IL-6 were the same in both DCs and macrophages (**Figures 5 and 6**), which indicates that the difference in the type of innate immune cells does not affect the efficacy of the CLI-095.

As mentioned earlier, TLR4 activation triggers several kinases, such as IRAK4, which results in translocation of NF-κB, the transcription of pro-inflammatory cytokines and chemokines, and DC maturation [60]. Furthermore, our results suggest that CLI-095 targets an event that occurs before the transcription of pro-inflammatory cytokines. Therefore, we examined the effect of CLI-095 on the IRAK4 and NF-κB signaling pathways in response to hypoxia and re-oxygenation. The IRAK4 and NF-κB expression levels were greatly reduced in CLI-095-treated DCs compared to cells that were not treated (**Figure 4**), indicating that CLI-095 can block the activation of the TLR4 signal in DCs induced by hypoxia and re-oxygenation.

Taken together, our study demonstrated the role of TLR4 in innate immunity activation in response to hypoxia and re-oxygenation. In addition, the results showed that untreated DCs were more matured than DCs pretreated with CLI-095: DC maturation and the secretion of cytokines and chemokines after exposure to inflammatory stimuli were decreased in pretreated DCs. Moreover, our results showed that CLI-095 inhibited the TLR4-signaling pathways, but not the TLR4 itself. Several studies have reported that CLI-095 could have a potential role in treating inflammation-related diseases. CLI-095 protects against acute cerebral IRI and could be considered as a potential therapeutic strategy to constrain the inflammatory process in other types of IRI, such as renal IRI [107].

In conclusion, we have characterized a new molecule, CLI-095, which specifically and significantly downregulates TLR4. In addition, we have shown that DCs are activated in hypoxia and re-oxygenation models, and that CLI-095 has a significant impact in reducing DC activation. Therefore, TLR4 is an important innate immune receptor implicated in hypoxia and re-oxygenation. Since hypoxia and re-oxygenation represent an in vitro model of IRI, CLI-095 might be useful as a potential therapeutic target for reducing renal IRI. Most immune cells are overloaded with TLR4 considering a more robust role of neutrophil in IRI and DCs in organ rejection, manipulating their TLR4 pathway could be a logical target in both.

5 Future direction

The future direction of this project involves determining CLI-095's role in modulating DCs and their TLR4 expression in an animal model of kidney IRI. To obtain such a model, C57BL/6 mice will be subjected to left kidney pedicle clamping for 50 min, and then the clamping will be released and the right kidney will be removed. Control groups will include untreated animals and animals subjected to laparotomy alone. The mice will be treated with CLI-095 for 1 hr or 18 hrs prior to clamping. The mice will be exsanguinated, and both kidneys will be collected 24 hrs after clamping and release. Kidney function (creatinine, proteinuria) will be assessed, and immunohistochemistry, *H&E*, TUNEL, and TLR4 staining will be performed.

In addition, the effect of CLI-095 in pretreated allograft kidneys will be determined. Kidneys will be taken from donor BALB/c mice and stored for 24 hrs in a cold standard UW preservation solution (mimics DCD=donation after cardiac death), which will be supplemented with group 1 CLI-095 and group 2 standard UW preservation solution only. Renal transplant with the treated kidney will be performed on C57BL/6 mice, and then all animals will receive a sub-therapeutic regimen of tacrolimus. Graft function (creatinine, proteinuria) and survival will be assessed on days 0, 8, and 30. Assessment for rejection (H&E), DCs (CD11c, CD80, CD86, TLR4, and 9), T cell infiltration, cytokines, and adhesion molecules will be assessed using qRT-PCR, Western blot, or immunohistochemistry. The use of animal models is an important step in the translation of our in vitro study to a clinical setting, and will allow us to confirm our results.

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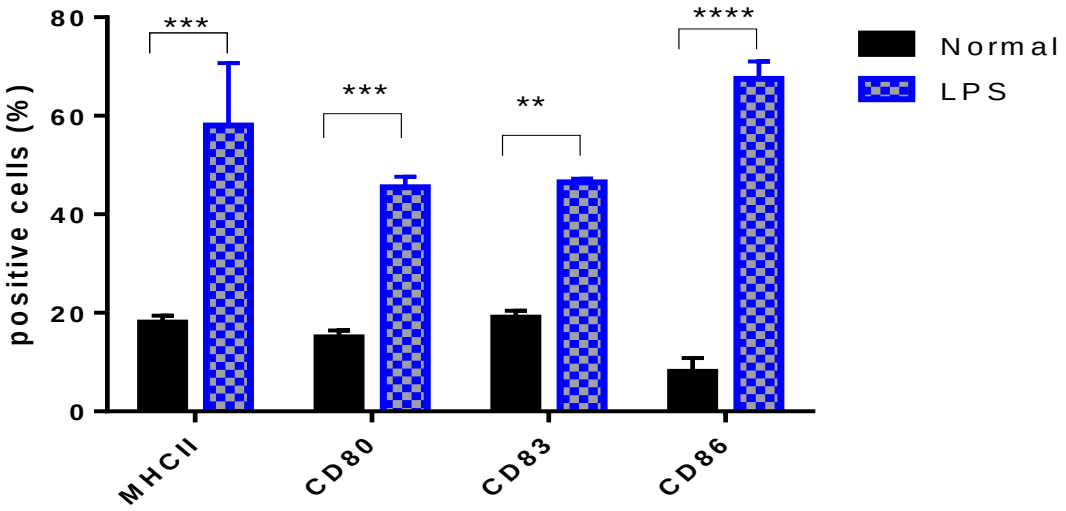
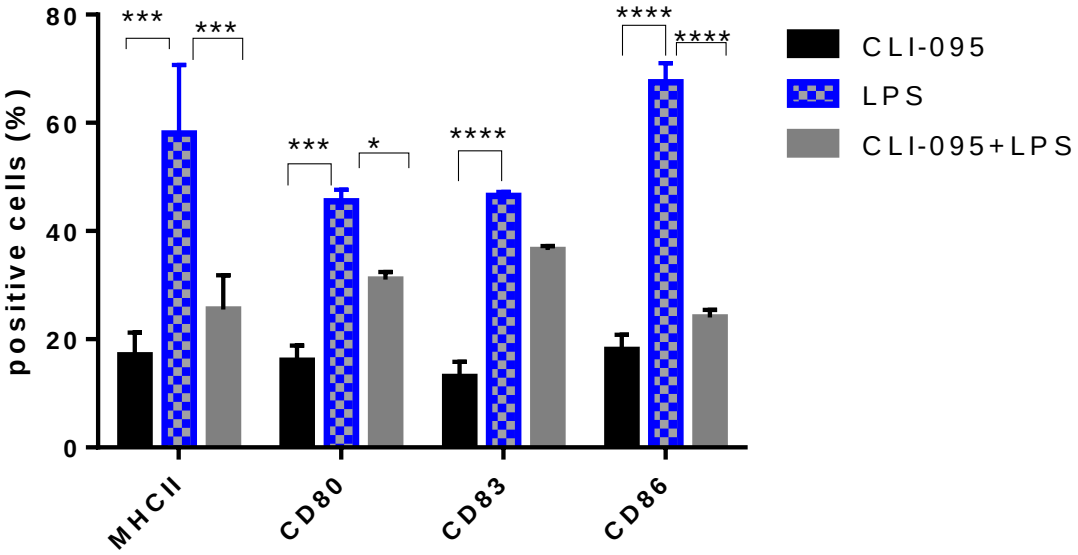
Appendices

A. Analysis of surface maturation marker expression in DC in response to LPS (TLR4-specific ligand)



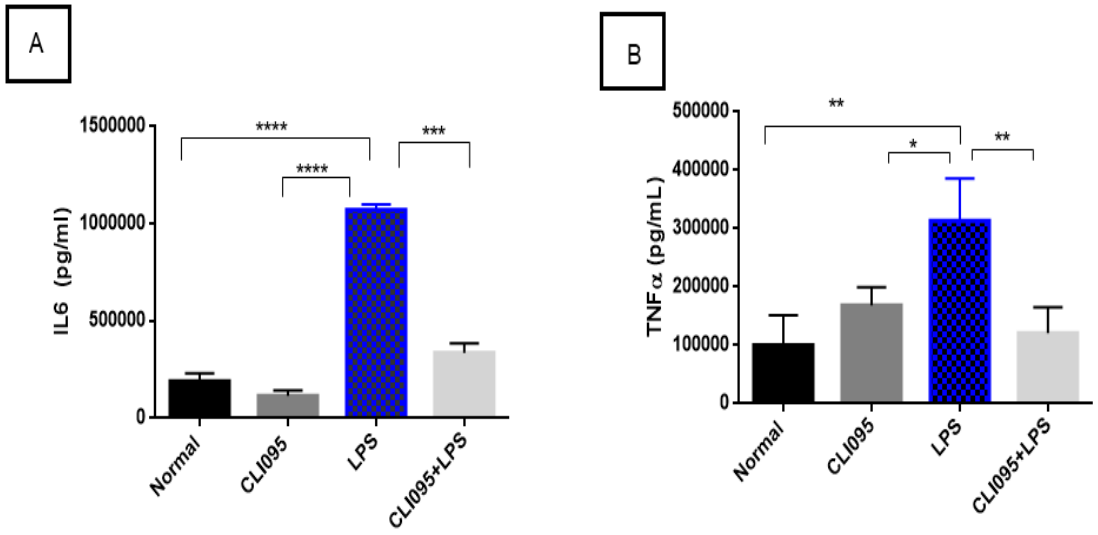
Cells stimulated with LPS (50 ng/ml) for 12 hrs and compared to normal cells (cells without treatment). Flow cytometry analysis of MHCII, CD80, CD83, and CD86 showed that LPS stimulation significantly increased the expression of these cytokines in LPS-stimulated DCs. Results are representative of five different experiments. Error bars represent SD, * $p < 0.05$, ** $p < 0.002$, *** $p < 0.001$, **** $p < 0.0001$.

B. Analysis of cell surface marker expression in DCs in response to LPS stimulation and CLI095 treatment



Cells stimulated with LPS (50 ng/ml) compared to cells that were treated with CLI095 (100 μg/ml) for 3 hrs before stimulation with LPS for 12 hrs (CLI095+LPS) and DCs treated with CLI095 and incubated for 3 hrs (CLI095). Flow cytometry analysis of MHCII, CD80, CD83, and CD86 showed that LPS stimulation significantly increased the expression of these cytokines compared to unstimulated cells. Cells treated with CLI095 for 3 hrs before stimulation showed significant reductions in MHCII, CD80, and CD86 compared to LPS-stimulated cells. Results are representative of five different experiments. Error bars represent SD, * $p < 0.05$, ** $p < 0.002$, *** $p < 0.001$, **** $p < 0.0001$.

C. CLI-095's protective effects on DC pro-inflammatory cytokines in response to LPS



Cytokine production by DCs stimulated with no treatment (normal), CLI-095 (100µg/ml), LPS (50 ng/ml), or both CLI-095+LPS. DCs were stimulated with LPS (50 ng/ml) for 12 hrs and their cytokine production was compared to that of untreated cells (normal), cells treated with CLI-095 (100µg/ml) for 3 hrs before stimulation with LPS for 12 h (CLI-095+LPS), and DCs treated with CLI-095 and incubated for 3 hrs (CLI095). The culture media were collected and IL6 (A) and TNF-α (B) were quantified by ELISA. Results are expressed as mean pg/ml ± SE from three independent experiments. Cells treated with CLI-095 showed reductions in pro-inflammatory cytokine levels (TNFα and IL6) compared to cells that were only subjected to LPS. * $p < 0.05$, ** $p < 0.002$, *** $p < 0.001$, **** $p < 0.0001$.

Curriculum Vitae

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Poster presentation:

Rabindra Bhattacharjee, Patrick Luke, Tony Jevnikar, Aaron Haig, Gediminas Cepinskas, Manujendra Saha, **Hajed Alharbi**, Lida Radan. Cli95 Inhibits Toll-Like Receptor 4 induced Inflammation and Maturation of Dendritic Cells Presented at 2016 CST-CNTRP-SQT joint Scientific Meeting, Québec City, Quebic, Oct 13 2016

Patrick Luke, Gediminas Cepinskas, **Hajed Alharbi**, Aaron Haig, Rabindra Bhattacharjee. Immunosuppressive Actions of CLI95 and CORM401 on TLR4 in Renal Ischemia Reperfusion Injury presented at American Transplant Congress, Boston, Massachusetts, June 11 2016

Alharbi H, Bhattacharjee RN, Radan L, Saha MN, Luke PW. *Immunological impact of CLI095 on ischemia reperfusion* Presented at the Annual Pathology and Laboratory Medicine Research Day, London, ON, April 7 2016

Alharbi H, Bhattacharjee RN, Radan L, Saha MN, Luke PW. *Immunological impact of CLI095 on ischemia reperfusion* Presented at the the fifth-annual London Health Research Day, London, ON, March 29 2016

Alharbi H, Bhattacharjee RN, Saha MN, Luke PW. *Role of Innate Immunity in Kidney Ischemia Reperfusion Injury* Presented at the Annual Pathology and Laboratory Medicine Research Day, London, ON, March 30 2015