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Activated Neutrophils Mediate KIM-1 Shedding and Renal Remodelling

Shreyas Lingadahalli
Cleveland State University

ACTIVATED NEUTROPHILS MEDIATE KIM-1 SHEDDING AND RENAL REMODELLING

SHREYAS LINGADAHALLI

Bachelor of Medicine, Bachelor of Surgery

Rajiv Gandhi University of Health Sciences

Bangalore India

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Cleveland State University

by

_____

Dr. Thomas McIntyre, Ph.D.

Department of Cellular and Molecular Medicine, Cleveland Clinic

_____

Date

_____

Dr. Crystal Weyman, Ph.D.

Department of Biological Geological and Environmental Science, Cleveland State University

_____

Date

_____

Dr. Christine Moravec, Ph,D.

Heart and vascular Institute, Cleveland Clinic

_____

Date

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ABSTRACT

Kidney performs a complex task of concentrating the urine, retention of salts and protein and excretion of metabolic toxins and by the virtue of its function, is always under the chemical stress and subject to constant tissue damage. Post injury, nephrons have the ability to regain their function by remodeling including, clearing of apoptotic and necrotic debris. Kidney Injury Molecule-1(KIM-1/TIM-1/HAVCR-1) is a phosphatidylserine receptor that recognizes the apoptotic bodies and directs them to the lysosomal degradation. KIM-1 a type I transmembrane glycoprotein, although not constitutively expressed, is expressed in injured epithelial cells. It is known that the extracellular domain is cleaved by, MMP-9 and MMP-3, and the cleaved protein can be detected in the urine, making it a sensitive and non-invasive biomarker of renal injury. In rat renal injury model we observed neutrophil infiltration at the site of injury. To understand the role of neutrophis and its relation to KIM-1, we hypothesized, **activated neutrophils increases shedding of the KIM-1 extracellular domain via MMP activity and thus reducing the uptake and clearing of apoptotic bodies.** The purpose of the study is to determine if activated neutrophils can cause KIM-1 shedding and identify the MMP involved in this cleavage. Immortalized human proximal tubular (HK2) cells were

treated with supernatant from PAF (pro-inflammatory lipid) activated neutrophils. We observed an increase shedding of KIM-1 on treatment with activated neutrophil supernatant and that shedding was blocked after pretreatment

of neutrophils with an MMP-9 inhibitor. Pretreatment with MMP-3 inhibitor did not show any change. We also found KIM-1 mediated uptake of apoptotic bodies is reduced after the KIM-1 ectodomain shedding .Our study suggests that activated neutrophils can cause KIM-1 shedding via MMP-9 that consequentially reduces the phagocytic activity of the epithelial cells and thus affect the kidney remodeling

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

1.1 Acute renal failure and its significance

Acute Renal Failure (ARF), previously known as Acute Renal Injury or Ischemic Renal Injury (IRI) (Bellomo et al., 2004), is a syndrome characterized by rapid loss (ranging from hours to days) of kidney function with the accumulation of metabolic wastes such as urea, creatinine, uric acid and many other metabolites. It is usually associated with reduction in the urine output (not in all cases), increased serum potassium and sodium concentrations, and increased metabolic acids (Bellomo et al., 2004). ARF carries a high risk of both short-term and long-term mortality and morbidity (Ali et al., 2007). Despite advances in pharmacological intervention, there is no marked reduction in mortality and morbidity. Over the years, research has stressed the need for detecting ARF in the early stages. Intervention at the early stages of the disease progression not only reduces mortality and morbidity, but also favors better prognosis with complete regeneration of the renal functions. Urinary MMP-9, IL18, NGAL, and KIM-1 are among

the renal injury biomarkers in different stages of investigation. Of these, KIM-1 has emerged to be a very sensitive and specific biomarker for renal injury; it is better than traditional markers such as serum creatinine and blood urea nitrogen (BUN) (Vaidya et al., 2010). The pathogenesis of ARF is complex and involves endothelial and epithelial cell death, alterations in microvascular blood flow, intra-tubular obstruction, and immunological and inflammatory processes. Of the many components involved in this complex mechanism, the role of infiltrating leuckocytes, especially neutrophils and the epithelial cell death by apotosis and its clearance, are of special interest to this study. This study ventures to explore the role of activated neutropils, their cross talk with other molecules, and their effects on pathophysioloy of AFR and renal repair. Ischemic reperfusion injury (IRI) is the most common etiology of ARF, and most of the understanding of ARF comes from the murine model of IRI. Recent studies in the murine model of IRI have demonstrated the role of apoptotic cells in inflammation, and the importance of rapid clearing of apoptotic cells to prevent further inflammation and immunological reactions (Kelly et al., 2001). KIM-1, apart from being a sensitive biomarker, is suggested to play an important role in the pathogenesis and subsequent renal repair. KIM-1 is a scavenger receptor, and is involved in the clearing of apoptotic/necrotic bodies (Ichimura et al., 2008). This study is focused towards understanding the role of activated neutrophils, medicated KIM-1 modulation, and its overall affect on apoptotic/necrotic cell uptake and renal repair.

1.2 Current treatment modalities for ARF

The current treatment modalities for ARF can be broadly divided into non-dialysis treatment and dialysis treatment. The non-dialysis treatment is focused on reversing the underlying cause and reversing its metabolic consequences, which include volume overload, solute overload (hyperkelemic acidosis; uremia), endocrine deficiencies, and non-renal disturbances like GI bleeding, delirium, sepsis, respiratory dysfunction, and cardiac arrest (Alkhunaizi and Schrier, 1996, Conger, 1995). Even with the newer pharmacological agents like loop diuretics (Nigwekar and Waikar, 2011), mannitol, Fenoldopam (dopamine-1 receptor agonist that decreases vascular resistance and increases renal blood flow) (Denton et al., 1996), atrial natriuretic peptide (ANP) (Allgren et al., 1997), and insulin-like growth factor -1 (Petrinec et al., 1996), there has not been any significant reduction in mortality and morbidity associated with ARF or improvement of renal regeneration (Westenfelder, 2011).

Dialysis therapy, or renal replacement therapy (RRT), includes continuous renal replacement therapy (CRRT) and intermittent renal replacement therapy (IRRT). 70% of all patients with ARF in the intensive care unit require RRT (Davenport et al., 1991). IRRT has been the only available mode of therapy. The disadvantage of this procedure is that it is not well tolerated by patients with hemodynamic instability. CCRT, which was first introduced in 1977, involves slow and continuous dialysis, and it is well tolerated by most patients. However, it has the disadvantage of being a complicated, expensive procedure and carries increased risk of infections. Many non-randomized and

retrospective studies in comparing CRRT vs. IRRT have shown no significant differences in the mortality rates of the patients (Kellum et al., 2002). Even with the RRT therapy, the mortality is as high as 50% in critically ill patients (Star, 1998). This suggests a need for better understanding of the molecular mechanism of ARF and developing novel therapies aimed at improving renal regeneration. KIM-1, a scavenger receptor, may play an important role in renal regeneration and hence, better understanding of its molecular mechanism and exploiting it as a treatment modality are prime goals of this study.

1.3 Hypothesis and specific aims

Neutrophils play an important role in the pathogenesis of ARF. The goal of this study is to understand the interaction of neutrophils with KIM-1 and its role in pathogenesis and epithelial cell repair. Hence we hypothesized: **activated neutrophils increase the KIM-1 ectodomain shedding via MMP-9 activity and thereby reduce the uptake of apoptotic bodies by renal tubular epithelial cells.**

Specific aim 1) Understand the role of neutrophils in KIM-1 modulation. Determine if activated neutrophils mediate KIM-1 ectodomain shedding.

Specific aim 2) Determine the effect of neutrophils on KIM-1 mediated apoptotic cell uptake.

Specific aim 3) Identify the matrix metalloproteinase (MMP) involved in the cleavage of the KIM-1 ectodomain.

1.4 Organization of thesis

Chapter II provides detailed background information on etiology and pathogenesis of ARF, and the role of leukocytes and apoptotic/necrotic cells in the inflammation process; KIM-1 structure, expression, and renal and extra-renal functions of KIM-1. The mechanism of renal regeneration, the role of KIM-1 in clearing the apoptotic/necrotic cells, and possible role in renal regeneration will be discussed. Relevant anatomy and the physiological role of the kidney will also be discussed. Materials and methods used in this study will be discussed in chapter III. The results obtained from these experiments will be discussed in chapter IV. Conclusions and future directions will be discussed in chapter V.

CHAPTER II

BACKGROUND

2.1 Kidney

2.1.1 Gross anatomy of the kidney

The kidney is one of the most versatile organs in the body, performing several essential and regulatory functions. It is one of the vital components of the human urinary system. Human healthy kidneys are reddish brown in color and are situated behind the peritoneum on either side of the vertebral column. Figure 1 depicts the gross anatomy of the kidney. The upper border of the kidney is marked at the level of the 12th thoracic vertebra and the lower border at the 3rd lumbar vertebra. The right kidney lies about 1cm lower than the right kidney to accommodate the liver. The dimensions of adult kidneys are approximately 11cm X 6cm X 3cm, and it weighs about 150 grams in males and 135 grams in females. The internal microstructure of the kidney can be divided into an internal medulla and an external cortex. The renal medulla consists of pale, striated, conical renal pyramids with a peripheral base and apices converging towards papillary sinuses. The subcapsular renal cortex arches over the base of the pyramids. It is divided

from the medulla by tangential blood vessels. The cortex can be histologically divided into outer and inner cortexes. The cortex closest to the medulla is sometimes referred to as the juxtamedullary cortex. The renal artery, a direct branch of the abdominal aorta, supplies about 20% of the total cardiac output to the pair of kidneys. The renal artery divides into lobar arteries, which supply each pyramid. These further divide into interlobar, arcuate, and interlobular arteries. The lateral rami of the interlobular arteries continue as afferent arterioles, which carry the blood through the glomuruli and efferent arterioles carry the filtered blood away from the glomeruli. The venous drainage is by the renal veins, which drain to the inferior vena cava (Standring S 2009).

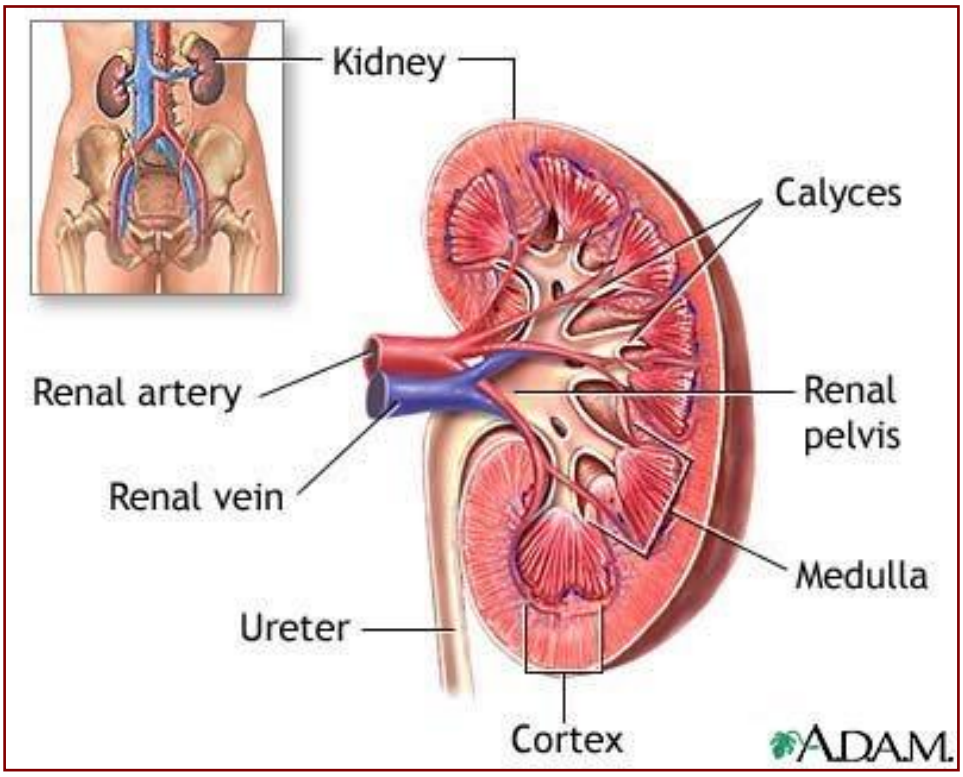

Figure 1: Gross anatomy of human kidney. Adopted from http://www.infobarrel.com/External_Anatomy_of_the_Kidney

2.2 Nephron

A nephron comprises the functional unit of the kidney, and each kidney consists of about 1 million nephrons. The parts of the nephron are shown in Figure 2.

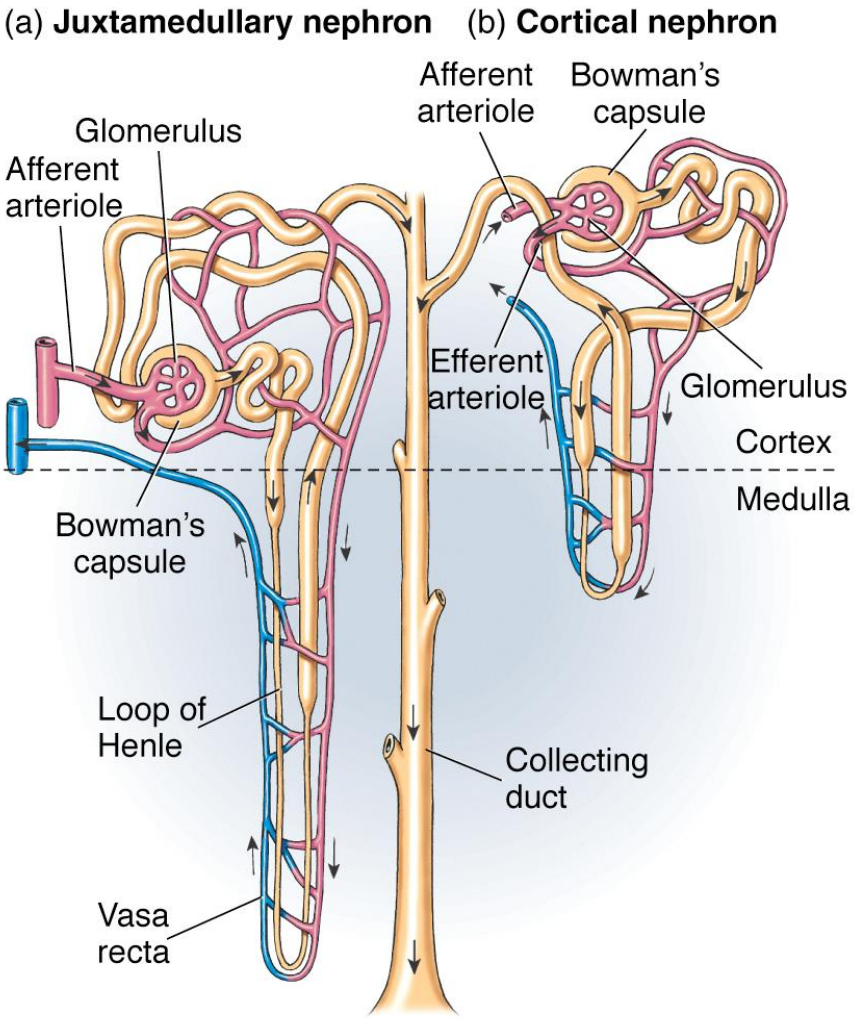

Figure 2: Parts of nephron. Adopted from www. holzbau-brunner.de

2.2.1 Glomerulus

A glomerulus is a collection of convoluted capillary blood vessels supported by a delicate mesh of mesangial matrix. The tuft of capillaries creates a mesh to filter the blood carried in by the afferent arteriole. Filtrate formed moves to the tubules and the filtered blood exits the glomerulus by the efferent arteriole (Standring S 2009).

2.2.2 Bowman's capsule

Bowman's capsule is the blind expanded end of a renal tubule. It consists of simple squamous epithelial cells on its outer wall and the inner wall is made of specialized epithelial podocytes. Podocytes are stellate cells with foot processes that branch and give rise to terminal pedicles. These pedicles form a tight junction between the neighboring pedicles, and form a narrow slit between two pedicles called the filtration slit. The filtration slit is lined by a dense membranous slit diaphragm, through which the filtrate must pass to enter the urinary space (Standring S 2009).

2.2.3 Renal tubule

The renal tubule consists of a glomerular capsule that continues as a proximal tubule, connected by a small neck. The proximal tubule continues as a coiled or convoluted tubule. As this approaches the medulla, it straightens and becomes the descending thin limb of the loop of Henle, and with an abrupt U-turn, continues as the ascending limb of Henle. Deep in the medulla, the tubules are narrower and the walls are thin. These sections are called descending and ascending thin segments of Henle's loop

respectively. The ascending thick limb of Henle continues as the distil tubule and re-enters the medulla. The distil tubule then continues as the convoluted distal tubule, and finally straightens out again to form the connecting tubule to join the collecting ducts.

The renal tubules are lined by single layered epithelial cells, and the type of epithelial cells varies according to the region and function. Proximal tubules are lined by cuboidal, or low columnar epithelial cells, with brush border of tall microvilli on its luminal surface, and basal cytoplasm, which is rich in mitochondria. The microvilli on the luminal surface increase the surface area in contact with the tubular fluid. Abundant mitochondria provide the ATP required for medullary transportation and reabsorption of ions and small molecules against the concentration gradient.

Renal tubules, based on their marked differences in structure, histology, and function, can also be divided into three segments: namely S1, S2, and S3. All three segments are characterized by unique cell type. S1 and most of the S2 segment comprises the proximal convoluted tubule. A sharp transition in the cell type from the convoluted part to the straight part of the tubule marks the boundaries of S3 segment (Standring S 2009).

2.3 Functions of kidney

Kidney performs a wide range of functions, from excretion of metabolic wastes to maintaining the normal homeostasis of the total body fluid, volume, or acid-base contents. These functions are carried out independently, or in orchestration with the many hormones secreted by the kidney. The major functions of the kidney are as follows:

A. Excretory functions: Kidney is involved in the production, concentration, and excretion of the metabolic wastes like urea, uric acid, and creatinine.

B. Osmolality regulation: Kidney regulates the osmolality, or total body fluid, by regulating the water contents of urine; i.e., when plasma osmolality drops, kidney increases water reabsorption, and secretes concentrated urine. Similarly, when the plasma osmolality increases, the kidney secretes dilute urine, and thus maintains the total body fluid. This function is under strict control of antidiuretic hormone, secreted by the posterior pituitary gland. The hormone increases the vascular permeability of the collecting duct, thus secreting dilute urine.

C. Acid base homeostasis: Kidney and lungs are the principle organs involved in the regulation of acid base balance and maintenance of pH in permissible levels. Kidneys maintain the pH levels by reabsorbing the bicarbonate ions or excreting H^+ ions.

D. Blood pressure regulation: Kidneys are involved in the maintenance and regulation of blood pressure by regulating the reabsorption of sodium chloride. This, in turn, is controlled by renin, a hormone secreted from kidney, which is a part of renin-angiotensin pathway.

E. Hormone secretion: Kidney secretes a variety of hormones, including erythropoietin and enzymes like renin. In response to hypoxia, erythropoietin is secreted, which stimulates the bone marrow for increased erythropoiesis. Calcitrol, an active form of vitamin D, facilitates absorption of calcium in the intestine and renal phosphate reabsorption. It is also involved in maintenance of bone calcium deposition.

F. **2.4 Epidemiology of acute renal failure**

The global burden of ARF and the complications associated with it are increasing. A recent epidemiological study from Scotland shows incidence of ARF as 2147 per million population per year (PMP) (Ali et al., 2007), and a large community based study in the United States shows incidence of ARF over 5000 PMP requiring no dialysis, and over 2915 PMP requiring dialysis (Hsu et al., 2007). The rate of ARF increases drastically in critically ill patients, with a reported rate of 67% in patients admitted to intensive care facility, and a frequency of 1.9% in all hospital admissions (Hoste et al., 2006). ARF is a very important cause for mortality and morbidity in hospitalized patients, with an adjusted mortality risk of 1.4 (Lafrance and Miller, 2010). Mortality of patients developing post-operative ARF ranges 24-100% and 50-70% in patients in intensive care facility on dialysis (Zanardo et al., 1994, Spiegel et al., 1991). 20-60% of patients with ARF require dialysis, and of the subgroup surviving the initial dialysis, only 25% required lifelong dialysis (Pascual et al., 1990). This shows that the syndrome can be reversed, and that the kidney possesses the property of regeneration.

2.5 Etiology of acute renal failure

ARF can result from a wide range of insults to the body. In a hospital setting, ARF is common in elderly, newborns, or patients with other serious debilitating conditions. It is usually associated with multi-organ failure, sepsis, patients on nephrotoxic drugs, and administration of radio contrast dye. The common etiological factors have been depicted in Figure 3.

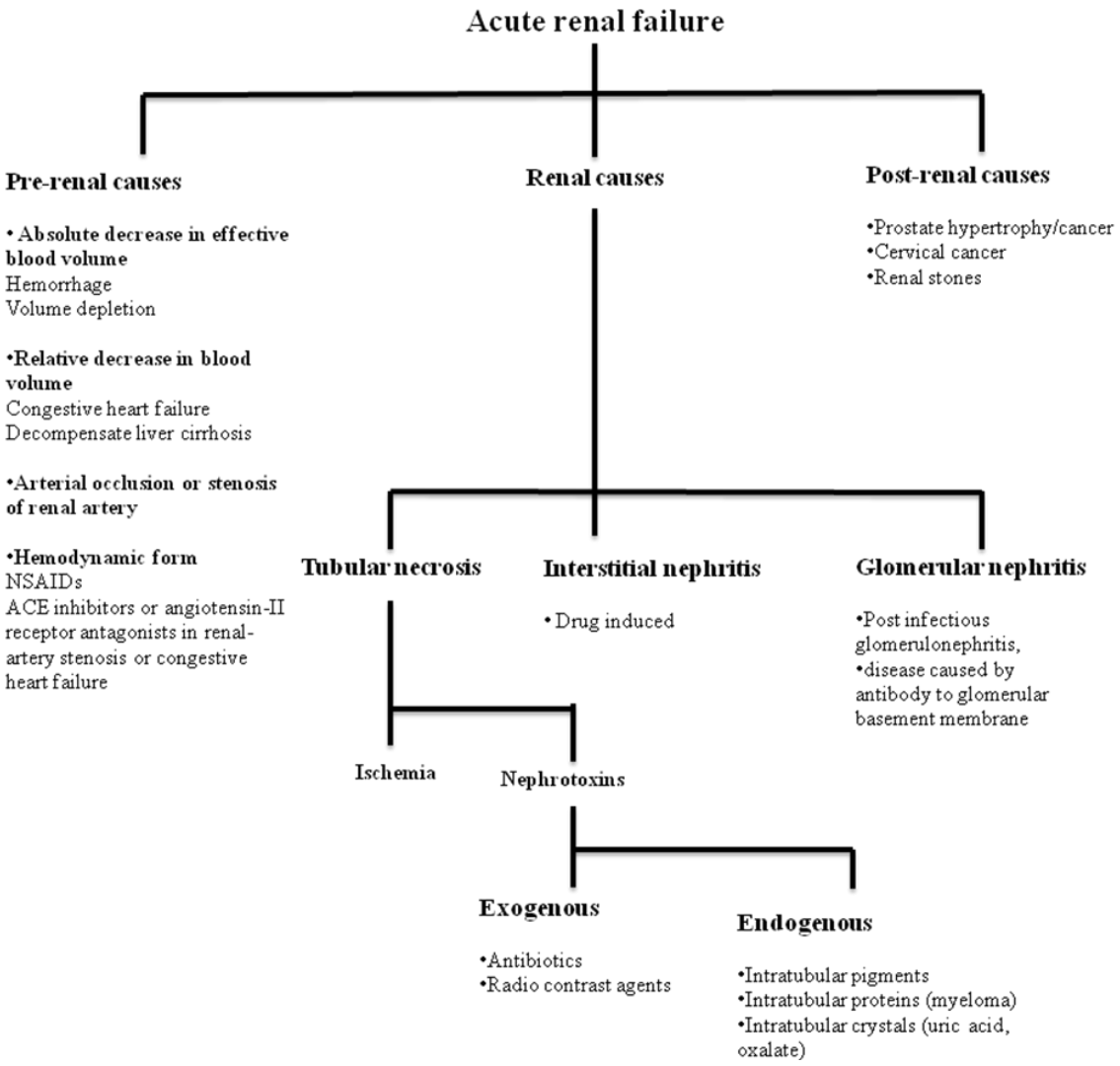

Figure 3 Etiology of acute renal failure. The figure shows, most important causes of ARF. Modified from (Lameire et al., 2005)

2.5.1 Pre-renal causes

Reduction in the perfusion of the kidney with intact tubular and glomerular function results in the reduction of renal clearance, is called pre-renal failure or pre-renal azotemia. The most common causes include severe dehydration due to diarrhea, use of diuretics, excessive vomiting, and cardiac failure (Brater, 2002, Gambaro and Perazella,

2003). Elderly people and newborns are particularly susceptible, owing to pre-existing hypovolemia state (Himmelfarb, 2009). The kidney responds to reduction in the renal perfusion or pressure, by auto-regulating glomerular filteration rate (GFR) and renal blood flow. When the pre-glomerular pressure drops gradually, the kidney maintains a constant glomerular capillary hydrostatic pressure by secreting vasodilatating agents like prostaglandin I2 (Baylis and Brenner, 1978) and NO_2 (De Nicola et al., 1992). A tubuloglomerular feedback mechanism maintains the GFR and the fluid delivery to the distil nephron. In case of acute fluid loss, there is an increased fluid absorbtion from the proximal tubule, and the tubuloglomerular feedback mechanism mitigates a reduction in GRF (Blantz, 1998, Badr and Ichikawa, 1988). In patients with reduced renal perfusion, non-steroidal anti inflammatory drugs (NSAIDS) are known to precipitate pre-renal azotemia (Shankel et al., 1992). Drugs like cyclosporine and tacrolimus cause vasoconstriction of small renal vessels leading to pre-renal azotemia (Textor et al., 1993). ACE inhibitors or ACE receptor blockers are also known to precipitate pre-renal azotemia (Franklin and Smith, 1986). In hospital settings, patients with septic shock, cardiac failure, liver diseases, or multi-organ failure are at high risk (MacDowall et al., 1998) to suffer from pre-renal azotemia. In surgical cases, postoperative and perioperative renal dysfunction, reduction in the mean arterial blood pressure, and reduction in the effective blood volume due to anesthesia, lead to pre-renal azotemia (Shusterman et al., 1987).

2.5.2 Renal or intrinsic causes.

Renal azotemia is associated with some cellular injury. The renal failure is characterized based on the site of the insult: glomerulus, vessels, interstitium, or tubules. And of these, tubular necrosis accounts for about 70% of all the cases (Nash et al., 2002), and 50% of tubular necrosis is caused by ischemic injuries. Tubular ischemic injury is usually a continuation of pre-renal azotemia, where the blood supply to the tubule is severely compromised, leading to necrosis of the tubular cells. The ischemic tubular necrosis can be reversed on early detection and by treating the root cause.

Toxic injury counts as the second most common cause of intrinsic renal failure, accounting for about 20% of all the renal failure cases (Pannu and Nadim, 2008). The most common drugs causing failure, but not limited to these, include aminoglycoside antibiotics, radio contrast dyes, heme pigments, NSAIDS, chemotherapeutic agents like cisplatin, and myeloid light chain proteins (Liangos, 2012). The toxins act directly on cells or through another mechanism, damaging them. The mechanisms of action of most common nephrotoxic drugs are discussed in Table 1. Ischemia and toxins often combine to cause acute renal failure in severely ill patients with conditions such as sepsis, hematologic cancers, or the acquired immunodeficiency syndrome (Rao and Friedman, 1995).

Mechanism of action	Drug
Reduction in renal perfusion through alteration of intrarenal hemodynamics	NSAIDs, angiotensin-convertingĞenzyme inhibitors, cyclosporine, tacrolimus, radiocontrast
Direct tubular toxicity	Aminoglycoside antibiotics, radiocontrast agents, cisplatin, cyclosporine, tacrolimus, amphotericin B, methotrexate, foscarnet, pentamidine, organic solvents, heavy metals, intravenous immune globulin
Heme-pigmentĞinduced tubular toxicity (rhabdomyolysis)	Cocaine, ethanol, lovastatin
Intratubular obstruction by precipitation of the agent or its metabolites or by-products	Acyclovir, sulfonamides, ethylene glycol, chemotherapeutic agents,methotrexate
Allergic interstitial nephritis	Penicillins, cephalosporins, sulfonamides, rifampin, ciprofloxacin, NSAIDs, thiazide diuretics, furosemide, cimetidine, phenytoin, allopurinol
HemolyticĞuremic syndrome	Cyclosporine, tacrolimus, mitomycin, cocaine, quinine, conjugated estrogens

Table 1: Mechanism of action of common nephrotoxic drugs. Adopted from (Thadhani et al., 1996)

Interstitial nephritis, glomerular nephritis, and vascular nephritis represent a very small percentage of all the ARF cases. Interstitial nephritis is most commonly caused by drug reaction. The other less frequent causes include autoimmune disease like lupus, infiltrating disease like amylodosis, and many viral infections (Cameron, 1988). Glomerular nephritis is mostly secondary to autoimmune diseases. Malignant

hypertension and vasculitis are the common reason for vascular nephritis (Graciano et al., 2007). Interstitial nephritis and glomerular nephritis are also reversible in most cases, and corticosteroids are known to hasten the recovery.

2.5.3 Post -renal causes

Obstruction to the urinary outflow at the level of the kidney or further down the urinary tract leads to increased backpressure on the kidney, resulting in structural and functional damage to the kidney. The most common causes include obstruction of the urethra by prostatic hypertrophy, prostatic cancer, or cervical cancer (Feest et al., 1993). Other less frequent causes include bilateral kidney stones, blood clots, fungal infections, retroperitoneal infection, colon and rectal carcinoma.

2.6 Pathophysiology of acute renal failure

Acute renal failure is a complex dynamic process, involving a cross-talk between the tubular epithelial cells (Ueda and Shah, 2000), basement membrane, extra cellular matrix, infiltrating leukocytes (Okusa, 2002), and vascular factors (Sutton et al., 2002). This discussion will be limited to acute tubular necrosis from ischemia/toxic injury, which, as mentioned in the section 2.5.2, accounts for more than 90% of all the ARF cases. Interestingly, tubular epithelial cell are particularly vulnerable to injury (Havasi and Borkan, 2011), more than any other cell in the nephron. In this section, the pathophysiology of acute tubular necrosis, especially resulting from ischemic renal

injury, will be discussed in detail. Pathophysiology of other non-common causes such as interstitial necrosis and glomerular nephritis, are complex and usually immune-cell-mediated (Falk and Jennette, 2010, Glassock, 2010, Lightstone, 2010) and will be beyond the scope of this study.

Most of our understanding of pathophysiology of acute tubular necrosis comes from the well-established mouse ischemia/reperfusion model (Wei and Dong, 2012). Although this model has its limitations, as the tubular injury resulting from sepsis or toxic insults are not explained (Ishikawa et al., 2010), it does give us a clear understanding of tubular necrosis secondary to pre-renal causes.

Following an ischemic insult, the complement system is activated; predominantly by an alternative pathway (Thurman et al., 2005). Complement up-regulates the endothelial binding proteins, receptors for collagen-like (cC1qR) and globular domains (gC1qR) of complement C1q (Guo et al., 1999). Complement also activates dendritic cells (DC) and C3 (macrophage derived complement protein) binding to the endothelial cells. The activated DCs in turn activate T cells (Sandor et al., 2009). Complement receptor 1-related protein y (Crry), a complement inhibitor, is expressed by the basement membrane of the proximal tubular cells (Thurman et al., 2006b). Loss of polarity and destruction of the basement membrane leads to altered expression of Crry. The inability of Crry to inhibit the complement is the key in activation of complement (Thurman et al., 2006a). Crry deficient mice, as would be expected, are more susceptible to IRI (Thurman et al., 2006a). In tubular necrosis, tubular epithelial cells are not innocent victims of injury, but play an active role in the inflammation process. They not only generate pro-inflammatory cytokines like TNF-α, MCP-1, IL-8, IL-6, IL-1β, TGF-β, RANTES, and

epithelial neutrophil-activating protein 78 (ENA-78), which activate inflammatory cells; they also express Toll-like receptors (TLRs), complement and complement receptors, and molecules that regulate T lymphocyte activity (Bonventre and Zuk, 2004). TLRs are transmembrane receptors widely expressed in leukocytes and renal epithelial cells. Increased expression of TLR-2 and TLR-4 and increased neutrophil infiltration has been observed during ischemic injury. TLR-4(-/-) mice with wild type mice bone marrow graft showed significantly less serum creatinine and histological tubular necrosis compared to wildtype mice with TLR-4(-/-) bone marrow graft after an IRI (Pulskens et al., 2008). Similarly, cisplatin treatment in mice with a targeted deletion of TLR-2, demonstrated reduced renal dysfunction, tubular necrosis serum, urinary cytokines, and chemokines compared to mice expessing TLR -2 (Zhang et al., 2008a).

Tubular epithelial cells undergo a series of alterations, as depicted in Figure 4, finally resulting in necrosis or apoptosis. Ischemia results in extensive damage to the basement membrane with a rapid loss of cytoskeletal integrity (Lameire et al., 2005). Apical membranes are damaged with the shedding of the proximal tubular brush border (Solez et al., 1979). This is followed by mislocalization of cell adhesion molecules and other membrane proteins like Na^+K^+ATPase and β-integrins (Gailit et al., 1993, Zuk et al., 1998). ATP depletion leads to an increase in intercellular free calcium concentrations, resulting in activation of proteases and phospholipases, which in turn contribute to further disruption of the cytoskeleton and impair mitochondrial energy metabolism interfering with production of ATP (Bonventre, 1993). Na^+K^+ATPase maintains the cell volume and cell polarity by maintaining a negative intracellular charge and low Na^+ concentrations. With depletion of oxygen and ATP, Na^+K^+ATPase ceases to function, leading to an

increase Na^+ and Cl^- concentration with a loss of cell polarity (Leaf, 1959, Flores et al., 1972). β-integrin dependent cell-cell, cell-matrix adhesions are disrupted by cytokines and with superimposed ATP depletion, results in disruption of cell–cell junctional complexes (Zuk et al., 1998). Disruption of the tight junction alters both cell permeability and cell polarity (Abbate et al., 1994). Loss of cell membrane integrity leads to swelling of the cells. The swollen cells block the lumen and further reduce the blood supply to already ischemic cells (Linshaw et al., 1991). This ultimately leads to activation of the apoptosis pathway. The dead and living cells slough into the lumen forming casts. The casts are known to block the lumen, increase the intertubular pressure, and reduce the glomerular filtration rate. The increase in permeability also results in back-leak of glomerular filtrates. In rat renal ischemia/reperfusion models, the majority of luminal cells were apoptotic or necrotic cell debris (Ichimura et al., 1995). These apoptotic and necrotic cells were gradually cleared as the kidney regenerated (Borregaard and Cowland, 1997).

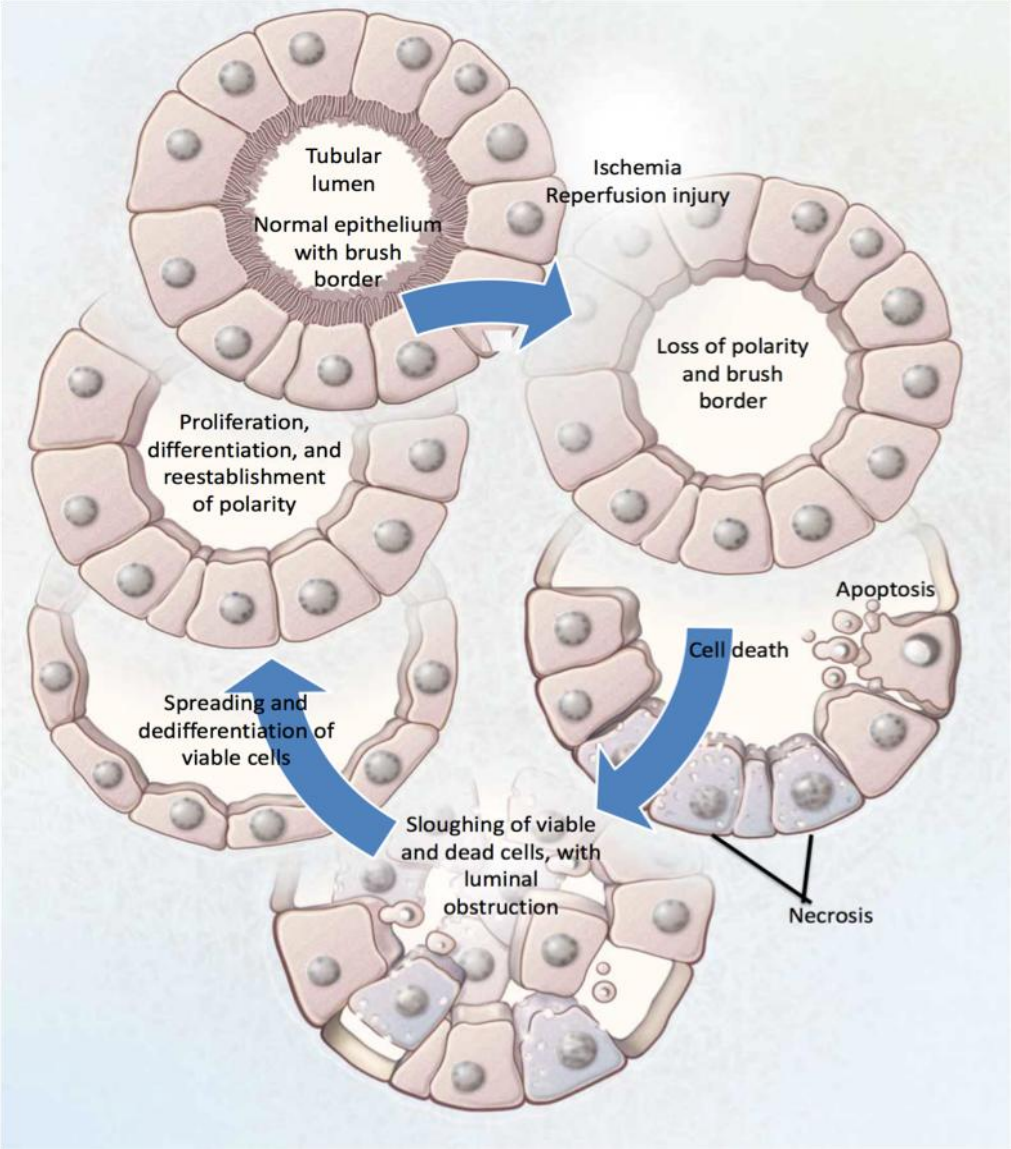

Figure 4: Tubular epithelial cell injury and repair in ischemic reperfusion injury. Modified from (Thadhani et al., 1996)

2.6.1 Role of inflammatory cells in pathogenesis of ARF

Inflammatory cells also play an important role in the pathogenesis of ARF. The roles of neutrophils, macrophages, dendritic cells, and lymphocytes in pathogenesis of

ARF are the subject of active research. However roles of lymphocytes, dendritic cells, T cells, and B cells remain controversial and are beyond the scope of this discussion. Only the role of neutrophils and macrophages in the pathogenesis of ARF will be discussed in detail.

Neutrophils constitute the majority of white blood cells, and are the first responders to the site of injury. Infiltration of neutrophils during ischemic injury has been demonstrated in large number mice and human renal biopsies (Furuichi et al., 2002, Miyazawa et al., 2002, Willinger et al., 1992, Linas et al., 1992). Though the role of neutrophils in the pathogenesis of ARF is still not clearly understood, mice depleted of peripheral circulating neutrophils were less susceptible to ischemic renal injury (Kelly et al., 1996). Neutrophils adhere to the endothelial cells through a complex mechanism involving several classes of adhesion molecules, which includes selectins, mucins, integrins, and the Ig superfamily of proteins (Rabb et al., 1997). After neutrophils adhere to the endothelial cells and chemotax ouside the vessel, they release reactive oxygen species (ROS), proteases, elastase, myeloperoxidase, and matrix metalloproteinases that further increase inflammation (Rabb et al., 1997). Chemokines and selectins are upregulated by cytokines like IL-1 and TNFα. Then chemokines recruit leukocytes and activate them, increasing adhesion (Rahman et al., 1998). Circulating or locally produced TNFα and ROS produced during reperfusion injury is also known to recruit neutrophils (Ishibashi et al., 1999). Transgenic mice overproducing anti-oxidants show less histological injury and reduced neutrophil infiltration compared to wildtype mice (Ishibashi et al., 1999). Of the many adhesion molecules involved, ICAM-1 has been studied in detail; blocking ICAM -1 by injecting anit-ICAM-1 antibody, beneficial effects

were observed in mice subjected to renal ischemia (Kelly et al., 1994). Similarly, ICAM-1 knockout mice also demonstrated reduced tubular necrosis and serum creatine, versus wild type mice subjected to ischemic injury (Kelly et al., 1996). After activation, neutrophils are known to secrete matrix metalloproteinases (MMP). In fact, neutrophils are considered the major source of MMPs in kidney, and are associated with many diseases like pylonephritis and renal tissue scarring (Tan and Liu, 2012). MMPs from neutrophils are particularly of great interest to this study, as some family members have the ability to cleave a transmembrane protein expressed from the injured tubular cells known as kidney injury molecule -1(KIM-1) (Ichimura et al., 1998). The role of KIM-1 and KIM-1 ectodomain shedding in relation to renal injury and regeneration will be discussed in detail in section 2.11.

Macrophages are derived from blood monocytes. Apart from their role as phagocytes, they are also known to secrete NO from iNOS and proinflammatory cytokines including IL-18, IL-1α, IL1β, TNF α, and Nk-kB (Akcay et al., 2009). Macrophage infiltration is observed at different stages of ARF. At early stages of AKI, they are known to increase inflammation due to their pro-inflammatory cytokines (Li et al., 2008); whereas, in the later stages they exhibit protective functions, clearing the apoptotic cells by phagocytosis and secreting anit-inflammatory cytokines (Lee et al., 2011). Depletion of macrophages using genetic techniques (Furuichi et al., 2003) or liposomal colodronate (Ferenbach et al., 2012) during AKI, has demonstrated beneficial effects; however, in a different study, depletion of macrophages in cisplatin-induced AKI had no protective effects (Lu et al., 2008). Macrophage phenotype also determines their role; M1 macrophages have pro-inflammatory effects, while M2 macrophages have

protective function (Lee et al., 2011). Hence, further studies are required to understand the exact functions of macrophages in AKI.

2.6.2 Apoptotic cells and inflammation

Apoptotic cells produced as a result of various insults need to be cleared by physical removal, and this represents the final stage in apoptosis programming (Kerr et al., 1972). Accumulation of apoptotic cells in the lumen not only blocks the lumen and reduces the GFR, but also promotes secondary inflammation. Apoptotic cells are self-contained, and thus prevent the release of intracellular contents. However, if they are not cleared rapidly, they lose their cell membrane integrity; and, over time, release their intracellular contents, which can provoke secondary inflammation and autoimmunity in the tissue (Daemen et al., 1999). Studies in the murine model of ischemic reperfusion injury show administration of IGF-1 (a growth and survival factor) and active caspase inhibitor reduced inflammation; and this is most likely because of their ability to inhibit apoptosis (Daemen et al., 1999). These findings strongly suggest that apoptotic cell death, either directly or indirectly, contributes to inflammation and autoimmune reaction. Professional phagocytes, such as macrophages, dendritic cells, and non-professional phagocytes surrounding epithelial and endothelial cells, perform an important role in clearing these cells. They are usually very efficient and rapid in clearing necrotic/apoptotic bodies (Lauber et al., 2004); and thereby reduce secondary inflammation. In the early stages of apoptosis, cells release ‘find-me’ signals, which include many chemokines. The chemokines attract the motile phagocytes to the cells

undergoing apoptosis. In the later stages of apoptosis, the cells express molecules like phosphatidylserine (PS), which are termed as 'eat-me' signals (Ravichandran and Lorenz, 2007). Among the array of eat-me signals, exposure of PS on the cell surface, which is otherwise on the inner surface of a healthy cell, is the key signal recognized by the phagocytic cells. The phagocytic cells identify and bind to these 'eat-me' signals on apoptotic bodies, and mediate their uptake (Peiser et al., 2002, Pearson, 1996). Macrophages also help in attenuating the inflammation by secreting the anti-inflammatory cytokine IL10, and reparative growth factors including TGF-β (Savill and Fadok, 2000). Uptake of apoptotic cells by non-professional phagocytes has also been shown to secrete growth-promoting factors and thus reduce further injury. Mouse mammary epithelial cells, involved in the clearing of the apoptotic Jurkat-T cells, secreted hepatocyte growth factor, TGF-β and VEGF, and also primed the cells to resist UV induced cell injury (Golpon et al., 2004). In a similar study in mice, administration of excessive apoptotic cells, or masking the PS via annexinV (to prevent the PS mediated uptake) in the mice, produced the hallmark of autoimmunity, such as autoantibody production and deposition of IgG in glomurulus (Asano et al., 2004). Defective clearing of apoptotic cells depicts strong relation with the development of autoimmune disorders like lupus, and it is due to development of antinuclear antibody against the chromatin from the ruptured apoptotic cells (Taylor et al., 2000). Hence, clearing of apoptotic/necrotic cells is necessary to attenuate the inflammation.

2.7 Tubular epithelial cell regeneration and apoptotic cell clearance

Unlike the heart or brain, kidney possesses the capability to recover completely from ischemic or toxic injury. As mentioned in earlier sections, proximal tubular necrosis comprises the majority of ARF cases. Hence, this discussion is limited to the events following tubular damage: i.e. regeneration and proliferation of the surviving proximal tubular epithelial cells, and the regaining of renal functions. After injury, surviving epithelial cells undergo a series of alterations before they can function at their normal capacity. Although the exact mechanism of the renal regeneration remains elusive, a series of studies over years has emphasized the role of apoptotic cell clearing and renal regeneration. Many theories have been proposed to identify the source of the cells that are involved in the replacement of the dead cells. First, the new cells could be produced from the resident stem cells (Oliver et al., 2004). Stem cells are self-renewing; i.e. can produce more cells of their own kind by division. They also have the capability to differentiate into different cell types by asymmetric division (Tanaka and Reddien, 2011). Extensive experiments using genetic–fate mapping techniques have demonstrated the absence of marrow-derived or renal interstitial cell-derived epithelial stem cells involved in the reparative process (Humphreys et al., 2008). Second, there are specialized progenitor cells, which have only a limited self-renewal property; they differentiate along one particular cell lineage. Using unbiased DNA analog-based lineage identification in a mouse model subjected to IRI, it was confirmed that no special progenitor cells were involved in the repair of the renal epithelial cells (Humphreys et al., 2011). Third, the surviving epithelial cells dedifferentiate to cover the loss of the neighboring cells (Pawar

et al., 1995). Of the three possible hypotheses, dedifferentiation and proliferation of surviving epithelial cells seem to the accepted mechanism.

2.8 Mechanism of renal proximal tubular epithelial cell regeneration

During homeostasis, kidney proximal tubular cells exhibit minimal proliferation as demonstrated by proliferative cell nuclear antigen (PCNA) and Ki-67 immunoreactivity (Nadasdy et al., 1994, Messier and Leblond, 1960). The proliferation is under a rigid control, and is limited to just replace the cells lost into the urine. Post injury, surviving epithelial cells rapidly enter the cell cycle and extensively proliferate to make up for the loss of the neighboring cells (Witzgall et al., 1994). The mechanism of renal repair is depicted in Figure 5. The crucial processes involved in the regeneration process are migration, proliferation, and recuperation of physiological functions.

The functional recovery of the nephron begins with the migration of the surviving epithelial cells. The mechanical scrape technique is the most widely used method to mimic the tubular epithelial cell loss. In this technique, a tract of cells is scraped from the confluent monolayer, and the capability of the surrounding cells to migrate and cover the tract is measured. Using these techniques, rabbit proximal tubular epithelial cells were scraped. It was observed that 77% of the scraped area recovered within 7 days with no treatment; whereas epidermal growth factor (EGF) stimulated more complete recovery, TGFβ inhibited the recovery. It was also observed that when the cells were treated with known neprotoxins like mercuric chloride or fumonoisin, they

displayed migratory defects and overall cytotoxicity. These results suggest the importance of migration in renal repair (Counts et al., 1995).

Dedifferentiation is a property of rapidly dividing epithelial cells. The cells dedifferentiate to attain the gene expression pattern seen in the developing nephron, which has a major implication for regulation of renal repair. The dedifferentiation process in renal tubular epithelial cells is associated with an up-regulation of genes encoding Egr-1, c-fos, NAK-1, c-myc, and heat shock protein-70 (HSP-70) (Counts et al., 1995). Several developmental genes like vimentine and Pax2, are also re-expressed (Basile et al., 1997). Vimentin is an intermediate filament protein found in the undifferentiated mesenchymal cell, otherwise not found in the adult kidney, and it is also a marker for dedifferentiated epithelial cells. Cell adhesion molecules also play an important role in the migration and dedifferentiation process. $\alpha_6\beta_1$ intigrin (Kreidberg and Symons, 2000), neural cell adhesion molecule (NCAM) (Acheson et al., 1991), and leukocyte-endothelial adhesion molecule are the most important cell adhesion molecules in the adult proximal tubular epithelial cell. After ischemic injury, β_1 integrin relocates to the lateral border, facilitating cell-cell and cell-matrix interaction (Zuk and Matlin, 2002). NCAM, a member of Ig superfamily of proteins, controls the cell polarity and cell shape (Walsh and Doherty, 1997). NCAM is also used as a marker for dediffrentation phenotype metanephric mesynchyme (Bokel and Brown, 2002).

The role of extra cellular matrix (ECM) cannot be overstressed. ECM not only provides a scaffold for the proliferating and migrating epithelial cells, many signaling pathways are also activated by the interaction of ECM molecules with integrins. Post-ischemic insult, there is an increased production of ECM components including type IV

collagen α1 chain and fibronectin, containing the extra type III (EIIIA) domain, and their activity is detected in the basement membrane of the proliferating epithelial cells (Basile et al., 1998). It is further confirmed by the finding that splice variants of fibrinonectin-EIIIA are minimal in the normal kidney, but significantly increased after IRI (Zuk et al., 2001), which suggests the fibrinonectin-EIIIA involvement in the renal regeneration. Similarly, lamilin 5, which is known to be involved in the regeneration of skin wounds, is proposed to be involved in renal regeneration. It is widely expressed along the basement membrane of the nephron.

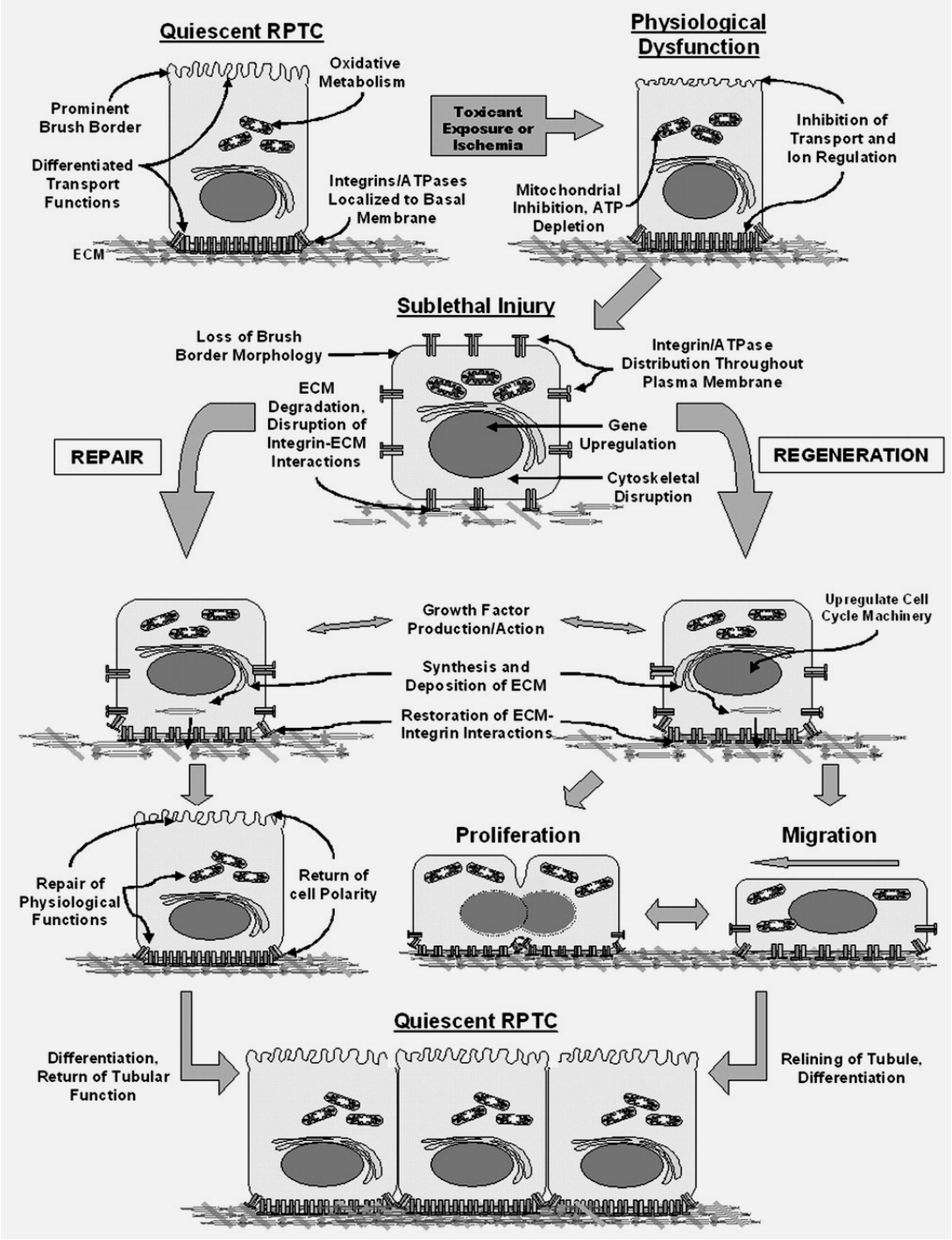

Figure 5: Mechanism of renal regeneration. Figure adopted from (Tadani, et.al., 2008) RTPC-Renal proximal tubular epithelial cells

2.9 KIM-1/TIM-1/HAVCAR-1

The same protein has been separately identified in different processes, and so identified by different names. When expressed in kidney, particularly proximal tubular epithelial cells, it is called kidney injury molecule -1(KIM-1); similarly, when expressed on T cells, it is called T cell immunoglobulin mucin domain-1 (TIM-1), and in liver it is known as hepatitis A virus cellular receptor -1. In the following sections, structure, function, and role of KIM-1 in renal regeneration will be discussed in detail.

2.9.1 Structure of KIM-1

KIM-1 is a type I transmembrane glycoprotein, and structurally resembles mucosal addressin cell adhesion molecule (MAdCAM-1) (Briskin et al., 1993). KIM-1 has an extra cellular domain, a transmembrane domain, and a cytoplasmic tail. The ectodomain, consisting of 100 residues, has an immunoglobulin V (Ig-V), made of highly conserved 6 cystine residues, similar to what is found in immunoglobulin superfamily (IgSF). It also contains a region rich in Thr/Ser/Pro with O-linked glycosylation, a characteristic of mucin like domain. Many N-linked glycosylation are also present (Bailly et al., 2002). The crystal structure of KIM-1 reveals the IgV domain to be made up of 2 anti-parallel β- sheets (BED and GFC) with four cystine residues joining a CC' loop to the GFC β- sheet which forms a pocket that recognizes phosphatidylserine (PS) (Su et al., 2008). This binding cleft is unique for KIM-1, and is not found in other molecules of immunoglobulin super family. The ability of KIM-1 to bind to the phosphatidylserine suggests an important function in recognition of apoptotic cells. The transmembrane

domain consists of 20 residues, but specific functions of the transmembrane domain are still unknown. The C terminal or the cytoplasmic region is short and made of 47 residues, and is ~10 KDa in size. It is also the most highly conserved region between the human and the mouse (Monney et al., 2002). Human KIM-1 has 2 splice variants that differ in the cytoplasmic domain; KIM-1a, consisting of 339 amino acids, is mainly expressed in the liver, and has no tyrosine kinase phosporylation site; KIM-1b (referred to as KIM-1 in this literature), consisting of 359 amino acids, is mainly expressed in the kidney, and has 2 highly conserved tyrosine residues and a predicted tyrosine kinase phosporylation motif at position 350 (Zhang et al., 2007). The extracellular region of KIM-1 can be cleaved at a proteolytic site close to transmembrane domain; thus shedding a physiologically active soluble protein of ~90kDa into the extracellular space. This carries a great clinical significance, as the ectodomain serves as a renal injury marker. The cells expressing KIM-1 display constitutive shedding of the ectodomain (Bailly et al., 2002). The shedding was inhibited by a broad-spectrum matrix metalloproteinase (MMP) inhibitor or by blocking of the proteoletic site by a site-specific antibody (Bailly et al., 2002). Thus, the cleavage of the extracellular domain is believed to be MMP mediated *in vivo*. Yet, a separate study has also shown that the constitutive ectodomain shedding is mediated by the activation of extra cellular signal regulated kinases (ERK) activation, and the shedding is accelerated by the activation of p38 mitogen activated protein (MAP) kinase, suggesting endogenous shedding is regulated. The secondary structure (and not the amino acid sequence) in the juxtamembrane region is important for the shedding of the ectodomain (Zhang et al., 2007).

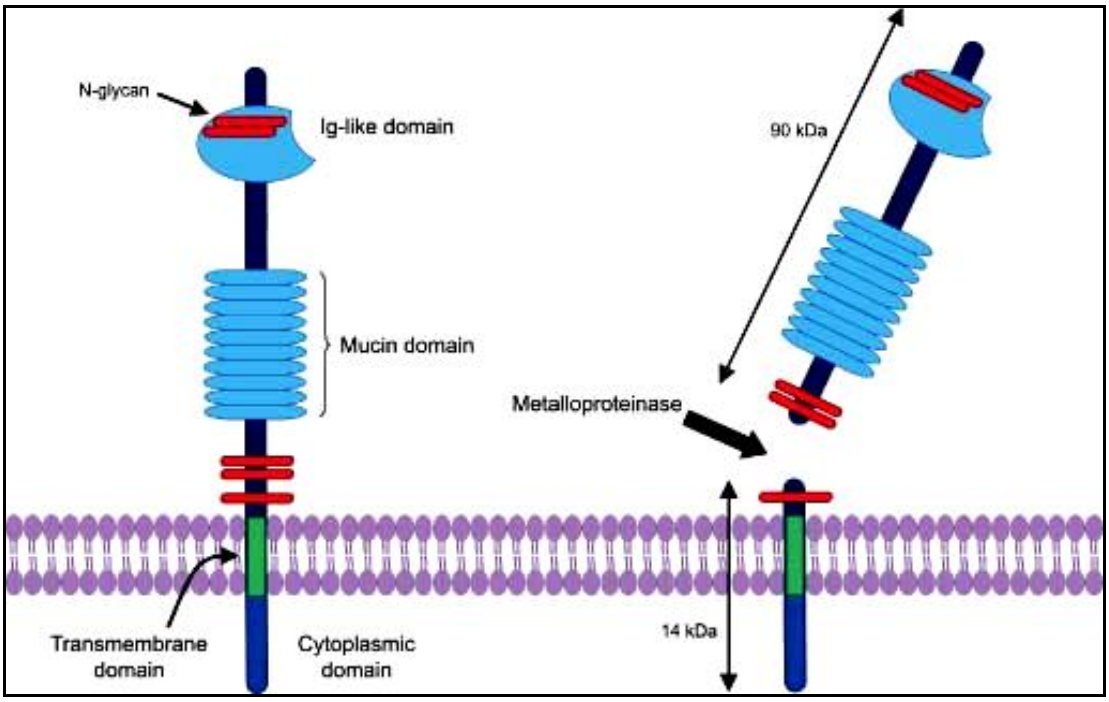

Figure 6: Structure of KIM-1. Adopted from (Waanders et al., 2009)

2.9.2 Expression of KIM-1

KIM-1 was first identified and characterized in 1998. It was noted that KIM-1 was not expressed in healthy murine kidney, but was abundantly expressed after different direct or indirect renal injuries (Ichimura et al., 1998). Similar findings were observed for human KIM-1 expression. While KIM-1 was undetectable in healthy human urine, it was abundant in patients suffering from various primary and secondary kidney injuries, allograft transplantation, and renal cell carcinoma. On further examination, with double labeling with aquaporin-1 (a marker for renal tubules), it was observed that KIM-1 was co-expressed with aquaporin-1 and was predominantly expressed by the proximal tubular epithelial cell (van Timmeren et al., 2007). KIM-1 is expressed on the apical membrane

of the injured tubules (van Timmeren et al., 2007). The expression of murine KIM-1, depending on the nature of the injury, varies between different segments of the proximal tubule. In the renal ischemic model, the expression was predominantly from the S3 segment of the corticomedullary tubules (Chiusolo et al., 2011). In conditions like polycystic kidney disease, where the injury is not primarily to the S3 segment, the expression was observed from the mid-cortical and superficial tubules (van Timmeren et al., 2006). However, in 90% of all the renal injuries, both acute and chronic, the expression was predominantly from the S3 segment of the proximal tubules (van Timmeren et al., 2007).

2.9.3 Function of KIM-1

KIM-1/TIM-1 has been a topic of extensive research over the past few years because of its varied roles in the kidney and immune system. In the renal system, most of the work is directed towards understanding KIM-1 as a sensitive renal injury marker. Similarly, extensive research has been carried out by immunologists to understand the role of TIM-1 in regulating the immune system; although a global knockout was without effect for immune responses (Barlow et al., 2011). In the following sections, the role of renal and extra renal functions of KIM-1/TIM-1 will be discussed in detail.

2.9.3.1 Renal functions of KIM-1

As mentioned earlier, the extracellular domain of KIM-1 is a receptor for PS. PS is present in the inner surface of the cell membrane, but when cells undergo apoptosis, PS is exposed on the outer surface. This is recognized by a scavenger receptor, which by definition, recognizes the PS, or oxidized LDL, and mediates their uptake. In vitro studies show rat tubular epithelial cells recognize the PS on the apoptotic cells, mediate its uptake, and direct it towards lysosomal degradation (Ichimura et al., 2008). Thus, the KIM-1 expressing epithelial cells act as 'semi-professional' phagocytes. Hence, KIM-1 expressing epithelial cells recognizes the 'eat me' signal from the apoptotic/necrotic cells, and so should mediate uptake, degradation, and clearance of the tubule of the dead and necrotic cells. The mechanism and role of KIM-1 in directing the apoptotic cells to the lysosomal degradation is not clear yet, but it is proposed, KIM-1 may be a co-receptor in mediating the uptake.

KIM-1 has also demonstrated protective functions in IRI. In a murine kidney IRI model, administration of monoclonal antibody against KIM-1 (RMT-10 clone) to the mice had a protective function (Nozaki et al., 2102). Similar results were also observed after liver ischemia/reperfusion injuries (Ueno et al., 2008). However, other groups using a different monoclonal antibody, directed towards different epitopes, failed to observe similar results. Hence, it is speculated that blocking different epitopes on the KIM-1 may lead to altered functions. Further studies are required to have a conclusive answer.

Early detection of renal injury has always been of great significance; it not only reduces the mortality and morbidity related to ARF, but also reduces the cost of the

treatment. Following an injury, renal cells are known to have altered expression or secretion of some molecules. They have been exploited for their use as renal injury markers. KIM-1, IL-18, MMP-9, osteopontin, clusterin, and NGAL are some of the molecules to be studied for use as renal injury markers (Bonventre et al., 2010). The ectodomain of KIM-1 shed to the extracellular space can be detected in the urine, and serves as a non-invasive marker for renal injury, and has been validated for its sensitivity and specificity (Vaidya et al., 2010). Urinary KIM-1 has the highest sensitivity and specificity compared to all the other biomarkers of renal injury (Vaidya et al., 2010). KIM-1 is more sensitive than traditional biomarkers like serum creatinine and blood urea nitrogen (BUN) (Liangos et al., 2007). KIM-1 shedding has also been considered a good prognostic marker of renal injury, where the reduced shedding in the urine signifies the reduction of the renal injury.

KIM-1 is also proposed to be involved in development of interstitial fibrosis. KIM-1 expression is observed around the atrophic tubules surrounding the fibrosis. Although there is no proof of increased expression of KIM-1 leading to fibrosis, a positive association between the KIM-1 expression and renal fibrosis exists (Kuehn et al., 2002). However, positive significant association is observed with tubular KIM-1 expression and interstitial damage (fibrosis and macrophage infiltration).

Extensive KIM-1 expression and shedding is observed in clear-cell type renal carcinoma (RCC). Though the tumor cells do not express KIM-1, abundant expression is observed in the neighboring epithelial cells (Cuadros et al., 2013). Many hypotheses have been proposed to explain the expression of KIM-1 in them. First, mechanical pressure exerted by the rapidly dividing tumor cell may cause ischemia in the neighboring cell or

may dedifferentiate them. Second, KIM-1 expressing cells may represent the early stages of the RCC (Cuadros et al., 2013). Conclusive answers remain elusive. Since RCC is an immunogenic tumor, KIM-1/TIM-1 may be involved in modulating the immunogenicity of the tumor.

KIM-1 is expressed in the primary cilia of the proximal tubular epithelia cells (Kotsis et al., 2007). Primary cilia play important and diverse functions in the cellular signaling. Defects in the primary cilia, usually due to the mutations in the genes encoding the ciliary proteins, are associated with diseases such as impaired mucociliary clearance, situs inverses, hydrocephalus, obesity, infertility, and polycystic kidney disease (Singla and Reiter, 2006). Autosomal dominant polycytic kidney (PKD) disease is usually associated with the mutations in PKD-1 gene, which encodes polycystin-1, or PKD-2 encoding TRPP-2 (Wu and Somlo, 2000). Renal cilia are non-motile, and lead to an increase in intracellular calcium in response to urine flow (Liu et al., 2003). It is now known that KIM-1 interacts with TRPP2 (Kotsis et al., 2007) and is dependent on the highly conserved tyrosine at 350 in the cytoplasmic tail, and mutation of the tyrosine residue results in defect of intracellular calcium change in response to flow (Kotsis et al., 2007). As KIM-1 holds structural homology to MaDCAM family of proteins, an endothelial integrin and selectin receptor, it is proposed to be involved in cell-to-cell or cell-to-matrix adhesion. The exact role of KIM-1 in this process is yet to be determined (Gordon, 2002).

2.9.3.2 Extra renal function of KIM-1

KIM-1, also known as HAVCR-1, is expressed in hepatocytes, and facilitates the cellular entry of hepatitis A virus (Silberstein et al., 2001).

KIM-1, also known as TIM-1, belongs to TIM family of proteins, which consist of TIM-1, TIM-3, and TMI-4 in humans, and 4 (TIM-2) members in mice. TIM plays important and complex roles in regulating the immune system. It is involved in the T cell activation differentiation, survival, and T cell tolerance (Rodriguez-Manzanet et al., 2009). TIM-1 is expressed on the active $CD4^+$ T cells, but not on the naïve cells. Post differentiation, TIM-1 is predominantly expressed on TH-2 cells; whereas, TH-1 cells show limited or no expression (Umetsu et al., 2005). On T cells, TIMs act as receptors. TIM-4 present on the antigen presenting cells or dentritic cells is the ligand for TIM-1, and mediates the differentiation of Th-2 cells (Meyers et al., 2005). TIM-3 is known to bind to gelatin-9 and regulate the duration of response of Th-1 (Umetsu et al., 2005).

Initial studies in interval-specific congenic mice (HBA) (McIntire et al., 2001) identified TIM-1 as a susceptible gene for asthma and hypersensitive airway disease. TIM-1 plays an important role in regulating the TH-2 cell responses, and thus regulates the airway inflammation by regulating the TH-2 response in the asthma mice model (Sizing et al., 2007). Epidemiological studies have also established a relation between the SNPs in TIM-1 gene to increased asthma and atopy (Graves et al., 2005).

KIM-1/TIM-1 is also known to play an important role in organ transplant rejection. Anti-KIM-1/TIM-1 monoclonal antibody administered to mice before the

cardiac allograft, reduced the rate of rejection; whereas, agonist KIM-1/TIM-1 antibody increased the rate of organ transplant rejection in the murine allograft kidney transplantation model. The above-mentioned studies and more similar studies depict a very strong association of KIM-1/TIM-1 in modulating some elements of the immune response in allograft tissue transplantation.

Cisplatin, a widely used chemotherapeutic agent, which is nephrotoxic, is also ototoxic. In cisplatin treated mice, increased expression of KIM-1 was observed in cochlear cells (Mukherjea et al., 2006). Possible cochlear injury molecule (CIM-1) is being explored.

2.10 Role of Matrix metalloproteinase in KIM-1 cleavage and acute renal failure

Matrix metalloproteinases are groups of Zn dependent endopeptidases. They were first discovered about 50 years ago in the metamorphosis of tadpole tail for their collagenolytic activity, degrading the extra cellular matrix. To date, 28 MMPs have been identified, which share structural similarities and a catalytic Zn^{+} domain (Rodriguez et al., 2010). Based on their substrates, MMPs are divides as collagenase (MMP -1, -8 and -13), stromelysins (MMP- 3,-10 and -11), gelatinases (MMP -2 and -9), and membrane type MMP (MT-MMP) (Back et al., 2010). MMPs and their natural inhibitors TIMPs are under a strict regulation, and play important and varied roles ranging from embryonic development to pathogenesis of various diseases. MMP-9 is also a predicted renal injury marker (Han et al., 2008). Apart from their role in the pathogenesis of various renal

diseases, as discussed in section 2.9.1**,** the KIM-1 ectodomain is known to be cleaved by MMP (Bailly et al., 2002), which makes it even more important for this discussion.

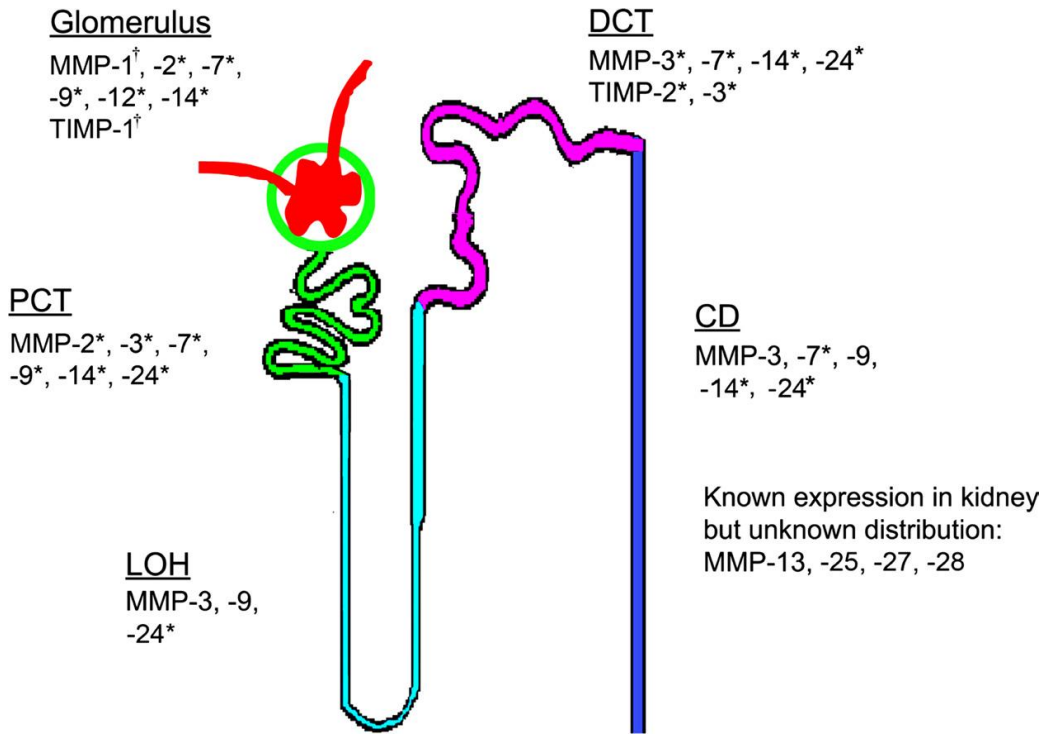

Figure 7: the expression profile of MMPs and their natural inhibitors TIMPs. The figure shows the complied data for the expression profile for different MMPs in relation to nephron in kidney. * denotes upregulation of MMPs in different pathological conditions. Adopted from (Tan and Lui, 2012)

Many recent studies have demonstrated the role of MMPs, especially MMP-2 and MMP-9, in the pathogenesis of renal ischemia-reperfusion injury. An increased expression of MMP-2 and MMP-9 has been observed after IRI (Basile et al., 2004, Caron et al., 2005, Catania et al., 2007). However, as contradictory results have also been reported, uncertainties still exist (Jain et al., 2000, Ziswiler et al., 2001, Bengatta et al.,

2009). The extracellular domain shedding in P769 cells was inhibited by preincubating the cells with broad-spectrum MMP inhibitor BB94 (Bailly et al., 2002); which suggested the role of MMP in endogenous KIM-1 shedding. Further, experiments carried out in primary tubular cell culture suggested the role of MMP-3 in KIM-1 shedding (Lim et al., 2012). Yet another study suggested the role of membrane-type 1 MMP (MT-MMP1) in KIM-1 shedding (Guo et al., 2012). These conflicting results suggest that MMP involved in KIM-1 shedding is still unclear or more than one MMP may be involved in the KIM-1 shedding. Hence, as mentioned in section 1.3, one of the specific aims of this study is to discover the MMP involved in the KIM-1 shedding in injured kidney.

2.11 Role of KIM-1 in apoptotic cell clearance and renal regeneration

The earlier sections discuss the role of apoptotic and necrotic cells in sustaining inflammation during ARF, and the importance of rapid clearing of the necrotic and apoptotic cells in renal regeneration. The role of professional phagocytes and non-professional phagocytes in clearing the apoptotic and necrotic cells has also been discussed in section 2.6. The studies from our lab, using a rat ethanol injury model, showed extensive renal damage with apoptotic bodies as determined by TUNEL staining (Latchoumycandane C, unpublished data). He observed extensive neutrophil infiltration, whereas macrophages at the site of injury were minimal. Similar findings of absence of infiltrating macrophages have been reported (Waanders et al., 2010). Hence, with absence of macrophages, the ‘semiprofessional phagocytes’ are all that can be involved in rapid clearing of apoptotic/necrotic cells. As discussed in section 2.9.3, KIM-1 confers

a phagocytic phenotype to the epithelial cells (Ichimura et al., 2008). Hence, KIM-1 might play role in clearing the apoptotic bodies and in turn, could contribute to attenuating the inflammation, preventing further inflammation, stalling immunological response, and remodeling and regeneration of kidney. Renal allograft kidney biopsies showed increased expression of KIM-1; interestingly, increased expression of KIM-1 was associated with better prognosis with allograft renal transplantation (Zhang et al., 2008b). This demonstrates, KIM-1 may not just be a prognosis marker, but involved in the renal repair and regeneration.

Hepatocyte growth factor (HGF), a well-characterized renal repair factor (Liu and Yang, 2006), is known to be upregulated in the epithelial cells that phagocytosed apoptotic cells. It was observed that over expressing KIM-1 in pig kidney epithelial cells also demonstrated increased mRNA expression of HGF (Ichimura et al., 2008). This suggests that KIM-1 expressing epithelial cells, apart from clearing the apoptotic bodies, may be involved in the renal regeneration by secreting growth/reparative factors.

KIM-1 is expressed in dedifferentiating epithelial cells and co-localizes with the other dedifferentiation markers. KIM-1 also shares spatial relationship with ostiopontin, a protein expressed from the tubular epithelial cells involved in renal repair (de Borst et al., 2007). All these observations point towards the role of KIM-1 as more than just a biomarker or a silent observer in renal injury and repair, but rather, play a role in renal repair.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell culture

Immortalized normal human proximal tubular epithelial (HK2) cells were purchased from ATCC (# CRL-2190); were revived in Dulbecco's minimum essential medium- F12 (DMEM-F12), cell culture media (Media core, Cleveland Clinic Foundation, Cleveland OH); supplemented with 10% v/v heat inactivated fetal bovine serum, (FBS Sigma Aldrich St. Louis MO) and 10000 IU penicillin streptomycin, (Sigma Aldrich St. Louis MO). The cells were cultured in controlled environment of 5% CO_2 at 37^0C. The cells were cultured to 80% confluence before passaging.

3.2 Isolation of neutrophils

Blood was collected from healthy volunteers in blood collection bag with the anti-coagulant, ACD. RBC, buffy coat, and plasma were separated by centrifugation for 20

min at 1300 RPM using Beckmen Coulter Allegra X15 R tabletop swinging bucket centrifuge. The buffy coat was carefully transferred to a different tube and diluted with equal volume of Hanks balanced salt solution (HBSS) w/o Mg^{++}, Ca^{++}, or phenol red (Cleveland clinic media core Cleveland OH). Equal volume of 3% dextran 500 (#31395, Sigma Aldrich St. Louis MO) solution in 1.9% NaCl was added and mixed well. It was left to stand in 4^0C for 45 minutes – 1 hour allowing the RBCs to settle to the bottom. The supernatant, which mainly contains neutrophils and monocytes, was carefully transferred to a different tube. The netrophils/monocytes were precipitated by centrifuging at 1100 rpm for 10 minutes in Beckmen Coulter Allegra X15 R tabletop centrifuges with swinging buckets. The carry over RBCs were lysed in 0.2% NaCl solution for 30 sec. Care was taken not to exceed the lysis over 30 sec. RBC free neutrophil/monocyte were resuspended in HBSS and loaded on top of Ficoll-Plaque plus (#17-1440-02, GE Life sciences, Pittsburgh PA) cushion in the ratio of 5:4 v/v. Neutrophils were separated from the monocytes by centrifuging for 30 mins at 1100 rpm. The neutrophils were counted using hemecytometry counter and 10^7 neutrophils were resuspended in 1mL of HBSS and used for further experiments.

3.3 Western blotting for KIM-1

Western blotting was performed to analyze total KIM-1 expression in the HK2 cells, and ectodomain shedding into the conditioned media. The cells were harvested in RIPA buffer with 10% v/v protinase inhibitor cocktail. The total protein content was estimated by Bio-Rad DC™ protein assay kit (#500-0111 Bio-Rad, Hercules CA) using

the manufactures protocol. 20µg of protein was mixed with the 2X Laemmli sample buffer (#161-0737EDU Bio-Rad, Hercules CA) containing 0.5% β-mercaptoethanol and boiled for 5 minutes at 95^0 C and loaded on to a 4-20% gradient Tris-HCl precast gel (#345-0032 Bio-Rad, Hercules CA). The electrophoresis was carried out at 120 V for 2 hours. The proteins were transferred on to nitrocellulose membrane using iBlot dry transfer system (Invitrogen Inc). The membrane was then blocked for 1 hour in 5% non-fat milk in PBST. Then the membrane was probed for KIM-1 using human anti-KIM-1 mouse monoclonal antibody (# MAB1750, R&D Minneapolis, MN) at the concentration of 1:1000 in 5% non-fat milk in PBST overnight at 4^0C. The primary antibody was decanted and washed with PBST for 10 minutes X 3 times followed by incubation with HRP conjugated goat anti-mouse secondary antibody (# Bio-Rad, Hercules CA) in the concentration of 1: 10000 in 5% non-fat milk in PBST for 1 hour at room temperature. The membrane was washed with PBST for 15 minutes 3 times.

3.4 Analysis of surface expression of KIM-1 on HK2 cells and uptake of lipid vesicles by FACS.

Flow cytometric analysis was utilized to quantify the surface expression and ectodomain shedding of KIM-1 in HK2 cells. HK2 cells were grown in complete media and at 70% confluence and serum starved overnight before treatment. Post treatment, the cells were harvested in 1X trypsin EDTA (Media core, Cleveland Clinic, Cleveland OH). The harvested cells were washed 3 times with filtered PBS (Sigma Aldrich, St Louis MO) and blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes on ice.

The cells were incubated with mouse monoclonal KIM-1 antibody (#MAB-1750 R&D Minneapolis, MN) in the dilution of 1:100 v/v in 1% BSA for 30 minutes followed by goat anti-mouse secondary antibody tagged with Alexa Fluor 488 IgG (#A-11029, Life Technologies, Carlsbad, CA) in the dilution of 1:100 in 1% BSA in PBS for 30 minutes to detect the surface KIM-1 expression. The cells were washed in PBS thrice after each step and fixed with 4% parformaldehyde and stored in the same fluid at 4^0C until utilized for flow analysis. 350000 cells were analyzed in FACScan (BD Biosciences). The live cells were gated and the KIM-1 expression was analyzed using FlowJo software.

The uptake of lipid vesicles containing DiI (synthesis of unilayer lipid to be explained in the next section) by HK2 cells used 3 hours incubation. The cells were harvested as mentioned before and washed with 1% BSA in PBS X 3 times. Then cells were resuspended in 300 µL of PBS and analyzed in FACScan (BD Bioscienses) in FL2 channel to detect the DiI taken up by the cells. The cells were gated for live cells and analyzed by FlowJo software.

3.5 siRNA mediated KIM-1 knockdown

KIM-1 specific siRNA was purchased from Dharmacon (#L-019856-00-0005, Thermo Fisher Scientific, Wlatam MA) and reconstituted in siRNA buffer purchased from Dharmacon (#B-002000-UB-100, Thermo Fisher Scientific, Wlatam MA). The working concentration of 25 µM was used to transfect the cells. HK2 cells were cultured in 6 well plates and allowed to reach ~ 50% confluence before transfection. Transfections were carried out according to manufactures protocol using lipofectamin RNAimax

(#13778030 Life technologies NY). The cells were incubated at 5% CO_2 at 37^0C for 72 hours. The cells were harvested and analyzed for KIM-1 expression using western blots.

3.6 Unilamellar lipid vesicle synthesis

In a live cell, PS is present in the inner leaflet, while in cells undergoing apoptosis/necrosis, the PS is exposed on the outer surface. The PS on the apaototic/necrotic cells is recognized by scavenger receptors that mediates uptake of the cells or particles. We mimicked this system by synthesizing unilamellar vesicle containing PS. Mini extruder from Avantis polar lipids was used to synthesize these liposomes. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were purchased from Avanti polar lipids, Alabaster AL (#770557 and 840034 respectively). POPC and POPS were mixed in the molar ratio of 7:3.Vibrant DiI (Life technologies, Carlsbad CA) at the concentration of 3:500 v/v was added and mixed well. The chloroform solvent was evaporated using a liquid nitrogen evaporator. The pellets were hydrated in 500 µL of sterile HBSS. Lipid vesicles were prepared by extrusion by repeatedly forcing the slurry through a 100 nm membrane. The unilammelar vesicles contained either POPC alone, or POPC and POPS in defined molar ratio, along with DiI.

3.7 Quantification of shed KIM-1 by ELISA

The shed ectodomain of KIM-1 in conditioned media was quantified by ELISA. Human KIM-1 ELISA duo kits were purchased from R&D (# DY1750 Minneapolis MN) and the experiment was performed using manufacture's protocol. In brief, Costar 96 well EIA plate (# 3590 Costar, Cambridge MA) was coated with goat anti-human KIM-1 capture antibody by incubation at the concentration of 72 µg/mL in PBS overnight at room temperature. Next day the plate was washed with PBS containing 0.5% Tween-20 (PBST) X 3 times followed by blocking with 1% BSA in PBS for 2 hours at room temperature. 100 µL of sample diluted in 1% BSA in PBS was added and incubated at room temperature for 1 hour. The washing step was repeated and 100 µL of biotinylated goat anti-human detection antibody at the concentration of 72 µg/mL was added and incubated for 1 hour at room temperature. The plate was washed again and 100 µL Streptavidin-HRP in the dilution of 1:200 v/v was added and incubated for 20 minutes. After washing, 100 µL substrate solutions (Thermo scientific, Wlatam MA) was added and incubated for 20 minutes in the dark. The reaction was stopped by 50 µL 2N H_2SO_4 and the optical density was determined at 450 nm in multi well plate reader.

3.8 Gelatin zymogram to determine MMP activity

Precast gelatinase zymogram gels were purchased from Bio-Rad (#161-1167, Bio-Rad, Hercules CA) to identify MMPs in cell culture and neutrophil supernatants. The supernatants were mixed with equal volumes of 2X Liemmli buffer and loaded on to the gel without boiling. After electrophoresis, the gel was renatured in 2.5% v/v Triton X 100

(#T9284 Sigma Aldrich, St Louis MO) for 1 hour in room temperature. The gel was then incubated in developing solution (# LC2671 Invitrogen, Carlsbad CA) for 48 hours at room temperature. The developing solution was decanted and the gel was stained with 3 % Commassie blue for 30 minutes at room temperature. Then the gels were destained by destaining buffer containing 40% methanol and 10% glacial acetic acid, until the bands were clear.

3.9 Immunocytochemistry to quantify uptake of unilamellar phosphatidylserine vesicles

HK-2 cells were cultured in 4 well chamber slides (# 70400 Lab-Tek®, Hatfield PA) to 60 % confluence. The cells were treated with unilamellar vesicles in the dilution of 3:100 v/v for 3 hours. After decanting the cell culture media, cells were washed in 1X PBS followed by fixation in 4 % paraformaldehyde for 10 minutes. The HK-2 cells were stained for KIM-1 using mouse monoclonal KIM-1 antibody (#MAB-1750 R&D Minneapolis, MN) in the dilution of 1:100 v/v in 1% BSA for 30 minutes followed by goat anti-mouse secondary antibody tagged with Alexa Fluor 488 IgG (#A-11029, Life Technologies, Carlsbad, CA) in the dilution of 1:100 in 1% BSA in PBS for 30 minutes. As the unilamellar vesicles were prestained with DiI; no additional staining was necessary. The cells were washed with 1X PBS and mounted on with a covering slip using mounting media containing DAPI (# H-500 Vector laboratories Peterborough UK). The slides were sealed to prevent drying and viewed under Olympus fluorescent microscope.

CHAPTER IV

RESULTS

4.1 Activated neutrophils increase KIM-1 shedding in HK-2 cells

As we hypothesized, activated neutrophils mediate KIM-1 shedding in human renal proximal tubular cells. The KIM-1 shedding was measured by 1) analyzing the surface expression of KIM-1 by FACS and 2) detection and quantification of the shed KIM-1 in the conditioned media by ELISA. Neutrophils were isolated freshly from human blood as mentioned in section 3.2. Neutrophils ($1 \text{ X } 10^7$) were resuspended in 1 mL of HBSS and were activated by 3 µM Platelet Activating Factor (PAF) for 1 hour at room temperature with gentle agitation (Kuijpers et al., 1991). Other activation factors like LPS was also tried, but discontinued, as neutrophils formed aggregates and adhered to the walls of the microcentrifuge tubes. Supernatant from activated neutrophils was used for the over-night treatment of HK2 cells (200 µL, for each well of a 6 well cell culture plate). Supernatent from unactivated neutrophils was used as control. The relative decrease in the surface expression of KIM-1 or increase in the shed KIM-1 into the conditioned media was measured against the untreated cells. Figure 8A depicts the

results from the FACS analysis. The untreated cells (control) show the maximum KIM-1 expression (shown in pink). As the cells were not permeabilised, the measured KIM-1 are expected on the cell surface. HK-2 cells treated with activated neutrophil supernatents show least relative fluorescent intensity denote the maximum shedding of KIM-1from the surface (shown in green). The mean fluorescent intensity analyzed by FACS show significant reduction on treatment with activated neutrophils compared to untreated HK-2 cells (Figure 8B). No significant changes in KIM-1 expression in HK-2 cells were observed on treating HK-2 cells with supernatants form unactivated netrophils which suggests the role of activation of neutrophils. Treatment with PAF, in absence of neutrophils, did not show any effect on KIM-1 expression by HK2 cells (data not shown). As the antibody detects only the N- terminal region, the reduction in KIM-1 expression signifies the shedding of KIM-1 ectodomain. Unactivated neutrophils also mediate some KIM-1 shedding (shown in blue); this is likely from unavoidable neutrophil activation.

To confirm that decreased surface expression of KIM-1was due to the shedding of the ectodomain, the shed ectodomain in the conditioned media was quantified by KIM-1 ELSIA. The results from ELISA are depicted in the Figure 8C. HK2 cells treated with supernatants from activated neutrophils showed significant increase in KIM-1 shedding into the conditioned media compared to untreated HK2 cells and HK-2 cells treated with supernatants from unactivated neutrophis. However there is no significant difference in KIM-1 shedding between untreated HK-2 cells and HK-2 cells treated with supernatants from unactivated neutrophil.

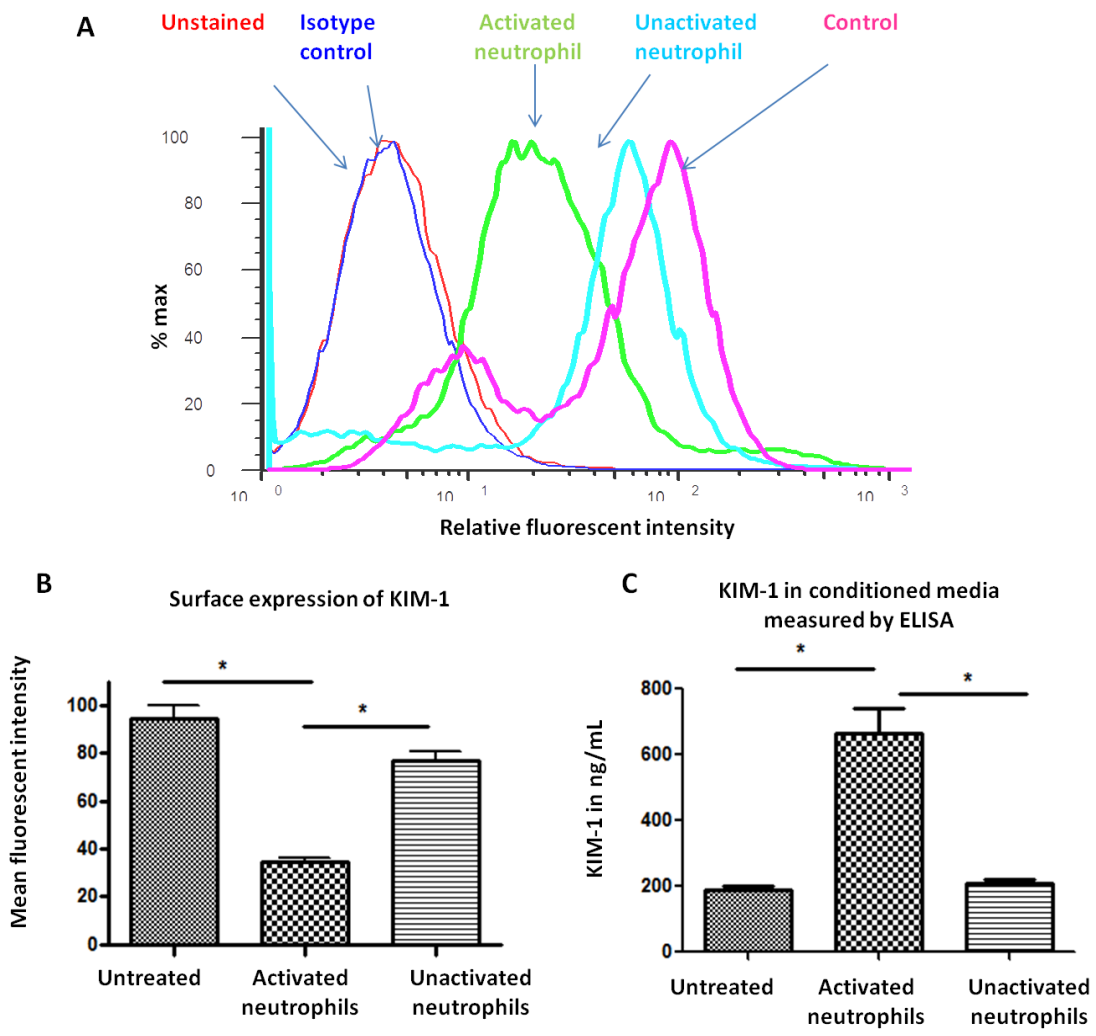

Figure 8: Activated neutrophils mediate KIM-1 ectodomain shedding in HK-2 cells. A) Surface expression of KIM-1 was analyzed by FACS and 35,000 HK-2 cells were counted per group. The relative fluorescent intensity profile is represented in the graph. B) The bar diagram shows the mean relative fluorescent intensity values from 4 independent experiments. HK-2 cells treated with supernatants from activated neutrophils show significant reduction in the mean fluorescent intensity value of KIM-1 compared to untreated cells, suggesting KIM-1 shedding mediated by activated neutrophils C) The shed KIM-1 ectodomain into conditioned media was quantified by ELISA. HK-2 cells treated with supernatants from activated neutrophils show significant increase in the KIM-1 compared to untreated HK-2 cells or HK-2 cells treated with

supernatants from unactivated neutrophils . The data are expressed as mean ± SEM (n=4), $p < 0.05$ was considered as significant.

4.2 Phosphatidylserine dependent uptake of lipid vesicles

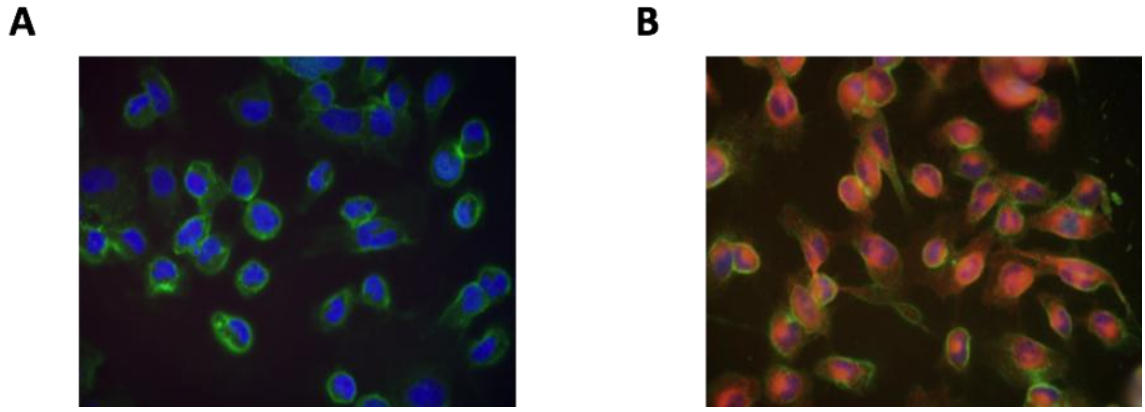

Figure 9: Immunofluorescence showing PS dependent uptake of lipid vesicles. HK-2 cells were treated with unilamellar lipid vesicles containing POPC and POPS in defined molar ratios and DiI. The pictures were taken at 60X maginifcation. HK-2 cells were also stained for KIM-1(green fluorescence) A) HK2 cells treated with liposomes containing only POPC, shown no red florescence indicating the absence of uptake of vesicles. B) HK2 cells treated with liposome contain POPS show red fluorescence indicating PS dependent uptake of vesicles.

Unilamellar lipid vesicles, containing POPC and POPS in defined molar ratios and labeled with Vibrant DiI, cell labeling solution (Life technologies Carlsbad CA) , were prepared as explained in section 3.6. The uptake of unilamellar lipid vesicles was examined by FACS (Figure 10), and immunofluorescence (Figure 9). To identify the uptake of unilamellar vesicles by immunofluorecence, HK2 cells cultured to 60% confluence in a 4 well chamber slide were treated for 3 hours with lipid vesicles containing only POPC, or, vesicles containing POPC + POPS in molar ratios of 7:3. The

HK2 cells treated with lipid vesicles containing no POPS show no red fluorescence suggesting the absence of uptake of the lipid vesicles (Figure 9A). Whereas HK-2 cells treated with lipid vesicles containing POPS (Figure 9A) show red fluorescence suggesting uptake of lipid vesicles.

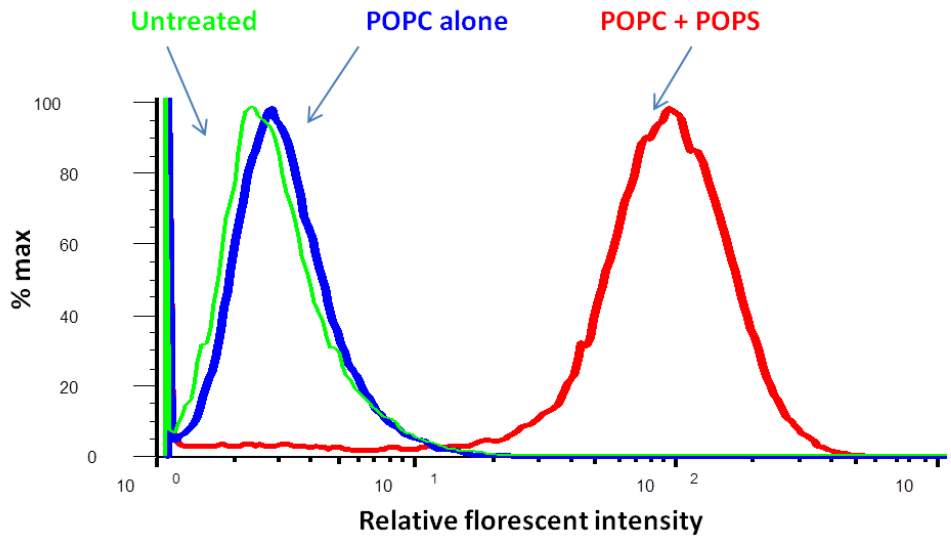

Figure 10: Phospatidylserine dependent uptake of lipid vesicles in HK-2 cells. Uptake of unilammelar vesicles by HK-2 cells was analyzed by FACS and 35,000 HK-2 cells were counted per group. HK-2 cells treated vesicles containing PS (red) show increased amount of relative fluorescent intensity compared to HK-2 cells treated with vesicles without PS (blue), suggests PS dependent uptake of lipid vesicles in HK2 cells.

The uptake of PS vesicles was also quantified by FACS. HK2 cells grown to 80% confluence were treated with unilamellar lipid vesicles consisting of POPC alone or vesicles containing POPC and POPS in the ratio of 7:3 for 3 hours in the dilution of 1 to 100 in DMEM-F12 media. The cells were harvested with trypsin EDTA and the excess lipids in the media were removed with 1% BSA in PBS and subjected to FACS analysis.

The cells treated with liposomes containing only POPC (shown in blue), show the least amount of relative fluorescent intensity, signifying minimal or no lipid vesicle uptake. Similarly, lipid vesicles containing PS (shown in red), show increased relative amount of relative fluorescent intensity, suggesting uptake of unilamellar lipid vesicles. Untreated cells (shown in green) served as control. This data suggests that uptake of lipid vesicles is PS dependent and the physiological uptake of apoptotic bodies is successfully mimicked.

4.3 The uptake of unilamellar lipid vesicles containing phospatidylserine is KIM-1 mediated

The above results show that uptake of lipid vesicles was PS dependent. Hence we wanted to examine if the KIM-1, a scavenger receptor, had any role in its uptake. To do this, a transient siRNA mediated KIM-1 knockdown in HK2 cells was created. The siRNA mediated KIM-1 knockdown was confirmed by western blotting which was probed for KIM-1. The results are depicted in Figure 11. Lane 1, 2 and 3 show KIM-1 expression in untreated cell, cells treated with transfection reagent, Lipofectamin 2000, and non-specific siRNA control respectively. Lane 4 and 5 shows KIM-1expression in HK-2 cells treated with KIM-1 specific siRNA at the concentration of 15 µM and 25 µM respectively. Lane 4 and 5 show appreciable reduction of KIM-1 (102 kDa) compared to the untreated cells, which signifies successful siRNA mediated KIM-1 knockdown.

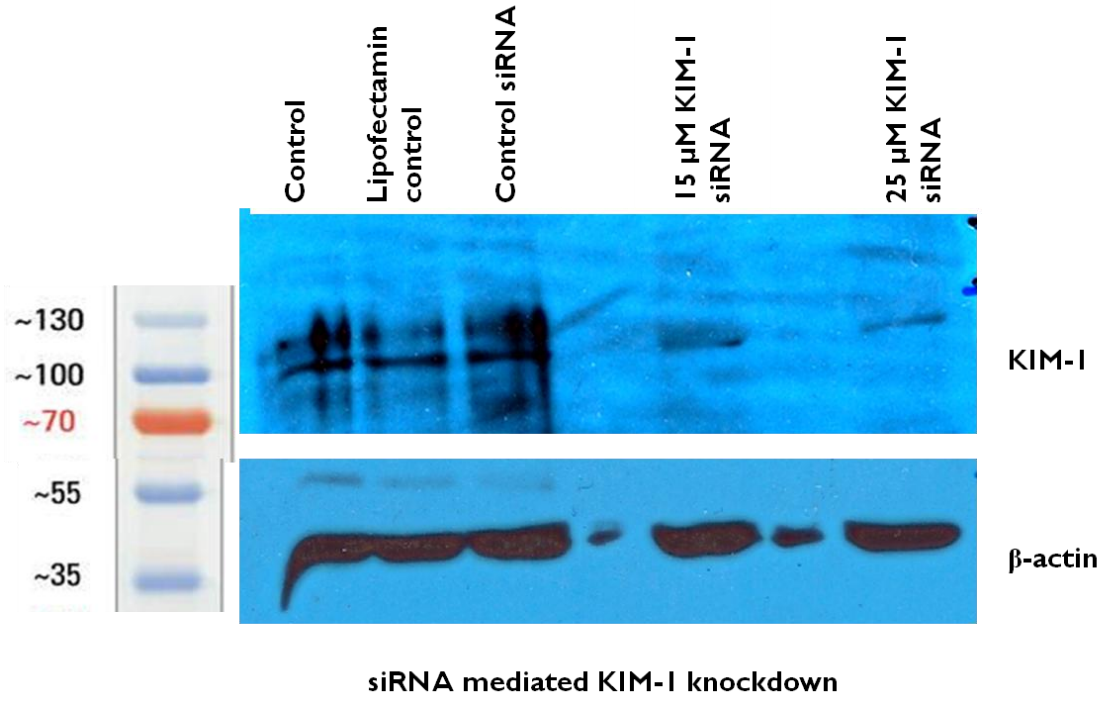

Figure 11: Western blots showing successful siRNA mediated knockdown of KIM-1in HK-2 cells. KIM-1 identified at 102kDa, show appreciable reduction in KIM-1 expression in KIM-1 siRNA transfected cells (lane 4 and lane 5) compared to untransfected cells (lane 1), non-specific control siRNA transfected cells (lane 3) and transfection reagent control (lane 2), suggesting successful knockdown of KIM-1 in HK2 cells using KIM-1 specific siRNA.

Further we examined the uptake of lipid vesicles containing PS in KIM-1 knockdown HK2 cells by FACS. As mentioned earlier, the cells were treated with the lipid vesicles in the ratio of 1: 100 v/v for 3 hours. The cells were harvested and subjected to FACS. The results of FACS analysis are shown in Figure 11. Control or untreated cells (shown in green) show the maximum amount of relative fluorescent intensity, which signify maximum lipid vesicle uptake. Cell treated with control siRNA (shown in blue) do not show any reduction in the amount of relative fluorescent intensity. However, HK-2 cells with KIM-1 knockdown (shown in pink and light blue), show significant reduction

in the amount of relative fluorescent intensity. This result gives us concrete evidence of KIM-1 mediated uptake of PS containing lipid vesicles. As apoptotic cells express PS on their surface, this result suggests KIM-1 mediated uptake apoptotic cells by renal tubular epithelial cells.

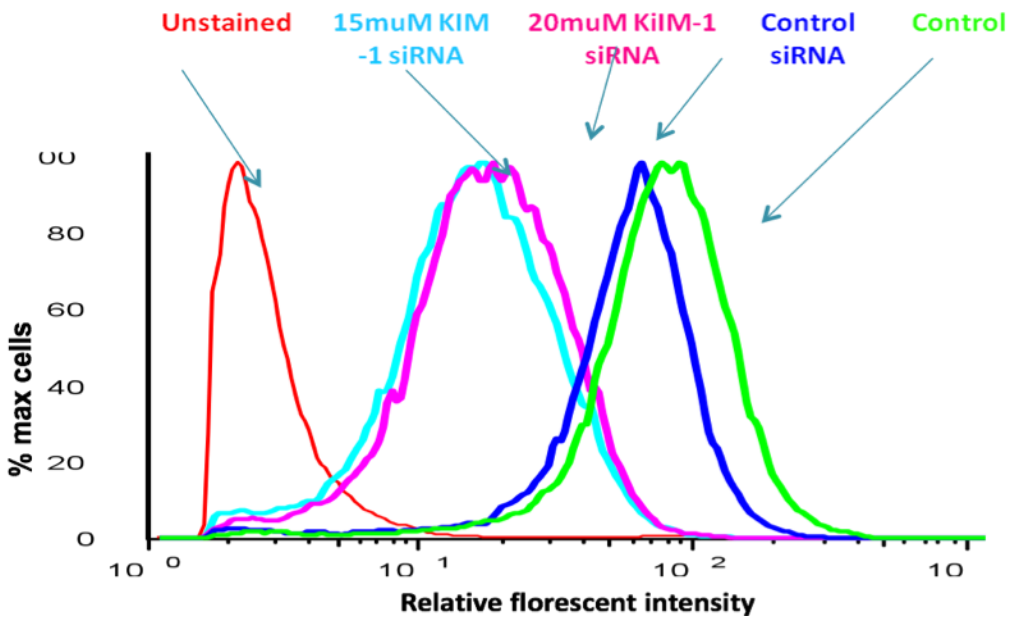

Figure 12: KIM-1 mediated uptake of PS vesicles by HK-2 cells. Uptake of unilammelar vesicles by HK-2 cells was analyzed by FACS and 35,000 HK-2 cells were counted per group. Relative fluorescent intensity profile in FL2 channel is represented in the graph. HK-2 cells lacking KIM-1 (shown in pink and blue) show reduced amount of relative fluorescent intensity compared to control (shown in green) or HK-2 cells transfected with control siRNA (shown in blue) which suggests KIM-1 dependent uptake of PS vesicles.

4.4 Activated neutrophils reduces phospatidylserine liposome uptake in HK-2 cells by increasing the KIM-1 shedding

In section 4.2 we observed an increase in KIM-1ectodomain shedding mediated by activated neutrophils. It is also known the PS binding site is situated in the ectodomain of KIM-1. Hence, effect of loss of KIM-1 ectodoamin on uptake of PS containing unilamellar vesicles was tested. HK2 cells were cultured to 80% confluence and treated overnight with the supernatant from neutrophils activated by 3 µM PAF. These cells were treated with PS containing unilamellar lipid vesicles. The cells were harvested with trypsin EDTA and washed with 1% BSA before the cells were fixed using 4% paraformaldehyde and analyzed by FACS. The results are depicted in Figure 13.

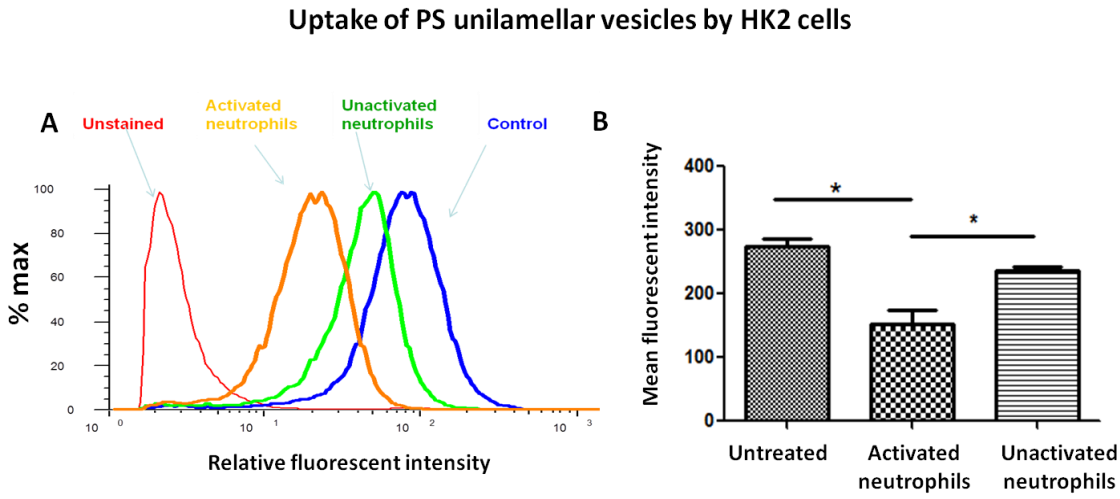

Figure 13: Activated neutrophils reduce PS liposome uptake in HK-2 cells. A) Uptake of PS vesicles by HK-2 cells was analyzed by FACS and 35,000 HK-2 cells were counted per group. The relative fluorescent intensity profile is represented in the graph. B) The bar graph represents the mean relative fluorescent intensity values from 4 independent experiments. HK-2 cells treated

with supernatants from activated neutrophils show significant reduction in relative fluorescent intensity values, suggesting reduced uptake of PS liposomes, compared to untreated HK-2 cell, and cells treated with supernatants from unactivated neutrophils. The data are expressed as mean ± SEM (n=4) $p < 0.05$ was considered significant.

HK-2 cells treated with activated neutrophil supernatants (orange) show reduced relative fluorescent intensity compared to untreated cells (blue) suggesting a reduction in the uptake of PS containing lipid vesicles. The reduction in the lipid vesicle uptake is due to increased shedding of KIM-1 ectodomain by activated neutrophils. Due to unavoidable activation of neutrophils, unactivated neutrophils also cause some reduction in the lipid vesicle uptake (green).

4.5 Neutrophils secrete Matrix metalloproteinas -9 upon activation

Activated neutrophils are known to secrete MMP-1, 2, 3, 7 and 9. Of theses, MMP-9, a gelatinase, is most important and well studied (Allport et al., 2002). Gelatin zymography explained in section 3.8 was employed to identify the MMP activity. The cell culture supernatents from untreated cells served as a control to compare the MMP activity in activated and unactivated neutrophil supernatants. The results from the gelatin zymography are depicted in Figure 14. Lane 1, MMP activity from the supernatant of untreated HK2 cells, shows minimal MMP-9 activity (92kDa). Lane 2 shows the MMP activity in the supernatant form unactivated neutrophils. There is no increase in MMP-9 activity compared to control. However on activation of neutrophils with PAF, as in lane 3, shows appreciable increase in the MMP-9 activity. The specificity of the increased

MMP activity was confirmed to be from MMP-9 by pre-treating the activated neutrophils with specific MMP-9 inhibitor (# 1177749-58-4, EMD Millipore, Billerica, MA) as shown in Lane 4. There is appreciable reduction in the band at 92 KDa, which suggest the MMP activity from activated neutrophil is from MMP-9.

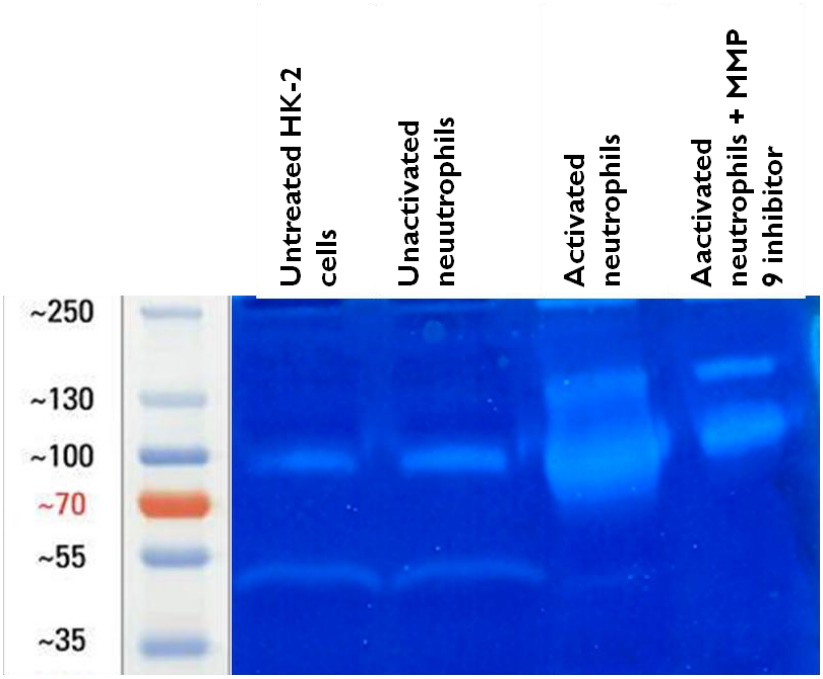

Figure 14: Activated neutrophils secrete MMP-9. Supernatants from PAF activated neutrophils, unactivated neutrophils, activated neutrophils treated with activated neutrophils, and HK-2 cells were loaded on to gelatin zymogram. Shown activated neutrophils (lane 3) show maximum MMP-9 activity. HK2 cell supernatants (lane 1) and unactivated neutrophils (lane 2), show minimal or no active MMP-9. The MMP-9 activity is reduced on pretreating the activated neutrophils with specific MMP-9 inhibitor (lane 4).

4.6 Activated neutrophils mediate KIM-1 shedding via MMP-9

Bitamastat or BB94 (#2961 Tocris bioscience Bristol UK) is a broad spectrum MMP inhibitor and this inhibits a range of MMPs when used at different concentrations. To identify the MMP involved in KIM-1 shedding, activated neutrophils were treated with different concentrations of BB-94 for 1 hour at room temperature; their releasates were added to HK2 cells and surface KIM-1 expressions in these HK-2 cells were quantified by FACS and shed KIM-1 in conditioned media by ELISA. Figure 14 shows the results from the analysis. BB-94 when used at the concentrations that inhibit, MMP-1 and MMP 2 there was no inhibition of KIM-1 shedding. When used in higher concentrations (50 nM) where MMP-1, -2, -9, - 7 and -3 were inhibited, inhibition of KIM-1 shedding was complete. Finally, a specific MMP-9 inhibitor (# 1177749-58-4, EMD Millipore, Billerica, MA) was used to confirm the role of MMP-9 in KIM-1 shedding. As shown in Figure 15A, HK-2 cells treated with supernatants from activated neutrophils pretreated with MMP-9 inhibitor (shown in green) shows increased amount of relative fluorescent intensity compared to cells treated with activated neutrophil supernatant or pretreated with MMP-1and -2 inhibitor (black) suggesting inhibition of KIM-1 shedding. Bar graph (Figure 15B) shows the mean relative fluorescent intensity from 4 independent experiments. HK-2 cells treated with supernatants from activated neutrophils pretreated with MMP-9 inhibitor show significant reduction in the KIM-1 shedding compared to HK-2 cells treated with supernatants from activated neutrophils without MMP inhibitor. Figure 15C show the quantification of shed KIM-1 ectodomain into the culture media by ELSIA..

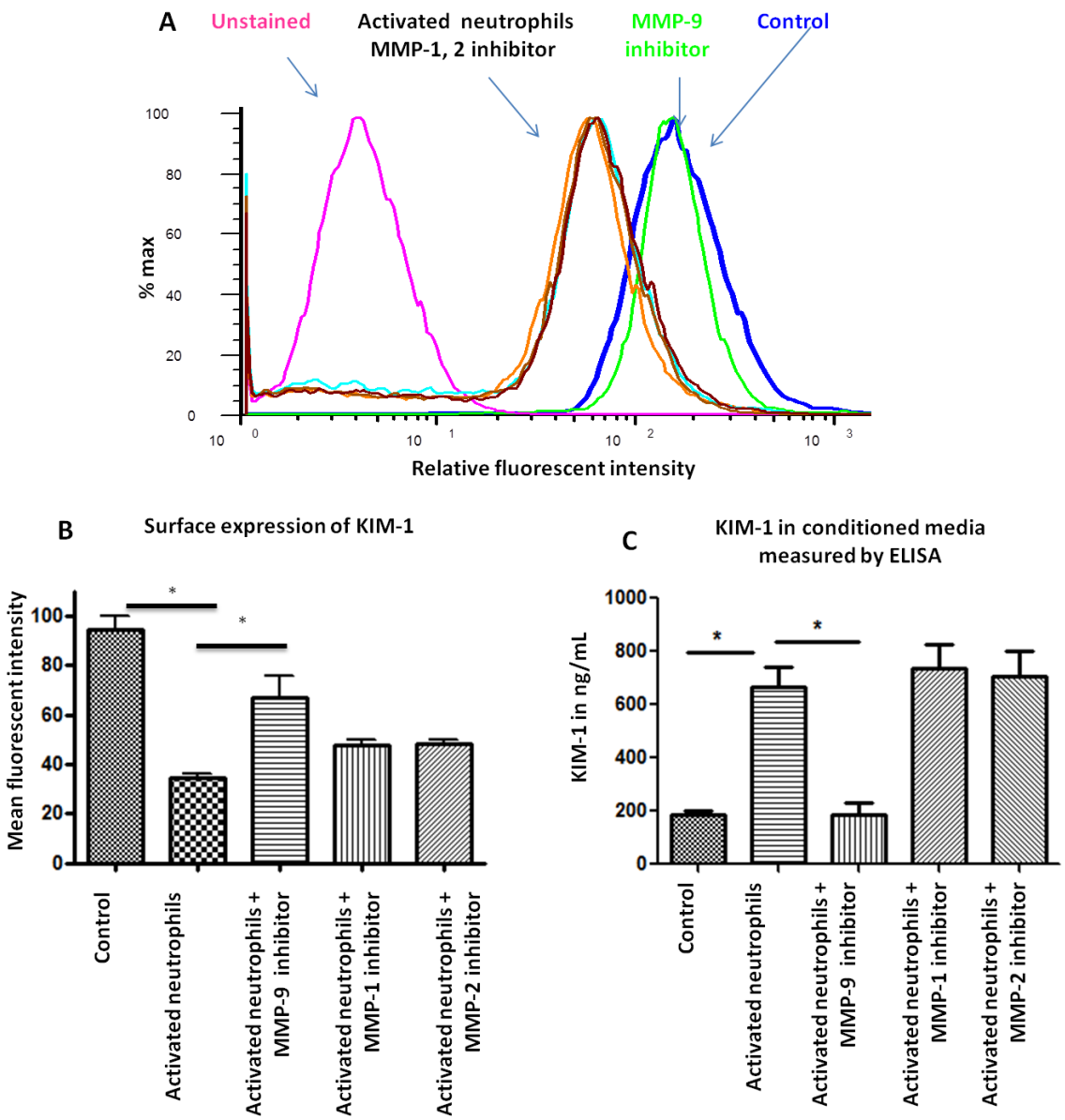

Figure 15: Pretreatment of activated neutrophils with MMP-9 inhibitor inhibits KIM-1 shedding in HK2 cells. A) Surface expression of KIM-1 was analyzed by FACS and 35,000 HK-2 cells were counted per group. The relative fluorescent intensity profile is represented in the graph. B) The bar diagram shows the mean relative fluorescent intensity values from 4 independent experiments. HK-2 cells treated with activated neutrophils show significant reduction in the KIM-1 expression and the shedding is significantly inhibited on pre-treating the

activated neutrophils with specific MMP-9 inhibitor. Pre-treating the activated neutrophils with MMP-1 and MM-2 inhibitor show now change in the KIM-1 expression. C) The shed KIM-1 ectodomain was quantified by ELISA. Shed KIM-1 in conditioned media quantified by ELISA. The data are expressed as mean ± SEM (n=4), $p < 0.05$ was considered significant.

A significant reduction in the shedding of KIM-1 ectodomain is observed on treating the HK-2 cells with activated neutrophils + MMP-9 inhibitor compared to activated neutrophils without MMP-9 inhibitor. However, HK-2 cells treated with MMP inhibitors did not show any changes in the surface expression of KIM-1, suggesting that the MMPs involved in KIM-1 ectodomain shedding are secreted by activated neutrophils and not by HK-2 cells (data not shown). According to our findings, even though MMP-9 secreted from activated neutrophils predominantly mediates KIM-1 ectodomain shedding, the role of other MMPs cannot be neglected.

4.7 Inhibiting secreted MMP-9 increases the uptake of vesicles containing phosphatidylserine in HK-2 cells

As discussed in section 4.6, we observed a significant reduction in the KIM-1 ectodomain shedding on inhibiting the secreted MMP-9 from activated neutrophils. As the KIM-1 ectodomain contains the PS binding site and is involved in the uptake of the unilamellar vesicles containing PS, we analyzed the effect of inhibiting MMP-9 in activated neutrophils on PS vesicle uptake in HK-2 cells by FACS. The results from the FACS analysis are represented in Figure 16. As described in section 3.4, HK-2 cells cultured to 80 % confluence were treated with activated neutrophils with or with not

pretreating them with specific MMP-9 inhibitor followed by treatment with unilamellar lipid vesicles containing PS. HK-2 cells treated with activated neutrophils show significant reduction in the amount of relative fluorescent intensity, suggesting reduced uptake of vesicles (green) compared to untreated cells (blue). On inhibiting MMP-9 in activated neutrophils, the amount of relative fluorescent intensity significantly increases, suggesting the regain of the lipid uptake capacity by HK-2 cells (orange). Inhibition of MMP-9 inhibits the shedding of KIM-1 ectodomain, which leads to the regain of the phogocytic property of HK-2 cells.

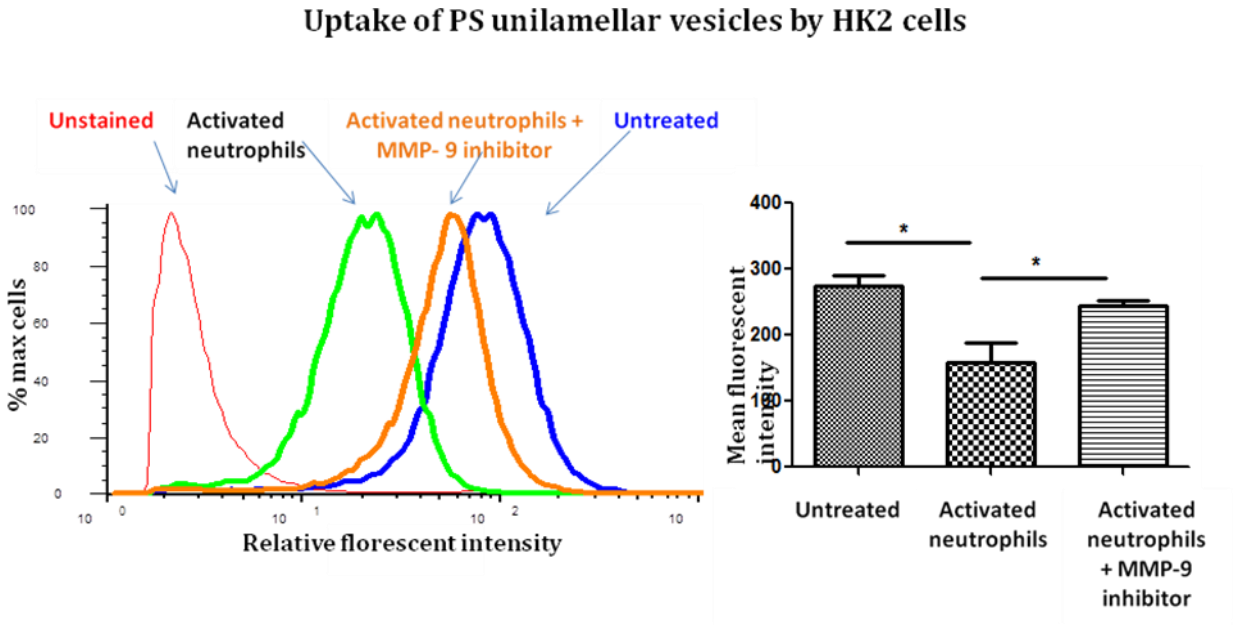

Figure 16: Inhibition of activated neutrophil secreted MMP-9 increases the uptake of unilamellar PS vesicles in HK-2 cells. A) Uptake of PS vesicles by HK-2 cells was analyzed by FACS and 35,000 HK-2 cells were counted per group. The relative fluorescent intensity profile is represented in the graph. B) The bar diagram shows the mean relative fluorescent intensity values from 4 independent experiments. HK-2 cells on treatment with supernatants from activated neutrophils pretreated with specific MMP-9 inhibitor show significant increase in uptake of

unilamellar vesicles containing PS compared to HK-2 cells treated with supernatants from activated neutrophils. There is also significant reduction in the uptake of PS vesicles in HK-2 cells treated with supernatents from activated neutrophils comaperd to untreated HK-2 cells.The data are expressed as mean ± SEM (n=4), $p < 0.05$ was considered significant.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

KIM-1, a sensitive, non-invasive urinary biomarker for kidney injury is also suggested to play a role in ARF pathogenesis, renal repair, and regeneration. In this study, we have examined the interaction of activated neutrophils with KIM-1 on HK-2 cells, relevant to its possible effects on kidney regeneration after acute injury. We have demonstrated activated neutrophils mediate significant KIM-1 ectodomain shedding in HK-2 cells. Activated neutrophils are known to secrete many proteases including MMPs. We have demonstrated a significant reduction in the KIM-1 ectodomain shedding in HK-2 cells on pretreating the activated neutrophils with a specific MMP-9 inhibitor, suggesting the role of neutrophil secreted MMP-9 in KIM-1 shedding. Even though role of other MMPs cannot be neglected, we have demonstrated that shedding of KIM-1ectodomain is predominantly by MMP-9 mediated.

KIM-1 is a scavenger receptor i.e. epithelial cells expressing KIM-1 obtain the properties of 'semi-professional' phagocytes. KIM-1 expressing epithelial cells

recognizes the PS on apoptotic/necrotic cells and mediates its uptake and directs it to degradation in lysosome. Rapid clearing of apoptotic/necrotic cells is necessary to prevent further inflammation and renal regeneration. We examined the neutrophil mediated KIM-1 shedding on the ability to take up the PS containing unilamellar vesicles which mimicked this process of apoptotic/necrotic cell uptake by renal tubular epithelial cells. In this study we have demonstrated a reduction in PS vesicle uptake by HK-2 cells by materials released from activated neutrophils. Inhibiting KIM-1 shedding in HK-2 cells by inhibiting neutrophil secreted MMP-9 prevents the neutrophil mediated loss of PS vesicle uptake capacity. Thus we conclude, KIM-1 may play a role in renal repair and regeneration by rapid clearing of apoptotic/necrotic cells from the lumen and preventing the shedding of KIM-1 by inhibiting MMP-9 form the neutrophils may hasten the renal regeneration.

5.2 Future directions

In this *in-vitro* study we have successfully demonstrated the role of activated neutrophils in KIM-1 shedding and its effects on uptake of apoptotic bodies. These results should be further validated in a mouse model. Though TIM-1(-/-) mouse has failed to show any changes in the phenotype, mice lacking renal KIM-1/TIM-1 will help to provide a better understanding of role of KIM-1 in renal pathogenesis and repair. A neutrophil depletion model will provide a better understanding of the interaction of neutrophils with KIM-1. These studies will provide us with an opportunity to develop treatment modalities, which involves prevention of KIM-1 shedding and hastening the renal recovery.

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