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DENDRITIC CELLS AND IL-10 IN NEPHROTOXIC ACUTE KIDNEY INJURY

A Dissertation in

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by

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ABSTRACT

Cisplatin is a highly effective chemotherapeutic agent used to treat a wide variety of tumors in humans. The key limitation with cisplatin therapy is nephrotoxicty affecting 25-35% of treated patients. Here we investigated the distribution of dendritic cells within the kidney and the role of dendritic cells and dendritic cell-produced IL-10 in cisplatin-induced acute kidney injury using a mouse model in which dendritic cells express green fluorescent protein and diphtheria toxin receptor. Dendritic cells were found interspersed between renal tubules throughout the tubulointerstitium but not in glomeruli. The functional significance of dendritic cells within their physiological context in cisplatin nephrotoxicity was examined by depleting dendritic cells using diphtheria toxin. Mice depleted of dendritic cells either before or coincident with cisplatin treatment, but not at later stages, exhibited more severe renal dysfunction, kidney tubular injury, neutrophil infiltration and greater mortality than dendritic cell non-depleted mice. Neutrophils and monocytes were abundant in the kidney at later stages of cisplatin nephrotoxicity. In contrast, infiltration of inflammatory dendritic cells was insignificant after cisplatin treatment. Through the use of bone marrow chimeric mice, we also confirmed that the exacerbated renal injury was mediated through the effect of diphtheria toxin on dendritic cells. Using mixed bone marrow chimeric mice, we showed that the worsening of cisplatin nephrotoxicity in dendritic cell-depleted mice occured independent of diphtheria toxin-mediated death of dendritic cells. Analysis of markers on renal dendritic cells after cisplatin treatment showed almost steady-state levels of expression of antigen presentation (MHC class I and MHC class II) and costimulatory molecules (CD40, CD80 and CD86), and an increase in expression of IL-10 and ICOS-L. Further investigation using mixed bone marrow chimeric mice lacking dendritic cell-derived IL-10 showed a moderate level of attenuation of kidney injury by dendritic cell-produced IL-10. These data demonstrate that dendritic cells protect mice from cisplatin nephrotoxicity.

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List of Abbrevations

AIF	: apoptosis-inducing factor
AKI	: acute kidney injury
ARF	: acute renal failure
BUN	: blood urea nitrogen
CD	: Cluster of Differentiation
CTLA-4	: cytotoxic T lymphocyte antigen-4
DAPI	: 4’,6-diamidino-2-phenylindole
DC	: dendritic cell
DC-LAMP	: dendritic cell-lysosome associated membrane protein
DMEM	: Dulbecco's-modified Eagle medium
DTR	: diphtheria toxin receptor
DTx	: diptheria toxin
EDTA	: ethylenediamine tetraacetic acid
FBS	: fetal bovine serum
Fc	: Fragment crystallizable
FcR	: Fc receptor
Flt-3	: Fms-like tyrosine kinase-3
Foxp3	: Forkhead box p3
G-CSF	: granulocyte colony stimulating factor
GFP	: green fluorescent protein
GM-CSF	: granulocyte macrophage colony stimulating factor
hbEGF	: heparin-binding EGF (epidermal growth factor)-like growth factor
HO-1	: heme oxygenase-1
HSP	: heat shock protein
IACUC	: Institutional Animal Care and Use Committee
ICAM	: intercellular adhesion molecule
ICOS	: inducible costimulator
ICOSL	: inducible costimulator ligand

IDO	: indoleamine 2,3-dioxygenase
IFNγ	: interferon-gamma
IGF	: insulin-like growth factor
IGIF	: IFN-γ-inducing factor
IL	: interleukin
IL-10R1	: interleukin-10 receptor 1
IL-10R2	: interleukin-10 receptor 2
IMDM	: Iscove's modified Dulbecco's medium
iNOS	: inducible nitric oxide synthase
IRI	: ischemia reperfusion injury
JAK	: Janus tyrosine kinase
KO	: knockout
LIF	: leukemia inhibitory factor
MCP-1	: monocyte chemotactic protein-1
MFI	: mean fluorescence intensity
MHC	: major histocompatibility complex
MIP	: macrophage inflammatory protein
MIP-1α	: macrophage inflammatory protein-1α
NK	: natural killer
PAF	: platelet activating factor
PAS	: periodic acid-Schiff
PCR	: polymerase chain reaction
PD-1	: programmed death
PD-L	: programmed death ligand
RANTES	: regulated on activation normal T cell expressed and secreted
ROS	: reactive oxygen species
SOCS	: suppressor of cytokine signaling
STAT	: signal transducers and activators of transcription protein
SV40	: Simian vacuolating virus 40
TBS	: TRIS-buffered saline

TGF-β : transforming growth factor – β

TKPTS : transgenic kidney proximal tubule

Th : T helper cell

TLR : Toll-like receptor

TNFR : tumor necrosis factor receptor

TNF-α : tumor necrosis factor-α

TRAF : tumor necrosis factor receptor-associated factor

T reg : regulatory T cells

Tyk : tyrosine kinase

VCAM : vascular cell adhesion molecule

Acknowledgements

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I dedicate this thesis to the memory of my late father, T. H. Kempegowda, without whose encouragement none of this would have been possible. I also dedicate this work to my mother, K. P. Lakshmi Kempegowda, who has always a solid support. I would not be what I am today if it were not for the support of my parents. I thank them for raising me to be a good person, and I hope that I have made them proud.

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Chapter 1: Introduction

1.1 Acute Renal Failure

Acute renal failure (ARF) occurs in 5-7% of hospitalized patients and results in about 50% mortality (Hou et al., 1983; Nash et al., 2002; Thadhani et al., 1996). ARF in hospitalized patients is usually acquired during hospitalization. The term ARF is usually used to denote acute tubular necrosis, a form of intrinsic ARF caused by ischemia or nephrotoxins (Haller and Schelling, 2000). Since the 1960's, attempts to substantially decrease the mortality associated with ARF have not been successful (Kelly and Molitoris, 2000; Vijayan and Miller, 1998). Although co-morbid conditions in ARF patients contribute to mortality, ARF by itself is an independent risk factor that increases the mortality rate by 10-15-fold as compared to patients without ARF (Chertow et al., 1998). The financial costs of ARF are estimated to be 8 billion dollars per year, or 130,000 dollars per life-year saved (Vijayan and Miller, 1998). Until a better understanding of the pathogenesis of renal injury is achieved, it is very unlikely that the high mortality and the cost associated with ARF will be reduced.

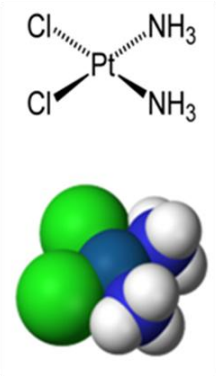
1.2 Cisplatin as an anti-cancer agent

Cisplatin is a highly effective chemotherapeutic agent used to treat certain forms of cancers (Lebwohl and Canetta, 1998). It remains as a standard component of treatment regimens of head and neck cancers (Planting et al., 1999), ovarian (Bolis et al., 1997; Hoskins et al., 2000) and cervical cancers (Rose et al., 1999), testicular cancer (Loehrer PJ et al., 1998), small-cell (Noda et al., 2002) and non small-cell lung cancers

(Gatzemeier et al., 2000), bladder cancer (Coppin et al., 1996) and others (Pritchard et al., 2000). However, cisplatin treatment also causes ARF in 25-35% of patients (Offerman et al., 1984; Ries and Klastersky, 1986; Safirstein et al., 1986). The nephotoxicity of cisplatin has been recognized as a major side effect since its introduction over 25 years ago. In spite of intense effort over the past few decades to find an equally effective and less toxic alternative, cisplatin continues to be widely used to treat cancers.

1.2.1 History of cisplatin discovery

Cisplatin, cis-diamminedichlorido platinum(II), is a platinum based compound (Fig. 1-1)(Reedijk and Lohman, 1985). It was first described by Michel Peyrone in 1893 and the structure was deduced by Alfred Werner in 1893 for which Werner won the Nobel prize for Chemistry in 1913. Later in the 1960s, Rosenberg et al. (1965) in a study to determine the effect of electric field on growth processes of bacteria, noticed inhibition of bacterial binary fission by cisplatin, which was released during electrolysis of a platinum electrode (Rosenberg et al., 1965). The bacteria formed long filamentous structures, up to 300 times its normal length, without cell division. Cisplatin was later tested for anti-tumor activity on different forms of tumors. After its approval for clinical use by the FDA in 1978, this drug revolutionized the treatment of certain types of cancer.



Cisplatin

Systematic (IUPAC) name
(*SP*-4-2)-diamminedichloroplatinum

Figure 1-1. Structure of Cisplatin

1.2.2. Synthesis of cisplatin

Cisplatin is commonly synthesized from potassium tetrachloroplatinate (II), $K_2[PtCl_4]$ (Alderden et al., 2006) (Fig.1-2). After adding the first NH_3 group to $K_2[PtI_4]$, the second NH_3 group can be added in *cis* or *trans* to the bound NH_3 group. Iodine has a larger *trans* effect than NH_3. Therefore, the second added NH_3 group preferably substitutes *trans* to an iodine ligand, and *cis* to the first added NH_3 group. The resulting compound $PtI_2(NH_3)_2$ is finally converted to $PtCl_2(NH_3)_2$ by treating with excess of KCl.

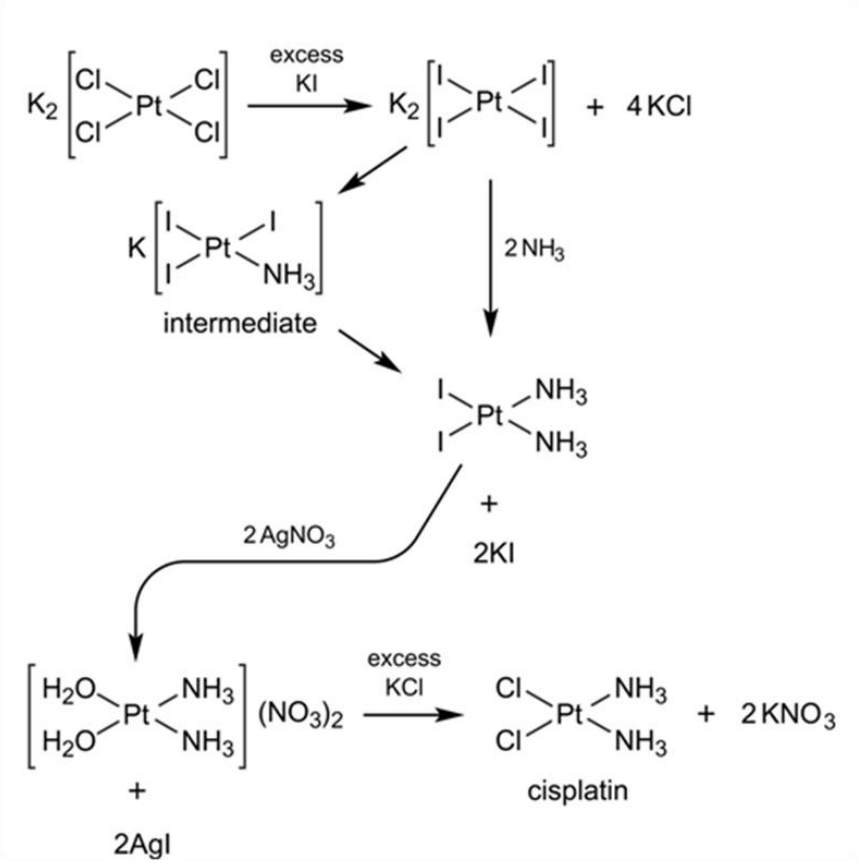


Figure 1-2. Synthesis scheme of cisplatin (Alderden et al., 2006).

1.2.3. Cisplatin uptake and action

Cisplatin is generally believed to exert antitumor effects by interacting with cellular DNA (Eastman A 1999, Alderdan et al., 2006). Following cisplatin administration, the high chloride concentration (approximately 100 mM) in the blood stream limits the replacement of chloride group of cisplatin by water molecules. However, proteins, especially those that contain thiol groups (serum albumin and amino acid cysteine), attack cisplatin in blood and make cisplatin less effective. For example, one day after cisplatin administration, 65-98% of the platinum in the blood is bound to plasma proteins. The cisplatin that is intact and not protein bound enters the cell by diffusion through the cell membrane (Gately and Howell, 1993). Cisplatin can also actively cross cell membranes through copper-transporting proteins (Ishida et al., 2002). Likewise, organic cation transporters have also been implicated in cisplatin uptake (Ludwig et al., 2004; Yonezawa et al., 2005). In the cell, because of the relatively low chloride concentration (4-20mM), one of the chloride molecules of cisplatin is replaced by a water molecule (Fig. 1-3). The resulting molecule of cisplatin is positively charged and cannot readily leave the cell. This hydrated cisplatin molecule coordinates to a basic site on DNA, usually guanine, forming a monofunctional DNA adduct. The platinum ($[PtCl(H2O)(NH3)2]^{+}$), subsequently, crosslinks to two bases by displacing the other chloride ligand. The bifunctional adduct, guanine-guanine or adenine-guanine, thus formed causes significant distortion of the DNA (Cohen et al., 1980). In rapidly dividing cells such as tumor cells, the distorted DNA results in defective DNA templates and arrests DNA synthesis and replication, which eventually leads to cell death (Fig. 1-3, path a) (Alderden et al., 2006, Pabla and Dong, 2008). However, in case of less DNA

damage, the cell is restored to normal cell cycle by the cellular nuclear excision repair apparatus (Fig. 1-3, path b).

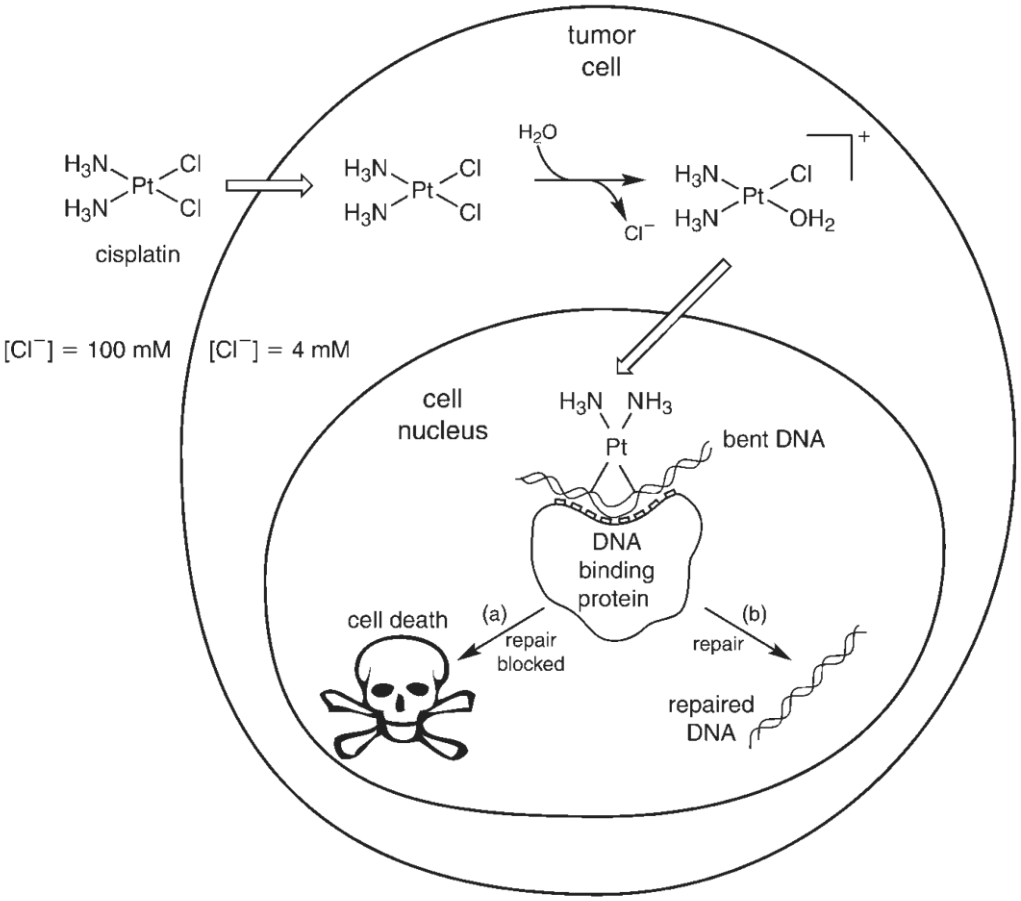


Figure 1-3. Schematic diagram showing cytotoxic pathways of cisplatin. Cisplatin after entering the cell is hydrated and binds to the cellular DNA. The DNA adducts thus formed by cisplatin, if not repaired by the cellular nucleotide excision repair mechanism (path b), induce apoptotic or necrotic cell death (path a) (Alderden et al., 2006).

Although cisplatin has been found to be a very effective chemotherapeutic agent in the treatment of certain types of cancers, its use is mainly limited by two factors: acquired resistance to cisplatin treatment and severe side effects in normal tissues (Pabla and Dong, 2008; Siddik, 2003; Wang and Lippard, 2005). Several tumors are intrinsically resistant to cisplatin treatment, while some acquire resistance after exposure to cisplatin over time. Resistance to cisplatin treatment can be due to decreased cellular uptake of cisplatin, increased efflux of cisplatin, increased capacity of cells to repair the damaged DNA, neutralization of cisplatin by glutathione, metallothionein and other thiol group-containing molecules and finally defective apoptotic signaling in response to DNA damage (Kartalou and Essigmann, 2001; Siddik, 2002; Wernyj and Morin, 2004). The other major limiting factor associated with cisplatin chemotherapy is toxicity to normal tissues such as neurotoxicity, ototoxicity and nephrotoxicity.

1.2.4. Cisplatin nephrotoxicity

Kidneys help in the excretion of metabolic wastes such as urea and ammonia. They are involved in the regulation of electrolytes, acid-base balance and blood pressure, and reabsorption of carbohydrates and amino acids. They are also involved in the production of vitamin D, renin and erythropoietin. Acute renal dysfunction occurs mainly in response to ischemic or toxic insults to the kidney. The incidence of renal dysfunction in cisplatin-treated patients is very high, occurring in about one-third of treated patients. Clinically, renal dysfunction in response to cisplatin treatment is manifested as an increase in the serum creatinine concentration, blood urea nitrogen (BUN), a reduction in

the glomerular filtration rate and decrease in serum magnesium and potassium levels (Gomez-Campdera et al., 1986; Gonzales-Vitale et al., 1977). Initially, the exposure of tubular epithelial cells to cisplatin causes activation of complex signaling pathways which eventually leads to activation of apoptosis pathways and cell death (Fig. 1-4). Meanwhile, cytokines and chemokines released by renal epithelial cells in response to cisplatin treatment attract leukocytes from the circulation and further exacerbate renal inflammation and kidney injury (Ramesh and Reeves, 2002). Cisplatin is also reported to cause damage to renal vasculature and reduce renal blood flow, where the ischemia by itself causes kidney injury (Dursun et al., 2006).

Cisplatin treatment causes injury and death of cells in renal tubules. Earlier studies indicated more death of cells in distal tubules in cisplatin nephrotoxicity (Megyesi et al., 1998; Pabla and Dong, 2008). However, recent studies indicate more injury in proximal tubules than in distal tubules (Li et al., 2004; Tsuruya et al., 2003). A recent study has addressed the site of kidney injury in response to cisplatin treatment in great detail using specific lectins that bind to proximal or distal convoluted tubules. The lectin phytohemagglutinin binds to proximal tubules, whereas peanut agglutinin binds to distal tubules. The ligands for these lectins in proximal and distal tubules are not known. Kidney sections from cisplatin treated mice showed more injury in the phyto-hemagglutinin-stained tubules than peanut agglutinin-stained tubules, implying that the renal proximal tubular cells are the major affected cell type in cisplatin nephrotoxicity (Wei et al., 2007).

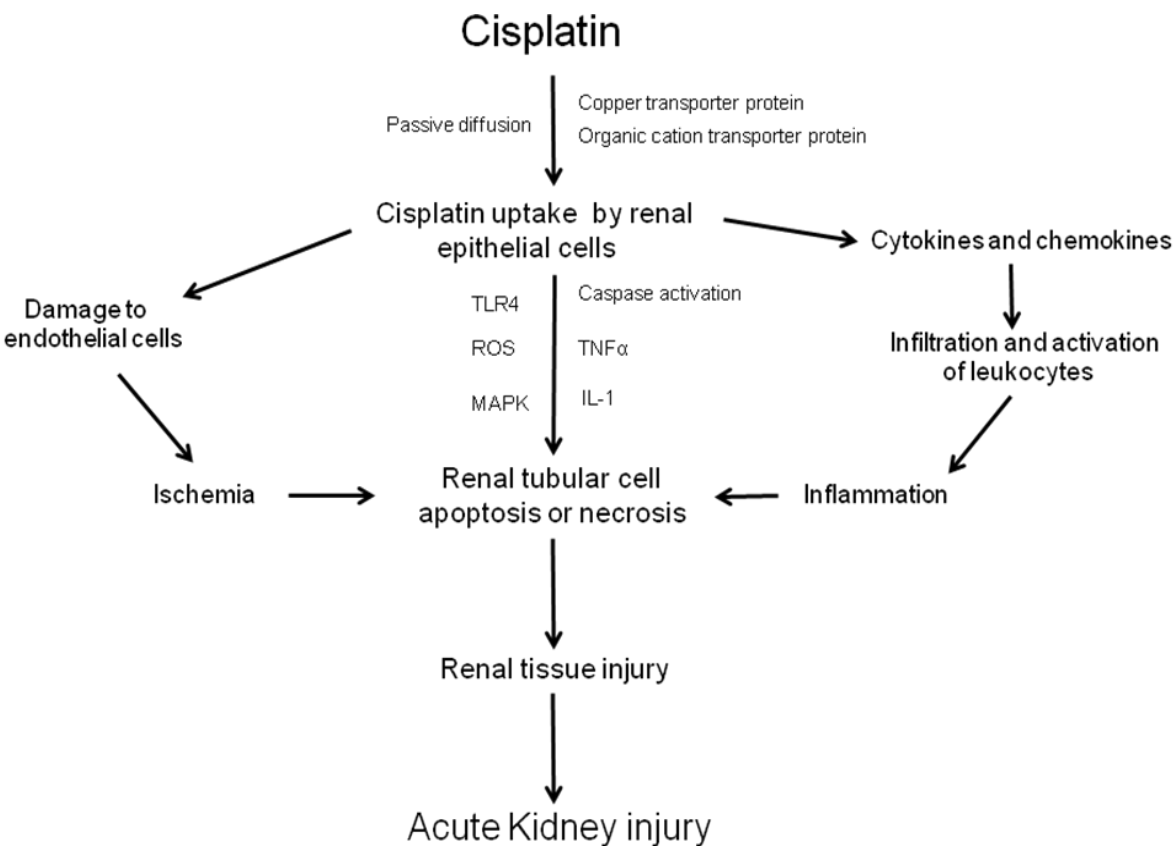


Figure 1-4. Pathophysiological events in cisplatin nephrotoxicity. Cisplatin enters the cell by passive diffusion and/or active transport. The active transport is mediated through the copper or organic cation transporter proteins. Cisplatin in tubular epithelial cells activates signaling pathways that cause activation/production of TLR4, ROS, MAPK, caspases, IL-1, TNFα and other factors that induce tubular injury. Cisplatin may also mediate vascular damage and cause ischemic tubular cell death. In response to cisplatin treatment, renal epithelial cells produce chemokines and cytokines that cause infiltration and activation of leukocytes. These infiltrated leukocytes, in turn, aggravate kidney injury and cause acute renal failure.

Most studies of cisplatin nephrotoxicity have focused on its direct toxicity to renal tubular epithelial cells. Renal epithelial cells treated with cisplatin undergo apoptosis at low concentrations while necrosis is seen at high concentrations (Lieberthal et al., 1996; Okuda et al., 2000). Administration of cisplatin *in vivo* causes both the apoptosis and necrosis in renal tubular cells. Apoptosis in renal epithelial cells in response to cisplatin treatment is caused by both the extrinsic pathway mediated by death receptors and the intrinsic pathway mediated by mitochondria and the endoplasmic reticulum stress pathway (Fig. 1-5).

In the extrinsic pathway, binding of death receptors by ligands such as TNFα or Fas activates downstream caspase-8 and mediates apoptosis (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003; Razzaque et al., 1999; Strasser et al., 2000). In this regard, Kaushal et al., (2001) found activation of caspase 8 and caspase 9 in response to cisplatin treatment in epithelial cells (Kaushal et al., 2001). Ramesh et al., (2002) and Zhang et al., (2007) investigated the effect of TNFα in cisplatin nephrotoxicity both *in vitro* and *in vivo* (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003; Zhang et al., 2007). Cisplatin treatment caused marked induction of TNFα in renal epithelial cells. Genetic or pharmacological inhibition of TNFα attenuated cytokine and chemokine production, and ameliorated cisplatin nephrotoxicity. Using bone marrow chimeric mice, Zhang et al., (2007) showed that the TNFα produced by renal parenchymal cells induced kidney injury in cisplatin nephrotoxicity (Zhang et al., 2007). Further studies by Ramesh and Reeves have shown the requirement of TNFR2 rather than TNFR1 in cisplatin nephrotoxicity (Ramesh and Reeves, 2003).

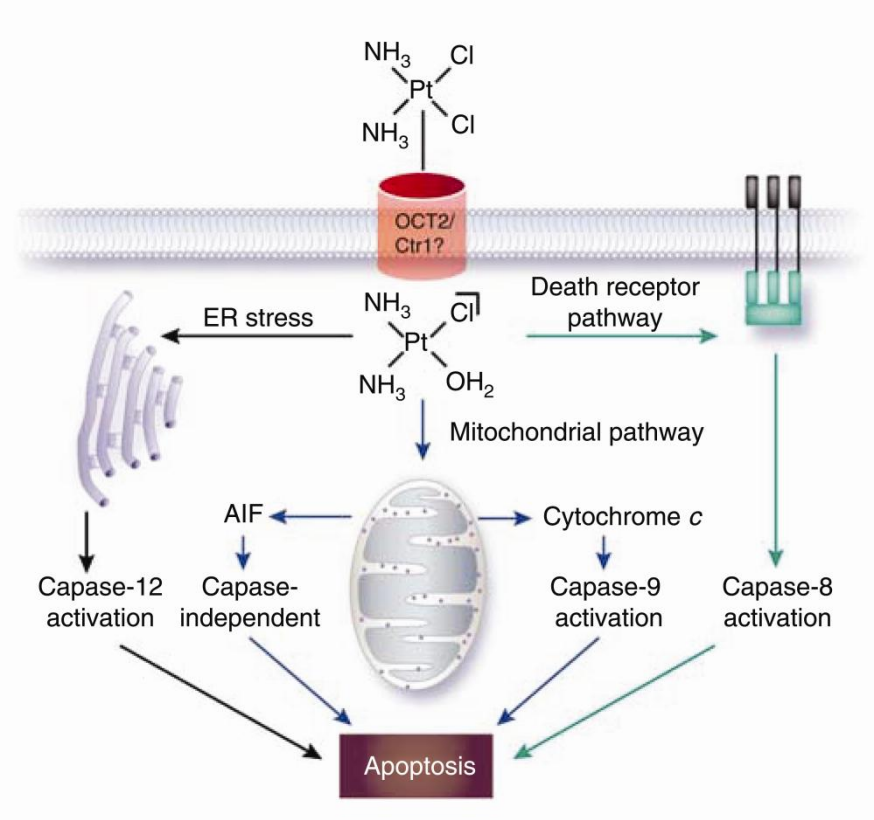


Figure 1-5. Cisplatin-mediated activation of apoptotic pathways in renal tubular cells. Cisplatin enters the cell through passive diffusion and/or active transport (copper transporter and organic cation transporter) and is hydrated in the low chlorine microenvironment in the cell. This hydrated cisplatin mediates apoptosis in cells by activating both the intrinsic mitochondrial and extrinsic death receptor pathways. In addition, the ER stress pathway can also be induced in response to cisplatin and cause apoptosis (Pabla and Dong., 2007).

In the intrinsic pathway, activated proapoptotic Bcl-2 family proteins, Bax and Bak, cause porous defects in the mitochondrial membrane and mediate release of apoptogenic factors from the organelles (Danial and Korsmeyer, 2004; Lee et al., 2001; Strasser et al., 2000). The factors released from mitochondria include AIF (apoptosis-inducing factor), cytochrome c, smac, endonuclease G, and others. Cytochrome c released from the mitochondria causes activation of caspase-9, which in turn activates downstream caspases and causes apoptosis. Smac indirectly mediates apoptosis by inhibiting the inhibitor of apoptosis proteins (IAPs). In contrast, AIF enters the nucleus after it is released from mitochondria and induces apoptosis in a caspase-independent manner. In the endoplasmic reticulum stress pathway of apoptosis, caspase-12, the initiator of apoptosis, is activated in response to cisplatin treatment (Liu and Baliga, 2005).

Cisplatin-mediated production of reactive oxygen species has also been implicated in its direct cellular toxicity (Baliga et al., 1998; Davis et al., 2001; Shiraishi et al., 2000a). In this regard, cisplatin injury to cells can be ameliorated by free radical scavengers (Appenroth et al., 1997), iron chelators (Baliga et al., 1998), superoxide dismutase (Davis et al., 2001) and heme oxygenase-1 induction (Shiraishi et al., 2000).

1.3 Role of inflammation in acute renal failure

Recent *in vivo* studies in different models of acute kidney injury, including cisplatin nephrotoxicity, show elevated levels of cytokines and chemokines and

infiltration of leukocytes in the kidney (Fig. 1-6) (Dong et al., 2007; Zhang et al., 2007). Genetic or pharmacological inhibition of these chemokines and cytokines are reported to cause attenuation of kidney injury (Kielar et al., 2005; Melnikov et al., 2001; Miura et al., 2001; Ramesh and Reeves, 2002). Recent studies indicate that TLR4 may trigger cytokine production in cisplatin nephrotoxicity. Zhang et al., (2008) examined the effect of TLR4 receptor signaling in cisplatin nephrotoxicity (Zhang et al., 2008). Their studies showed TLR4-mediated aggravation of kidney injury in cisplatin nephrotoxicity. Likewise, ablation of different populations of leukocytes ameliorated kidney injury in various models of ARF (Dong et al., 2007; Dong et al., 2008; Li et al., 2007).

The process of adhesion and extravasation of leukocytes to inflamed renal interstitium is mediated by selectins, integrins and members of the immunoglobulin superfamily, including intercellular adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecule (VCAM) (Bonventre and Zuk, 2004; Takada et al., 1997). For example, inhibition of ICAM-1 decreases renal ischemia-reperfusion injury (Kelly et al., 1994; Takada et al., 1997). The sequestered leukocytes increase renal injury further by producing more inflammatory mediators such as TNFα, IL-1, IL-6, ROS and others.

Recent studies have implicated T cells in mediating acute renal injury. Blocking B7-CD28 costimulation pathway of T cells reduces renal dysfunction and mononuclear cell infiltration in ischemic injury (Chandraker et al., 1997; Takada et al., 1997). Likewise, T cells are also shown to mediate ischemia reperfusion injury of liver, lung, heart and intestine (Rabb, 2006; Shigematsu et al., 2002; Zwacka et al., 1997)

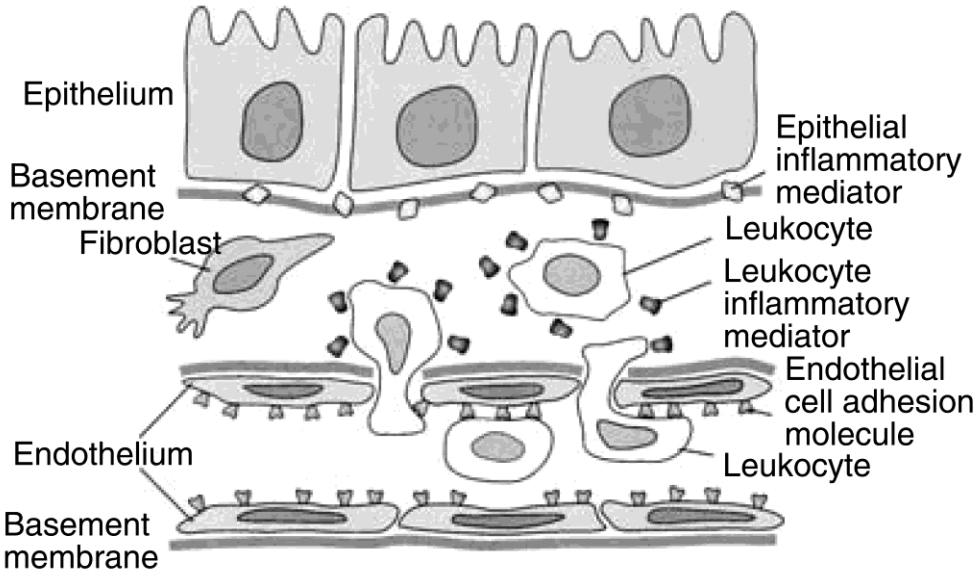


Figure 1-6. Schematic illustration of inflammatory mediators produced by renal epithelial cells and leukocytes in acute kidney injury. In response to ischemic or toxic insult, tubular epithelial cells produce IL-1, IL-6, IL-8, TNFα, TGF-β, MCP-1, KC and RANTES. Upon injury, expressions of adhesion molecules are increased on endothelial cells and also on the basolateral surface of epithelial cells. These adhesion molecules, along with chemokines, aid the infiltration of leukocytes to the sites of injury. The recruited leukocytes produce cytokines and chemokines such as IL-1, IL-6, IL-8, TNFα, MCP-1, including ROS and eicosanoids. The release of these cytokines and chemokines in the injured renal tissue serve as positive feedback pathway, which enhances inflammation and cell injury (Bonventre and Zuk., 2004).

In addition, T cells are shown to cause injury in cardiac ischemia and stroke (Arumugam et al., 2005; Hansson, 2005). In ischemia reperfusion injury of kidney, T cell-deficient mice show protection with reduced renal infiltration of leukocytes and cytokine and chemokine production (Burne et al., 2001b; Rabb et al., 2000). Further studies have shown that $CD4^+$ T cells, rather than $CD8^+$ T cells, mediate ischemic injury in mice. However, in contrast to this finding, mice lacking T and B lymphocytes failed to show protection against ischemic injury (Park et al., 2002). Taken together, the types of leukocytes that are important in renal injury remain uncertain (Ysebaert et al., 2004). In this regard, there is also growing evidence that dendritic cells mediate kidney injury in different renal diseases (Dong et al., 2005; Dong et al., 2008; Fiore et al., 2008; Kurts et al., 2007; Lee et al., 2008), including ischemic injury (Dong et al., 2007), transplant rejection (Coates et al., 2004; Loverre et al., 2007) and glomerulonephritis (Heymann et al., 2009; Scholz et al., 2008).

1.4 Dendritic cells

1.4.1 Dendritic cells in immunity

Host immune system defense against external pathogens depends on a combined action of both the antigen non-specific innate immune system and antigen specific adaptive immune system (Banchereau et al., 2000). The important functions of the innate immune system are the rapid recognition of pathogens or tissue injury and signaling the presence of danger to the adaptive immune system. The innate immune system includes phagocytic cells, natural killer cells, interferons and complement. Dendritic cells are specialized migratory antigen presenting cells with the ability to induce a primary

immune response. They are found in peripheral tissues and organs. Tissue dendritic cells are of the myeloid class and express high levels of CD11c compared to plasmacytoid dendritic cells. Immature dendritic cells have high phagocytic activity with low level expression of MHC class I, MHC class II and costimulatory molecules, CD40, CD80 and CD86. Dendritic cells recognize microbes through their pattern recognition and scavenger receptors, such as Toll-like receptors, DEC-205, mannose receptor and others. Dendritic cell internalization of pathogenic material is mediated though endocytosis, phagocytosis and macropinocytosis (Norbury, 2006). Upon maturation, in response to microbial components, cytokines or products of tissue injury, dendritic cells downregulate phagocytic activity and upregulate expression of MHC class I, MHC class II, CD40, CD80 and CD86 (Fig. 1-7, 1-9A). These matured dendritic cells migrate to the regional lymph node and activate T cells (Fig. 1-8, 1-9A) (Banchereau and Steinman, 1998). In addition, products that induce maturation of dendritic cells trigger them to produce large amounts of immune enhancing factors, such as cytokines IL-1, IL-6, IL-12 and interferon α (Dalod et al., 2002; Reis et al., 1997). Dendritic cells also activate other innate immune cells such as NK and NKT cells (Fernandez et al., 1999; Fujii et al., 2002). The mature dendritic cells induce protective adaptive immunity by activating T cells (Ludewig et al., 1998; Maldonado-Lopez et al., 1999; Schuler-Thurner et al., 2002). Activated T lymphocytes migrate to the injured tissue and mediate clearance of pathogens. These T cells can also secrete cytokines and activate other leukocyte populations such as macrophages, NK cells and eosinophils.

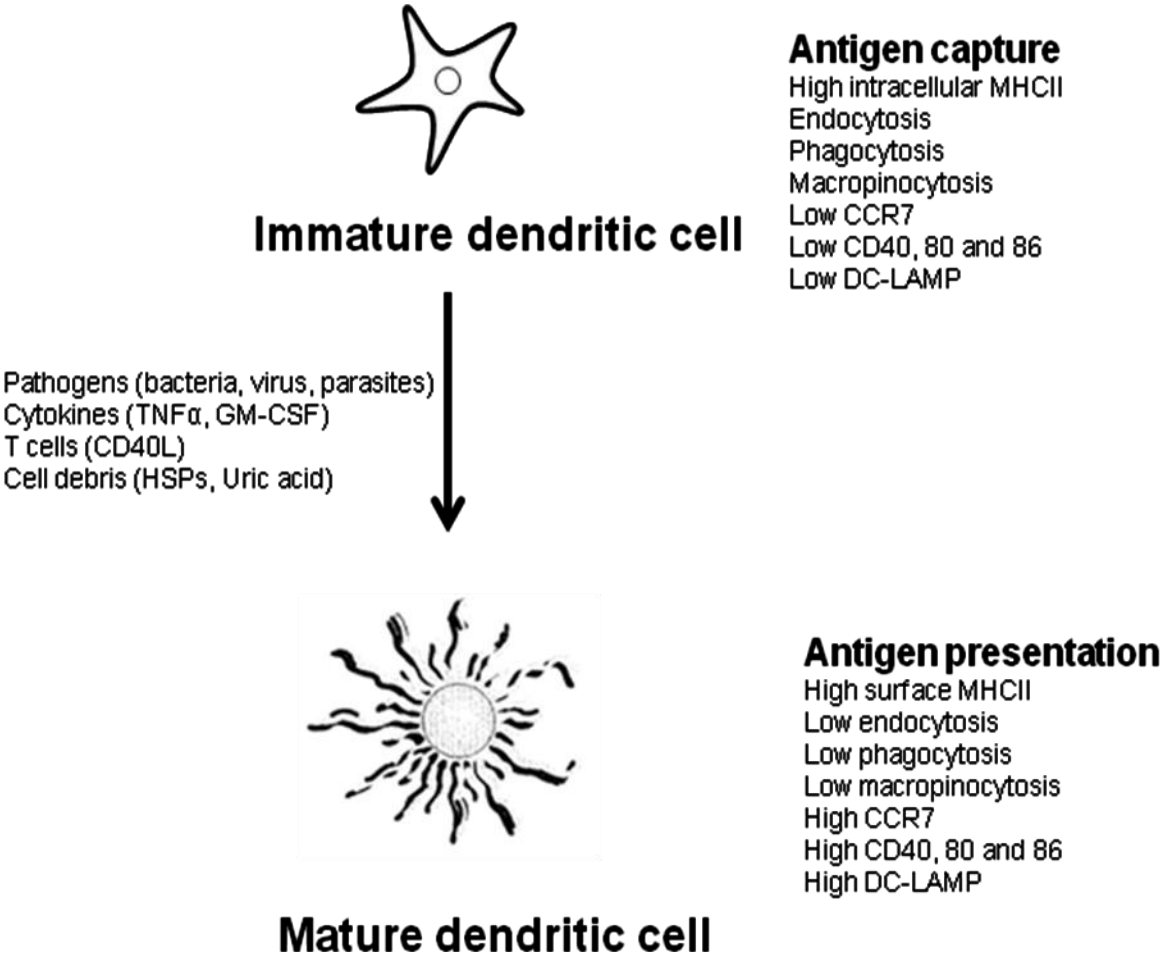


Figure 1-7. Maturation of dendritic cells. Immature dendritic cell in peripheral tissues and organs possess high endocytic capability with high intracellular MHC class II, and low CCR7, CD40, CD80, CD86 and DC-LAMP (lysosome-associated membrane protein) expression. Upon maturation in response to pathogens, cytokines and/or products of tissue injury, dendritic cells down regulate phagocytosis and up regulate expression of MHC class II, CD40, CD80, CD86 and DC-LAMP among others.

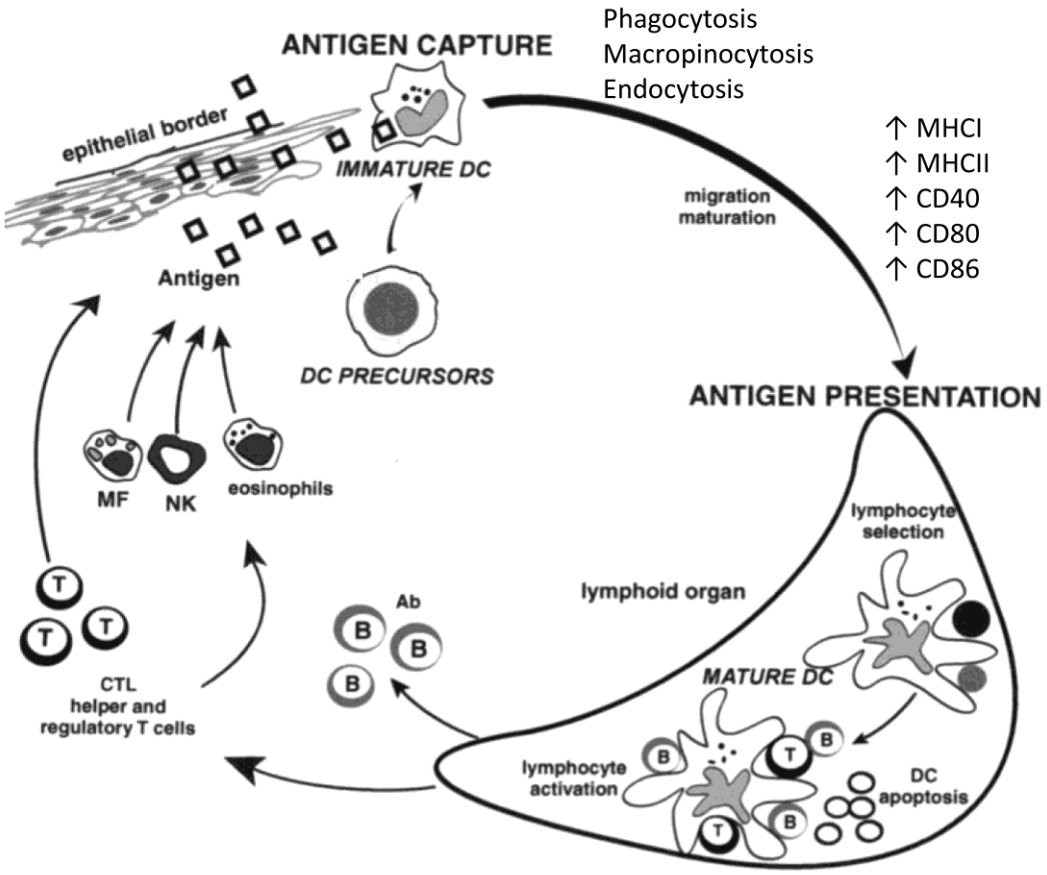


Figure 1-8. Dendritic cells in induction of immune response. Dendritic cell (DC) precursors in circulation enter tissues and differentiate into immature dendritic cells. These immature dendritic cells located in peripheral tissues and organs recognize pathogens or the products of tissue injury through their pattern recognition and scavenger receptors and capture them. During this process they are activated with increased expression of CD40, CD80, CD86, MHC class I and MHC class II. Upon maturation, dendritic cells upregulate expression of chemokine receptor CCR7 and migrate to the regional lymph node on a concentration gradient of Secondary Lymphoid Chemokine (SLC) and MIP-3β. Matured dendritic cells in the lymph node activate T cells and B cells though their secreted cytokines and antigen presentation and costimulatory molecules. Activated T helper cells and cytotoxic T cells (CTL) proliferate in larger numbers and migrate to the site of injury. Helper T cells secrete cytokines and activate leukocytes such as NK cells, macrophages and eosinophils, whereas cytotoxic T cells lyse pathogen infected cells. B cells activated by T cells and dendritic cells differentiate into plasma cells and produce antibody that can neutralize initial pathogen (modified from Banchereau et al., 2000).

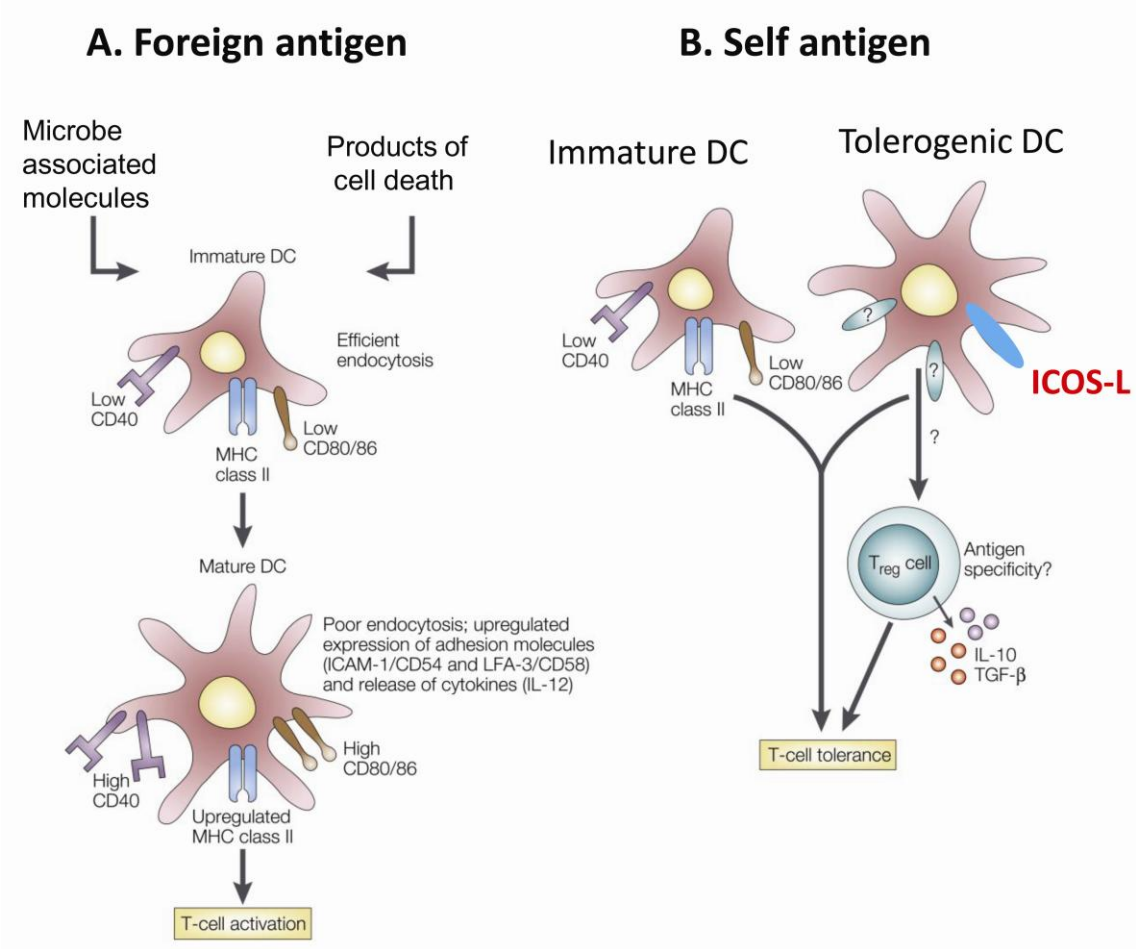


Figure 1-9. Dendritic cells at the junction of immunity and tolerance. A. Dendritic cells exposed to products of tissue injury or microbial constituents undergo changes in their surface phenotype and antigen processing capability. The upregulated antigen presentation and costimulatory molecules on dendritic cells interact with their ligands on T cells and activate them to initiate an immune response. B. Immature dendritic cells or a distinct subset of tolerogenic dendritic cells in tissues continuously sample the proteins and present them to self reactive T cells. The low expression of antigen presentation and costimulatory molecules leads to T cell tolerance rather than activation. Immature or tolerogenic dendritic cells can also induce T cell tolerance by activating T regulatory cells through molecules such as ICOS-L (modified from Walker and Abbas., 2002).

In addition to pathogens, dying mammalian cells stimulate cells of the innate immune system (Albert et al., 1998; Fonteneau et al., 2001; Gallucci et al., 1999; Matzinger, 1998; Shi and Rock, 2002; Shi et al., 2003). Dendritic cells are known to ingest cellular debris during tissue injury and mature in response to endogenous ligands, such as heat shock proteins (HSP) and uric acid, released by dead cells (Buttiglieri et al., 2003; Fonteneau et al., 2001; Matzinger, 1998; Shi and Rock, 2002). Thus endogenous ligands released during cell death can also alert the innate immune system and mediate inflammation and immune response.

1.4.2 Dendritic cells in tolerance induction

Dendritic cells that are matured or activated by different mechanisms, including TLR ligands, cytokines and CD40 ligation activate T cells of the adaptive immune system (Steinman and Nussenzweig, 2002). However, dendritic cells are also known for their role in the induction of immune tolerance (Fig. 1-9) (Steinman et al., 2003). Immune tolerance is the mechanism by which the body resists development of immune responses against self antigens. Dendritic cells, in addition to thymic epithelial cells, are known to induce central tolerance in the thymus by depleting self-reactive T cells during development (Steinman et al., 2003; Steinman et al., 2002). Central tolerance is efficient, but not complete. This is because of the enormous repertoire of T cells in the body (approximately against 25 million specificities) (Walker and Abbas, 2002). Negative selection in the thymus may not deplete all T cells that are able to recognize self antigens in the periphery.

Peripheral tolerance is an alternative mechanism to deplete self reactive T cells that are not silenced in the thymus. Under steady-state conditions, dendritic cells in the peripheral lymphoid organs and tissues capture proteins and dying cells in their immediate microenvironment and present them to T cells constitutively through their major histocompatability complex class I and class II molecules. The presentation of these molecules to T cells at low doses with self antigens without adequate expression of costimulatory molecules can lead to clonal deletion of autoreactive T cells (Hawiger et al., 2001). In addition, peripheral dendritic cells induce T regulatory cells that suppress effector T cell responses by a variety of mechanisms, including signaling via the ICOS/ICOS-ligand pathway (Akbari et al., 2002; Lohning et al., 2003; Mahnke et al., 2003; Scholz et al., 2008). In the periphery, dendritic cells can also induce tolerance by other means (Banchereau et al., 2000; Coombes et al., 2007; Fehervari and Sakaguchi, 2004; Mahnke and Enk, 2005; Ohnmacht et al., 2009; Steinman et al., 2003; Yamazaki et al., 2007) including production of TGF-β, IL-10 or indoleamine 2,3-dioxygenase (IDO) (Akbari et al., 2001; Laouar et al., 2008; Munn et al., 2002; Travis et al., 2007) and expression of ICOSL, PD-L1, PD-L2 and FcγR2B (Brown et al., 2003; Desai et al., 2007).

Costimulatory signals from antigen presenting cells are required for optimal activation of T cells (Fig. 1-10). The costimulatory molecules of antigen presenting cells that interact with their receptors on T cells include CD40, CD80, CD86, PDL-1, PDL-2 and ICOSL. Inducible costimulator ligand (ICOSL) is a B7 family member expressed on dendritic cells, macrophages, B cells, endothelial cells and epithelial cells (Aicher et al.,

2000; Aicher et al., 2000; Greenwald et al., 2005; Ling et al., 2000; Swallow et al., 1999; Yoshinaga et al., 1999), whereas its ligand ICOS is only expressed on activated T cells, particularly Th2 cells (Coyle et al., 2000; McAdam et al., 2000). In this regard, Th2 cells are one of the major sources of IL-10. Although, Th1 cells express ICOS upon activation, their expression is less compared with Th2 cells. In contrast, T regulatory (T reg) cells show high expression of ICOS even in the steady-state. The ICOS/ICOSL co-stimulatory pathway is crucial in T cell activation, differentiation, and effector function. ICOS serves distinct roles at different intervals during the pathogenesis of autoimmune diseases (Greenwald et al., 2005; Rottman et al., 2001; Sporici et al., 2001). For example, blockade of ICOS during the initial phase of experimental allergic encephalitis-aggravated disease, whereas ICOS blockade during the effector phase ameliorated disease (Dong and Nurieva, 2003; Rottman et al., 2001). ICOS engagement stimulates the production of IL-10 by lymphocytes and controls T regulatory cell functions. Dendritic cells are reported to express ICOSL and regulate T reg cells and their IL-10 production (Akbari et al., 2002; Herman et al., 2004; Scholz et al., 2008). Patients with ICOS deficiency show severe reduction in IL-10 production by T cells in response to different stimuli, indicating a critical role for ICOS in IL-10 production (Warnatz et al., 2006). In this regard, T reg cells and IL-10 were showed to attenuate kidney injury in different models of renal diseases (Gandolfo et al., 2009; Hochegger et al., 2005; Scholz et al., 2008; Tipping et al., 1997; Wolf et al., 2005; Yin et al., 2002). A recent study by Odobasic et al., (2006) suggests that ICOSL protects kidney during the induction and effector phases of crescentic glomerulonephritis (Odobasic et al., 2006), indicating

different cells with ICOSL expression, including dendritic cells, can have a protective effect against acute kidney injury.

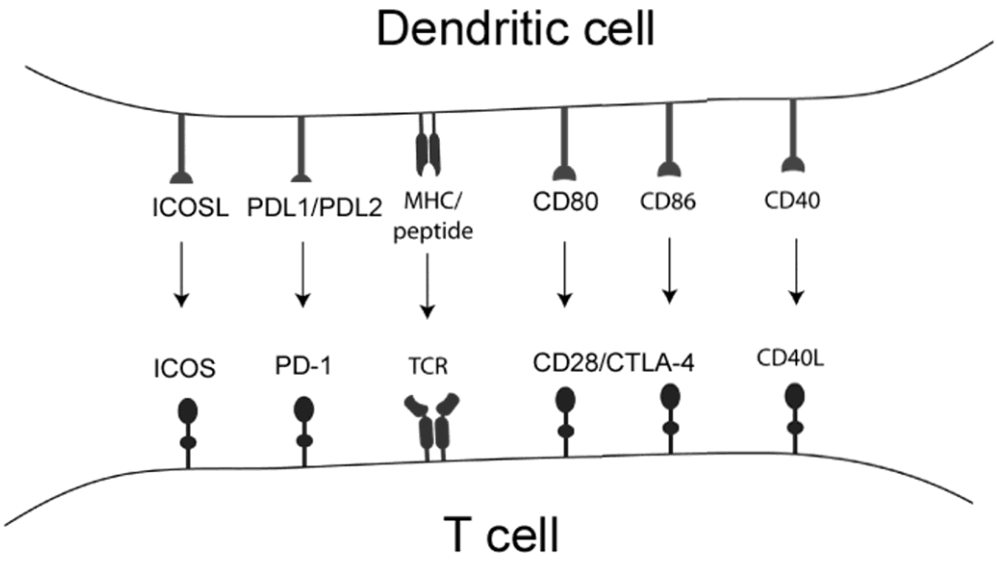


Figure 1-10. Dendritic cell costimulatory molecules and their receptors. Dendritic cells with their antigen presentation (MHC class I, MHC class II) and costimulatory molecules (CD40, CD80, CD86, PDL1/2, ICOSL) interact with T cells. Both CD28 and CTLA-4 can bind to CD80 and CD86 ligands. In contrast to CD28, CTLA-4 is a negative regulator of T cell activation. CTLA-4 expression is tightly regulated, with most of them residing within cytoplasmic vesicles.

Although less is known about the signaling pathways of ICOS, the co-receptor shares similarility with CD28 in expression of a motif that can bind to the SH2 domain of phosphatidylinositol 3-kinase (PI3K) (Rudd and Schneider, 2003). PI3K is known to activate phosphatidylinositide kinase 1, protein kinase B and glycogen synthase kinase 3, which regulate cellular metabolism, protein translation and apoptosis.

T regulatory (T reg) cells are lymphocytes that are required to maintain tolerance against self antigens (Kim et al., 2007; Sakaguchi et al., 2008). T reg cells are mainly comprised of two subsets namely T regulatory type 1 (Tr 1) and natural T reg cells (Roncarolo et al., 2006). Tr 1 cells are induced from naïve T cells, and thus represent a population of adaptive immune responders, whereas natural T reg cells are selected in the thymus, and usually possess predefined antigen specificity. Loss of T reg cells leads to fatal autoimmunity that involves multiple organs (Kronenberg and Rudensky, 2005; Sakaguchi et al., 1995). These T reg cells inhibit development of autoimmune diseases such as type 1 diabetes (Tarbell et al., 2004) and inflammatory bowel disease (Izcue et al., 2008; Powrie et al., 1993). Dendritic cells regulate T reg cell homeostasis under steady-state conditions (Coombes et al., 2007; Fehervari and Sakaguchi, 2004; Mahnke and Enk, 2005; Yamazaki et al., 2007). Constitutive loss of dendritic cells causes spontaneous fatal autoimmunity (Ohnmacht et al., 2009). Break in immune tolerance against self antigens in the absence of dendritic cells might be mediated through T reg cells because ablation of dendritic cells in mice causes a drastic reduction in T reg cell number (Darrasse-Jeze et al., 2009). Increasing dendritic cell numbers leads to an increase in T reg cell proliferation and accumulation that is mediated through MHC class II expression on dendritic cells. T reg cell-mediated attenuation of inflammation or mediation of self tolerance is achieved by different mechanisms such as production of TGF-β, IL-10 or adenosine, or CTLA-4-mediated inhibition of T cell activation (Kinsey et al., 2009; Kobie et al., 2006; Watanabe et al., 2008; Yamazaki et al., 2007). As mentioned above, T reg cells show high expression of ICOS. Blocking of ICOS in these T reg cells abrogates their regulatory capacity in diabetes (Herman et al., 2004) and

asthma, probably through lack of anti-inflammatory IL-10 production (Akbari et al., 2002). Studies in a murine model of chronic kidney disease show attenuation of kidney injury by T reg cells (Wolf et al., 2005). Likewise, recently Kinsey et al., (2009) and Gandolfo et al., (2009) showed T reg cell-mediated suppression of innate immunity and amelioration of kidney injury in renal ischemia reperfusion injury (Gandolfo et al., 2009; Hochegger et al., 2005; Kinsey et al., 2009).

1.4.3 Inflammatory dendritic cells

Dendritic cells are heterogenous and all subtypes of dendritic cells are capable of antigen uptake, processing and presentation to T cells (Shortman and Liu, 2002; Shortman and Naik, 2007). However, these subtypes differ in their location, migratory pathways and function, and dependence on inflammatory stimuli for their generation. Inflammatory dendritic cells are a novel population of dendritic cells that are generated as a consequence of infection or inflammation (Geissmann et al., 2003; Geissmann et al., 2008). They are not found under normal steady-state conditions, but appear, for example, after infection with *Listeria monocytogenes* and at later stages of glomerulonephritis (Heymann et al., 2009; Serbina et al., 2003). These inflammatory dendritic cells are also called Tip dendritic cells because of their ability to produce TNF and inducible nitric-oxide synthase (iNOS) (Geissmann et al., 2003; Geissmann et al., 2008). Inflammatory dendritic cells are derived from inflammatory monocytes. The inflammatory monocytes do not contribute to the generation of steady-state dendritic cells because inflammatory monocytes injected into normal wild-type (WT) produce no detectable dendritic cell progeny (Geissmann et al., 2003; Naik et al., 2006; Sunderkotter et al., 2004). In contrast,

these monocytes differentiate into inflammatory dendritic cells in mice subjected to inflammatory stimuli. Inflammatory monocytes deficient in the GM-CSF receptor fail to differentiate into inflammatory dendritic cells, indicating the requirement of GM-CSF for the generation of inflammatory dendritic cells (Shortman and Naik, 2007). These monocyte-derived inflammatory dendritic cells can be distinguished from steady-state conventional dendritic cells by their high expression of CD11b and Gr-1 (Heymann et al., 2009; Serbina and Pamer, 2006).

1.4.4 Dendritic cells in the kidney

Most of the information regarding the role of dendritic cells in the pathophysiology of different conditions is extrapolated from *in vitro* and *in vivo* analysis of secondary lymphatics and skin, where the dendritic cells are copious and easily accessible (Kurts, 2006b). In spite of the potential for dendritic cells to contribute to renal inflammation, relatively little work has been done on renal dendritic cells due to technical difficulties in their detection and isolation. One major controversy has been the significance of low to intermediate level expression of the macrophage marker, F4/80, on renal $CD11c^{+}$ MHC II^{+} dendritic cells. However, $CD11c^{+}$ cells of kidney resemble splenic dendritic cells, but not peritoneal macrophages, in morphology, phagocytic activity, lysosomal content, microbicidal effector functions, expression of T cell costimulatory molecules, and the ability to activate T cells (Kruger et al., 2004). Renal dendritic cells respond to systemic and local acute injury by producing cytokines and chemokines (Dong et al., 2005). Recent findings in $CX3CR1^{GFP/+}$ mice show an intricate tubulointerstitial network of GFP-positive cells in kidney (Soos et al., 2006) . These GFP-

positive cells constantly probed the environment with their dendrites. These cells were later shown to express CD11c.

1.4.5 Dendritic cells in renal diseases

Dendritic cells constitute an abundant population of leukocytes in normal kidney (Heymann et al., 2009; Kruger et al., 2004; Soos et al., 2006). However, their role in renal diseases is largely unknown. Most of the reported studies were based on measures of dendritic cell infiltration in kidneys subsequent to injury rather than their functional relevance within their physiological context. Segerer et al., (2008) showed renal tubulointerstitial compartment-specific infiltration of dendritic cells in glomerulonephritis (Segerer et al., 2008). Studies on allograft rejection and immunoglobulin A nephropathy showed a change in the number and anatomical distribution of dendritic cells in kidney as compared with normal individuals (Woltman et al., 2007). Similarly, in transplanted kidney biopsies with delayed graft function, Loverre et al., (2007) noticed infiltration of dendritic cells as compared with pre-transplated biopsies (Loverre et al., 2007). These studies indicate a likely contribution of dendritic cells in renal inflammation and aggravation of kidney injury.

1.4.6 Dendritic cell ablation mouse models

Dendritic cells play a central role at the crossroads of immunity and tolerance. Investigation of dendritic cell involvement in different pathophysiological conditions requires assessment of their function within their physiological context. To study dendritic cell function *in vivo,* the preferred method is their ablation in the intact

organism (Bennett and Clausen, 2007; Sapoznikov and Jung, 2008). Liposomal clodronate injection has been used to deplete dendritic cells (Dong et al., 2007; Dong et al., 2008). Uptake of clodronate encapsulated liposomes depends on the phagocytic activity of the cell. Cellular release of clodronate from liposomes causes inhibition of cell metabolism and ultimately cell death (Van Rooijen and Sanders, 1994). However, phagocytic intake of clodronate is not restricted to dendritic cells. Macrophages and monocytes are also depleted during the process (Blazar et al., 2001; Swirski et al., 2007). The use of cell-specific antibodies is an alternative method used extensively to deplete different types of leukocytes (Kirimanjeswara et al., 2005; Kuwajima et al., 2006a; Li et al., 2007). However, this conventional technique is hindered by the reason that myeloid dendritic cells interspersed in peripheral tissues might be less susceptible to antibody-mediated phagocytosis and cytotoxicity.

1.4.7 Conditional and constitutive dendritic cell ablation

Constitutive depletion of dendritic cells can be achieved through expression of cytotoxic proteins in dendritic cells, such as diphtheria toxin (Birnberg et al., 2008; Ohnmacht et al., 2009). Diphtheria toxin (DTx), produced by *Corynaebacterium diphtheriae,* is a potent toxin that upon internalization through diphtheria toxin receptor (DTR) blocks protein synthesis and causes rapid cell death (Holmes, 2000). Diptheria toxin is composed of A and B subunits. The cell surface DTR, the hbEGF (heparin-binding EGF (epidermal growth factor)-like growth factor), binds the B subunit of DTx and mediates internalization (Naglich et al., 1992) (Fig. 1-11). In the cell, the A subunit causes ADP ribosylation and, in turn, inactivation of elongation factor 2.

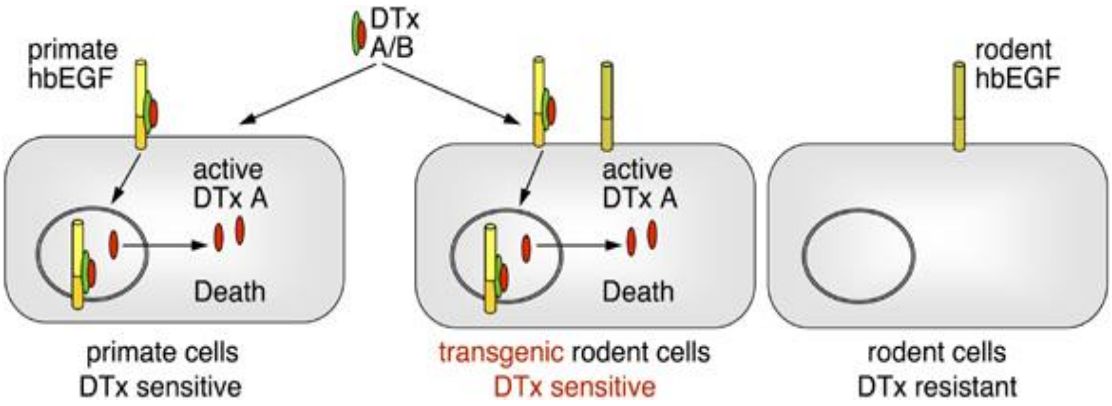


Figure 1-11. Schematic illustration of diphtheria toxin (DTx) mediated depletion of primate diphtheria toxin receptor (DTR)-expressing cells. The cell surface primate hbEGF binds the B subunit of DTx with high affinity and mediates internalization of toxin. In the cell, the A subunit released in the cytoplasm inhibits protein synthesis and causes apoptotic cell death. Rodent cells are resistant due to the difference in 3 amino acids in the extracellular domain of hbEGF that is required for binding to B subunit of DTx (Sapoznikov and Jung, 2008)

The inactivation of elongation factor 2 causes termination of protein synthesis and apoptotic cell death. Rodents are comparatively resistant to DTx compared to primates, owing to the change in 3 amino acids in the extracellular domain of DTR that binds the B subunit of DTx (Mitamura et al., 1997). Constitutive depletion of myeloid dendritic cells is achieved by breeding mice expressing transgene Cre recombinase that is under the transcriptional control of CD11c promoter with mice carrying the DTx A subunit under control of a loxP-flanked stop cassette in the ubiquitously expressed ROSA26 locus (Birnberg et al., 2008; Ohnmacht et al., 2009). Dendritic cells are depleted constitutively in the progeny of these mice that express DTx in dendritic cells. Recently, Ohnmacht et al., (2009) and Birnberg et al., (2008) used this constitutive dendritic cell ablation method to determine the role of dendritic cells during development and subsequent homeostasis in mice (Birnberg et al., 2008; Ohnmacht et al., 2009). However, the depletion of

dendritic cells throughout the lifetime in these mice induces compensatory mechanisms which obscure the role of dendritic cells. For example, mice with constitutive depletion of dendritic cells develop a fatal myeloproliferative disorder.

Unlike constitutive depletion, conditional depletion is temporally inducible and carries less chance of the induction of compensatory processes that may affect the parameter under investigation. This method of ablation of dendritic cells is achieved using a CD11c promoter to drive the transgenic expression of simian DTR receptor (Jung et al., 2002). CD11c is highly expressed by all conventional dendritic cells and has been used to target transgenes to dendritic cells (Brocker et al., 1997; Lindquist et al., 2004). DTx injection to CD11c-DTR transgenic (CD11c-DTRtg) mice causes rapid, efficient and transient depletion of dendritic cells (Jung et al., 2002). Ablation of dendritic cells occurs within 24 hr after DTx injection and the cells begin to replenish 3 days later. In these transgenic mice, DTR is expressed as a fusion protein with GFP. This allows visualization of dendritic cells by both fluorescent and intravital microscopy. One major disadvantage with this model is that repeated injection of DTx to these mice causes mortality (Scholz et al., 2008; Zammit et al., 2005). This mortality has been attributed to the expression of CD11c-DTR on non hematopoietic cells because WT mice reconstituted with CD11c-DTRtg bone marrow are resistant to high or multiple doses of DTx (Zaft et al., 2005). Thus, mortality in these CD11c-DTRtg mice can be prevented by making CD11c-DTRtg to WT mice chimera. CD11c-DTR transgenic mice have been used extensively to study the function of dendritic cells *in vivo* in different viral, bacterial, parasitic and prion infections (Bennet and Clausen, 2007; Sapoznikov and Jung,

2008). Likewise, this mice model has been used to investigate the function of dendritic cells in tolerance, glomerulonephritis, NK cell responses and antitumor immunity.

Although the CD11c promoter used to create the transgene in CD11c-DTRtg mice had been considered to be dendritic cell-specific, low levels of CD11c expression are also reported in NK cells, plasmacytoid dendritic cells, activated T cells, alveolar macrophages, and splenic marginal zone and metallic macrophages (Huleatt et al., 1995; Lucas et al., 2007; Probst et al., 2005; Sapoznikov et al., 2007; Sapoznikov et al., 2008; Van-Rijt et al., 2005). However, as the CD11c promoter fragment used in CD11c-DTRtg mice does not carry the entire promoter sequence of CD11c, its expression is more restricted to CD11c high expressing cells (Sapoznikov et al., 2007). For example, the CD11c-DTR/GFP transgene is inactive in plasmacytoid dendritic cells and NK cells (Lucas et al., 2007; Sapoznikov et al., 2007). Also considering the efficiency of DTx that a single molecule of DT is enough to arrest protein translation (Pappenheimer et al., 1982; Bennett et al., 2007), DTx-mediated direct depletion of plamacytoid dendritic cells and NK cells is very less likely to occur in CD11c-DTRtg mice.

1.4.8 Conditional gene and cell ablation method

Dendritic cell ablation by conditional or constitutive depletion methods is useful for defining the functions of dendritic cells within their physiological context. Further investigation of dendritic cells at the molecular level requires more sophisticated approaches to achieve specific mutations in dendritic cells. Mutations in the gene of interest in dendritic cells can be accomplished by conditional gene ablation or conditional

cell ablation methods (Fig. 1-12) (Caton et al., 2007; Sapoznikov and Jung, 2008; Sapoznikov et al., 2008; Travis et al., 2007). Conditional gene ablation relies on the combination of dendritic cell-specific expression of Cre and loxp sites flanking the gene of interest (Caton et al., 2007; Travis et al., 2007). This causes cell-restricted rearrangement of the conditional mutant allele with loss of function of that gene. In conditional gene ablation, the loss of the gene in cells can be achieved in both hematopoietic and non hematopoietic cell compartments. The major disadvantage with this method is the requirement of mice having loxp site flanked gene of interest and mice expressing Cre under the transcriptional control of cell-specific marker. In addition, this method requires long-term breeding to achieve homozygous or hemizygous expression of Cre and homozygous loxp site flanked alleles in the required cell compartment.

In the conditional cell ablation method, mutation within the gene of interest in dendritic cells requires generating mixed bone marrow chimeras in WT mice using bone marrow of candidate mutant mice and CD11c-DTRtg mice (Darrasse-Jeze et al., 2009; Kuwajima et al., 2006; Sapoznikov and Jung, 2008; Sapoznikov et al., 2008). The resulting chimeric hematopoietic compartment harbors a mixed dendritic cell compartment consisting of DTx-resistant mutant dendritic cells and DTx-sensitive WT dendritic cells. Injection of DTx to these chimeric mice causes depletion of DTR positive dendritic cells carrying the WT gene leaving behind dendritic cells having the mutant gene. As the conditional cell ablation technique is based on making bone marrow chimeric mice, the ablation of the gene is restricted to hematopoietic cell lineage.

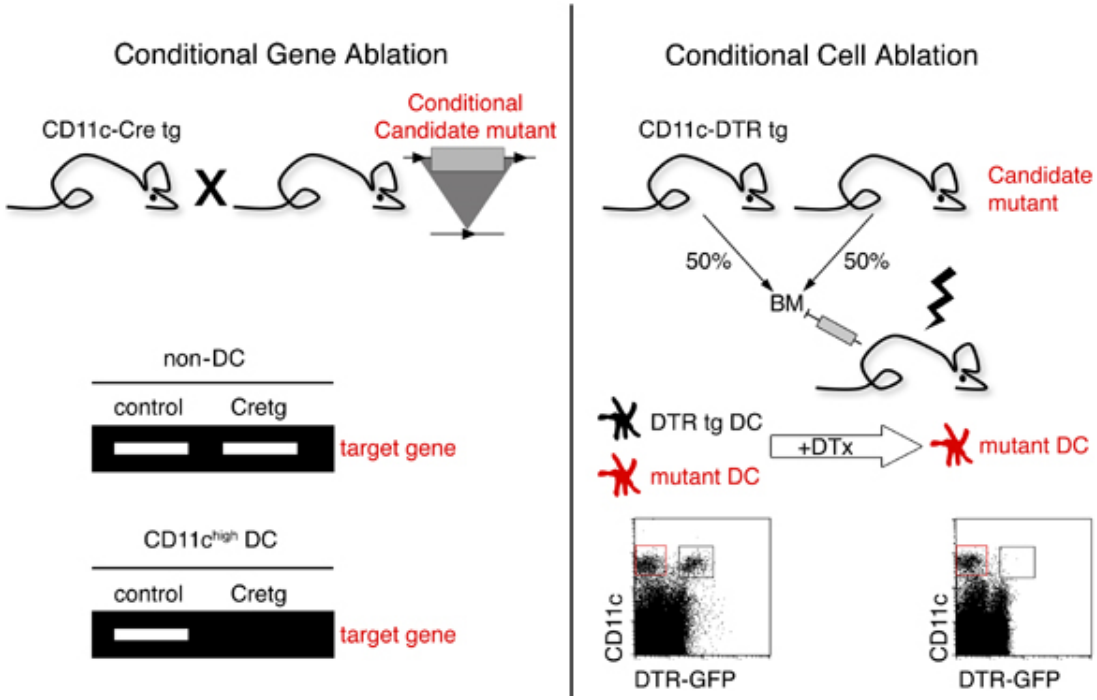


Figure 1-12. Conditional gene ablation and conditional cell ablation methods to achieve specific mutations in the dendritic cells. Conditional gene ablation relies on dendritic cell-restricted expression of Cre, and loxp sites flanking the gene of interest. This causes cell-restricted rearrangement and loss of function of that gene. The specificity of the approach is confirmed by analyzing genomic DNA of dendritic cells and non dendritic cells for the presence of the target gene. Conditional cell ablation requires the generation of mixed bone marrow chimeras by injecting equal numbers of bone marrow cells from CD11c-DTRtg mice and candidate mutant mice to lethally irradiated WT mice. The mutation is induced in these mixed chimeras by DTx-mediated ablation of the CD11c-DTRtg dendritic cells which leaves behind mutant dendritic cells. The specificity of this approach can be confirmed by flow cytometry (Sapoznikov and Jung, 2008).

The conditional cell ablation approach provides a faster and less expensive alternative to conditional gene ablation approach. This method has been used to investigate the function of different secreted factors or molecules of dendritic cells, including IL-15 (Kuwajima et al., 2006), B cell activating factor, macrophage migration inhibition factor (Sapoznikov et al., 2008) and MHC class II (Darrasse-Jeze et al., 2009), in normal immune homeostasis, immunity and tolerance.

1.5 Interleukin -10

1.5.1 Interleukin-10, Interleukin-10 receptor 1 and Interleukin-10 receptor 2

Traditionally, cytokines are classified as pro- or anti-inflammatory based on their effects in different pathophysiological conditions (Ramesh and Reeves, 2002; Scholz et al., 2008; Tipping et al., 1997; Travis et al., 2007; Yin et al., 2002; Zhang et al., 2007). IL-10, also known as cytokine synthesis inhibitory factor, was first described as a Th2 cytokine that inhibited the activation and cytokine production by Th1 cells (Fiorentino et al., 1989). IL-10 inhibits the production of a wide range of cytokines and other soluble mediators of inflammation including IL-1α, IL-1β, IL-6, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, LIF and PAF (de Waal Malefyt et al., 1991; Fiorentino et al., 1991; Fiorentino et al., 1991). Chemokines are secreted at sites of inflammation and mediate recruitment of leukocytes such as neutrophils, monocytes, macrophages and T cells. IL-10 inhibits the production of many chemokines including both CC (MCP-1, MCP-5, Mip-1, RANTES) (Berkman et al., 1995; Marfaing-Koka et al., 1996) and CXC chemokines (IL-8, IP-10, KC, MIP-2) (Kopydlowski et al., 1999). IL-10-mediated attenuation of inflammation is also mediated through the increased production of anti-inflammatory molecules such as soluble p55 and p75 TNFR and IL-1R antagonist, and inhibition of expression of receptors for proinflammatory cytokines (Dickensheets and Donnelly, 1997; Hart et al., 1996; Jenkins et al., 1994; Joyce and Steer, 1996; Linderholm et al., 1996; Linderholm et al., 1996).

In mice, IL-10 is a 178 amino acid non-covalent homodimer of 2 interpenetrating glycosylated polypeptides of 17-21KDa (Fig. 1-13) (Moore et al., 1990; Wakkach et al., 2000). Human IL-10 is also 178 amino acids in length but is not glycosylated (Dumoutier and Renauld, 2002; Vieira et al., 1991). Human IL-10 is effective on mouse cells, whereas mouse IL-10 is active only on mouse cells (Tan et al., 1993a; Vieira et al., 1991). IL-10 is mainly produced by Th2 cells, T reg cells, dendritic cells and macrophages (Iwasaki and Kelsall, 1999; Panuska et al., 1995; Schmidt-Weber et al., 1999). Other cells reported to produce IL-10 include monocytes (Frankenberger et al., 1996), keratinocytes (Becherel et al., 1997), mast cells (Masuda et al., 2002), microglia (Chabot et al., 1999), hepatic stellate cells (Wang et al., 1998), NK cells (Mehrotra et al., 1998), B cells (Spencer and Daynes, 1997) and eosinophils (Nakajima et al., 1996).

The IL-10 receptor is a heterodimer that consist of two subunits, R1 and R2, and belongs to the class II cytokine receptor family (Wang et al., 2002; Zdanov et al., 1996). IL-10 and IL-10 receptor interaction can be multimeric, consisting of at least two R1 and R2 chains forming a signaling receptor complex (Kotenko and Pestka, 2000).

IL-10R1 is a 90-120KDa polypeptide chain expressed by most hematopoietic cells (Liu et al., 1997; Tan et al., 1993). Expression of IL-10 receptor in non-hematopoietic cells such as fibroblasts, keratinocytes or epidermal cells is more often induced (Michel et al., 1997; Michel et al., 1997; Weber-Nordt et al., 1994). Constitutive IL-10R1 expression is reported in colonic epithelium and placental cytotrophoblasts (Bourreille et al., 1999; Denning et al., 2000). Upon ligand binding, IL-10R1 mainly

serves as a STAT3 docking port, the first step in signal transduction (Kotenko and Pestka, 2000).

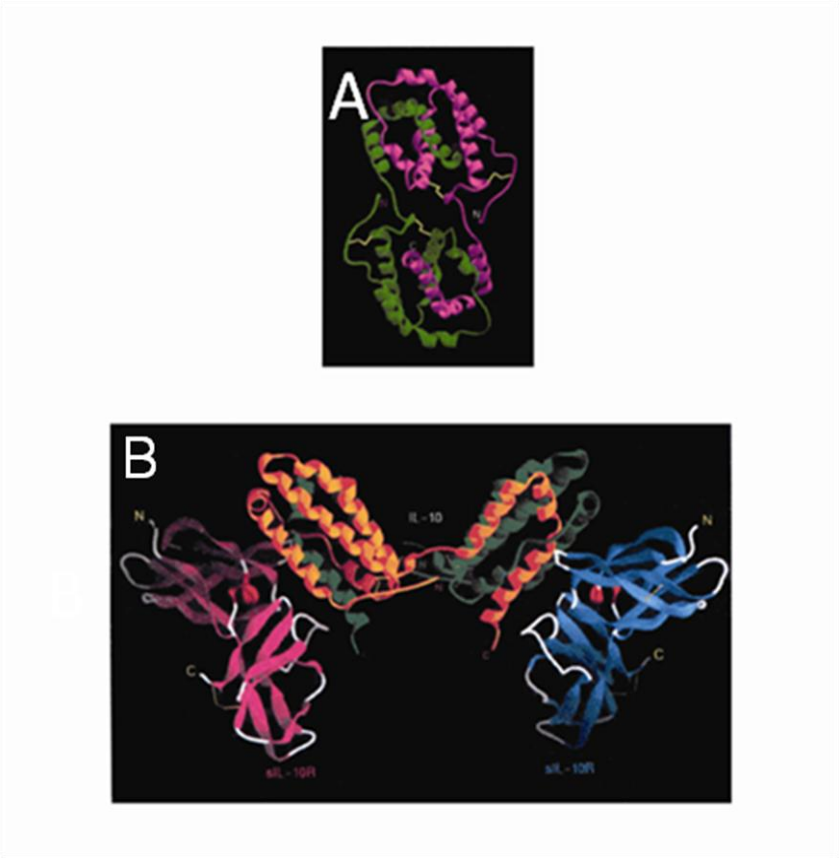


Figure 1-13. Structure of IL-10 and IL-10/IL-10 receptor complex. A. Ribbon diagram of IL-10 dimer. C and N termini are marked and disulfide bonds are shown in yellow. B. Model structure of IL-10/IL-10 receptor 1 dimer complex. Loops and coil regions of the IL-10 receptor 1 dimer are shown in white with a short interdomain helix in red (Zdanov et al., 1996).

Like other members of the class II cytokine receptor family, IL-10R1 utilizes a 60 KDa R2 subunit for signaling (Moore et al., 2001). IL-10R2 is also a subunit of IL-22, IL-26 and IFN-γ receptor complex (Donnelly et al., 2004; Wang et al., 2002). IL-10R2 expression in most cells and tissues is constitutive (Gibbs and Pennica, 1997; Lutfalla et

al., 1993). The principal role of IL-10R2 is the recruitment of tyrosine kinase (Tyk) 2 into the IL-10 receptor complex for signaling (Kotenko et al., 1997; Moore et al., 2001; Spencer et al., 1998). The binding affinity of IL-10 to IL-10R1 is not affected by IL-10R2 (Kotenko et al., 1997). Binding of IL-10 to IL-10R1 induces a conformational change that enables IL-10R2 to interact with IL-10/IL-10R1 complex (Fig. 1-14) (Donnelly et al., 2004). This activates the receptor associated Janus Tyrosine Kinases (JAK) 1 and Tyk2 (Finbloom and Winestock, 1995). JAK1 is associated with IL-10R1 whereas Tyk2 is associated with IL-10R2. These activated kinases catalyze phosphorylation of tyrosine residues on the intracellular domain of the IL-10R1 chain (Weber-Nordt et al., 1996). These phosphotyrosine residues serve as docking sites for the latent cytosolic transcription factor STAT. IL-10R1 bound STAT3 is in turn phosphorylated on tyrosine by the activated receptor complex associated Janus kinases. The resulting phosphorylated STAT3 homodimers translocate to the nucleus and bind to STAT-binding elements in the promoter region of various IL-10 responsive genes (Donnelly et al., 1999). IL-10-inducible genes include anti-inflammatory, anti-apoptotic and cell-cycle progression genes such as SOCS-3, BCLXL (B-Cell Lymphoma Extra Large), Cyclin A, Cyclin D1, Cyclin D2, Cyclin D3, Pim1 and c-Myc. BCLXL is an antiapoptotic gene whereas Pim1 and c-Myc are oncogenes. Suppressor of cytokine signaling-3 (SOCS-3) is a member of newly identified gene family that inhibits JAK/STAT-dependent signaling. The ability of IL-10 to inhibit proinflammatory cytokines and chemokines, at least in part, depends on the induction of SOCS-3 (Donnelly et al., 1999).

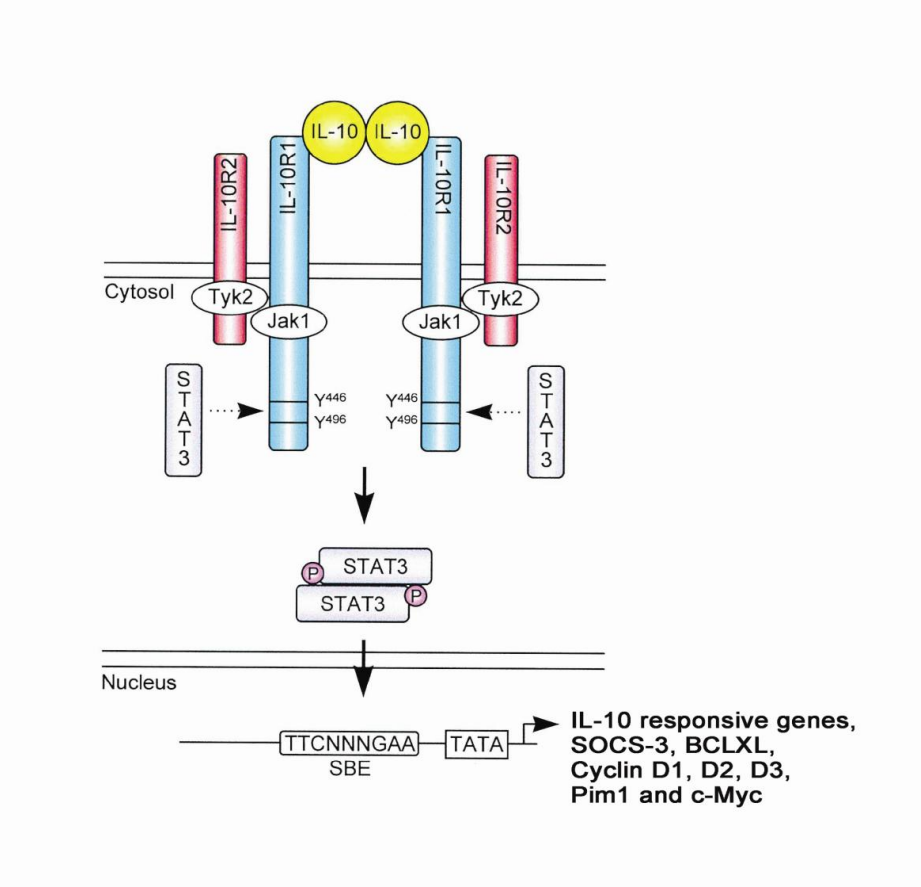


Figure 1-14. IL-10 signaling. IL-10 signaling is initiated by binding of IL-10 homodimer to two adjoining IL-10R1 molecules. Binding of IL-10 to IL-10R1 activates the receptor complex associated JAK1 and Tyk2, which in turn phosphorylates the intracellular domains of the IL-10R1 chains on specific tyrosine residues. These phosphorylated tyrosine residues serve as docking sites for the latent cytosolic, transcription factor, STAT3. The IL-10R1 bound STAT3 is in turn phosphorylated by JAK. This activated STAT3 dissociates and homodimerizes with other STAT3 molecules, and translocates to the nucleus. In the nucleus, the homodimer of STAT3 binds with high affinity to STAT-binding elements (SBE) in the promoters of IL-10 responsive genes (modified from Donnelly et al., 1999).

IL-10 is a potent inducer of heme oxygenase I (HO-1). IL-10 has been shown to mediate production of HO-1 and induce anti-inflammatory effects in mice (Lee and Chau, 2002). HO-1 is a rate limiting enzyme in heme metabolism that catalyzes heme into carbon monoxide, iron and biliverdin (Ponka, 1999). These metabolites of heme have antioxidant, antiapoptotic, anti-inflammatory, and cytoprotective properties (Jarmi and Agarwal, 2009). Biliverdin possesses potent anti-oxidant properties, whereas iron sequestered in ferritin exhibits cytoprotective effects (Balla et al., 1992). Carbon monoxide has anti-inflammatory, vasodilatory, and antiapoptotic effects.

In kidney, HO-1 is expressed at very low levels in normal conditions (da Silva et al., 2001). However, HO-1 is induced in kidney in different renal diseases, such as renal ischemia reperfusion injury, nephrotoxic serum nephritis and contrast induced nephropathy. In response to ischemic or toxic insult, HO-1 is induced primarily in proximal tubules of kidney. In this regard, cisplatin causes injury predominantly in proximal tubules, the major site of HO-1 induction in acute kidney injury. HO-1 has been shown to protect kidney in different models of renal failure such as ischemia reperfusion injury (Shimizu et al., 2000), transplantation (Nakao et al., 2005), contrast induced nephrotopathy (Goodman et al., 2007) and cisplatin nephrotoxicity (Shiraishi et al., 2000b). In cisplatin nephrotoxicity, HO-1-deficient mice treated with cisplatin shows exacerbated structural and functional renal injury as compared with WT mice. These findings indicate a protective effect of HO-1 in acute kidney injury. The HO-1-induced in kidneys in renal diseases could be mediated through IL-10.

1.5.2 Interleukin-10 in renal diseases

A number of endogenously produced factors during inflammation have been shown to attenuate kidney injury (Daemen et al., 1999; Scholz et al., 2008). IL-10 is a pleiotropic cytokine with anti-inflammatory, immunosuppressive and immunostimulatory properties (Moore et al., 2001). Renal injury is mediated by production of variety of proinflammatory cytokines (Dong et al., 2007; Melnikov et al., 2001; Ramesh et al., 2007) and chemokines (Miura et al., 2001), and infiltration and activation of leukocytes (Ascon et al., 2006; Awad et al., 2009; Dong et al., 2005). IL-10 inhibits the production of these cytokines and chemokines, expression of adhesion molecules and activation of leukocytes (Moore et al., 2001). Many studies have reported the beneficial effects of IL-10 in different renal diseases. Exogenous IL-10 prevents crescent formation and glomerular injury in experimental glomerulonephritis (Tipping et al., 1997a). Likewise, administration of IL-10 attenuated murine lupus (Yin et al., 2002) and experimental mesangial proliferative glomerulonephritis (Kitching et al., 2002a). Macrophages or adenoviruses genetically engineered to produce IL-10 *in vivo* attenuated glomerulonephritis (El-Shemi et al., 2004; Wilson et al., 2002). Exogenous IL-10 ameliorated ischemic and cisplatin-induced kidney injury, and helped renal recovery after transplantation (Deng et al., 2001). Consistent with exogenous IL-10 findings, endogenous IL-10 protected kidney from renal ischemia reperfusion injury (Daemen et al., 1999). However, the role of endogenous IL-10 in cisplatin nephrotoxicity is not known.

1.6 Rationale, Hypothesis and Specific aims

The long term goal of our laboratory is to reduce morbidity and mortality associated with ARF. The objective of this proposal is to investigate the dendritic cell role in toxin-mediated ARF. The central hypothesis is that renal dendritic cells ameliorate kidney injury in cisplatin nephrotoxicity by inducing the production of anti-inflammatory cytokines, such as IL-10. The rationale behind this study is that once the functions of renal dendritic cells in kidney injury are identified, these observations can be extended to human studies for the development of innovative approaches for the prevention and treatment of acute renal failure. The objective of this study was accomplished under the following two specific aims:

1. **Determine the role of dendritic cells in cisplatin nephrotoxicity (Chapter 2).** Dendritic cells are the most abundant renal leukocyte population. Studies during the last decade indicate that renal dendritic cells play a vital role in a variety of renal pathophysiological conditions. Liposomal clondronate-mediated depletion of dendritic cells was the most common model employed to study the role of renal dendritic cells within their physiological context. However, clondronate is known to deplete monocytes and macrophages in addition to dendritic cells. Therefore, we first characterized renal dendritic cells by comparing them with splenic dendritic cells. We also studied renal dendritic cell-localization in kidney, which is largely unknown. We then characterized a DTR-based dendritic cell ablation mice model that can be used to study the role of renal dendritic cells *in vivo.*

Finally, we investigated the impact of renal dendritic cell depletion on cisplatin nephrotoxicity using CD11c-DTRtg transgenic mice.

2. **Determine the effect of endogenous IL-10 and dendritic cell-derived IL-10 in cisplatin nephrotoxicity (Chapter 3).** Using IL-10-deficient mice, we investigated the role of endogenous IL-10 as a protective mechanism in cisplatin nephrotoxicity. Next, using a conditional cell ablation approach, we determined the role of dendritic cell-derived IL-10 in cisplatin nephrotoxicity.

Chapter 2: Role of renal dendritic cells in cisplatin nephrotoxicity

2.1 Introduction

Innate immune responses are pathogenic in both ischemic and toxic acute renal failure. In response to renal injury, inflammatory chemokines and cytokines are produced both by renal parenchymal cells, such as proximal tubule epithelial cells, and resident or infiltrating leukocytes (Dong et al., 2007; Li et al., 2007; Ramesh and Reeves, 2003; Zhang et al., 2007). The elaborated chemokines and cytokines, including TNF-α, IL-18, KC and MCP-1, subsequently recruit additional immune cells to the kidney, such as neutrophils, T cells, monocytes and inflammatory dendritic cells, which may cause further injury through pathways which are not yet fully defined (Ascon et al., 2006; Furuichi et al., 2003; Heymann et al., 2009; Kielar et al., 2005; Li et al., 2007; Liu et al., 2006; Melnikov et al., 2001; Miura et al., 2001; Ramesh and Reeves, 2002). Dendritic cells are sentinels of the immune system and, under steady-state conditions, induce tolerance by various mechanisms including production of TGF-β, IL-10 or indoleamine 2,3-dioxygenase (Akbari et al., 2001; Laouar et al., 2008; Munn et al., 2002; Travis et al., 2007) expression of PDL-1, PDL-2 or FcγR2B (Brown et al., 2003; Desai et al., 2007), clonal deletion of autoreactive T cells (Hawiger et al., 2001) and induction of T reg cells via the ICOS-ICOS-ligand pathway (Akbari et al., 2002; Lohning et al., 2003; Mahnke et al., 2003; Scholz et al., 2008). In addition, upon activation, dendritic cells can become tolerogenic, rather than immunogenic, and restrict inflammation (Akbari et al., 2001; Scholz et al., 2008). In response to pathogens or products of tissue injury, dendritic cells mature and initiate immunity or inflammatory diseases (Jung et al., 2002; Van-Rijt et al.,

2005). Monocytes recruited to inflamed tissues can also differentiate into inflammatory dendritic cells and mediate defense against pathogens or contribute to inflammatory tissue responses (Geissmann et al., 2008; Heymann et al., 2009; Serbina and Pamer, 2006; Shortman and Naik, 2007).

Although dendritic cells represent a major population of immune cells in the kidney (Soos et al., 2006), their role in renal disease is poorly defined. Liposomal clodronate has been used to study the pathophysiologic role of phagocytic cells, which include dendritic cells, monocytes and macrophages (Bennett and Clausen, 2007; Dong et al., 2007; Dong et al., 2008; Jo et al., 2006). An alternative dendritic cell-specific approach uses expression of the simian diptheria toxin receptor (DTR) driven by the CD11c promoter to target dendritic cells for DTx-mediated cell death (Jung et al., 2002). This model has been used extensively to study the role of dendritic cells in various physiologic and pathophysiologic contexts (Bennett and Clausen, 2007; Sapoznikov and Jung, 2008). However, its application in kidney disease has been limited to two recent studies of immune complex-mediated glomerulonephritis (Heymann et al., 2009; Scholz et al., 2008).

We have reported that inflammation plays an important role in the pathogenesis of cisplatin-induced acute renal injury (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003; Zhang et al., 2007; Zhang et al., 2008). The present study examined the renal dendritic cell population and the impact of its depletion on cisplatin nephrotoxicity. We show that dendritic cells are the most abundant population of renal resident leukocytes,

and form a dense network throughout the tubulointerstitium. Using a conditional dendritic cell depletion model, we determined that dendritic cell depletion markedly exacerbates cisplatin-induced renal dysfunction, structural injury and infiltration of neutrophils.

2.2 Methods

2.2.1 Mice

Experiments were performed using 6- to 14-week old C57BL/6 (CD45.2) or congenic C57BL/6 mice, B6.SJL-PtprcaPep3b/BoyJ (CD45.1), and CD11c-DTRtg mice (B6.FVB-Tg Itgax-DTR/GFP 57Lan/J) harboring a transgene encoding a simian DTR/GFP fusion protein under the transcriptional control of mouse CD11c promoter. This transgene has been mapped on mouse chromosome 1 by the Jackson Laboratory. All mice used for experiments were purchased from ther Jackson Laboratory. The mice were bred in the barrier facility at The Penn State Hershey College of Medicine. The transgenic mice (B6.FVB-Tg Itgax-DTR/GFP 57Lan/J) were screened by PCR using the primers; forward 5' GGG ACC ATG AAG CTG CTG CCG 3', reverse 5' TCA GTG GGA ATT AGT CAT GCC 3' for transgene, and forward 5' CAA ATG TTG CTT GTC TGG TG 3', reverse 5' GTC AGT CGA GTG CAC AGT TT 3' for an internal positive control.

PCR conditions

Step	**Temperature (^{o}C)**	**Time**	**Note**
1	94	3 min	-

2	94	30 sec	-
3	62	1 min	-
4	72	1 min	repeat steps 2-4 for 35 cycles
5	72	2 min	-
6	10	-	hold

Mice bearing the transgene were used for the experiments. WT littermates of the transgenic mice, negative for the transgene, were used as controls.

Bone marrow chimeras were generated as reported previously (Sapoznikov et al., 2008; Zhang et al., 2007). Donor mice were euthanized with sodium pentobarbital and the femurs were removed and flushed with DMEM medium containing 10% FBS to obtain bone marrow cells, and finally suspended in normal saline. The recipient mice were lethally irradiated using Gammacell irradiator (two doses of 600 rads, 4h apart). Within 2-3 hr after irradiation, 10 million donor bone marrow cells were injected into the tail vein of recipients. The following bone marrow chimera were generated: WT mice (CD45.2) reconstituted with WT bone marrow (CD45.1); WT mice (CD45.1) reconstituted with CD11c-DTRtg bone marrow (CD45.2); CD11c-DTRtg mice (CD45.2) reconstituted with WT bone marrow (CD45.2) and WT mice reconstituted with equal number of WT (CD45.1) and CD11c-DTRtg (CD45.2) bone marrow cells. Mice were maintained in specific pathogen-free conditions. Experimental protocols used in this study were approved by the IACUC of The Penn State Hershey College of Medicine.

2.2.2 Diptheria toxin-mediated dendritic cell ablation

Dendritic cells were depleted in CD11c-DTRtg mice by a single intra-peritoneal injection of 4 ng DTx/gm of body weight (Sigma Aldrich). Ablation of dendritic cells was confirmed by staining for $CD11c^{+}$ $MHCII^{+}$ dendritic cells in spleen and kidney after perfusing the mice through the heart with 20 ml of saline. CD11c-DTRtg littermates negative for the transgene were used as controls. Cisplatin was injected in CD11c-DTRtg mice 24 hr after DTx injection. In WT mice reconstituted with CD11c-DTRtg bone marrow, 4 ng/gm of body weight of DTx was injected twice, 24 hr before and 24 hr after cisplatin injection.

2.2.3 Drug administration and renal function assessment.

Cisplatin (MP Biomedicals) was dissolved in normal saline at a concentration of 1mg/ml and filtered through a 0.2μM filter. To induce ARF, mice were given a single intraperitoneal injection of either vehicle (saline) or cisplatin (20 mg/kg body weight). Blood was collected by tail vein bleeding at different time points after cisplatin injection using heparin-coated microvette tubes (Sarstedt). Blood cells were removed by centrifugation at 1000 g for 5 min and renal function was determined by measuring blood urea nitrogen (VITROS DT60II chemistry slides, Ortho-Clinical Diagnostics, Rochester, NY) and serum creatinine (DZ072B, Diazyme Labs, CA).

2.2.4 Culture of bone marrow-derived dendritic cells and TKPTS cells.

C57BL/6 mice were euthanized using Nembutal® (pentobarbital sodium injection, 100 mg/kg body weight) and bone marrow from femur and tibia was flushed with IMDM medium containing 5% FBS, penicillin (100U/ml) and streptomycin (100µg/ml). Bone marrow cells were centrifuged at 300 g for 5 min and RBCs were lysed using RBC lysis buffer (Sigma). The cells were washed and suspended in IMDM medium containing 10% FBS, penicillin (100IU/ml), streptomycin (100µg/ml), GM-CSF (10ng/ml) and IL-4 (4ng/ml) in petri-dishes (100 X 20 mm). After removing one-half of old medium, fresh medium was added on day 2 and day 5. Non-adherent dendritic cells were harvested from petri-dishes on day 7 and used for the experiment.

TKPTS were grown using IMDM medium containing 10% FBS, penicillin (100IU/ml), streptomycin (100µg/ml). For coculture experiments, bone marrow-derived dendritic cells were added (1.5 X 10^6 cells/well) to the TKPTS cells (approximately 75% confluence) grown in 6 well tissue culture plates (Corning).

2.2.5 Histological examination

Kidneys were fixed in buffered formalin for 24 hr, embedded in paraffin, sectioned (4uM thickness), and stained with periodic acid-Schiff stain (Histology core facility, The Penn State Hershey College of Medicine). The extent of tubular injury in PAS-stained kidney sections was assessed using a semi-quantitative scale by calculating the percentage of tubules in the cortex that displayed tubular dilatation, loss of brush border, desquamation of epithelial cells, cell necrosis and cast formation as follows: 0 = none; 0.5 = <10%; 1=10-25%; 2 = 26-50%; 3 = 51-75%; 4 = >75% (Liu et al., 2006).

Ten 40X fields from each kidney were examined. The scoring was performed blinded to the treatment and strain of the animal.

2.2.6 Immunostaining

Formalin-fixed and paraffin-embedded kidney tissues were sectioned (4µM thickness) onto SuperFrost Plus microscope slides (Fischer HealthCare). Tissue sections were deparaffinized and dehydrated using xylene and decreasing concentrations of ethanol (100%, 95% and 70%) and finally rinsed in tap water. The sections were boiled for 30 min in 10mM sodium citrate buffer to expose antigens which have been masked by the tissue fixation process. After washing with water, endogenous peroxidases were quenched using 3% hydrogen peroxide. An avidin-biotin blocking kit (Vector Laboratories) was used to block endogenous biotin. Immunohistochemistry for neutrophils were performed using rat anti-murine primary antibody (MCA 771 GA, Serotec) followed by biotinylated anti-rat secondary antibody. After washing, the sections were incubated with ABC reagent (Vectastain ABC kit, vector laboratories) followed by diaminobenzidine (DAB reagent, Pierce Laboratories). The sections were washed and counterstained with Harris hematoxylin (Sigma Aldrich) for 1 min. After staining, sections were dehydrated using increasing concentrations of ethanol (70%, 95% and 100%) followed by xylene, and mounted with permount (Fischer Scientific). Ten 40X fields were examined in the cortex of immunostained kidney sections for quantification of neutrophils. For confocal analysis, saline-perfused kidneys were fixed in 2% paraformaldehyde for 2 hr at room temperature immediately after excision followed by

incubation in 30% sucrose overnight at 4^{o}C. Tissue sections stained with 4',6-diamidino-2-phenylindole (DAPI) were imaged using a Leica confocal microscope.

2.2.7 Preparation of single-cell suspensions and flow cytometric analyses.

Mice were anaesthetized using pentobarbital sodium injection (75 mg/kg body weight) and perfused with 20 ml of saline to remove intravascular leukocytes. Spleen and kidneys were minced into fragments of 1mm^3 and digested with 2 mg/ml of collagenase D and 100U/ml of DNase I in DMEM/5% FBS for 40 min at 37^{o}C. After washing the cells with flow cytometry buffer (PBS/0.5% BSA/5mM EDTA/0.1% sodium azide), the digested tissues were passed sequentially through 100µM and 40µM mesh (BD Biosciences). The filtrate containing the renal cell suspension was centrifuged at 300 g for 5 min, and RBCs in the resulting pellet were lysed using RBC lysis buffer (Sigma).

After blocking Fc receptors with rat anti-FcR from 2.4G2 hybridoma supernatant, spleen and renal cells were stained in flow cytometry buffer (PBS/0.5% BSA/5mM-EDTA/10% mouse serum/0.1% sodium azide) using the following FITC, PE, PE-Cy5.5 or APC-labeled antibodies; anti-CD45 (clone 30-F11), CD45.1 (A20), CD11c (HL3), F4/80 (BM8), MHC class I (34-1-2S), MHC class II (AF6-120.1), CD11b (M1/70), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), PDL-1 (MIH5), PDL-2 (122), ICOSL (HK5.3), 7/4 (AbD serotech), Gr-1 (RB6-8C5), Ly-6G (1A8, BioLegend), CD4 (GK1.5), CD8 (53-6.7), B220 (RA3-6B2), NK1.1 (PK136), CD3 (145-2C11) and PDCA-1 (eBio 927). Unless otherwise indicated, the antibodies were obtained from eBioscience or PharMingen. Flow cytometry was performed on a FACSCaliber and analyzed using

CellQuest (BD PharMingen) or WinMDI 2.8 software (http://facs.scripps.edu/software.html).

2.2.8 Statistical analysis

Results were expressed as mean ± SE. All data were analyzed using an unpaired, two-tailed Student t test. A value of $P<0.05$ was considered significant.

2.3 Results

2.3.1 Dendritic cells upregulate maturation markers following cisplatin treatment *in vitro*.

Cisplatin treatment causes apoptosis and necrosis of renal epithelial cells. Necrotic cells are reported to activate immature dendritic cells. Gallucci et al., (1999) showed activation of bone marrow-derived dendritic cells in response to their coculture with necrotic syngenic cells with upregulation of MHC class II, CD40 and CD86 (Gallucci et al., 1999). In order to determine the effect of renal epithelial cell injury and necrosis on dendritic cell activation, we cocultured bone marrow-derived dendritic cells with cisplatin-treated transgenic kidney proximal tubule (TKPTS) cell line and examined the expression of dendritic cell major histocompatability complex and costimulatory molecules by measuring mean fluorescene intensity (MFI) (Fig 2-1). MFI is the average of fluorescence elicited by fluorochrome-conjugated antibodies that are bound to their epitopes on cells. TKPTS cells were developed from renal proximal tubules of transgenic mice expressing the large T antigen of SV40 virus (Ernest et al., 1995).

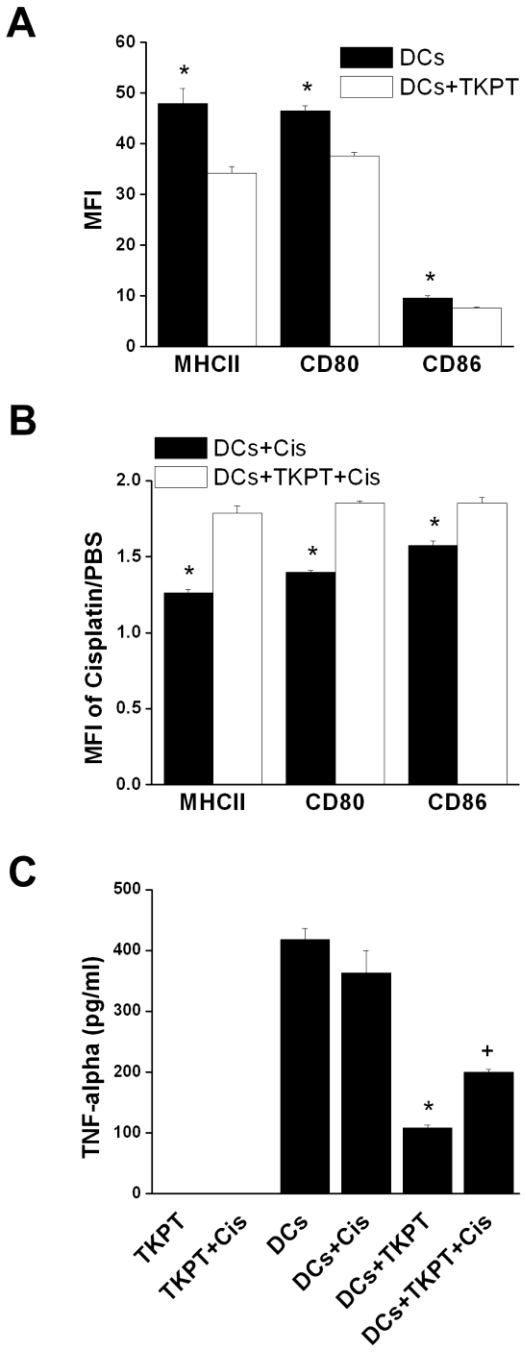


Figure 2-1. Dendritic cell (DC) expression of maturation markers and production of TNFα in response to cisplatin and cisplatin treated TKPT cells. Bone marrow-derived DC were co-cultured with TKPT cells, and treated with 25uM of cisplatin for 24 hr. The cells were stained for CD11c, MHCII, CD80 and CD86, and mean fluorescence intensity (MFI) was determined by flow cytometry (A and B). TNFα production by DCs in response to cisplatin or TKPT cells and cisplatin (C). $*,^{+}$ $P<0.05$ vs. other group (A and B) or groups (C), n = 4/group.

Because the T antigen confers cell immortality *in vitro*, cells isolated from the kidney have been used to derive different cell lines, such as glomerular epithelial, mesengial, and endothelial cell lines. In the presence of healthy TKPTS cells, dendritic cells downregulated their expression of maturation markers and TNFα production (Fig 2-1A), consistent with the findings of Gallucci et al., (1999). In contrast, dendritic cells upregulated MHC class II, CD80 and CD86 in response to cisplatin and cisplatin-treated TKPTS cells (Fig 2-1B). Likewise, dendritic cells produced TNFα in response to cisplatin-treated TKPTS cells. (Fig 2-1C). These results indicate that the cisplatin-treated TKPTS cells stimulate bone marrow-derived dendritic cells under *in vitro* conditions. However, the effect of cisplatin on renal dendritic cells *in vivo* is not known. Therefore, we decided to investigate ARF in the presence and absence of dendritic cells *in vivo*.

2.3.2 Properties of renal dendritic cells.

Flow cytometric analysis of tissue digests, revealed a distinct population of cells expressing high levels of CD11c and MHC class II in both kidney and spleen (Fig. 2-2A). Dendritic cells were the most abundant population of leukocytes in the kidney (46.15% ± 2.5% of all $CD45^+$ cells, n=4). $9.4 \pm 0.6 \times 10^4$ $CD11c^+$ $MHCII^+$ dendritic cells were obtained from each kidney compared with $6.8 \pm 0.3 \times 10^5$ from the spleen of normal C57BL/6 mice (n=12). Comparative analysis of cell surface markers of renal and splenic dendritic cells (Fig. 2-2B) showed similar expression of CD11b, antigen presentation molecules (MHC class I and MHC class II), and costimulatory molecules (CD40, CD80 and CD86). In contrast to splenic dendritic cells, renal dendritic cells were negative for CD4 and CD8 expression. Renal dendritic cells also differed from the bulk of splenic

conventional dendritic cells by their prominent expression of F4/80, a surface marker of monocytes and macrophages (Fig. 2-2B and 2C).

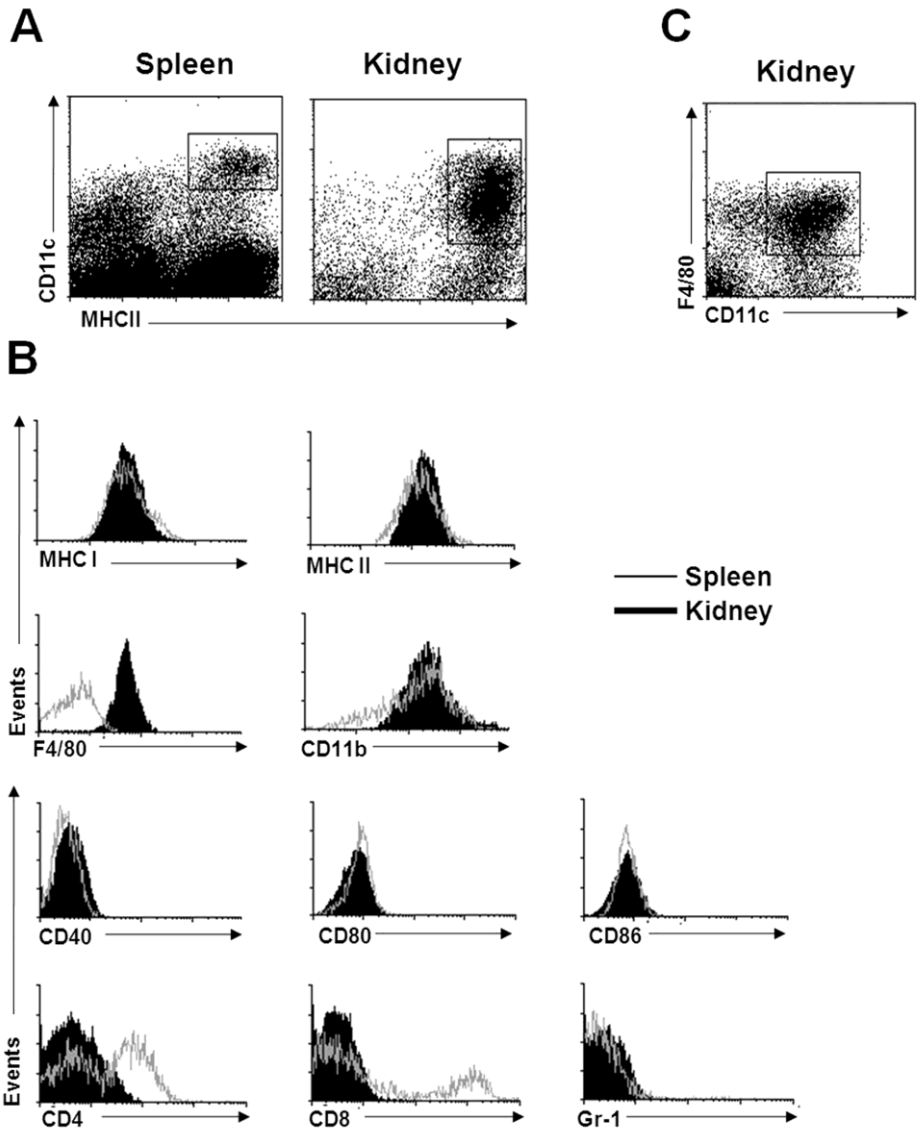


Figure 2-2. Characterization of mouse renal dendritic cells. A. Flow cytometry of spleen and kidney leukocytes gated on $CD45^+$ for the identification of $CD11c^+$ and $MHCII^+$ dendritic cells. B. Comparison of cell surface markers of renal and splenic dendritic cells gated as $CD11c^+$ $MHCII^+$ cells. C. Expression of CD11c and F4/80 by kidney dendritic cells gated as $CD45^+$ cells.

2.3.3 Dendritic cell localization in kidney

Although dendritic cells form an abundant population of renal leukocytes, their anatomical localization in kidney is largely unknown. Here to study renal dendritic cell distribution, we used transgenic mice (CD11c-DTRtg) which express GFP and the simian diphtheria toxin receptor (DTR) driven by the CD11c promoter (Fig. 2-3A, C and D). Confocal imaging revealed GFP-positive cells throughout the tubulointerstitium but not in glomeruli (Fig. 2-3D and E). GFP-positive cells had a stellate shape typical of dendritic cells.

2.3.4 Inducible ablation of kidney dendritic cells *in vivo*

To selectively deplete dendritic cells *in vivo*, we employed CD11c-DTRtg mice in which expression of the DTR/GFP fusion protein in dendritic cells renders the normally resistant murine cells sensitive to DTx induced cell death without affecting $CD11c^-$ $F4/80^+$ macrophages (Jung et al., 2002; Sapoznikov and Jung, 2008). We first examined the efficiency of depletion of renal and splenic dendritic cells in response to DTx treatment. Similar to the recent study by Scholz et al (Scholz et al., 2008), DTx administration to CD11c-DTRtg mice caused a marked transient depletion of $CD11c^+$ $MHCII^+$ cells in both kidney and spleen (Fig. 2-4), but not $CD11c^-$ $F4/80^+$ macrophages in kidney (Fig. 2-5). These findings indicate the specificity of dendritic cell depletion in CD11c-DTRtg mice. Ablation of dendritic cells was maximal at 24 hr in the spleen and at 48 hr in the kidney (Fig. 2-4A-D). Dendritic cell depletion in the kidney was more prolonged than in spleen.

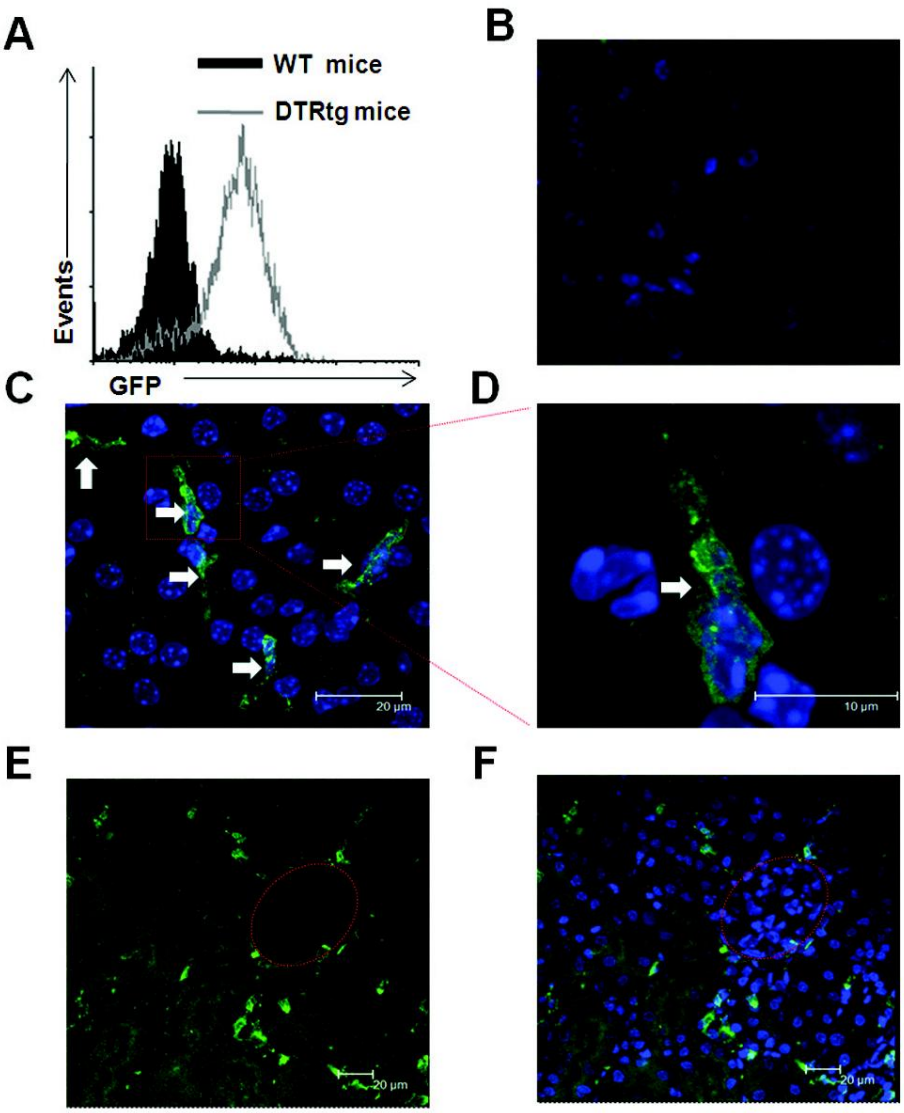


Figure 2-3. Dendritic cells in the kidney. A. Flow cytometry of renal $CD11c^{+}$ $MHCII^{+}$ dendritic cells from WT and CD11c-DTRtg mice for GFP expression. Confocal microscopy of kidney sections of WT (B) and CD11c-DTRtg mice (C, D, E, F) for GFP-positive dendritic cell localization. D. Magnification of boxed area in panel (C) showing GFP-positive cells between renal tubules. E, F. Absence of GFP-positive cells (dendritic cells) in glomerulus. Arrows point to GFP-positive dendritic cells. Nuclei are stained with DAPI.

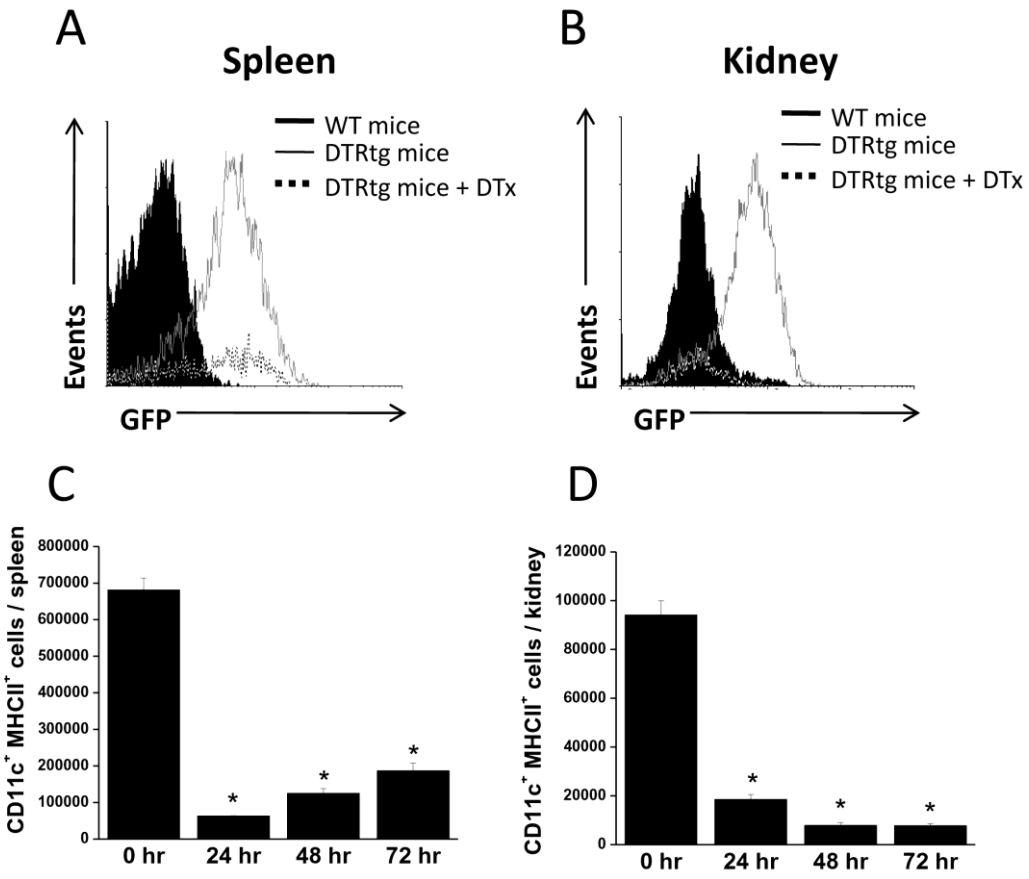


Figure 2-4. Dendritic cell depletion in CD11c-DTRtg mice after DTx treatment. Histogram of GFP expression by splenic (A) and renal (B) $CD11c^+$ $MHCII^+$ dendritic cells in WT and CD11c-DTRtg mice and their depletion at 24 hrs after DTx treatment. Flow cytometric quantification of spleen (C) and kidney (D) $CD11c^+$ $MHCII^+$ dendritic cells obtained from CD11c-DTRtg transgenic mice at different time points after DTx injection. *P<0.003, n = 4-12/group.

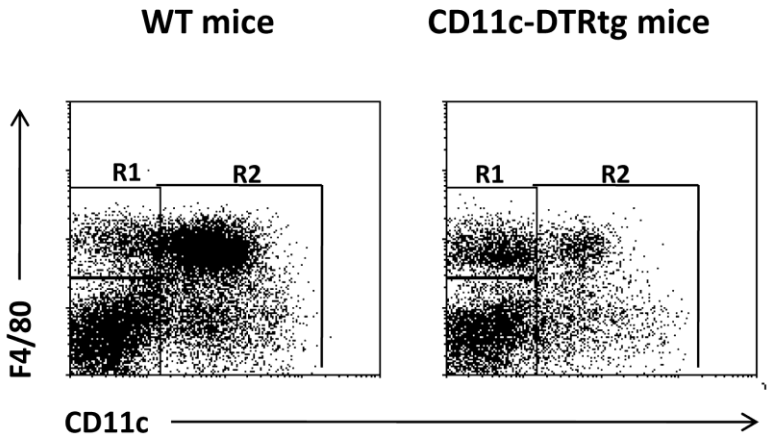


Figure 2-5. Renal macrophages and dendritic cells subsequent to DTx treatment. WT and CD11c-DTRtg mice treated with DTx were sacrificed at 24 hrs and single cell kidney suspensions were analyzed by flow cytometry gating on $CD45^+$ leukocytes for $F4/80^+$ macrophages (R1) and $CD11c^+$ $F4/80^{+/-}$ dendritic cells (R2). In CD11c-DTRtg mice, DTx reduced the number of dendritic cells (R2), but not macrophages (R1) relative to WT mice.

2.3.5 Dendritic cell depletion exacerbates cisplatin nephrotoxicity

Although *ex vivo* studies in humans indicate a likely contribution of dendritic cells in renal inflammation, renal dendritic cell function within their physiological context is largely unknown (Loverre et al., 2007, Segerer et al., 2008, Woltman et al., 2007). Here we used CD11c-DTRtg mice to ablate dendritic cells and investigate the effect of dendritic cell depletion on cisplatin nephrotoxicity. DTx was injected 24 hr before cisplatin injection to deplete dendritic cells. Treatment of either CD11c-DTRtg mice or their WT littermates with DTx alone did not result in renal dysfunction as assessed by levels of BUN and serum creatinine (Fig. 2-6A and B).

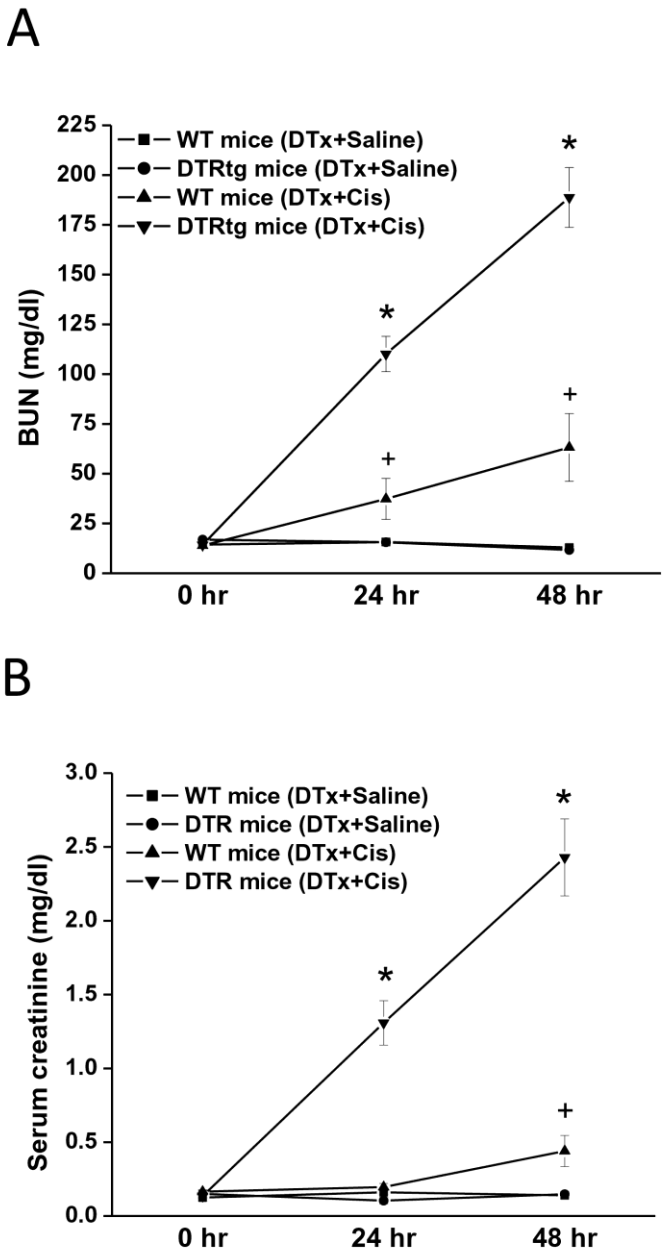


Figure 2-6. Dendritic cell depletion increases susceptibility to cisplatin nephrotoxicity. WT and CD11c-DTRtg mice were treated with either DTx or DTx and cisplatin. Blood collected at different time points with respect to cisplatin injection was analyzed for BUN (A) and serum creatinine (B) as a measure of renal function. **P*<0.00001 vs. all other groups, $^{+}$ P<0.05 vs. all other groups. n = 9-17/group.

WT mice injected with DTx followed by cisplatin showed minimal elevations in levels of BUN and serum creatinine at 24 hr which increased by 48 hr. This time course of cisplatin nephrotoxicity is the same as observed in our prior studies (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003; Ramesh and Reeves, 2005). In comparison to WT mice, DTx and cisplatin-injected CD11c-DTRtg mice showed both earlier and more dramatic increases in serum creatinine and BUN. The survival at 72 hr was only 20% in the CD11c-DTRtg mice and 100% in WT mice treated with DTx and cisplatin (n=5).

2.3.6 Renal morphology in cisplatin nephrotoxicity

Renal dysfunction in reponse to ischemic or toxic insult is associated with renal tubular damage (Zhang et al., 2007, Zhang et. al., 2008). In order to determine the extent of kidney injury in dendritic cell-depleted and non-depleted mice treated with cisplatin, kidney sections obtained at 24 hr and 48 hr after cisplatin treatment were stained with PAS stain and examined for tubular injury. In our studies, the worsening renal dysfunction in response to cisplatin treatment in dendritic cell-depleted mice was associated with an increase in tubular necrosis. Kidneys from DTx-treated WT mice and CD11c-DTRtg mice did not exhibit any visible tubular damage (Fig. 2-7A, B and E). WT mice treated with DTx followed by cisplatin displayed moderate tubular injury characterized by dilation of tubules, loss of brush border and sloughing of epithelial cells (Fig. 2-7C and E). CD11c-DTRtg mice treated with DTx followed by cisplatin showed more extensive renal tubular damage compared with mice not depleted of dendritic cells (Fig. 2-7D and E).

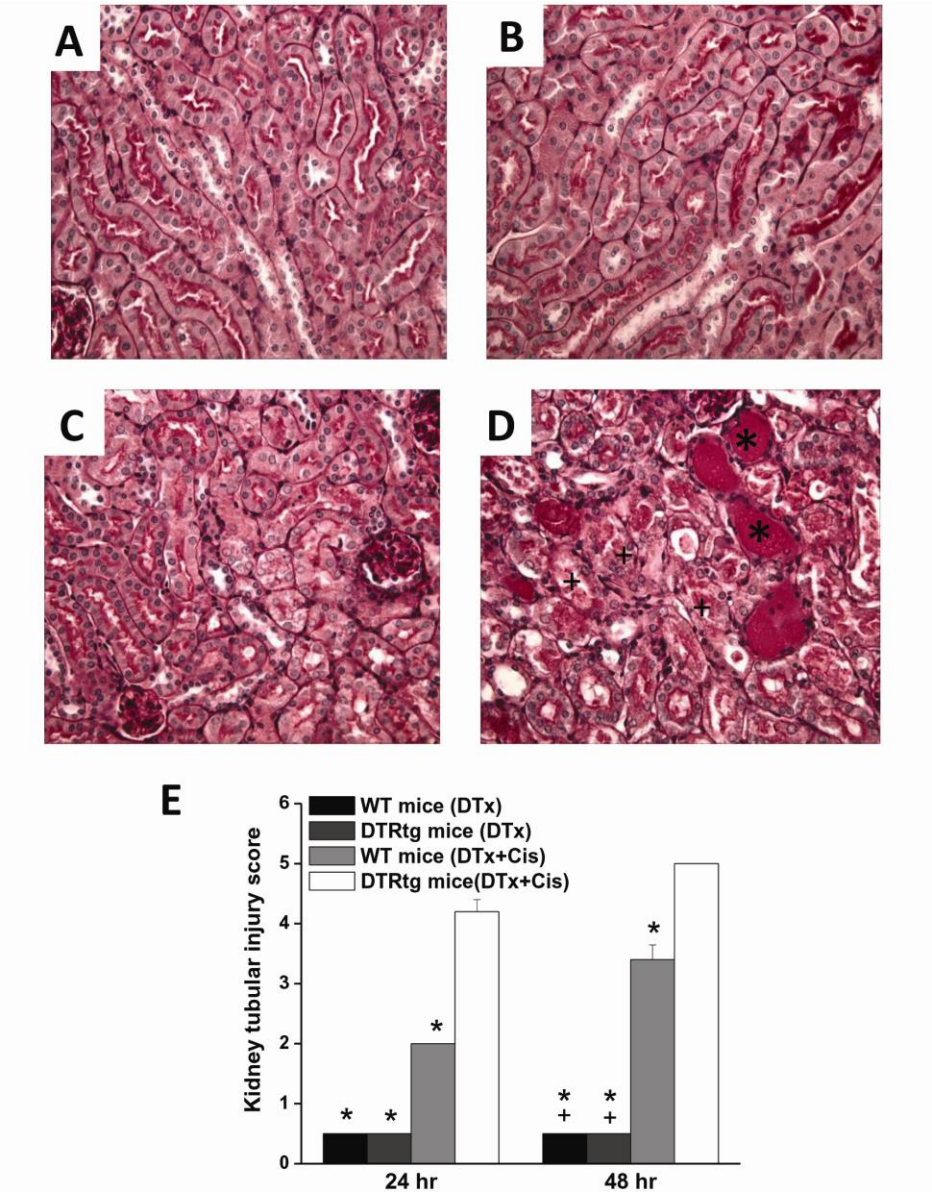


Figure 2-7. Effect of dendritic cell ablation on renal morphology in cisplatin nephrotoxicity. WT mice (A and C) and CD11c-DTRtg mice (B and D) were treated with DTx (A and B) or DTx and cisplatin (C and D). Cisplatin was injected 24 hr after DTx injection (C and D). Kidneys were harvested at 48 hr after cisplatin injection and stained with PAS. E. Tubular injury scoring for renal tubular cell necrosis (+) and cast formation (*) (A-D) in the renal cortex of PAS-stained kidney sections harvested at 24 hr and 48 hr after cisplatin injection. *P<0.003 vs. CD11c-DTRtg mice (DTx + Cis), ^{+}P<0.001 vs. WT mice (DTx + Cis), n = 4-5/group.

2.3.7 Renal leukocyte infiltration in cisplatin nephrotoxicity

Tissue injury is associated with inflammation and infiltration of leukocytes (Ramesh and Reeves, 2002). Neutrophils are mobilized to the sites of tissue injury under the influence of chemokines and represent the hallmark of inflammation and tissue damage. Neutrophils are also reported to mediate sterile inflammation (Chen et al., 2007) and impair tissue healing (Dovi et al., 2003). The extent of neutrophil infiltration into kidney coincides with the magnitude of kidney injury (Ramesh et al., 2007; Zhang et al., 2008). Since dendritic cell ablation exacerbated renal dysfunction as early as 24 hr after cisplatin injection, we investigated whether dendritic cell depletion altered the influx of different leukocyte populations, including neutrophils, in the kidney. DTx-treated CD11c-DTRtg mice showed a significant reduction in total kidney leukocytes compared with DTx-treated WT mice (Fig. 2-8). This decrease in total leukocyte population is due to depletion of dendritic cells, which constitute a major leukocyte population in the kidney. Cisplatin treatment resulted in a striking increase in leukocyte infiltration in dendritic cell-depleted CD11c-DTRtg mice, with neutrophils constituting the major infiltrating leukocyte population. WT mice treated with cisplatin showed only a moderate increase in neutrophils. The number of T cells, B cells, NK cells and plasmacytoid dendritic cells infiltrating the kidney were not dramatically altered by either DTx or cisplatin in either WT or CD11c-DTRtg mice. Immunohistochemistry for neutrophils confirmed the results obtained by flow cytometry (Fig. 2-9).

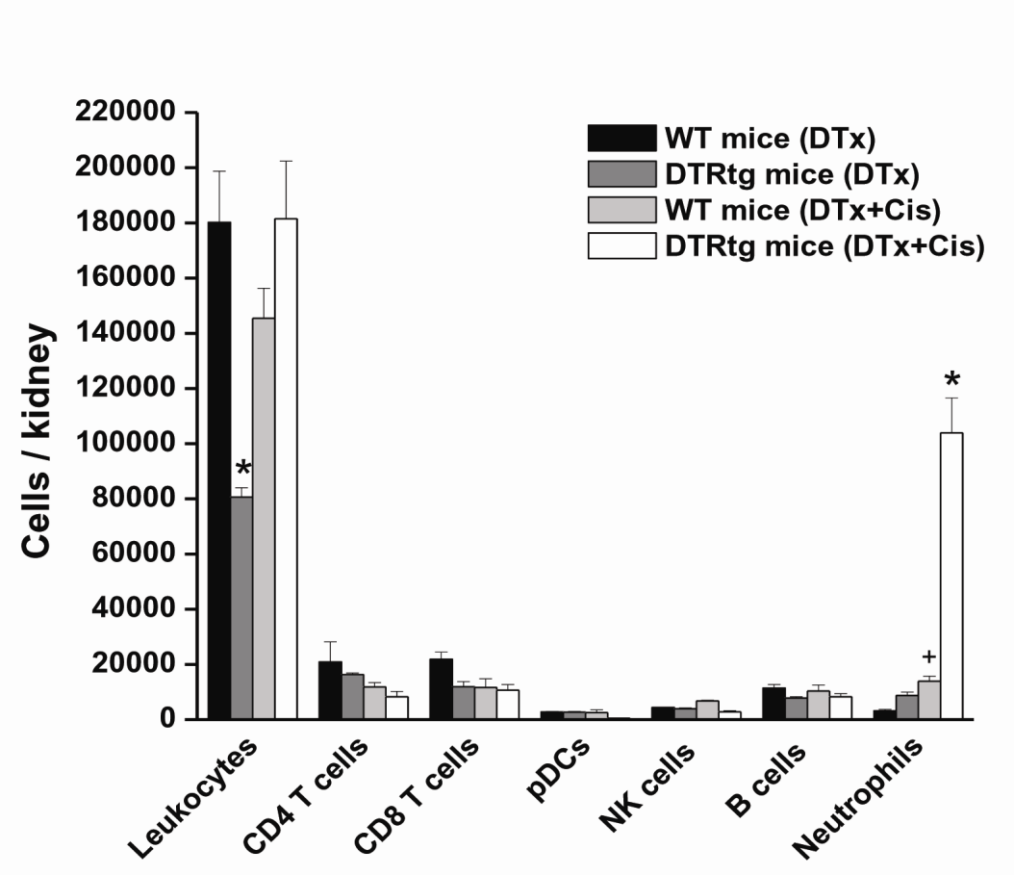


Figure 2-8. Effect of dendritic cell depletion on renal leukocyte infiltration in cisplatin nephrotoxicity. WT and CD11c-DTRtg mice treated with DTx or DTx and cisplatin were sacrificed at 24 hr and single cell kidney suspensions were analyzed by flow cytometry gating on $CD45^+$ leukocytes for total numbers of $CD4^+$ and $CD8^+$ T cells, $CD11b^-$ $PDCA\text{-}1^+$ plasmacytoid dendritic cells (pDCs), $CD3^-$ $NK1.1^+$ NK cells, $CD11c^-$ $B220^+$ B cells and $F4/80^-$ $Ly\text{-}6G^+$ neutrophils per kidney. **P*<0.05 vs. all other groups, ^+P<0.05 vs. WT mice (DTx), n = 4-5/group.

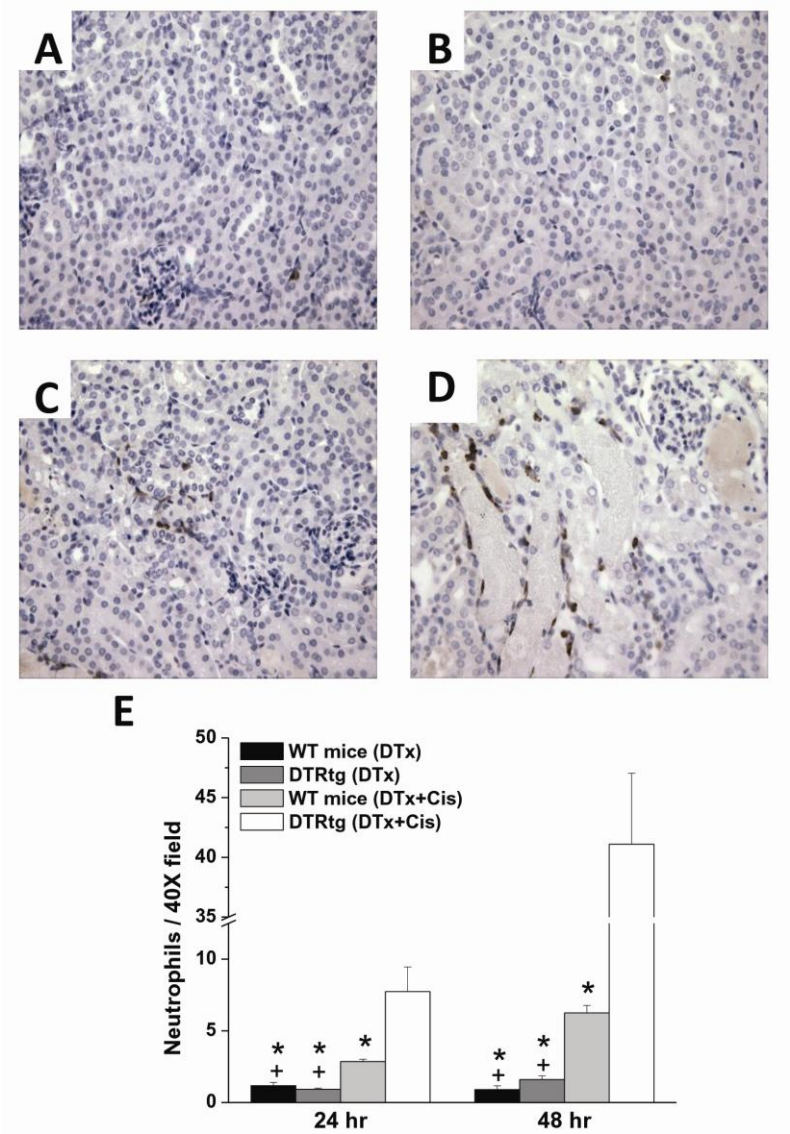


Figure 2-9. Effect of dendritic cell depletion on renal neutrophil infiltration and localization in cisplatin nephrotoxicity. WT mice (A and C) and CD11c-DTRtg mice (B and D) were treated with DTx (A and B) or DTx and cisplatin (C and D). Cisplatin was injected 24 hr after DTx treatment (C and D). Kidneys harvested 48 hr after cisplatin injection were stained for neutrophils. E. Enumeration of neutrophils in renal cortex of kidney sections harvested at 24 hr and 48 hr after cisplatin treatment. **P*<0.001 vs. CD11c-DTRtg mice (DTx + Cis), $^{+}$*P*<0.05 vs. WT mice (DTx + Cis), n = 4-5/group.

2.3.8 Renal dendritic cell role at different stages in cisplatin nephrtoxicity

Our studies indicate that dendritic cell depletion before cisplatin treatment aggravates cisplatin nephrotoxicity. In order to determine the impact of dendritic cell depletion at later intervals of cisplatin nephrotoxicity, DTx was injected to WT and

DTRtg mice either 24 hr or 1 hr before or 24 hr after cisplatin injection (Fig. 2-10). Injection of CD11c-DTRtg mice with DTx either 24 hr or 1 hr before cisplatin exacerbated renal injury to a similar extent whereas injection of CD11c-DTRtg mice with DTx 24 hr after cisplatin injection had no impact on the severity of renal dysfunction (Fig. 2-10A-D). These results indicate that dendritic cell depletion at late stages does not influence the ongoing kidney injury in cisplatin nephrotoxicity.

2.3.9 Cisplatin nephrotoxicity is attenuated by hematopoietic CD11c-expressing cells

High or repeated doses of DTx are lethal to CD11c-DTRtg mice (Scholz et al., 2008; Zammit et al., 2005). It has been postulated that this toxicity is due to the CD11c-driven expression of DTR in some vital non-hematopoietic cells (Zammit et al., 2005). To exclude the involvement of DTR expressed on non-hematopoietic cells in the previous results, we created chimeric mice in which CD11c-DTR/GFP bone marrow was transplanted into irradiated WT recipients, thereby restricting DTR expression to the hematopoietic compartment (Fig 2-11B and 2-13A). Administration of high doses or repeated doses of DTx to these mice is not lethal (Bennett and Clausen, 2007; Zammit et al., 2005). We also made WT to CD11c-DTRtg chimeras to determine the efficiency of renal dendritic cell depletion in response to the irradiation protocol used to make the chimeric mice. Injection of DTx to WT to CD11c-DTRtg chimeric mice caused almost complete depletion of resident GFP-positive dendritic cells in kidney by 8 weeks after bone marrow transplantation (Fig. 2-11A). In contrast, WT mice which received CD11c-DTRtg bone marrow showed numerous GFP-positive renal dendritic cells in the kidney (Fig. 2-11B). These results indicate that the resident renal dendritic cells in bone marrow

chimeras are almost exclusively of donor origin. Finally, as expected, administration of DTx caused a marked depletion of GFP-positive dendritic cells in the CD11c-DTRtg to WT chimeric mice (Fig. 2-11C).

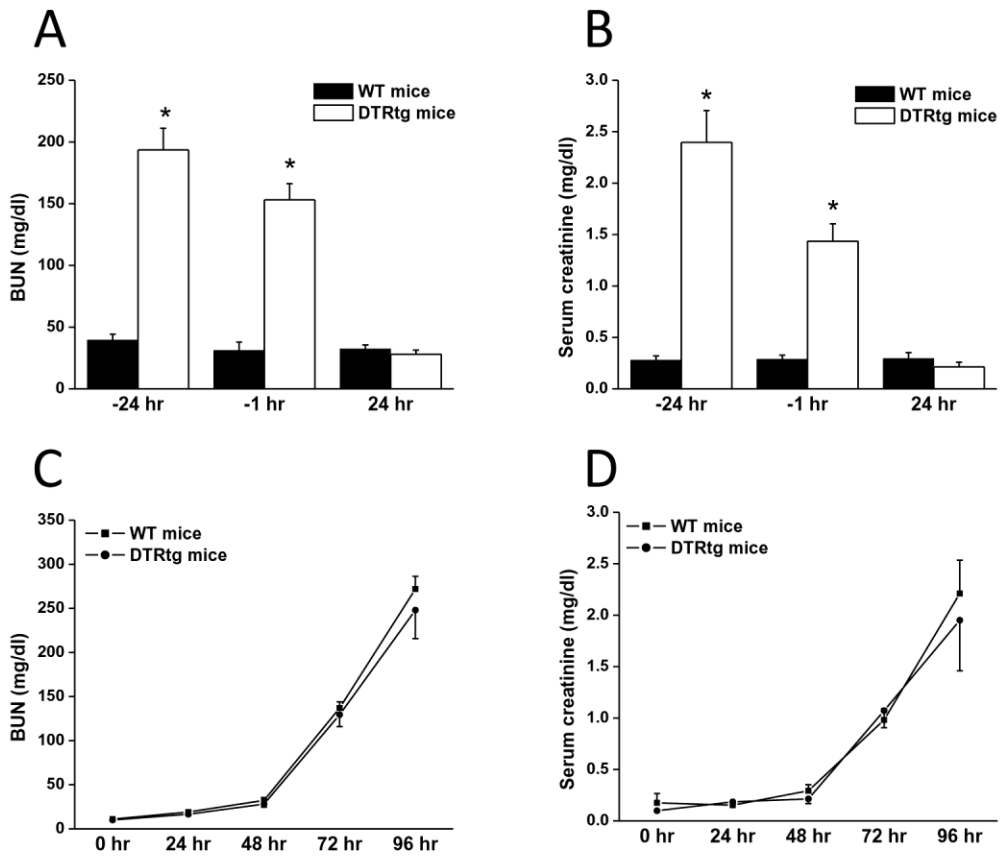


Figure 2-10. Kinetic analysis of renal dendritic cell role in cisplatin nephrotoxicity. WT and CD11c-DTRtg mice were treated with DTx at -24 hr, -1 hr or 24 hr with respect to cisplatin injection. Blood collected at 48 hr after cisplatin injection was analyzed for BUN (A) and serum creatinine (B). WT and CD11c-DTRtg mice were injected with cisplatin followed by DTx at 24 hr (C and D). Blood collected at different time points with respect to cisplatin injection was measured for BUN (C) and serum creatinine (D). **P*<0.001 vs. WT mice, n = 7-10/group.

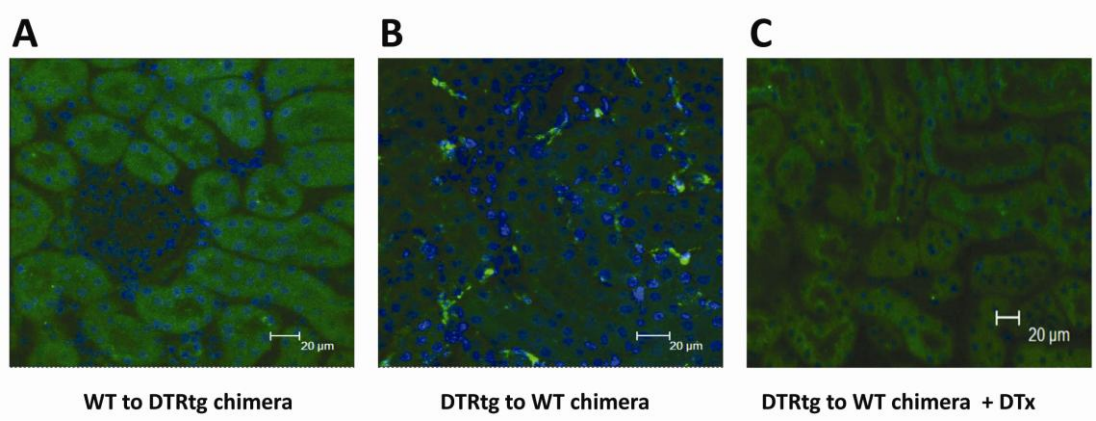


Figure 2-11. Renal dendritic cell depletion after irradiation, replenishment by donor bone marrow-derived dendritic cells and ablation of dendritic cells to DTx treatment. Lethally-irradiated CD11c-DTRtg mice (A) or WT mice (B) were injected with bone marrow of WT mice (A) or CD11c-DTRtg mice (B). After 8 weeks, confocal microscopy was performed on renal sections to determine GFP-positive renal dendritic cells. C. Confocal microscopy of renal sections of CD11c-DTRtg to WT chimera mice obtained 24 hr after DTx injection to determine efficiency of depletion of GFP-positive renal dendritic cells.

Having established that the kidney dendritic cells in CD11c-DTRtg to WT chimeric mice are of donor (CD11c-DTRtg) origin, DTx and cisplatin were administered to CD11c-DTRtg to WT chimeric mice. To ensure maximal depletion of dendritic cells, the mice received DTx 24 hr before and 24 hr after cisplatin injection (Bennett and Clausen, 2007; Sapoznikov and Jung, 2008; Sapoznikov et al., 2008; Zammit et al., 2005). Consistent with our earlier observation in CD11c-DTRtg mice, the CD11c-DTR to WT chimeric mice depleted of dendritic cells showed more severe renal dysfunction as measured by BUN (Fig. 2-12A) and serum creatinine (Fig. 2-12B) than non-depleted chimeric mice treated with cisplatin alone. Likewise, CD11c-DTRtg to WT chimeric mice treated with DTx and cisplatin exhibited increased mortality compared to CD11c-DTRtg to WT chimeric mice which received cisplatin alone (Fig. 2-12C). Treatment of CD11c-DTRtg to WT chimeric mice with DTx alone did not produce any mortality.

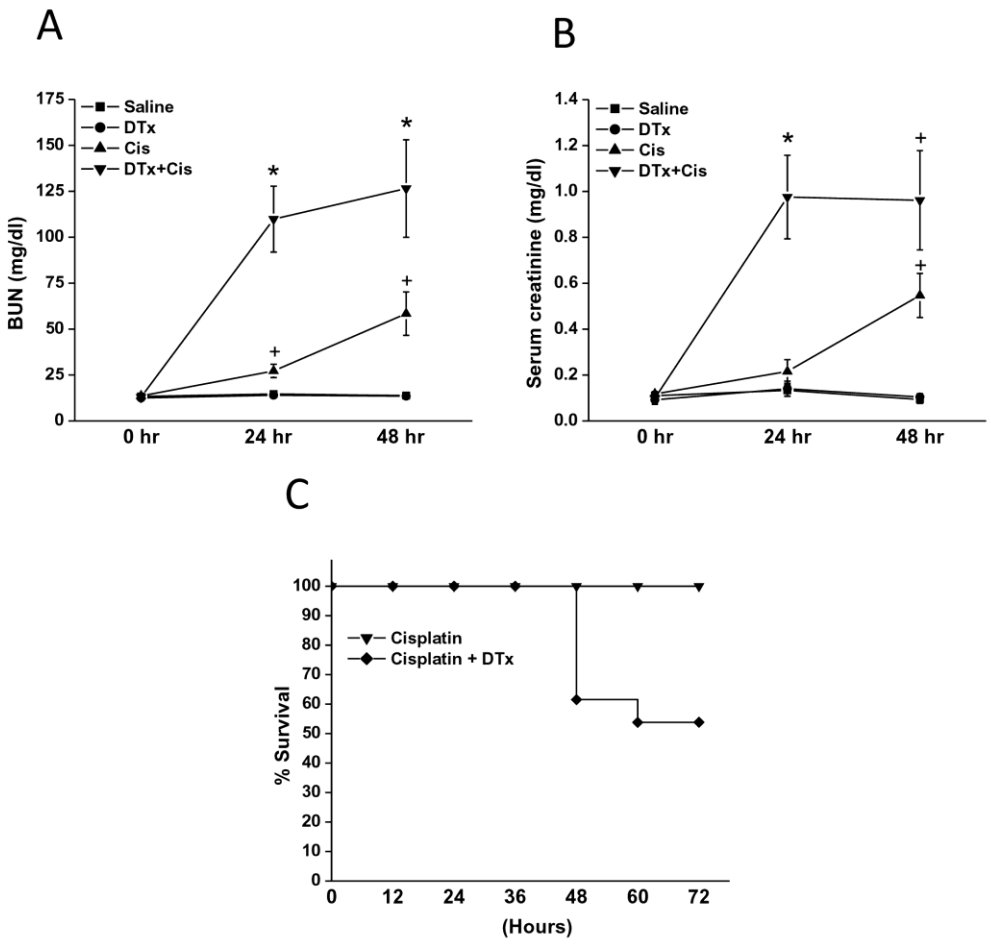


Figure 12-12. Dendritic cell-mediated protection is independent of DTx effect on non-hematopoietic cells. CD11c-DTRtg to WT chimera mice were injected with saline, DTx, cisplatin or DTx and cisplatin. Blood collected at different time points with respect to cisplatin injection was analyzed for BUN (A) and serum creatinine (B). (C) Survival rate was determined in CD11c-DTRtg to WT chimeric mice following the administration of cisplatin or DTx and cisplatin, n=10-13/group. $^{+}P<0.01$ vs. all other groups, * $P<0.05$ vs. all other groups, n = 8-13/group.

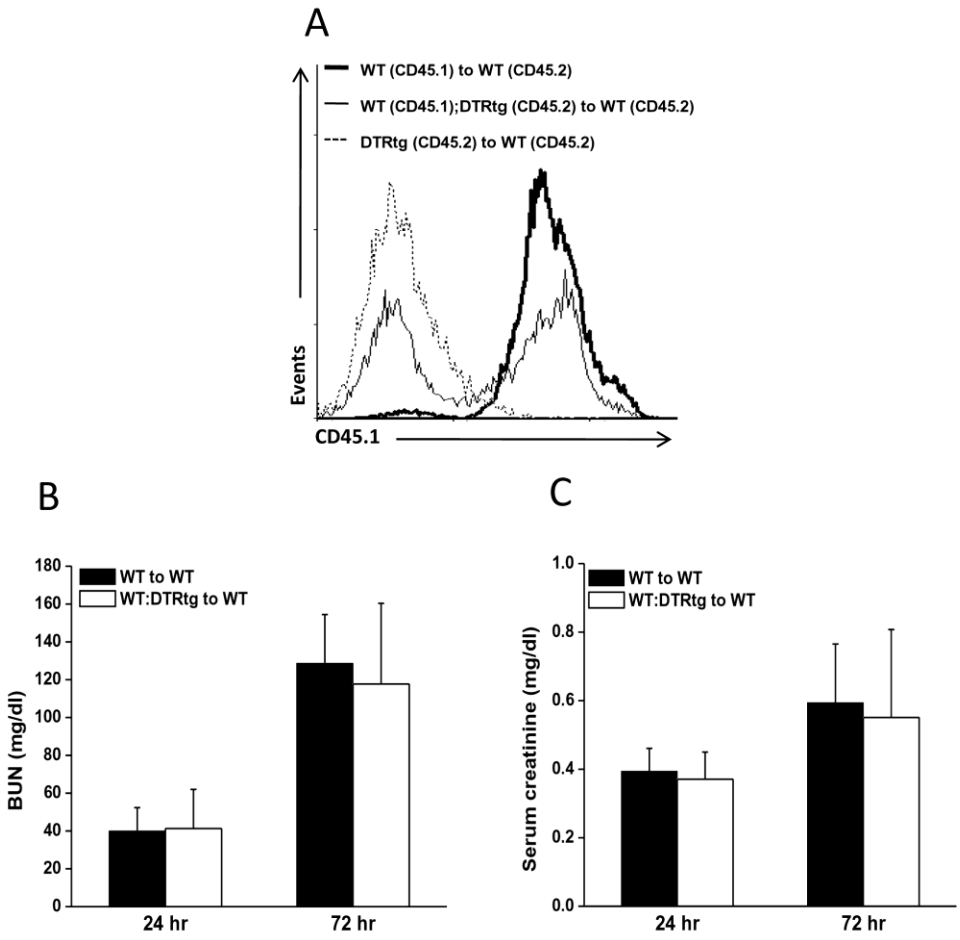


Figure 2-13. Effect of dead cells on dendritic cell-mediated protection in cisplatin nephrotoxicity. A. Flow cytometry of blood leukocytes obtained from WT(CD45.1) to WT (CD45.2), WT(CD45.1):DTRtg(CD45.2) to WT(CD45.2) and DTRtg(CD45.2) to WT(CD45.2) chimera to demonstrate the efficiency of donor bone marrow replenishment and the mixed chimerism of the DTRtg(CD45.2):WT(CD45.1) to WT(CD45.2) mice. Blood collected from WT(CD45.1) to WT (CD45.2) and WT(CD45.1):DTRtg(CD45.2) to WT(CD45.2) chimera mice injected with DTx and cisplatin at different time points with respect to cisplatin injection was measured for BUN (B) and serum creatinine (C). n = 6-7/group.

2.3.10 Exacerbation of kidney injury by DTx is independent of dead dendritic cells

Dendritic cells are abundant throughout the tubulointerstitium and administration of DTx to CD11c-DTRtg mice or DTR to WT chimeric mice results in death of dendritic cells. The exacerbation of cisplatin nephrotoxicity by DTx, then, could be the result of these dead dendritic cells, perhaps serving as a danger signal to the immune system,

rather than the absence of dendritic cells. To address this possibility, we produced mixed bone marrow chimeric mice in which 50% of the dendritic cells are WT and 50% express DTR and are susceptible to DTx-induced cell death (Fig. 2-13A). Treatment of these mice with DTx produces dendritic cell death but does not fully deplete dendritic cells. DTx was injected into these mixed chimeric mice 24 hr before and 24 hr after cisplatin treatment. DTx treatment had no impact on the severity of cisplatin-induced renal dysfunction in these mixed chimeras as determined by levels of BUN (Fig. 2-13B) and serum creatinine (Fig. 2-13C). These results indicate that dead or dying dendritic cells, per se, do not account for the worsening of cisplatin nephrotoxicity in DTx-treated mice.

2.3.11 Inflammatory dendritic cell-recruitment in cisplatin nephrotoxicity

In nephrotoxic nephritis, $CD11b^{hi}$ $Gr\text{-}1^{hi}$ $CD11c^{+}$-expressing monocyte-derived proinflammatory dendritic cells are recruited to the kidney (Heymann et al., 2009). We quantified $CD11b^{hi}$ $Gr\text{-}1^{hi}$ $CD11c^{+}$ dendritic cells in the kidney after cisplatin treatment (Fig. 2-14A and C). Cisplatin-injected WT mice showed a very minimal infiltration of $CD11b^{hi}$ $Gr\text{-}1^{+}$ $CD11c^{+}$ inflammatory dendritic cells at 48 hr and 72 hr compared to saline-treated mice. However, kidneys of cisplatin treated mice showed a time-dependent increase in the number of $CD11b^{hi}$ $Gr\text{-}1^{hi}$ $CD11c^{-}$ cells. Gr-1 is a surface marker detected by monoclonal antibody RB6-8C5 that binds to both Ly6C and Ly6G (Fleming et al., 1993; Serbina and Pamer, 2006). By morphology, neutrophils are $Ly6G^{hi}$ $7/4^{hi}$ and monocytes are $Ly6G^{-}$ $7/4^{hi}$, where 7/4 detects a polymorphic 40KDa antigen expressed on monocytes and neutrophils (Tsou et al., 2007). To characterize these

infiltrating leukocytes further, cells were stained for neutrophil and monocyte markers (Fig. 2-14 B and D). We observed a time-dependent increase in neutrophils.

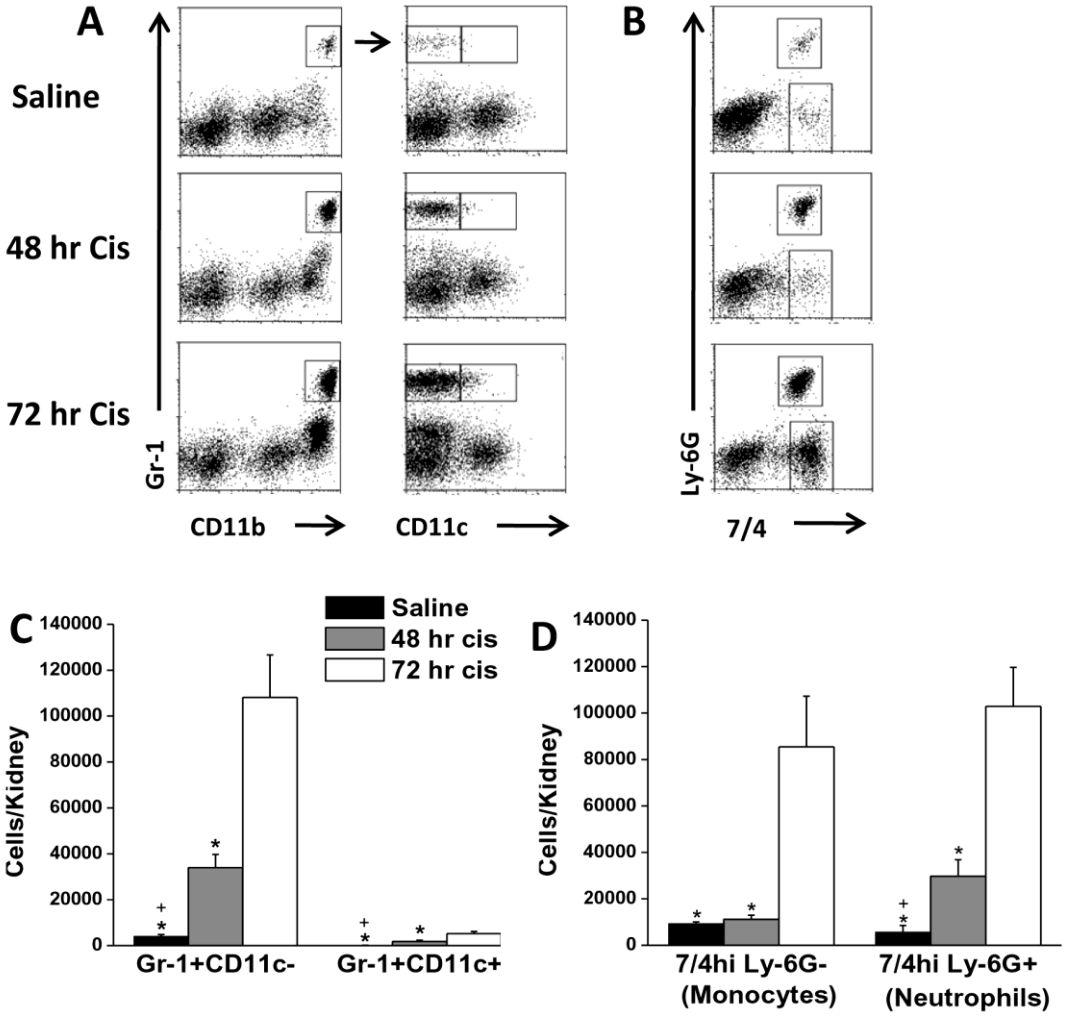


Figure 2-14. Renal infiltration of inflammatory dendritic cells, neutrophils and monocytes in cisplatin nephrotoxicity. WT mice injected with saline or cisplatin were sacrificed at 48 hr or 72 hr and single cell renal suspensions were analyzed by flow cytometry gating on $CD45^+$ leukocytes for (A) the expression of Gr-1 versus CD11b and CD11c and (B) Ly-6G versus 7/4. (C) Absolute number of $Gr\text{-}1^+$ $CD11b^+$ $CD11c^-$ leukocytes and $Gr\text{-}1^+$ $CD11b^+$ $CD11c^+$ inflammatory dendritic cells. (D). Absolute number of $7/4^{hi}$ $Ly\text{-}6G^-$ monocytes and $7/4^{hi}$ $Ly\text{-}6G^+$ neutrophils. $^*P<0.05$ vs. 72 hrs cisplatin, $^+P<0.05$ vs. 48 hrs and 72 hrs cisplatin, n=3-4/group.

Likewise, the number of $Ly6G^{-}$ $7/4^{hi}$ monocytes (Tsou et al., 2007) increased at 72 hr in cisplatin-treated mice. Thus, neutrophils and monocytes, rather than inflammatory dendritic cells, are increased in cisplatin nephrotoxicity.

2.3.12 Activation state of renal dendritic cells in cisplatin nephrotoxicity

Dendritic cell maturation is an important control point in the initiation of inflammation and immunity. Maturation of dendritic cells is characterized by a decrease in phagocytic activity and upregulation of antigen presentation and costimulatory molecules (Banchereau et al., 2000; Steinman et al., 2003a). In addition, dendritic cells elicit regulatory functions through a variety of cell membrane-associated and secreted factors including expression of ICOSL (Akbari et al., 2001; Akbari et al., 2002; Laouar et al., 2008a). To elucidate the mechanisms underlying the dendritic cell-mediated protection against cisplatin nephrotoxicity, renal dendritic cells from cisplatin treated mice were examined for the changes in the expression of MHC class I, MHC class II, CD40, CD80 and CD86 (Fig. 2-15A), the maturation markers that are required for the induction of inflammatory response, and ICOSL (Fig. 2-15B and C), which can induce IL-10 production by T cells (Akbari et al., 2002). Kidney dendritic cells from WT mice treated with saline or cisplatin showed similar expression of MHC class I, CD40, CD80 and CD86. A slight decrease in MHC class II expression was noticed in kidney dendritic cells obtained from cisplatin treated mice. These results indicate that renal dendritic cells instead of maturing in response to products of tissue injury (Dong et al., 2007), maintained their steady-state expression of antigen presentation and co-stimulatory molecules in cisplatin-treated mice, at least when examined 24 hr after cisplatin

treatment. The renal dendritic cells from WT mice treated with saline showed high expression of ICOSL (Fig. 2-15B and C), which increased significantly in response to cisplatin treatment, indicating activation of dendritic cell regulatory functions.

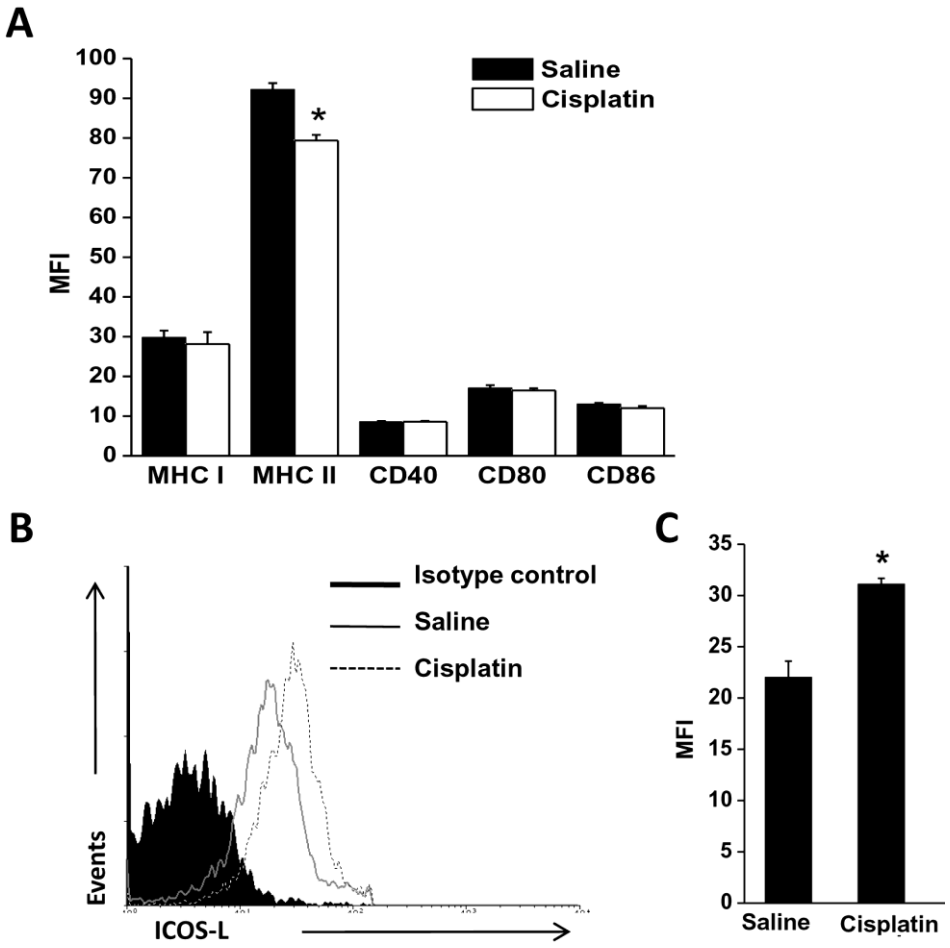


Figure 2-15. Renal dendritic cell expression of surface markers in response to cisplatin treatment. WT mice treated with saline or cisplatin were sacrificed at 24 hr and renal dendritic cells ($CD45^+$ $CD11c^+$ $NK1.1^-$ $PDCA^-$) were analyzed for the expression of MHCI, MHCII, CD40, CD80 and CD86 (A) and ICOS-L (B and C) by flow cytometry. **P*<0.02 vs. saline, n=3-5/group.

2.4 Discussion

A pathogenic role for inflammation in acute kidney injury (AKI) is well-established. Compelling evidence indicates that renal dysfunction in different forms of AKI is the outcome of secretion of immune mediators (Furuichi et al., 2003; Kielar et al., 2005; Melnikov et al., 2001; Miura et al., 2001; Ramesh and Reeves, 2002) and the activation of renal resident and recruited leukocytes (Ascon et al., 2006; Dong et al., 2007; Li et al., 2007; Liu et al., 2006). However, recent studies also indicate that certain cytokines (Deng et al., 2001; Kitching et al., 2002; Kluth et al., 2001; Yin et al., 2002) and immune cells (Kanamaru et al., 2006; Mahajan et al., 2006; Scholz et al., 2008) may limit kidney injury by reducing the immune-mediated inflammatory response (Lee et al., 2008). Dendritic cells constitute an abundant population of leukocytes in the normal kidney. During renal injury, dendritic cells are thought to contribute to renal inflammation and exacerbate kidney injury (Dong et al., 2007; Fiore et al., 2008; Segerer et al., 2008). These studies were based on measures of dendritic cell infiltration and cytokine production but did not determine the functional relevance of dendritic cells within their physiological context *in vivo*. Contrary to above findings, the present work used a conditional dendritic cell ablation mice model to demonstrate that resident renal dendritic cells mediate protection in a nephrotoxic model of AKI.

Dendritic cells were abundant in the kidney, accounting for almost one-half of all renal leukocytes. These $CD11c^{+}$ cells expressed high levels of MHC class II, another feature of dendritic cells, and had stellate morphology typical of dendritic cells. Dendritic

cells in kidney are heterogenous with the majority of renal dendritic cells expressing F4/80, a marker of macrophages. Expression of F4/80 on dendritic cell subsets has also been reported in many other tissues (Belz et al., 2004; Iijima et al., 2007; Sapoznikov et al., 2008). It is possible that $CD11c^+$ $F4/80^-$ dendritic cells differentiate into $CD11c^+$ $F4/80^+$ dendritic cells in kidney. This can be determined by injecting WT mice (CD45.1) subjected to sublethal irradiation with bone marrow cells obtained from congenic mice (CD45.2) and investigating the course of reconstitution of renal dendritic cells ($F4/80^+$ or $F4/80^-$) from donor derived bone marrow cells at different time intervals by flow cytometry. In this experiment, the appearance of $F4/80^+$ and $F4/80^-$ populations of renal dendritic cells at the same time will indicate that these two populations arise independently from each other from their precursors. However, if the increase in $F4/80^-$ renal dendritic cells preceeeds the occurrence of $F4/80^+$ renal dendritic cells, the $F4/80^+$ dendritic cells may be derived from $F4/80^-$ dendritic cells in kidney and vice versa.

The distribution of dendritic cells within the kidney has been determined using various surface markers. Using CX3CR1 as a marker, Li et al., (2008) and Soos et al., (2006) showed dendritic cells in glomeruli and an intricate network throughout the tubulointerstitium .Using CD11c-DTRtg mice, we also observed a network of dendritic cells in the tubulointerstitium but not in glomeruli. The observed distribution of dendritic cells throughout the interstitium, but not glomerulus, as well as the overlap of dendritic cell and macrophage markers is consistent with a recent report in human kidney (Segerer et al., 2008). We suspect the glomerular cells seen in the $CX3CR1^{GFP/-}$ mice may

represent other CX3CR1-positive cells, such as NK cells, monocytes or T cells (Fong et al., 1998; Imai et al., 1997).

While a number of studies have documented changes in the numbers, trafficking or maturation state of renal dendritic cells in different pathophysiological conditions, the role of these renal dendritic cells in their physiological context remains poorly defined. The functional role of dendritic cells in AKI has been examined using liposomal clodronate (Dong et al., 2007; Lu et al., 2008). In ischemic AKI, clodronate reduced the severity of renal injury (Day et al., 2005; Jo et al., 2006) while in the cisplatin model clodronate did not affect renal injury although the infiltration of the kidney by macrophages was significantly reduced (Lu et al., 2008). Unfortunately, clodronate is not specific for dendritic cells. In the present study, we used the CD11c-DTR/GFP transgenic mouse model to study the course of cisplatin nephrotoxicity in the absence of dendritic cells. In these mice, dendritic cells express DTR driven by the CD11c promoter. Injection of DTx caused marked transient depletion of dendritic cells in spleen and kidney while renal macrophages were spared from DTx treatment. Depletion of dendritic cells was more prolonged in kidney compared to spleen. Dendritic cells can mediate both central and peripheral tolerance (Steinman et al., 2003). In this regard, Ohnmacht et al., (2009) using a constitutive ablation mouse model, showed that constitutive depletion of dendritic cells caused spontaneous fatal autoimmunity with cellular infiltrates in different organs, including kidney. In our studies, depletion of dendritic cells in the DTRtg mice did not result in renal dysfunction or infiltration of immune cells in the kidneys, consistent with findings by others (Heymann et al., 2009; Scholz et al., 2008). This finding may imply

that a prolonged, rather than transient, period of dendritic cell depletion is required to elicit a break in immune tolerance and induce autoimmunity.

The principal stimuli of inflammation are injury and infection. Cells dying in large numbers *in vivo* stimulate a potent inflammatory response (sterile inflammation). In response to the mediators of inflammation produced at the site of tissue injury, there is a rapid influx of neutrophils, followed by monocytes. Sterile inflammatory responses to injured cells of different organs and tissues can cause substantial tissue injury and disease. Neutrophils infiltrating into tissues during sterile inflammation contribute to the pathogenesis of different diseases, including ischemic or toxic injuries to heart, liver and lung (Chen et al., 2007; Romson et al., 1983). Several laboratories have shown a prominent infiltration of neutrophils both in the kidney cortex and medulla subsequent to ischemic or toxic insults (Burne et al., 2001; Linas et al., 1992; Zhang et al., 2008). Faubel et al., (2007) and Melnikov et al., (2002) investigated the effect of neutrophil-depletion on kidney injury in cisplatin and ischemic ARF in mice, respectively (Faubel et al., 2007; Melnikov et al., 2002). Ablation of neutrophils using the RB6-8C5 monoclonal antibody did not confer functional or histologic protection against cisplatin-induced ARF. However, in ischemic renal injury, depletion of neutrophils resulted in a reduction of serum creatinine. In addition to neutrophils, RB6-8C5 monoclonal antibody recognizes monocytes (Fleming et al., 1993; Serbina and Pamer, 2006). Therefore, injection of the RB6-8C5 antibody might have depleted both monocytes and neutrophils. Macrophages and dendritic cells are derived from monocytes. In the studies of Faubel et al., (2007) and Melnikov et al., (2002) on the effects of neutrophil-depletion in ARF, it is possible that

antibody RB6-8C5 affected both macrophages and dendritic cells. In our studies, ablation of dendritic cells resulted in a marked increase in the infiltration of neutrophils with exacerbation of injury in cisplatin nephrotoxicity. However, whether the increase in infiltration of neutrophils was due to depletion of dendritic cells or to the aggravated kidney injury is not unraveled from our experiments. Likewise, whether the exacerbated kidney injury in dendritic cell-ablated mice is due to neutrophils or some other protective effects of dendritic cells is not known. The effects of infiltrated-neutrophils, in the above scenario, can be determined by depleting them using Ly-6G antibody in the absence of dendritic cells. In this experiment, a decrease in renal injury in dendritic cell and neutrophil-ablated mice compared with only dendritic cell-ablated mice would indicate that the neutrophils mediate the exacerbated cisplatin nephrotoxicity in the absence of dendritic cells.

Dendritic cell depletion prior to cisplatin treatment caused a marked increase in the severity of renal dysfunction, structural damage and neutrophil infiltration compared with dendritic cell non-depleted mice, indicating that dendritic cells protect the kidneys from cisplatin nephrotoxicity. This observation is consistent with a recent finding in glomerulonephritis that dendritic cells attenuate nephrotoxic nephritis in mice (Scholz et al., 2008). Cisplatin treatment in the presence or absence of dendritic cells had no impact on infiltration of T cells into kidneys compared from saline treated mice as determined by flow cytometry of perfused kidneys. Recent studies suggest T reg cell-mediated suppression of innate immunity and amelioration of kidney injury in acute renal failure (Gandolfo et al., 2009; Kinsey et al., 2009). It is possible that T cells, particularly T reg

cells, present in renal vasculature are removed by the high pressure perfusion used in our protocol to remove circulating leukocytes. Thus, investigation into the infiltration of T reg cells subsequent to exsanguination rather than perfusion is required. It is also possible that non-renal dendritic cells and T reg cells, including these cells in renal lymph nodes, are ameliorating kidney injury in ARF by suppressing the systemic inflammatory response.

The major limitation of the CD11c-DTRtg mouse model is that repeated or high doses of DTx cause death in these mice (Scholz et al., 2008; Zammit et al., 2005). Death in CD11c-DTRtg mice is particularly important in studies where dendritic cell ablation causes more morbidity and mortality than attenuation and survival of these mice. This appears to be due to expression of DTR on non-hematopoietic cells because CD11c-DTRtg mice reconstituted with WT mice bone marrow were also found susceptible to DTx induced death (Zaft et al., 2005). We therefore made CD11c-DTRtg to WT chimeric mice to restrict expression of DTR to the hematopoietic compartment and investigated the effect of dendritic cell ablation on cisplatin nephrotoxicity. Through the use of these CD11c-DTRtg to WT chimeric mice, we determined that the exacerbation of cisplatin-induced AKI was referable to DTx effects on CD11c-expressing hematopoietic dendritic cells rather than ectopic expression of the DTR on other cells. These results are consistent with the view that resident renal dendritic cells play a protective role against cisplatin nephrotoxicity. Using WT to CD11c-DTRtg chimeric mice, we also determined that renal dendritic cells, in contrast to dendritic cells in skin (Merad et al., 2002), are radiosensitive and that bone marrow transplantation effectively replaces the renal dendritic cell

population with donor cells. This finding has important implications for the design and interpretation of bone marrow chimera studies which are being increasingly applied to the study of kidney disease (Wu et al., 2007; Zhang et al., 2007; Zhang et al., 2008).

The mechanism whereby depletion of dendritic cells exacerbated cisplatin mediated AKI is not certain. Products released from dying cells can stimulate immune cells and induce inflammation (Kono and Rock, 2008; Shi et al., 2003). These findings is particularly relevant in light of recent studies from several laboratories (Leemans et al., 2005; Wu et al., 2007), including our own (Zhang et al., 2008), which have identified a role for TLR signaling in AKI. However, using a mixed chimeric mice approach in which WT mice were reconstituted with bone marrow from WT and CD11c-DTRtg mice, we were able to demonstrate that dead dendritic cells themselves were not responsible for the exacerbation of AKI.

Tubular epithelial cells undergo apoptotic and/or necrotic cell death in AKI (Lieberthal et al., 1996; Lieberthal et al., 1998; Padanilam, 2003). Dendritic cells coming in contact with many of the endogenous ligands, including HSPs and uric acid, released from dead cells can undergo activation and mediate inflammation (Gallucci et al., 1999; Shi et al., 2003). Also during tissue injury, inflammatory dendritic cells are recruited into the sites of inflammation (Geissmann et al., 2008; Heymann et al., 2009; Serbina and Pamer, 2006; Shortman and Naik, 2007). In our studies, dendritic cell depletion before cisplatin injection exacerbated kidney injury in cisplatin nephrotoxicity. This finding is consistent with the demonstrated protective role of dendritic cells in nephrotoxic nephritis

(Scholz et al., 2008). However, after cisplatin treatment, inflammatory dendritic cells may infiltrate the kidney or resident renal dendritic cells could become proinflammatory in response to products released during cisplatin nephrotoxicity (Gallucci et al., 1999; Heymann et al., 2009; Serbina et al., 2003; Serbina and Pamer, 2006). In order to determine the role of these dendritic cells in cisplatin nephrotoxicity, we injected DTx at the time of cisplatin injection or 24 hr later. Dendritic cell ablation with DTx 1 hr before cisplatin injection showed exacerbated renal dysfunction similar to DTRtg mice treated with DTx 24 hr before cisplatin treatment. However, depletion of dendritic cells 24 hr after cisplatin treatment had no effect on renal injury. These results imply that dendritic cells provide protection at early but not at late stages of cisplatin mediated kidney injury. Likewise, the recruitment of inflammatory dendritic cells was insignificant. Instead, neutrophils and monocytes infiltration was abundant at later stages of cisplatin nephrotoxicity. This finding contrasts with a recent report of inflammatory dendritic cell recruitment at later stages of nephrotoxic nephritis (Heymann et al., 2009). This difference in inflammatory dendritic cell infiltration may likely reflect differences in the pathogenesis between these two models of kidney injury.

In order to explore the mechanism of dendritic cell-mediated protection in cisplatin nephrotoxicity, we examined the activation state of renal dendritic cells before and after cisplatin injection. It is documented that renal dendritic cells are functionally immature and tolerogenic (Coates et al., 2004; Heymann et al., 2009; Kruger et al., 2004; Scholz et al., 2008). In response to pathogens, dendritic cells undergo maturation, a terminal differentiation process characterized by upregulation of antigen presentation

molecules, MHC class I and MHC class II, and T cell costimulatory molecules such as CD40, CD80 and CD86. Cisplatin treatment did not result in any difference in the expression of MHC class I, CD40, CD80 and CD86, except a slight decrease (10%) in the expression of MHC class II. This finding indicates that the products of tissue injury are not inducing maturation in these immature renal dendritic cells. Renal dendritic cells possess high phagocytic potential under steady-state conditions (Lukacs-Kornek et al., 2008). Thus, it is possible that renal dendritic cells are limiting inflammation by capturing apoptotic renal epithelial cells. Our studies to determine renal dendritic cell uptake of injured renal cells by staining for epithelial cell specific marker, pan cytokeratin, in dendritic cells by intracellular flow cytometry was not successful, which might be due to problem with the technique or due to rapid degradation of dead cells subsequent to phagocytosis.

Bone marrow-derived dendritic cells in coculture with TKPTS downregulated the expression of maturation markers and basal level of TNFα production. TKPTS are an established continuous cell line of murine proximal tubule epithelial cells (5018 Ernest,S. 1995). Despite their transformed phenotype, the TKPTS maintain features characteristic of their normal counterparts in kidney. However, culturing of TKPTS for many generations may cause changes in their features. In our experiment, the decrease in expression of maturation markers on bone marrow-derived dendritic cells in coculture with TKPTS is less likely contributed by changes in TKPTS functions. This conclusion is based on reported findings in which bone marrow-derived dendritic cells cocultured with primary culture of fibroblasts showed a decrease in dendritic cell expression of MHC

class II, CD40 and CD86 (Gallucci et al., 1999). Likewise, dendritic cells-cocultured with intestinal epithelial cells showed downregulation of MHC class II, CD80 and CD86 expression, and inflammatory cytokine production (Butler el al., 2005). The decrease in bone marrow-derived dendritic cell expression of maturation markers in the presence of TKPTS can be due to decrease in spontaneous activation of dendritic cells in artificial environment, perhaps by factors secreted by TKPTS cells. In contrast to renal dendritic cell expression of antigen presentation and costimulatory molecules in response to cisplatin treatment, bone marrow-derived dendritic cells showed upregulation of MHC class II, CD80 and CD86 expression in response to cisplatin or cisplatin and TKPT cells. Likewise, cisplatin-stimulated TNFα production by dendritic cells in the TKPT cell and dendritic cell coculture experiment. However, cisplatin treatment alone did not induce TNFα production in dendritic cells compared with non cisplatin-treated dendritic cells. This difference between the *in vivo* and vitro experiments might be due the differences in the type of dendritic cell subset and the microenvironment under which they encounter injured renal epithelial cells

Renal dendritic cells were found to express high levels of ICOSL, which increased further after cisplatin treatment, consistent with the findings reported on dendritic cell-mediated amelioration of nephrotoxic nephritis in mice (Scholz et al., 2008). The observations that renal dendritic cells maintain their steady-state status with respect to expression of antigen presentation and costimulatory molecules, and upregulate ICOSL in response to cisplatin treatment indicate that these regulatory mechanisms may have largely contributed to tissue protection in cisplatin nephrotoxicity.

It is possible that production of IL-10 (Akbari et al., 2001), or other anti-inflammatory factors (Laouar et al., 2008; Munn et al., 2002; Travis et al., 2007) by renal dendritic cells is an endogenous protective mechanism in AKI. In our studies, infiltration of T cells into the kidney in response to cisplatin treatment was low and comparable with that of saline treated mice. It is possible that dendritic cells in other organs, such as in spleen and lymph nodes, are activating T cells to produce IL-10 and/or regulating T reg cell functions (Darrasse-Jeze et al., 2009). For example, ICOSL stimulates IL-10 production by T cells (Akbari et al., 2002) and IL-10 and ICOSL are reported to ameliorate kidney injury (Deng et al., 2001; Odobasic et al., 2006). In this regard, T reg cells show high expression of ICOS even in the steady-state compared from Th2 cells which upregulate ICOS upon activation. Resident dendritic cells producing anti-inflammatory factors may also modulate the infiltration of inflammatory cells into the kidney (Akbari et al., 2001; Mashimo et al., 2008). Finally, it should be recognized that DTx causes a global depletion of dendritic cells in the CD11c-DTRtg mice. Therefore, we cannot exclude the possibility that the exacerbation of cisplatin AKI was due to the depletion of extrarenal dendritic cells rather than, or in addition to, resident renal dendritic cells.

In summary, we determined the features and distribution of dendritic cells within the kidney and examined their role in a model of toxin-induced AKI. Dendritic cells are protective against cisplatin nephrotoxicity since conditional depletion of dendritic cells accelerates and exacerbates the course of cisplatin-induced AKI. Although this study has

not determined the mechanism, it supports our central hypothesis that dendritic cells protect kidney from cisplatin nephrotoxicity. Elucidation of the mechanism whereby dendritic cells reduce cisplatin-induced AKI may reveal opportunities for pharmacologic or cell-based interventions. Studies of dendritic cells in other forms of AKI are also warranted.

Chapter 3: Endogenous and dendritic cell-derived IL-10 in cisplatin nephrotoxicity

3.1 Introduction

Cisplatin is one of the most effective chemotherapeutic agents used for the treatment of a variety of solid tumors. A major disadvantage with cisplatin chemotherapy is nephrotoxicity affecting approximately 25-35% of treated patients (Hou et al., 1983; Nash et al., 2002; Thadhani et al., 1996). Compelling evidence indicates the involvement of inflammatory mechanisms in the pathogenesis of acute kidney injury (Ascon et al., 2006; Day et al., 2005; Dong et al., 2005; Dong et al., 2007; Dong et al., 2008; Heymann et al., 2009; Jo et al., 2006). Soluble mediators such as cytokines (TNFα, IL-6, IL-1β, IL-17, IFNγ and IL-18) (Dong et al., 2007; Ramesh and Reeves, 2002; Zhang et al., 2007), chemokines (RANTES, MIP-2, MCP-1, IP-10 and KC) (Li et al., 2008), and other factors (proteases and reactive oxygen species) (Bond et al., 2005; Bylander et al., 2008; Matsushima et al., 1998), are released by renal resident cells in response to ischemic or toxic insults and recruit leukocytes from the circulation and activate them, which further complicates the ongoing kidney injury (Ascon et al., 2006; Awad et al., 2009; Day et al., 2005; Heymann et al., 2009; Jo et al., 2006; Liu et al., 2006; Loverre et al., 2007; Lu et al., 2008). This process is mediated by expression of integrins, selectins, and endothelial adhesion molecules (Bonventre and Zuk, 2004; Takada et al., 1997). Cisplatin nephrotoxicity is caused due to the production of various proinflammatory cytokines and chemokines, and direct toxic effects of cisplatin on renal epithelial cells including cisplatin mediated DNA damage, mitochondrial dysfunction (Sugiyama et al., 1989),

production of reactive oxygen species (Matsushima et al., 1998), caspase activation (Kaushal et al., 2001) and cell death.

A number of anti-inflammatory agents are reported to decrease renal infiltration of leukocytes and ameliorate ischemic and cisplatin induced renal injury (Deng et al., 2001; Goodman et al., 2007; Jarmi and Agarwal, 2009; Ogawa et al., 2001; Scholz et al., 2008; Wang et al., 2008). IL-10 is an anti-inflammatory cytokine produced mainly by Th2 cells, T reg cells, dendritic cells and macrophages. IL-10 inhibits production of various cytokines and chemokines, and activation of immune cells in many pathological conditions (Akbari et al., 2001; Eppinger et al., 1996; Hayward et al., 1997; Kitching et al., 2002; Moore et al., 2001). IL-10 regulates the growth and differentiation of a variety of cells including, T cell, B cells, NK cells, macrophages, dendritic cells, granulocytes, mast cells, keratinocytes, epithelial cells and endothelial cells (Moore et al., 2001). IL-10 inhibits the production of many proinflammatory cytokines and chemokines, and expression of adhesion molecules (Berkman et al., 1995, Fiorentino et al., 1991, Kopydlowski et al., 1999, Marfaing-koka et al., 1996). IL-10 is reported to play a major role in the attenuation of tissue injury in different pathophysiological conditions. Administration of IL-10 attenuates kidney injury in lupus nephritis, nephrotoxic nephritis and anti-Thy1.1-nephritis (Kitching et al., 1997; Kitching et al., 2002b; Tipping et al., 1997b; Yin et al., 2002). Exogenous IL-10 inhibits ischemic and cisplatin-induced kidney injury, and helps renal recovery after transplantation (Deng et al., 2001). Consistent with these effects of exogenous IL-10, endogenous IL-10 protects the kidney from renal

ischemia reperfusion injury (Daemen et al., 1999). However, the source and role of endogenous IL-10 in nephrotoxic acute renal failure is not known

Dendritic cells are antigen presenting cells known for their ability to inhibit inflammation under steady-state conditions by various mechanisms including production of IL-10, TGF-β or IDO, regulation of T reg cells and clonal deletion of autoreactive T cells (Akbari et al., 2001; Darrasse-Jeze et al., 2008; Laouar et al., 2008; Munn et al., 2002; Travis et al., 2007). In addition, studies also indicate that IL-10 produced by dendritic cells themselves or by cells under the influence of dendritic cells ameliorates inflammatory immune response (Akbari et al., 2001; Mashimo et al., 2008; Scholz et al., 2008). In this regard, IL-10 produced by pulmonary dendritic cells is reported to inhibit inflammation in response to antigens exposed in the respiratory tract (Akbari et al., 2001). Dendritic cells in kidney represent the most abundant population of leukocytes. Recently, in nephrotoxic nephritis, dendritic cells were shown to exert a renoprotective function, possibly by inducing IL-10 production by infiltrating T cells (Scholz et al., 2008).

Using a DTR-based dendritic cell ablation mice model, we showed that dendritic cells protect the kidney from cisplatin nephrotoxicity. It is possible that production of IL-10 by dendritic cells is an endogenous protective mechanism in cisplatin mediated nephrotoxicity. To test this hypothesis, we first investigated the role of endogenous IL-10 in cisplatin nephrotoxicity. To determine the role of dendritic cell-derived IL-10 in cisplatin nephrotoxicity, we employed a conditional cell ablation method (Sapoznikov

and Jung, 2008). We created mixed bone marrow chimera containing hematopoietic cells equally derived from CD11c-DTRtg and IL-10KO mice. DTx treatment in these mice depletes IL-10 positive dendritic cells leaving behind dendritic cells negative for the IL-10 gene. Our results from these experiments indicate that endogenous IL-10 and dendritic cell-derived IL-10 protect mice from cisplatin nephrotoxicity.

3.2 Methods

3.2.1 Mice

Experiments were performed using 6- to 14- week old C57BL6 mice and IL-10KO mice (B6.129P2-Il10tm1Cgn/J), and CD11c-DTRtg mice (B6.FVB-Tg Itgax-DTR/GFP 57Lan/J) harboring a transgene encoding a simian DTR/GFP fusion protein under the transcriptional control of mouse CD11c promoter (section 2.2.1). IL-10KO mice were generated by replacing codons 5-55 with a linker containing a termination codon followed by a neomycin cassette. In addition a termination codon was also introduced into exon 3. These mice, obtained from Jackson Laboratory, were housed in a specific pathogen-free barrier facility. IL-10KO mice were used within two weeks after their arrival from Jackson Laboratory because these mice maintained in unhygienic or conventional housing conditions develop chronic enterocolitis. For making bone marrow chimeras, donor mice were euthanized with sodium pentobarbital and the femurs were removed and flushed with DMEM medium containing 10% FBS to obtain bone marrow cells. After centrifugation at 300 g for 5 min, the bone marrow cells were suspended in normal saline. The recipient mice were lethally irradiated using a Gammacell irradiator (two doses of 600 rads, 4 hr apart). Within 2-3 hr after irradiation, 10 million donor bone

marrow cells were injected into the tail vein of each recipient. Two sets of bone marrow chimeric mice were generated: WT mice reconstituted with CD11c-DTRtg bone marrow and WT mice reconstituted with equal amounts of IL-10KO and CD11c-DTRtg mice bone marrow. Dendritic cells were ablated in chimeric mice by intraperitoneal injection of DTx, twice, 24 hr before and 24 hr after cisplatin injection. Animal were used according to protocols approved by the IACUC of The Penn State Hershey College of Medicine.

3.2.2 Drug administration and renal function assessment

Cisplatin administration and renal function assessment was performed as described earlier (2.2.3).

3.2.3 Immunostaining

Formalin fixed renal tissue sections were stained for neutrophils as mentioned before using Ly-6G antibody (2.2.5). Ten 20X fields were examined in the cortex of each immunostained kidney section for quantification of neutrophils.

3.2.4 Western blot analysis

Kidney was homogenized in lysis buffer (20mM Tris-HCl, 150mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 1mM Na_3VO_4) containing aspartic, cysteine and serine protease and alkaline, serine and threonine phosphatase inhibitors (Calbiochem). The homogenate was centrifuged at 8000 g for 5 min at 4^{o}C and protein concentration of the supernatant was quantified using the BCA protein assay kit (Pierce). Samples of

protein (50µg) were separated on 10% SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane. After blocking the membrance with 5% BSA in Tris-Buffered Saline (50mM Tris-HCl, 150mM NaCl) containing 0.1% Triton X-100, the membrane was incubated with rabbit anti-pSTAT3 and anti-STAT3 antibody (Cell Signalling) followed by HRP conjugated goat anti-rabbit antibody. After washing the membrane with Tris Buffered Saline containing 0.1% Triton X-100, proteins on the membrane were detected using enhanced chemiluminescene detection reagent (Amersham Pharmacia Biotech).

3.2.5 Renal dendritic cell isolation by flow cytometry

Single cell suspensions of renal cells were prepared and stained for dendritic cells using anti-CD45, MHC class II and CD11c antibodies (BD Pharmingen) as described previously (2.2.6). Renal dendritic cells were sorted by flow cytometry using a MoFlo cell sorter in the Flow Cytometry core facility of The Penn State Hershey College of Medicine.

3.2.6 Quantification of mRNA by real-time PCR

Real-time PCR was performed using the Applied Biosystem 7700 sequence detection system. For RNA isolation from renal dendritic cells, the cells were first lysed with Trizol reagent (Invitrogen). After adding chloroform, phase separation was carried out by centrifuging the samples at 11,000 g for 15 min at 4^0C. The supernatant containing RNA was precipitated with isopropyl alcohol and centrifuged at 11,000 g for 10 min at 4^0C. Finally, the RNA pellet was washed with 75% ethanol and suspended in DNase and

RNase free water. The RNA obtained from renal dendritic cells was reverse transcribed using Omniscript reverse transcription kit (Qiagen) and random primers. PCR conditions: 37^0C for 1 hr 15 min, 70^oC for 10 min. The cDNA was amplified using the SYBR Green PCR amplification kit (Qiagen) in the Genomics core facility of The Penn State Hershey College of Medicine. PCR conditions: 95^oC for 15 min and 40 cycle of 94^oC for 15 sec,53^oC for 30 sec, 72^oC for 40 sec. The primers used were: IL-10R1 (forward: 5'-AGG CAG AGG CAG CAG GCC CAG CAG ATT GCT-3'; reverse: 5'-TGG AGC CTG GCT AGC TGG TCA CAG TAG GTC-3') IL-10R2 (forward: 5'-GCC AGC TCT AGG AAT GAT TC-3'; reverse: 5'-ATT GTT CTT CA A GGT CCA C-3') IL-10 (forward: 5'-CCA AGC CTT ATC GGA AAT GA-3"; reverse: 5'-AGG G GA GAA ATC GAT GAC AG-3') and β-Actin (forward: 5'- TGT TAC CAA CTG GGA CGA CA-3'; reverse: 5'-GGG GTG TTGA AGG TCT CAA A-3'). The amplification specificity of the PCR reactions was confirmed by melting-curve analysis. Qunatitative levels of IL-10 mRNA were normalized to β-actin expression.

3.2.7 Statistical analysis

Results were expressed as mean ± SE. All data were analyzed using an unpaired, two-tailed Student t test. A value of $P<0.05$ was considered significant.

3.3 Results

3.3.1 Expression of IL-10 receptor and STAT3 signaling in kidney in response to cisplatin treatment.

The heterodimer complex of the IL-10 receptor is composed of two subunits, R1 and R2 (Wang et al., 2002). IL-10 selectively binds to IL-10R1 independent of IL-10R2 (Kotenko et al., 1997). However, IL-10R2 binding to IL-10 and IL-10R1 complex is required for efficient signaling through the members of Signal Transducers and Activators of Transcription (STAT) family (Finbloom and Winestock, 1995). IL-10R2 also serves as a second subunit for signaling of IL-22, IL-26 and IFNγ, other members of the class II cytokine family (Donnelly et al., 2004; Wang et al., 2002). Here, we investigated the expression of IL-10, IL-10R1 and IL-10R2, and activation of STAT3 signaling in response to cisplatin treatment (Fig. 3-1) by quantitative RT-PCR and Western blot analysis, respectively. Mice injected with cisplatin showed a dramatic upregulation of IL-10R1, but not of IL-10 or IL-10R2, in kidney at 24 hr compared with saline treated mice (Fig. 3-1A). In addition, kidneys from mice treated with cisplatin showed phosphorylation of STAT3 at 24 hr and 48 hr (Fig. 3-1B). However, in addition to IL-10, other factors, such as IL-5, IL-6, epidermal growth factor and IFNs, may also cause phosphorylation of STAT 3. Phosphorylation of STAT3 was almost absent in saline treated kidneys.These results indicate activation of IL-10 receptor signaling in kidney after cisplatin treatment and possibly a role for endogenous IL-10 in cisplatin nephrotoxicity.

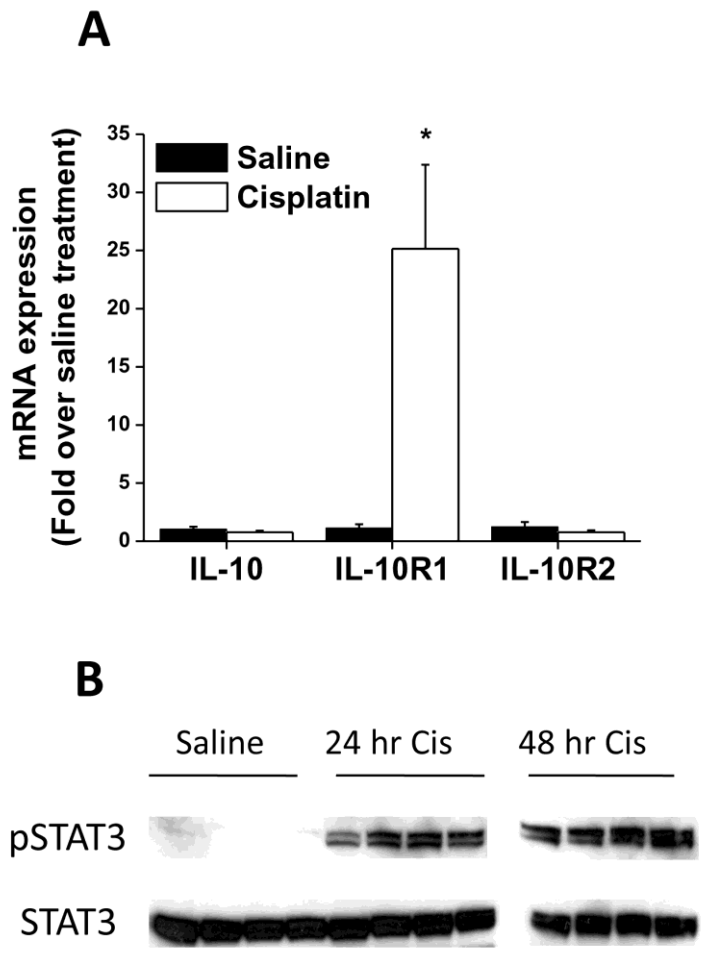


Figure 3-1 Effect of cisplatin on renal IL-10 receptor expression and phosphorylation of STAT3. A . WT mice treated with saline or cisplatin were sacrificed at 24 hr and kidneys were used to determine expression of IL-10, IL-10R1 and IL-10R2 by quantitative RT-PCR, *P<0.05, n = 3/group. B. Kidney from WT mice obtained at 24 hr and 48 hr after saline or cisplatin treatment were used to determine phosphorylation of STAT3 by western blot analysis n = 4/group.

3.3.2 Endogenous IL-10 in cisplatin nephrotoxicity.

Different renal pathologies are ameliorated by exogenous or endogenous IL-10 (Kitching et al., 1997; Kitching et al., 2002; Tipping et al., 1997; Yin et al., 2002). Exogenous administration of IL-10 attenuates cisplatin nephrotoxicity (Deng et al., 2001). Having determined that cisplatin treatment causes upregulation of IL-10R1 and

phosphorylation of STAT3, we then investigated the role of endogenous IL-10 in the pathogenesis of cisplatin-mediated acute renal failure. WT and IL-10 KO mice were treated with cisplatin and renal function was assessed by measuring the level of BUN and serum creatinine. As shown in Figure 3-2, WT mice treated with cisplatin showed minimal increase in the levels of BUN and serum creatinine at 24 hr which elevated by 48 hr and 72 hr. In comparison to WT mice, IL-10 KO mice treated with cisplatin showed earlier and more marked increases in the levels of BUN and serum creatinine. WT and IL-10KO mice treated with saline showed basal normal levels of BUN and serum creatinine. These findings indicate that endogenous IL-10 protects the kidney from cisplatin nephrotoxicity.

3.3.3 Neutrophil infiltration in IL-10KO mice in cisplatin nephrotoxicity.

Cisplatin-mediated renal injury is associated with cytokine and chemokine production and infiltration of leukocytes, especially neutrophils (Zhang et al., 2007, Zhang et al., 2008). IL-10 inhibits neutrophil chemotaxis and their production of pro-inflammatory cytokines (Cassatella et al., 1993; Cassatella et al., 1994; Moore et al., 2001; Wang et al., 1994). Since the absence of endogenous IL-10 exacerbated kidney injury, we investigated renal neutrophil infiltration in WT and IL-10KO mice 72 hr after cisplatin treatment. The number of neutrophils in kidney sections of IL-10KO mice treated with saline was comparable to that of saline treated WT mice (Fig. 3-3A, B and E). WT mice treated with cisplatin showed marginal infiltration of neutrophils (Fig. 3-3C and E). However, IL-10KO mice treated with cisplatin showed a severe influx of neutrophils into kidney (Fig. 3-3 D and E). This observation is consistent with the

findings of Daemen *et al.,* (1999) that endogenous IL-10 attenuates kidney injury and infiltration of neutrophils in renal ischemia reperfusion injury (Daemen et al., 1999).

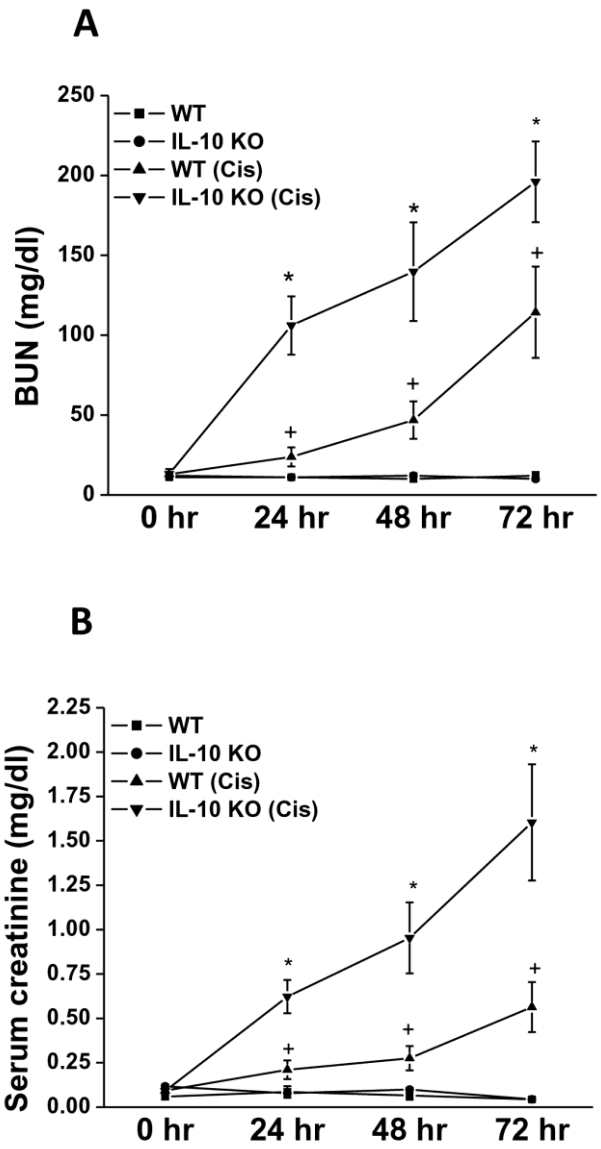


Figure 3-2. Effect of endogenous IL-10 in cisplatin nephrotoxicity. WT and IL-10 KO mice were treated with saline or cisplatin. Blood collected at different time points with respect to cisplatin injection was analyzed for the level of BUN (A) or serum creatinine (B) as a measure of renal function. *, $^{+}P < 0.05$. vs. all other groups, n = 4-18/group.

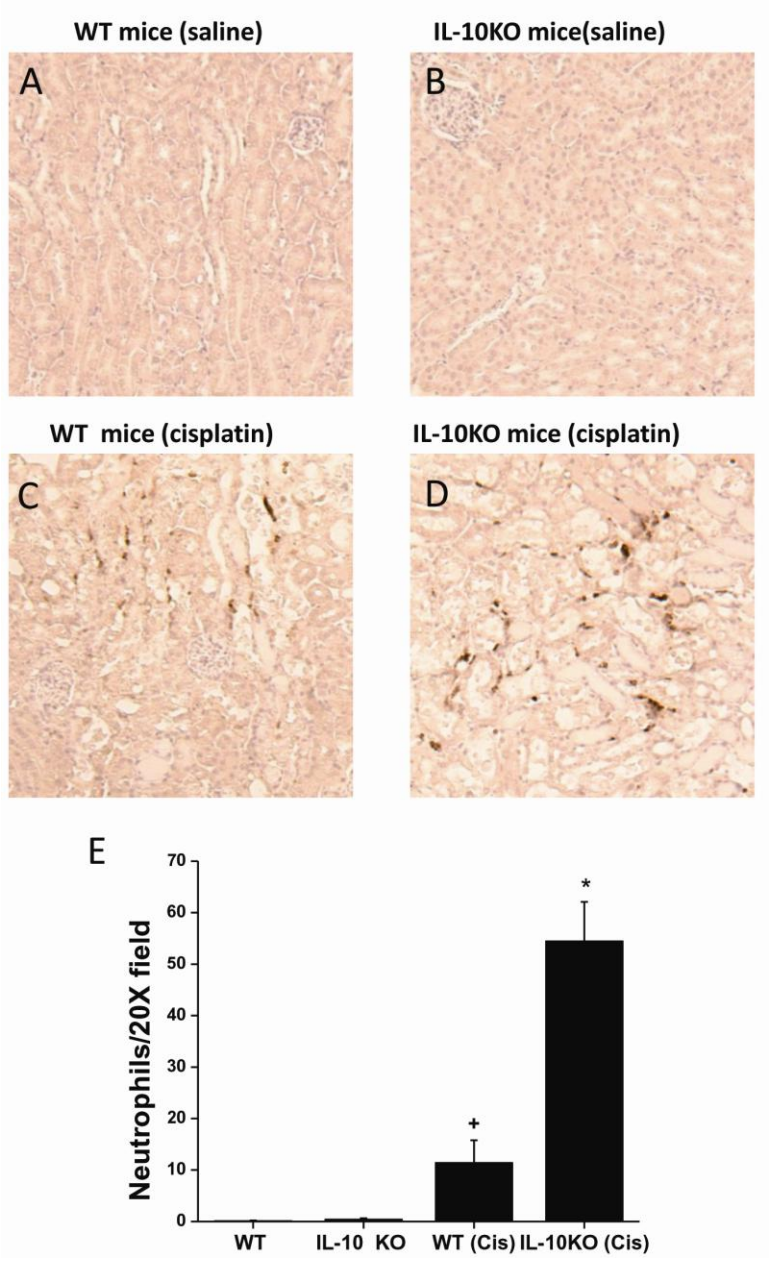


Figure 3-3. Effect of endogenous IL-10 on renal neutrophil infiltration. WT mice (A and C) and IL-10KO mice (B and D) were treated with saline (A and B) or cisplatin (C and D). Kidneys harvested at 72 hr after cisplatin treatment were stained for neutrophils. E. Enumeration of neutrophils in the cortex of kidney sections harvested at 72 hr after cisplatin treatment. $^{+,}$ *$P < 0.002$ vs. all other groups, n = 4-7/group.

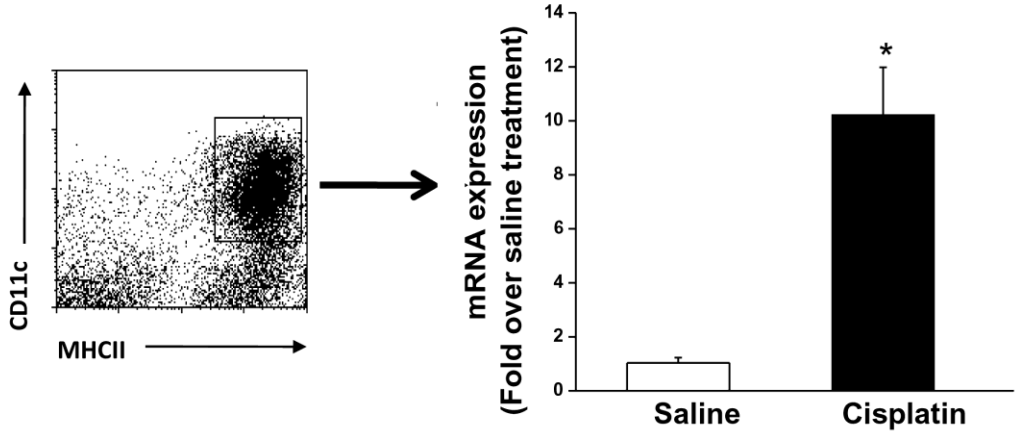


Figure 3-4. Renal dendritic cell expression of IL-10 in response to cisplatin treatment. WT mice treated with saline or cisplatin were sacrificed at 24 hrs and renal dendritic cells were sorted by flow cytometry and expression of IL-10 was determined by quantitative RT-PCR. *P<0.05. n=3/group.

3.3.4 Dendritic cell expression of IL-10 to cisplatin treatment.

Dendritic cells form an abundant population of leukocytes in kidney, and are known to attenuate nephrotoxic nephritis in mice (Scholz et al., 2008). IL-10 is an anti-inflammatory cytokine produced by many cell types, including dendritic cells (Akbari et al., 2001; Moore et al., 2001). Studies in CD11c-DTRtg mice showed a protective function for dendritic cells in cisplatin nephrotoxicity. It is possible that dendritic cells produce IL-10 in response to cisplatin treatment and protect the kidney from cisplatin nephrotoxicity. In order to determine dendritic cell responsiveness to cisplatin with respect to IL-10 production, we sorted renal dendritic cells from saline or cisplatin treated mice 24 hr after cisplatin treatment, and measured the expression of IL-10 by quantitative real-time PCR (Fig. 3-4). Renal dendritic cells from cisplatin-treated mice showed a 10-fold increase in IL-10 expression as compared to saline treated mice, indicating a

potential protective role for dendritic cell IL-10 production in cisplatin induced nephrotoxicity.

3.3.5 Role of dendritic cell-derived IL-10 in cisplatin nephrotoxicity

Dendritic cells have been reported to produce IL-10 and attenuate inflammation in allergic asthma and endotoxin-induced uveiitis (Akbari et al., 2001; Mashimo et al., 2008). Having determined that renal dendritic cells and endogenous IL-10 protect kidneys from cisplatin nephrotoxicity and that renal dendritic cells express IL-10 in response to cisplatin treatment, we investigated a possible role of dendritic cell-derived IL-10 production in the attenuation of cisplatin nephrotoxicity. We used a conditional cell ablation method to determine the effect of dendritic cell-produced IL-10 in cisplatin nephrotoxicity. This method has been used to investigate the function of different secreted factors or molecules of dendritic cells, including IL-15 (Kuwajima et al., 2006), B cell activating factor, macrophage migration inhibition factor (Sapoznikov et al., 2008) and MHC class II (Darrasse-Jeze et al., 2009), in normal immune homeostasis, immunity and tolerance. In this technique, equal numbers of IL-10KO and CD11c-DTRtg mice bone marrow cells were injected into irradiated WT mice. After injection of DTx, these chimeric mice selectively lack dendritic cell derived IL-10. First, as a control to determine the effect of 50% dendritic cell depletion on cisplatin nephrotoxicty, we made mixed chimeric mice containing hematopoietic cells equally derived from WT and CD11c-DTRtg mice, and WT to WT mice chimera (Fig 2-12A). These chimeric mice were injected with DTx and cisplatin and renal function was determined by measuring BUN and serum creatinine (Fig 2-12 B and C). 50% depletion of dendritic cells in mixed

chimeric mice had no impact on the extent of renal dysfunction compared to dendritic cell non-ablated WT to WT chimeric mice. This finding also indicates that depletion of 50% of dendritic cells in IL-10KO and CD11c-DTRtg mixed chimeric mice by itself does not contribute to cisplatin nephrotoxicity. Then, to determine the role of dendritic cell IL-10 in cisplatin nephrotoxicity, we injected mixed chimeric mice containing IL-10KO and CD11c-DTRtg mice derived hematopoietic cells with cisplatin or cisplatin and DTx and compared the extent of renal dysfunction with dendritic cell depleted and non-depleted CD11c-DTRtg to WT chimeric mice treated with cisplatin (Fig 3-5). DTx was injected twice, 24 hr before and 24 hr after cisplatin injection. Mixed chimeric mice depleted of IL-10-producing dendritic cells by injection of DTx showed a moderate increase in BUN (Fig. 21A) and serum creatinine (Fig 3-5B) as compared with dendritic cell non-depleted mice at 48 hr after cisplatin treatment. In contrast, dendritic cell-depleted CD11c-DTRtg to WT chimeric mice showed severe renal dysfunction compared with dendritic cell non-depleted mice. These results indicate that IL-10 of dendritic cells attenuates kidney injury from cisplatin nephrotoxicity. However, considering the difference in the extent of renal dysfunction in dendritic cell depleted and non-depleted mixed chimeric mice and CD11c-DTRtg to WT chimeric mice, dendritic cell derived IL-10 may not be a major factor in dendritic cell mediated protection of cisplatin nephrotoxicity.

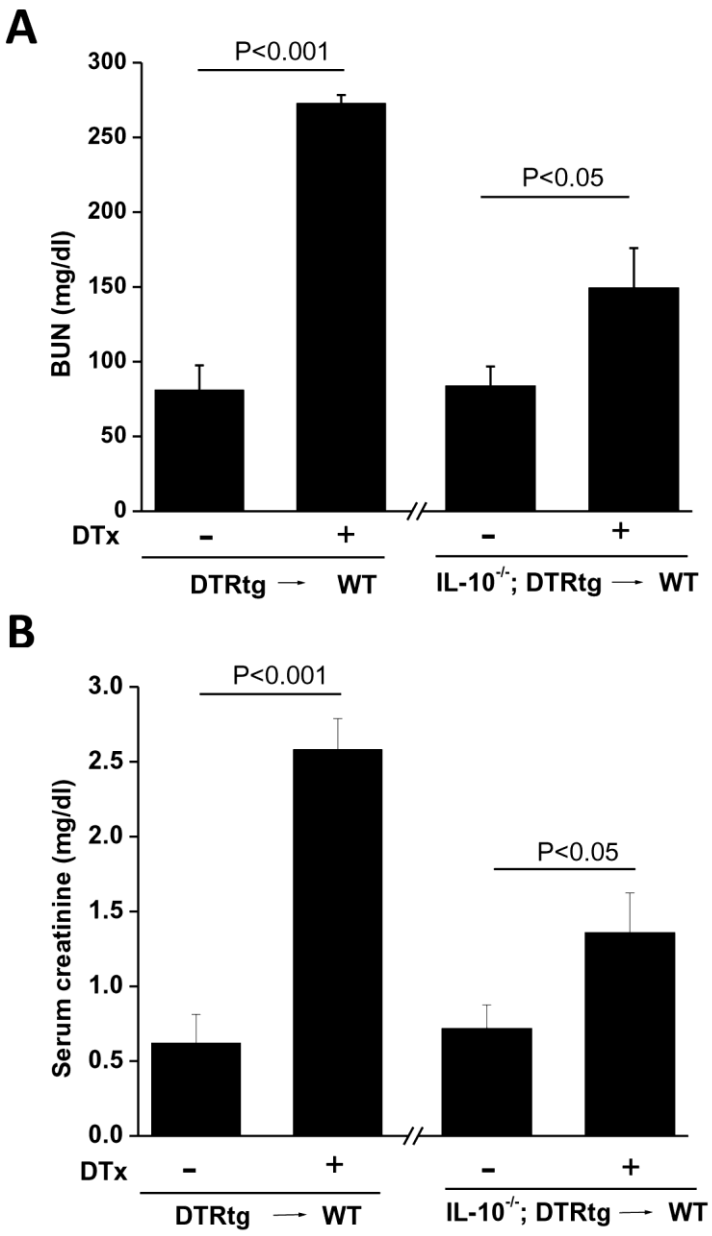


Figure 3-5. Effect of dendritic cell-derived IL-10 in cisplatin nephrotoxicity. WT mice were reconstituted with bone marrow from CD11c-DTRtg mice (n = 5/group) or a mixture of CD11c-DTRtg and IL-10KO bone marrow (n=10-13/group) and were treated with cisplatin or DTx and cisplatin. Blood collected at 48 hr after DTx treatment was analyzed for BUN (A) and serum creatinine (B).

3.4 Discussion

Studies from many laboratories over the past decade have firmly established the role of inflammation in the pathogenesis of renal diseases of various origins, including ischemic and toxic kidney injury. Renal cells and resident leukocytes, in response to ischemic or toxic insult, secrete a wide range of chemokines and cytokines (Dong et al., 2007; Li et al., 2007; Ramesh and Reeves, 2003; Zhang et al., 2007). These mediators of inflammation up-regulate the expression of adhesion molecules and attract different populations of leukocytes that include neutrophils, macrophages, T cells, NK cells and dendritic cells, which may further exacerbate injury by producing cytokines, chemokines, ROS, eicosanoids, and others (Ascon et al., 2006; Furuichi et al., 2003; Heymann et al., 2009; Kielar et al., 2005) . Concurrent with the induction of a stress activated proinflammatory response, many agents with anti-inflammatory properties (e.g., adenosine, nitric oxide, netrin-1, IL-10, VEGF, IGF-1, and heme oxygenase) are produced that may prevent tissue injury, or help in tissue repair/remodeling subsequent to injury in differerent organs and tissues, including the kidneys (Deng et al., 2001; Garcia et al., 2008; Goodman et al., 2007; Jarmi and Agarwal, 2009; Li and Okusa, 2006; Ogawa et al., 2001; Scholz et al., 2008; Wang et al., 2008). IL-10 is a multifunctional anti-inflammatory cytokine that has been reported to attenuate different renal pathologies (Kitching et al., 1997; Kitching et al., 2002; Tipping et al., 1997; Yin et al., 2002). Here we investigated the role of endogenous IL-10 in cisplatin nephrotoxicity using IL-10KO mice. Our studies using a cell ablation mouse model have established that renal dendritic cells protect the kidney from cisplatin mediated injury. We also explored the function of

endogenous IL-10 and dendritic cell-derived IL-10 in cisplatin nephrotoxicity. Our findings indicate that endogenous IL-10 and dendritic cell-derived IL-10 protect mice from cisplatin nephrotoxicity

Exogenous administration of IL-10 attenuates kidney injury in lupus nephritis, experimental glomerulonephritis, and mesangial proliferative glomerulonephritis (Kitching et al., 1997; Kitching et al., 2002; Tipping et al., 1997; Yin et al., 2002). Likewise, administration of IL-10 protects kidneys from cisplatin and ischemic kidney injury and helps kidney recovery after transplantation (Deng et al., 2001). In the present study, cisplatin treatment caused increased expression of IL-10R1 and activation of the IL-10 signaling pathway in kidneys. These findings indicate a possible role for endogenous IL-10 in cisplatin nephrotoxicity. To investigate the functional relevance of endogenous IL-10 in AKI, we studied the course of cisplatin nephrotoxicity in IL-10 deficient mice. Cisplatin-injected IL-10KO mice showed aggravation of renal dysfunction, indicating a protective role for endogenous IL-10 in cisplatin nephrotoxicity. IL-10 is produced by many cell types, including Th2 cells, T reg cells, macrophages and dendritric cells (Moore et al., 2001). Dendritic cells serve several functions including induction of immunity and tolerance (Banchereau et al., 2000). Under steady-state conditions dendritic cells suppress inflammation by different anti-inflammatory mechanisms including production of IL-10 (Akbari et al., 2001; Steinman et al., 2003). In allergic asthma and endotoxin-induced uveiitis, dendritic cells produce IL-10 and ameliorate inflammation (Akbari et al., 2001; Mashimo et al., 2008). Our studies to determine the impact of dendritic cell ablation on kidney injury showed a protective

function of dendritic cells in cisplatin nephrotoxicity. As dendritic cells are known to produce IL-10, we investigated renal dendritic cell production of IL-10 after cisplatin treatment. IL-10 expression by renal dendritic cells was increased up to 10-fold after cisplatin treatment compared to saline treated mice, indicating a possible function of dendritic cell-derived IL-10 in protection against cisplatin nephrotoxicity. Kidneys obtained from saline or cisplatin treated mice did not show a difference in IL-10 expression as determined by real-time PCR. This similarity in IL-10 expression in kidneys, in contrast to renal dendritic cells, might be due to substantial dilution of mRNA from renal dendritic cells by mRNA from other cells within the kidney. In this regard, renal dendritic cells constitute less than 0.1% of total kidney cells.

Establishing a direct link between dendritic cell-derived IL-10 and cisplatin nephrotoxicity requires a system in which dendritic cells lack the capacity to produce IL-10. This system can be achieved either by constitutive gene ablation or conditional cell ablation methods (Caton et al., 2007; Sapoznikov and Jung, 2008; Sapoznikov et al., 2008; Travis et al., 2007). We employed the conditional cell ablation system because this approach is faster and more economical. Also, because the ablation is only temporary, the possibility of development of compensatory pathways is relatively low compared to conditional gene ablation method. Injection of DTx into chimeric mice having leukocytes equally derived from CD11c-DTRtg and IL-10KO bone marrow causes depletion of CD11c-DTRtg dendritic cells (around 50%), leaving behind only the IL-10KO dendritic cells. We then investigated the role of IL-10 produced by dendritic cells by injecting cisplatin or DTx and cisplatin to 1:1 CD11c-DTRtg and IL-10KO chimeric mice. Our

studies showed a protective function for dendritic cell-derived IL-10 in cisplatin nephrotoxicity. However, considering the moderate attenuation of kidney injury by dendritic cell-derived IL-10, the contributions of other dendritic cell mechanisms may be more important mediators of the DC protective function in cisplatin nephrotoxicity.

Endogenous IL-10 showed marked protection in cisplatin nephrotoxicity. It is possible that IL-10 produced by other cells, such as T reg cells, protect the kidneys from cisplatin induced nephrotoxicity (Cools et al., 2008; Fu et al., 2008; Gandolfo et al., 2009; Hochegger et al., 2005; Kinsey et al., 2009; Scholz et al., 2008; Wolf et al., 2005). T reg cells are regulated by dendritic cells through their cell surface and secreted molecules, including MHC class II and ICOS-L (Akbari et al., 2002; Herman et al., 2004; Lohning et al., 2003; Scholz et al., 2008). In support of this notion, a recent study showed a drastic reduction in T reg cell number after depletion of dendritic cells in mice (Darrasse-Jeze et al., 2009). Likewise, constitutive depletion of dendritic cells produced a break in self tolerance and a spontaneous fatal autoimmunity (Ohnmacht et al., 2009). These studies indicate a possible role for dendritic cell-regulated T reg cells in cisplatin nephrotoxicity. In our studies, depletion of dendritic cells showed exacerbation of kidney injury, and renal dendritic cells showed high levels of ICOSL expressioin that were elevated further upon cisplatin treatment. In nephrotoxic nephritis, blockade of ICOSL is reported to aggravate kidney injury and reduce IL-10 production in splenocytes (Odobasic et al., 2006), indicating a protective role of ICOSL in renal diseases. Unlike Th2 cells which upregulate ICOS upon activation, T reg cells express high levels of ICOS even at steady-state conditions. It is possible that renal dendritic cells and/or

dendritic cells in spleen and lymph nodes, through their ICOSL regulate T reg cells and their IL-10 production. In this regard, studies in a murine model of chronic kidney disease showed attenuation of kidney injury by T reg cells (Wolf et al., 2005). Likewise, recently Kinsey et al., (2009) and Gandolfo et al., (2009) reported T reg cell-mediated suppression of innate immunity and amelioration of kidney injury in renal ischemia reperfusion injury (Gandolfo et al., 2009; Hochegger et al., 2005; Kinsey et al., 2009).

In summary, we have determined the effect of cisplatin on renal IL-10 signaling and investigated the role of endogenous IL-10 and dendritic cell IL-10 in cisplatin-induced acute kidney injury. Endogenous IL-10 is protective in cisplatin nephrotoxicity, and dendritic cell-derived IL-10 is marginally involved in dendritic cell mediated attenuation of cisplatin nephrotoxicity. The protective role of dendritic cells and endogenous IL-10 may be linked to the regulation of T reg cells. Further studies are warranted on dendritic cell-mediated regulation of T reg cells in cisplatin nephrotoxicity. Elucidation of these mechanisms may be exploited for pharmacologic or cell-based interventions to treat immune-mediated acute kidney injury.

Chapter 4 : General discussion and conclusion

4.1 Discussion

In the kidney, dendritic cells form an abundant population of leukocytes. Renal dendritic cells respond to local and systemic injury by producing cytokines and chemokines, and migrating to regional lymph nodes to activate the adaptive immune system (Dong et al., 2005). Most of the reported studies on renal dendritic cells are based on measures of dendritic cell infiltration, migration or cytokine production subsequent to injury rather than on the functional relevance of renal dendritic cells within their physiological context *in vivo*. Earlier findings in renal diseases included changes in renal dendritic cell anatomical distribution and number in glomerulonephritis, immunoglobulin A nephropathy and transplantation (Loverre et al., 2007; Segerer et al., 2008; Woltman et al., 2007). Although these few studies indicate a likely contribution of dendritic cells in renal inflammation, our understanding of renal dendritic cells in health and disease within their physiological context is rudimentary when compared with dendritic cells in other organs and tissues. In the present study, we used a murine conditional dendritic cell ablation model to characterize these largely unknown renal dendritic cells and to determine their role in a nephrotoxic model of acute kidney injury.

In this study, we first characterized renal dendritic cells by comparing them with well-studied splenic dendritic cells. Phenotypic characterization of renal dendritic cells showed both similarities and differences in the expression of cell surface markers as compared with their splenic counterparts. Anatomical localization of renal dendritic cells

showed a dense network within the interstitium but not in the glomerulus. This is consistent with findings in human kidneys where dendritic cells were found in the interstitium but not in the glomerulus (Segerer et al., 2008).

Although characterization of dendritic cells in different renal pathologies provides some clues regarding their probable role in kidney injury, the exact function of renal dendritic cells in terms of attenuation or exacerbation of kidney injury can only be determined in experimental models in which they can be depleted *in vivo*. Instead of using liposomal clodronate to deplete dendritic cells, which depletes monocytes and macrophages preferentially to dendritic cells (Blazar et al., 2001; Jo et al., 2006; Swirski et al., 2007), we used a dendritic cell-specific depletion method. This approach uses the expression of DTR on dendritic cells that are subsequently ablated in response to DTx treatment (Sapoznikov and Jung, 2008; Sapoznikov et al., 2008; Scumpia et al., 2005). Using this approach, we first investigated the kinetics of renal dendritic cell depletion after DTx treatment. These CD11c-DTRtg mice treated with DTx showed a marked transient depletion of dendritic cells in spleen and kidney whereas renal macrophages were spared from DTx treatment. Depletion of dendritic cells was more prolonged in kidney as compared to spleen. Transient ablation of dendritic cells in these CD11c-DTRtg mice did not cause any renal dysfunction or immune cell infiltration in kidney (Jung et al., 2002; Sapoznikov et al., 2008; Scholz et al., 2008).

Dendritic cell depletion prior to cisplatin treatment caused a marked increase in the severity of cisplatin-induced renal dysfunction, structural damage and neutrophil

infiltration, indicating that resident dendritic cells that are present in kidney under steady-state conditions protect the kidney from cisplatin nephrotoxicity. One limitation of this model is the death of transgenic mice in response to high or repeated doses of DTx injection due to expression of DTR on some vital non-hematopoietic cells (Scholz et al., 2008; Zammit et al., 2005). In order to restrict DTR expression on hematopoietic cells, we made CD11c-DTRtg to WT mice chimera and tested our hypothesis that dendritic cells protect the kidneys from cisplatin nephrotoxicity. Depletion of dendritic cells in these chimeric mice caused exacerbated renal injury with elevated levels of BUN and serum creatinine, and decreased survival rate. In order to prove that this exacerbated kidney injury in cisplatin nephrotoxicity is due to the absence of dendritic cells rather than dead dendritic cells (Kono and Rock, 2008; Matzinger, 1998; Shi et al., 2003), we employed a mixed chimeric mice approach in which DTx treatment caused death of about 50% of the dendritic cells. With this approach, we demonstrated that dead dendritic cells themselves were not responsible for exacerbation of kidney injury in cisplatin nephrotoxicity. Using WT to CD11c-DTRtg and CD11c-DTRtg to WT mice chimera, we also determined that renal dendritic cells are radiosensitive and bone marrow transplantation could be used to effectively replace the resident renal dendritic cell population with donor cells. In light of increased use of bone marrow chimeras to determine the relative role of hematopoietic and parenchymal cells in different renal diseases, the later findings are very important in interpretation of bone marrow chimera results (Wu et al., 2007; Zhang et al., 2007; Zhang et al., 2008).

Ischemic or toxic insults cause acute tubular necrosis in AKI (Lieberthal et al., 1996; Lieberthal et al., 1998; Padanilam, 2003). In our studies, steady-state dendritic cells provided protection against cisplatin nephrotoxicity. However, resident renal dendritic cells coming in contact with products of tissue injury can undergo maturation and mediate inflammation at later stages of cisplatin nephrotoxicity (Beg, 2002; Gallucci et al., 1999). Monocytes recruited during renal injury may also differentiate into inflammatory dendritic cells and mediate kidney injury (Geissmann et al., 2008; Heymann et al., 2009; Serbina and Pamer, 2006; Shortman and Naik, 2007). In order to address the role of dendritic cells in these scenarios, we depleted dendritic cells at early or late stages after cisplatin treatment and assessed the extent of renal dysfunction in response to cisplatin treatment. Our findings indicate that dendritic cells protect the kidney at early but not at late stages of cisplatin mediated kidney injury. However, as this experiment did not give information about the infiltration of inflammatory dendritic cells to the kidney in cisplatin nephrotoxicity, we examined their infiltration at later stages of kidney injury. To our surprise, the infiltration of inflammatory dendritic cells was insignificant, rather, neutrophils and monocytes were found in abundance at later stages of cisplatin nephrotoxicity at least when examined at 48 hr and 72 hr after cisplatin treatment. This observation contrasts with a recent report of inflammatory dendritic cell recruitment in nephrotoxic nephritis (Heymann et al., 2009) and likely reflects differences in the pathogenesis between these two models of kidney injury.

Concurrent to the induction of a proinflammatory response in response to ischemic or toxic insult, many different endogenous anti-inflammatory agents, including

IL-10 are produced (Deng et al., 2001; Garcia et al., 2008; Goodman et al., 2007; Jarmi and Agarwal, 2009; Li and Okusa, 2006). These endogenously produced anti-inflammatory factors may prevent renal injury or help kidney repair/remodeling subsequent to kidney injury. IL-10 is a pleuripotent cytokine that inhibits the production of proinflammatory cytokines and chemokines, expression of adhesion molecules, and activation of immune cells (Moore et al., 2001). Exogenous or endogenous IL-10 has been shown to ameliorate renal injury in different models of renal pathologies (Kitching et al., 1997; Kitching et al., 2002; Tipping et al., 1997; Yin et al., 2002). Dendritic cells are known to produce IL-10 and attenuate inflammation (Akbari et al., 2001; Mashimo et al., 2008). Likewise, we observed very high expression of IL-10 mRNA by renal dendritic cells after cisplatin treatment.

Exogenous administration of IL-10 provides protection from cisplatin-mediated kidney injury (Deng et al., 2001). Before addressing the role of dendritic cell derived IL-10 in cisplatin nephrotoxicity, we investigated the function of endogenous IL-10 with the notion that endogenous IL-10, similar to exogenous IL-10 (Denj et al., 2001), might protect the kidneys from cisplatin mediated kidney injury. We studied the course of cisplatin nephrotoxicity in mice genetically deficient in IL-10. Cisplatin injection into IL-10KO mice resulted in aggravated kidney injury with infiltration of neutrophils, indicating a beneficial function of endogenous IL-10 against cisplatin nephrotoxicity.

In order to determine the role of dendritic cell IL-10 production in cisplatin nephrotoxicity, we used a conditional cell ablation method in which injection of

DT to chimeric mice having leukocytes equally derived from CD11c-DTRtg and IL-10KO mice bone marrow causes depletion of CD11c-DTRtg dendritic cells leaving behind dendritic cells lacking the IL-10 gene (Caton et al., 2007; Sapoznikov and Jung, 2008; Sapoznikov et al., 2008; Travis et al., 2007). Depletion of IL-10-producing dendritic cells in these mixed chimeric mice resulted in an increase in renal dysfunction, indicating a protective role for dendritic cell IL-10 in cisplatin nephrotoxicity (Fig 4-1). However, the protection was marginal. This finding indicates that other dendritic cell mechanisms, in addition to IL-10 production, might be contributing to their protective role in cisplatin nephrotoxicity. Therefore, we investigated other regulatory mechanisms of dendritic cells that might have contributed to attenuation of cisplatin nephrotoxicity in mice.

Under steady-state conditions, immature dendritic cells in tissues and organs are highly phagocytic and that helps them to take up proteins and clear dying cells in different organs and tissues (Banchereau et al., 2000; Steinman et al., 2003). Likewise, renal dendritic cells are also known to possess high phagocytic potential under steady-state conditions (Lukacs-Kornek et al., 2008). In order to induce immunity, these cells must undergo a terminal differenciation process called maturation. This process is characterized by dendritic cell upregulation of antigen presentation and costimulatory molecules to stimulate T cell response. Cisplatin treatment did not cause much difference in the expression of these markers, indicating that the products of kidney injury are not inducing maturation in these immature renal dendritic cells. Considering the findings that phagocytic activity of immature dendritic cells is very high (Banchereau et al., 2000;

Steinman et al., 2003), it is possible that renal dendritic cells limit inflammation by capturing apoptotic renal epithelial cells at early phases of kidney injury. Our attempts to determine renal dendritic cell uptake of injured renal cells by staining for the epithelial cell specific marker, pan cytokeratin, in dendritic cells by intracellular flow cytometry were not successful. This might be due to problems with the technique or due to rapid degradation of dead cells subsequent to phagocytosis.

In the present study, we determined that renal dendritic cells and endogenous IL-10 protect the kidneys from cisplatin nephrotoxicity. Although our studies indicated that dendritic cell IL-10 is not a critical factor in dendritic cell-mediated amelioration of cisplatin nephrotoxicity, they did not rule out an indirect role for dendritic cells in endogenous IL-10 mediated protection against cisplatin induced nephrotoxicity. In the mixed chimera experiments to study the role of dendritic cell-derived IL-10 in cisplatin nephrotoxicity, DTx treatment caused depletion of DTR expressing dendritic cells leaving behind IL-10 negative dendritic cells. It is possible that these dendritic cells, mostly non renal dendritic cells, regulate other cells, such as T reg cells, to produce IL-10 and attenuate kidney injury (Fig 4-1). In this regard, dendritic cells are known to regulate T reg cells and inhibit inflammation (Darrasse-Jeze et al., 2009). Renal dendritic cells express a high level of ICOSL that is further elevated in response to cisplatin treatment. ICOSL has been shown to attenuate kidney injury (Odobasic et al., 2006). Recently, T reg cells were reported to protect the kidneys from ischemic insults (Gandolfo et al., 2009; Hochegger et al., 2005; Kinsey et al., 2009).

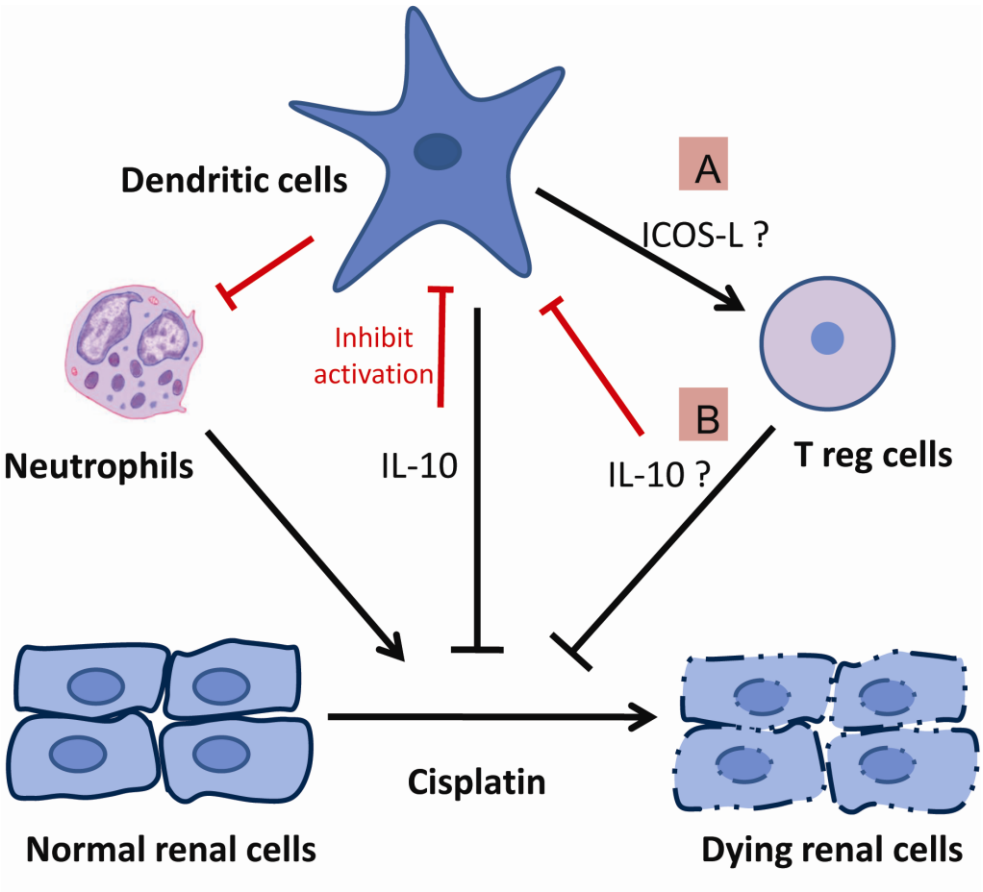


Figure 4-1. Hypothetical model of dendritic cell-mediated protection in cisplatin nephrotoxicity. Dendritic cells attenuate kidney injury partly by producing IL-10. The IL-10 thus produced in response to cisplatin treatment, in turn, can inhibit maturation of dendritic cells. Other possibilities of dendritic cell mediated amelioration of cisplatin nephrotoxicity include phagocytosis or macropinocytosis of tissue debris and dead cells, inhibition of neutrophil infiltration, and ICOSL mediated activation of T reg cells. (A and B are for future directions).

Considering all of these observations, it is possible that dendritic cells, through their ICOSL and /or other molecules, are regulating T reg cell function and their IL-10 production. Further studies are required to determine whether kidney dendritic cell regulation of T reg cells attenuate cisplatin nephrotoxicity, and if it does, additional studies are warranted to determine the extent of protection that is mediated through T reg cell IL-10.

4.2 Conclusion

In summary, we have determined the features and distribution of renal dendritic cells and examined their function in a mice model of toxin-induced acute kidney injury. We also investigated the role of endogenous IL-10 and dendritic cell IL-10 in acute kidney injury. Our results indicate that dendritic cells and endogenous IL-10 are protective against cisplatin nephrotoxicity and prove our hypothesis that dendritic cells protect the kidney from cisplatin nephrotoxicity. Based on our findings and earlier reports, we consider that the contiguous network of dendritic cells present in the tubulointerstitium compartment of kidney is functionally protective in kidney injury. Under steady-state conditions, these highly phagocytic renal immature dendritic cells take up proteins and apoptotic cells in their immediate microenvironment and help in the suppression of inflammation. Considering the phagocytic potential of steady-state immature renal dendritic cells and extensive tubular injury in dendritic cell ablated mice caused by cisplatin, it is possible that renal dendritic cells with their high phagocytic activity limit inflammation by ingesting dead renal epithelial cells. In the present study, although dendritic cell-derived IL-10 protected the kidneys from cisplatin nephrotoxicity, it was not critical considering the extent of protection. In the light of ICOSL of dendritic cell-mediated regulation of T reg cells, it is also possible that dendritic cells, through their ICOSL and /or other molecules, are regulating T reg cell functions and their IL-10 production. Further studies are warranted to examine dendritic cell regulation of T reg cells and its role in the attenuation of cisplatin mediated nephrotoxicity. Elucidation of the mechanisms whereby dendritic cells reduce cisplatin-induced kidney injury may be

exploited for pharmacologic or cell-based interventions to treat immune-mediated acute kidney injury.

4.3 Limitations

The kidney is a complex organ comprised of many different cell types and structures, such as blood vessels, tubules and glomeruli, which function in a highly coordinated fashion to achieve various goals such as regulation of electrolytes, acid-base balance, blood pressure, reabsorption of glucose and amino acids, excretion of urea, and production of vitamin D, renin and erythropoietin. The use of whole animal, isolated organ, as well as tissue and cell culture models has helped elucidate these renal functions at the biochemical, cellular and molecular levels in different pathophysiological conditions (Lieberthal and Nigam, 2000). Simple models of renal injury, such as cultured tubular cells, provide the opportunity for precise manipulation, control of individual variables and mechanistic understanding. However, as compared to patients with renal failure or animal models, they are less complex and quite often cannot be reproduced under *in vivo* settings (Fig. 4-2). For example, we began our studies by examining the effect of cisplatin on renal dendritic cells using primary cell culture of bone marrow-derived dendritic cells. They are relatively easy to culture and manipulate at the molecular level. These dendritic cells, in response to cisplatin or cisplatin treated renal epithelial cells, showed maturation with increased expression of antigen presentation and costimulatory molecules, and production of TNFα. In contrast, *in vivo* studies, renal dendritic cells showed more steady-state phenotype with expression of IL-10 in response

to cisplatin treatment. Because of this difference, we decided to study renal dendritic cell function in cisplatin nephrotoxicity *in vivo* using a mouse model, which is more relevant to the findings in patients with acute renal failure than cell culture model systems. Therefore, *in vitro* results must be compared with *in vivo* findings before extensive mechanistic studies can be performed. Also, the information obtained from different models, especially the simple ones like cell culture, requires better understanding of its strength and weakness.

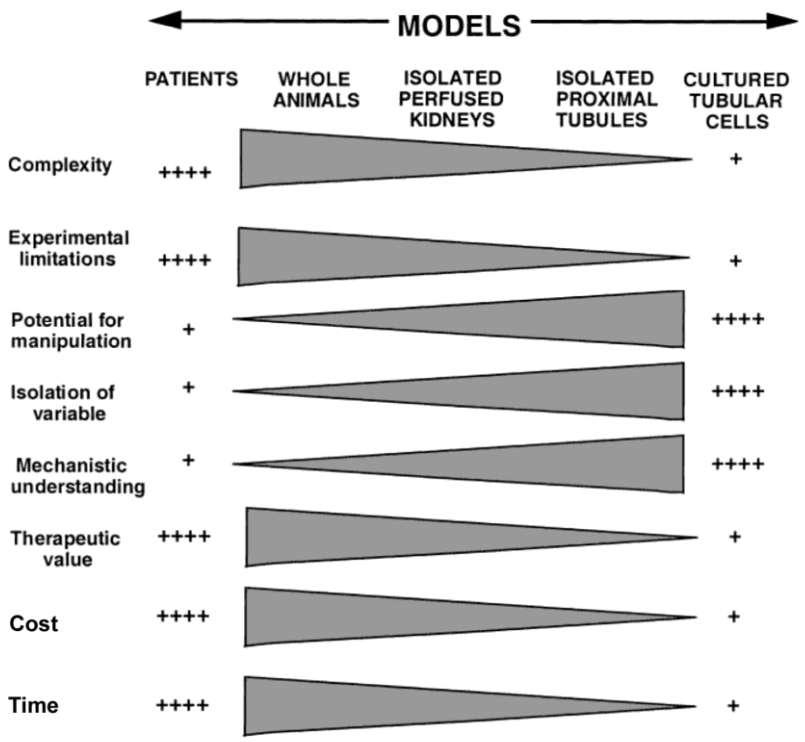


Figure 4-2. A comparison of the advantages and disadvantages of available models of acute kidney injury using a relative scale (modified from Lieberthal et al., 2000)

The pathogenesis of human acute renal failure involves complex interactions between various humoral, hemodynamic and toxic factors (Brezis and Rosen, 1995; Heyman et al., 2002; Lieberthal and Nigam, 2000; Myers and Moran, 1986). Most of the models developed to unravel the complexity of renal pathophysiology and develop strategies for therapeutics are based on single insult models such as acute kidney injury induced by toxins, ischemia, radiocontrast agents, drugs, or rhabdomyolysis. However, the pathogenesis of renal injury in these models is far different from the features of acute kidney injury observed in humans. The major reason might be the difference in physiology of animal models compared with humans. In humans, sublethal and reversible injury to renal tubular cells, by itself, causes a marked decrease in renal tubular function (Heyman et al., 2002). Compared to this, in animal models, extensive renal injury with renal tubular necrosis is required to cause significant renal dysfunction. For example in cisplatin nephrotoxicity, very limited necrosis is noticed in humans compared to relatively extensive necrosis in mice. Clinically, cisplatin nephrotoxicity is seen 10 days after cisplatin treatment in humans and may lead to subclinical but permanent renal dysfunction (Pabla and Dong, 2008). In contrast to this in mice, cisplatin mediated nephrotoxicity is noticed within 48 hr with increase in serum creatinine which may lead to mortality by 3 to 5 day after cisplatin treatment. However, cisplatin induced nephrotoxicity in mice models have some similarities with humans (Heyman et al., 2002); the dose of 6-20 mg/kg of body weight is appropriate for use in humans, induction of cisplatin nephrotoxicity in mice model is simple and reproducible, acute kidney injury occurs after a single administration of cisplatin as generally seen in humans, the pathology of proximal tubules S3 segment involvement, the tubular dysfunction such as

glycosuria, hypokalemia and hypomagnesemia, and the recovery phase in mice are comparable with that seen in humans, and also factors such as volume depletion that predispose humans to acute kidney injury are replicated in mice.

Dendritic cells are professional antigen presenting cells that function at the crossroads of immunity and tolerance. In our studies, we noticed a contiguous network of dendritic cells in the renal interstitium. To determine the function of these renal dendritic cells *in vivo* in cisplatin nephrotoxicity, we used CD11c-DTRtg transgenic mice in which renal dendritic cells can be depleted by injecting DTx. Mice depleted of these renal dendritic cells showed exacerbated renal dysfunction and structural damage with infiltration of neutrophils. Based on this, we concluded that renal dendritic cells protect the kidney from cisplatin nephrotoxicity. However, injection of DTx to CD11c-DTRtg transgenic mice causes global depletion of dendritic cells. In this regard, dendritic cells are seen in various tissues and organs. Therefore it is possible that the exacerbation of kidney injury in cisplatin nephrotoxicity was due to the depletion of extrarenal dendritic cells rather than, or in addition to, resident renal dendritic cells. Dendritic cells can be depleted in CD11c-DTRtg mice in specific tissue and organs. However, this depends on the anatomical location and relative access to them. For example, van Rajt et al., et al (2005) administered DTx intratracheally to deplete dendritic cells in lung and showed amelioration of allergen-induced asthma in dendritic cell-depleted mice (van Rijt et al., 2005). In this approach, the DTx was more restricted to the luminal space of the respiratory tract. Likewise, Denning et al., (2008) depleted dendritic cells in intestine by administering DTx orally, and showed lamina propria dendritic cell-mediated production

of IL-17 by T cells. In this model, orally administered DTx was restricted to the lumen of the gastrointestinal tract. In the kidney, renal dendritic cells are known to sample the glomerular filtrate in the tubules (Lukacs-Kornek et al., 2008). Injection of DTx into renal tubules of the nephron may deplete renal dendritic cells. However, considering the complexity of renal tubules and the difficulty in accessing the luminal side of renal tubules, it is highly impracticable. The other approach may be to deposit DTx below the renal capsule. In this approach renal dendritic cells might be specifically depleted. In addition to renal dendritic cells, renal lymph node dendritic cells may also get depleted, as DTx in the interstitium is eventually drains to the regional lymph node. However, it is possible that DTx entering into the circulation from lymph through the thoracic duct or through absorption into the renal microcirculation might deplete dendritic cells in other organs and tissues.

4.4 Future directions

Although our studies showed dendritic cell-mediated attenuation of kidney injury in cisplatin nephtotoxicity, we did not determine the mechanism other than marginal protection by IL-10 of dendritic cells. Therefore, in future studies, our objective will be to determine the other most probable means by which dendritic cells attenuate kidney injury in cisplatin nephrotoxicity. Our hypothesis is that dendritic cells, in addition to their IL-10-mediated protection, regulate T reg cells and attenuate kidney injury in cisplatin nephrotoxicity. This hypothesis is based on our studies and the recent findings on the

dendritic cell role in T reg cell homeostasis and T reg cell function in amelioration of kidney injury. To achieve our objective, future studies will be focused on:

1. **ICOSL of dendritic cells in** T reg **cell homeostasis and cisplatin nephrotoxicity (Fig. 4-1 A).** Recent studies indicate dendritic cell-mediated regulation of T reg cell homeostasis (Darrasse-Jeze et al., 2009). Darrasse-Jeze et al., (2009) noticed a marked decrease in T reg cells in response to ablation of dendritic cells using CD11c-DTRtg mice. Using *in vivo* models of autoimmunity, they showed dendritic cell-mediated increase in T reg cells and prevention of autoimmunity. Likewise, in humans, influenza peptide-pulsed immature dendritic cells caused a marked induction of IL-10 producing T reg cells (Dhodapkar et al., 2001; Steinman and Nussenzweig, 2002). In our studies, renal dendritic cells and/or non-renal dendritic cells ameliorated kidney injury in cisplatin nephrotoxicity. In addition, dendritic cells showed high expression of ICOSL in response to cisplatin treatment. In this regard, Akbari et al., (2002) showed that dendritic cells through their ICOSL regulate T reg cell functions and ameliorate airway hyperreactivity. ICOSL has been shown to ameliorate kidney injury in mice (Deng et al., 2001; Odobasic et al., 2006). T reg cells express ICOS even in the steady-state. Thus in our studies, amelioration of cisplatin nephrotoxicity by dendritic cells might be occurring indirectly through ICOSL-mediated regulation of T reg cells. In our studies, T cells were not found to be abundant in perfused kidneys before and after cisplatin treatment as determined by flow cytometry. It is possible that T cells, particularly T reg cells, present within the kidney vasculature were removed by the high pressure of perfusion. Thus, investigation into the infiltration of T reg cells in non-perfused kidneys is required. It is also possible

that non-renal dendritic cells, including dendritic cells in renal lymph nodes, regulate the function of T reg cells and ameliorate kidney injury in acute renal failure. To investigate the function of ICOSL of dendritic cells in cisplatin nephrotoxicity, first, we will deplete dendritic cells in CD11c-DTRtg mice and determine the T reg cell population in circulation, spleen, kidneys and renal lymph nodes at different time intervals after ablation of dendritic cells Then, we will explore the significance of ICOSL expression by dendritic cells on homeostasis of T reg cells using a conditional cell ablation approach in which irradiated WT mice will be injected with equal numbers of bone marrow cells derived from CD11c-DTRtg mice and ICOSL-deficient mice. Injection of DTx into these mice depletes ICOSL-expressing dendritic cells leaving behind dendritic cells deficient in ICOSL. We would determine the T reg cell population in different organs and tissues after injection of DTx. To determine the effect of ICOSL expression by dendritic cells in cisplatin nephrotoxicity, we will use ICOSL-deficient dendritic cell chimeric mice. Using these mixed chimeric mice, we will determine the course of cisplatin nephrotoxicity by analyzing serum for the level of BUN and serum creatinine, renal sections for structural changes and kidneys for infiltration of leukocytes. In this study we expect to see a decrease in the number of T reg cells after dendritic cell depletion in CD11c-DTRtg mice and CD11c-DTRtg:ICOSL KO to WT mixed chimeric mice. In mixed chimeric mice with dendritic cells deficient in ICOSL gene, we expect to see exacerbated kidney injury in response to cisplatin treatment compared with their controls.

2. T reg cells and IL-10 of T reg **cells in cisplatin nephrotoxicity (Fig 4-1B).**

T reg cells are essential for maintaining peripheral tolerance (Kim et al., 2007; Sakaguchi et al., 2008). Depletion of T reg cells causes fatal autoimmune syndrome affecting multiple organs, including development of type 1 diabetes and inflammatory bowel disease. Recently, T reg cells have been shown to attenuate kidney injury in a mouse model of renal ischemia reperfusion injury. Kinsey et al., (2009) found exacerbation of kidney injury in response to partial depletion of T reg cells (Kinsey et al., 2009). In addition, they found attenuation of kidney injury after adoptive transfer of T reg cells, but not of IL-10 deficient T reg cells, suggesting that the IL-10 of T reg cells may protect kidney in cisplatin nephrotoxicity. Likewise, Gandolfo et al., (2009) noticed swift kidney repair after transfer of T reg cells in ischemic acute kidney injury (Gandolfo et al., 2009). In our studies, we determined the protective effect of endogenous IL-10 in cisplatin nephrotoxicity. Based on reported findings in ischemic acute kidney injury, it is likely that the protection observed in the WT mice compared with IL-10 deficient mice was mediated by IL-10 of T reg cells. To investigate the possibility of T reg cell-mediated protection, we will make Foxp3-DTR/GFP to WT chimeric mice (Kim et al., 2007) and use these mice to determine the role of T reg cells in cisplatin nephrotoxicity. Foxp3 (Forkhead box P3) is a transcription factor expressed in T reg cells. In Foxp3-DTR/GFP knockin mice, cDNA encoding human DTR fused to GFP with an internal ribosome entry site is inserted into the 3' untranslated region of Foxp3 gene. T reg cells in Foxp3-DTR/GFP to WT chimeric mice will be depleted by injecting DTx. The extent of renal dysfunction in T reg cell-depleted and non-depleted mice in response to cisplatin treatment will be determined by assessing BUN and serum creatinine, renal structural

damage, and infiltration of leukocytes in kidney. By depleting T reg cells at different time intervals with respect to cisplatin treatment, we will also investigate their importance at different stages of cisplatin nephrotoxicity. T reg cells attenuate inflammation by different mechanisms, including production of IL-10. Although in our studies IL-10 of dendritic cells showed attenuation of kidney injury in cisplatin nephrotoxicity, the protection was modest. It is possible that IL-10 of T reg cells is mediating protection from cisplatin nephrotoxicity, similar to the findings in renal ischemia reperfusion injury (Kinsey et al., 2009). In this regard, T reg cell-derived IL-10 has been shown to inhibit inflammation at environmental interfaces (Rubtsov et al., 2008). To investigate the possibility of IL-10 of T reg cells protecting kidney from cisplatin nephrotoxicity, we will use a tissue specific IL-10KO model in which IL-10 is deleted in T reg cells by cre recombinase expressed under the control of Foxp3, a T reg cell-specific promoter. Cisplatin will be injected to these mice and into non-cre expressing littermates and the extent of renal dysfunction will be determined by measuring the level of BUN and serum creatinine. In response to cisplatin treatment, we expect to see an increase in renal dysfunction in T reg IL-10 KO mice compared with the non-depleted mice.

4.5 Clinical implications.

Certain immune cells, such as T reg cells and dendritic cells, have been implicated in protection against kidney injury in different models of acute renal failure (Gandolfo et al., 2009; Lee et al., 2008; Scholz et al., 2008). Likewise, administrations of immune cells that have been modified to produce certain cytokines are also reported to attenuate kidney injury in mice (Kluth et al., 2001; Wilson et al., 2002). However,

therapies using these regulatory immune cells to prevent or treat renal diseases have not been tested in humans. Considering the extent of protection observed in animal models, there is a great potential of transferring the technology from bench to bedside.

In our study, we uncovered a protective function of dendritic cells in cisplatin nephrotoxicity. Cisplatin is a chemotherapeutic agent used to treat cancer. Dendritic cells are used in cancer immunotherapy in humans (Fong and Engleman, 2000). For example, dendritic cells have been used in clinical trails to treat tumors of breast, skin, lung, colorectal, pancreas, prostate, kidney and others. Thus, it is possible to use dendritic cell as a "two-edged sword" to treat cancer and also to ameliorate kidney injury in cisplatin nephrotoxicity.

Although the results obtained in clinical trials about dendritic cell immunotherapy are provocative, the procedure used to isolate precursors of dendritic cells and to grow them in sufficient numbers is tedious. In this regard, several growth factors have been tried to culture dendritic cells in large numbers from their precursors *in vivo* (Dong et al., 2002). Flt-3 ligand is a growth factor that captured the interest of numerous researchers because of its ability to induce proliferation of dendritic cells in both *in vivo* and *in vitro*.

Flt-3 ligand (fms like-tyrosine kinase), is a 30KDa glycoprotein that causes a dramatic increase in the proliferation of dendritic cells (Dong et al., 2002). The capacity to induce dendritic cells by Flt-3 ligand is significantly high compared with other hematopoietic growth factors such as GM-CSF, G-CSF and M-CSF in mice

(Maraskovsky et al., 1996). Mice treated with Flt-3 ligand for 9 days have shown a 17-fold increase in dendritic cells in spleen, 4-fold increase in lymph nodes and 6-fold increase in peripheral blood. Systemic administration of Flt-3 ligand is reported to cause an antitumor response in different cancer models (Esche et al., 1998; Lynch et al., 1997). In humans, Flt-3 was shown to induce development of dendritic cells. (Maraskovsky et al., 2000; Marroquin et al., 2002). In a phase 1 clinical study, administration of Flt-3 ligand up to 100mg/kg/day for 14 days was well tolerated in healthy humans. Also, Flt-3 ligand produced an over 40-fold increase in the absolute number of dendritic cells in humans. Thus, it is possible that administration of dendritic cells or Flt-3 ligand to humans before cisplatin therapy can attenuate kidney injury with concomitant protection from cancer.

IL-10 is a pleiotropic cytokine with anti-inflammatory, immunosuppressive and immunostimulatory properties (Moore et al., 2001). IL-10 is generally considered a suppressor of cell-mediated immune responses and activator of humoral immune responses. The protective or deleterious effects of endogenous or administered IL-10 depend on the stage and type of disease. For example, in infections with intracellular pathogens, where cell-mediated immune response is very critical to eliminate the pathogen, low IL-10 increases resistance and high IL-10 increases susceptibility to disease. Conversly, in rheumatoid arthritis, where cell-mediated immune responses are believed to play a pathogenic role, endogenous or administered IL-10 attenuates injury to joints. The beneficial effects of IL-10 therapy may also depend on the timing of administration. For example, IL-10 pretreatment, in contrast to posttreatment, causes

improved graft acceptance in transplantation. In most studies which have examined the role of endogenous IL-10 in modulating immune responses and, in turn, disease outcome, the cell types which produce IL-10 and the surrounding microenvironment where IL-10 is produced were not determined. As IL-10 is produced by different cell types, such as Th2 cells, T reg cells, dendritic cells, macropahges, monocytes and epithelial cells, it is possible that the microenvironment where these cells produce IL-10 determines the outcome of the disease. Likewise, exogenous administration of IL-10 may have different effects compared with endogenously produced IL-10. For example, exogenous IL-10 may inhibit many of the adhesion molecules and chemokines that are required for the recruitment of anti-inflammatory cells, such as T reg cells, to the site of inflammation.

Studies in animal models have reported the beneficial effects of IL-10 in certain renal diseases, including glomerulonephritis and renal ischemia reperfusion injury (Daemen et al., 1999; Tipping et al., 1997). Likewise, administration of IL-10 has been shown to attenuate cisplatin nephrotoxicity (Deng et al., 2001). Consistent with the effects of exogenous IL-10, in our studies, endogenous IL-10 protected kidneys from cisplatin-induced ARF. In contrast to these findings, some studies found no effect or even aggravation of kidney injury due to this cytokine (Chadban et al., 1997; Ishida et al., 1994). IL-10 has been tested in patients with Crohn's disease, rheumatoid arthritis, psoriasis and other conditions. However, little information is available pertaining to IL-10 use in human renal diseases. Considering the protective functions of IL-10 in different

animal models of renal failure, its use in therapy to suppress kidney injury is very promising.

In future directions, based on previous findings on the role of T reg cells in the attenuation of kidney injury (Gandolfo et al., 2009; Kinsey et al., 2009), we hypothesized that dendritic cells regulate T reg cells and inhibit cisplatin-mediated kidney injury. So far, therapies using human T reg cells have not been tested in humans to either prevent or treat renal injury in kidney diseases (Lee et al., 2008). Many forms of kidney disease are mediated by inflammation. Considering the anti-inflammatory properties of T reg cells, there is a great potential for using these cells in treating kidney injury. However, many hurdles need to be overcome before T reg cells can be used for treatment in humans. Most of the phase 1 trials conducted in USA and Germany utilized antibody-coated magnetic-bead separation techniques to isolate T reg cells from blood (Riley et al., 2009). Blood leukocytes were first depleted of non-T cells by negative isolation. T reg cells show high expression of CD25 under steady-state conditions. The leukocytes depleted of non-T cells were enriched for T reg cells by positive selection using CD25 antibody. As CD25 is also moderately expressed on other conventional T cells, a subsaturating concentration of CD25 antibody is used to enrich T reg cells. The T reg cells isolated from this procedure gives ~50% purity as determined by flow cytometry for the expression of T reg cell markers, CD4, CD25 and Foxp3. Although Foxp3 is a specific marker for natural T reg cells, further enrichment can not be performed based on Foxp3 expression because of the intracellular expression of Foxp3. The contamination of conventional non-T reg cells, especially memory T cells, is also a major obstacle in T reg

cell therapy in humans. In addition, obtaining T reg cells in adequate numbers for treatment requires *in vitro* expansion, where proliferation of inflammatory conventional T cells at the expense of T reg cells further compromises the purity of the T reg cells. Infusion of T reg cells in large numbers is also associated with risks of immunosuppression. This suppression of immune system may precipitate infections and malignancy in the recipient. Also, isolated T reg cells can be contaminated with infectious agents and tumors of the donor. Therefore, greater understanding of therapeutic strategies of T reg cells, particularly their isolation and expansion, are required before the promise of T reg cell therapy can be transformed from benchside research to bedside treatment to humans with kidney diseases.

Appendix: TNFα receptors in ischemia reperfusion injury

A. 1. Introduction

Acute kidney injury is a serious complication affecting 5-7% of hospitalized patients with over 50% mortality. Apart from nephrotoxins, ischemia reperfusion injury (IRI) is the major cause of acute renal failure in native kidneys and in kidney allografts (Rabb, 2002; Rabb, 2006). An ischemic event of organs and subsequent restoration of blood flow (reperfusion) induce an acute inflammatory response. Studies during the past two decades, both *in vitro* and *in vivo* on renal ischemia reperfusion injury show the association of intrarenal inflammation with ARF (Bonventre and Zuk, 2004; Rabb, 2002). However, the primary event through which the cascade of inflammatory processess in the kidney is initiated has not yet been elucidated. Since renal ischemia is the major cause of ARF, there is a need to understand the inflammatory pathogenesis involved in IRI. This knowledge may eventually help us to develop strategies to ameliorate ARF through possible clinical interventions.

TNFα is a proinflammatory cytokine produced by many cell types, including renal cells in response to inflammation, infection and injury (Ernandez and Mayadas, 2009; Tracey and Cerami, 1994; Ware, 2005). TNFα was originally identified based on its ability to induce necrosis in tumors and cachexia in chronic infections. TNFα is a 26 KDa homotrimer transmembrance protein (Kriegler et al., 1988; Perez et al., 1990) that may be cleaved into a 17KDa soluble form by a metalloproteinase, the TNFα converting enzyme (Fig A1) (TACE or ADAM-17) (Black et al., 1997). TNFα is primarily released

by stimulated dendritic cells, macrophages and T cells (Agarwal et al., 2001; Dong et al., 2007). TNFα elicits a wide variety of biological functions including induction of immunity, inflammation, cell differentiation, cell proliferation and cell death.

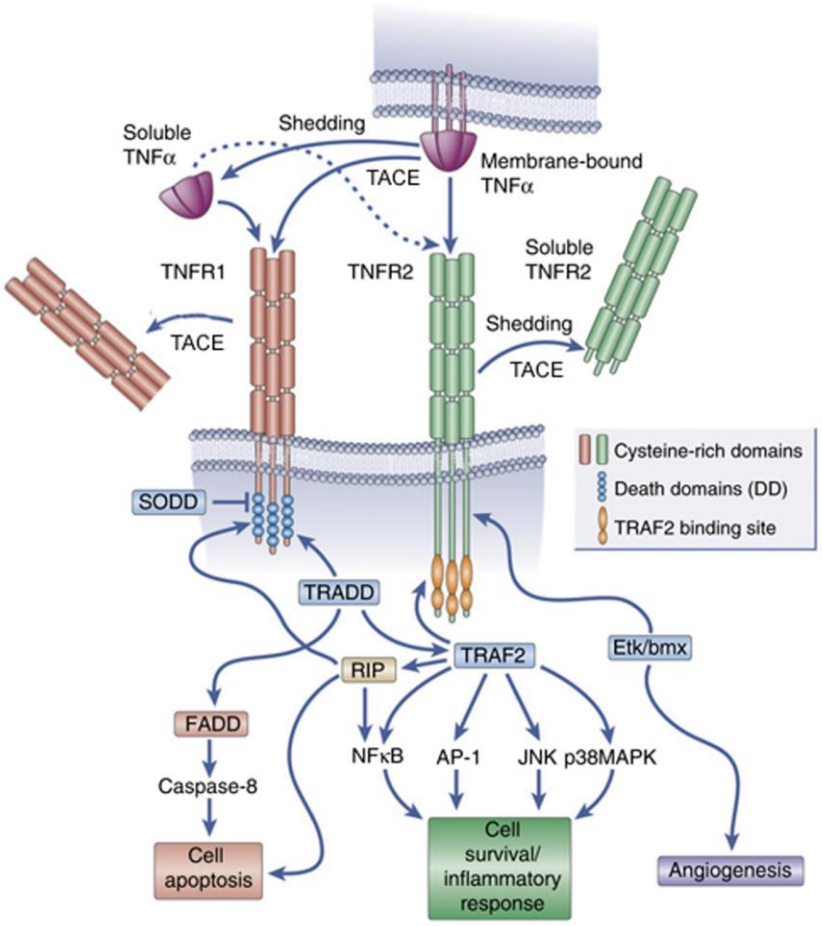


Figure A-1. TNFα signaling pathways. TNFR1, TNFR2 and TNFα are noncovalent homotrimers that can be released by metalloproteinase, TACE. TNFα binding to TNFR1 releases inhibitor SODD from death domains and allows TRADD to bind to TNFR1. TRADD can signal through FADD and induce cell apoptosis, or through TRAF2 and induce cell survival and inflammatory response genes. TRAF2 can interact with RIP (receptor interacting protein) and mediate cell apoptosis. TNFα binding to TNFR2 may induce angiogenesis by activating Etk/bmk (modified from Ernandez and Mayadas, 2009).

TNFα increases the expression of major histocompatability complex I and II molecules that are required to present antigen to T cells. It also induces the expression of adhesion molecules which aid in the migration of leukocytes to the site of inflammation. However, in addition to proinflammatory responses, TNFα can also possess anti-inflammatory properties. In this regard, TNFα causes apoptosis of activated T cells and inhibits autoimmunity (Kontoyiannis and Kollias, 2000). Production of TNFα is stimulated by bacteria, viruses and parasites commonly known as pathogen associated molecular patterns, and products released from tissue injury (Tracey and Cerami, 1994; Ware, 2005). TNFα is also produced in response to cytokines such as IL-1, IL-2, IFNγ, and by TNFα itself (Agarwal et al., 2001). Hyperthermia, certain chemicals, such as phorbol esters, phosphatase inhibitors, UV or X-ray irradiation may also stimulate TNFα production. Production of TNFα is inhibited by phosphodiesterase inhibitors, metalloprotease inhibitors, cytokines such as IL-6, IL-10 or TGFβ, inhibitors of NFκB activation, anti-oxidants, prostaglandin synthase inhibitors and a variety of other agents (Agarwal el al., 2001).

In the kidney, glomerular, mesangial and tubular epithelial cells produce TNFα in response to injury (Ernandez and Mayadas, 2009). Patients with renal diseases show significantly increased levels of TNFα. Elevated levels of TNFα are noticed in ischemia, acute transplant rejection, and in some forms of glomerulonephritits. The expression of TNFα is reported to depend on the site of renal injury. For example, TNFα is produced by renal tubular cells in renal obstruction, cisplatin nephrotoxicity, allograft rejection and IRI (Donnahoo et al., 2001; Misseri et al., 2004; Ramesh and Reeves, 2002), whereas

TNFα is induced in glomerular cells in idiopathic membranous nephropathy, IgA nephropathy and membranous nephropathy (Neale et al., 1995; Takemura et al., 1994).

TNFα mRNA and protein levels in kidney are increased very early after renal ischemia (Donnahoo et al., 1999). Neutralization of TNFα in renal ischemia reperfusion injury with TNF binding protein attenuated kidney injury. Daeman et al., (1999) noted that administration of TNFα neutralizing antibodies cause reduction in renal ischemic injury, Likewise, neutralization of IL-10, a known suppressor of TNFα production, in renal ischemia reperfusion injury exacerbated kidney injury (Daemen et al., 1999b). Exogenous administration of TNFα causes renal cell apoptosis, glomerular injury, leukocyte infiltration and finally renal failure (Bertani et al., 1989; Tomosugi et al., 1989). Studies in our laboratory have shown TNFα mediation of acute renal failure in cisplatin nephrotoxicy (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003). Cisplatin treatment causes an increase in renal TNFα mRNA and protein levels. Further studies have shown that pharmacologic or genetic inhibition of TNFα markedly ameliorates renal injury in cisplatin nephrotoxicity.

The biological activities of TNFα are mediated through two functional distinct receptors, TNFR1 (p55, CD120a) and TNFR2 (p75, CD120b) (Fig. A1). Similar to TNFα, TNF receptors are expressed on the surface of many cell types as non-covalent homotrimers, and can be proteolytically released as soluble molecules capable of binding to TNFα (Ernandez and Mayadas, 2009; Gruss and Dower, 1995). Of the two receptors, TNFα has higher binding affinity to TNFR1 than TNFR2. With respect to binding to

soluble and membrane bound forms of TNFα, TNFR1 binds equally well to both, whereas TNFR2 binds to the membrane bound form of TNFα with higher affinity compared to the soluble form of TNFα (MacEwan, 2002). TNFR1 is widely expressed by different cell types, whereas TNFR2 is primarily expressed by hematopoietic cells (Santee and Owen-Schaub, 1996).

The cytoplasmic domains of both TNFR1 and TNFR2 do not possess intrinsic enzymatic activity and need to recruit intermediate proteins to transduce signals (Ernandez and Mayadas, 2009; MacEwan, 2002). The major difference in the cytoplasmic domains between these receptors is the presence of a death domain (DD) in TNFR1, which under normal conditions is occupied by the silencer of DD protein (SODD) to prevent TNFα independent activation of TNFR1 (Tartaglia et al., 1993). Binding of TNFα to TNFR1 induces a conformational change in the receptor and allows TRADD (TNF receptor associated death domain protein) to bind to DD. TRADD can recruit FADD (Fas associated death domain protein), which can activate caspase 8 and induce apoptosis (Ermolaeva et al., 2008). TRADD can also associate with TRAF2 (TNF receptor associated factor 2 protein) and activate downstream signaling pathways, such as NFkB, AP-1, JNK and p38MAPK. These pathways are implicated in cell survival signaling and inflammatory responses. TRAF2 can also interact with RIP (receptor interacting protein) and mediate cell apoptosis. TNFR2 signaling is also been implicated in angiogenesis regulation through endothelial/epithelial tyrosine kinase (Etk/bmx) independently of TRAF2 (Pan et al., 2002).

Studies using receptor-specific blocking antibodies, ligands and genetically-deficient TNFR1 or TNFR2 mice indicate that many of the detrimental functions of TNFα are mediated by TNFR1 (Barbara et al., 1994; Pfeffer, 2003). However, TNFR2 is known to support TNFR1 activation by forming heterocomplex with TNFR1 (Declercq et al., 1998; Lucas et al., 1998; Pinckard et al., 1997) . In addition, TNFR2 may also recruit TNFα for signaling through TNFR1 (Tartaglia et al., 1993). Depending on the initial insult, TNFα may vary in its ability to mediate renal injury. For instance, in endotoxemia-induced acute renal failure, TNFα acting directly on TNFR1 exacerbates kidney injury (Cunningham et al., 2002). In contrast, TNFα binding to TNFR2 causes kidney injury in cisplatin nephrotoxicity (Ramesh and Reeves, 2003). Likewise, TNFα binding to TNFR2 expressed on renal cells mediates development of glomerulonephritis in mice (Vielhauer et al., 2005).

Although TNFα and TNFR1/R2 have been implicated in the pathogenesis of different renal diseases (Bertani et al., 1989; Ramesh and Reeves, 2003; Tomosugi et al., 1989), the effect of TNFR1 and TNFR2 signaling with respect to renal function in ischemic renal injury remains largely unknown. Previous studies in our laboratory have shown TNFα mediation of cisplatin nephrotoxicity through TNFR2 (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003; Ramesh et al., 2007). The present study examined the effect of TNFR1, TNFR2 or TNFR1/R2 deficiency in renal ischemia reperfusion injury. We show that both TNFR1 and TNFR2 mediate renal ischemic acute kidney injury in mice.

A.2. Methods

A.2.1 Mice

Experiments were performed using 8- to 10-week old TNFR1 knockout (B6.129-Tnfrsf1atm1Mak/J), TNFR2 knockout (B6.129S2-Tnfrsf1b$^{tm1Mwm/J}$) mice, and TNFR1/TNFR2 double knockout (B6;129S-Tnfrsf1a^{tm1Imx} Tnfrsf1b$^{tm1Imx/J}$). For TNFR1KO and TNFR2KO experiments, C57BL/6J mice were used as controls, whereas B6129SF2/J mice were used as controls for TNFR1/R2 KO experiments. All mice were purchased from Jackson Laboratory and housed in a barrier facility at The Penn State Hershey College of Medicine. Mice were used within two weeks after they were purchased.

TNFR1KO mice were made by inserting a neomycin cassette at position 535 of the coding sequence of TNFR1 gene (Pfeffer et al., 1993). TNFR2KO mice were made by disrupting exon 2 of TNFR2 gene by inserting neomycin cassette (Erickson et al., 1994). The double-receptor knockout mice (TNFR1/R2) were generated by intercrossing single receptor-deficient mice. Male mice were used for the experiments. Animal were used according to protocols approved by the IACUC of The Penn State Hershey College of Medicine.

A.2.2 Induction of renal ischemia reperfusion injury

To induce renal IRI, mice were anesthetized using pentobarbital sodium injection (50 mg/kg body weight), intraperitoneally and right and left flank incisions were made to

expose the kidneys. Both renal pedicles were clamped using non-traumatic microvascular clamps for 28 min. Renal ischemia was confirmed visually by a change in colour of the kidneys. During the procedure animals were kept at a constant temperature (approximately 37^{o}C) on a heating pad with warm saline-soaked gauze covering the surgical site. After removing the clamp, the muscle layer was closed with suture and the skin with stainless steel staples. Similarly, sham surgery was performed except for the clamping of renal vessels. One ml of saline was administered after surgery to replace fluid loss by subcutaneous route. Mice were observed in a warm cage until they awoke and started drinking.

A.2.3 Renal function assessment

Blood was collected from tail vein at different time points after cisplatin injection using heparin coated microvette tubes (Sarstedt). Blood cells were removed by centrifugation at 2000 g for 5 min and renal function was determined by measuring blood urea nitrogen (VITROS DT60II chemistry slides, Ortho-Clinical Diagnostics, Rochester, NY) and serum creatinine (DZ072B, Diazyme Labs, CA).

A.2.4 Statistical analysis

Results were expressed as mean±SE. All data were analyzed using an unpaired, two-tailed Student t test. A value of $P<0.05$ was considered significant.

A.3 Results

A.3.1 TNFR1 deficiency in renal ischemia reperfusion injury.

TNFR1 signaling is widely implicated to cause inflammatory response, particularly in sepsis (Cunningham et al., 2002; Vassalli, 1992; Zanotti et al., 2002)). In order to determine the effect of TNFR1 signaling in renal ischemic injury, we induced renal ischemia in TNFR1-deficient mice and assessed renal function at different time points during reperfusion by measuring BUN and serum creatinine (Fig. A2). TNFR1-deficient mice showed levels of BUN and serum creatinine at 0 hr that were comparable with those of WT mice. This finding indicates that TNFR1-deficient mice were normal with respect to renal function before induction of renal ischemia. Likewise, sham-operated TNFR1KO mice showed similar levels of BUN and serum creatinine compared with sham operated WT mice. This finding indicates that the surgery, by itself, did not cause renal injury in these mice. Renal ischemia reperfusion injury induced WT mice showed an increase in the level of BUN and serum creatine, indicating that the ischemic insult and reperfusion caused renal injury. TNFR1KO with renal ischemia reperfusion injury developed renal dysfunction to a similar extent as did WT mice, indicating that TNFR2 alone can mediate ischemic acute renal failure in mice.

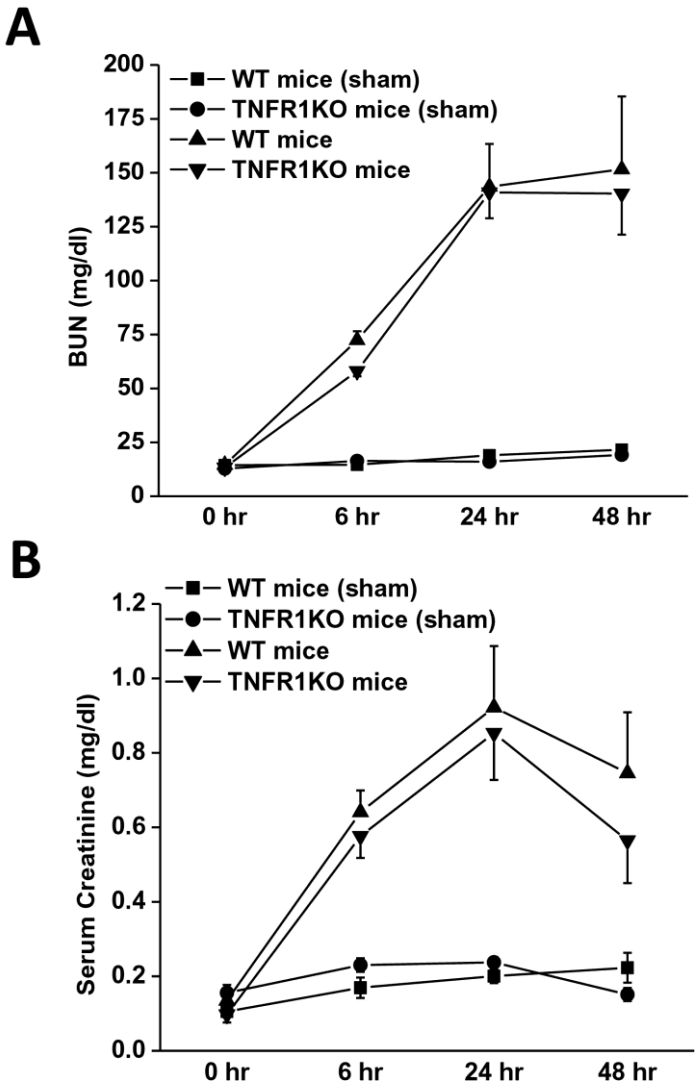


Figure A-2. Effect of TNFR1 deficiency on renal ischemia reperfusion injury. WT and TNFR1KO mice were subjected to renal ischemia or sham surgery. Blood collected at different time points with respect to renal ischemia or sham surgery was analyzed for BUN (A) and serum creatinine (B) as a measure of renal function. n = 5-12/group.

A.3.2 TNFR2 deficiency in renal ischemia reperfusion injury.

TNFR2 is reported to cause renal injury in cisplatin nephrotoxicity (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003). Vielhauer et al., (2005) showed that renal cell expressed TNFR2 is essential for the development of glomerulonephritis in mice (Vielhauer et al., 2005). Having found no amelioration of renal ischemia reperfusion injury in TNFR1KO mice, we investigated the effect of TNFR2 deficiency on renal ischemia in mice (Fig. A3). The level of BUN and serum creatinine in TNFR2KO mice measured before induction of ischemic renal injury or after sham surgery was low and comparable with that of WT mice, indicating normal renal function in TNFR2KO mice. TNFR2 deficient mice with renal ischemic injury showed renal dysfunction similar to that of WT mice, indicating that both TNFR1 and TNFR2 can cause ischemic acute renal failure in mice.

A.3.3 TNFR1/R2 deficiency in renal ischemia reperfusion injury.

TNFα is known to induce renal injury in different models of acute renal failure either through TNFR1 or TNFR2 (Ramesh and Reeves, 2002; Ramesh and Reeves, 2003; Vielhauer et al., 2005). In our studies, neither TNFR1 nor TNFR2 deficiency alone ameliorated ischemic renal injury in mice. We thus investigated the effect of deficiency of both TNFα receptors on renal function in ischemic injury (Fig. A4). The levels of BUN and serum creatinine in TNFR1/R2KO mice determined before induction of ischemic renal injury, or after sham surgery were low and comparable with those of WT mice, indicating normal renal function in TNFR1/R2KO mice and after sham surgery. WT mice showed renal dysfunction with elevated level of BUN and serum creatinine in

response to the ischemic insult. In contrast, TNFR1/R2 deficient mice showed attenuation of kidney injury as determined by lower levels of BUN and serum creatinine compared with WT mice. This indicates the requirement for both TNFR1 and TNFR2 deficiency for the attenuation of kidney injury in ischemic acute renal failure.

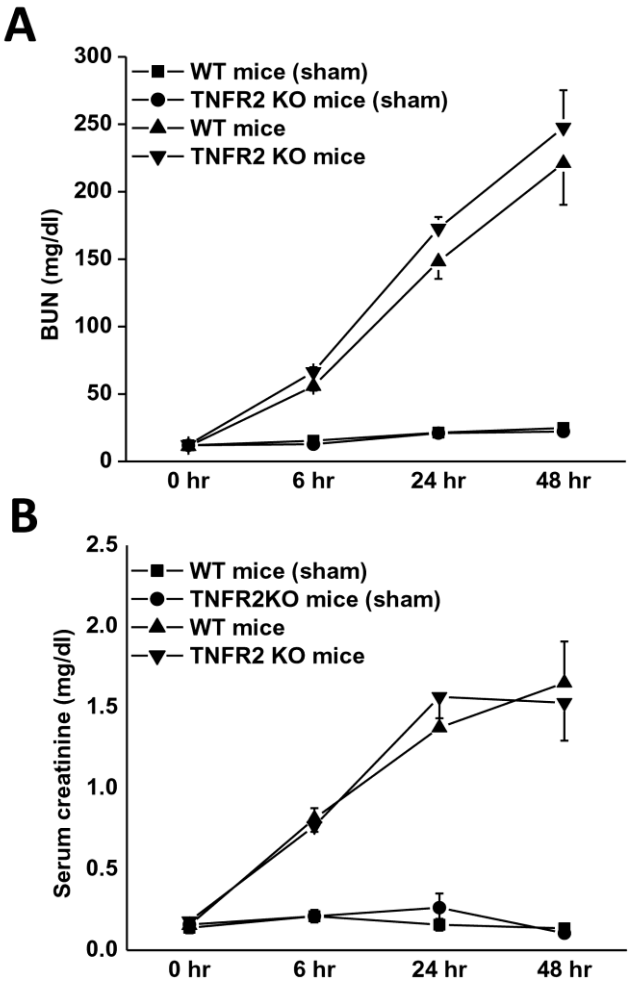


Figure A-3. Effect of TNFR2 deficiency on renal ischemia reperfusion injury. WT and TNFR2KO mice were subjected to renal ischemia or sham surgery. Blood collected at different time points with respect to renal ischemia or sham surgery was analyzed for BUN (A) and serum creatinine (B) as a measure of renal function. n = 5-11/group.

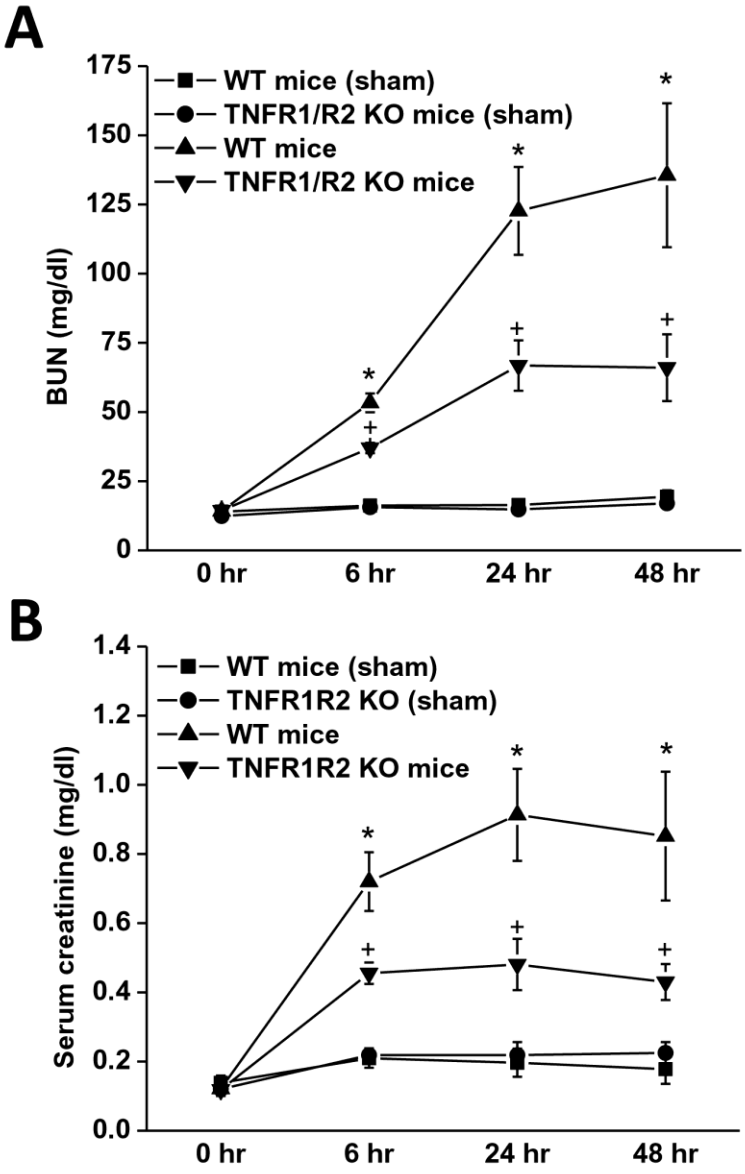


Figure A-4. Effect of TNFR1/R2 deficiency on renal ischemia reperfusion injury. WT and TNFR1/R2 KO mice were subjected to renal ischemia or sham surgery. Blood collected at different time points with respect to renal ischemia or sham surgery was analyzed for BUN (A) and serum creatinine (B) as a measure of renal function. $^{*,+}P<0.05$ vs. all other groups. n = 5-10/group.

A.4. Discussion

TNFα is an important mediator of inflammatory immune mediated disorders and plays a vital function in the induction of acute or chronic renal failure ((Donnahoo et al., 1999; Ramesh and Reeves, 2002; Ramesh and Reeves, 2003; Vielhauer et al., 2005). Renal ischemia reperfusion injury is the major cause of renal dysfunction in acute kidney injury. Although TNFα has been implicated in the pathogenesis of ischemic renal failure (Donnahoo et al., 1999), the role of the TNFα receptors in renal ischemia reperfusion injury has not been established. In this study, we investigated the effect of TNFR1, TNFR2 or TNFR1/R2 deficiency on renal function in ischemia reperfusion injury. Using TNFα receptor deficient mice, we determined that neither TNFR1 nor TNFR2 deficiency alone protects mice from renal ischemia reperfusion injury. We also examined the effect of both TNFR1 and TNFR2 deficiency on renal ischemic injury. Our results indicate that deletion of both TNFα receptors is required to ameliorate ischemic kidney injury Thus, TNFα actions on either of the receptors is sufficient to cause renal dysfunction in renal ischemia reperfusion injury.

TNFR1 and TNFR2 are expressed by most cell types. Mice deficient in one or both TNF receptors are overtly normal, indicating that signaling through these receptors is not required for normal mouse development (Peschon et al., 1998). In our studies,both single and double receptor deficient mice showed basal levels of BUN and serum creatinine that were comparable with those of WT mice, indicating normal renal function in these mice. Likewise, WT, TNFR1KO, TNFR2KO and TNFR1/R2KO mice that

underwent the sham operation showed no appreciable renal dysfunction which indicates that the surgery by itself did not induce impairment in renal function in our studies.

In many pathological conditions, including some renal diseases, the deleterious effects of TNFα are mediated through TNFR1. For instance, Cunningham et al., (2002) noticed attenuation of kidney injury in TNFR1KO mice in response to LPS induced acute renal failure (Cunningham et al., 2002). In their studies TNFR1KO mice showed less apoptosis in renal cells and less neutrophil infiltration following LPS administration, indicating that TNFR1 mediates kidney injury in endotoxemia. In our studies, deficiency of TNFR1 did not protect kidneys from renal ischemic injury, indicating that TNFR1 is redundant in the presence of TNFR2 in renal ischemia reperfusion injury. The differences in our observations with those of LPS induced renal injury might be due to the difference in pathogenesis between these two models of acute renal failure. For example, LPS causes a severe systemic inflammatory response, whereas in renal ischemia reperfusion injury, the inflammation is largely restricted to kidneys.

TNFR2 has been implicated to mediate injury in different diseases. For example, TNFR2 deficient mice show attenuation of inflammation in chronic intestinal inflammation in mice (Mizoguchi et al., 2002). In collagen induced arthritis, TNFR2 mediates induction of collagen induced arthritis in mice (Tada et al., 2001). Veilhauer et al., (2005) reported renal cell expressed TNFR2 mediation of kidney injury in glomerulonephritis (Vielhauer et al., 2005). Likewise, Akassoglou et al., (2003) found that TNFR2 mediated an inflammatory response to ischemia in the central nervous system (Akassoglou et al., 2003). As our studies using TNFR1KO mice did not show

attenuation of ischemic kidney injury, we investigated the effect of TNFR2KO deficiency on renal ischemia reperfusion injury. Similar to the findings for TNFR1KO, TNFR2 deficiency was found not to be protective against renal ischemia.

The results from TNFR1KO and TNFR2KO mice studies indicated a possible redundancy for TNFα signaling in renal ischemia reperfusion injury. Previous studies by Daeman et al., (1999) on renal ischemia reperfusion injury have shown amelioration of kidney injury to anti- TNFα antibody treatment (Daemen et al., 1999). However, anti TNFα antibody such as infliximab and adalimumab induce cell lysis when bound to the membrane-bound form of TNFα (Ernandez and Mayadas, 2009; Lugering et al., 2001; Reimold, 2003). Macrophages, monocytes and dendritic cells produce TNFα and their ablation using liposomal clodronate attenuates renal ischemia reperfusion injury (Dong et al., 2007; Jo et al., 2006). In Daeman et al. (1999) studies it is possible that anti TNFα antibody treatment eliminated monocytes, macrophages and dendritic cells, at least to some extent. It is possible, then, that the attenuation of kidney injury they observed may have been due to changes in these cell populations rather than to disruption of TNFα signaling. Therefore, we investigated the effect of both TNFR1 and TNFR2 deficiency on renal ischemia reperfusion injury. Our results demonstrated an attenuation of kidney injury in TNFR1/R2 deficient mice, indicating for the first time that TNFα signaling through both TNFR1 and TNFR2 mediates kidney injury in renal ischemia reperfusion injury.

In summary we have determined the course and effect of TNFα signaling through TNFR1, TNFR2 or TNFR1/R2 in renal ischemia reperfusion injury in mice. TNFα signaling through either TNFR1 or TNFR2 can induce acute renal failure in response to renal ischemia. Deficiency of both receptors is required for attenuation of kidney injury in ischemia reperfusion injury.

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HONORS AND AWARDS

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1996-1998	Junior Research Fellowship from Indian Veterinary Research Institute
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RESEARCH EXPERIENCE

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PUBLICATIONS

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