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Kidney Injury Molecule-1 Signalling in Ischemic Acute Kidney Injury and Phagocytosis

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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(Thesis format: Integrated Article)

by

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I. ABSTRACT

Acute kidney injury (AKI) is defined by the rapid loss of kidney function due to tissue damage. It affects 10-30 % of hospitalized patients and is independently associated with increased morbidity and mortality. Ischemia-reperfusion injury (IRI) is the most common pathoetiological mechanism of AKI, whereby tissue injury is mediated by reactive oxygen species. Ischemic AKI leads to the rapid upregulation of a transmembrane protein, kidney injury molecule-1 (KIM-1) on the apical membrane of proximal tubular epithelial cells (TECs). Previous work from our group and others demonstrated that the extracellular domain of KIM-1 specifically binds to phosphatidylserine on apoptotic cells, thereby transforming KIM-1-expressing TECs into semi-professional phagocytes for apoptotic corpses. The pathophysiological role of KIM-1 in AKI and relevant signalling mechanisms have not yet been elucidated. Using an *in vivo* model of AKI in mice genetically deficient in Kim-1, we reveal that Kim-1 expression protects mice from tissue damage and renal dysfunction after AKI. To uncover the signalling pathways downstream of KIM-1 in TECs, we performed protein-protein interaction studies to uncover two signalling partners, the alpha subunit of the heterotrimeric G12 protein (Gα12), and the dynein light chain protein (Tctex-1). We provide evidence that KIM-1 interacts with both Gα12 and Tctex-1, using co-immunoprecipitation, GST-pull down and confocal microscopy co-localization studies. We show that KIM-1 upregulation during IRI suppresses endogenous Gα12 activation and down-stream effectors. Further, we reveal that KIM-1-inhibition of Gα12, and the down-stream mediator RhoA, are crucial for TEC-mediated clearance of apoptotic cells. Tctex-1 is bound to KIM-1 during the early stages of phagocytosis becoming dissociated at later stages. Here, siRNA mediated studies informed us that Tctex-1 expression is necessary for phagocytic uptake of apoptotic cells. Finally, given our data showing that TECs isolated from Kim-1-deficient mice were virtually incapable of phagocytosis, it can be inferred that our findings regarding Gα12 and Tctex-1 do not solely apply to phagocytosis by KIM-1, but to all other mechanisms of uptake of apoptotic cells by TECs. In summary, our results suggest an important protective role of KIM-1 in ischemic AKI that is mediated via non-phagocytic and phagocytic mechanisms. The work described in this thesis provides several novel mechanistic insights into the functional role of KIM-1 and suggest potential therapeutic targets for future development.

II. KEYWORDS

Kidney Injury Molecule-1 (KIM-1)

Gα12

G proteins

Tctex-1

Kidney

Acute Kidney Injury

Ischemia-Reperfusion Injury

Reactive oxygen species

Phagocytosis

Actin cytoskeleton

Microtubules

Rho GTPase

Apoptotic cells

III. CO-AUTHORSHIP STATEMENT

Data presented in Chapter 2 are published in the American Journal of Pathology (2015 May;185(5):1207-15. doi: 10.1016/j.ajpath.2015.02.003. Epub 2015 Mar 7). In this chapter, Drs. Xizhong Zhang and Junjun Wei (Matthew Mailing Centre for Translational Transplant Studies, London, ON) performed bilateral renal artery clamping experiments on wild type and Kim-1-deficient mice. Dr. Aaron Haig (Lawson Health Research Institute, London, ON) stained the histology tissue samples and graded them. Dr. Bradley M. Denker (Harvard Medical School, Boston, MA) provided Gα12 plasmids constructs to carry out these experiments. Dr. Rita S. Suri (the Centre de Recherche, Centre Hospitalier de l'Université de Montréal, Université deMontréal, Montréal, QC) performed statistical analyses on the data presented in the manuscript. Dr. Alp Sener (Western University, the Matthew Mailing Centre for Translational Transplant Studies, Lawson Health Research Institute, London, ON) aided in editing the manuscript for publication. All other experiments were performed by myself.

For Chapter 3, I carried out all of the experiments. Dr. Xizhong Zhang provided reagents and technical support. Dr. Bonventre (Harvard Medical School, Boston, MA) provided us with reagents to carry out the experiment. The manuscript describing this work is under revision at the Am J. Physiol-Renal Physiology.

In Chapter 4, I performed all of the experiments. Dr. Xizhong Zhang helped with reagent preparation. The yeast-two hybrid screening was done by Dr. Zervos (University of Central Florida, Orlando, FL).

Dr. Lakshman Gunaratnam (Western University, the Matthew Mailing Centre for Translational Transplant Studies, Lawson Health Research Institute, London, ON) supervised me and provided funding necessary to carry out all of the work presented in this thesis. He also conceptualized and designed the research described in this thesis.

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V. ABBREVIATIONS

Abbreviation	Full Name
769-P	Human renal cell carcinoma
AC(s)	Apoptotic cell(s)/Apoptotic thymocyte(s)
ADAM 17	A disintegrin and metalloproteinase -17
AKAP-	A-kinase anchoring protein,
AKG7	KIM-1 antibody binding to the mucin domain of KIM-1
AKI	Acute kidney injury
ATP	Adenosine triphosphate
BMPR-II	Bone morphogenetic receptor type II
BTK	Bruton's tyrosine kinase
CD 5	Cluster of differentiation 5
CKD	Chronic Kidney Disease
Co-IP	Co-Immunoprecipitation
C-terminal	Carboxy terminal
DAMPs	Damage associated molecular patterns
DCs	Dendritic cells
DHC	Dynein heavy chain
DIC	Dynein intermediate chain
DLC	Dynein light chain
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
ESRD	End-Stage Kidney Disease
FBS	Fetal bovine serum
G proteins	Guanine nucleotide-binding proteins
GAPs	GTPase or Guanine activating proteins
GDP	Guanosine diphosphate
GEF-H1	Rho Guanine nucleotide exchange factor
GFR	Glomerular Filtration Rate
GPCRs	G protein-coupled receptors
GPNMB	Transmembrane glycoprotein NMB
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GTPγS	Guanosine 5'-O-[gamma-thio] triphosphate (non-hydrolyzable GTP)
H_2O_2	Hydrogen peroxide

HA	Hemagglutinin
HAVcr-1	Hepatitis A virus cellular receptor 1
HEK-293	Human embryonic kidney 293
HMGB1	High mobility group box 1
Hsp	Heat shock protein
ICAM-1	Intracellular adhesion molecule-1
ICU	Intensive care unit
Ig	Immunoglobulin
IL-6/8/18	Interlukin-6 or 8 or 18
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
IRI	Ischemia-reperfusion injury
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
KDIGO	Kidney Disease: Improving Global Outcomes
KIM-1	Kidney injury molecule-1 (Human)
Kim-1	Kidney injury molecule-1(mouse)
Kim-$1^{-/-}$	Kim-1 knock out mice
Kim-$1^{+/+}$	Kim-1 expressing mice (wild type)
LARG	Leukaemia-associated RhoGEF
L-FABP	L-type fatty acid-binding protein
Lfc	Lbc's first cousin
LLC-PK1	Porcine proximal tubule epithelial cells
LPA	Lysophosphatidic acid
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MCP-1	Monocyte chemoattractant protein-1
MDCK	Madin-Darby canine kidney
MILIBS	Metal-ion-dependent ligand-binding site
NaCl	Sodium chloride
NF-κB	Nuclear factor-κB
NGAL	Neutrophil gelatinase-associated lipocalin
N-terminal	Amino terminal
PAS	Periodic acid–Schiff
PBS	Phosphate buffer solution
pHrodo Red	pH-sensitive dye pHrodo™ Red succinimidyl ester
PI3K	phophoinositide-3-kinase
PMA	Phorbol 12-Myristate 13-Acetate
PP 5	Ser/Thr protein phosphatase 5

PP2A	Protein phosphatase 2A
PP5	Ser/Thr protein phosphatase type 5
PRRs	Pattern recognition receptors
PS	Phosphatidylserine
PVDF	Polyvinylidene difluride
pY419-Src	Active (phosphorylated) Src
QL	Constitutively active
QL Gα12	constitutively active Gα12
Rac	Ras-related C3 botulinum toxin substrate
RANTES	Regulated on activation, normal T cell expressed and secreted
RasGAP1	Ras specific GTPase activating protein
RGS	Regulator of G protein signalling
RhoA	Ras homolog gene family, member A
RIFLE	Risk, Injury, Failure, Loss and End-stage
ROS	Reactive oxygen species
SIGLECs	Sialic acid-binding immunoglobulin-type lectins
siRNA	Small interfering RNA
SNAP	Soluble NSF-associated protein
Src	Rous sarcoma oncogene cellular homolog
TACE	TNF-α-converting enzyme
Tctex-1	T complex-testis expressed-1
Tctex-1-T94A	Unphosphorylated mimic Tctex-1
Tctex-1-T94E	Phosphorylated mimic Tctex-1
TECs	Tubular epithelial cells
TGF-β	Transforming growth factor beta
Thr-94	Threonine-94
TIM-1	T-cell immunoglobulin and mucin domain-1
Tim-1$^{\Delta mucin}$	Tim-1 mucin domain mutant
TLR2/4	Toll-like receptors 2 or 4
TNF-α	Tumor necrosis factor alpha
TPR	Tetratricopeptide repeat
Trk	Tropomyosin receptor kinase or Receptor tyrosine kinase
TRPP2	Polycystin2
VDAC1	Voltage-dependent anion-selective channel 1
WT	Wild type
WT Gα12	Wild type Gα12
ZO-1 or ZO-2	Zonula occludens 1 or 2

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Chapter 1

Introduction

The introduction was written by Ola Ziyad Ismail and edited by Dr. Gunaratnam

1.1. THE KIDNEY

The kidney is one of the most important organs in the body, as it is responsible for filtering the body's metabolic waste while retaining precious nutrients. Each kidney is made up of about 1 million functional units, referred to as nephrons (Kurts *et al.*, 2013). Structurally, each nephron consists of a glomerulus and a long tubular structure. The glomerulus is the filtration unit of the kidney and consists of fenestrated endothelial cells, a glomerular basement membrane, and visceral epithelial cells called podocytes, which collectively form the glomerular filtration barrier (Kurts *et al.*, 2013). This barrier is highly selective, allowing only water and small molecules (less than 60 kDa) to pass from the bloodstream into what is known as Bowman's space that leads into the tubular lumen. The process of filtration of blood through the glomerulus is known as ultrafiltration or glomerular filtration, which is often quantified as the glomerular filtration rate or GFR. A decrease in kidney function is defined by a reduction in the GFR. Changes in serum creatinine concentration, an inert metabolite that is filtered freely with no distal reabsorption and minimal tubular secretion, can be used to estimate the GFR (Bellomo *et al.*, 2004).

The tubule of the nephron consists of various differentiated segments: from proximal to distal these are the proximal convoluted tubule, the loop of Henle, the distal convoluted tubule, and the collecting duct. The collecting ducts ultimately lead to the renal pelvis where urine collects (Eckardt *et al.*, 2013). The tubular lumen is surrounded by renal tubular epithelial cells (TECs). These TECs are responsible for reabsorbing the majority of filtered water, small proteins, amino acids, carbohydrates, electrolytes, and water soluble vitamins. Other organic molecules and drug metabolites are non-absorbed along with some water to form the urine. The secretory and reabsorptive function of TECs allow for tight regulation of plasma osmolality, blood pressure, acid-base and electrolytes balance. Distal parts of the tubule also partake in this process. In addition to the reuptake of filtered macromolecules, the tubular epithelium also plays a role in hormone homeostasis (Eckardt *et al.*, 2013). The kidneys' fibroblasts are also responsible for producing erythropoietin, a vital hormone that is induced in response to hypoxia and regulates erythropoiesis. Finally, the kidney produces the hormone renin, which induces angiotensin and aldosterone production to regulate electrolyte and acid-base balance and blood pressure. In summary, the kidneys play excretory, metabolic and endocrine functions that dictate homeostasis. Importantly, life cannot be sustained in the absence of functional kidneys and thus patients with end-stage kidney disease (ESRD) require some form of dialysis or kidney transplantation to sustain life.

The main cell type in the kidney is tubular epithelial cells (TECs), with the remainder consisting of endothelial cells, smooth muscle cells, podocytes, and various cells within the interstitium, such as resident innate immune cells and fibroblasts. Proximal TECs form a "leaky" epithelium due to the expression of pore-forming claudin proteins (Claudins 2 and 10) in the tight junctions (Muto *et al.*, 2010). Proximal TECs also express abundant energy-dependent transporters, whose high energy demand is met by oxidative phosphorylation (Gstraunthaler *et al.*, 1985). This is a key feature that causes the proximal tubular region to be most susceptible to ischemic injury.

1.2. ACUTE KIDNEY INJURY

1.2.1. Definition

Acute kidney injury (AKI), previously known as acute renal failure, is defined as the abrupt decrease in renal function over a period of hours to days, leading to the inability of the kidney to excrete waste products and maintain fluid and electrolyte homeostasis (Thadhani *et al.*, 1996). However, a precise clinically diagnostic definition of AKI is still in development. Currently, most clinical definitions rely on serum creatinine, but newer definitions are being developed to identify early injury using biomarkers well before perturbations in serum creatinine are noticed (Kellum *et al.*, 2002; Levey *et al.*, 1988). To date, finding perfectly sensitive and specific markers for early AKI have been problematic, given the significant variation in etiology and patient populations affected. The first evidence-based consensus study to define AKI was done in 2004 (Bellomo *et al.*, 2004; Kellum *et al.*, 2002). It encompasses what is known as RIFLE criteria, which stands for three levels of increasing AKI severity (Risk, Injury, Failure), and outlines two outcome variables (Loss and End-stage) based on urine output and GFR level. The RIFLE criteria was later modified by the Acute Kidney Injury Network (AKIN) (Mehta *et al.*, 2007) to improve sensitivity of AKI diagnosis (Akcan-Arikan *et al.*, 2007; Mehta *et al.*, 2007). In 2012, the Kidney Disease: Improving Global Outcomes group (KDIGO) released yet newer guidelines for diagnosing and staging AKI (Work *et al.*, 2012) (Table 1.1).

Table 1. 1. The current staging of AKI as determined by the Kidney Disease: Improving Global Outcomes (KDIGO) foundation.

Modified from source; Work, Kidney Disease: Improving Global Outcomes (KDIGO) Acute Kidney Injury, & Group. (2012). KDIGO Clinical Practice Guideline for Acute Kidney Injury. Kidney Int. Supplementary, 2(1), 138.

Stage	Serum Creatinine	Urine Output
1	1.5-1.9 times baseline OR, ≥0.3 mg/dl (≥26.5 µmol/l) increase	<0.5 ml/kg/h for 6-12 hours
2	2.0-2.9 times baseline	<0.5 ml/kg/h for ≥12 hours
3	3.0 times beaseline OR, Increase in serum creatinine to ≥4.0 mg/dl (≥353.6 µmol/l) OR, Initiation of renal replacement therapy OR, In patients <18 years, decrease in estimated GFR to <35 ml/min per 1.73 m^2	<0.3 ml/kg/h for ≥24 hours OR, Anuria for ≥12 hours

1.2.2. Etiology

Ischemia-reperfusion injury (IRI) and nephrotoxins are the most common causes of AKI in humans (Thadhani *et al.*, 1996). IRI occurs with the significant reduction or cessation of blood flow (ischemia), resulting in immediate oxygen deprivation (hypoxia) and accumulation of toxic metabolic products, followed by restoration of blood flow (reperfusion) with production of reactive oxygen species (J. V. Bonventre *et al.*, 2011). In patients, the reduction of blood flow is most commonly caused by low blood pressure (hypotension). Septic shock is the most common cause of AKI in hospitalized patients, accounting for >50 % of AKI cases in intensive care unit (ICU) patients (Hoste *et al.*, 2007;Lopes *et al.*, 2010; Uchino *et al.*, 2005). Sepsis results in altered blood flow to vital organs including the kidneys, and is responsible for inadequate delivery of oxygen and metabolic substrates to cells. In this setting, ischemic AKI is exacerbated by the administration of vasoconstrictive drugs that are the cornerstone of treatment for septic shock (Sharfuddin *et al.*, 2011). Another common cause of ischemic AKI is cardiovascular surgery, where up to one fifth of patients will develop the condition (Pickering *et al.*, 2015). Overall, ischemia is the major cause of AKI and can be caused by many specific conditions (Figure 1.1), and is influenced heavily by the health status of a patient or the existence of co-morbidities.

Ischemic AKI can also occur due to secondary causes that influence systemic haemodynamics. Examples include cardiorenal syndrome, whereby a primary cardiac disorder compromises cardiac output and renal perfusion (McCullough *et al.*, 2013; Ronco, 2010), and hepatorenal syndrome in which acute or chronic liver failure results in exaggerated splenic vasodilation and renal hypoperfusion (Garcia-Tsao *et al.*, 2008; Gines *et al.*, 2009).

In addition, AKI may also be caused by extrinsic toxins (Joseph V. Bonventre *et al.*, 2004; Lameire *et al.*, 2008). Nephrotoxic agents can cause injury either by directly damaging TECs, or indirectly by altering renal hemodynamics (Perazella, 2012). For example, aminoglycosides are directly toxic to proximal tubule cells (Perazella, 2012; Schetz *et al.*, 2005), while angiotensin converting enzyme inhibitors decrease GFR by causing vasoconstriction of the glomerular blood vessels. About ten percent of AKI is caused by post-renal etiologies that are defined by obstruction to urine flow beyond the kidney (Basile *et al.*, 2012). Examples of this are benign prostatic hyperplasia compressing the urethra, or metastatic cancer compressing the ureters (Campbell *et al.*, 2014). Other rarer causes of AKI include renal inflammation (i.e. interstitial nephritis) due to medication allergy, infection, or autoimmunity (Choudhury *et al.*, 2006; Basile *et al.*, 2012).

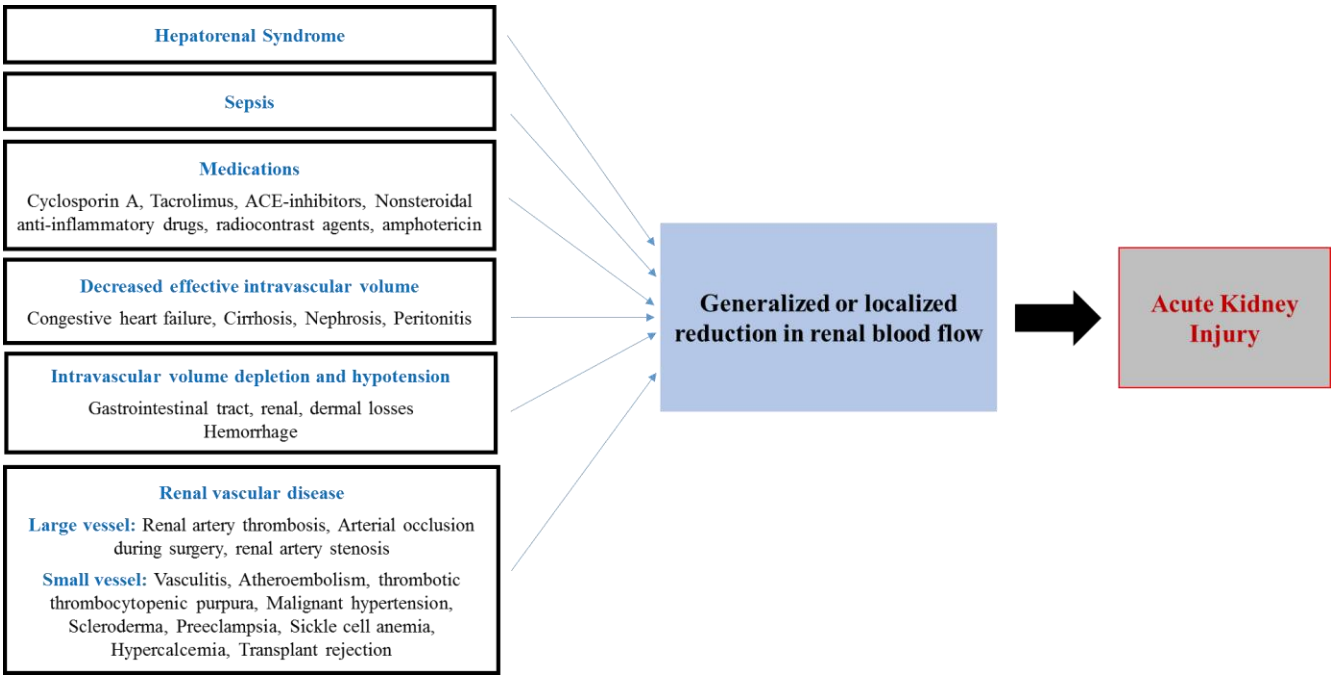


Figure 1.1. Pathophysiological conditions and medications leading to acute kidney injury

Modified from Bonventre, J. V and Yang, L. Cellular pathophysiology of ischemic acute kidney injury. Review. *J Clin Invest.* 2011: 121 (11): 4210-21

1.2.3. Animal models

The most widely used animal model of human AKI is the rodent ischemia-reperfusion model. It consists of transiently clamping and un-clamping the renal vascular pedicle to mimic IRI in humans (Heyman *et al.*, 2009; Heyman *et al.*, 2002). Two types of ischemia can be employed; i) cold ischemia to mimic what happens during organ preservation in renal transplantation, and ii) warm ischemia to mimic ischemic injury that occurs during shock or organ retrieval of the native kidney (Heyman *et al.*, 2002; Prowle *et al.*, 2009). In renal transplantation, cold ischemia starts when the organ is perfused with cold perfusion solution after procurement, and lasts until the temperature of the organ reaches the physiologic temperature (Jang *et al.*, 2015). Warm ischemia begins when perfusion is restored after anastomosis (Alejandro *et al.*, 1995; Halazun *et al.*, 2007). An alternative model to IRI is the nephrotoxic model where animals are administered a nephrotoxic agent such as cisplatin, a chemotherapeutic agent, which causes nephrotoxic AKI with some pathophysiological similarities to the IRI model (Yap *et al.*, 2012). A limitation of animal models is that they cannot capture the impact of co-morbidities and demographics that are well recognized to influence the development, course, and prognosis of AKI in humans (Bihorac *et al.*, 2009; Zarjou *et al.*, 2011; Grams *et al.*, 2015; James *et al.*, 2015). For example, one of the most important risk factors for the development of AKI is prior history of chronic kidney disease (Ishani *et al.*, 2009; Xue *et al.*, 2006).

1.2.4. Epidemiology of acute kidney injury

Despite advances in the diagnosis and management of AKI, it remains associated with high mortality and morbidity for patients. AKI affects between 10-20 % of all hospitalized patients worldwide depending on the definition used, with prevalence in the intensive care unit reaching over 30 % (Chertow *et al.*, 2005; Susantitaphong *et al.*, 2013). Almost 25 % of patients with AKI die, with in-hospital mortality and prolonged hospitalization being proportionally increased with the degree of kidney dysfunction in AKI (Hoste *et al.*, 2007; Chertow *et al.*, 1995). Worldwide, AKI affects approximately 13.3 million people each year, and is believed to contribute to 1.7 million deaths annually (Lewington *et al.*, 2013; Mehta *et al.*, 2015). Population-based studies suggest that AKI confers a large economic burden to the health care system. Greater than one million hospitalizations in the United States are complicated by AKI, accounting for ten billion in excess costs (Wald *et al.*, 2015).

In spite of advances in current therapeutic strategies to resolve AKI, the high mortality rate associated with AKI remains unchanged (Bihorac *et al.*, 2009; Chertow *et al.*, 2005; Chronopoulos *et al.*, 2010). While the metabolic derangements resulting from severe AKI can be managed with dialysis, there is little evidence that renal replacement therapies change long-term prognosis (Lo *et al.*, 2009; Metcalfe *et al.*, 2002; Wald *et al.*, 2009). Further, a significant proportion of surviving patients suffer long-term sequelae, including the development of chronic kidney disease (CKD) and end-stage renal disease (ESRD) (Chawla *et al.*, 2012; Hsu, 2012; Coca *et al.*, 2012). One recent study found that patients who appear to recover from their need for dialysis after an initial AKI episode have a 3-fold risk of eventually needing dialysis again for ESRD, compared to patients without AKI (Wald *et al.*, 2009). There is now strong experimental evidence suggesting a pathogenic link between AKI and CKD (Coca *et al.*, 2012; Venkatachalam *et al.*, 2015).

Whether the increase in mortality observed with AKI is causal or due to other factors is unclear, but emerging evidence suggests that AKI may lead to systemic inflammation and contribute to remote organ dysfunction (Awad *et al.*, 2009; Klein *et al.*, 2008; Vieira *et al.*, 2007). Animal studies show that AKI can lead to remote organ dysfunction in the lungs (K. D. Liu, 2009; Rabb *et al.*, 2003), the heart (K. J. Kelly, 2003; Kramer *et al.*, 1999), the liver, the intestines, and the brain (M. Liu *et al.*, 2008; Pratschke *et al.*, 2001).

1.2.5. Pathophysiology

The pathophysiology of AKI is highly complex and varied depending on its etiology (J. V. Bonventre *et al.*, 2011). As discussed above, the major pathoetiologies include ischemia, toxins, obstruction, and inflammation. For the purposes of this thesis, we will focus on ischemic AKI, the major cause of AKI in humans. The clinical course of ischemic AKI has been divided into several phases: initiation, extension, maintenance, and recovery. The cellular alterations taking place in the kidney at each phase are summarized in Table 1.2 (Molitoris *et al.*, 2004; Sutton *et al.*, 2002).

Table 1. 2. Summary of the different phases of acute kidney injury and the cellular pathophysiology occurring at the tubular and vascular endothelial cell levels.

AKI- Acute Kidney Injury, ATP- Adenosine Triphosphate, ROS- Reactive Oxygen Species, KIM-1- Kidney injury molecule-1, GPNMB -Transmembrane glycoprotein NMB.

Phases of AKI	Initiation	Extension	Maintenance and Recovery
Main initiator of damage	ATP Depletion	Inflammation	
Tubular Epithelial Cells	↑ Intracellular calcium		
	↑ Production of ROS		
	Apical and basolateral cytoskeleton disruption	Injured cells upregulate pro-survival mechanism (ie. KIM-1, GPNMB)	Some of these pro-survival signals play a role in clearance of apoptotic or necrotic cells
	Loss of cell polarity		
	Shed cells and cellular debris	Tubular construction Backleakage Altered transport	
	Proinflammatory cytokines production	Recruitment of leukocytes	Some recruited cells such as regulatory T cells modulate pro-inflammatory cytokines produced by other T cells
	↑ Toll-like receptor 2 and 4		
			Proliferation and differentiation by mesenchymal cells or tubular cells
Vascular Endothelial Cells	Disruption of actin cytoskeleton and junctional complex		
	Collapse of perivascular matrix	Impaired capillary flow	
	↑ Permeability		
	↑ Vasconstrictors production		
	↑ Adhesion molecules	Recruitment of leukocytes	
	↑ Proinflammatory cytokines		
			Proliferation by endothelial progenitor cells

During the initiation phase, cells are exposed to the ischemic insult. This directly leads to pathogenic effects that occur during the extension phase, resulting in a reduced GFR and altered kidney function. Depending on the severity of the injury (for example, the duration of ischemia), cells may experience different fates ranging from sub-lethal injury to different forms of cell death. One of the main mechanisms of cell death during IRI is via ATP depletion and altered tubule cell metabolism (Devarajan, 2006; Togel *et al.*, 2014). The proximal tubular epithelial cells of the S3 segment of the nephron are most sensitive to ischemic injury, due to their high metabolic rate, dependence on oxidative phosphorylation, and the unique architecture of the blood supply in the kidney (Basile *et al.*, 2012; Bastin *et al.*, 1987; Uchida *et al.*, 1988). When oxidative stress occurs, tubular cells fail to efficiently convert from oxidative to glycolytic metabolism, resulting in ATP depletion and cell injury through multiple mechanisms. ATP depletion leads to an increase in free intracellular calcium (Schrier *et al.*, 1987), which in turn causes activation of proteases, phospholipases, and cytoskeletal derangements (Devarajan, 2005). Importantly, ATP depletion causes the production of reactive oxygen molecules (Paller *et al.*, 1984). Reactive oxygen molecules activate many intermediates that cause protein oxidation (Andreoli *et al.*, 1997), lipid peroxidation (Zager, 1996), and DNA damage, triggering apoptotic cell death (Safirstein, 1997; Ueda *et al.*, 1992). ATP depletion also results in the loss of cell polarity, due to apical and basolateral mislocalization of polarized proteins (Devarajan, 2006; Molitoris, 2004). For example, mislocalization of the vital Na^+/K^+-ATPase pumps (Molitoris, 1993; Molitoris *et al.*, 1991), causes bidirectional transport of sodium and water across the epithelial membrane, leading to high fractional excretion of sodium often seen early in ischemic AKI in patients (Sharfuddin *et al.*, 2011). Mislocalization of β1 integrin, a basolateral protein (Zuk *et al.*, 1998), leads to subsequent detachment of viable cells from the tubular basement membrane during the extension phase. Experimental evidence suggests that this process of detachment contributes to cast the formation and obstruction within the tubular lumen, which is believed to further impair GFR (Molina *et al.*, 2005; Yang *et al.*, 2015).

In addition, ATP depletion causes alteration of the apical cytoskeleton due to dissociation of actin-stabilizing proteins, including tropomyosin and ezrin (Ashworth *et al.*, 2003; Ashworth *et al.*, 2004). The dissociation of these proteins allows the actin depolymerizing factor, cofilin, to sever apical F-actin. Sequentially, this leads to the formation of membrane-bound or free floating extracellular vesicles that are either internalized or released into the tubular lumen (Ashworth *et*

al., 1999). Disruption of the apical cytoskeleton also results in the loss of tight and adherence junctions (Devarajan, 2006; Sharfuddin *et al.*, 2011). Specifically, the loss of interaction between the tight junction proteins Neph1 and zonula occludens-1 (ZO-1) results in a loss of tubular integrity, which is associated with proteinuria in rat model of renal ischemic injury (Wagner *et al.*, 2008). The loss of tight junctions is important as it leads to impaired barrier function of the tubular epithelium, which magnifies the pathological effects of kidney dysfunction during the extension phase by contributing to the back leak of glomerular filtrate (e.g. filtered creatinine or urea) (Kwon *et al.*, 1998).

The initiation phase also damages vascular endothelial cells (Basile *et al.*, 2012), where disruption of actin cytoskeleton and junctional complexes occurs in a similar manner to that described above for TECs (Devarajan, 2006; Molitoris *et al.*, 2004). This results in collapse of the perivascular matrix, increasing microvascular permeability, thus allowing fluids to be lost into the interstitium and impairing capillary flow during the extension phase (J. V. Bonventre *et al.*, 2011; Sutton *et al.*, 2002). Injured endothelial cells also increase production of vasoconstrictors such as endothelin, and decrease the production of vasodilators such as nitric oxide (Conger *et al.*, 1991; Goligorsky *et al.*, 2004). Additionally, stressed endothelial cells express a variety of adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), P-selectin and E-selectin, which promote endothelial-leukocyte interactions to promote inflammation (Devarajan, 2006; K. J. Kelly *et al.*, 1994; Linas *et al.*, 1995). The combination of endothelial cell expression of adhesion molecules, production of vasoconstrictors, and increased vascular permeability significantly contributes to progression into the extension phase.

It was previously thought that if the injury was sublethal, complete restitution and recovery could occur after the cessation of the IRI insult. However, new evidence illustrates that dysfunctional endothelial and tubular cells substantially contribute to the extension phase, propagating tissue damage (Devarajan, 2006; Molitoris *et al.*, 2004). The extension phase is marked by inflammatory responses that are not only initiated by endothelial dysfunction, but according to recent evidence, by injured TECs (Joseph V. Bonventre *et al.*, 2004; Friedewald *et al.*,2004; Yang *et al.*, 2015). Together, these injured cells are responsible for the generation of proinflammatory cytokines (e.g. TNF-α, TGF-β, IL-6) and chemotactic cytokines (e.g. monocyte chemoattractant protein-1 (MCP-1), RANTES, IL-18) (Joseph V. Bonventre *et al.*, 2004; Daha *et al.*, 2000; Donnahoo *et al.*, 2000; Ishibashi *et al.*, 1999; Segerer *et al.*, 2000). In humans, plasma

levels of the proinflammatory cytokines IL-6 and IL-8 have been shown to predict mortality in AKI patients (Kielar *et al.*, 2005; Simmons *et al.*, 2004). Injured TECs also express toll-like receptors 2 and 4 (TLR2 and TLR4) that enable them to recognize extracellular danger signals and trigger inflammatory responses from within (Leemans *et al.*, 2005; Wu *et al.*, 2007). The sum of effects mediated by the proinflammatory mediators produced by TECs and endothelial cells causes an escalation of inflammation via recruitment of leukocytes and inflammatory cells (Friedewald *et al.*, 2004). Ultimately, the ensuing inflammatory response to the initial ischemic injury to cells serves to cause secondary tissue damage.

1.2.6. Mechanisms of cell death during acute kidney injury

During the extension phase, lethally injured TECs die either by apoptosis, regulated necrosis, or necrosis, depending on the level of ATP depletion and extent of cellular injury (Figure 1.2) (Basile *et al.*, 2012; Lieberthal *et al.*, 1998; Schumer *et al.*, 1992).

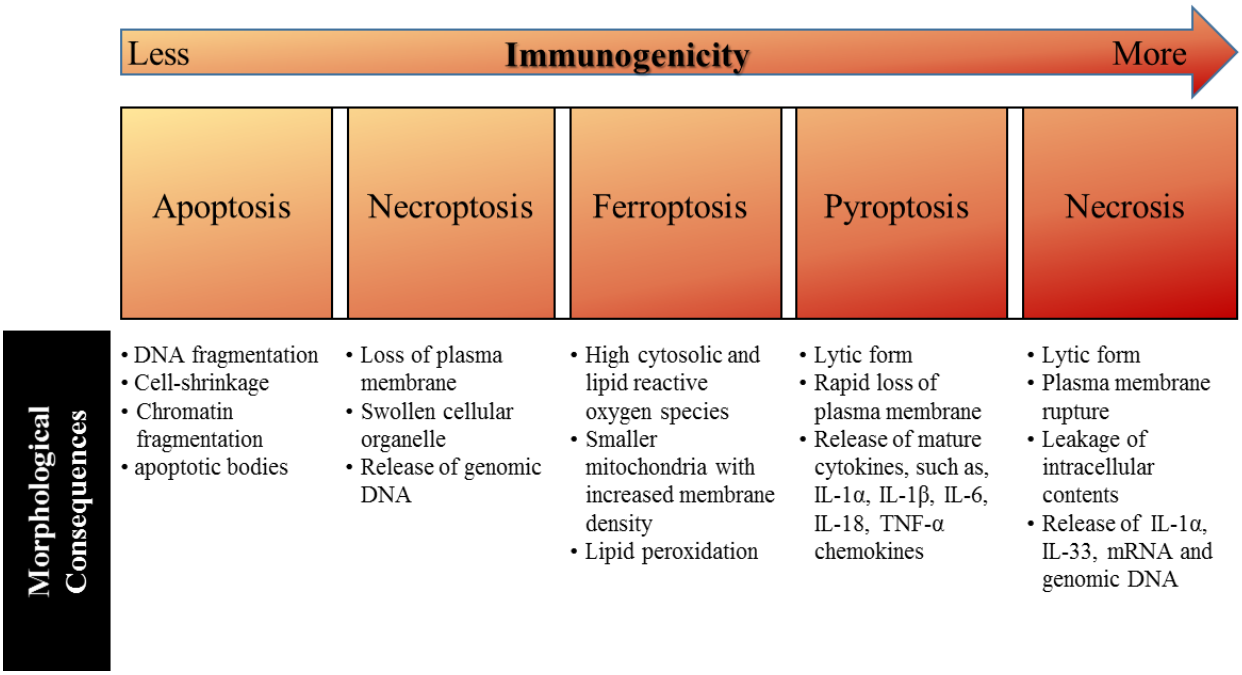


Figure 1.2. Different forms of cell death occurring in ischemic acute kidney injury and their contribution to inflammation.

The different forms of cell death occurring during ischemic acute kidney injury are divided into: programmed cell death (apoptosis), regulated necrosis (necroptosis, ferroptosis, or pyroptosis), and necrosis. The immunogenicity (ability to provoke an immune response) of each of these forms of death is based on the ensuing morphological consequence to the dying cell. All forms of cell death, except apoptosis, are known to release damage associated molecular patterns (DAMPs), such as High mobility group box 1 (HMGB1). Additional DAMPs released are indicated with morphological consequences of cell death forms. This figure likely oversimplifies the entire spectrum of cell death that occurs during tissue injury.

Apoptosis or programmed cell death is an energy-dependent mechanism that results in nuclear shrinkage, chromatin fragmentation and cell breakdown (J. F. Kerr, 2002; Padanilam, 2003; Wyllie *et al.*, 1980). The apoptotic cell membrane remains intact but often several proteins or modified lipids that are usually localized on the inner cellular membrane are exposed on the plasma membrane outer leaflet. These serve as "eat me" signals to allow for their phagocytic clearance by professional or semi-professional phagocytes (Fadok *et al.*, 1998; Ravichandran *et al.*, 2007). For example, the membrane phospholipid, phosphatidylserine, which is displayed by apoptotic cells before cellular fragmentation and formation of apoptotic bodies, is the most widely studied "eat me" signal to date (Fadok *et al.*, 1998; Fadok *et al.*, 1992). Since apoptosis usually occurs during normal cell turnover, there is little effect of this form of cell death on the surrounding tissue and it is largely considered to be a non-immunogenic form of cell death (Figure 1.2). Interestingly, blocking apoptotic pathways in mice subjected to ischemic AKI resulted in reduced inflammation and tissue damage (Daemen *et al.*, 1999).

In contrast, necrosis is considered to be the most immunogenic form of cell death. It begins with cellular and organelle swelling, followed by loss of plasma membrane integrity, with the consequent release of cytoplasmic and nuclear material outside the cell (Majno *et al.*, 1960). The intracellular material released from necrotic cells contains highly immunogenic proteins, referred to as damage associated molecular patterns (DAMPs), such as ATP, IL-1α, and HMGB1, double-stranded DNA (Kono *et al.*, 2008; Leach *et al.*, 1998). Extracellular DAMPs are recognized by specific pattern recognition receptors (PRRs), such as TLR-4 expressed by various cell types including tubular cells (Jang *et al.*, 2009). Cells responding to the DAMPs via PPRs produce even more proinflammatory mediators further amplifying the renal inflammatory response during ischemic AKI (Wu *et al.*, 2007; B. Zhang *et al.*, 2008).

Recently, many forms of regulated necrosis have been linked to ischemic AKI. Necroptosis is one type of regulated necrosis and is defined as necrotic cell death that is dependent on receptor-interacting protein kinase 3 (RIPK3) (Linkermann *et al.*, 2012; Tait *et al.*, 2013). Upon the activation of RIPK3, it phosphorylates the Mixed Lineage Kinase domain-Like protein (MLKL) which leads to subsequent plasma-membrane rupture. Blocking RIPK3 signaling has been shown to be protective in ischemic AKI in animal models (Linkermann *et al.*, 2012; Linkermann, *et al.*, 2013; Tristao *et al.*, 2012; L. Zhang *et al.*, 2013). Other regulated necrotic cell death pathways implicated in AKI include pyroptosis and ferroptosis (Linkermann, Chen, *et al.*, 2014). Pyroptosis

is unique in that it results in the maturation of proinflammatory cytokines, such as IL-1β and IL-18, via caspase-1 activation which brings about more inflammatory responses than necroptosis (Miao *et al.*, 2011; Yang *et al.*, 2014). Following 12 hours of IRI, the levels of pyroptosis-related proteins, such as caspase-1, were significantly elevated in renal tubular epithelial cells (Yang *et al.*, 2014). Ferroptosis is an iron-dependent, oxidative, non-apoptotic form of cell death which involves glutathione metabolism (Dixon *et al.*, 2012; Skouta *et al.*, 2014). Ferroptosis was shown to mediate necrotic tubular cell death in renal IRI, since blocking ferroptosis via chemical inhibitor (ferrostatin) resulted in the protection from severe IRI as seen by lower serum creatinine and less histological damage (Linkermann, Skouta, *et al.*, 2014). On the other hand, mice deficient in the proximal tubular cell-specific ferritin heavy chain showed greater mortality and worse renal injury following cisplatin-induced AKI (Zarjou *et al.*, 2013). Due to the different forms of cell death involved and their respective roles in promoting tissue damage in AKI via promoting inflammation, the extension phase is thought to be a promising window for early diagnosis and active therapeutic interventions in AKI.

1.2.7. Various inflammatory cells in acute kidney injury

During the extension phase, various immune cells infiltrate the kidney. Based on animal studies, neutrophils are the earliest leukocytes to be recruited into injured endothelium (Willinger *et al.*, 1992), followed by macrophages (Jang *et al.*, 2015; Ysebaert *et al.*, 2000). These macrophages are known to produce proinflammatory cytokines that stimulate the activity of other leukocytes and parenchymal cells (Duffield, 2010). Interestingly, depletion of kidney and spleen macrophages in mice prior to renal IRI was shown to prevent AKI, while adoptive transfer of macrophages re-established the AKI response (Day *et al.*, 2005). Additionally, monocytes have been shown to migrate to inflamed kidney tissues and differentiate into M1-type macrophages or dendritic cells, which further participate in inflammatory responses (L. Li *et al.*, 2008). Dendritic cells (DCs) are also thought to have a role in AKI; depletion of DCs leads to a lower level of TNF produced by kidneys (Dong *et al.*, 2007; Tadagavadi *et al.*, 2010). Another innate immune cell that has been seen to infiltrate ischemic kidney during AKI is the natural killer cell, which has been shown to orchestrate more neutrophil infiltration via production of interferon-gamma (IFN-γ) (L. Li *et al.*, 2007).

T cells have also been shown to be involved in AKI. Depletion of T cells was shown to protect mice from ischemic AKI (Yokota *et al.*, 2002; Ysebaert *et al.*, 2004). Interestingly, T-

regulatory cells have been shown to traffic into the ischemic kidney (M. Liu *et al.*, 2009), and have been proposed to promote repair during the maintenance phase though modulation of proinflammatory cytokines by other T-cells subsets (Kinsey *et al.*, 2010; Kinsey *et al.*, 2009). The inflammatory response triggered at the initiation stage involves the activation of complement systems, which contributes to the amplification of tissue damage from inflammation (Arumugam *et al.*, 2004; Quigg, 2003). The majority of the existing research has implicated the alternative pathway (Thurman *et al.*, 2003) and mannose-binding lectin pathway of complement activation in AKI (de Vries, Kohl, *et al.*, 2003; De Vries, Matthijsen, *et al.*, 2003; de Vries *et al.*, 2004).

1.2.8. Mechanisms of repair and regeneration after acute kidney injury

While there are numerous injurious pathways activated in AKI, several intra-renal protective mechanisms are also upregulated during the extension phase that help to limit tissue damage and potentiate repair during the maintenance and recovery phases. Injured but surviving TECs upregulate many genes that promote tissue repair and curtail inflammatory responses (Wen *et al.*, 2010). For example, Kidney Injury Molecule-1 (KIM-1) is specifically upregulated on TECs following ischemic injury (Ichimura *et al.*, 1998). KIM-1 is a phagocytic receptor that recognizes phosphatidylserine on apoptotic and necrotic cells, allowing their uptake by TECs (Ichimura *et al.*, 2008). This uptake has been shown to clear apoptotic cells, preventing them from undergoing secondary necrosis, and mediating down-stream anti-inflammatory responses triggered by extracellular DAMPs in proximal TECs (see section 1.3.4). These included the NF-κB pathway, infiltration of immune cells, and production of inflammatory cytokines (Yang *et al.*, 2015). Another important repair protein that is upregulated on injured TECs is the transmembrane glycoprotein NMB (GPNMB) (B. Li, Castano, *et al.*, 2010; Sharfuddin *et al.*, 2011). GPNMB is important in the recruitment of autophagy protein LC3 to phagosomes and the fusion of lysosomes with phagosomes. The latter plays an important role in uptake and degradation of apoptotic cells. Mice with mutated GPNMB exhibited increased inflammation, impaired repair and more apoptotic cell accumulation in response to renal ischemia (B. Li, Castano, *et al.*, 2010). Other proteins involved in repair and regeneration during the maintenance and recovery phase expressed by TECs include heme oxygenase (Nath, 2006) and heat shock proteins (Z. Wang *et al.*, 2011).

Additionally, recruitment of regulatory T cells has been shown to promote repair through the modulation of proinflammatory cytokines produced by other T cells (Kinsey *et al.*, 2010; Kinsey *et al.*, 2009; Sharfuddin *et al.*, 2011), whereby the depletion of regulatory T cells subsets

(Foxp3^{+}) resulted in increased accumulation of leukocytes and inflammatory cytokines within the kidney (Gandolfo *et al.*, 2009).

During the maintenance phase, the fate of the kidney is likely determined by a balance between cells that survive injury or die from it, as only surviving cells can promote repair (Humphreys *et al.*, 2011; Humphreys *et al.*, 2008, Berger *et al.*, 2014). The recovery phase aims to improve GFR, and regenerate the vascular and tubular epithelium (Basile *et al.*, 2012). Understanding the process of regeneration and proliferation after ischemic AKI is still a work in progress, with much debate surrounding the source of these proliferating cells (Kramann *et al.*, 2015). Mesenchymal stem cells were thought to be the source of proliferative cells, since they could differentiate into renal epithelium and endothelium *in vitro* (Bussolati *et al.*, 2005). Several groups have previously shown that infusing mesenchymal stem cells improved renal function following injury and even protected from injury if cells were infused before the ischemic insult (De Broe, 2005; Lange *et al.*, 2005; Togel *et al.*, 2005). More recently, research by Duffield *et al.* and Humphrey *et al.* have contradicted these findings. Their groups showed that terminally differentiated proximal TECs were solely responsible for the dedifferentiation, proliferation and redifferentiation necessary to regenerate the kidney tubular epithelium (Duffield *et al.*, 2005; Duffield *et al.*, 2011; Humphreys *et al.*, 2011; Humphreys *et al.*, 2008; Kramann *et al.*, 2015). Whether endothelial progenitor cells originate from hematopoietic stem cells or mesenchymal stem cells and their role remains unclear (Reinders *et al.*, 2006; Tongers *et al.*, 2007). Regardless of the source of the endothelial progenitor cells, they are recruited to injured renal microvasculature to repair and improve the functional recovery of the kidney following AKI (Becherucci *et al.*, 2009; B. Li, Cohen, *et al.*, 2010).

Emerging evidence strongly suggests that repair mechanisms following AKI are mostly incomplete and, when injury is severe, could lead to CKD (Basile *et al.*, 2012; Devarajan, 2006). Hallmarks of incomplete recovery are persistent tubulointerstitial inflammation, fibroblast proliferation, excess extracellular matrix deposition, and chronic macrophage and fibrosis activation (Duffield, 2010; Hsu, 2012; Ko *et al.*, 2008; Venkatachalam *et al.*, 2010; Venkatachalam *et al.*, 2015). Overall, the cellular pathophysiology of ischemic AKI is a highly complex field of research that is continuously developing. It is hoped that understanding the injury and repair pathways involved in ischemic AKI will eventually lead to the development of novel therapeutic strategies to ameliorate AKI and potentially prevent its systemic consequences.

1.2.9. Biomarkers for acute kidney injury

A biomarker is defined as a biological "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers Definitions Working, 2001). When patients develop AKI, they are asymptomatic until most of the kidney function is compromised. To date, a rise in serum creatinine and/or fall in urine output have been the most widely used markers of kidney function (Levey *et al.*, 1988; Work *et al.*, 2012). Unfortunately, the rise in serum creatinine is often delayed until much damage has occurred and is generally an unreliable indicator of kidney dysfunction (Coca *et al.*, 2008). Other biomarkers traditionally used in conjunction with serum creatinine and urine output have been serum urea (Beier *et al.*, 2011), and the chemical analysis of urine, including urine sediment (Kanbay *et al.*, 2010), fractional excretion of sodium (Chawla *et al.*, 2008) and fractional excretion of urea (Carvounis *et al.*, 2002). None of the currently used diagnostic markers provide information about the severity or nature of kidney injury or whether the repair and recovery process has started. This information might aid clinicians to make better therapeutic decisions when it comes to AKI in patients.

This knowledge gap has promoted a new area of research that aims to implement specific biomarkers to detect kidney injury prior at the onset of AKI, well before it is detected by increased serum creatinine (Bellomo *et al.*, 2012; Devarajan *et al.*, 2010). Several new biomarkers, such as cystatin C (Ahlstrom *et al.*, 2004; Herget-Rosenthal *et al.*, 2004; Moore *et al.*, 2010), L-type fatty acid-binding protein (L-FABP) (Deitch, 1998; Nakamura *et al.*, 2009; Zarjou *et al.*, 2011), Interleukin-18 (IL-18) (Bagshaw *et al.*, 2007; Bellomo *et al.*, 2007; Parikh *et al.*, 2005), Netrin-1 (Ramesh *et al.*, 2010; Reeves *et al.*, 2008), neutrophil gelatinase-associated lipocalin (NGAL) (Haase *et al.*, 2009; Mishra *et al.*, 2005), and Kidney injury molecule-1 (KIM-1) (Ichimura *et al.*, 2004; Sabbisetti *et al.*, 2013; Vaidya *et al.*, 2009) have been proposed as early biomarkers for AKI diagnosis and prognostication (Figure 1.3).

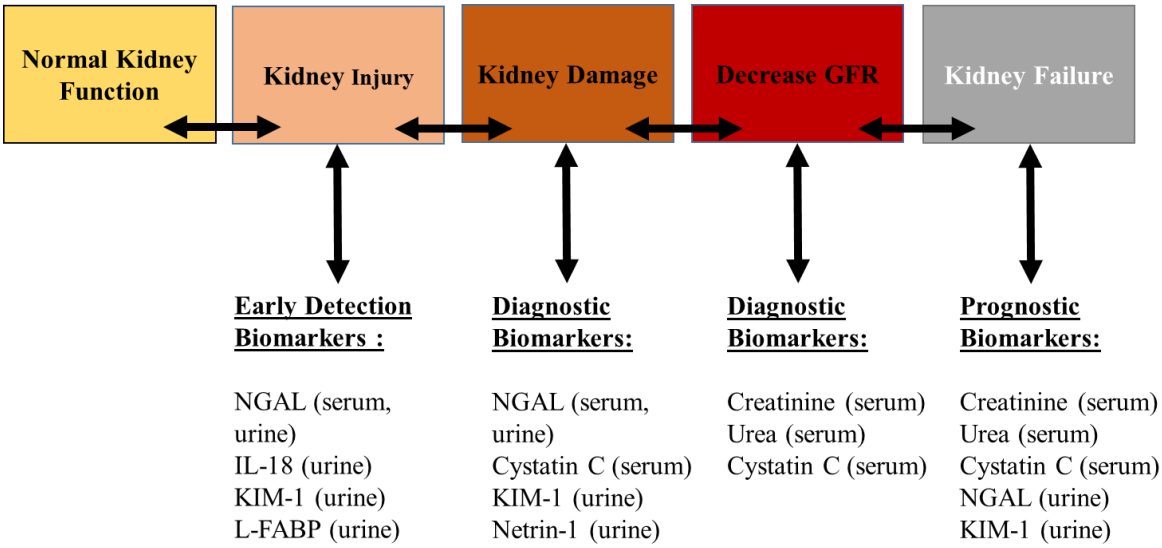


Figure 1.3. Emerging and traditional biomarkers of acute kidney injury during the different stages of disease progression

Kidney injury occurs before the kidney function is lost, as determined by the decreased glomerular filtration rate (GFR). Different markers detected in serum or urine during early kidney injury due to ischemic, toxic or septic insults serve as early detection markers. An increase in diagnostic markers might be parallel or prior to the loss of kidney function as determined by traditional biomarkers of decreased GFR, increased levels of serum creatinine and decreased urea. Following the loss of kidney injury, some traditional and new biomarkers could provide a prognostic assessment of outcome after AKI and possibility of kidney failure in patients. NGAL= neutrophil gelatinase-associated lipocalin, KIM-1= kidney injury molecule-1, IL-18= interleukin-18, L-FABP= liver fatty-acid-binding protein. *Modified from Bellomo, R., Kellum, J.A, Ronco, C. Acute kidney injury. Lancet 2013:280:756-66*

A few of these biomarkers have shown promising results in detecting AKI of various etiologies (Bagshaw *et al.*, 2010; Parikh *et al.*, 2005). Both NGAL and KIM-1 are among the highly upregulated genes in the early post-ischemic kidney and after nephrotoxic injury (Mishra *et al.*, 2005; Mishra *et al.*, 2003; Mishra, Mori, Ma, Kelly, Barasch, *et al.*, 2004). NGAL is an iron-transporting protein that is co-localizaed with endosomal markers (Bao *et al.*, 2010). Adminstration of recombinant NGAL has been shown to protect from apoptosis and enhance tubular cell proliferation following murine IRI (Mishra, Mori, Ma, Kelly, Yang, *et al.*, 2004; Mori *et al.*, 2005). In addition, NGAL is expressed by tubular cells undergoing proliferation following injury, which suggests that is might play a role in the mainentance and recovery phase of AKI (Yang *et al.*, 2002). These exciting functional data have led to a number of human translational studies to evaluate blood and urine level of NGAL as novel biomarker for AKI (Bolignano *et al.*, 2008; Hirsch *et al.*, 2007). A standardized commercial bedside biomarker kit for NGAL has now been approved by the Food and Drug Administration for the early detection of AKI in the United States.

KIM-1 is another promising candidate biomarker of AKI, as its expression is dependent on kidney injury (Ichimura *et al.*, 2008; Ichimura *et al.*, 1998). This type 1 transmembrane receptor undergoes spontaneous ectodomain shedding upon expression on the apical membrane of proximal TECs which enables its detection in the urine of patients and rodents with AKI (Han *et al.*, 2002; Han *et al.*, 2009; Ichimura *et al.*, 2004; Sabbisetti *et al.*, 2013). Recently, Sabbisetti, *et al.*, showed that KIM-1 is also elevated in the plasma of patients who developed AKI following cardiac surgery (Sabbisetti *et al.*, 2014). In addition, KIM-1 levels also predicted the progression to end-stage renal disease in diabetic patients with elevated serum KIM-1, presumably due to recurrent AKI in the setting of CKD. In conclusion, both NGAL and KIM-1 are sensitive and specific biomarkers of tubular injury, and are considered to be part of the potential panel of AKI biomarkers to be used at the bedside in the future. This thesis will focus on understanding the physiological function of KIM-1 and its downstream cell signalling in proximal TECs in AKI.

1.3. KIDNEY INJURY MOLECULE-1

1.3.1. Classification and structure

Kidney injury molecule-1 (referred to as KIM-1 in humans and Kim-1 in rodents) was first identified by *Ichimura, et al.* in a cDNA screen for genes upregulated in the post-ischemic rat kidney (Ichimura *et al.*, 1998). KIM-1 mRNA and protein expression is undetectable in the normal kidney, but increases dramatically during ischemic or toxic AKI (Ichimura *et al.*, 2004; Vaidya *et al.*, 2009; Vaidya *et al.*, 2010). It belongs to the T cell immunoglobulin mucin (TIM) gene family, which includes TIM-1, TIM-3 (DeKruyff *et al.*, 2010; Nakayama *et al.*, 2009), and TIM-4 (Kobayashi *et al.*, 2007; Santiago, Ballesteros, Martinez-Munoz, *et al.*, 2007) in humans, and eight members (Tim 1-8) in mice (Kuchroo *et al.*, 2003). Human TIM-1, TIM-3 and TIM-4 are functional orthologs of mouse Tim-1, Tim-3 and Tim-4, respectively. KIM-1 is referred to as T-cell immunoglobulin and mucin domain-containing protein-1 (TIM-1) when expressed by immune cells, and KIM-1 when expressed on cells that originate from the kidney (including kidney cancers), but both are in fact the product of the same gene (HAVCR1) (Kuchroo *et al.*, 2003). KIM-1 was first discovered as the hepatitis A virus cellular receptor 1 (HAVcr-1) in monkey cells (Kaplan *et al.*, 1996). HAVcr-1 was later found to exist in humans, serving as the cellular receptor for *Hepatotropic picornavirus*, which causes acute liver disease in humans (Feigelstock *et al.*, 1998). In 1998, murine Kim-1 was identified on injured renal proximal TECs and exhibited some degree of homology to the *HAVcr1* gene (Ichimura *et al.*, 1998). In 2001, McIntire *et al.* performed positional cloning to identify a novel susceptibility gene locus for asthma and allergy (T-cell and airway phenotype regulator), and discovered TIM-1 within the *Tapr* locus as the susceptibility gene (McIntire *et al.*, 2001). The genetic sequence of TIM-1 indicated that both TIM-1 and HAVcr-1 encoded for the same protein. In mice, the Tim gene family is located on chromosome 11B1.1, and in humans it is located on chromosome 5q33.2 (McIntire *et al.*, 2001). Both regions have been linked to asthma, allergy and autoimmunity (McIntire *et al.*, 2004; McIntire *et al.*, 2003; Umetsu *et al.*, 2005).

Notably, TIM-1 is an extremely polymorphic gene with over one thousand identified genetic variants, accounted for by single nucleotide polymorphisms, insertions or deletions (McIntire *et al.*, 2003; Rodriguez-Manzanet *et al.*, 2009). TIM-1 variants have been linked to an individual's susceptibility to allergy, asthma (Gao *et al.*, 2005; Matricardi *et al.*, 1997; Matricardi *et al.*, 2000), eczema (Graves *et al.*, 2005), Hepatitis A infection (H. Y. Kim *et al.*, 2011), HIV

progression to AIDS (Wichukchinda *et al.*, 2010) and filovirus infection (Kuroda *et al.*, 2014). Given that TIM-1 is expressed on different immune cells, it is perhaps not surprising that polymorphisms of TIM-1 have also been associated with susceptibility to other autoimmune diseases such as rheumatoid arthritis (Chae, Song, Heo, *et al.*, 2003; Chae, Song, Lee, *et al.*, 2003; Chae *et al.*, 2004), multiple sclerosis (Grabmer *et al.*, 2010; Khademi *et al.*, 2004), and systemic lupus erythematosus leading to lupus nephritis (W. X. Li *et al.*, 2011; Y. Wang *et al.*, 2008).

Structural studies reveal that all TIM proteins (including KIM-1) are similar. They are all type I cell surface glycoproteins made up of an N-terminal immunoglobulin variable domain (IgV) with six cysteines, a mucin domain with O-linked and N-linked glycosylation close to the cellular membrane, a single transmembrane domain, and a cytoplasmic domain with tyrosine phosphorylation motif(s) (Ichimura *et al.*, 1998; Santiago, Ballesteros, Martinez-Munoz, *et al.*, 2007) (Figure 1.4). In addition, the mucin domain is rich in threonine, serine, and proline residues that play a role in O-linked glycosylation (Kuchroo *et al.*, 2003). TIM-4 is unique in that it contains an Arg-Gly-Asp (RGD) motif in its IgV domain and has a shorter cytoplasmic domain that lacks a key cell signalling motif (Meyers, Chakravarti, *et al.*, 2005; Santiago, Ballesteros, Martinez-Munoz, *et al.*, 2007; Shakhov *et al.*, 2004). Hence, TIM-4 is believed to interact with a signalling intermediate or a co-receptor to transduce signals in cells (Flannagan *et al.*, 2014; D. Park *et al.*, 2009). The N-terminal cysteine-rich region of the Ig domain of TIM-1, along with the mucin-domain account for the high sequence identity of about 40 % between mouse and human TIM-1 (Ichimura *et al.*, 1998; Kuchroo *et al.*,2003; Thompson *et al.*, 1998). The number of O-linked glycosylation sites varies from 37 in mouse Tim-1 to 56 in human TIM-1, which contributes to the size difference seen between them (Human KIM-1 ~119 kDa and mouse Kim-1 ~60 kDa) (Bailly *et al.*, 2002; Freeman *et al.*, 2010).

The extracellular domain of KIM-1 bears similarity to the immunoglobulin superfamily, which is characterized by an extracellular domain containing an immunoglobulin (Ig), and a mucin domain (Shimizu *et al.*, 1993; Van Klinken *et al.*, 1995). Molecules belonging to the immunoglobulin superfamily usually function as membrane receptors or adhesion molecules. This group includes mucosal addressin cell adhesion molecule 1 (MAdCAM-1), CD34, TACTILE (CD96), and HAVcr-1 which is the homolog of KIM-1 (Ichimura *et al.*, 1998; Van Klinken *et al.*, 1995). The MAdCAM-1 protein is most similar to KIM-1, as it contains four cysteine residues in the Ig domain and KIM-1 contains six cysteines (Ichimura *et al.*, 1998). The IgV-like domain of

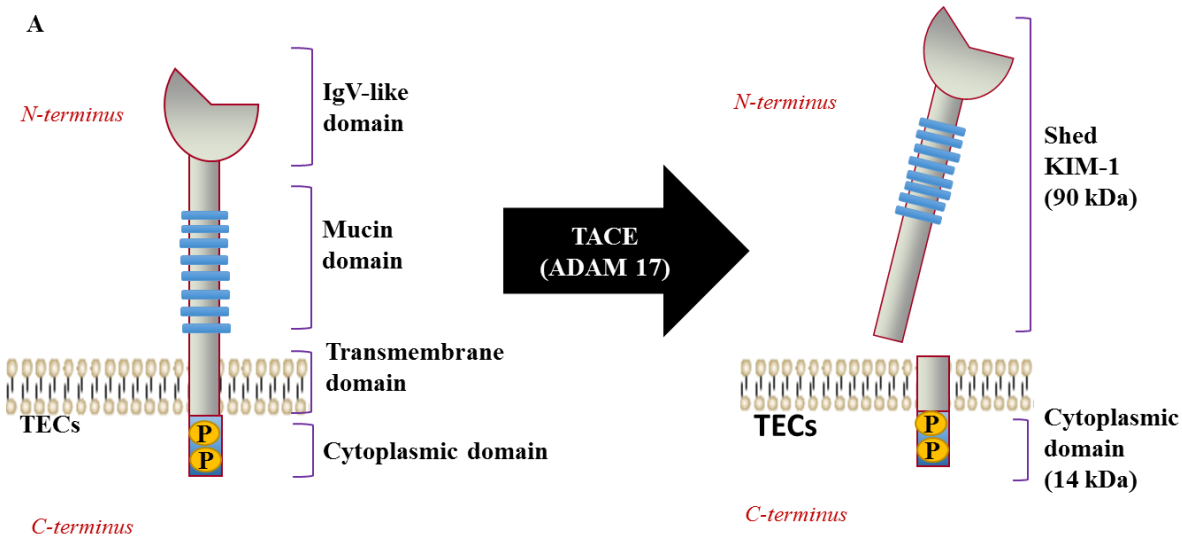
KIM-1 shares functional characteristics with sialic acid-binding immunoglobulin-type lectins (SIGLECs) and C-type-lectins (Wilker *et al.*, 2007). These similarities could suggest a potential role for KIM-1 in cell trafficking and adhesion. Surprisingly, TIM-1/KIM-1 has been shown to contain a signal peptide for trafficking through clathrin-coated vesicles from the endoplasmic reticulum to the cell surface (Balasubramanian *et al.*, 2012). The signal peptide-deleted TIM-1 mutant failed to undergo glycosylation and failed to enter vesicles for proper targeting to the cell surface.

The crystal structure of TIM-1 has been solved. The IgV domain contains a distinct narrow cleft that is formed by the four cysteines residues that bridge the CC' loop to the GFC β sheet (Santiago, Ballesteros, Tami, *et al.*, 2007). Additionally, the crystallographic analysis uncovered that both murine and human TIM-1 can partake in homophilic interaction (TIM-1-TIM-1 interactions), which may be a reflection of its function as an adhesion molecule. When the crystal structure of phosphatidylserine (TIM-1 ligand) bound to TIM-1 was solved, the IgV domain was found to form a metal-ion-dependent ligand-binding site (MILIBS), where the ion is predicted to be calcium (Santiago, Ballesteros, Martinez-Munoz, *et al.*, 2007). This MILIBS has asparagine and aspartic acid residues that engage in metal coordination, which is highly conserved in all TIM family members, except for mouse Tim-2 (Santiago, Ballesteros, Martinez-Munoz, *et al.*, 2007). The open confirmation of the IgV domain requires that the hydrophobic aromatic phenylalanine and tryptophan residues remain on the tip of the CC'FG loop to allow the lipid chain of phosphatidylserine (PS) to penetrate the narrow cleft for binding of PS to TIM-1 (Kobayashi *et al.*, 2007; Santiago, Ballesteros, Martinez-Munoz, *et al.*, 2007; Santiago, Ballesteros, Tami, *et al.*, 2007). Mutations in the aromatic residues of the FG loop within the MILIBS abrogate its binding to PS (Kobayashi *et al.*, 2007; Santiago, Ballesteros, Martinez-Munoz, *et al.*, 2007). Mutations in MILIBS also affect TIM-1 trafficking to the cell-surface, resulting in the accumulation of mutant TIM-1 on the plasma membrane (Balasubramanian *et al.*, 2012).

KIM-1 has been shown to undergo spontaneous membrane-proximal cleavage, releasing a soluble 90 kDa fragment of KIM-1 into the urine or the conditioned media of the TECs expressing the full-length protein (Z. Zhang *et al.*, 2007) (Figure 1.4). It is this property that has made soluble KIM-1 an attractive non-invasive diagnostic biomarker for tubular injury (Han *et al.*, 2002). In cells expressing KIM-1, the cleavage was found to be mediated by ERK activation and that activation of p38 MAP kinase resulted in accelerated cleavage of KIM-1 (Bailly *et al.*, 2002; Z.

Zhang *et al.*, 2007). Recently, our lab showed that KIM-1 is cleaved by TNF-α-converting enzyme (TACE) or ADAM 17 (*a d*isintegrin *a*nd *m*etalloproteinase), which is a Zn^{2+}-dependent cell surface protease (Gandhi *et al.*, 2014). We also showed that reactive oxygen species generated during IRI, accelerate KIM-1 shedding. Interestingly, we found that excess apoptotic cells can accelerate KIM-1 cleavage, and that the shed KIM-1 binds to apoptotic cells, competitively inhibiting their phagocytic uptake by TECs expressing KIM-1. This may be akin to a negative feedback loop. When KIM-1 is shed, a 14 kDa fragment containing its transmembrane and cytosolic domains remains bound (retained transmembrane KIM-1) to the plasma membrane (Z. Zhang *et al.*, 2007). This cytoplasmic domain of human KIM-1 contains two putative tyrosine phosphorylation sites at position 350 and 356. Phosphorylation at tyrosine 350 is required for TIM-1 to mediate co-stimulatory signalling in T cells (Binne *et al.*, 2007; de Souza *et al.*, 2005) and likely in renal proximal TECs (Yang *et al.*, 2015).

It is important to note that human KIM-1 has two splice variants, KIM-1a and KIM1b (Bailly *et al.*, 2002). The KIM-1a variant is expressed in liver and contains a short cytosolic domain which lacks the above tyrosine phosphorylation motif, whereas, the KIM-1b variant encodes the full-length protein and is expressed elsewhere including the kidney. HAVcr-1 corresponds to the KIM-1a splice variant, while TIM-1 on immune cells corresponds to KIM-1b (Bailly *et al.*, 2002; Kaplan *et al.*, 1996; Rees *et al.*, 2008). In this thesis, we will be focusing on the KIM-1b variant and refer to it henceforth as KIM-1.



B

KIM-1b/ KIM-1: 312 KKYFFKKEVQQLSVSFSSLQIKALQNAVEKEVQAEDNIYIENSLYATD 359
KIM-1a/HAVcr-1: 312 KKYFFKKEVQQLRPHKSCIHQRE 334

Figure 1.4. Human KIM-1 structural domains and the peptide sequence of its alternatively spliced variants.

A: KIM-1 is type-1-transmembrane glycoprotein with an IgV like domain, mucin domain (glycosylation indicated by blue lines in the mucin domain), transmembrane domain and a cytoplasmic domain that contains tyrosine phosphorylation motifs (indicated as P). KIM-1 (KIM-1b) is expressed on renal proximal tubule epithelial cells after injury. It is cleaved by TNF-α-converting enzyme (TACE) or ADAM 17 (a disintegrin and metalloproteinase) into soluble shed KIM-1 (90 kDa) and a membrane-bound cytoplasmic fragment (14 kDa). ***B:*** Peptide sequence alignment of two human KIM-1 variants, KIM-1a (HAVcr-1) and KIM-1b (KIM-1). KIM-1b has a tyrosine-phosphorylation motif, QAEDNIY, highlighted in orange. This motif is missing in the KIM-1a variant. The human KIM-1b (KIM-1) variant size is 359 amino acids, whereas the human KIM-1a (HAVcr-1) protein is 334 amino acids. Modified from (Bailly *et al.*, 2002).

1.3.2. KIM-1 expression

When KIM-1 was first identified in the kidney, it was noted to be expressed on the proliferating (bromodexoyurindine-positive) and dedifferentiated (vimentin-positive) epithelial cells in regenerating proximal tubules in post-ischemic rat kidney (Ichimura *et al.*, 1998). KIM-1 is expressed on the apical membrane of injured proximal tubule cells, predominantly in the S3 segment within the corticomedullary region (van Timmeren, Vaidya, *et al.*, 2007; van Timmeren, van den Heuvel, *et al.*, 2007), where injury is most likely to occur as mentioned earlier. Since its identification, a large number of studies have been conducted on KIM-1 in humans with different etiologies of AKI. These have confirmed that KIM-1 expression on injured tubules or shedding in the urine serves as a sensitive and specific biomarker for AKI (Han *et al.*, 2002; Sabbisetti *et al.*, 2013; Sabbisetti *et al.*, 2014; Vaidya *et al.*, 2010; Vaidya *et al.*, 2006; Vaidya *et al.*, 2008). Various studies in humans and mice show that KIM-1 is also highly upregulated following AKI caused by nephrotoxins, (Ichimura *et al.*, 2004), such as cisplatin (Amin *et al.*, 2004), folic acid (Waanders *et al.*, 2009), cyclosproine (Perez-Rojas *et al.*, 2007), and iodinated contrast agents (Jost *et al.*, 2009). This has made it an attractive biomarker to screen for nephrotoxicity of therapeutic compounds that are in development by the pharmaceutical industry (Han *et al.*, 2002; Ichimura *et al.*, 2004; van Timmeren, Vaidya, *et al.*, 2007).

Both tubular KIM-1 (~119 kDa) and urinary soluble KIM-1 (~90 kDa) have also been detected in patients with different proteinuric, inflammatory and fibrotic renal diseases. KIM-1 has been detected in CKD patient biopsies, and its expression is often associated with tubulo-interstitial damage, inflammation, fibrosis and a dedifferentiated tubular phenotype (Rennert, 2011; Vaidya *et al.*, 2011; van Timmeren, van den Heuvel, *et al.*, 2007). In the setting of CKD, whether KIM-1 is a marker of acute injury, inflammation, or fibrosis is not known. Recent evidence of KIM-1-expression on renal transplants with interstitial fibrosis and tubular atrophy supports the notion that it is involved in chronic kidney injury (Nogare *et al.*, 2015; van Timmeren, Vaidya, *et al.*, 2007; Yeung *et al.*, 2011; Schroppel *et al.*, 2010). In murine models, Kim-1 is upregulated in the kidneys of mice subjected to obstructive AKI (fibrosis model) (Humphreys *et al.*, 2013) and polycystic kidney disease (Kuehn *et al.*, 2002). Consequently, the upregulation of KIM-1 expression following renal injury and its persistent expression in the case of chronic injury has made it a useful as a non-invasive biomarker. Interestingly, KIM-1 has been shown to be highly expressed in human renal cell carcinoma specimen and cell lines isolated from cancer patients,

suggesting that it might additionally be related to cancer progression (Han *et al.*, 2005; Shalabi *et al.*, 2013).

The wide range of stimuli that lead to KIM-1 upregulation makes it a very interesting protein to study from the point of view of gene regulation. Recently, some of the transcriptional regulators that control KIM-1 expression in the kidney were identified (Ajay *et al.*, 2014). Following tubular injury in rat and human kidneys, or oxidative stress in human TECs, the checkpoint kinase 1 (Chk1) is phosphorylated and this allows it to interact with the signal transducer and activator of transcription-3 (STAT3). STAT3 is subsequently phosphorylated and binds to the KIM-1 promoter to upregulate its transcription.

Interestingly, when Kim-1 expression was measured in various mouse tissues by quantitative RT-PCR, its RNA expression was highest in the lymph node and kidney, weakly expressed in the spleen, lung, and thymus, and undetectable in the heart or liver (Umetsu *et al.*, 2005). This suggests that Kim-1/Tim-1 expressing immune cells are enriched in lymph nodes. In terms of expression by immune cells, mouse Tim-1 is expressed on activated T cells, but not naïve $CD4^+$ T cells (Freeman *et al.*, 2010; McIntire *et al.*, 2001; Umetsu *et al.*, 2005). Following differentiation of T-cells into effector cells, Tim-1 is expressed by Th2, Th1 and Th17 cells (Angiari *et al.*, 2014; Nakae, Iikura, *et al.*, 2007; Nakae, Iwakura, *et al.*, 2007; Sizing *et al.*, 2007). Tim-1 is also expressed by germinal B cells and is considered a hallmark characteristic of these cells (S. H. Wong *et al.*, 2010). A number of groups have recently demonstrated that Tim-1 is a marker of regulatory B cells that produce IL-10 (Ding *et al.*, 2011; Xiao *et al.*, 2015; Xiao *et al.*, 2012). In regards to expression by innate immune cells, Tim-1 is expressed on mast cells (Nakae, Iikura, *et al.*, 2007) and invariant natural killer T cells (H. S. Kim *et al.*, 2010; Lee *et al.*, 2010). The wide expression pattern of Tim-1 might explain the different functions carried by KIM-1/TIM-1. Whether the *HAVCR1* gene is regulated differently in immune cells compared to the kidney remains unknown.

1.3.3. Extrarenal KIM-1 function

A significant body of work supporting an extra-renal function of KIM-1/TIM-1 was published long before its role in the kidney began to be elucidated. TIM-1 was first shown to be a co-stimulatory molecule for naïve $CD4^+$ T cell activation and proliferation (de Souza *et al.*, 2005; Encinas *et al.*, 2005; Umetsu *et al.*, 2005) and an inhibitor of Th2 cell differentiation (Xiao *et al.*, 2007). Most of the studies that supported TIM-1's co-stimulatory function focussed on

rodent models and used indirect strategies (e.g. monoclonal antibodies to Tim-1) to determine its function. These studies are somewhat limited, since the amount of anti-Tim-1 antibody administered, the binding affinity of the antibody, and antibody binding site on Tim-1 could have affected the interpretation of the findings (Meyers, Sabatos, *et al.*, 2005; Sizing *et al.*, 2007; Waanders *et al.*, 2010). For example, in the mouse model of OVA-induced lung inflammation, administration of monoclonal antibody against the mucin domain of Tim-1 exacerbated immune responses (Sizing *et al.*, 2007). This was proposed to occur through the activation of T-cells to produce Th2 cytokines, which drive eosinophil and mast cell activation, and trafficking into the lung thereby amplifying the inflammation. On the other hand, administering Tim-1 antibodies against the IgV domain blocked inflammation through inhibition of T-cells activation. Additionally, Kim-1 was found to be expressed on natural killer T (NKT) cells, which allowed NKT to bind to apoptotic cells triggering their activation, proliferation, and cytokine production (H. S. Kim *et al.*, 2010; Lee *et al.*, 2010). This in turn led to exacerbated airway hyper-reactivity in a mouse model of asthma. Surprisingly, despite expressing Kim-1, NKT cells have not been reported to mediate phagocytosis of apoptotic cells. Nonetheless, these experiments did reinforce the role of Tim-1 in asthma and airway hypersensitivity previously proposed by genetic studies placing Tim-1 in the *Tapr* (asthma susceptibility) locus (McIntire *et al.*, 2001).

In 2010, the first sets of genetically modified mice with either Tim-1 deficiency or Tim-1 transgene expression on either T or B cells were generated (S. H. Wong *et al.*, 2010). The findings in this paper were surprising, because very low levels of Tim-1 were found to be expressed on activated T cells or Th2 cells, which was contradictory to what had been published previously. The expression of Tim-1 on T-cells did not stimulate Th2 differentiation and production of Th2 cytokines when activated. However, Tim-1 expression was seen on B-cells following B-cell receptor stimulation, and this was shown to be dependent on phosphoinositide-3-kinase and the nuclear factor-κB pathway (NF-κB). Interestingly, Tim-1 was found to be expressed on germinal center B cells which was previously unknown at that time. These new findings have brought the previous findings using monoclonal antibodies to study Tim-1 function into question.

The discovery of Tim-1 expression on B-cells sparked interest in its possible role in B-cell biology. Administration of a low-affinity TIM-1-specific antibody promoted prolonged islet survival in an allograft transplant model (Ding *et al.*, 2011). Tim-1 is expressed by a majority of

IL-10 producing regulatory B-cells. Given that: 1) Tim-1 is expressed on B regulatory cells (S. H. Wong *et al.*, 2010); 2) Tim-1 is a phosphatidylserine receptor for apoptotic cells (Ichimura *et al.*, 2008; Ichimura *et al.*, 1998; Kobayashi *et al.*, 2007); and 3) apoptotic cell-binding induces generation of B regulatory cells, but not phagocytosis (Gray *et al.*, 2007; Yeung *et al.*, 2015), the role of Tim-1 binding to apoptotic cells on B regulatory cells to mediate immune response has been studied. The generation of mice lacking the mucin domain of Tim-1 (Tim-$1^{\Delta mucin}$) provided an in-depth understanding of the role of Tim-1 in immune tolerance. B regulatory cells expressing Tim-$1^{\Delta mucin}$ were unable to bind apoptotic cells leading to a reduction in IL-10 production and accelerated allograft rejection (Yeung *et al.*, 2015). In another study, these Tim-$1^{\Delta mucin}$ mice developed autoimmune disease as characterized by the increase in T-cells, activation of dendritic cells, elevation of serum Ig, and generation of autoantibodies such as those to dsDNA (Xiao *et al.*, 2012). These results suggest that the role of Tim-1 in mediating immune responses is dependent on binding apoptotic cells. Recently, it was shown that defective (Tim-$1^{\Delta mucin}$) or mutant Tim-1 led to more inflammatory mediators with increased proinflammatory cytokines, increased in Th1 and Th17 production, and reduced generation of regulatory T-cells ($FoxP3^{+}$) (Xiao *et al.*, 2015). This caused an enhancement of the severity of experimental autoimmune encephalomyelitis. Therefore, Tim-1 expression on B regulatory cells plays a critical role in maintaining self-tolerance and inhibiting the development of autoimmune disease (Nozaki *et al.*, 2014; Sattler *et al.*, 2014; Xiao *et al.*, 2015; Xiao *et al.*, 2012; Yeung *et al.*, 2015).

The extracellular domain of TIM-1 characterizes it as a member of the immunoglobulin superfamily, and was recently implicated in leukocyte adhesion and trafficking akin to SIGLECs and C-type lectins (Angiari *et al.*, 2014). Tim-1 expression on Th1 and Th17 cells enables them to bind to endothelial P-selectin, increasing their recruitment into inflamed tissues. In addition, Th1 and Th17 cells from the Tim-1-mucin domain mutant (Tim-$1^{\Delta mucin}$), as well as the Ig-like domain mutant of Tim-1, showed reduced ability to traffic into inflamed tissue of the skin and central nervous systems in models of skin hypersensitivity and experimental autoimmune encephalomyelitis, respectively.

In conclusion, Tim-1 plays complex and varied roles in mammals that are largely dependent on which tissues it is expressed. The function of Tim-1 also depends on the microenvironment in which Tim-1 is expressed, and whether this microenvironment contains any

of its ligands, such as apoptotic cells. Moreover, Tim-1 seems to have a double-edged function in immune cells, where it could mediate development of inflammatory responses or tolerance.

1.3.4. Renal function of KIM-1

KIM-1 was first identified as a gene that is specifically upregulated following renal IRI (Ichimura *et al.*, 1998). Given its expression pattern on proximal TECs, it was proposed to have a role in the restoration of the morphological integrity of renal tubules (Ichimura et al., 1998). Almost 10 years after its identification in the kidney, several groups identified KIM-1 as a receptor for the "eat me" signal, phosphatidylserine, which enables proximal tubular cells expressing KIM-1 to recognize and engulf apoptotic cells (Ichimura *et al.*, 2008; Kobayashi *et al.*, 2007). Because KIM-1 also binds to a variety of other ligands, including low-density lipoprotein, phosphatidylethanolamine, and necrotic cell debris, KIM-1 is classified as a type B scavenger receptor (Ichimura *et al.*, 2008). Based on *in vitro* data, it was hypothesized that KIM-1 functions to remove dead tubular cell debris, preventing them from obstructing the lumen and triggering inflammation, which would impede tissue repair (Ichimura *et al.*, 2012; Waanders *et al.*, 2010).

A vastly different role for KIM-1 in promoting survival has been proposed by another group: KIM-1 was shown to protect against cell death using an *in vitro* model of ischemic injury via depletion of ATP and glucose (Balasubramanian *et al.*, 2012). During ischemic stress, KIM-1 was shown to interact with and target the pro-apoptotic orphan nuclear receptor, NUR77, for lysosomal degradation, thereby preventing apoptosis. Most importantly, silencing KIM-1 or mutating the phosphatidylserine binding pocket of KIM-1 resulted in increased NUR77 abundance and decreased renal cell survival following ischemic injury *in vitro*. While this study hinted at a potential effective mechanism for KIM-1 in AKI, the *in vivo* relevance of this finding is uncertain.

Remarkably, transgenic overexpression of Kim-1 in kidney TECs in the absence of a cellular injury stimulus had been shown to cause rapid kidney failure in mice by promoting progressive renal fibrosis (Humphreys *et al.*, 2013). These transgenic mice spontaneously developed interstitial kidney inflammation with fibrosis, marked by tubular dedifferentiation, microcytic tubular dilation and fibrosis. The chronic expression of Kim-1 on TECs was shown to spontaneously increase the level of proinflammatory cytokines such as monocytes chemotactic protein-1 (MCP-1), which was believed to have caused the abnormal leukocyte infiltration in the absence of tissue injury. The development of kidney failure in these mice was associated with the classic extrarenal manifestations that can be seen in patients with CKD, such as hypertension,

anemia, and early death. The conflicting findings observed between the Tim-1$^{\Delta mucin}$ and the Kim-1 transgenic overexpression mice suggest that both the level and context of renal KIM-1 expression profoundly influences whether it has a protective or damaging effects.

Following renal insult and upregulation of KIM-1 (Ichimura *et al.*, 1998), there is evidence of low levels of immune cell infiltration into the kidney (Nozaki *et al.*, 2012; Nozaki *et al.*, 2011; Rong *et al.*, 2011). Most of these infiltrating immune cells are located in the outer medulla, tubular wall and tubular basement membrane (Yang *et al.*, 2015). Surprisingly, it was identified that KIM-1 is a ligand for the leukocyte mono-immunoglobulin (Ig)-like receptor 5 (LMIR5) or CD300B, which is expressed by myeloid cells (Yamanishi *et al.*, 2010). LMIR5-TIM-1 interaction increased the accumulation of LMIR5 expressing neutrophils in the renal interstitium of mice subjected to renal IRI. This, in turn, led to the activation of LMIR5 expressing mast cells, which augmented tissue damage following IRI (Yamanishi *et al.*, 2010). Several groups have utilized inhibitory monoclonal antibodies against Tim-1 to determine whether inhibiting Tim-1 would have an effect on kidney injury (Nozaki *et al.*, 2014; Nozaki *et al.*, 2012; Nozaki *et al.*, 2011). In a mouse model of cisplatin-induced nephrotoxicity, inhibiting Tim-1 on T- cells resulted in less renal injury and histological damage, reduced leukocyte infiltration, and lower levels of proinflammatory cytokines (Nozaki *et al.*, 2011). Similarly, in a model of induced crescentic glomerulonephritis or glomerular injury, inhibition of Tim-1 resulted in reduced injury, leukocyte accumulation, and inflammatory response mediated by Th1 and Th17 (Nozaki *et al.*, 2012). These studies all suggest a role for KIM-1 in the recruitment of inflammatory cells and propagation of further tissue damage, either via Tim-1 expressed on immune or kidney epithelial cells.

Though the above studies provided much insight into the potential roles of KIM-1 in kidney injury by using Tim-1$^{\Delta mucin}$ mice or antibodies blocking of KIM-1, none of them have directly addressed whether KIM-1 expression plays a role in ischemic AKI *in vivo.* Given that these studies have proposed opposing roles for KIM-1, it remains to be determined whether KIM-1 is protective or harmful in AKI. In this thesis, we aim to determine the function of KIM-1 using Kim-1-deficient mice (S. H. Wong *et al.*, 2010).

1.3.5. Renal KIM-1 signalling

Though much research has been done to help understand the physiological role of KIM-1 in the kidney, very little is known about its capacity to mediate intracellular signalling in TECs. Understanding these pathways might be key for designing novel therapeutic targets needed to

ameliorate acute kidney injury. As discussed above, the extracellular domains of KIM-1 have been shown to bind to phosphatidylserine on apoptotic and necrotic cell debris (Ichimura *et al.*, 2008), nuclear receptor NUR77 (Balasubramanian *et al.*, 2012), and leukocyte mono-immunoglobulin (Ig)-like receptor 5 (LMIR5)/CD300b (Yamanishi *et al.*, 2010) in TECs. However, none of these studies uncovered KIM-1 intracellular signalling proteins that mediate the renal functions of KIM-1. To uncover the signalling mechanism by which KIM-1 binds and engulfs apoptotic cells, we immunoprecipitated KIM-1 from human embryonic kidney-293 (HEK-293) cells expressing HA-tagged KIM-1 after stimulation with PS containing liposomes and performed mass spectrometry to determine associated proteins. Using this method, we identified the alpha subunit of the heterotrimeric G12 protein Gα12 (M. P. Strathmann *et al.*, 1991), a novel KIM-1 interacting protein. Independently, using a yeast two-hybrid system (in collaboration with Dr. Tony Zervos, University of Central Florida) and a HeLa cell cDNA library, we identified another novel KIM-1 interacting protein, the dynein light chain protein Tctex-1 (Lader *et al.*, 1989). Both of these proteins are interesting candidates that we will briefly cover in the rest of the introductory chapter. In Chapter 2 and 3, we will study the role of Gα12 in KIM-1 expressing cells in ischemic AKI and the phagocytic process. In Chapter 4, we will study the role of Tctex-1 in KIM-1-expressing cells.

1.4. ALPHA SUBUNIT OF HETEROTRIMERIC G12 PROTEIN

1.4.1. Classification, structure and expression

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are signal transducers that link extracellular stimuli to intracellular effectors that mediate cellular responses to the stimulus (Suzuki *et al.*, 2009). Extracellular signals are typically received via members of the seven-transmembrane domain receptors known as G protein-coupled receptors (GPCRs). Alternatively, they can be associated with other receptors or modulators to mediate non-canonical functions. As heterotrimers, these proteins consist of a guanine-nucleotide binding subunit Gα, and closely associated β and γ subunits (usually referred to as Gβγ) (Neer, 1995). In the inactive state, a single α subunit is bound to a GDP molecule in association with a Gβγ subunit (Figure 1.5). Upon ligand binding to the associated GPCR or indirect activation of the G protein by other modulators, the GDP molecule is exchanged for a GTP molecule on the α subunit. A family of proteins called GTP-exchange factors or GEFs catalyzes this process. The GDP-GTP exchange causes a conformation changes in the switch regions of the α subunit (Dhanasekaran *et al.*, 1996; Fields *et*

al., 1997), allowing it to dissociate from the Gβγ subunit. Both the GTP-bound α subunit and Gβγ subunits are "free" to interact and activate various downstream effectors. Meanwhile, the activated α subunit hydrolyzes the bound GTP to GDP via its intrinsic GTPase activity, which can be accelerated by GTPase activating proteins (GAPs). Once Gα is in an inactive form (GDP-bound), it dissociates from its effectors and reassociates with Gβγ subunits to start the cycle again.

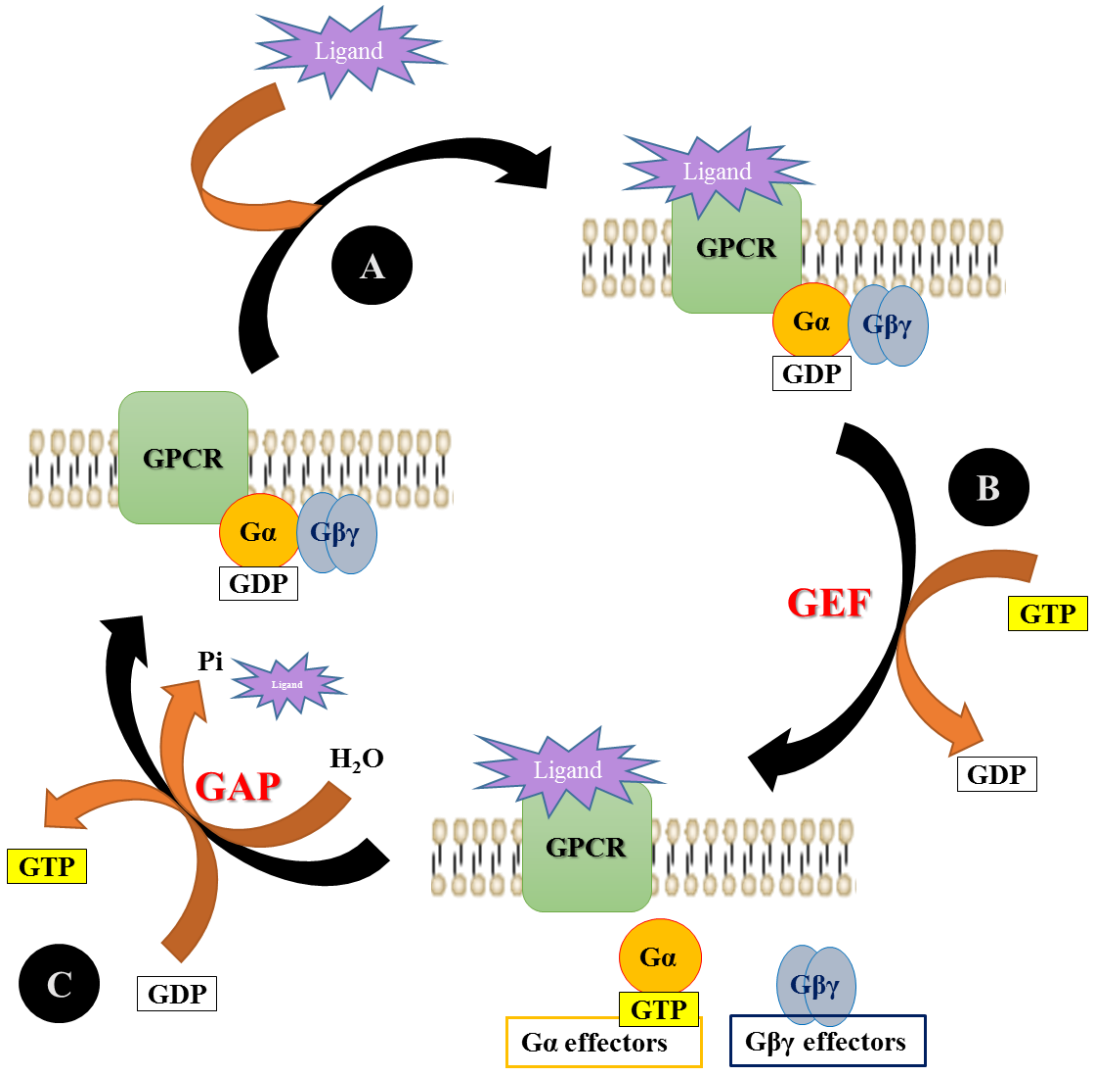


Figure 1.5. The G protein cycle.

A: In the inactive state, the G-protein coupled receptor (GPCR) is not associated with ligand. The G protein α subunit is bound to GDP molecule and is associated with Gβγ subunit close to the plasma membrane. ***B:*** Upon ligand binding to GPCR, GPCR undergoes a conformational change that allows the exchange of GDP to GTP on the α subunit. This change causes dissociation of the α subunit from the Gβγ subunit. Both the GTP-bound α subunit and Gβγ subunit can then interact with and activate various downstream effectors. Activation of the Gα subunit can also occur via a guanine nucleotide exchange factor (GEF) mechanism, which is independent of the GPCR (non-canonical pathway). ***C:*** Once the ligand dissociates from the GPCR, the Gα subunit hydrolyzes the bound GTP to GDP through its intrinsic GTPase activity, which is accelerated by GTPase activating proteins (GAPs). When the Gα subunit becomes inactive, it dissociates from its effectors and reassociates with the Gβγ subunit, ending the activation of downstream effectors.

In humans, there are at least 21 Gα subunits encoded by 16 genes, six Gβ subunits encoded by five genes, and 12 Gγ subunits encoded by 18 genes (Downes *et al.*, 1999; Oldham *et al.*, 2008). The heterotrimeric G proteins are divided into four subfamilies based on the amino acid sequence similarity of the Gα subunits: Gαs, Gαi, Gαq and Gα12 (Simon *et al.*, 1991; M. Strathmann *et al.*, 1989). The G12 class of G proteins consists of Gα12 and Gα13, which share about 67 % amino acid sequence identity between them and less than 45 % identity with other α subunit groups (Dhanasekaran *et al.*, 1996; M. P. Strathmann *et al.*, 1991; Vara Prasad *et al.*, 1994). In 1991, the G12 class of proteins became the last and the fourth class to be identified (M. P. Strathmann *et al.*, 1991). They both have an approximate molecular mass of 44 kDa and are insensitive to ADP-ribosylation by pertussis toxin. Both Gα12 and Gα13 are phosphorylated by protein kinase C, which serves to block any interaction with the Gβγ subunit (Kozasa *et al.*, 1996; Offermanns *et al.*, 1996; Suzuki *et al.*, 2009). In contrast to other Gα subfamilies, both Gα12 and Gα13 are only post-translationally modified by palmitoylation near their N-termini (Jones *et al.*, 1998; Ponimaskin *et al.*, 2000; Veit *et al.*, 1994). This modification is important for the association of Gα13 with plasma membrane (Bhattacharyya *et al.*, 2009) and for the localization of Gα12 to lipid rafts (Waheed *et al.*, 2002). The N-terminal regions of both Gα12 and Gα13 share only 16 % amino acid identity (Dhanasekaran *et al.*, 1996). This sequence difference is believed to determine their relative subcellular localization. Gα12 is mostly localized to the plasma membrane (Waheed *et al.*, 2002), but contains a mitochondrial targeting sequence that allows it to be associated with mitochondria under certain circumstances, where it is involved in mitochondrial motility and membrane permeability (Andreeva *et al.*, 2008). On the other hand, Gα13 is localized to the cytosol and only translocate to the plasma membranes upon activation (Yamazaki *et al.*, 2005). In addition to being involved in localization, the N-terminal sequences have also been shown to determine their selective coupling to various receptors (Yamaguchi *et al.*, 2003). Although Gα12 and Gα13 interact with different effectors and receptors, they are both activated by thrombin and lysophosphatidic acid via the protease-activated receptor and the lysophosphatidic acid receptors (Aragay *et al.*, 1995; Hains *et al.*, 2006; Q. Wang *et al.*, 2004). Both Gα12 and Gα13 have a relatively slow rate of intrinsic nucleotide exchange and GTP hydrolysis, which suggests that they partake in prolonged signalling (Dhanasekaran *et al.*, 1996; Kozasa *et al.*, 1995). Though both proteins can activate common downstream signalling pathways (Kurose, 2003), significant differences have been observed between Gα12 and Gα13 knockout mice. Transgenic mice that

lack Gα13 are embryonically lethal due to their defect in angiogenesis, whereas mice deficient in Gα12 develop normally (Gu *et al.*, 2002; Offermanns, 2001; Offermanns *et al.*, 1997). This indicates that Gα12 and Gα13 regulate distinct signalling pathways and cannot completely compensate for each other in a physiologically significant manner.

Gα12 is similar in structure to all other Gα subfamilies, as it has a GTPase and a helical domain (Oldham *et al.*, 2006). The GTPase domain is composed of six-stranded β-sheets surrounded by five α-helices. The five α-helices contain three flexible loops, named Switch I, II and III (Lambright *et al.*, 1994; Lambright *et al.*, 1996; Wall *et al.*, 1995). These switch regions undergo significant structural changes when in the GDP (Lambright *et al.*, 1994; Mixon *et al.*, 1995) or non-hydrolyzable GTP (GTPγS) bound forms (Coleman *et al.*, 1994; Noel *et al.*, 1993). The GTPase domain also contains sites for binding to the Gβγ dimer, GPCRs, and downstream effector proteins (Lambright *et al.*, 1996; Oldham *et al.*, 2008; Sondek *et al.*, 1996). On the other hand, the helical domain, consisting of six α-helices, serves to increase the affinity of Gα for guanine nucleotides (Remmers *et al.*, 1999; Warner *et al.*, 1998) and increases the GTP hydrolysis activity of the protein (Markby *et al.*, 1993). It also plays an important role in coupling specific G proteins to specific effectors (W. Liu *et al.*, 1998). Based on mutagenesis studies, both the amino and carboxyl termini are structurally and functionally important components of the protein (Suzuki *et al.*, 2009; Yamaguchi *et al.*, 2003).

The identification of the interaction between Ser/Thr protein phosphatase type 5 (PP5) and both Gα12 and Gα13 was key to the development of a tool to study the biology of G12 proteins (Yamaguchi *et al.*, 2002; Yamaguchi *et al.*, 2003). Specifically, Yamaguchi, *et al.* demonstrated that the N-terminal regulatory tetratricopeptide repeat (TPR) within PP5 binds selectively to the GTP-bound forms of Gα12 or Gα13. This feature of PP5 allowed the use of the TPR domain conjugated to GST (GST-TPR) in the form of a GST pull-down assay to detect the level of active Gα12 and Gα13 *in vitro* or *ex-vivo* as we have done in this thesis (Sabath *et al.*, 2008; Yanamadala *et al.*, 2007). In addition, the constitutively active Gln (Q)→Leu (L) mutant of the α subunit for G12 (*QL* Gα12), which is incapable of GTP hydrolysis, and the wild-type (*WT*) Gα12 have been routinely used to test the sufficiency of Gα12 in driving various cellular processes (Vara Prasad *et al.*, 1994; K. Wong *et al.*, 2006; Zhu *et al.*, 2004).

Gα12 is ubiquitously expressed in all tissues, with lower levels of expression in the intestine (M. P. Strathmann *et al.*, 1991). This expression pattern is different from Gα13, as its

expression levels vary between different tissues. The eye, kidney, liver, lung and testis have exhibited a higher levels of expression of Gα13 compared to other tissues.

1.4.2. Function

Gα12 interacts with many down-stream effectors that have overlapping functions (P. Kelly *et al.*, 2007) (summarized in Table 1.2). This allows Gα12 to be involved in a myriad of cellular processes, such as cell growth, cellular transformation, cell polarity, cell migration, paracellular permeability, apoptosis, and membrane localization of different proteins (Refer to Table 1.2 for more information). We will be focusing on the function of Gα12 in the kidney for the purpose of this thesis.

Table 1. 3. Various proteins demonstrated to interact with Gα12 and the functional outcome(s) of these interactions.

Proteins with an asterisk (*) have been shown to interact with both Gα12 and Gα13. *Abbreviation:* PP-protein phosphatase, eNOS-endothelial nitric oxide synthase, RGS-Regulator of G protein signalling, AKAP-A-kinase anchoring protein, ZO-Zonula occludens, BTK-Bruton's tyrosine kinase, Hsp- Heat shock protein, GAP-Guanine activating protein, SNAP-Soluble NSF-associated protein, GEF-Guanine nucleotide exchange factor.

Protein	**Function**	**Reference**
*Rho specific GEFs (work as GAP for Gα12) LARG PDZ-RhoGEF P115RhoGEF	Stimulate RhoA activation leading to many down-stream protein activation effects, such as, phosphorylation of focal adhesion kinase, paxillin and p130 Crk-associated substrate	(Tanabe *et al.*, 2004), (Fukuhara *et al.*, 2000), (Kuner *et al.*, 2002), (Iguchi *et al.*, 2008), (Needham *et al.*, 1998)
*Protein Serine/Threonine phosphatases 5 (PP5)	Activates many different downstream mediators leading to cell growth, differentiation, and DNA damage	(Yamaguchi *et al.*, 2002)
Protein phosphatase 2A (PP2A)	Activates apoptosis	(Zhu *et al.*, 2004)
Zonula occludens 1 or 2 (ZO-1 or ZO-2)	Disrupts tight junction to increase paracellular permeability	(Meyer *et al.*, 2003; Meyer *et al.*, 2002)
*Cadherins	Promotes cell migration via release of the transcription activator β-catenin	(Meigs *et al.*, 2002; Meigs *et al.*, 2001)
p120-catenin	Regulates cadherin and β-catenin interaction	(Noren *et al.*, 2000)
α-SNAP	Regulates localization of cadherins	(Andreeva *et al.*, 2005)
Heat shock protein 90 (Hsp90)	Localizes Gα12 into lipid rafts	(Vaiskunaite *et al.*, 2001; Waheed *et al.*, 2002)
*Radixin	Serves as a cross-link between plasma membrane proteins and the actin cytoskeleton	(Gohla *et al.*, 1999; Vaiskunaite *et al.*, 2000)
Axin (RGS protein)	Mediates WNT signalling to control cell growth and differentiation	(Stemmle *et al.*, 2006)

A-kinase anchoring protein (AKAP) Lbc	Translocates Gα12 into plasma membrane to activate RhoA	(Diviani *et al.*, 2001; Niu *et al.*, 2001)
Bruton's tyrosine kinase BTK	Mediates Rho activation by tyrosine kinase activity (BTK)	(Jiang *et al.*, 1998; Suzuki *et al.*, 2003))
Ras specific GTPase activating protein (RasGAP1)	Downregulates Ras signalling	(Jiang *et al.*, 1998)
*Socius	Associates with Rho-like Rand protein to regulate stress-fiber disassembly	(Tateiwa *et al.*, 2005)
*Endothelial nitric oxide synthase (eNOS)	Regulates nitric oxide biosynthesis	(Andreeva *et al.*, 2006)
Polycystin-1	Regulates apoptosis and cystic cell growth	(Xu *et al.*, 2015; Yu *et al.*, 2010; Yu *et al.*, 2011; Yuasa *et al.*, 2004)

Madin-Darby canine kidney (MDCK) cells engineered to allow the inducible expression of wild type or the constitutively active Gα12 (*QL* Gα12) have been instrumental in helping investigators understand the signalling processes downstream of Gα12 in kidneys (Meyer *et al.*, 2002). Using these cells, Denker and colleagues identified that the tight junction scaffolding protein, zonula occludens 1 (ZO-1) is a downstream target of Gα12. Moreover, they demonstrated that constitutively active Gα12 increases paracellular permeability in cells by disrupting the localization of tight junction proteins (ZO-1, occluding and claudin-1) and adherens junction proteins (E-cadherin and β-cateinin) (Meyer *et al.*, 2003). Consequently, this leads to significant reorganization of actin cytoskeletons, and loss of polarity with mislocalization of Na-K-ATPase molecules. These Gα12-mediated changes were later shown to occur through the Gα12-dependent activation of Src kinase, and subsequent Src-mediated phosphorylation of the tight junction proteins and their disassembly from the epithelial barrier (Meyer *et al.*, 2003; Sabath *et al.*, 2006; Sabath *et al.*, 2008).

The involvement of Gα12 in tight junction dynamics is very important in renal development and survival after injury (Denker *et al.*, 2011). There are large differences in the paracellular permeability across the nephron segments due to the diversity of claudin proteins throughout the tubule. The proximal tubule has the lowest permeability, reflecting the importance of preventing the filtrate from reentering the circulation via the intercellular spaces (Denker *et al.*, 2011; Gonzalez-Mariscal *et al.*, 2000; Kiuchi-Saishin *et al.*, 2002; Muto *et al.*, 2010). In AKI, ischemic and toxic insults lead to the disruption of tight junctions (Kwon *et al.*, 1998; Meyer *et al.*, 2001; Tsukamoto *et al.*, 1999). Once dysregulated by injury, the tight junctions must reassemble to re-establish a functional tubule and to avert fibrosis (Denker *et al.*, 2011; Yu *et al.*, 2008).

Interestingly, Gα12 directly interacts with polycystin-1, encoded by the *PKD1* gene (Yu *et al.*, 2010; Yu *et al.*, 2011; Yuasa *et al.*, 2004), which is mutated in autosomal dominant polycystic kidney disease. Silencing polycystin-1 (or mutating the *PKD1* gene) causes the activation of Gα12 followed by ADAM-10 metalloproteinase maturation and activation to increase shedding of E-cadherin (Xu *et al.*, 2015). This eventually causes the disruption of adherens junction proteins and loss of polarity causing cystic growth of renal epithelial cells.

As discussed earlier, renal IRI results in TECs undergoing detachment and apoptosis. Activation of Gα12 via thrombin or constitutively active Gα12 has been shown to stimulate

apoptosis via its interaction with and activation of protein phosphatase 2A (PP2A) (Yanamadala *et al.*, 2007;Zhu *et al.*, 2004). This leads to the activation of JNK (c-Jun N-terminal kinase), followed by degradation of the anti-apoptotic protein Bcl-2, and finally to upregulation of IκBα, which potently stimulates the apoptosis pathway. Additionally, Gα12 interaction with polycystin-1 was found to modulate Gα12/JNK stimulated apoptosis in TECs (Yu *et al.*, 2010; Yu *et al.*, 2011; Yuasa *et al.*, 2004). The role played by activated Gα12 in triggering apoptosis suggests that Gα12 might be pathogenic under ischemic conditions. Indeed, the activation of Gα12 after IRI was recently shown to exacerbate tissue damage and renal dysfunction (Yu *et al.*, 2012). Denker and colleagues elegantly showed that ROS generated during IRI is a potent activator of Gα12 and its downstream effector, Src kinase (Yu *et al.*, 2012). This study demonstrated that ROS-activated Src subsequently phosphorylates ZO-1 and occludin, leading to disruption of tight junctions. Accordingly, Gα12-deficient mice were protected from renal IRI, whereas mice with TECs-specific transgenic overexpression of constitutively active Gα12 (Gα12 *QL*) exhibited more severe tissue damage and delayed recovery after IRI (Yu *et al.*, 2012). Collectively these findings reflect on the adverse effects of Gα12 activation on renal epithelial cells during ischmic AKI and naturally led us to an interesting avenue of research to understand whether there is a link between KIM-1 and Gα12 signalling during AKI.

In this thesis, we will test if KIM-1 might be protective against renal IRI by inhibiting Gα12 activation by ROS. We will also explore a novel role for Gα12 in the phagocytosis of apoptotic cells by proximal TECs via KIM-1.

1.5. TCTEX-1

1.5.1. Structure and expression

Tctex-1, short for t complex-testis expressed-1, was identified in a cDNA screen looking for candidate genes in the mouse t complex whose aberrant expression may be functionally related to sterility and transmission ratio distortion (Lader *et al.*, 1989). Later, it was defined as a component of the light chain of the cytoplasmic dynein motor complex (Hirokawa, 1998; Hirokawa *et al.*, 1998; Lader *et al.*, 1989; Vallee *et al.*, 2004). Cytoplasmic dynein is a minus-end directed motor complex on microtubules (Vallee *et al.*, 2004). It is involved in the retrograde or apical transport of the bound cargoes that take place during processes such as nuclear migration, golgi and vesicular transport, and mitotic spindle orientation (Vale, 2003). Dynein is comprised of

two heavy chains (~530 kDa), each with ATPase and motor activity (Mazumdar *et al.*, 1996; Vallee *et al.*, 1990), two or three intermediate chains (~74 kDa), and a group of light intermediate (57-61 kDa) and light chain proteins (~8-22 kDa) (S. J. King *et al.*, 2002). Other light chain proteins include LC8 and LC/roadblock. Human Tctex-1 and murine Tctex-1 share about 94 % sequence identity (Roux *et al.*, 1994; Watanabe *et al.*, 1996). Typically, the cargo is carried by dynein on the microtubules by association with another protein, dynactin (Vaughan *et al.*, 1995). Tctex-1 and LC8 are two of the three dynein light chain subunits that are tightly associated with dynein intermediate chains to carry out the dynein function (Makokha *et al.*, 2002; Nyarko *et al.*, 2004).

Based on the crystal structure, Tctex-1 exists in dimeric forms consisting of two α-helices that are flanked by four β-strands of each monomer (Williams *et al.*, 2005). One β strand crosses between the monomer to form an intersubunit β sheet at the dimer interface. Tctex-1 exhibits a tightly controlled dissociation-coupled unfolding mechanism, which indicates the absence of stable monomer form of Tctex-1under normal circumstances (Talbott *et al.*, 2006). At the dimer interface, a water channel exists that contains a threonine residue at position 92 (Song *et al.*, 2007). The phosphorylation of Tctex-1 at Threonine-92 was shown to directly mask the binding site to the dynein intermediate chain, which mediates the dissociation from dynein as a phosphorylated dimer (Song *et al.*, 2007). Additionally, another Tctex-1 phosphorylation site exists at Threonine-94 (Thr-94), which can be phosphorylated by protein kinase C *in vitro* (Chuang *et al.*, 2005). This phosphorylation site allows for the dissociation from the dynein complex to mediate dynein-independent functions. Another important site of phosphorylation is Serine-82 of Tctex-1, and phosphorylation at this site has been shown to reduce its binding to dynein, but not its interaction with specific targets (e.g. rhodopsin) (Yeh *et al.*, 2006). The N-terminus of Tctex-1 consists of 8-13 amino acids and usually appears splayed away from the structure to mediate binding to interacting partners (Williams *et al.*, 2005). In addition, Tctex-1 contains four tyrosine residues that could be post-translationally modified by phosphorylation; however, the role of phosphorylation at these sites is still unknown (Campbell *et al.*, 1998; Mok *et al.*, 2001).

Tctex-1 has been shown to be differentially expressed in various tissues; the kidney, spleen and testis have higher levels of Tctex-1 than brain as determined by Western blot (S. M. King *et al.*, 1996). Immunofluorescence staining of endogenous Tctex-1 suggests that it is localized to the Golgi apparatus and along microtubules (Tai *et al.*, 1998). This can be disrupted if the cells are

treated with microtubule depolymerizating drugs (nocodazole) or a Golgi apparatus disruptor (e.g. Brefeldin A).

1.5.2. Function and interacting partners

Tctex-1 has diverse dynein-dependent and -independent functions in cells (Hirokawa, 1998; Hirokawa *et al.*, 1998; Lader *et al.*, 1989) (Table 1.3). Through dynein-dependent functions, Tctex-1 is associated with microtubules in order to mediate a range of intracellular motility events, such as retrograde trafficking in neurons and receptor trafficking (Sachdev *et al.*, 2007; Tai *et al.*, 1998). On the other hand, Tctex-1 also functions independently of dynein to regulate actin cytoskeletal dynamics (Chuang *et al.*, 2005). These studies found that phosphorylation of Tctex-1 at Threonine-94 or Serine-82 (Chuang *et al*., 2005; Machado *et al.*, 2003; Song *et al.*, 2007) promoted its dissociation from the dynein light chain allowing it to bind to other non-dynein proteins in order to mediate various effector functions (Chuang *et al.*, 2005; Song *et al.*, 2007). Interstingly, the role of Tctex-1 in renal TECs or in the kidney has not been elucidated. Our studies of the interaction of KIM-1 with Tctex-1 will be the first to implicate a role for it in the kidney.

Table 1. 4. Tctex-1 interacting partners and the functional outcome of their interactions.

Proteins with an asterisk (*) have been shown to mediate functions independently of dynein. Abbreviation: Bone morphogenetic receptor type II (BMPR-II), Cluster of differentiation 5 (CD5), Tropomyosin receptor kinase (Trk), Voltage-dependent anion-selective channel 1 (VDAC1), Rho-specific exchange factor (GEF-H1), Lbc's first cousin (Lfc)

Protein	Function	Reference
Dynein intermediate chain	Mediates cargo binding and trafficking	(Mok *et al.*, 2001)
Fyn kinase	Involves in mitosis of lymphocytes	(Campbell *et al.*, 1998; Kai *et al.*, 1997)
Doc2	Intracellular vesicle transports of cathepsin D	(Nagano *et al.*, 1998)
Small GTPase FIP-1	Involves in adenoviruse life cycle and adenovirus mediated inhibition of tumor necrosis factor alpha-induced apoptosis	(Lukashok *et al.*, 2000)
Rhodopsin	Transports of rhodopsin containing vesicles	(Tai *et al.*, 1999; Tai *et al.*, 2001; Yeh *et al.*, 2006)
Bone morphogenetic receptor type II (BMPR-II)	Regulates vascular remodelling in primary pulmonary hypertension	(Machado *et al.*, 2003)
Parathyroid hormone receptor	Regulates agonist induced receptor internalization	(Sugai *et al.*, 2003)
Polio virus receptor (CD 155)	Retrograde axonal transport of virus along neuronal cell body	(Mueller *et al.*, 2002; Ohka *et al.*, 2004)
Cluster of differentiation (CD 5)	Regulates vesicles transport in T and B cells	(Bauch *et al.*, 1998)
Tropomyosin receptor kinase (Trk) family, also known as receptor Tyrosine Kinase Trk A, TrkB, TrkC	Regulates Trafficking of vesicles containing nerve growth factor (ligand for Trk) in neuron	(Yano *et al.*, 2004; Yano *et al.*, 2000; Yano *et al.*, 2001)
Voltage-dependent anion-selective channel 1 (VDAC1)	Involves in trafficking of the VDAC1 into the membrane and regulation of mitochondria permeabilization	(Fang *et al.*, 2011; Schwarzer *et al.*, 2002)

Tastin (Trophinin-binding protein)	Regulates spindle assembly and centrosome integrity during mitosis	(Nadano *et al.*, 2002; Yang *et al.*, 2008)
*βγ subunit of G protein	Regulates neurite outgrowth Regulates RhoA and Rac1 mediated actin cytoskeletal changes	(Meiri *et al.*, 2014; Sachdev *et al.*, 2007; Takesono *et al.*, 1999; Yeh *et al.*, 2013)
Rho-specific exchange factor (GEF-H1) (Human) or Lbc's first cousin (Lfc) (murine)	Regulates RhoA and Rac1 activity Regulates actin and microtubule assembly Involves in mitotic spindle assembly	(Conde *et al.*, 2010; Meiri *et al.*, 2009; Meiri *et al.*, 2012)

Earlier studies regarding Tctex-1 revealed that its interaction with Rhodopsin, a GPCR, is necessary for vesicular transport of rhodopsin from the Golgi apparatus to the apical cell-surface (Tai *et al.*, 1999; Tai *et al.*, 2001). This Tctex-1-dependent transport mechanism was found to require the dynein intermediate chain. Phosphorylation of Tctex-1 at Serine-82 causes a reduced affinity for dynein, but not to rhodopsin (Yeh *et al.*, 2006). This suggested that Tctex-1 is modulated by phosphorylation to control cargo release once it delivers it to the apical membrane. This could be the case for KIM-1, where Tctex-1 could be involved in trafficking between the cell surface and the lysosome or endoplasmic reticulum via clathrin-coated vesicles.

Tctex-1 also plays an important role in actin and microtubule dynamics. This was first evident in embryonic hippocampal neurons development, where Tctex-1 mediated Rac1 activation through dynein independent function (Chuang *et al.*, 2005; Sachdev *et al.*, 2007). More recently, Tctex-1 was shown to be a negative regulator of the Rho GEF; Lfc (in mice) or GEF-H1 (in humans) (Meiri *et al.*, 2009). Tctex-1 interacts with Lfc and this inhibits cortical neurogenesis and induces disruption of proper mitotic spindle orientation (Conde *et al.*, 2010; Gauthier-Fisher *et al.*, 2009). Lfc and GEF-H1 are Rho-specific guanine nucleotide exchange factors (Rho GEF), which interact with microtubules, and their overexpression promotes stress fiber formation through RhoA activation (Birukova *et al.*, 2006; Krendel *et al.*, 2002; Ren *et al.*, 1998). Tctex-1 binding to Lfc or GEF-H1 represses their nucleotide exchange activity indirectly through sequestering of Lfc or GEF-H1 to microtubules (Meiri *et al.*, 2012). In addition, the phosphorylation of Lfc or GEF-H1 at Serine-885 by protein kinase A is an important step required to create a high affinity 14-3-3 binding site that inhibits their exchange activity (Meiri *et al.*, 2009). Once Lfc or GEF-H1 are activated, they are released from microtubules to activate and modulate actin structures. Hence, Lfc or GEF-H1 mediates cross-talk between actin and microtubules (Krendel *et al.*, 2002).

Interestingly, Tctex-1 has been identified as an activator of G protein signalling 2 (AGS2), a G protein coupled receptor-independent activator of the heterotrimeric G proteins in yeast (Takesono *et al.*, 1999). Tctex-1 activates this G protein by binding to the Gβγ subunit independently of dynein (Sachdev *et al.*, 2007; Takesono *et al.*, 1999). This binding seems to free the α subunit of the G protein to mediate other down-stream effects, such as neurite outgrowth in primary hippocampal neurons (Sachdev *et al.*, 2007). Recently, Tctex-1 was specifically implicated in Gα12 signalling by the Rottapel group in Toronto (Meiri *et al.*, 2014).

They elegantly showed that activation of the GPCR for Gα12 allows the interaction between PP2A and GEF-H1. This interaction leads to dephosphorylation of GEF-H1 on Serine-885 which triggers the release of GEF-H1 from the inhibitory dynein complex. Subsequently, released GEF-H1 becomes free to bind to the Gα12, leading to the disassembly of the heterotrimeric complex. Meanwhile, the Gβγ subunit binds to Tctex-1, disrupting the anchoring function of Tctex-1 that sequesters GEF-H1 to microtubules. Ultimately, this leads to the activation of RhoA and stress fiber formation, which is a hallmark of Gα12 agonists and GEF-H1 activation (Figure 1.6). This interesting interplay between Gα12 and Tctex-1 may have implications for our work linking KIM-1 to both Gα12 and Tctex-1 during phagocytosis of apoptotic cells, wherein both actin and microtubules are suggested to be important. In professional phagocytes, both actin and microtubules are continuously remodelled during phagocytosis. During engulfment, localized actin polymerization occurs underneath the cell corpse to help in forming the phagocytic cup and in the extension of pseudopods, all of which require activation of Rho family of GTPases, RhoA, Rac and Cdc42 (Castellano *et al*., 2001; Hochreiter-Hufford et al., 2013). Microtubules are also involved in the initial process of engulfment since microtubules disruption reduces pseudopods dynamic (Rosania *et al.,* 1996). Following particle uptake, disassembly of actin filament is more pronounced with increasing microtubule networks surrounding the engulfed particle (Castellano *et al*., 2001; Harrison *et al.,* 2002). This allows for the maturation of the phagosome where microtubules function as tracks for delivery of the engulfed target to the degradation machinery. In KIM-1 mediated uptake of apoptotic cells, the exact dynamics of actin and microtubules is not known. We aim throughout this thesis to understand the link between KIM-1, its interacting partners and the actin/microtubules cytoskeleton.

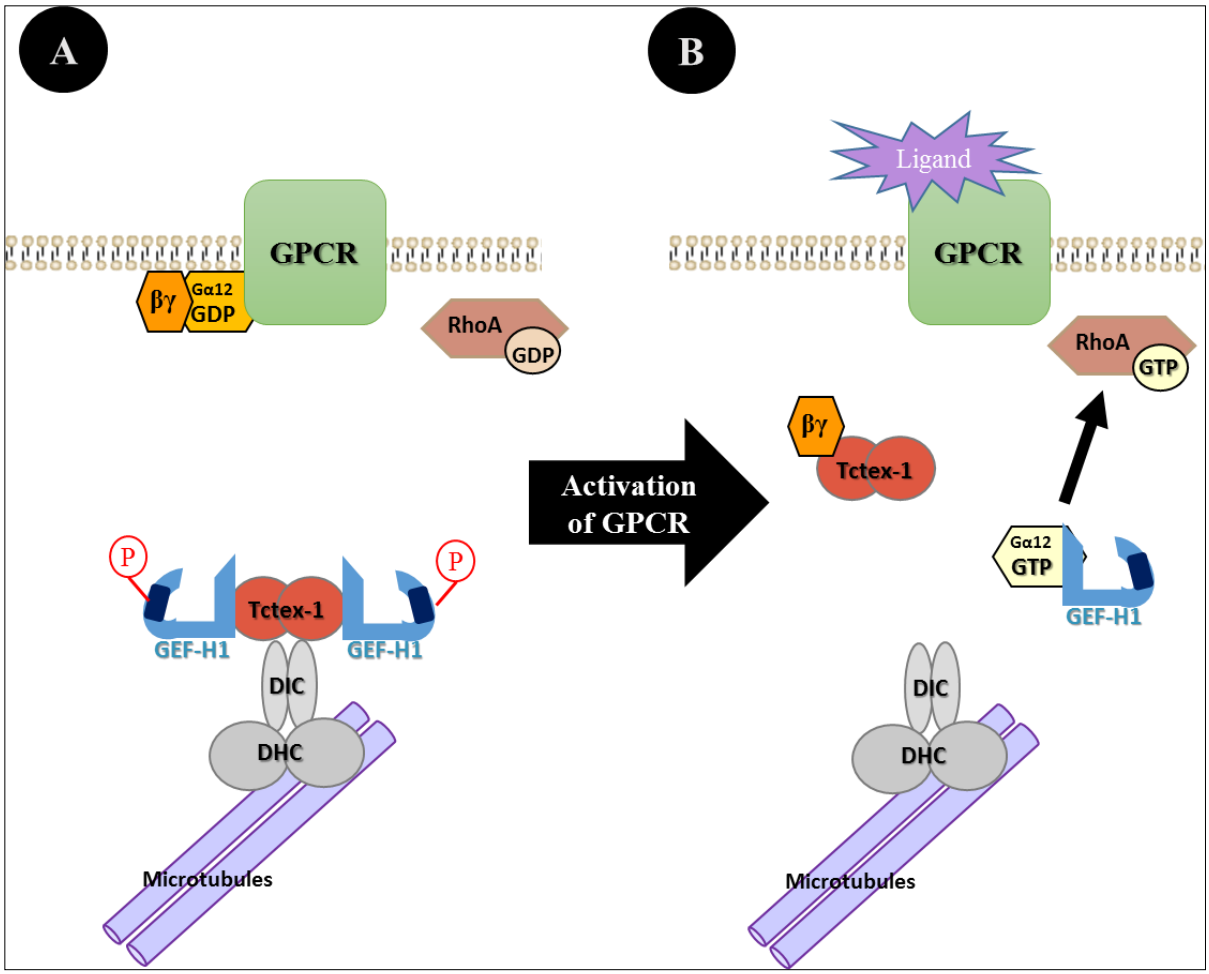


Figure 1.6. Regulation of actin cytoskeleton dynamics by Gα12 or Tctex-1 via Rho GEF (GEF-H1).

A: In the absence of GPCR stimulation, Tctex-1 is bound to microtubules via the dynein intermediate chain (DIC). Lfc or GEF-H1 is phosphorylated at Serine-885 by protein kinase. This creates an inhibitory site (dark blue) which allows for Lfc or GEF-H1 to be tethered to microtubules via Tctex-1. Under these conditions, heterotrimeric G12 protein is intact. As such, the α-subunit is in an inactive form (GDP-bound) and the βγ subunit is at the membrane. B: Upon GPCR stimulation (e.g. Thrombin), PP2A (not shown) interacts with Lfc or GEF-H1 and this triggers its dephosphorylation and release from the Tctex-1-dynein complex. LFc or GEF-H1 then binds to active Gα12, which was activated as a response to ligand dependent activation of the GPCR. Binding of Lfc or GEF-H1 to Gα12 allows the activation of the down-stream effector, RhoA. On the other hand, the released βγ subunit binds to Tctex-1 to stabilize it and prevent it from rebinding to Lfc or GEF-H1.

1.6. PURPOSE, HYPOTHESIS AND SPECIFIC AIMS

Over the past few years, several research groups have independently provided evidence suggesting that KIM-1 is sufficient to mediate the phagocytic clearance of apoptotic cells and necrotic debris (Ichimura *et al.*, 2008; Kobayashi *et al.*, 2007). Until now, *in vivo* evidence for a functional role for KIM-1 in AKI has been lacking. Moreover, there has been limited information regarding whether KIM-1 mediates intracellular signalling in TECs during AKI.

Our hypothesis is that KIM-1 upregulation on TECs protects mice from ischemic AKI and mediates phagocytic signalling via downstream interaction with Gα12 and Tctex-1. The purpose of the work described in this thesis is to understand the physiological role of KIM-1 during ischemic acute kidney injury and uncover the potential signalling mechanisms involved. In addition, we aim to understand mechanistically how KIM-1 carries out its phagocytic function. During this study, we have identified two novel KIM-1 interacting proteins, Gα12 and Tctex-1. The identification of these KIM-1 targets allowed us to focus on three main aims:

1. Role of KIM-1 and Gα12 in renal ischemia-reperfusion injury
2. Role of KIM-1 and Gα12 in KIM-1-mediated phagocytosis
3. Role of KIM-1 and Tctex-1 in KIM-1-mediated phagocytosis

The data presented in Chapters 2, 3 and 4 of this thesis will summarize our research designed to address these aims.

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Chapter 2

Kidney Injury Molecule-1 protects against Gα12 activation and tissue damage in renal ischemia-reperfusion injury

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Dr. Aaron Haig[2] stained the histology tissue samples and graded them.
Dr. Bradley M. Denker[3] provided Gα12 plasmids constructs to carry out these experiments.
Dr. Rita S. Suri performed statistical analysis on the data presented in the manuscript and generated some figures.
Dr. Alp Sener[1,5,6] provided help with editing the manuscript for publication.
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2.1. SUMMARY

Ischemic acute kidney injury (AKI) is a serious untreatable condition. Activation of the G-protein alpha-12 (Gα12) subunit by reactive oxygen species (ROS) is a major cause of tissue damage during renal ischemia-reperfusion injury. Kidney Injury Molecule-1 (KIM-1) is a transmembrane glycoprotein that is highly upregulated during AKI, but the physiologic significance of this is unclear. In this chapter, we show for the first time that KIM-1 inhibits Gα12 activation, and protects mice against renal ischemia-reperfusion injury. We reveal that KIM-1 physically interacts with and inhibits cellular Gα12 activation following inflammatory stimuli including ROS, by blocking GTP binding to Gα12. Compared to wild type (Kim-$1^{+/+}$) mice, Kim-1 knockout (Kim-$1^{-/-}$) mice exhibited greater Gα12 and downstream Src activation, both in primary tubular epithelial cells after *in vitro* stimulation with H_2O_2, and in whole kidneys after unilateral renal artery clamping. Finally, we show that Kim-1-deficient mice had more severe kidney dysfunction and tissue damage after bilateral renal artery clamping, compared to wild type mice. Our results suggest that KIM-1 functions as part of an endogenous protective mechanism against renal ischemia-reperfusion injury through inhibition of Gα12.

2.2. INTRODUCTION

Acute kidney injury (AKI) is a serious medical condition that most often results from ischemia-reperfusion injury (IRI) and for which there is no effective treatment (Bonventre *et al.*, 2011; Chertow *et al.*, 2005). Kidney injury molecule-1 (KIM-1) is a cell-surface glycoprotein receptor (Freeman *et al.*, 2010) that is specifically upregulated on the apical surface of proximal tubular epithelial cells (TECs) following AKI (Ichimura *et al.*, 1998). It is a highly sensitive and specific biomarker of tubular injury that is virtually absent in healthy kidneys (Vaidya *et al.*, 2010). Both mouse and human KIM-1 [also known as T cell immunoglobulin, mucin domain-1 protein (TIM-1), and hepatitis A virus cellular receptor-1 (HAVCR-1)] are small type I transmembrane glycoproteins that belong to the TIM gene family (Freeman *et al.*, 2010). Structurally, KIM-1 is made up of an IgV-like domain, a mucin domain, a transmembrane domain, and an intracellular domain that has been implicated in T cell signaling (de Souza *et al.*, 2005; Z. Zhang *et al.*, 2007). The pathophysiologic role of KIM-1 signaling in AKI remains unknown.

Here we reveal a novel interaction between KIM-1 and Gα12. Gα12 is a ubiquitously expressed G protein belonging to the G12 family of G proteins that has pleiotropic effects on cells

including inducing proliferation, focal adhesion assembly, cytoskeletal reorganization, apoptosis and disruption of tight junctions (Dhanasekaran *et al.*, 1996). Recently, Yu *et al.,* (Yu *et al.*, 2012) demonstrated that activated Gα12 is a pivotal mediator of TEC injury caused by ischemia-reperfusion. ROS generated during ischemia-reperfusion stimulates Gα12 to activate Src-dependent injury pathways. Specifically, Gα12-deficient mice were protected from renal IRI, whereas mice with TEC-specific transgenic overexpression of constitutively active Gα12 (Gα12 QL) exhibited worse tissue damage and delayed recovery after IRI (Yu *et al.*, 2012). Given the relevance of KIM-1 and Gα12 in AKI, we investigated the biological relationship between these proteins using *in vitro* and *in vivo* models of ischemic AKI.

2.3. EXPERIMENTAL PROCEDURES

2.3.1. Animal preparation and induction of ischemia-reperfusion injury

All mouse experiments were performed according to the animal experimental guidelines issued by the Animal Care and Use Committee at Western University (refer to the Appendix for approval certificate). C57BL/6 wild type (Kim-$1^{+/+}$) mice were obtained from the Charles Rivers Laboratory. Kim-1 knock out (Kim-$1^{-/-}$) mice were generously provided to us by Dr. Andrew N. J. McKenzie (MRC laboratory of Molecular biology, Cambridge, UK). The Kim-$1^{-/-}$ mice were backcrossed over generations to the C57BL/6 strain and homozygous knockout mice (Kim-$1^{-/-}$) were obtained by inter-breeding the heterozygotes (Kim-$1^{+/-}$). Genomic DNA was isolated from mice tail digests and genotypes were determined by PCR using primers ASEQ965 5'-ATATCTCAGGAA TGGGATTG TGAC-3' and ASEQ966 5'-CTACTGTATTT AACTGATTTGAAG-3'. 6-9 week-old male Kim-$1^{+/+}$ or Kim-$1^{-/-}$ mice weighing 20-25 grams were subjected to unilateral or bilateral renal pedicle ligation for 25-35 minutes (as indicated) at 32 °C, as described previously (Z. X. Zhang et al., 2010). To assess Gα12 activity in the ischemic and contralateral kidneys, following reperfusion, the kidneys were removed after being flushed with cold PBS. Sham controls were treated with the same operative procedure as in the injury group, but kidneys were not clamped.

2.3.2. Renal function and histology

Serum creatinine was detected by a Jaffe reaction method with an automated CX5 clinic analyzer (Beckman, Pasadena, CA). Kidney sections (5–6 microns) from animals at 24 and 48 hours (h) after reperfusion or sham surgery were stained with Periodic acid–Schiff (PAS) by a

trained pathologist-blinded to sample type (Dr. Aaron Haig) and the degree of tubular injury was graded by light microscopy by a single pathologist blinded to mouse strain using a semiquantitative score, that examined proximal tubule dilation, brush-border damage, proteinaceous casts, interstitial widening, and necrosis (0: none, 1: <10 %, 2: 11~25 %; 3: 26-45 %; 4: 46-75 %; 5: >75 %) as described previously (Bakker *et al.*, 2014).

2.3.3. Mass spectrometry

KIM-1 immune complexes were derived from human embryonic kidney-293 (HEK-293) cells stably overexpressing HA-tagged KIM-1 (Z. Zhang *et al.*, 2007) or vector alone (Otsuka *et al.*, 2005). Plasmid expressing KIM-1-HA was a kind gift from Prof. Joseph Bonventre (Brigham and Women's Hospital, Harvard Institutes of Medicine, Boston, MA) and it was generated in a similar manner as described in Zhang, et al (Z. Zhang *et al.*, 2007). Briefly, the full-length human KIM-1 with an N-terminal hemagglutinin (HA) tag was generated by PCR using phKIM1.2 as template and then sub-cloned into the EcoRV site of eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA). Upon transfection of KIM-1-HA, protein and surface expression was comparable to endogenous KIM-1 expressed in human renal adenocarcinoma cells (769P) (Figure 2.1A, B). Immunoprecipitation was done with anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA). SDS-PAGE was used to identify unique bands that co-immunoprecipitated with KIM-1 and a target protein band of approximately 38 kDa was excised and subjected to in-gel tryptic digestion followed by mass spectrometry (MALDI TOF MS) and analyzed by the Taplin mass spectrometry facility (Harvard Medical School, Cambridge, MA).

2.3.4. Cell lines and culture

Human embryonic kidney-293 (HEK-293) cells stably expressing control vector or human KIM-1 were cultured at 37 °C in 5 % (vol/vol) CO_2 and maintained in DMEM (Invitrogen, Carlsbad, CA) containing 5 % (vol/vol) fetal bovine serum (FBS) and 800 μg/mL Geneticin (G418) Sulfate (Santa Cruz Biotechnology, Santa Cruz, CA). Expression of KIM-1 was confirmed by Western blotting. HEK-293 cells were transfected with truncated version of KIM-1 containing its cytosolic domain fused to transmembrane domain using Lipofectamine 2000® (Invitrogen, Carlsbad, CA). Briefly, this plasmid was generated by PCR using the Not1 restriction sites of the human KIM-1 plasmid (Z. Zhang *et al.*, 2007). The resulting construct containing the transmembrane and cytosolic domain of KIM-1 was sub-cloned into pFLAG-CMV™-3 (Sigma-Aldrich, St. Louis, MO, USA). The expression of the construct was confirmed using Western

blotting (Figure 2.1C). Kidneys isolated from 3-4 week old C57BL/6 Kim-$1^{+/+}$ or Kim-$1^{-/-}$ mice were cultured to select for proximal tubular epithelial cells (TECs) as described previously (Sharpe *et al.*, 2012). Briefly, kidneys were homogenized and the cells isolated were cultured for the first 6 days in serum free DMEM/F-12 mixed media (1:1) (Invitrogen, Carlsbad, CA) supplemented with 5 % insulin-transferrin-selenium solution, 5 % penicillin-streptomycin solution (Invitrogen, Carlsbad, CA), 0.5 µg/ml of mouse epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ), 50 ng/ml hydrocortisone (Invitrogen, Carlsbad, CA). After 6 days, cells were cultured with the same media mixture supplemented with 5 % FBS as described earlier.

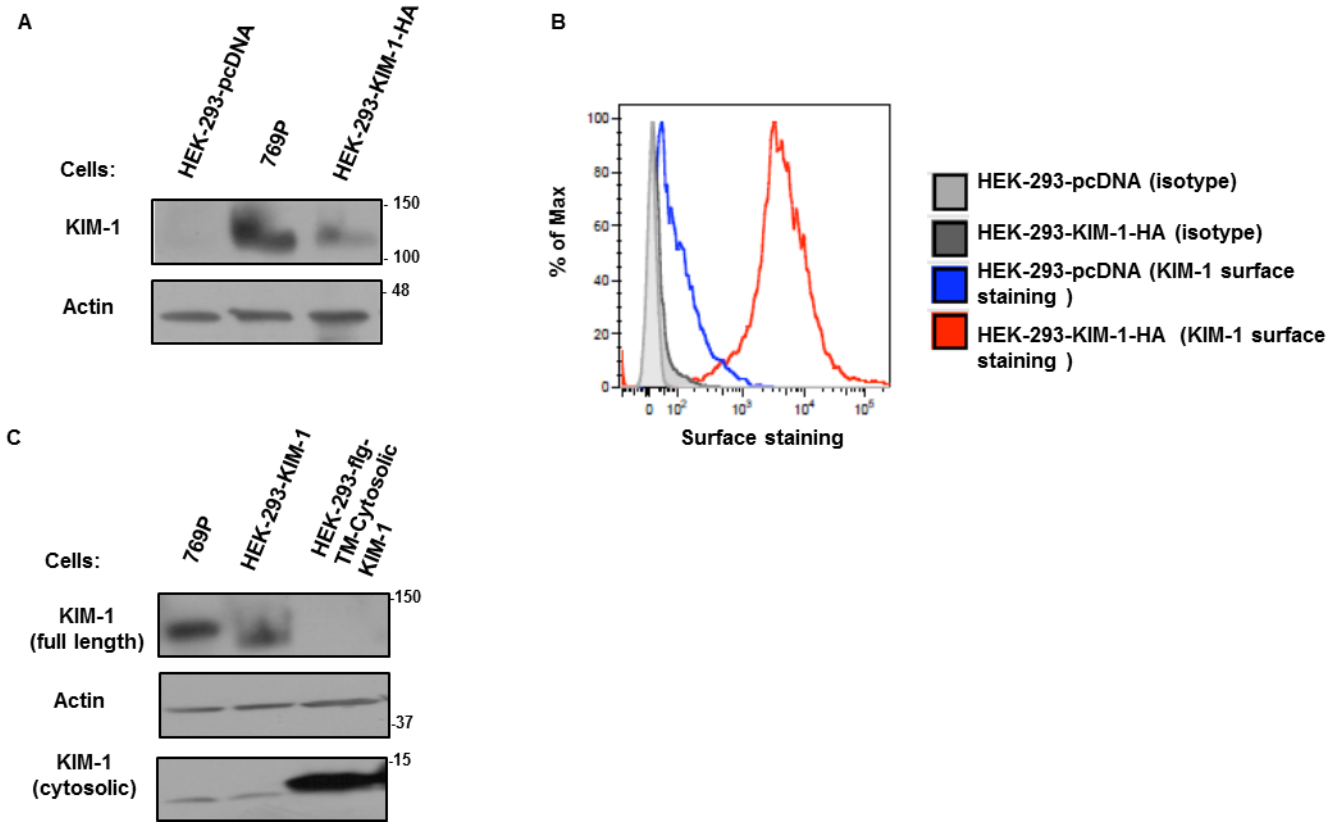


Figure 2.1. Protein expression of KIM-1-HA and KIM-1 with truncated KIM-1 containing its transmembrane and cytosolic domains in HEK-293 cells

A: Human embryonic kidney-293 (HEK-293) cells were transfected with either control vector (pcDNA3) or HA tagged KIM-1-expressing vector (KIM-1-HA). Cell lysates were compared to the endogenous level of KIM-1-expression in human renal adenocarcinoma cell line (769P) by Western blotting using antibodies against full length KIM-1 (extracellular domain), and actin. ***B***: Surface staining for KIM-1 in HEK-293 cells expressing pcDNA3 or KIM-1-HA as determined by flow cytometry. ***C***: HEK-293 cells were transfected with either full-length KIM-1 or truncated flag-tagged KIM-1 construct containing only the transmembrane (TM) and the cytosolic domain of KIM-1. Lysates from these cells were run on Western blot and probed for full-length KIM-1 (antibody against extracellular domain of KIM-1), the cytosolic domain of KIM-1 (antibody against cytosolic domain of KIM-1), and actin. Lysate from human renal adenocarcinoma cells (769P) was used as a positive control. Data represent three independent experiments.

2.3.5. Western blot

Confluent monolayers of cell lines (HEK-293 or TECs) or kidney cortices were homogenized and lysed in ice cold lysis buffer and were centrifuged at 15,000 g for 10 minutes. The supernatant was collected for Western blot analysis. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL), and 50 µg of protein was used as input for the experiment. 1000 µg of protein was used for immunoprecipitation or GST-TPR pull-down experiments. Input and immunopreciptated samples were electrophoresed on Mini-PROTEAN TGX gels 4-20 % (Bio-Rad Laboratories, Hercules, CA) and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Membranes were blocked with blocking buffer (Tris-buffered saline, 0.1 % Tween-20, and either 5 % nonfat dried milk (Bioshop, Burligton, ON) or 5 % bovine serum albumin solution (Sigma Aldrich, St. Louis, MO) for 30 minutes and were then incubated overnight at 4 °C with one of the following primary antibodies: cytosolic domain of human KIM-1 (PA 4145, Custom antibody, Thermo Fisher Scientific, Rockford, IL) (Ichimura *et al.*, 1998), extracellular domain of human KIM-1 (Dr. Bonventre, Harvard medical School, Cambridge, MA)(Bailly *et al.*, 2002), extracellular domain of mouse Kim-1 (AF1817, R&D systems, Minneapolis, MN), Gα12 (S-20)/actin (I-19)/GAPDH (V-18) or c-Src (N-16) (Santa Cruz Biotechnology, Santa Cruz, CA), and active Src (pY419-Src) antibody (44-660G, Invitrogen, Carlsbad, CA). Membranes were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (dilution 1:30000, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature in blocking buffer. Proteins were visualized using SuperSignal West Femto chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL). The resulting protein bands were scanned using a Brother scanner (Brother Electronics, Dollard-des-Ormeaux, QC) and the integrated density of each protein band was determined using ImageJ Software. Each densitometric graph represents at least three independent experimental results. To normalize for protein loading, the integrated density of each band was divided by the integrated density of the actin band in the same lane from the same membrane (Kerr *et al.*, 1972).

2.3.6. Immunoprecipitation and GST-Gα12 pull-down assay

For immunopreciptation (IP), cells were lysed with ice-cold lysis buffer (25 mM Hepes (Sigma Aldrich, St. Louis, MO), 150 mM NaCl, 15 mM $MgCl_2$, 1 % Triton X-100, and Mini-protease inhibitors tablet (Roche Diagnostic, Basel, Switzerland)) (Meyer *et al.*, 2003). Following

protein quantification, immunoprecipitation of 1000 µg of each lysate was done using antibodies against Gα12, the cytosolic domain of KIM-1 (Z. Zhang *et al.*, 2007), or rabbit IgG control (sc-2027, Santa Cruz Biotechnology, Santa Cruz, CA) and protein-A/G Sepharose beads (Santa Cruz Biotechnology). Lysates (representing 5 % of total lysate) and immunoprecipitation samples were analyzed by SDS-PAGE and Western blot as described above. For GST-Gα12 pull-downs, KIM-1-HA was *in vitro* translated and S^{35}-labelled in rabbit reticulocytes lysates as described previously (Zhu *et al.*, 2004). Protein lysates were incubated with 1 gram of GST-Gα12 or GST alone and eluted after 3 hours incubation. Samples were analyzed by SDS-PAGE and autoradiography (Biomax; Denville Scientific, Metuchen, NJ).

2.3.7. Gα12 activation assay (GST-TPR pull down)

A construct of GST-fused to the tetratricopeptide repeat (TPR) domain of Ser/Thr protein phosphatase type 5 (PP5) was kindly provided by Dr. N Dhanasekaran (Temple University, USA) (Zhu *et al.*, 2004). GST-TPR was purified from *Escherichia coli* and conjugated to glutathione-agarose beads (Thermo Fisher Scientific, Rockford, IL) as described (Yamaguchi *et al.*, 2002). GST-TPR conjugated beads were freshly made for each experiment. Kidney homogenates following ischemia-reperfusion injury or sham surgery, as well as unstimulated cells or cells stimulated with 5 mM H_2O_2 (Bio Basic, Amherst, NY) or 2 U/ml alpha-thrombin (Enzyme Research Laboratories, South Bend, IN) were lysed with ice-cold lysis buffer (50 mM Hepes pH 7.5) (Sigma Aldrich, St. Louis, MO), 1 mM EDTA, 3 mM DTT, 2 mM $MgSO_4$, 1 % Polyoxyethylene (10) lauryl ether ($C_{12}E_{10}$), and mini-protease inhibitor tablet (Roche Diagnostic, Basel, Switzerland) (Yu *et al.*, 2012). Supernatants containing 1 mg of the protein were incubated with GST-TPR coupled glutathione-agarose beads at 4 °C for 5 hours (Zhu *et al.*, 2004). Where indicated, cell lysates were loaded with 1 µM non-hydrolyzable GTP analog (GTPγS) (Cytoskeleton, Denver, CO) following protein quantification and incubated for 15 minutes at room temperature prior to adding GST-TPR coupled glutathione-agarose beads. Samples were centrifuged at 5000g for 5 minutes and washed with 1x PBS twice before eluting the bound active Gα12. Both 50 µg of protein (input) and pull-down samples were analyzed by SDS-PAGE and Western blotting by probing for Gα12 to represent total (input) and active Gα12 (pull-down) in these samples.

2.3.8. Immunofluorescence and confocal microscopy

HEK-293 cells were cultured at subconfluent density on poly-DL-lysine hydrobromide (Sigma Aldrich, St. Louis, MO) coated glass cover slips. Cells were transfected with GFP-tagged Gα12 construct (Andreeva *et al.*, 2008) using Lipofectamine 2000® (Invitrogen, Carlsbad, CA). Cells were fixed with 4 % paraformaldehyde (Sigma Aldrich, St. Louis, MO) and stained for surface KIM-1 using antibody against extracellular domain of KIM-1 overnight at 4 °C. The slides were washed and incubated with Alexa555 conjugated secondary antibody (Molecular probes, Invitrogen, Carlsbad, CA). Coverslips were mounted using Shandon-Mount® (Thermo Fisher Scientific, Rockford, IL) permanent mounting medium. Mice kidneys following ischemia-reperfusion injury or sham surgery were extracted and quartered. The sections were fixed in 4 % paraformaldehyde overnight for subsequent paraffin embedding. Kidney cortex sections (10 µm) were sliced at 50-µm intervals using a Leica RM2125 microtome (Leica Microsystems, Buffalo Grove, IL) and were mounted on glass slides. Sections were blocked with 5 % horse serum (Invitrogen, Carlsbad, CA) and hybridized overnight at 4 °C with antibodies specific for either the extracellular domain of mouse Kim-1 (SAB3500252, dilution 1:50, Sigma Aldrich, St. Louis, MO), or Gα12 (S-20, dilution 1:50, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then washed and incubated with the appropriate fluorophore-conjugated secondary antibodies (dilution 1:100; Invitrogen, Carlsbad, CA). Cover slips were applied to all the sections with Shandon-Mount® permanent mounting medium before imaging. Samples were viewed with FLUOVIEW X83I confocal microscopy (Olympus, Tokyo, Japan). Data were acquired and analyzed using FLUOVIEW FV10 ASW 4.0 viewer and ImageJ software (National Institutes of Health, Bethesda, MD) was used to determine Pearson's coefficient for colocalization of KIM-1 and Gα12. Quantification of colocalization was determined for 5 random fields per sample and was done in 4 independent experiments.

2.3.9. Statistical analysis

Statistical analysis was done using Graph Pad Prism (Graph Pad Software Inc., La Jolla, CA). Mean or median differences between knockout and wild type mice were compared using unpaired t-tests or Mann-Whitney-U tests as appropriate; proportions were compared using Chi-squared tests, and 95 % confidence intervals were calculated using the method of Wilson (Wilson, 1942). P-values of < 0.05 were considered significant. Error bars represent the standard error of the mean unless otherwise indicated.

2.4. RESULTS

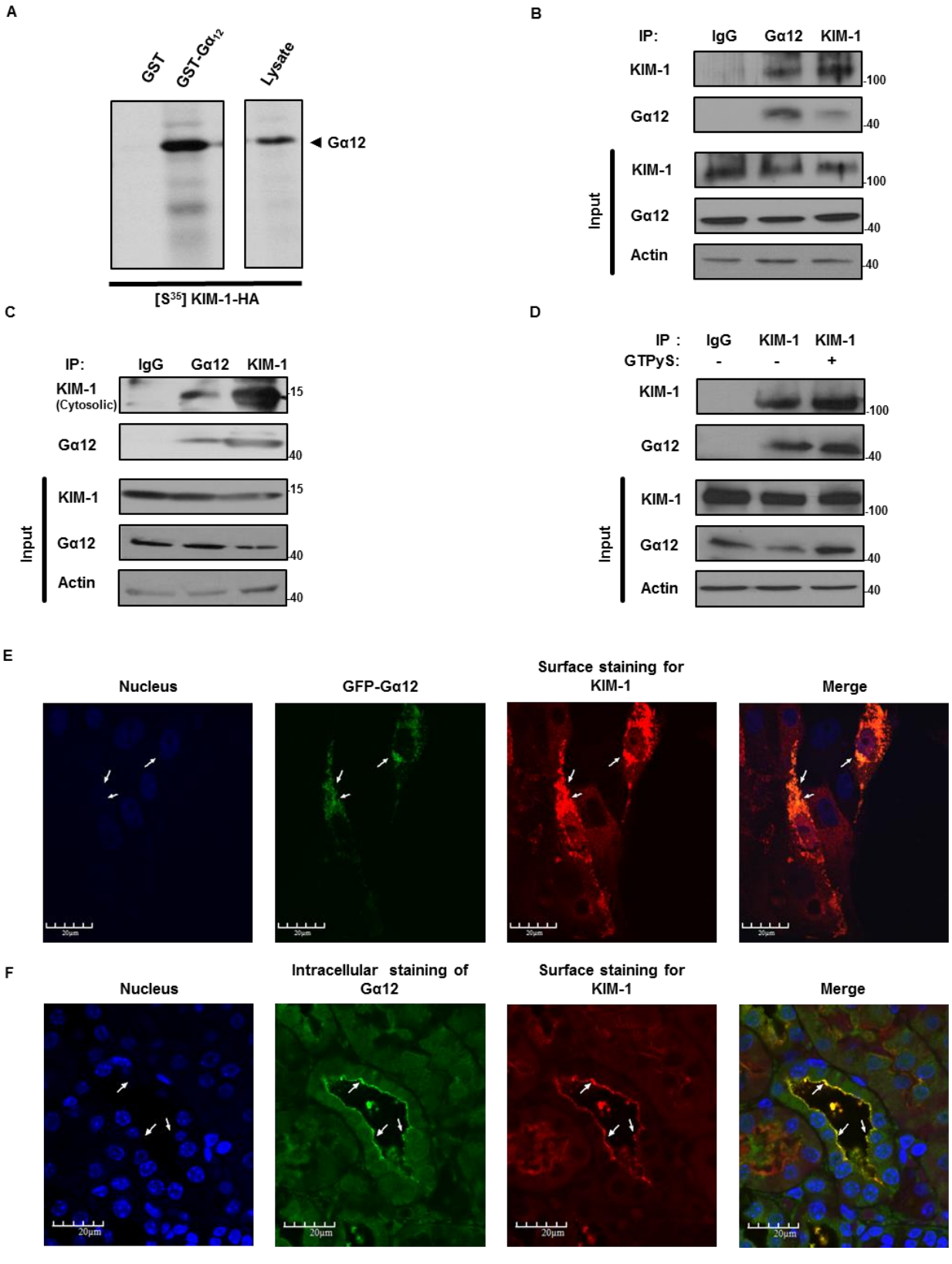
2.4.1. KIM-1 interacts with Gα12

To identify putative signalling proteins downstream of KIM-1, we performed mass-spectrometric analysis on KIM-1 (anti-HA) immunoprecipitates from HEK-293 cells stably overexpressing KIM-1 fused to a c-terminal HA tag (Z. Zhang *et al.*, 2007) or vector alone as described in the methods. Of the several target proteins identified at 40 kDa (Table 2.1), we focused on Gα12 because of its previously described roles in the biology of TECs and AKI (Yu *et al.*, 2012; Yanamadala *et al.*, 2007).

Table 2.1. Potential KIM-1 interacting proteins as determined by mass spectrometry.

Interacting proteins	**Accession number**	**Peptide sequence identified**
Homo sapiens guanine nucleotide-binding protein, alpha-12 subunit (g alpha 12)	Q03113	ILLLGAGESGK
Homo sapiens arginase 1 (liver-type arginase)	P05089	TGLLSGLDIMEVNPSLGK
Homo sapiens set protein (phosphatase 2a inhibitor i2pp2a) template activating factor i (taf-i)	Q01105	VEVTEFEDIK
Homo sapiens fructose-bisphosphate aldolase c	P09972	GVVPLAGTDGETTTQGLDGLSER
Homo sapiens ig kappa chain c region	P01834	TVAAPSVFIFPPSDEQLK
Homo sapiens nucleophosmin numatrin	P06748	FINYVK, GPSSVEDIK, VDNDENEHQLSRLR, MSVQPTVSLGGFEITPPVVLR
Homo sapiens phosphoserine aminotransferase	Q9y617	FGVIFAGAQK
Homo sapiens heterogeneous nuclear ribonucleoprotein a3	P51991	SSGSPYGGGYGSGGGSGGYGSR

To confirm the mass-spectrometry results, we performed pull-down experiments using S^{35}methionine-labelled KIM-1 from reticulocyte lysates with glutathione S-transferase (GST) fused to Gα12 to determine if Gα12 can interact directly with KIM-1 (Zhu *et al.*, 2004). We found that KIM-1 with a c-terminal HA tag was bound to GST-Gα12, but not unconjugated GST (Figure 2.2A). Co-immunoprecipitation of Gα12 and KIM-1 using both anti-KIM-1 and anti-Gα12, but not the control antibody confirmed this interaction (Figure 2.2B). The cytosolic domain of KIM-1 was sufficient to mediate binding to Gα12, as demonstrated by co-immunoprecipitation of Gα12 with a truncated version of KIM-1 containing its cytosolic domain fused to its transmembrane domain and an extracellular flag tag (Figure 2.2C) (Z. Zhang *et al.*, 2007). To determine if activated Gα12 can also interact with KIM-1, we treated KIM-1 expressing HEK-293 cell lysates with non-hydrolyzable GTP (GTPγS) prior to performing co-immunoprecipitation (Ohga *et al.*, 1989); GTPγS treatment of Gα12 did not alter the interaction with KIM-1 (Figure 2.2D). To visualize the interaction of Gα12 with KIM-1, HEK-293 cells were co-transfected with KIM-1 and GFP-Gα12 and co-localization of both proteins was assessed by confocal microscopy. A significant proportion of KIM-1 was associated with Gα12 in cells expressing both proteins, as indicated by Pearson's coefficient (0.409 ± 0.098, n=7) (Figure 2.2E). In addition, we found co-localization of both murine Kim-1 and Gα12 in kidney tissue sections isolated from C57BL/6 wild type (Kim-$1^{+/+}$) mice that were subjected to renal ischemia-reperfusion injury (IRI) (bilateral renal pedicle clamping for 30 minutes followed by 24 hours of reperfusion) in order to cause Kim-1 upregulation (Figure 2.2F). Taken together, the above data suggest that KIM-1 interacts constitutively with Gα12 via its cytosolic domain, independent of Gα12 activation.


A
GST
GST-Gα12
Lysate
Gα12
[S35] KIM-1-HA
B
IP:
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Gα12
Input
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IgG
Gα12
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IgG
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KIM-1
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KIM-1
Gα12
Input
KIM-1
Gα12
Actin
100
40
100
40
40
E
Nucleus
GFP-Gα12
Surface staining for KIM-1
Merge
F
Nucleus
Intracellular staining of Gα12
Surface staining for KIM-1
Merge
20μm

Figure 2.2. KIM-1 interacts with Gα12.

A: Autoradiogram of pull-downs with purified GST-Gα12 or GST of *in vitro* translated S^{35}-methionine-labelled HA-tagged-KIM-1. ***B:*** Immunopreciptation (IP) of KIM-1 and Gα12 from lysates of HEK-293 cells stably expressing human KIM-1 using anti-Gα12, anti-KIM-1 (against the cytosolic domain), or rabbit IgG control antibodies. ***C:*** Lysates of HEK-293 cells expressing a truncated KIM-1 protein comprised of only the transmembrane and cytosolic domains were subjected to IP with the indicated antibodies. ***D:*** Lysates of HEK-293 cells stably expressing KIM-1 were pretreated with non-hydrolysable GTP (GTPγS) to activate Gα12 and then subjected to IP. The input lane represents 5 % of the lysate. Both lysates and IP samples were analyzed by SDS-PAGE and Western blotting for KIM-1 (using an antibody against the extracellular domain), Gα12, and actin. Results represent three independent experiments. ***E:*** Co-localization of both KIM-1 and Gα12 proteins in HEK-293 cells co-transfected with KIM-1 and GFP-tagged Gα12 constructs. KIM-1 was detected using an antibody against its extracellular domain (600x, bar represents 20 μm). ***F:*** Kidney cortex cross-section from wild type (Kim-1+/+) mice that were immunofluorescently stained with anti-Kim-1 (Red) and anti-Gα12 antibody following renal artery clamping for 30 minutes followed by reperfusion for 24 hours (400x, Bar 100μm). Arrows indicate areas with a high degree of co-localization between KIM-1 or Kim-1 and Gα12.

2.4.2. KIM-1 inhibits Gα12 activation by blocking GTP-binding

It has long been hypothesized that KIM-1 might play a protective role in AKI (Ichimura *et al.*, 1998). Given that Gα12 activation instigates injury pathways in AKI, we hypothesized that KIM-1 might suppress endogenous Gα12 activation in TECs. Because Gα12 is activated by guanine nucleotide exchange, we studied its activation status using a GST-TPR pull-down assay (Yamaguchi *et al.*, 2002). Figure 2.3A demonstrates that endogenous Gα12 activity is inhibited in cells expressing KIM-1, but not the control vector (pcDNA). Next, to test if KIM-1 expression could inhibit Gα12 activation by physiologic ligands that stimulate its cognate GPCRs, we measured the level of active Gα12 after stimulating HEK-293 cells transiently expressing KIM-1 or control vector with alpha-thrombin (Figure 2.3B) (Yu *et al.*, 2012; Yanamadala *et al.*, 2007). Given that ROS stimulate Gα12 activation in IRI, we tested whether KIM-1 expression affected Gα12 activation by H_2O_2; Gα12 activation was significantly blunted in KIM-1-expressing cells compared to control cells upon stimulation with either alpha-thrombin or 5 mM H_2O_2 (Figure 2.3B). Additionally, we didn't observe any effect of the level of H_2O_2 used on cell viability (Figure 2.3C and D).

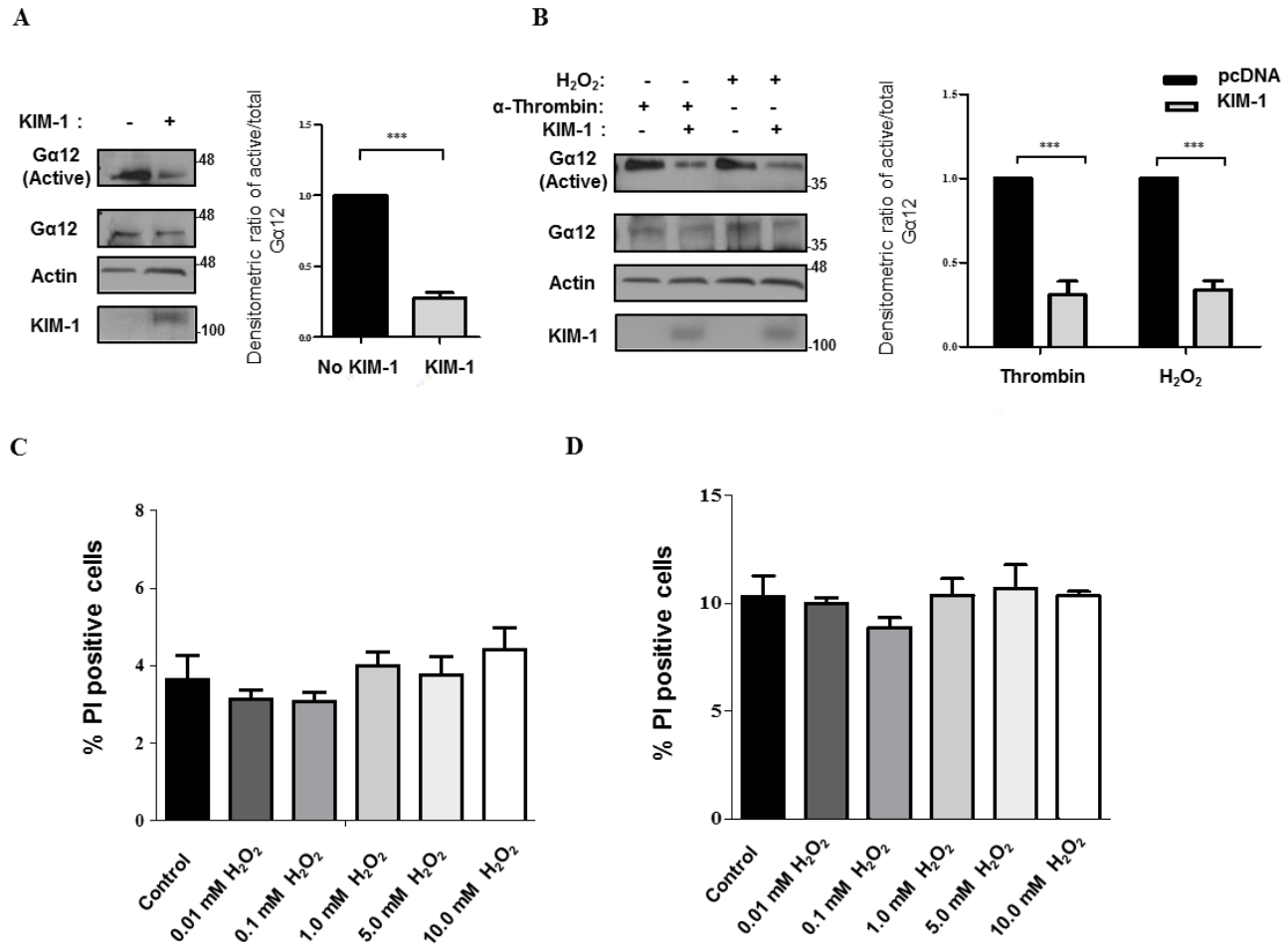
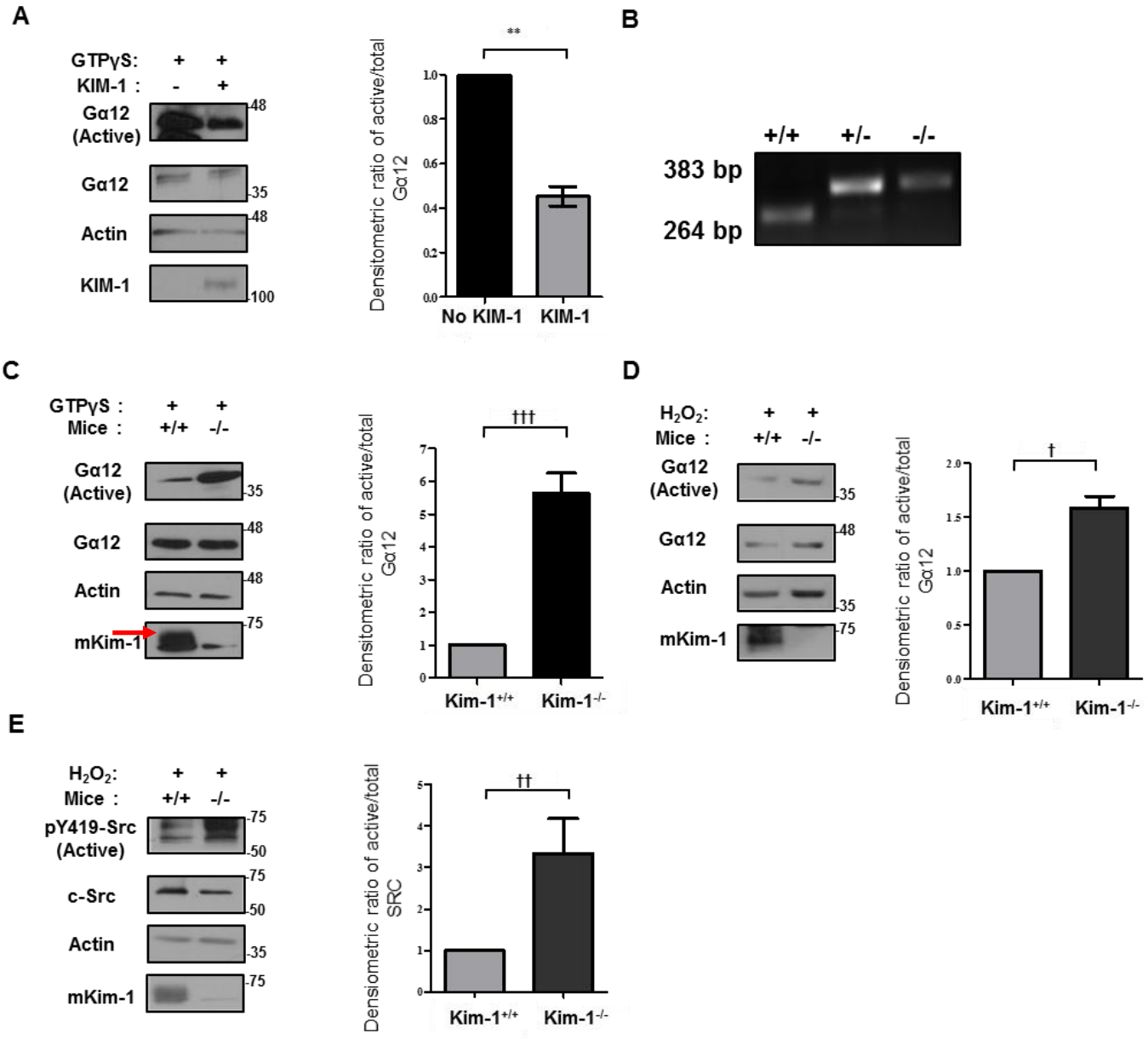


Figure 2.3. KIM-1 expression inhibits cellular Gα12 activation.

HEK-293 cells were transfected with either control vector (pcDNA) or KIM-1 plasmid in both panels A and B. ***A:*** Cells were left untreated. ***B:*** Cells were stimulated with either α-thrombin (2 U/ml) or H_2O_2 (5 mM) for 30 minutes. Samples in ***A*** and ***B*** were subjected to the GST-TPR pull-down assay to measure the level of Gα12 activation. Lysates (total) and pull-down (active) samples were analyzed by SDS-PAGE and Western blotting with antibodies against Gα12, KIM-1 and actin as indicated. Densitometric analyses of the ratio of active to total Gα12 relative to control vector transfected cells are shown as a representation of three independent experiments (n=3, ***p<0.001). ***C* and *D***: Proximal tubule epithelial cells isolated from either wild type **(*C*)** or Kim-1 knock out **(*D*)** mice were treated with increasing concentrations of H_2O_2 (mM) as indicated. Cell death was calculated as the percentage of propidium iodide (PI) positive cells as detected by flow cytometry in comparison to control treatment (water). n=3, no significant difference was found between the treatments.

Based on the above data, we suspected that KIM-1 might block nucleotide exchange on Gα12, thereby preventing its activation. To test this, we exposed lysates from HEK-293 cells transiently transfected with either control vector or vector encoding KIM-1 to GTPγS to irreversibly lock Gα12 in the active conformation. We observed significantly less GTP-bound Gα12 in KIM-1 expressing cells compared to cells not expressing KIM-1 (Figure 2.4A). Similar results were obtained when we used HEK-293 cells stably expressing KIM-1 or control vector. To extend these findings to a physiologically relevant *in vitro* model, we compared Gα12 activation in primary TECs isolated from previously generated Kim-$1^{-/-}$ mice (Figure 2.4B) to Kim-$1^{+/+}$ mice. Concordant with previous reports (Wong *et al.*, 2010), Kim-$1^{-/-}$ mice were phenotypically normal. *Ex vivo* culture of TECs from Kim-$1^{+/+}$ mice resulted in KIM-1 upregulation as previously reported (Ichimura *et al.*, 1998). As expected, this was absent in TECs isolated from Kim-$1^{-/-}$ mice as indicated by Western blot of mouse Kim-1 (Figure 2.4 C, D, E). Consistent with data in HEK-293 cells, we observed significantly higher levels of active Gα12 in TEC-lysates from Kim-$1^{-/-}$ mice compared to TEC-lysates from Kim-$1^{+/+}$ mice loaded with GTPγS (Figure 2.4C). Similarly, the level of Gα12 activation was significantly higher in TEC-lysates from Kim-$1^{-/-}$ mice compared to that from Kim-$1^{+/+}$ mice upon stimulation with H_2O_2 (Figure 2.4D). Given that Src was shown to mediate ROS/Gα12 mediated epithelial injury (Yu *et al.*, 2012), we examined Src activation using pY419 antibodies (which recognize phosphorylated Src) in the total cell lysates from Kim-$1^{-/-}$ and Kim-$1^{+/+}$ TECs after treatment with H_2O_2. Figure 2.4E shows significantly enhanced Src activation in TECs from Kim-$1^{-/-}$ mice compared to those from Kim-$1^{+/+}$ mice after H_2O_2 stimulation. Taken together, these data suggested that KIM-1 constitutively inhibits Gα12/Src activation by blocking GDP-GTP exchange by Gα12.


A
GTPγS: + +
KIM-1 : - +
Gα12 (Active)
Gα12
Actin
KIM-1
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48
100
Densitometric ratio of active/total Gα12
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No KIM-1
KIM-1
B
+/+
+/-
-/-
383 bp
264 bp
C
GTPγS : + +
Mice : +/+ -/-
Gα12 (Active)
Gα12
Actin
mKim-1
35
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Densitometric ratio of active/total Gα12
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Kim-1+/+
Kim-1-/-
D
H2O2: + +
Mice : +/+ -/-
Gα12 (Active)
Gα12
Actin
mKim-1
35
48
35
75
Densiometric ratio of active/total Gα12
†
Kim-1+/+
Kim-1-/-
E
H2O2: + +
Mice : +/+ -/-
pY419-Src (Active)
c-Src
Actin
mKim-1
75
50
75
50
35
75
Densiometric ratio of active/total SRC
††
Kim-1+/+
Kim-1-/-

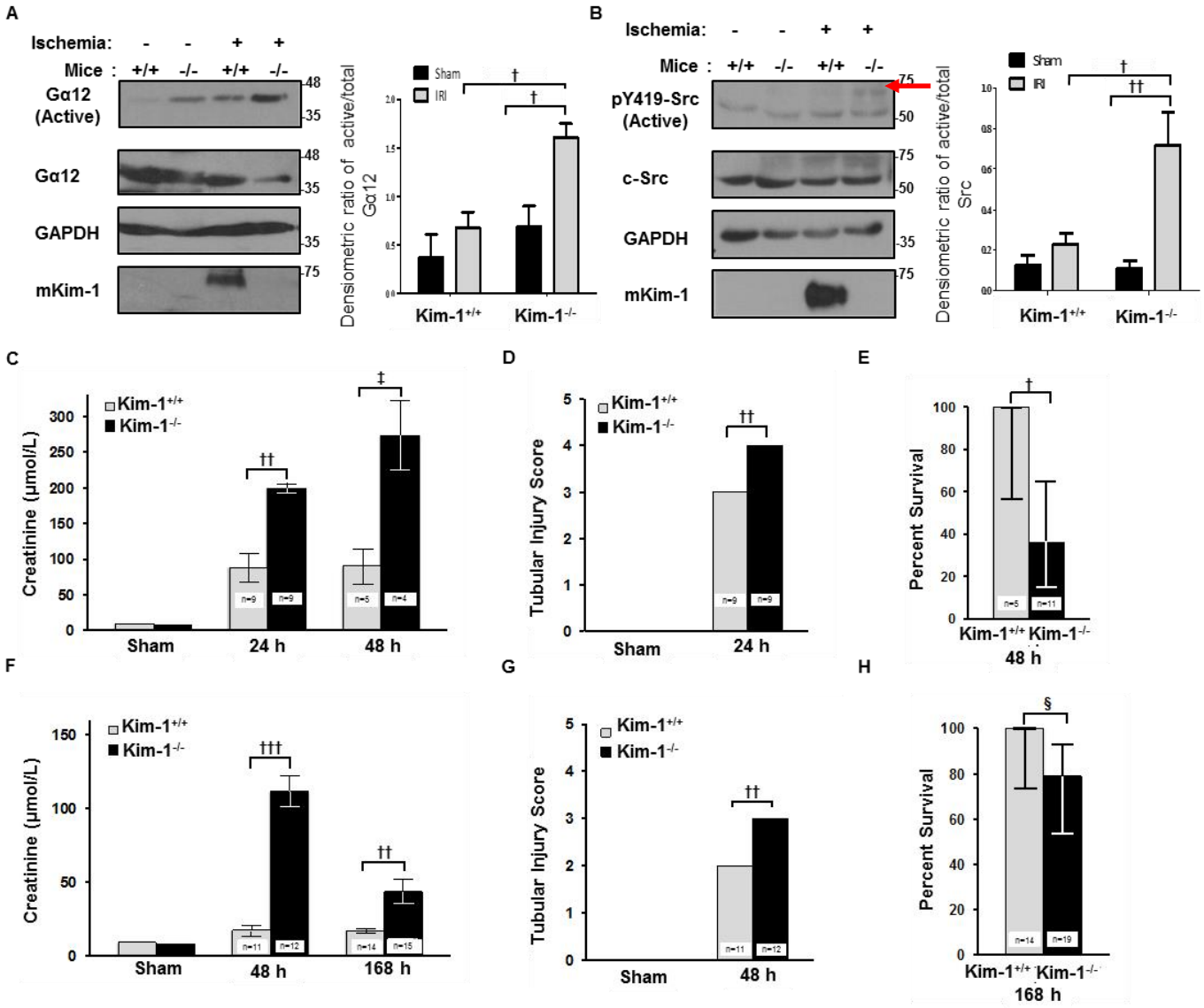
Figure 2.4. KIM-1 inhibits Gα12 activation and its down-stream effector Src.

A: HEK-293 cells were transfected with either control vector (pcDNA) or KIM-1 plasmid and were stimulated with the non-hydrolysable GTP analog (GTPγS) for 15 minutes. ***B***: Confirmation of genotype using genomic DNA of wild type (+/+), heterozygotes (+/-) and homozygotes Kim-1 knock out (-/-) C57BL/6 mice by PCR. ***C***: Tubular epithelial cells (TECs) isolated from wild type (Kim-$1^{+/+}$) and Kim-1-deficient (Kim-$1^{-/-}$) mice were stimulated with GTPγS for 15 minutes. ***D***: TECs were stimulated with H_2O_2 (5 mM) for 30 minutes. Samples in *A, C,* and *D* were subjected to the GST-TPR pull-down assay to measure the level of Gα12 activation. Lysates (total) and pull-down (active) samples were analyzed by SDS-PAGE and Western blotting with antibodies against Gα12, human KIM-1 (KIM-1) or mouse Kim-1 (mKim-1) and actin where indicated. ***E***: Activated and total Src were detected in total cell lysates from TECs stimulated with H_2O_2 by Western blotting using an anti-p-Src (Py419-Src) phosphospecific antibody and anti-c-Src antibody, respectively. Densitometric analysis of the ratio of active to total Gα12 (or pY419-Src to c-Src) relative to non-KIM-1 expressing cells or wild type TECs (+/+). Figure shown is representative of three independent experiments (n=3, *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control vector (pcDNA), †$p<0.05$, ††$p<0.01$ and †††$p<0.001$ compared to Kim-$1^{-/-}$). Red arrow point to the mouse Kim-1 band.

2.4.3. Kim-1-deficient mice exhibit higher Gα12 and Src activation and worse tissue damage after ischemia-reperfusion injury

To extend these findings to a physiologically important model, we next determined whether upregulation of KIM-1 by TECs during renal IRI would block Gα12 activation in the kidneys by ROS. To test this, we subjected Kim-$1^{+/+}$ and Kim-$1^{-/-}$ mice to 35 minutes of unilateral renal artery clamping followed by 24 hours of reperfusion and measured Gα12 activation in the affected and contralateral kidney tissues (Yu *et al.*, 2012). As expected, we observed both an increase in Gα12 activation and an upregulation in Kim-1 expression (Ichimura *et al.*, 1998; Vaidya *et al.*, 2010) in the reperfused kidneys, but not the contralateral (non-ischemic) kidneys as determined by Western blot (Figure 2.5A). Predictably, the degree of Gα12 activation after IRI was significantly higher in the kidneys from Kim-$1^{-/-}$ mice compared to those from the Kim-$1^{+/+}$ mice. Though there was a small difference in active Gα12 levels in the contralateral kidneys between Kim-$1^{-/-}$ and Kim-$1^{+/+}$ mice, this was not significant ($p>0.05$). In a parallel set of experiments, we also observed increased Src activation in the total cell lysates from Kim-$1^{-/-}$ kidneys compared to Kim-$1^{+/+}$ kidneys subjected to ischemia-reperfusion, which is in keeping with previous work (Figure 2.5B) (Meyer *et al.*, 2003; Yu *et al.*, 2012).

In view of the pathogenic role of activated Gα12 in IRI (Yu *et al.*, 2012), and the above data demonstrating that KIM-1 inhibits Gα12 activation, we hypothesized that Kim-1-deficient mice would show more severe IRI compared to Kim-$1^{+/+}$ mice. As shown in Figure 2.5C, after 30 minutes of renal ischemia and 24 hours of reperfusion, renal function was significantly worse in Kim-$1^{-/-}$ compared to Kim-$1^{+/+}$ mice (mean creatinine 198 vs. 87 μmol/L, respectively, $p<0.01$, n=9 per group). Kim-$1^{-/-}$ mice also exhibited worse histological renal damage compared to Kim-$1^{+/+}$ mice (Figure 2.5D). After 48 hours of reperfusion, 7/11 Kim-$1^{-/-}$ while 0/5 Kim-$1^{+/+}$ mice had died ($p<0.05$) (Figure 2.5E). When we reduced ischemia time to 25 minutes, Kim-$1^{+/+}$ mice maintained near-normal renal function (mean creatinine 17 μmol/L, n=11), while Kim-$1^{-/-}$ mice developed severe renal impairment (mean creatinine 111 μmol/L, n=12) ($p<0.001$) after 48 hours of reperfusion (Figure 2.5F). Tissue injury was also greater in Kim-$1^{-/-}$ than Kim-$1^{+/+}$ mice (Figure 2.5G). After 168 hours of reperfusion, there remained a significant difference in renal function between Kim-$1^{+/+}$ mice (mean creatinine 17 μmol/L) and Kim-$1^{-/-}$ mice (mean creatinine 43 μmol/L) ($p<0.01$), though 4/19 Kim-$1^{-/-}$ while 0/14 Kim-$1^{+/+}$ mice had died ($p=0.067$) (Figure 2.5H). Histology is provided in Figure 2.5I.


A
Ischemia: - - + +
Mice : +/+ -/- +/+ -/-
Gα12 (Active)
Gα12
GAPDH
mKim-1
48
35
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Densiometric ratio of active/total Gα12
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IRI
†
†
Kim-1+/+
Kim-1-/-
B
Ischemia: - - + +
Mice : +/+ -/- +/+ -/-
pY419-Src (Active)
c-Src
GAPDH
mKim-1
75
50
75
50
35
75
Densiometric ratio of active/total Src
Sham
IRI
†
††
Kim-1+/+
Kim-1-/-
C
Creatinine (μmol/L)
Kim-1+/+
Kim-1-/-
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††
Sham
24 h
48 h
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Tubular Injury Score
Kim-1+/+
Kim-1-/-
††
Sham
24 h
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Percent Survival
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Kim-1+/+ Kim-1-/-
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Creatinine (μmol/L)
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Sham (Kim-1+/+)
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Kim-1+/+
Kim-1-/-

Figure 2.5. Kim-1-deficient mice exhibit increased renal Gα12 activation and tissue damage after IRI.

A: Wild type (Kim-$1^{+/+}$) and Kim-1-deficient (Kim-$1^{-/-}$) mice underwent unilateral renal pedicle clamping for 35 minutes, followed by 24 hours of reperfusion. Active Gα12 was measured in renal cortical lysates obtained from clamped and contralateral kidneys by GST-TPR pull-down assay, followed by SDS-PAGE and Western blotting for active and total Gα12, mouse Kim-1 (mKim-1) and GAPDH. ***B***: The level of active phosphorylated Src (pY419) compared to total Src was measured by Western blot in renal cortical lysates from mice treated as in (***A***). Samples were analyzed by SDS-PAGE and Western blot for indicated antibodies. Densitometric analysis of the ratio of active Gα12 to total Gα12 (or pY419-Src to c-Src) is shown as a representation of three independent experiments (n=3/group, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$). ***C-I***: Independent groups of Kim-$1^{+/+}$ and Kim-$1^{-/-}$ mice underwent sham surgery or bilateral renal pedicle clamping for 30 minutes (n=5-11/ group, C-E) or 25 minutes (n=7-12/ group, F-I), followed by 24, 48 or 168 hours (h) of reperfusion. ***C and F***: Kidney function was determined by plasma creatinine levels. ***D and G***: Quantification of tubular damage in whole kidneys after 48 hours of reperfusion (n=9/group). ***E and H:*** Percent survival after reperfusion. $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ and $^{\dagger\dagger\dagger}p<0.001$, $^{\S}p=0.078$, $^{\ddagger}p=NS$. ***I:*** Representative PAS stained kidney sections after 48 hours of reperfusion (Sham represents Kim-$1^{-/-}$) (200x, Bar=0.10mm). Arrows indicate a major histological difference (tubule dilation) between Kim-$1^{+/+}$ and Kim-$1^{-/-}$ following injury.

2.5. DISCUSSION

AKI is a serious medical condition for which the pathogenic mechanisms are not well understood and for which there is no known treatment. It has been recently discovered that Gα12 has a crucial pathogenic role in renal IRI (Meyer *et al.*, 2003; Yu *et al.*, 2012). Specifically, ROS stimulates Gα12 to activate tubular injury pathways, including disruption of tight junctions via Src activation. To our knowledge, regulation of this pathway has never been described. Here, we show that KIM-1 blocks GTP binding onto Gα12, and thus functions to reduce the level of active Gα12. In support of this finding, we found that Kim-$1^{-/-}$ mice exhibited exaggerated Gα12 and Src activation *in vivo* during renal IRI, compared to Kim-$1^{+/+}$ mice. In addition, we observed worse renal dysfunction and histology after bilateral pedicle clamping in Kim-$1^{-/-}$ as compared to Kim-$1^{+/+}$ mice. Taken together, our findings suggest that KIM-1 protects against Gα12-mediated tissue damage during ischemic AKI. Whether KIM-1 has additional, Gα12-independent effects on TECs that protect from IRI is an area of further study (Ichimura *et al.*, 2012).

Our results provide particular insight into the regulation of G-protein signalling. Gα12, like all G proteins, is a molecular switch that is activated by GTP binding (upon GDP dissociation) and inactivated when bound-GTP is hydrolyzed to GDP (Dhanasekaran *et al.*, 1996). We found that KIM-1 reduces binding of GTPγS to Gα12. To our knowledge, KIM-1 is the first guanine nucleotide dissociation inhibitor against Gα12 to be identified (Siderovski *et al.*, 2005). Interestingly, the cytosolic domain of KIM-1 does not contain the 19-amino-acid sequence GoLoco motif that is typical of guanine nucleotide dissociation inhibitors (Kimple *et al.*, 2002). The residues responsible for KIM-1 inhibition of Gα12 are unknown. It is conceivable that targeting Gα12 using an exogenous chemical inhibitor might ameliorate renal IRI (Yu *et al.*, 2012). However, such an inhibitor does not exist and may in fact be toxic given that Gα12 is expressed ubiquitously and also regulates many crucial cellular functions (Dhanasekaran *et al.*, 1996).

Our results suggest that KIM-1 represents a natural, endogenous mechanism to protect against tissue damage during renal IRI. Given that KIM-1 is expressed by TECs only during the injury and returns to undetectable baseline levels upon renal recovery, after day seven post-IRI (Ko *et al.*, 2010), Gα12 inhibition by KIM-1 is likely transient. Interestingly, the upregulation of KIM-1 seems to overlap with Gα12 activation by ROS during ischemia-reperfusion. The down regulation of KIM-1 after AKI is equally important as conditional overexpression of KIM-1 in TECs in the absence of an injury stimulus results in kidney fibrosis (Humphreys *et al.*, 2013). It

has been proposed that antagonizing KIM-1 signalling may represent a novel therapeutic target to ameliorate renal fibrosis in chronic kidney disease (CKD). However, our identification of KIM-1 in regulating Gα12 and in protection against renal dysfunction during IRI suggest that caution is warranted in targeting KIM-1 in CKD, as such a therapeutic agent may exacerbate AKI episodes which have been linked to progression of CKD (Chawla *et al.*, 2014). It would also be interesting to know if transiently overexpressing KIM-1 during IRI would further protect against tissue damage. We speculate that strategies to enhance KIM-1 expression or function may be particularly important in patients with polymorphic variants of KIM-1 that might confer reduced ability to inhibit Gα12.

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Chapter 3

G protein α 12 (Gα12) is a negative regulator of kidney injury molecule-1-mediated phagocytosis

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3.1. SUMMARY

Kidney injury molecule-1 (KIM-1) is a receptor for the "eat me" signal phosphatidylserine, which is present on apoptotic cells. The specific upregulation of KIM-1 by injured tubular epithelial cells (TECs) enables them to clear apoptotic cells thereby protecting from AKI. Recently, we discovered that KIM-1 binds directly to the alpha subunit of heterotrimeric G12 protein (Gα12) and inhibits its activation by reactive oxygen species during renal ischemia-reperfusion injury (Chapter 2). Here we investigated if Gα12 has a role in KIM-1-mediated phagocytosis. We showed that KIM-1 remains bound to Gα12 and suppresses Gα12 activity during phagocytosis. When we silenced Gα12 expression by siRNA, KIM-1-mediated engulfment of apoptotic cells was increased significantly; in contrast overexpression of constitutively active Gα12 (*QL* Gα12) resulted in the opposite effect. Inhibition of RhoA, a key effector of Gα12, using a chemical inhibitor or expression of dominant-negative RhoA had the same effect as inhibition of Gα12 on phagocytosis. Consistent with this, silencing Gα12 suppressed active RhoA in KIM-1-expressing cells. Finally, using primary TECs from Kim-$1^{+/+}$ and Kim-$1^{-/-}$ mice, we confirmed that phagocytosis depends on KIM-1 expression, and that this process is enhanced by silencing Gα12. Our data reveal a previously unknown role of Gα12 in regulating phagocytosis in renal TECs. These results may have many implications given the known harmful role of Gα12 in acute kidney injury.

3.2. INTRODUCTION

The process of apoptosis or programmed cell death serves to rid the body of damaged and senescent cells that arise during both physiologic and pathologic conditions, such as acute tissue injury (Elliott *et al.*, 2010). The final common step of apoptosis is the removal of the cell corpses by phagocytes without triggering inflammation (Manfredi *et al.*, 2002). Renal proximal tubular epithelial cells (TECs) are particularly susceptible to apoptosis and necrosis during acute kidney injury (AKI) caused by ischemia and toxins (Sanz *et al.*, 2008; Shiratsuchi *et al.*, 1997). During AKI (Yang *et al.*, 2015; Ichimura *et al.*, 1998), proximal tubular epithelial cells (TECs) become transformed into semi-professional phagocytes and rapidly clear apoptotic cells through a process known as phagocytosis or efferocytosis (deCathelineau *et al.*, 2003). This process is crucial to regulate inflammation, as uncleared apoptotic cells obstruct the tubular lumen or undergo secondary necrosis and trigger inflammation which can impede repair (deCathelineau *et al.*, 2003;

Erwig *et al.*, 2006; Fadok *et al.*, 1998; Ren *et al.*, 1998; Rosen *et al.*, 1999; Yang *et al.*, 2015).

Phagocytes recognize apoptotic cells via cell-surface engulfment receptors that directly or indirectly bind to "eat me" signals displayed on the surface of apoptotic cells (Erwig *et al.*, 2008; Lauber *et al.*, 2004). Ligand-binding leads to receptor activation, which then brings about a series of complex downstream signalling pathways that ultimately result in reorganization of the cell cytoskeleton enabling engulfment, internalization and degradation of the bound corpses (Castellano *et al.*, 2001; Hochreiter-Hufford *et al.*, 2013). The Rho family of small GTPases such as RhoA and Rac1 have been implicated in the regulation of cytoskeletal remodelling in professional and semi-professional phagocytes (Lauber *et al.*, 2004; Machacek *et al.*, 2009; Nakaya *et al.*, 2006; Ren *et al.*, 1998). Like all G proteins, RhoA and Rac1 cycle between GDP-bound inactive, and GTP-bound active conformations regulated by GTP exchange factors (GEFs) or GTPase activating proteins (GAPs), respectively (Geyer *et al.*, 1997; Ridley, 1999, 2001). Although both RhoA and Rac1 belong to same family, they differ in their action: Rac1 induces polymerization of actin leading to membrane ruffles and filopodia formation, while RhoA induces actin assembly into bundles and stress fibers (Hall *et al.*, 2000). While the activation of Rac1 seems to be an evolutionarily conserved event in phagocytosis (including apoptotic cell engulfment) (Kinchen *et al.*, 2005; Kinchen *et al.*, 2007; Ravichandran *et al.*, 2007), there is some controversy regarding the inhibitory role for RhoA in phagocytosis (Erwig *et al.*, 2006; Olazabal *et al.*, 2002; Ravichandran *et al.*, 2007; Zhou *et al.*, 2001).

Several groups have previously identified kidney injury molecule-1 (KIM-1) as a phagocytosis receptor that is upregulated on the apical surface of proximal tubular proximal cells (TECs) following injury (Han *et al.*, 2002; Ichimura *et al.*, 1998). TIM-1 is a receptor for phosphatidylserine, one of the most well characterized apoptotic cell "eat-me" signals (Fadok *et al.*, 1998; Ravichandran *et al.*, 2007; Wang *et al.*, 2003). Both mouse (termed Kim-1) and human KIM-1, also known as T cell, immunoglobulin and mucin domain-1 protein (TIM-1), are small type I transmembrane glycoproteins that belongs to the TIM gene family (Freeman *et al.*, 2010). The human TIM family consists of TIM-1 (Feigelstock *et al.*, 1998; Ichimura *et al.*, 1998; McIntire *et al.*, 2001; Santiago *et al.*, 2007), TIM-3 (DeKruyff *et al.*, 2010; Nakayama *et al.*, 2009), and TIM-4 (Kobayashi *et al.*, 2007; Santiago *et al.*, 2007), which are conserved between mouse and man, and they all bind directly to phosphatidylserine (Freeman *et al.*, 2010; Ichimura *et al.*, 2008; Kobayashi *et al.*, 2007; Santiago *et al.*, 2007). KIM-1/TIM-1 is expressed on mast cells, regulatory

B cells, activated CD4 T cells, and TECs following AKI (Freeman *et al.*, 2010; Han *et al.*, 2002). KIM-1 contains an IgV-like domain, a mucin-like domain, a transmembrane region, and an intracellular tail that has been implicated in cell signalling in differentiated T cells (A. J. de Souza *et al.*, 2005; Anjali J. de Souza *et al.*, 2008; Ichimura *et al.*, 1998; Santiago *et al.*, 2007) and B cells (Yeung *et al.*, 2015). The crystal structure of the IgV-like domain of KIM-1/TIM-1 has been solved, and reveals that it forms a conserved binding pocket termed the metal ion-dependent ligand-binding site (MILIBS) (Cao *et al.*, 2007; DeKruyff *et al.*, 2010; Santiago *et al.*, 2007) that allows highly specific recognition of phosphatidylserine exposed on the surface of apoptotic cells (Freeman *et al.*, 2010; Santiago *et al.*, 2007). Cell-surface KIM-1 concentrates at the point of contact with an apoptotic cell and forms the phagocytic cup (Freeman *et al.*, 2010). We previously showed that Kim-1-deficient mice succumb to worse tissue injury and mortality after ischemic AKI (Ismail *et al.*, 2015). Moreover, TECs from mice expressing a mucin-domain deletion mutant of Kim-1 are unable to bind apoptotic cells, exhibit impaired phagocytic function by TECs and also succumb to worse AKI (Yang *et al.*, 2015). These data underline the biological importance of Kim-1-mediated clearance of apoptotic cells by TECs in limiting tissue damage during AKI.

Recently, we uncovered that KIM-1 interacts with the alpha subunit of heterotrimeric G12 proteins (Gα12) and inhibited its activation by ROS during ischemic AKI (renal ischemia-reperfusion injury), which is crucial to limiting downstream injury pathways triggered by Gα12 activation in TECs (Ismail *et al.*, 2015). Gα12 belongs to the G12 class of G proteins (Dhanasekaran *et al.*, 1996; Vara Prasad *et al.*, 1994). It is ubiquitously expressed and has pleiotropic effects on cells, including inducing proliferation, focal adhesion assembly/disassembly and cytoskeletal reorganization (Kurose, 2003; Needham *et al.*, 1998). Gα12-driven cytoskeletal reorganization, including actin stress fiber formation, is mediated through activation of the RhoA GTPase via Rho guanine nucleotide exchange factors (GEFs) (Tanabe *et al.*, 2004), including LARG (Fukuhara *et al.*, 2000), PDZ-RhoGEF (Kuner *et al.*, 2002), and p115 RhoGEF (Iguchi *et al.*, 2008).

Given that Gα12 activation and KIM-1 upregulation occur simultaneously during ischemic AKI, we investigated the role of Gα12 in KIM-1-mediated phagocytosis using cell culture models of TECs and primary TECs isolated from mice kidneys. We reveal that activated Gα12 inhibits phagocytosis and that KIM-1-suppression of Gα12 activation is crucial for TECs to mediate phagocytosis of apoptotic cells. In addition, we show that the inhibitory effect of Gα12 on

phagocytosis is mediated by its downstream effector, RhoA. Finally, our data demonstrating that TECs absolutely require KIM-1 expression for phagocytosis implies that altering Gα12 activity might be a viable therapeutic strategy to enhance clearance of apoptotic cells following AKI.

3.3. EXPERIMENTAL PROCEDURES

3.3.1. Cell culture and materials

Human Embryonic Kidney 293 (HEK-293) cells and porcine proximal tubule epithelial cells (LLC-PK1) were cultured at 37 °C in 5 % (vol/vol) CO_2 and maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10 % FBS (Invitrogen, Carlsbad, CA). HEK-293 cells stably expressing KIM-1-GFP (herein referred to as HEK-293-KIM-1-GFP), KIM-1 (no tag) (HEK-293-KIM-1), pcDNA-GFP (HEK-293-pcDNA), as well as, LLC-PK1 stably expressing KIM-1 (LLC-PK1-KIM-1) and pcDNA (LLC-PK1-pcDNA) were generated by transfecting with plasmid constructs encoding either KIM-1 or control vector using Lipofectamine® 2000 (Life technologies, Thermo Fisher Scientific, Rockford, IL). Several single cell clones were selected using 800 µg/ml Geneticin (G418) Sulfate (Santa Cruz Biotechnology, Santa Cruz, CA). Cells expressing a high level of KIM-1 protein determined by Western blotting were used for further experiment and were maintained in DMEM containing 10 % FBS and 800 µg/ml G418. Primary proximal tubule epithelial cells (TECs) were isolated from 2-4 week old C57BL/6 Kim-$1^{+/+}$ and Kim-$1^{-/-}$ mice and cultured for the first 6 days in serum free DMEM/F-12 mixed media (1:1) supplemented with 5 % insulin-transferrin-selenium solution (Invitrogen, Carlsbad, CA), 5 % penicillin-streptomycin solution (Invitrogen, Carlsbad, CA), 0.5 µg/ml of mouse EGF (Peprotech, Rocky Hill, NJ), and 50 ng/ml hydrocortisone (Thermo Fisher Scientific, Rockford, IL). After 6 days, TECs were cultured with same media mixtures supplemented with 5 % FBS as described earlier (Sharpe *et al.*, 2012). Chemicals were purchased from Sigma (Sigma Aldrich, St. Louis, MO) unless stated. The pH-sensitive dye pHrodo™ Red succinimidyl ester (pHrodo™ Red, SE), Alexa Fluor® 555 Goat Anti-Mouse antibody and DAPI were purchased from Life Technologies (Molecular probes, Invitrogen, Carlsbad, CA), and Rhodamine phalloidin from Cytoskeleton (Cytoskeleton, Denver, CO). Gα12 (S-20, 1:500 dilution) and actin (I-19, 1:1000 dilution) antibodies were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA), PE-conjugated anti-human KIM-1 (1D12, 1:100 dilution) and PE-conjugated rat IgG2b isotype control (RTK 4530, 1:100 dilution) were purchased from Biolegend. Allophycocyanine (APC)-conjugated

anti-mouse Tim-1 rat IgG2B (AF1817A, 1:100 dilution) and APC-conjugated rat IgG2B isotype control (1:100 dilution) were obtained from R&D system Inc. (R&D Systems Inc., Minneapolis, MN). RhoA specific monoclonal antibodies (ARH03, 1:500 dilution) and non-hydrolysable GTP analog were purchased from Cytoskeleton (Cytoskeleton, Denver, CO). A custom anti-KIM-1 (PA 4145, cytosolic domain) antibody was generated by immunizing rabbits with a peptide (CKEVQAEDNIYIENSLYATD) contained within the cytosolic domain of KIM-1 and conjugated to KLH as previously described (Life technologies, Thermo Fisher Scientific, Rockford, IL) (Bailly *et al.*, 2002; Gandhi *et al.*, 2014). A KIM-1 antibody against the mucin domain of KIM-1 (AKG7) was kindly provided by Dr. Bonventre (Harvard Medical School, Brigham and Women's Hospital, Boston, MA). Complete Mini EDTA-free protease inhibitor cocktail tablets were purchased from Roche (Roche Diagnostic, Basel, Switzerland). Rho inhibitor (cell permeable C3 transferase) was purchased from Cytoskeleton, Rho kinase (ROCK) inhibitor (Y27632) and Rac1 Inhibitor (NSC23766) were purchased from EMD Millipore (EMD Millipore, Billerica, MA). Plasmid constructs for Gα12, RhoA and Rac1 were kindly provided by Dr. Bradley Denker (Harvard Medical School, Brigham and Women's Hospital, Boston, MA), while the plasmid constructs for KIM-1 were provided by Dr. Bonventre (Harvard Medical School, Brigham and Women's Hospital, Boston, MA). Lipofectamine® 2000 (Life Technologies, Thermo Fisher Scientific, Rockford, IL) and Dharmafect1 (Thermo Fisher Scientific, Rockford, IL) were used for transfecting plasmid and siRNA, respectively.

3.3.2. Mice

Wild-type C57BL/6 (Kim-$1^{+/+}$) mice were obtained from the Jackson Laboratory. C57BL/6 Kim-1-deficient mice (Kim-$1^{-/-}$) were obtained from Dr. Andrew N. J. McKenzie (MRC laboratory of Molecular biology, Cambridge, UK) and these were generated by targeted disruption of the mouse *havcr1* gene in mouse embryonic stem cells as described previously (Wong *et al.*, 2010). All animal procedures were approved by the Western University Animal Use Subcommittee in accordance with the regulations of the Canadian Council on Animal Care (refer to appendix for certificate of approval).

3.3.3. Phagocytosis assay and FACS analysis

To prepare apoptotic cells for the phagocytosis assays, thymocytes were harvested from 3 to 6 week old C57BL/6 mice, and apoptosis was induced by UV exposure for five minutes followed by incubation overnight at 37 °C in 5 % CO_2 in DMEM media supplemented by 10 %

FBS and 1 % penicillin-streptomycin solution as described previously (Gandhi *et al.*, 2014). Apoptosis of the thymocytes were verified by flow cytometry analysis showing positive staining for Annexin V and negative staining for propidium iodide. Apoptotic thymocytes were stained with pH-sensitive dye pHrodo™ Red succinimidyl ester (pHrodo™ Red, SE) at a final concentration of 150 nM for 30 minutes at room temperature. Labelled apoptotic cells were washed twice with 1x PBS to remove excess dye. The pHrodo dye helped in distinguishing between bound and internalized apoptotic cells, where apoptotic cells exhibit higher fluorescence upon entry into the acidic compartment of phagolysosomes (Gandhi *et al.*, 2014; Miksa *et al.*, 2009; Park *et al.*, 2009). The fluorescently-labelled apoptotic cells were counted and $3x10^6$ were added to each well of 6-well plate ($1.5x10^7$ for 10 cm plates) containing phagocytes cells (HEK-293 cells, LLC-PK cells or isolated TECs) and incubated for various time points at 37 °C in 5 % CO_2. Cells were then placed on ice for 30 minutes to reduce non-specific binding of apoptotic cells. The plates were washed three times with ice-cold PBS, and cells were collected with 5 mM EDTA-PBS, and resuspended with FACS buffer (1x Phosphate-buffered saline (PBS), 2 % calf serum, and 0.1 % sodium azide) for flow analysis. For KIM-1-surface staining, the cells were blocked in PBS and 10 % goat serum for 15 minutes, followed by incubation for 30 minutes at room temperature with either PE-conjugated anti-KIM-1 (1D12, human), or APC-conjugated anti-Tim-1 (AF1817A, mouse) targeting extracellular domain of KIM-1 or Kim-1 and their respective isotype control antibodies at a ratio of 1:100. All samples were run on a BD LSR II flow cytometer using various filters, such as, YG 582/15 (pHrodo red, PI) and B 530/30 (GFP, FITC) (BD Biosciences, San Jose, CA). The percentage of phagocytosis represents the number of cells that have internalized the pHrodo red labelled apoptotic cell(s), as indicated by the higher fluorescence of pHrodo red dye (10^4-10^5 on logarithmic scale). Cells with low pHrodo red fluorescence ($<10^4$) were excluded from the percentage of phagocytosis calculation since they are not internalized.

3.3.4. Immunoprecipitation and Western blot

HEK-293 cells were lysed with ice-cold lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 1 % Triton X-100, and Complete Mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostic, Basel, Switzerland). For immunoprecipitation, cell extracts containing 1.0 –1.5 mg of the protein/mL were incubated with 10 ug of Gα12 (S-20), KIM-1 (PA4145), or rabbit IgG antibody (sc-2027) and 20 µl of protein-A/G Sepharose beads (Santa Cruz

Biotechnology, Santa Cruz, CA) at 4 °C overnight. Beads were centrifuged, washed three times, and suspended in 20 µL of SDS sample buffer and heated at 100 °C for five minutes. Lysates (representing 5 % of total lysate) and immunoprecipitation samples were separated by electrophoresis under reducing condition and transferred to polyvinylidene difluroide (PDVF) membranes (EMD Millipore, Billerica, MA). PDVF-membranes were probed with antibodies specific to surface KIM-1 (AKG7, 1:3 dilution), the cytosolic domain of KIM-1 (PA 4145, 1:1,500 dilution), Gα12 (1:500 dilution), actin (1:1,000 dilution), or RhoA (1:500 dilution). The signal was visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies and ECL western blot detection reagent (Luminata forte, EMD Millipore, Billerica, MA) and visualized by autoradiography (Biomax; Denville Scientific, South Plainfield, NJ).

3.3.5. GST-TPR pull down

The construct encoding the GST-fused TPR domain of PP5 was kindly provided by Dr. N Dhanasekaran (Temple University, PA, USA). GST-TPR protein was purified from *Escherichia coli* as described earlier (Yoshiaki Yamaguchi *et al.*, 2002). Cells were lysed with ice-cold lysis buffer (20 mM HEPES pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5 % Triton x-100, and complete Mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostic, Basel, Switzerland). 1 mg of protein lysate and ≈1 µg of GST-TPR coupled glutathione-agarose beads were incubated together at 4 °C for five hours. Both lysate and pull-down samples were analyzed by SDS-PAGE and Western blotting to detect total and active Gα12 in these samples, respectively. Lysates loaded with non-hydrolyzable GTP analog (GTPγS) or GDP (Cytoskeleton, Denver, CO) were considered as a positive or negative controls, respectively.

3.3.6. Immunofluorescence and confocal microscopy

HEK-293 cells were cultured at subconfluent density on poly-D-lysine hydrobromide (Sigma Aldrich, St. Louis, MO) coated glass cover slips, and were transfected with either different wild type or mutant versions of GFP-tagged Gα12 or siRNA against Gα12. Cells were fixed with 4 % paraformaldehyde, followed by counterstaining of the nucleus with DAPI (0.5 µg/ml). Cell-surface staining for KIM-1 was performed using the monoclonal antibody against the mucin domain of KIM-1 (AKG). For this, cells were blocked with 1 % BSA in 1x PBS for 1 hour at room temperature, followed by incubation with anti-KIM-1 overnight. Bound KIM-1 was labelled with Alexa 555 conjugated anti-mouse at concentration of 1:1000. For actin cytoskeleton staining, cells were permeabilized with 0.25 % Triton in 1x PBS for five minutes, and then stained with

rhodamine phalloidin for 0.5 hours according to the manufacturer's instructions (Cytoskeleton, Denver, CO). Coverslips were mounted using Shandon-Mount® permanent mounting (Thermo Fisher Scientific, Rockford, IL) and viewed with a FLUOVIEW X83I confocal microscopy (Olympus, Tokyo, Japan). Data were acquired and analyzed using FLUOVIEW FV10 ASW 4.0 viewer and ImageJ software (National Institutes of Health, Bethesda, MD) to determine the Van Steensel's score for colocalization of KIM-1 and Gα12. Colocalization was quantified based on five random fields per sample and three independent experiments. Quantification of the number of apoptotic cells bound was assessed in eight random fields per sample and was done in four independent experiments. Approximately 100 to 120 cells were counted for each field as determined by DAPI staining of nucleus.

3.3.7. siRNA of Gα12 in HEK-293 and proximal tubular epithelial cells

ON Target plus® Smart pool siRNA against Gα12-specific and non-targeting pool (Control siRNA) were purchased from Dharmacon (Thermo Fisher Scientific, Rockford, IL). Cells were transfected with 50 nM of siRNA using Dharmafect1® for 24 hours before further analysis of knockdown.

3.3.8. RhoA activation

RhoA activation was determined using Rhotekin-RBD (Rho-binding domain) beads according to the manufacturer's instructions (Cytoskeleton, Denver, CO) and as described before (Yu *et al.*, 2012). Both lysates, and pull-down samples were analyzed by SDS-PAGE and Western blotting to determine the levels of total and active RhoA in these samples, respectively. Controls included incubating lysates with GDP (1 mM, negative control) or GTPγS (200 µM, positive control) for 15 minutes and then adding the beads. The ratio of active-to-total RhoA was determined based on densitometric analysis of Western blot images. For RhoA activation measurement after siRNA-mediated knockdown of Gα12, we used the G-LISA RhoA activity assay kit (Cytoskeleton, Denver, CO), due to its high sensitivity and the low level of the total protein required to perform the assay.

3.3.9. Quantification and statistics

Western blots were scanned and band intensity quantified using Image J software (National Institutes of Health, Bethesda, MD). Background was subtracted in these analyses, normalized to actin and the linear range of response was determining using a standard sample that was serially diluted. Statistics were done in GraphPad Prism (Graph Pad Software Inc., La Jolla, CA) or IBM

SPSS statistic 22 (IBM, Armonk, NY). The significance for the percentage of phagocytosis or immunoprecipitation densitometric analysis over phagocytosis time course was determined using one-way ANOVA (as indicated). The significance of the results comparing active and total Gα12 or RhoA for cells stimulated with apoptotic cells over a period of time (15, 30, 60 and 90 minutes) was determined using the analysis of covariance (ANCOVA). All other analyses were done using an unpaired t-test where indicated. P-value of <0.05 was considered significant. Error bars represent the standard error of the mean unless otherwise indicated.

3.4. RESULTS

3.4.1. KIM-1 interacts with Gα12 during phagocytosis

KIM-1 is not expressed by healthy TECs, but is upregulated more than any other protein after AKI (Ajay *et al.*, 2014;Ichimura *et al.*, 1998; Ko *et al.*, 2010). Because it is virtually impossible to perform mechanistic studies on TEC-phagocytosis *in vivo*, we did this *in vitro* using human embryonic kidney-293 (HEK-293) cells overexpressing KIM-1. Exogenous expression of KIM-1 in cultured cells is routinely used to mimic KIM-1 that is upregulated on TECs during AKI *in vivo* and HEK-293 cells are widely used to study the phagocytic signalling and phagocytic function of KIM-1 (Gandhi *et al.*, 2014; Ichimura *et al.*, 2008; kobayashi et al., 2007). In addition, HEK-293 cells were ideal for our studies since they express adequate level of Gα12 for detection by Western blotting (Figure 3.1A). We failed to detect endogenous Gα12 by Western blotting in cells that endogenously express KIM-1, such as the human renal adenocarcinoma cell line (769P), which is also routinely used to study KIM-1-mediated phagocytosis (Gandhi *et al.*, 2014; Ichimura *et al.*, 2008; Kobayashi *et al.*, 2007). Next, we established assay conditions for our study by creating HEK-293 cells that stably express C-terminal GFP-tagged human KIM-1 and determining their efficiency and kinetics of apoptotic engulfment (Ichimura *et al.*, 2008; Ichimura *et al.*, 1998; Ismail *et al.*, 2015). To distinguish bound from internalized apoptotic cells when using flow cytometry to detect phagocytosis, targets were labelled with a pH-dependent fluorescent dye (pHrodo) that exhibits higher fluorescence upon entry of apoptotic cells into the acidic compartment of phagolysosomes (Gandhi *et al.*, 2014; Miksa *et al.*, 2009; Park *et al.*, 2009). Stable transfection with GFP-tagged human KIM-1 enabled efficient engulfment of apoptotic cells (40 % vs. 8 %, $p<0.05$) relative to cells transfected with GFP-tagged control vector (pcDNA-GFP) (Figure 3.1B). Phagocytosis was further confirmed using confocal microscopy, where we were able to visualize the formation of phagocytic cups after co-culture of HEK-293-KIM-1-GFP cells

with apoptotic cells over the course of phagocytosis (Figure 3.1C). Phagocytic cups were formed at the cell-surface within 15 minutes of the addition of apoptotic cells. Pretreatment of the phagocytic cells with the actin polymerization inhibitor cytochalasin D significantly reduced the uptake of apoptotic cells by KIM-1-expressing HEK-293 cells (Figure 3.1D).

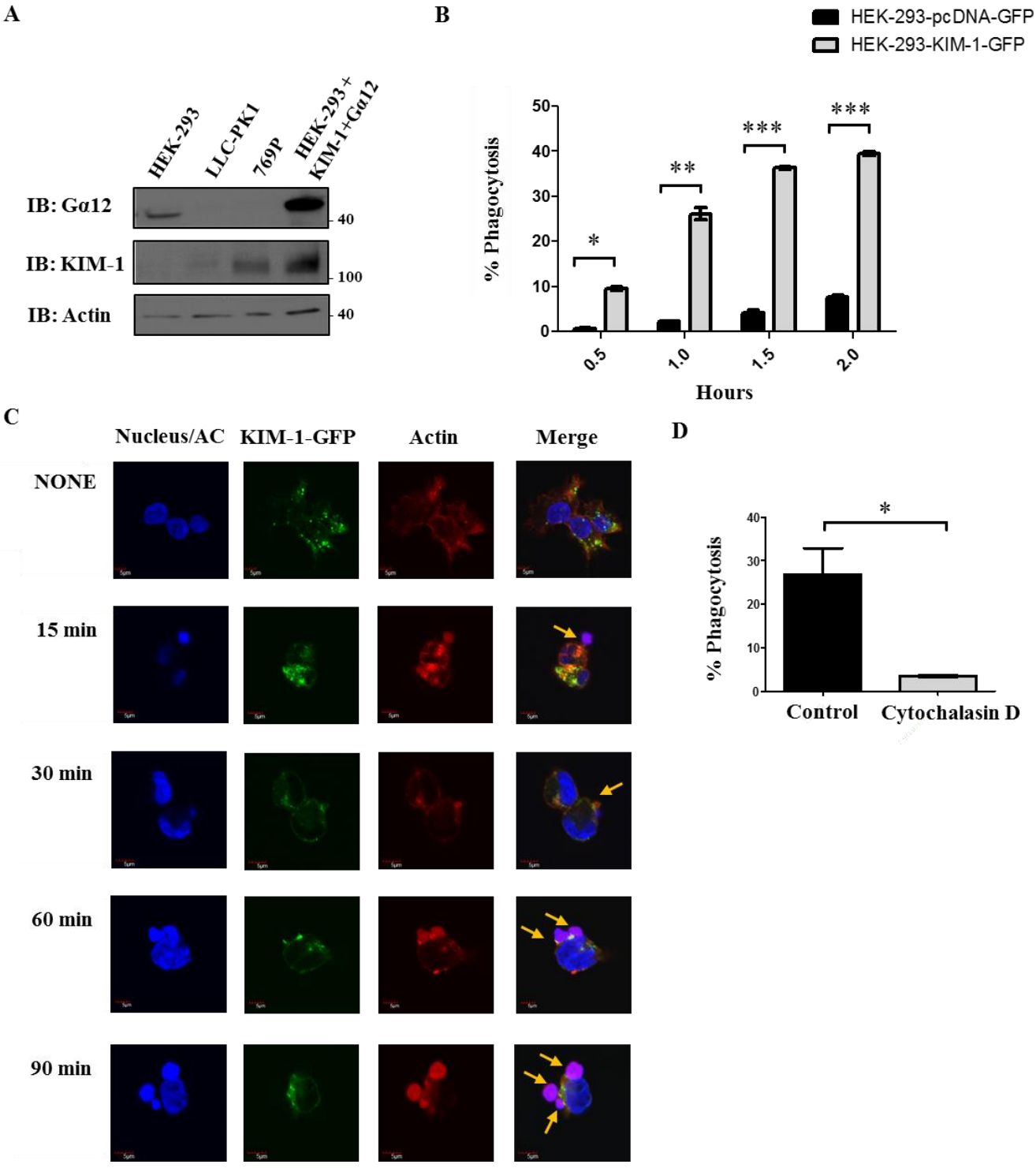

A
HEK-293
LLC-PK1
769P
HEK-293+ KIM-1+Gα12
IB: Gα12
IB: KIM-1
IB: Actin
40
100
40
B
HEK-293-pcDNA-GFP
HEK-293-KIM-1-GFP
% Phagocytosis
50
40
30
20
10
0
*
**
***
***
0.5
1.0
1.5
2.0
Hours
C
Nucleus/AC
KIM-1-GFP
Actin
Merge
NONE
15 min
30 min
60 min
90 min
D
% Phagocytosis
40
30
20
10
0
*
Control
Cytochalasin D

Figure 3.1. KIM-1-mediates the uptake of apoptotic cells.

A: Various cell lines were screened for KIM-1 and Gα12 expression via Western blot. These cells include; human embryonic kidney-293 cells (HEK-293), porcine proximal tubular epithelial cells (LLC-PK1), human renal adenocarcinoma cells (769P), HEK-293 cells transfected with human KIM-1 and Gα12 served as a positive control. Lysates were obtained, separated by SDS-PAGE and immunoblotted (IB) for KIM-1, Gα12 and actin. ***B:*** HEK-293 cells stably expressing either control vector (pcDNA-GFP) or KIM-1-GFP were fed pHrodo-labelled (red) apoptotic cells for various time points as indicated (hours). The percentage of phagocytosis was determined by flow cytometry measurement of GFP and high pHrodo red double positive cells (n=3, *p<0.05, unpaired t-test). ***C:*** HEK-293 cells stably expressing KIM-1-GFP were fed pHrodo-labelled apoptotic cells (red) for various time points (minutes or min). Cells were fixed and stained with rhodamine-phalloidin to visualize F-actin (red) and DAPI to visualize the nucleus and apoptotic cells (blue) (600x, bar represents 5 μm). Apoptotic cells labelled with both pHrodo red and DAPI (blue) appear as purple/pink coloured cells in the merged image. Arrows indicate the apoptotic cell(s) in the vicinity of KIM-1 expressed on the cells. ***D:*** HEK-293 cells stably expressing KIM-1-GFP were treated with vehicle control (DMSO) or the actin polymerization inhibitor cytochalasin D (10 μM), and fed fluorescently labelled apoptotic cells for 90 minutes. The percentage of phagocytosis represents the percentage of KIM-1-GFP positive cells with high fluorescence of pHrodo red of engulfed apoptotic cells as measured by flow cytometry (n=3,*p<0.05, unpaired t-test).

Based on our previous work demonstrating that the cytosolic domain of KIM-1 interacts with Gα12 in TECs (Ismail *et al.*, 2015), we sought to investigate how this was affected by phagocytosis. We performed co-immunoprecipitation of Gα12 with KIM-1 during the course of phagocytosis and found that the interaction is not dependent on apoptotic cell stimulation of HEK-293-KIM-1-GFP cells (Figure 3.2A). Importantly, apoptotic cell-stimulation was also not required for the interaction of KIM-1 with GST-Gα12 in HEK-293 cells stably expressing KIM-1 (Figure 3.2B).

In order to visualize the interaction of Gα12 with KIM-1 in the phagocytic cells, we co-transfected HEK-293 cells with KIM-1 and GFP-tagged Gα12 and examined the kinetics of Gα12-KIM-1 co-localization before and after adding apoptotic cells (Figure 3.2C). In keeping with our previous work (Ismail *et al.*, 2015), we observed co-localization of KIM-1 with Gα12 prior to and after the addition of apoptotic cells. As determined by quantification of the change in co-localization following stimulation with apoptotic cells using the Van Steensel's approach (Bolte *et al.*, 2006), we failed to see any significant difference between unstimulated and stimulated cells (Figure 3.2D). The above results suggested that KIM-1 constitutively interacts with Gα12 during the course of corpse engulfment.

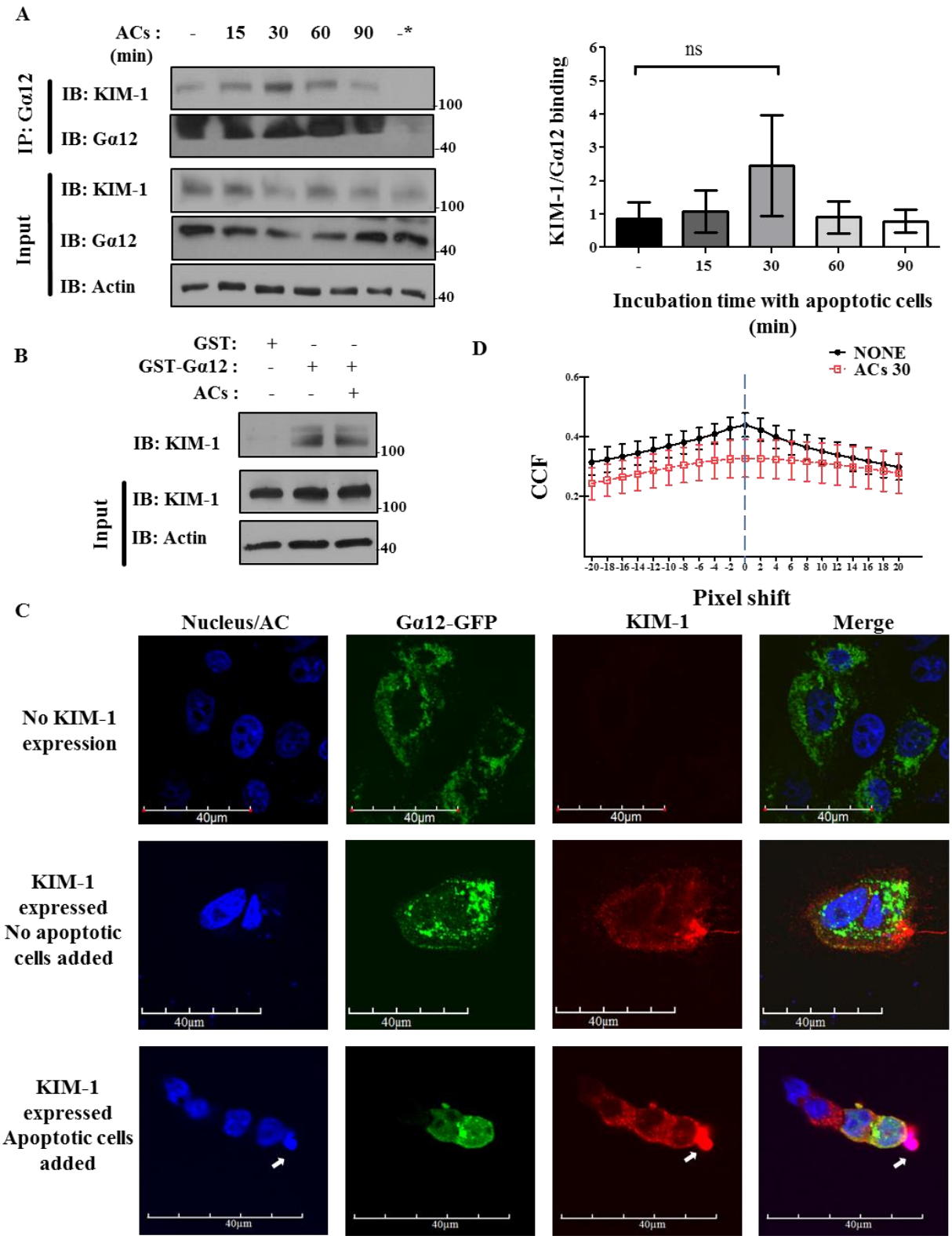

A
ACs : - 15 30 60 90 -*
(min)
IP: Gα12
IB: KIM-1
IB: Gα12
Input
IB: KIM-1
IB: Gα12
IB: Actin
100
40
100
40
40
B
GST: + - -
GST-Gα12 : - + +
ACs : - - +
IB: KIM-1
Input
IB: KIM-1
IB: Actin
100
100
40
KIM-1/Gα12 binding
ns
Incubation time with apoptotic cells (min)
D
NONE
ACs 30
CCF
Pixel shift
C
Nucleus/AC
Gα12-GFP
KIM-1
Merge
No KIM-1 expression
KIM-1 expressed No apoptotic cells added
KIM-1 expressed Apoptotic cells added
40μm

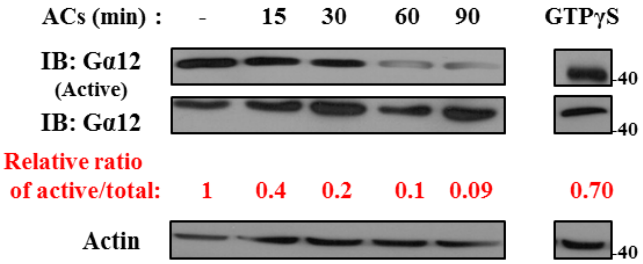
Figure 3.2. KIM-1 interacts with Gα12 during phagocytosis.

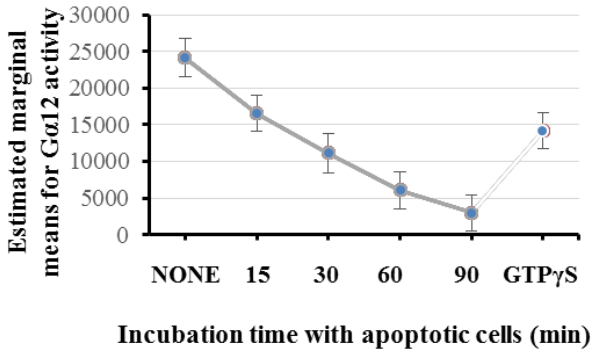
A: Human embryonic kidney-293 (HEK-293) cells stably expressing human KIM-1-GFP with high level of endogenous Gα12 were stimulated with apoptotic cells for indicated time and used for immunoprecipitation (IP) using antibody against Gα12. The ratio of bound KIM-1 to Gα12 was determined based on densitometric values obtained by Western blotting during apoptotic cell stimulation (n=3, ns= not significant, one-way ANOVA).***B:*** GST and GST-Gα12 pull-down was performed on extracts from HEK-293-KIM-1-GFP cells that were left unstimulated or stimulated with apoptotic cells for 90 minutes. All samples were analyzed by SDS-PAGE followed by immunoblotting (IB) with antibodies against KIM-1, Gα12 and actin. The input lane represents 5 % of the lysate. The data represents three independent experiments. ***C:*** HEK-293 cells were transfected with wild type KIM-1-and GFP-tagged Gα12 construct and fed pHrodo red fluorescently labelled apoptotic cells (AC) (blue/red) for 30 minutes prior to fixation. Surface KIM-1 staining was performed using anti-KIM-1 antibody against the mucin domain (AKG) and Alexa555-conjugated secondary antibody (600x, bar represent 40 μm). Arrows indicate where phagocytic cups formed. ***D:*** Images of colocalization of both KIM-1 and Gα12 were analyzed using the Van Steensel's approach, where the cross correlation function (CCFs) was calculated with a pixel shift of ±20. The results shown are representative of four images from three independent experiments (ns= not significant, one-way ANOVA).

3.4.2. KIM-1 down regulates Gα12 activity during KIM-1-mediated phagocytosis

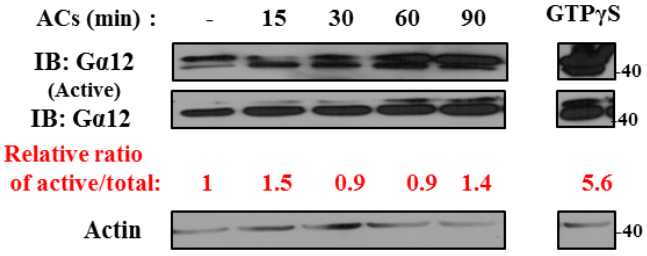
Phagocytosis involves a series of events that starts with the recognition of ligands on apoptotic cells by phagocytic receptors on the phagocytic cell (e.g. phosphatidylserine recognition by KIM-1), which then triggers phagocytic cup formation and subsequently a cascade of intracellular signalling events that include activation of various GTPases (Elliott *et al.*, 2010; Kinchen *et al.*, 2007; Ravichandran *et al.*, 2007). Since Gα12 is a GTPase, which cycles between active (GTP-bound) and inactive (GDP-bound) status, we sought to investigate if Gα12 was activated during engulfment of apoptotic targets. We measured the ratio of active-to-total Gα12 in cell lysates using a routinely used active-Gα12 pull-down assay that utilizes the tetratricopeptide repeat (TPR) domain of a downstream effector of Gα12, Ser/Thr phosphatase type 5 (PP5), fused to GST (Y. Yamaguchi *et al.*, 2002). Subsequently, we used Western blotting to detect both active and total Gα12. In KIM-1-expressing cells, we observed a steady decrease (from baseline) in the level of active Gα12 during phagocytosis (Figure 3.3A). The decrease in active Gα12 was more pronounced during later stages of phagocytosis (60-90 minutes after adding apoptotic cells). On the other hand, we observed no significant difference or trend in Gα12 activity in HEK-293 cells not expressing KIM-1 over the same period of observation (Figure 3.3B). We observed similar kinetics of Gα12 inactivation when porcine renal TECs (LLC-PK1) stably expressing a C-terminal HA-tagged KIM-1 were transfected with a Gα12 expression construct, but not in control vector (pcDNA) transfected cells (Figure 3.3C and D). Together, these data suggested that Gα12 activity is progressively suppressed in the HEK-293 cells in a KIM-1-dependent manner during phagocytosis.

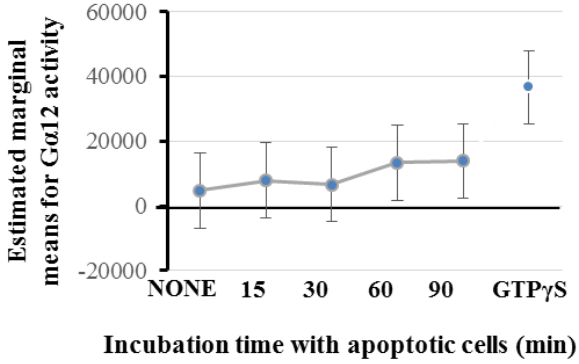
A


ACs (min) : - 15 30 60 90 GTPγS
IB: Gα12 (Active)
IB: Gα12
Relative ratio of active/total: 1 0.4 0.2 0.1 0.09 0.70
Actin
40
40
40

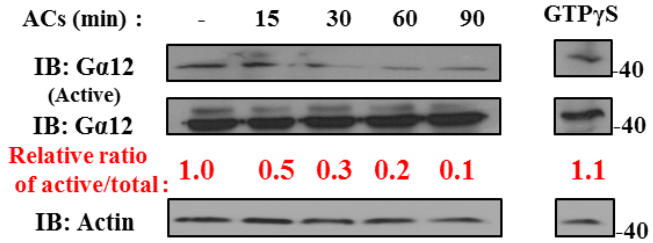

Estimated marginal means for Gα12 activity
30000
25000
20000
15000
10000
5000
0
NONE 15 30 60 90 GTPγS
Incubation time with apoptotic cells (min)

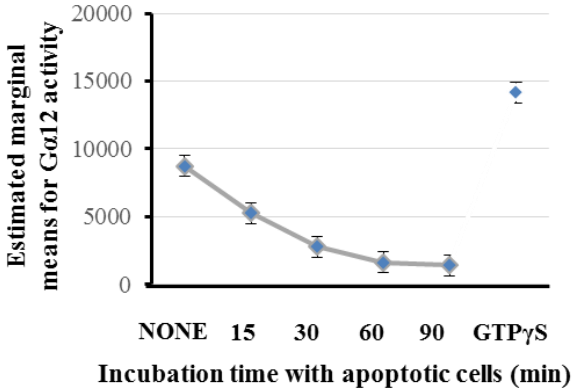
B


ACs (min) : - 15 30 60 90 GTPγS
IB: Gα12 (Active)
IB: Gα12
Relative ratio of active/total: 1 1.5 0.9 0.9 1.4 5.6
Actin
40
40
40

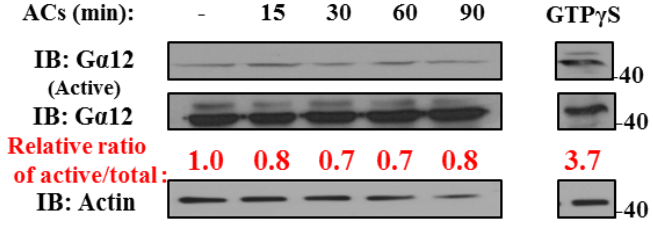

Estimated marginal means for Gα12 activity
60000
40000
20000
0
-20000
NONE 15 30 60 90 GTPγS
Incubation time with apoptotic cells (min)

C


ACs (min) : - 15 30 60 90 GTPγS
IB: Gα12 (Active)
IB: Gα12
Relative ratio of active/total: 1.0 0.5 0.3 0.2 0.1 1.1
IB: Actin
40
40
40


Estimated marginal means for Gα12 activity
20000
15000
10000
5000
0
NONE 15 30 60 90 GTPγS
Incubation time with apoptotic cells (min)

D


ACs (min): - 15 30 60 90 GTPγS
IB: Gα12 (Active)
IB: Gα12
Relative ratio of active/total: 1.0 0.8 0.7 0.7 0.8 3.7
IB: Actin
40
40
40

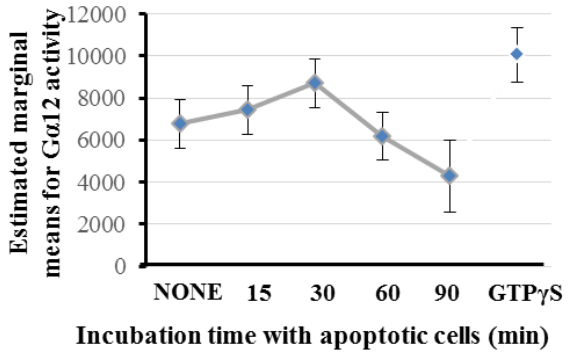

Estimated marginal means for Gα12 activity
12000
10000
8000
6000
4000
2000
0
NONE 15 30 60 90 GTPγS
Incubation time with apoptotic cells (min)

Figure 3.3. KIM-1 down regulates Gα12 activity during KIM-1-mediated phagocytosis.

HEK-293 cells stably expressing KIM-1-GFP ***(A)*** or pcDNA (control vector) ***(B)*** were fed apoptotic cells (ACs) for various time points (15, 30, 60, and 90 minutes). Similarly, porcine renal proximal TECs (LLC-PK1) stably expressing a C-terminal HA-tagged KIM-1 ***(C)*** or control vector (pcDNA) ***(D)*** were transfected with a wild-type Gα12 expression construct and fed apoptotic cells. Cell lysates were subjected to the GST-TPR pull-down assay to measure active Gα12 as described in the experimental procedure section. Samples were analyzed by SDS-PAGE followed by immunoblotting (IB) with antibodies against Gα12 and actin. The total Gα12 lanes represent 5 % of the lysate that used for GST-TPR pull-down. The numbers below blots represent the respective densitometric ratio of the active Gα12 band to total Gα12 band on the Western blot image normalized to the untreated sample. Graphs represent the estimated marginal means for active Gα12 as determined by the analysis of covariance (ANCOVA). In KIM-1-expressing cells (A and C), a statistically steady significant decrease in the level of active Gα12 during phagocytosis was observed (n=3, p=0.015 (A), p=0.001 (C)). Cells in B and D show no significant change (n=3, p=0.445 (B), p=0.278(D)).

3.4.3. Gα12 is a negative regulator of KIM-1-mediated phagocytosis

Though data presented thus far demonstrate that Gα12 activity is inhibited during phagocytosis, they do not necessarily imply a functional role for Gα12 in KIM-1-mediated phagocytosis. To study this, HEK-293-KIM-1-GFP cells were transfected with a control vector, vector encoding wild type Gα12 (*WT* Gα12), or vector encoding a constitutively active (GTPase-deficient) mutant of Gα12 (*QL* Gα12) (Meyer *et al.*, 2002), and phagocytosis efficiency was measured using flow cytometry. Overexpression of *QL* Gα12 significantly inhibited engulfment of apoptotic cells, whereas *WT* Gα12 had little effect on phagocytosis of apoptotic cells (Figure 3.4A). We obtained similar results when transfecting KIM-1-expressing LLC-PK1 cells with *QL* Gα12 (Figure 3.4B). To verify these findings, we performed confocal imaging of these cells and counted the number of apoptotic cells in the vicinity of the KIM-1 expressing HEK-293 cells for each condition. This analysis shows that there was a significant decrease in the number of apoptotic cells present inside cells transfected with the constitutively active mutant (Figure 3.4C).

In order to test how suppression of Gα12 expression affected phagocytosis, we silenced Gα12 using siRNA in HEK-293-KIM-1-GFP cells (Figure 3.4D) and performed phagocytosis assays. We found a significant increase in the uptake of apoptotic cells (Figure 3.4E) in conjunction with effective knockdown of Gα12 in these cells. Evaluation of phagocytosis by confocal microscopy once again confirmed these results (Figure 3.4F). The above data suggested that active Gα12 has a negative effect on KIM-1-mediated engulfment of apoptotic cells.

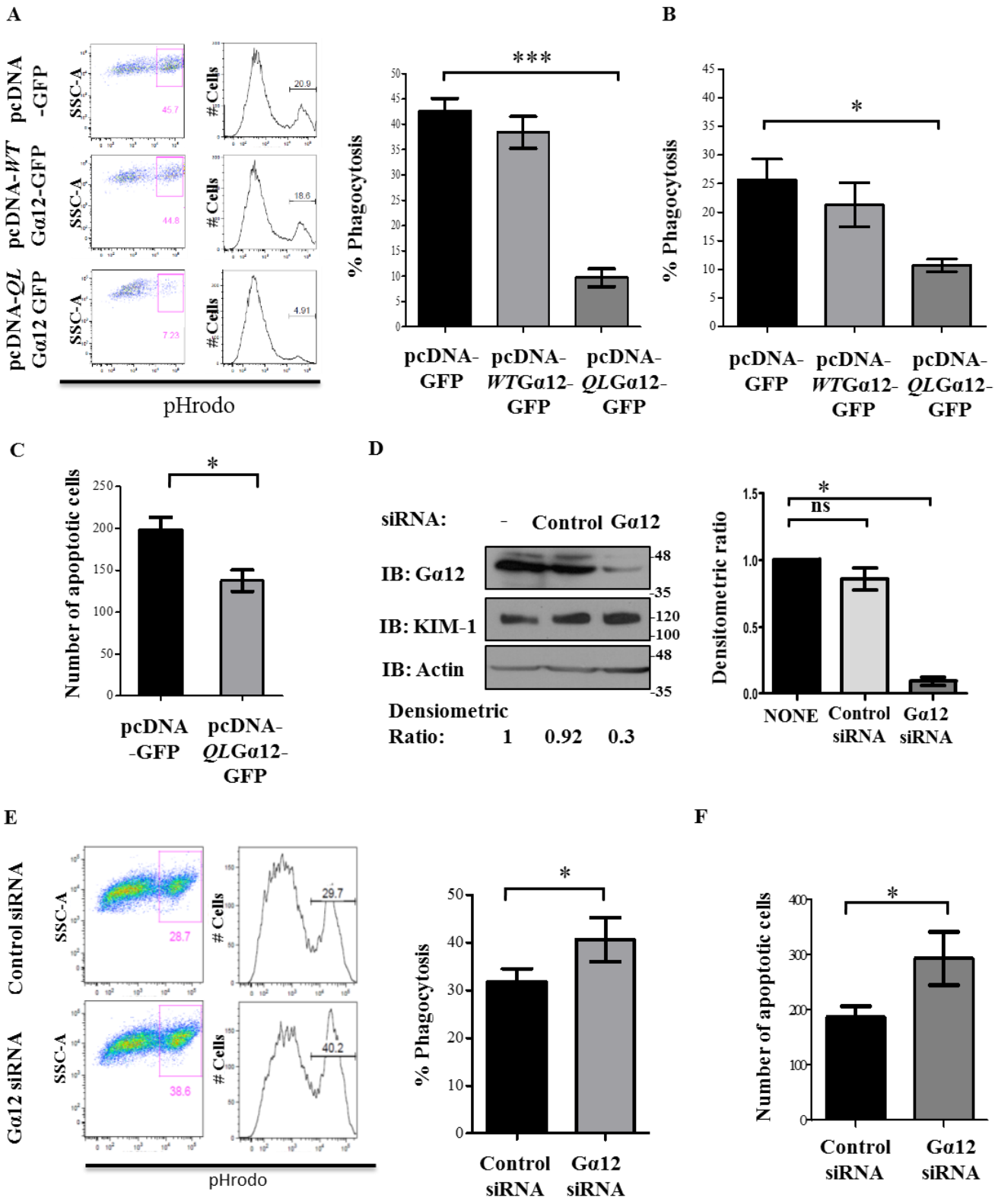

A
pcDNA -GFP
pcDNA-WT Gα12-GFP
pcDNA-QL Gα12 GFP
SSC-A
Cells
45.7
20.9
44.8
18.6
7.23
4.91
pHrodo
% Phagocytosis
***
pcDNA-GFP
pcDNA-WTGα12-GFP
pcDNA-QLGα12-GFP
B
*
C
Number of apoptotic cells
pcDNA -GFP
pcDNA-QLGα12-GFP
D
siRNA: - Control Gα12
IB: Gα12
IB: KIM-1
IB: Actin
48
35
120
100
Densiometric Ratio: 1 0.92 0.3
Densitometric ratio
ns
NONE
Control siRNA
Gα12 siRNA
E
Control siRNA
Gα12 siRNA
28.7
29.7
38.6
40.2
F

Figure 3.4. Gα12 negatively regulates KIM-1-mediated phagocytosis.

A: HEK-293 cells stably expressing wild type KIM-1 were transfected with either control vector encoding GFP (pcDNA-GFP), wild type GFP-tagged Gα12 (pcDNA-*WT* Gα12-GFP) or constitutively active GFP-tagged Gα12 (pcDNA-*QL* Gα12-GFP). ***B:*** LLC-PK1 cells stably expressing KIM-1 were transfected with the same constructs as in *A*. Cells in *A* and *B* were fed apoptotic cells that are fluorescently labelled with pHrodo red for 90 minutes. The percent of uptake of apoptotic cells was determined by flow cytometry after gating on GFP positive cells. The ratio of phagocytosis compared to control vector was determined (n=3, *p<0.05, ***p<0.001, unpaired t-test). ***C:*** HEK-293 cells stably expressing wild type KIM-1 were grown on cover slips and transfected with either pcDNA-GFP or pcDNA-*QL* Gα12-GFP and fed fluorescently labelled (pHrodo red) apoptotic cells for 90 minutes. Confocal microscopy images were taken to quantify the number of apoptotic cells bound to the phagocytic cells. The results were plotted as a representation of eight sections from four independent experiments with approximately 120 cells counted for each section (n=4, *p<0.05, unpaired t-test). ***D:*** HEK-293 cells stably expressing KIM-1 were transfected with 50 pmol of control siRNA or siRNA against Gα12 for 24 hours, and the levels of Gα12, KIM-1 and actin were determined by immunoblotting (IB) with the indicated antibodies. The graph showing the densitometric ratio of Gα12 in siRNA treated samples as compared to non-transfected samples represents data from four independent experiments (n=4, *p<0.05, ns= not significant, unpaired t-test). ***E:*** HEK-293-KIM-1-GFP expressing cells transfected with either control or Gα12 siRNA were fed fluorescently labelled (pHrodo red) apoptotic cells to measure phagocytosis as in *A* and *B* (n=5, *p<0.05, unpaired t-test). ***F:*** cells in *E* were visualized with confocal microscopy to count the number of apoptotic cells. The number of apoptotic cells bound to the cells that were seen in each field was plotted based on analysis of eight representative sections from each of 4 independent experiments with approximately 120 cells counted for each section (n=4, *p<0.05, unpaired t-test).

3.4.4. RhoA is a down-stream effector in KIM-1-mediated phagocytosis

Gα12 has been shown to transduce signals from GPCR to activate RhoA via Rho GEFs (Tanabe *et al.*, 2004). RhoA belongs to the Rho family of GTPases (Chimini *et al.*, 2000; Hall, 1998; Tosello-Trampont *et al.*, 2003) that are master regulators of the actin cytoskeleton and has been implicated in phagocytosis (Morimoto *et al.*, 2006; Nakaya *et al.*, 2006; Ravichandran *et al.*, 2007). To determine the role of RhoA in KIM-1-dependent engulfment of apoptotic cells, we inhibited RhoA signalling using a Rho inhibitor (cell-permeable C3 transferase) (Fritz *et al.*, 1994) or its downstream effector Rho-associated Protein Kinase (ROCK) using Y27632 (Ishizaki *et al.*, 1997; Ishizaki *et al.*, 2000). The inhibition of either RhoA or ROCK significantly increased the uptake of apoptotic cells when compared to control treatments respectively (Figure 3.5A). The comparable effects of both inhibitors on apoptotic cell-uptake suggested that ROCK might be a primary RhoA effector in mediating the inhibition of phagocytosis in KIM-1 expressing cells. In contrast, KIM-1-expressing HEK-293 cells transfected with a vector encoding either wild type RhoA or constitutively activated RhoA mutant (Q62L) exhibited significantly reduced phagocytic efficiency when compared to the cells transfected with a control vector (Figure 3.5B). However, when KIM-1-expressing cells were transfected with dominant negative RhoA (T19N), there was a significant increase in phagocytosis (Figure 3.5).

The parallel and negative effects of both activated Gα12 and RhoA on KIM-1-mediated phagocytosis suggested that RhoA likely mediated the Gα12-inhibitory effect on phagocytosis. To formally test if endogenous RhoA activity was regulated by Gα12 in our cells, we silenced endogenous Gα12 expression in HEK-293-KIM-1-GFP cells and measured the RhoA activation using a commercially available pull-down assay that employs the binding domain of Rho effector protein (Rhotekin) fused to GST (Ishizaki *et al.*, 1997). Endogenous RhoA activity was significantly inhibited in HEK-293-KIM-1-GFP cells treated with Gα12 siRNA, but not control siRNA, at baseline, at 30 minutes (early phagocytosis) and 90 minutes (late phagocytosis) after of exposure to apoptotic cells (Figure 3.5C). Additionally, transfection of HEK-293-KIM-1-GFP cells with a constitutively active mutant of Gα12 (*QL* Gα12) led to a significant increase in RhoA activity (Figure 3.5D).

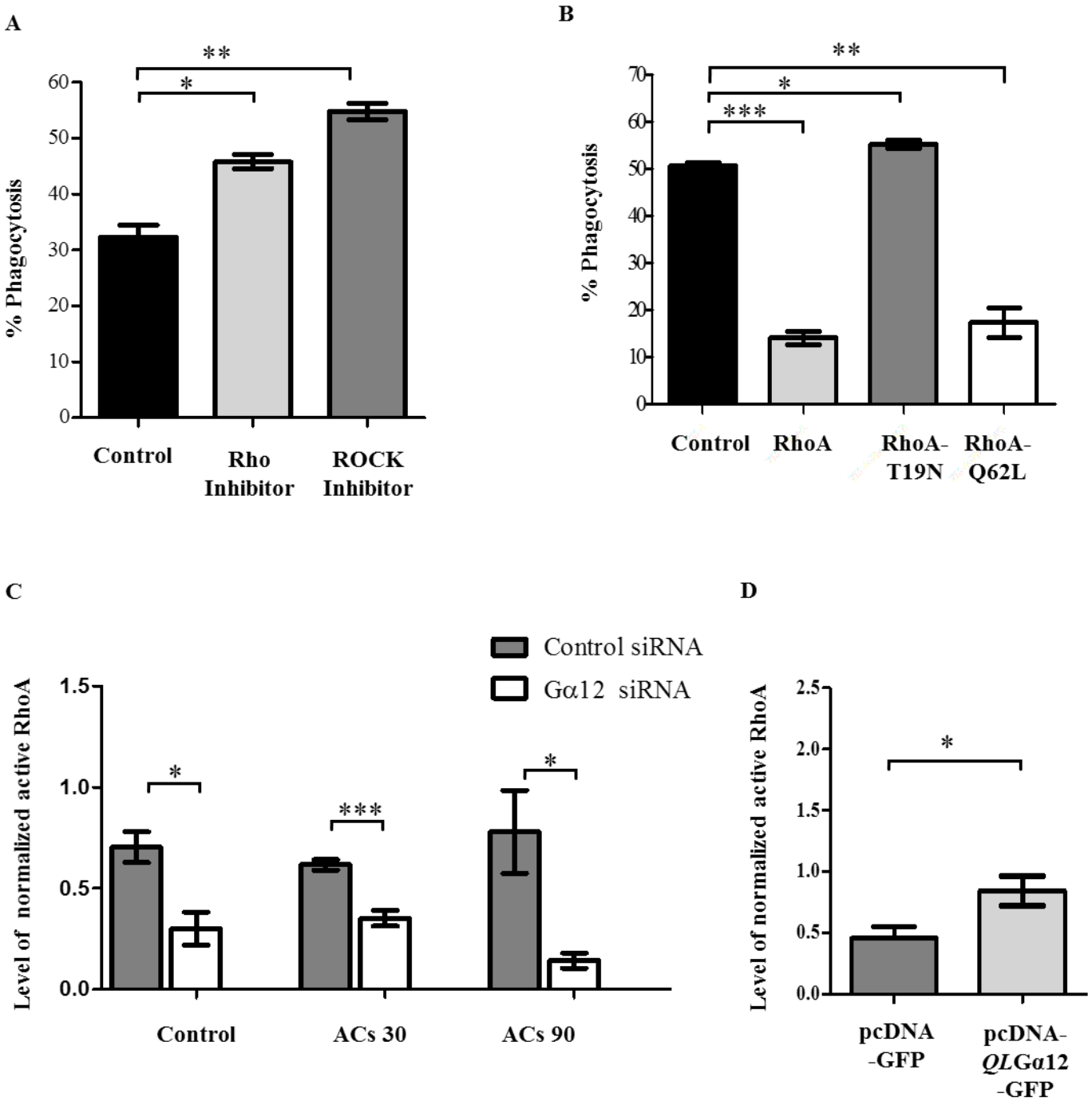

A
% Phagocytosis
Control
Rho Inhibitor
ROCK Inhibitor
B
% Phagocytosis
Control
RhoA
RhoA-T19N
RhoA-Q62L
C
Level of normalized active RhoA
Control siRNA
Gα12 siRNA
Control
ACs 30
ACs 90
D
Level of normalized active RhoA
pcDNA-GFP
pcDNA-QLGα12-GFP

Figure 3.5. RhoA is a down-stream effector of Gα12 in KIM-1-mediated phagocytosis.

A: HEK-293 cells stably expressing KIM-1 were treated for 4 hours with DMSO (control), Rho inhibitor (cell permeable C3 transferase) (0.5 µg/ml), or Rho kinase (ROCK) inhibitor Y27632 (10 µM). ***B:*** Cells were transfected with CFP-tagged control vector, CFP-tagged wild type RhoA, CFP-tagged dominant negative RhoA (T19N) or CFP-tagged constitutively active RhoA (Q62L). These cells *(A, B)* were incubated with pHrodo red-labelled apoptotic cells for 90 minutes and the percentage of phagocytosis by CFP-positive cells was measured by flow cytometry (n=3, *p<0.05, **p<0.01, ***p<0.001, unpaired t-test). ***C:*** HEK-293 cells stably expressing KIM-1 were transfected with either control siRNA or siRNA against Gα12 for 24 hours then stimulated with apoptotic cells (ACs) for 30 or 90 minutes. ***D:*** HEK-293-KIM-1 cells were transfected with either control vector or constitutively active Gα12 (*QL* Gα12). Levels of active RhoA *(in C and D)* were measured using the G-LISA RhoA activation assay kit (Cytoskeleton Inc.). Total RhoA levels in lysates were measured using RhoA ELISA kit (Cytoskeleton Inc.). The level of normalized active RhoA were calculated and graphed (n=3, *p<0.05, ***p<0.001, unpaired t-test).

We also examined dynamic RhoA activity during phagocytosis. Following stimulation with apoptotic cells, a statistically gradual significant increase in the level of active RhoA during KIM-1-mediated phagocytosis was observed in cells, with a peak activity occurred around 90 minutes after the addition of apoptotic cells (Figure 3.6A). There was no discernible pattern to RhoA activation observed in cells not expressing KIM-1 that were also fed apoptotic cells (Figure 3.6B). The difference in the trend between RhoA activation and Gα12 activation during KIM-1-mediated phagocytosis could suggest the involvement of independent proteins affecting the level of active RhoA and Gα12. Taken together, the above results suggested that Gα12-RhoA signalling negatively regulates KIM-1-dependent phagocytosis. Our data are consistent with reports showing that RhoA and ROCK are negative regulators of phagocytosis mediated by other phagocytic receptors (Nakaya *et al.*, 2006;Tosello-Trampont *et al.*, 2003).

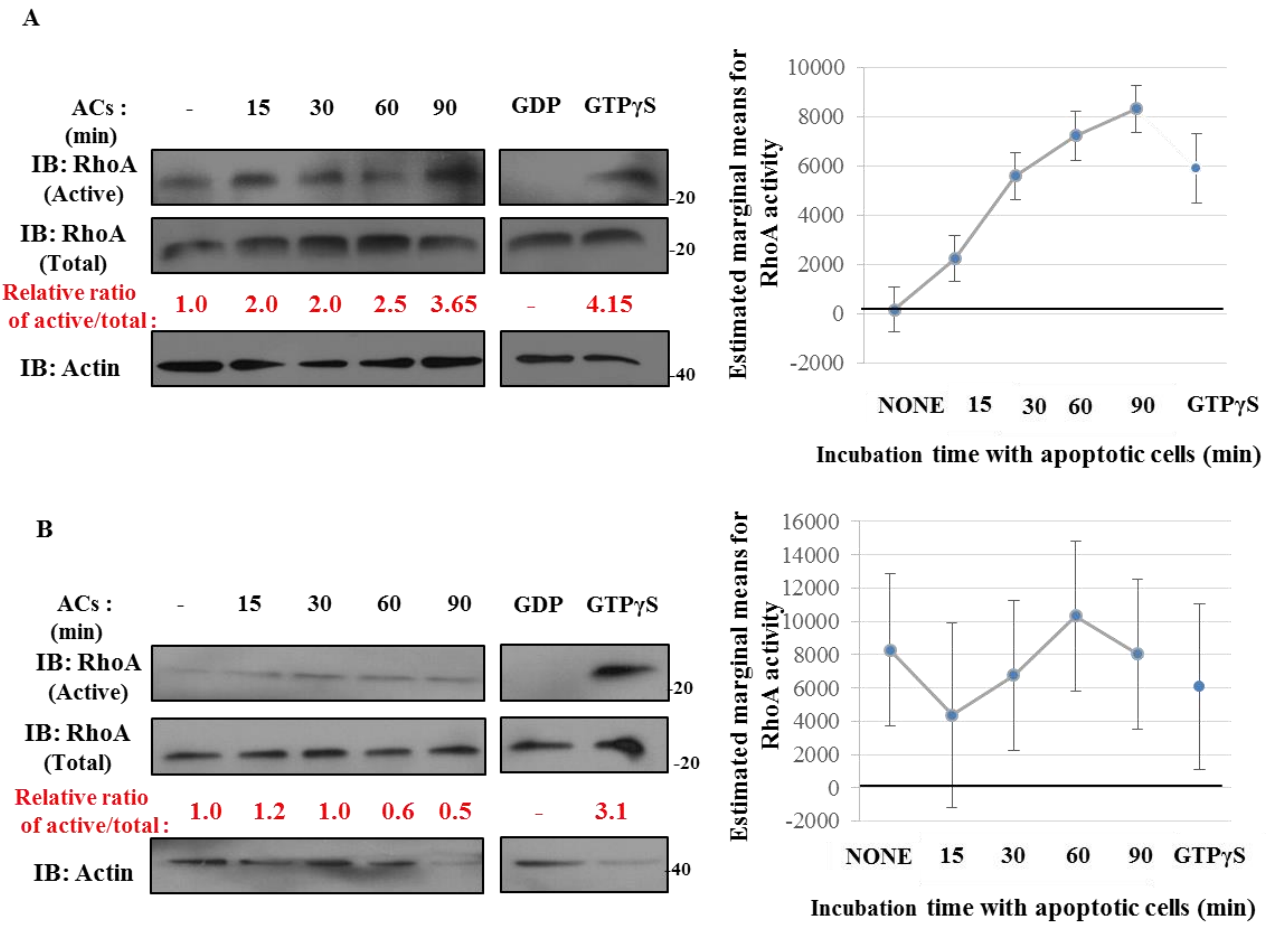


Figure 3.6. RhoA activity is increased during the course of KIM-1-mediated phagocytosis.

HEK-293 cells stably expressing KIM-1-GFP ***(A)*** or pcDNA-GFP (control vector) ***(B)*** were fed apoptotic cells for various time points (15, 30, 60, and 90 minutes). The level of active RhoA was measured using active RhoA pull-down assay (Cytoskeleton Inc.). Cell lysate loaded with non-hydrolyzable GTP (GTPγS) or GDP were used as a positive or negative control, respectively. Samples were analyzed by SDS-PAGE followed by immunoblotting (IB) with antibodies against RhoA and actin. Densitometric analysis of the relative ratio of the active RhoA to their respective total fractions in comparison to the non-treated sample is shown as numbers below blots. Graphs represent the estimated marginal means for active RhoA as determined by the analysis of covariance (ANCOVA) (n=3, p=0.009 (A), p=0.965 (B)).

Unlike RhoA, Rac1 has been shown to have a conserved and positive role in phagocytosis by both professional and semi-professional phagocytes regardless of which phagocytic receptor was involved (Ravichandran, 2011; Ravichandran *et al.*, 2007). Thus, we next sought to determine the role of Rac1 in KIM-1-mediated phagocytosis. Consistent with these reports, transfection of KIM-1-expressing cells with a vector encoding dominant negative Rac1 (T17N) or treatment of KIM-1-expressing cells with a Rac1 inhibitor significantly inhibited the phagocytic uptake of apoptotic cells compared to controls (Figure 3.7A and B). Hence, we reasoned that Rac1 is also required for KIM-1-dependent phagocytosis and has the opposite effect to RhoA on KIM-1-dependent corpse engulfment in our cell culture models.

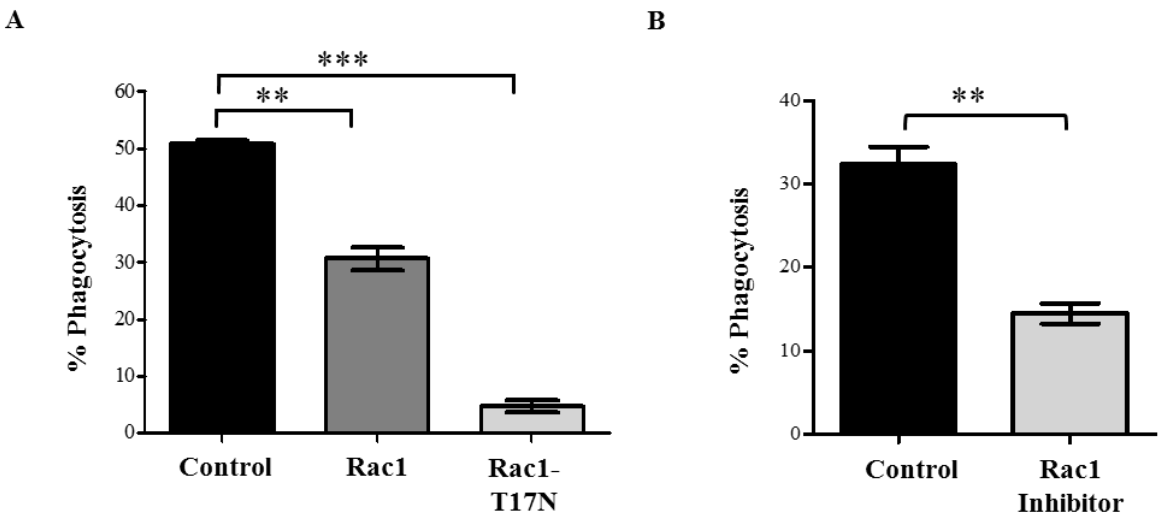


Figure 3.7. Rac1 is involved in KIM-1-mediated phagocytosis.

A: HEK-293 cells were co-transfected with construct encoding wild type KIM-1 and either CFP-tagged control vector (control), CFP-tagged wild type Rac1 or CFP-tagged dominant negative Rac1 (Rac1-T17N). ***B:*** HEK-293-KIM-1 cells were treated for 4 hours with DMSO (control) or Rac1 inhibitor (NSC23766) (50 µM). Cells in *(A)* and *(B)* were fed pHrodo-labelled apoptotic cells for 90 minutes and the percentage of uptake was measured by flow cytometry. In A, the percentage of pHrodo red high fluorescent cells was determined for CFP-positive cells (n=3, **$p<0.01$, ***$p<0.001$, unpaired t-test).

3.4.5. KIM-1 is the major phagocytic receptor in primary proximal tubule epithelial cells that control Gα12 activity

In the context of our previous report (Ismail *et al.*, 2015) demonstrating that KIM-1 binds directly to and suppresses Gα12 activation by blocking GTP-loading, our present data suggest that KIM-1-suppression of endogenous Gα12 activation in cells is required for efficient phagocytosis of apoptotic cells. To confirm the inhibitory role of Gα12 in a physiologically relevant cell type, we studied primary tubular epithelial cells (TECs) isolated from mice kidneys. First, we aimed to determine if KIM-1 is dispensable for phagocytosis in primary renal TECs or if its expression is absolutely required for phagocytosis. For this, we compared the phagocytic efficiency between primary TECs isolated from wild type (Kim-$1^{+/+}$) C57BL/6 and previously generated Kim-1-deficient mice (Kim-$1^{-/-}$) (Wong *et al.*, 2010). *Ex vivo* culturing of TECs from Kim-$1^{+/+}$ mice readily resulted in spontaneous Kim-1 upregulation, as previously reported (Ajay *et al.*, 2014; Humphreys *et al.*, 2013; Ichimura *et al.*, 2008), suggesting that cell culture condition mimics injury induced by IRI in AKI. As expected, compared to Kim-$1^{+/+}$ TECs, there was an absence of both total Kim-1 protein level and Kim-1 cell-surface expression in TECs isolated from Kim-$1^{-/-}$ mice, as determined by Western blot and flow cytometry, respectively (Figure 3.8A and B). When apoptotic cells were fed to TECs over 24 hours to stimulate phagocytosis, we found a significant difference between Kim-$1^{-/-}$ and Kim-$1^{+/+}$ TECs in phagocytosis as measured by flow cytometry (Figure 3.8C). Surprisingly, TECs deficient in Kim-1-expression were virtually defective in the uptake of apoptotic cells. To verify these findings using an alternate method, TECs were fed apoptotic cells for three hours and cells were stained for surface Kim-1 and imaged by confocal microscopy (Figure 3.8D). In contrast to Kim-1-deficient TECs and in keeping with our flow cytometry data, Kim-1-expressing TECs readily bound apoptotic cells and formed phagocytic cups around them (arrows, Figure 3.8D). Finally, to test if Gα12 negatively regulated KIM-1-dependent phagocytosis in primary TECs, we pretreated TECs isolated from Kim-$1^{+/+}$ mice with Gα12 siRNA or control siRNA, and compared their relative phagocytic efficiencies (Wong *et al.*, 2010). In keeping with what was observed in KIM-1-expressing HEK-293 cells, silencing Gα12 expression significantly increased the phagocytic capacity of TECs and its ability to mediate engulfment of apoptotic cells (Figure 3.8E and F).

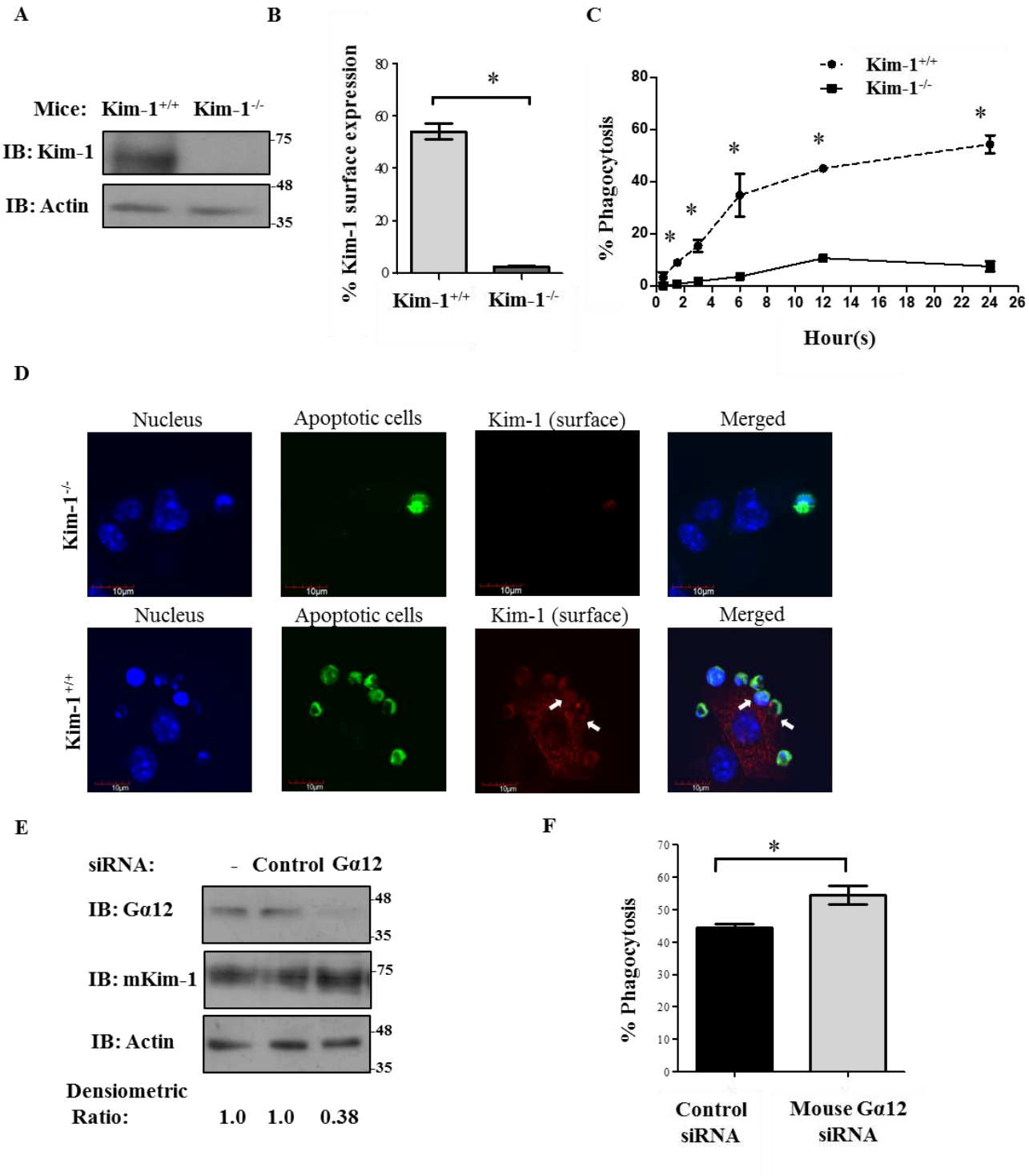

A
Mice: Kim-1+/+ Kim-1-/-
IB: Kim-1
IB: Actin
75
48
35
B
% Kim-1 surface expression
Kim-1+/+
Kim-1-/-
C
% Phagocytosis
Kim-1+/+
Kim-1-/-
Hour(s)
D
Nucleus
Apoptotic cells
Kim-1 (surface)
Merged
Kim-1-/-
Kim-1+/+
E
siRNA: - Control Gα12
IB: Gα12
IB: mKim-1
IB: Actin
Densiometric Ratio: 1.0 1.0 0.38
F
% Phagocytosis
Control siRNA
Mouse Gα12 siRNA

Figure 3.8. KIM-1 is a major phagocytic receptor in primary proximal tubular epithelial cells and Gα12 is a negative regulator of phagocytosis.

A: Proximal tubular epithelial cells (TECs) isolated from wild type (Kim-$1^{+/+}$) and Kim-1 knock out (Kim-$1^{-/-}$) mice were cultured and the expression levels of mouse Kim-1 were measured by Western blot. ***B:*** Surface expression of mouse Kim-1 on TECs was determined by flow cytometry using PE-conjugated anti-mouse Tim-1/Kim-1 antibody (R&D Systems Inc.) (n=3, *$p<0.05$, unpaired t-test). ***C:*** TECs were fed pHrodo red-labelled apoptotic cells for various time points (hour(s)) and the percent phagocytosis of fluorescently labelled apoptotic cells uptake by TECs (as determined by high fluorescence staining ($>10^4$)) was measured by flow cytometry (n=3, *$p<0.05$, one-way ANOVA). ***D:*** TECs were cultured on glass cover slips and fed green-labelled apoptotic cells for 3 hours. Cells were fixed and surface stained for mouse Kim-1 using anti-mouse Kim-1 antibody and Cy5-conjugated secondary antibody (600x, bar 10µm). ***E:*** TECs isolated from wild type C57BL/6 mice were transfected with 50 pmol of control siRNA or siRNA against mouse Gα12 for 24 hours and the levels of Gα12, mouse Kim-1 and actin were determined by Western blot. ***F:*** TECs transfected with either control siRNA or Gα12 siRNA were subjected to the phagocytosis assay and the percentage of phagocytosis of pHrodo-labelled apoptotic cells was measured by flow cytometry (n=4, *$p<0.5$, unpaired t-test).

3.5. DISCUSSION

KIM-1 is a phosphatidylserine-binding phagocytosis receptor (Freeman *et al.*, 2010; Ichimura *et al.*, 2008) that is specifically upregulated by tubular epithelial cells (TECs) during ischemic or toxic AKI (Ichimura *et al.*, 2008; Ichimura *et al.*, 1998; Ichimura *et al.*, 2012). However, the relevant intracellular signalling pathway(s) that it regulates had not been appreciated till now. Here, we reveal for the first time that Gα12 negatively regulates KIM-1-depedent phagocytosis. Further, we demonstrated that Gα12 mediates this inhibition through downstream activation of RhoA and likely via ROCK. Given that KIM-1-mediated phagocytosis prevents AKI (Yang *et al.*, 2015), our data demonstrating that KIM-1 is indispensable for phagocytosis by renal TECs implies that the Gα12-RhoA signalling axis plays an important role in regulating phagocytosis by TECs *in vivo* during AKI.

While we did not specifically address how Gα12 is activated in TECs during phagocytosis or the molecular mechanisms of Gα12 inhibition by KIM-1, our findings are supported by our previous work which suggested that KIM-1, when upregulated following ischemia-reperfusion injury (IRI), binds directly to Gα12 and inhibits its activation by ROS (Ismail *et al.*, 2015). This inhibition is mediated by the ability of KIM-1 to block GDP-GTP exchange onto Gα12. As a result, we showed that Kim-1-deficient mice exhibited a higher level of active Gα12 and worse kidney function following renal ischemic injury when subjected to renal IRI. This demonstrates that KIM-1 expression protected mice from tissue damage caused by ROS-mediated activation of injury pathways downstream of Gα12 (Yu et al., 2012). These downstream injury pathways include activation of Src kinase and inhibition of tight junctions assembly and reassembly that are required for tubular repair (Meyer *et al.*, 2002; Sabath *et al.*, 2008). Our current findings here suggest that ability of KIM-1 to suppress Gα12 activation is also crucial for TECs to carry out phagocytosis during AKI. This finding is particularly relevant because it was later shown that KIM-1-mediated phagocytosis contributes to protection from AKI by curtailing immune responses (Yang *et al.*, 2015). Thus, aberrant Gα12 activation by ROS during AKI not only destabilizes tight junctions (via Src kinase) (Meyer *et al.*, 2003; Yu *et al.*, 2012), but also impedes phagocytic clearance of dying cells. Thus, we propose that upregulation of KIM-1 on TECs during AKI and subsequent inhibition of Gα12 protects from renal damage during renal IRI via at least two potential mechanisms. The first one is by blocking ROS-mediated activation of injury pathways via activated Gα12 that leads to worse tissue damage and impaired repair (Yanamadala *et al.*, 2007;

Yu *et al*., 2012). The second mechanism is by enhancing phagocytic clearance of apoptotic and necrotic cells by surviving TECs (Gandhi *et al.*, 2014; Ichimura *et al.*, 2008) via suppression of Gα12. This inhibitory mechanism of KIM-1 on Gα12 would help regulate inflammation caused by spillage of the intracellular contents of necrotic and secondary necrotic cells into the extracellular milieu within the kidney (Elliott *et al.*, 2010; Ren *et al.*, 1998; Silva, 2010).

Actin cytoskeletal rearrangement is crucial for the engulfment of apoptotic cells (Castellano *et al.*, 2001; Chimini *et al.*, 2000). Rac1 and RhoA belong to the Rho family of GTPases that regulate actin cytoskeletal reorganization (Ridley, 2001) and have long been implicated in phagocytosis (Ravichandran *et al.*, 2007). The reciprocal role for Rac1 and RhoA in KIM-1-dependent phagocytosis in TECs proposed by our work is novel for KIM-1-mediated uptake of apoptotic cells and is consistent with what has been reported by other groups studying additional phosphatidylserine receptors (Erwig *et al.*, 2008; Nakaya *et al.*, 2006; Tosello-Trampont *et al.*, 2003). In those studies, the inhibitory effect of RhoA on apoptotic cell-clearance was mediated by ROCK, as was also the case in our experiments (Tosello-Trampont *et al.*, 2003). Activated RhoA increases the kinase activity of ROCK, which in turn phosphorylates myosin light chain (MLC) and promotes contractility via actin stress fiber formation (Riento *et al.*, 2003). The latent activation of RhoA in our cells might lead to an increase in contractility, causing stress fiber formation, which has been postulated to inhibit phagocytosis (Deery *et al.*, 1993; Ravichandran *et al*., 2007). The later has not been tested in this paper and requires future experimental work. In contrast to RhoA, activated Rac1 has an evolutionarily conserved positive role in promoting phagocytosis (Henson, 2005; Kinchen *et al.*, 2005; Nakaya *et al.*, 2008). It is generally accepted that Rac1 is recruited to help form phagocytic cups and generate membrane ruffles that are necessary to facilitate corpse engulfment (Kraynov *et al.*, 2000; Nakaya *et al.*, 2008). Rac1 is then down regulated when the phagocytic cup closes in conjunction with abrupt disruption of the associated actin patches. Our data are consistent with a requirement for Rac1 in phagocytosis. Whether one or more of the conserved phagocytic signalling pathways regulates Rac1 activation downstream of KIM-1 in TECs remains to be tested (Kinchen *et al.*, 2005; Tosello-Trampont *et al.*, 2007). Moreover, the opposing roles of RhoA and Rac1 in KIM-1-mediated phagocytosis might suggest a spatiotemporal pattern of activation. This may be better visualized in future studies through the use of biosensors with fluorescence resonance energy transfer (FRET) that respond to RhoA and Rac1 activation (Nakaya *et al.*, 2008; Wong *et al.*, 2006)

To our knowledge, this is the first report describing any aspect of phagocytic signalling by KIM-1, despite the fact that other members of the TIM family of phosphatidylserine receptors, such as TIM-3 and TIM-4, have been the subjects of much research (Balasubramanian *et al.*, 2012; Binne *et al.*, 2007; Curtiss et al., 2011; Freeman *et al.*, 2010; Mariat *et al.*, 2009; Yeung *et al.*, 2015). Studies on TIM-1 signalling thus far have focused primarily on the non-phagocytic functions of KIM-1 (that is TIM-1), for instance in its role in co-stimulation of T cell responses (Balasubramanian *et al.*, 2012; Bonventre *et al.*, 2011; Anjali J. de Souza *et al.*, 2008; Lee *et al.*, 2010; Mariat *et al.*, 2009). Balasubramanian *et al*. recently reported that TIM-1 mediates the degradation of NUR77, a nuclear receptor implicated in apoptosis and cell survival, but did not investigate phagocytic signalling (Balasubramanian *et al*., 2012). Additionally, Yang *et al*. demonstrated that KIM-1 interacts with p85 in TECs and down-modulates NF-κB signalling (Yang *et al.*, 2015). On the other hand, TIM-3 is expressed by dendritic cells, where it mediates phagocytosis of apoptotic cells, but very little is known regarding how it mediates phagocytic signalling (Miyanishi *et al.*, 2007; Nakayama *et al.*, 2009; Sanchez-Fueyo *et al.*, 2003). Tim-4 does not mediate phagocytic signalling directly, but has been postulated to signal via a yet unidentified co-receptor (Park *et al.*, 2009).

Although various GTPases make up fundamental components of the phagocytic signalling machinery (Hochreiter-Hufford *et al.*, 2013; Ravichandran *et al.*, 2007), to our knowledge, our study is the first one to implicate Gα12 in phagocytosis. Given the various fundamental processes that Gα12 regulates, such as motility (Kelly *et al*., 2006), modulation of cell-cell junctions (Meyer *et al*., 2003), cellular transformation (Dermott *et al.*, 1999) and contractility (Buhl *et al.*, 1995; Gavard *et al.*, 2008), our study suggests a relationship between these processes and phagocytosis in TECs. For example, pathological activation of Gα12 during ischemic-reperfusion injury results in activation of injury pathways via destabilization of tight junctions (Samarin *et al.*, 2009) through its interaction with zonula occludens-1 (ZO-1) and Src kinase (Meyer *et al.*, 2003; Meyer *et al.*, 2002; Sabath *et al.*, 2008). This disruption of the tight junction has been shown to be a hallmark of epithelial cell damage and is believed to be a contributor to kidney dysfunction (Denker *et al.*, 1998; Meyer *et al*., 2001). The relationship between tight junction assembly and disassembly during phagocytosis has not yet been studied and will be of major interest to our research.

Deciphering KIM-1 signalling pathway(s) may have future clinical implications in AKI. Our work presented here and previously (Ismail *et al.*, 2015) suggests that targeting Gα12 might

be a potential strategy to treat AKI by enhancing clearance of apoptotic cells and simultaneously inhibiting downstream injury pathways. Unfortunately, there is no specific Gα12 inhibitor available to currently test this *in vivo*. Nonetheless, our detailed work uncovering the importance of KIM-1 in TEC-phagocytosis and how it is regulated offers important insights into TEC biology that might be relevant to AKI.

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Chapter 4

Interaction of kidney injury molecule-1 with the dynein light chain Tctex-1 and its implication for KIM-1-mediated phagocytosis

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4.1. SUMMARY

In this chapter, we identify and study another novel KIM-1-interacting protein. Using a yeast two-hybrid system, we describe the identification of the 14-kDa light chain of cytoplasmic dynein motor protein, Tctex-1, as a potential KIM-1 interacting protein. Given that Tctex-1 is involved in microtubule and actin cytoskeletal dynamics, we hypothesized that Tctex-1 functions to enhance KIM-1-mediated engulfment of apoptotic cells. First, we established that Tctex-1 interacts with KIM-1 via co-immunoprecipitation. When we stimulated KIM-1-expressing cells with apoptotic cells, we observed that this interaction is significantly reduced upon longer incubation with apoptotic cells (90 minutes). The time dependent reduction in the interaction between Tctex-1 and KIM-1 was further confirmed by GST-Tctex-1 pull-down assay and immunofluorescence staining. When we inhibited KIM-1-mediated phagocytosis through inhibition of actin cytoskeleton changes by treatment with cytochalasin D or nocodazole, we restored the interaction between KIM-1 and Tctex-1 during the later stages of phagocytosis. In addition, we tested whether phosphorylation at Threonine-94 of Tctex-1 influenced the interaction with or phagocytic efficiency of KIM-1. Surprisingly, a phosphomimetic mutant of Tctex-1 at Threonine-94 (T94E) displayed a decreased ability to interact with KIM-1 without influencing KIM-1-mediated uptake of apoptotic cells. To determine the role of Tctex-1 during KIM-1-mediated phagocytosis, we silenced Tctex-1 expression via siRNA and found a significant reduction in uptake of apoptotic cells that was accompanied by highly disorganized actin structures. Furthermore, in a search for down-stream mediators of Tctex-1 signalling in KIM-1-expressing cells, we failed to find a link between Tctex-1 and RhoGTPase. This suggest that Tctex-1 might exhibit its function through different RhoGTPases that are novel to KIM-1.

4.2. INTRODUCTION

The process of programmed cell death, or apoptosis, is characterized by distinct morphological characteristics that serve as a mechanism for removing damaged or unwanted cells from the tissues of an organism (Kerr, 2002; Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Phagocytosis of apoptotic cells, termed phagocytosis (Vandivier *et al.*, 2006), prevents the release of noxious and immunogenic materials from dying cells, which reduces tissue damage associated with inflammation or inappropriate autoimmune response (Ren *et al.*, 1998). Phagocytosis is increasingly being recognized to be important in tissue repair after acute organ injury (Juncadella

et al., 2013; Yeung *et al.*, 2015). The process of phagocytosis is usually carried out by professional phagocytes (Albert *et al.*, 1998; Duvall *et al.*, 1985). However, emerging data suggests that amateur phagocytes, such as fibroblasts, retinal pigment epithelial cells, sertoli cells, and more recently renal proximal tubular epithelial cells, also play roles in this process (Finnemann *et al*., 1997; Ichimura *et al.*, 2008; Monks *et al.*, 2002; Patel *et al*., 2010; Shiratsuchi *et al.*, 1997). The direct or indirect recognition of apoptotic cells by phagocytes involves the recognition of "eat-me" signals that are newly (e.g. exposed or modified lipids or proteins) exposed on the surface of apoptotic cells. One of the most well characterized "eat me" signals is phosphatidylserine (PS) (Ravichandran *et al.*, 2007).

Kidney injury molecule-1 (KIM-1) is a well characterized phagocytic receptor that is expressed on renal proximal tubular epithelial cells (TECs). It recognizes phosphatidylserine on apoptotic cells (Ichimura *et al.*, 2008). KIM-1 was first identified in a cDNA screen looking for genes that are upregulated in the post-ischemic rat kidney (Ichimura *et al.*, 1998). KIM-1 is mostly localized to the apical membrane of TECs following injury to kidney commonly caused by ischemia or toxic insult (Ichimura *et al.*, 2004). Structurally, KIM-1 is a type I transmembrane glycoprotein consisting of an N-terminal immunoglobulin V (IgV) domain, a mucin domain, a transmembrane domain and a cytoplasmic tail (Ichimura *et al.*, 1998; Santiago *et al.*, 2007). The signalling mechanisms underlying KIM-1-mediated uptake of apoptotic cells are still not well understood. Recently, KIM-1 was shown to interact with p85 following apoptotic cell binding (Yang *et al.*, 2015). This was shown to lead to down-regulation of the NF-κB pathway and subsequent inhibition of inflammatory signalling during AKI. In addition, we have uncovered another protein that KIM-1 interacts with to carry its phagocytic function, known as the α subunit of heterotrimeric G12 protein (Gα12). Despite the fact that several proteins have been identified to interact with KIM-1, the overall signalling mechanisms by which KIM-1 regulates the uptake of apoptotic cells has not yet been fully elucidated.

Here we uncovered another a novel KIM-1 interacting protein, Tctex-1 (t-Complex testis-expressed-1), using a yeast two hybrid screen that exploited the cytoplasmic tail of KIM-1 as bait and a HeLa cell cDNA library (done by Dr. Zervos, University of Central Florida, Orlando, Florida). Tctex-1 was initially identified in a cDNA screen looking for candidate genes in the mouse t complex whose aberrant expression maybe functionally related to sterility and transmission ratio distortion (Lader *et al.*, 1989). Tctex-1 is a key component of the cytoplasmic

light chain of the dynein motor complex (King *et al.*, 1996). Cytoplasmic dynein is a multicomponent, microtubule-based, ATP-dependent motor unit that has several roles in intracellular retrograde transport, mitotic spindle localization and centrosome separation (Barton *et al.*, 1996; Schroer, 1994; Steuer *et al.*, 1990). Tctex-1 has diverse dynein-dependent and -independent functions (Hirokawa, 1998; Hirokawa *et al.*, 1998; Lader *et al.*, 1989). Through dynein-dependent functions, Tctex-1 is associated with microtubules in order to mediate a range of intracellular motile events that are important in the intracellular targeting or sorting of proteins (Mueller *et al.*, 2002; Sachdev *et al.*, 2007; Tai *et al.*, 1999). For example, Tctex-1 interaction with rhodopsin, a GPCR, and transports vesicles containing rhodopsin from the Golgi apparatus to the apical cell surface via tethering of Tctex-1 to the dynein complex on microtubules (Tai *et al*., 1999; Tai *et al.*, 2001). On the other hand, Tctex-1 has been shown to function independently of dynein, to regulate actin cytoskeletal dynamics (Chuang *et al.*, 2005). Phosphorylation of Tctex-1 at Threonine-94 (Thr-94) has been shown to promote its dissociation from the dynein light chain and may allow it to bind to other non-dynein proteins to mediate dynein-independent functions (Chuang *et al*., 2005; Song *et al.*, 2007). In addition, Tctex-1 expression is restricted to certain tissues, where immunoprecipitation of kidney, spleen and testis tissues lysate showed higher levels of Tctex-1 than brain tissue lysate (King *et al.*, 1996). Despite advances in the research regarding Tctex-1, most of the research on this protein has focused on the role of Tctex-1 in neurogenesis, with little information about its role in the kidney.

In this report, we uncovered a novel interaction between Tctex-1 and KIM-1 and we investigated the role of Tctex-1 in the phagocytic uptake of apoptotic cells. Our data show that when KIM-1 is upregulated, it interacts with Tctex-1 to enable phagocytosis. Altering actin or microtubules polymerization leads to a restoration of interaction that is lost during later stages of phagocytosis. When Tctex-1 is phosphorylated at Threonine-94, its interaction with KIM-1 is severely decreased. Furthermore, Tctex-1 appears to be important in KIM-1-mediated phagocytosis, since silencing Tctex-1 decreases the uptake of apoptotic cells. Altogether, our findings support the idea that Tctex-1 is important in KIM-1-mediated phagocytosis and that its interaction with KIM-1 is a very dynamic process. The mechanisms by which Tctex-1 affects phagocytosis remains to be studied.

4.3. EXPERIMENTAL PROCEDURES

4.3.1. Cell culture and materials

Human Embryonic Kidney 293 (HEK-293) cells and human renal adenocarcinoma cells (769-P) were cultured at 37 °C in 5 % (vol/vol) CO_2 and maintained in DMEM or RPMI media (Invitrogen, Carlsbad, CA) containing 10 % FBS (Invitrogen). Chemicals were purchased from Sigma (Sigma Aldrich, St. Louis, MO) unless stated. The pH-sensitive dye pHrodo™ Red succinimidyl ester (pHrodo™ Red, SE), Alexa Fluor® 555 goat anti-mouse, Alexa Fluor® 488 goat anti-rabbit and DAPI (4',6-diamidino-2-phenylindole) were purchased from Life Technologies (Molecular probes, Invitrogen, Carlsbad, CA). Rhodamine phalloidin stain was purchased from Cytoskeleton Inc. (Cytoskeleton, Denver, CO). The Tctex-1/DYNLT-1 antibody used for confocal microscopy was purchased from Proteintech Group (11954-1-AP, Proteintech Group Inc., Chicago, IL) and was used at a dilution of 1:50. Antibodies for Western blot against Tctex-1 (D9944, 1:800 working dilution), actin (SAB4301137, 1:1000 working dilution) were all purchased from Sigma (Sigma Aldrich, St. Louis, MO). PE-conjugated anti-human KIM-1 (1D12, 1:100 working dilution) and PE-conjugated rat IgG2b isotype control (RTK4530, 1:100 working dilution) were purchased from Biolegend (Biolegend, San Diego, CA). Anti-KIM-1 (cytosolic domain) antibody was custom generated by immunizing rabbits against the cytosolic domain of KIM-1 (PA 4145, Life technologies, Thermo Fisher Scientific, Rockford, IL) as previously described (Bailly *et al.*, 2002; Gandhi, *et al*, 2014). A monoclonal KIM-1 antibody binding against to the mucin domain of KIM-1 (AKG7) was kindly provided by Dr. Bonventre (Harvard Medical School, Brigham and Women's Hospital, Boston, MA). Complete Mini EDTA-free protease inhibitor cocktail tablets were purchased from Roche (Roche Diagnostic, Basel, Switzerland). Cytochalasin D and Nocodazole were purchased from EMD Millipore (EMD Millipore, Billerica, MA). The plasmid constructs encoding Tctex-1 were kindly provided by Dr. Robert Rottapel (University of Toronto, Toronto, ON) and the plasmid constructs for KIM-1 were from Dr. Bonventre (Harvard Medical School, Brigham and Women's Hospital, Boston, MA). Lipofectamine® 2000 (Life Technologies, Thermo Fisher Scientific, Rockford, IL) and INTERFERin® (Polyplus-transfection Inc., New York, NY) were used for transfecting plasmid DNA and siRNA, respectively.

4.3.2. Immunoprecipitation and Western blot

Cells were lysed with ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM $NaVO_4$, 1 mM NaF, 1 % Triton X-100, and complete mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostic, Basel, Switzerland). For immunoprecipitation, cell extracts containing 1.0–1.5 mg of protein/mL were incubated with 10 µg of anti-KIM-1 antibody (PA 4145, cytosolic domain), or rabbit IgG antibody (sc-2027, control) and 20 µl of protein-A/G Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. Beads were centrifuged, washed three times, suspended in 20 µL of SDS sample buffer and heated at 100 °C for five minutes. Lysates (representing 5 % of total lysate) and immunoprecipitated samples were separated under reducing condition and transferred to polyvinylidene difluroide (PVDF) membranes (EMD Millipore, Billerica, MA). PDVF-membranes were probed with antibodies specific to mucin domain of KIM-1 (AKG7, 1:1 working dilution), cytosolic domain of KIM-1 (PA 4145, 1:1,500 working dilution), Tctex-1 (D9944, 1:800 working dilution) or actin (SAB4301137, 1:2,000 working dilution). The primary antibody was visualized using the appropriate horseradish peroxidase-conjugated secondary antibodies and ECL Western blot detection reagent (Luminata forte, EMD Millipore, Billerica, MA) by autoradiography (Biomax; Denville Scientific, South Plainfield, NJ).

4.3.3. GST-Tctex-1 pull down

The GST-Tctex-1 construct (provided by Dr. King, (King, *et al.*, 1996)) was transformed into *E.coli* strain BL21 and expression of the fusion protein was induced by the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for three hours at 37 °C. After lysing the bacterial cells by sonication, GST-fusion proteins were purified by incubation with 1 ml of glutathione-Sepharose beads (Thermo Fisher Scientific, Rockford, IL) overnight at 4 °C. This was followed by three washes with 1x PBS and aliqouts of GST-Tctex-1 conjugated to glutathione-Sepharose beads were kept at -80 °C for future use. Cells were lysed with ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM $NaVO_4$, 1 mM NaF, 1 % Triton X-100) supplemented with complete mini EDTA-free protease inhibitor cocktail tablets (Roche Diagnostic, Basel, Switzerland). One milligram of protein lysate and 30 µl of GST-Tctex-1 coupled glutathione-sepharose beads were incubated together at 4 °C for overnight. Beads were washed to reduce non-specific binding and eluted using 20 µL of SDS sample buffer and heated

at 100 °C for five minutes. For each condition, both lysate and pull-down samples were analyzed by SDS-PAGE and Western blotting to detect total and pull-down KIM-1, respectively.

4.3.4. Immunofluorescence and confocal microscopy

HEK-293 cells were cultured at subconfluent density on poly-D-lysine hydrobromide (Sigma Aldrich, St. Louis, MO) coated glass cover slips, and were transfected with constructs encoding KIM-1 and flag-tagged Tctex-1. 769-P cells were grown on glass cover slips (without coating) and fed apoptotic cells for the indicated time points (15-90 minutes). Cells were washed three times with 1x PBS and fixed with 4 % paraformaldehyde, followed by counterstaining of the nucleus with DAPI (0.5 µg/ml), which stained both apoptotic cells and HEK-293 or 769-P. Cells were then permeabilized with 0.25 % Triton X-100 in 1x PBS for five minutes, and then blocked for 1 hour at room temperature with 1 % bovine serum albumin (BSA) and 0.05 % Triton X-100 in 1x PBS. Cells were then incubated with anti-Tctex-1 antibody (11954-1-AP, 1:50 working dilution) (Proteintech Group Inc., Chicago, IL) in 0.5 % BSA/PBS at 4 °C overnight. Coverslips were washed three times with 1x PBS and incubated with Alexa Fluor® 488 goat anti-rabbit (1:500 working dilution) at 37 °C for 1 hour. Coverslips were washed three times with PBS and surface staining of KIM-1 was done using an antibody against the mucin domain of KIM-1 (AKG7, 1:1 working dilution) at 4 °C overnight. Bound KIM-1 was labelled with Alexa 555 conjugated anti-mouse (1:1000 working dilution) at room temperature for 1 hour. The specificity of immunostaining was demonstrated by the absence of signal in samples processed using non-specific rabbit or mouse IgG followed by staining with the corresponding secondary antibody. For actin cytoskeleton staining, cells were permeabilized with 0.25 % Triton in 1x PBS for five minutes, and then stained with rhodamine phalloidin for 30 minutes according to the manufactures instructions (Cytoskeleton, Denver, CO). Coverslips were mounted using Shandon-Mount® permanent mounting (Thermo Fisher Scientific, Rockford, IL) and viewed with a FLUOVIEW X83I confocal microscopy (Olympus, Tokyo, Japan). Data were acquired and analyzed using the FLUOVIEW FV10 ASW 4.0 viewer and ImageJ software (National Institutes of Health, Bethesda, MD) to determine the Pearson's coefficient and Van Steensel's score for colocalization of KIM-1 and Tctex-1. Quantification of the colocalization score was assessed in three random fields per sample and was done in three independent experiments.

4.3.5. Phagocytosis assay and FACS analysis

To prepare apoptotic cells for the phagocytosis assay, thymocytes were harvested from 3-6 week old C57BL/6 mice, and apoptosis was induced by UV exposure for five minutes followed by incubation overnight at 37 °C in 5 % CO_2 in DMEM media supplemented by 10 % FBS and 1 % penicillin-streptomycin solution (Ichimura *et al.*, 2008). Apoptotic thymocytes were stained with pH-sensitive dye pHrodo™ Red succinimidyl ester (pHrodo™ Red, SE) at a final concentration of 150 nM for 30 minutes at room temperature. Labelled apoptotic cells were washed twice with 1x PBS to remove excess dye. The cells were counted and $3x10^6$ were added to each well of 6-well plate ($15x10^6$ for 10 cm plates) and incubated for various time points at 37 °C in 5 % CO_2 incubator. Cells were then placed on ice for 30 minutes to reduce non-specific binding of apoptotic cells. The plates were then washed three times with ice-cold 1x PBS, and cells were collected with 5 mM EDTA-PBS, and resuspended with FACS buffer (1xPhosphate-buffered saline (PBS), 2 % calf serum, and 0.1 % sodium azide) for flow analysis. For KIM-1-surface staining (or TIM-1), the cells were blocked in PBS and 10 % goat serum for 15 minutes, followed by incubation for 30 minutes at room temperature with PE-conjugated anti-TIM-1 (1D12, human) and respective isotype control antibody (RTK4530) at concentration of 1:100. All samples were run on BD LSR II flow cytometer (BD Biosciences, San Jose, CA). Percentage of phagocytosis represents the number of cells that have internalized the apoptotic cell(s) as indicated by higher pHrodo red fluorescence (10^4-10^5 on logarithmic scale). Engulfed cells with low fluorescence were excluded from analysis (Gandhi *et al.*, 2014).

4.3.6. siRNA targeting of Tctex-1

ON Target plus® Smart pool siRNA against Tctex-1 and a non-targeting pool (Control siRNA) were purchased from Dharmacon (Thermo Fisher Scientific, Rockford, IL). Cells were transfected with 100 nM of siRNA using INTERFERin® for 24 hours before further analysis of knockdown using Western blotting and flow cytometry.

4.3.7. Rac1 and RhoA activation

Rac1 or RhoA activation was determined using the G-LISA Rac1 or RhoA activity assay according to the manufacturer's instructions (Cytoskeleton, Denver, CO). Total RhoA was determined using Total RhoA ELISA kit (Cytoskeleton, Denver, CO). About 40 µg of protein was used for the activation assay and 20 µg of protein was used for the total assay. Treatment with 5 % serum after serum starvation overnight served as positive control for Rac1 activation (Curtiss *et*

al., 2011). Treatment of cells with 10 µM Nocodazole for 15 minutes served as positive control for RhoA activation as indicated previously (Martin *et al*., 2001).

4.3.8. Quantification and statistics

Western blots were scanned and band intensity quantified using Image J software (National Institutes of Health, Bethesda, MD). Background was subtracted in these analyses and the linear range of response was determining using a standard sample that was serially diluted. The level of interaction between two proteins was determined by diving the intensity value (obtained from Western blotting) of protein being co-immunoprecipitated (Tctex-1) by the intensity value of protein being immunoprecipitated (KIM-1). Graphs obtained were generated by using GraphPad Prism software (Graph Pad Software Inc., La Jolla, CA). Statistics were done in GraphPad Prism (Graph Pad Software Inc., La Jolla, CA) or IBM SPSS statistic 22 (IBM, Armonk, NY). The significance for the percentage of phagocytosis or immunoprecipitation densitometric analysis over phagocytosis time course was determined using one-way ANOVA (as indicated). All other analyses were done using an unpaired t-test where indicated. P-value of <0.05 was considered significant. Error bars represent the standard error of the mean.

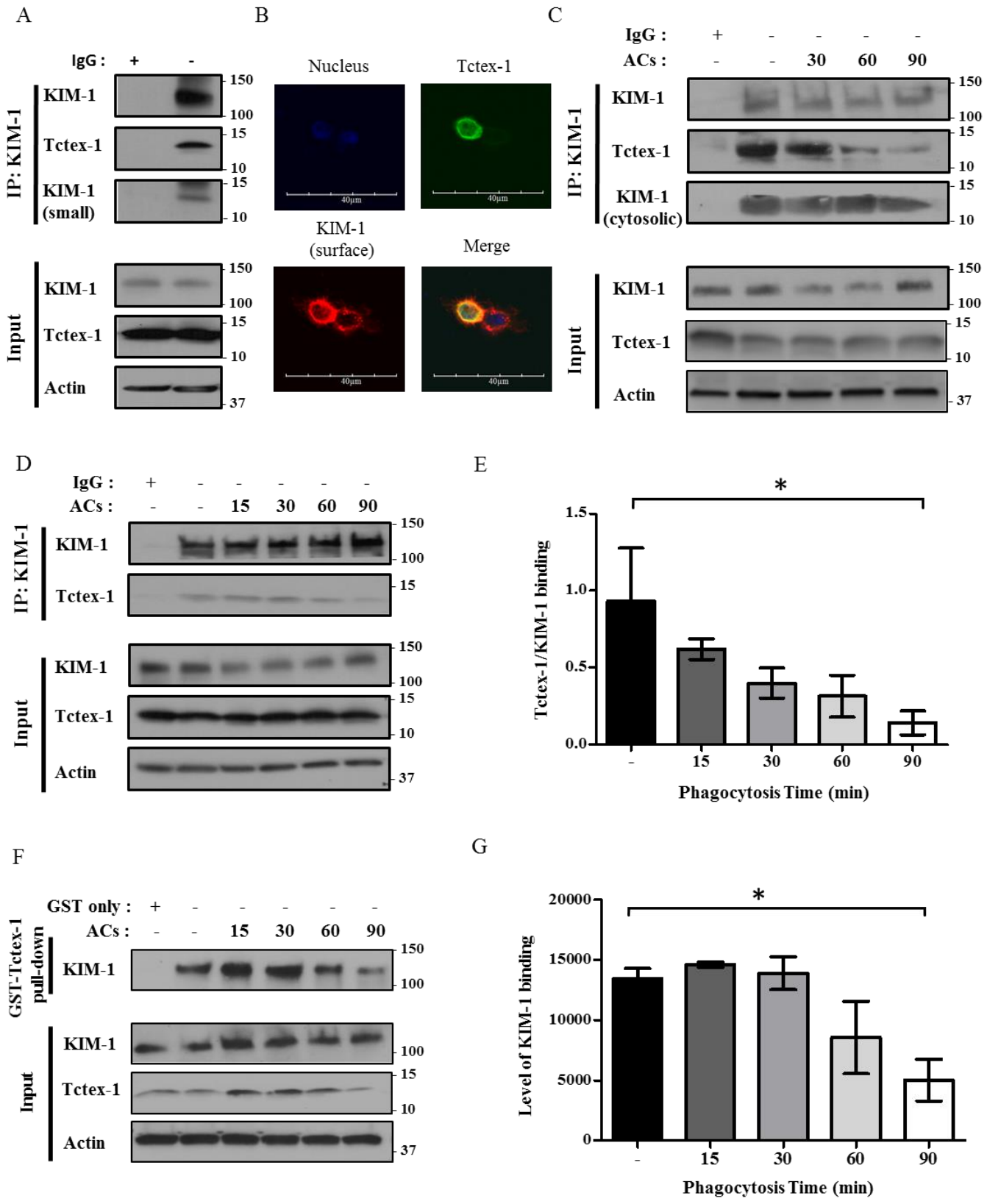
4.4. RESULTS

4.4.1. KIM-1 interacts with Tctex-1 during the early phase of phagocytosis

Previously, we had identified the alpha subunit of the heterotrimeric G12 protein as a novel KIM-1 interacting protein (Ismail *et al.*, 2015). As an alternative strategy to identify other potential KIM-1-interacting proteins, we performed a yeast two-hybrid screening using the cytoplasmic tail of KIM-1 as a bait plasmid and a HeLa cell cDNA library as a "fish/prey" plasmid (in collaboration with Dr. Zervos, Burnett School of Biomedical Sciences, University of Central Florida, Orlando, Florida). This identified two cDNA clones, one of which encoded for the dynein light chain protein, Tctex-1 (Lader *et al.*, 1989). Because of the important role that Tctex-1 plays in the modulation of actin cytoskeletal dynamics (Chuang *et al.*, 2005;Meiri *et al.*, 2012), and the requirement of the cytoskeleton for the formation of phagocytic arms in phagocytosis (Chimini *et al.*, 2000), we selected this candidate protein for further studies. To validate the results from the yeast two-hybrid screen, we performed co-immunoprecipitation (co-IP) experiments where HEK-293 cells were transfected with both human GFP-tagged KIM-1 and human flag-tagged Tctex-1 (Figure 4.1A). We further confirmed this interaction by detecting the co-localization of both

proteins when overexpressed by HEK-293 cells via immunofluorescent labelling and confocal microscopy imaging (Figure 4.1B).

Given the role of KIM-1 in the uptake of apoptotic cells (Ichimura *et al.*, 2008), we sought to determine whether the interaction between KIM-1 and Tctex-1 remains intact during the course of phagocytosis. Using co-IP experiments, we found a significant decrease in the interaction between KIM-1 and with Tctex-1 between 60 and 90 minutes after stimulation with apoptotic cells (later stages of phagocytosis) (Figure 4.1C). This exogenous overexpression of both proteins in HEK-293 cells provided evidence of the interaction, but did not necessarily reflect physiologic relevance. To address this, we utilized human renal adenocarcinoma cells (769-P) which endogenously and constitutively express both human KIM-1 (Bailly *et al.*, 2002) and Tctex-1. This confirmed the co-IP results obtained above, including the observed diminishment in interaction between KIM-1 and Tctex-1 during the later stages of phagocytosis (Figure 4.1D, E). To substantiate the interaction between KIM-1 and Tctex-1 *in vitro*, GST pull-down assays were performed. A fusion protein consisting of GST fused to full-length Tctex-1 (GST-Tctex-1) was used to test GST-Tctex-1 interaction with KIM-1 during the course of phagocytosis (Gauthier-Fisher *et al.*, 2009). As observed before, a significant reduction in the interaction between KIM-1 and GST-Tctex-1 was observed during the later stages of uptake of apoptotic cells (90 minutes) (Figure 4.1F, G). These data confirm that the interaction between KIM-1 and Tctex-1 is dynamic and appears to decline during the course of KIM-1-dependent phagocytosis.


A
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KIM-1
Tctex-1
KIM-1 (small)
Input
KIM-1
Tctex-1
Actin
150
100
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B
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Tctex-1
KIM-1 (surface)
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40μm
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IgG : + - - - -
ACs : - - 30 60 90
IP: KIM-1
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Tctex-1
KIM-1 (cytosolic)
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KIM-1
Tctex-1
Actin
D
IgG : + - - - - -
ACs : - - 15 30 60 90
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KIM-1
Tctex-1
Input
KIM-1
Tctex-1
Actin
E
Tctex-1/KIM-1 binding
1.5
1.0
0.5
0.0
*
- 15 30 60 90
Phagocytosis Time (min)
F
GST only : + - - - - -
ACs : - - 15 30 60 90
GST-Tctex-1 pull-down
KIM-1
Input
KIM-1
Tctex-1
Actin
G
Level of KIM-1 binding
20000
15000
10000
5000
0
*
- 15 30 60 90
Phagocytosis Time (min)

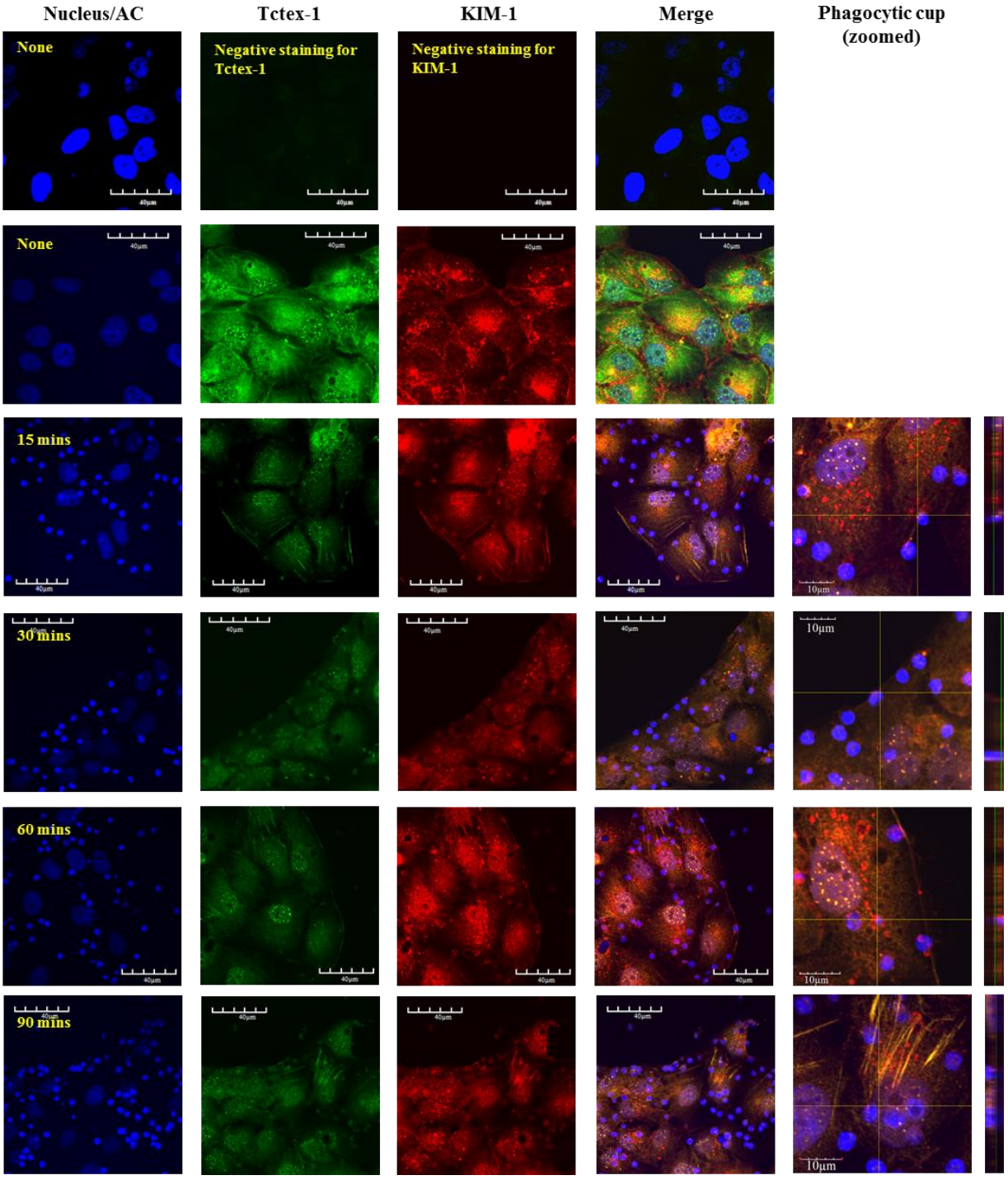
Figure 4.1. KIM-1 interacts with Tctex-1 during the early stages of phagocytosis.

A: Human embryonic kidney-293 (HEK-293) cells transfected with human KIM-1-GFP and flag-tagged Tctex-1 were lysed for immunoprecipitation (IP) using antibody against the cytosolic domain of KIM-1 or control non-specific mouse immunoglobulin antibody (IgG). ***B:*** Cells in A were visualized for interaction using immunostaining for KIM-1 (red) and Tctex-1 (green) as indicated in the experimental procedures section. Nuclei was stained with DAPI (blue) (600x, bar represents 40 μm). ***C:*** Cells in A were stimulated with apoptotic cells for various time points (minutes) and IP was performed using KIM-1 antibody or control mouse IgG. ***D:*** 769-P cells were stimulated with apoptotic cells and IP was performed similarly to C. ***E:*** The level of bound Tctex-1 to KIM-1 was determined based on densitometric values obtained by Western blotting (n=3, **p<0.01, ***p<0.001, one-way ANOVA). ***F:*** GST and GST-Tctex-1 pull-down was performed on 769-P cells that were left unstimulated or stimulated with apoptotic cells for indicated time (in minutes). ***G:*** The level of KIM-1 bound to GST-Tctex-1 was determined based on densitometric values obtained from Western blotting (n=3, **p<0.01, one-way ANOVA). All samples in A, C, D and F were analyzed by SDS-PAGE followed by immunoblotting with antibodies against KIM-1, Tctex-1 and actin. The input lane represents 5 % of the lysate. The data represents three independent experiments.

4.4.2. Intracellular colocalization of Tctex-1 and KIM-1 occurs during the early stages of phagocytosis

Confocal immunofluorescence analysis was performed to determine the degree of localization of both KIM-1 and Tctex-1 during the course of phagocytosis triggered by apoptotic cells. Using an antibody directed against the extracellular domain of KIM-1 to stain for endogenous KIM-1 expression in 769-P cells, we found KIM-1 to be distributed on the cell-surface and within the intracellular compartments reminiscent of the golgi apparatus as previously reported (Machacek *et al.*, 2009). On the other hand, immunostaining for endogenous Tctex-1 revealed diffuse cytoplasmic staining with some staining concentrated in the perinuclear region as previously shown (Campbell *et al*., 1998). Double staining showed that both Tctex-1 and KIM-1 colocalized in the cytoplasmic compartment of the cells prior to adding apoptotic cells (Figure 4.2A, none). During the course of the uptake of the apoptotic cells (15-90 minutes after addition of apoptotic cells), the colocalization between KIM-1 and Tctex-1 appears to decrease during later stages (Figure 4.2A). In order to quantify the colocalization images of KIM-1 and Tctex-1, we analyzed the results using JACoP software (as indicated in the experimental procedures section) to determine the Pearson's coefficient. We concluded that the level of colocalization decreased significantly during the later stages of phagocytosis (Figure 4.2B). Given the fact that some consider evaluating colocalization using Pearson's coefficient to be ambiguous since it incorporates noise and fluorescence intensity variation in analysis (Li *et al.*, 2011), we also analyzed the data according to Van Steensel's approach (Wong *et al.*, 2006). Similar to Pearson's coefficient, we found that the colocalization decreased significantly during later stages of apoptotic cells uptake (Figure 4.2C). These results suggest that during the process of KIM-1-mediated phagocytosis, there is a temporal decrease in the colocalization between KIM-1 and Tctex-1 in later stages.

A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Tctex-1
KIM-1
Merge
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Negative staining for KIM-1
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30 mins
60 mins
90 mins
40μm
10μm

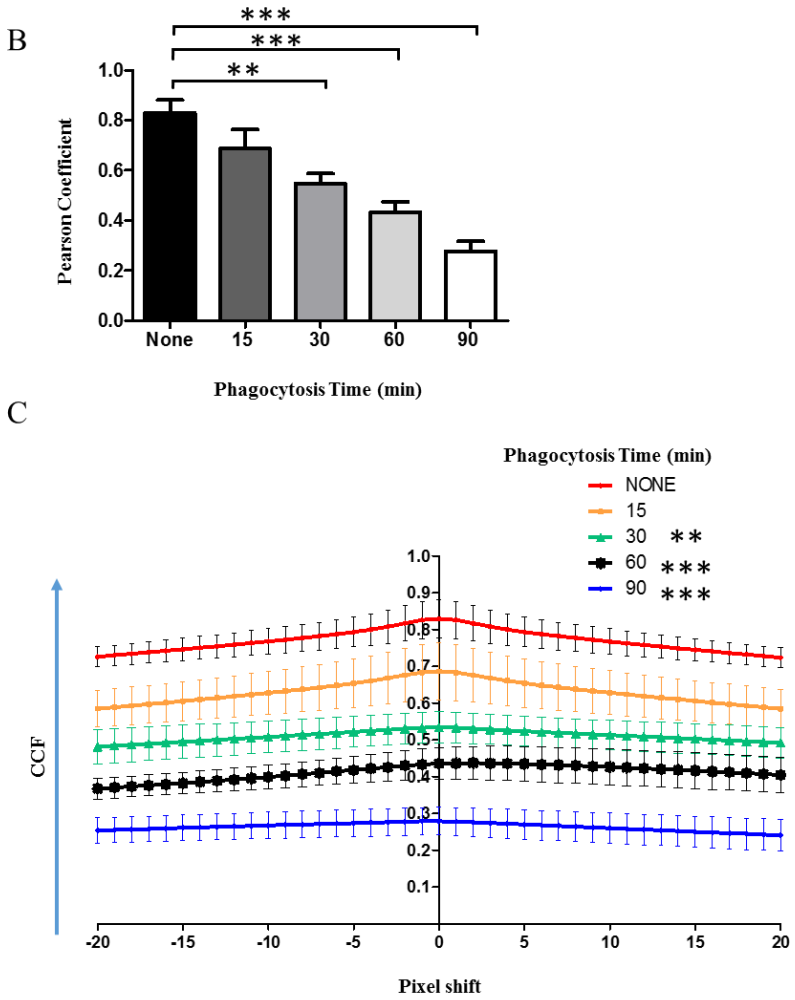


Figure 4.2. KIM-1 co-localizes with Tctex-1 during the early stages of phagocytosis.

A: 769-P cells were stimulated with apoptotic cells (blue) for various time points. Both KIM-1 and Tctex-1 were visualized using immunostaining for Tctex-1 (green) and KIM-1 (red) using Alexa-488 and Alex-555 labelled secondary antibodies as indicated in the experimental procedures section. Nuclei was counter-stained with DAPI (blue) (600x, bar represents 40μm). Further magnification is shown for the phagocytic cup formed by KIM-1 and apoptotic cells (bar represents 10 μm). Vertical lines appearing at 90 minutes of apoptotic cells-stimulation are suggestive of Tctex-1 trafficking along microtubules as indicated previously (Campbell *et al*., 1998). ***B:*** Images of colocalization of both KIM-1 and Tctex-1 over the time course of phagocytosis were analyzed using Pearson's coefficient (n=3, **p<0.01, ***p<0.001, one-way ANOVA). ***C:*** Colocalization of both KIM-1 and Tctex-1 during the course of uptake of apoptotic cells was analyzed using the Van Steensel's approach, where the cross correlation function (CCF) was calculated with a pixel shift of ±20 (n=3, **p<0.01, ***p<0.001 compared to none stimulated cells, one-way ANOVA). Quantification of colocalization score was assessed based on three random fields per sample and was done in three independent experiments.

4.4.3. Inhibition of KIM-1-mediated phagocytosis restores the KIM-1 and Tctex-1 interaction during the later stages of phagocytosis

Previous results showed that the interaction between KIM-1 and Tctex-1 was influenced by events occurring at later stages of phagocytosis. Generally, during later stages of the uptake of apoptotic cells, local remodeling of the underlying actin cytoskeleton and microtubule network was shown to be crucial for corpse uptake (Freeman *et al.*, 2014; Harrison *et al.*, 2002; Toda *et al.*, 2012). To determine the importance of actin and microtubule stability during KIM-1-mediated phagocytosis, we pretreated 769-P cells with cytochalasin D and nocodazole, inhibitors of actin filament polymerization and microtubule formation, respectively. These 769-P cells were subsequently fed fluorescently-labelled apoptotic cells and the level of uptake was determined by flow cytometry. As expected, there was a significant reduction in the level of the uptake of apoptotic cells compared to the no treatment control cells (Figure 4.3A). In order to determine whether actin and microtubule changes during phagocytosis contributed to the loss of KIM-1 and Tctex-1 interaction at later stages of phagocytosis, we performed co-IP following pretreatment with either vehicle, nocodazole or cytochalasin D and stimulated with apoptotic cells for 90 minutes. We observed a restoration of the interaction between KIM-1 and Tctex-1 following cytochalasin D and nocodazole treatment, which occurred at a similar level to non-stimulated cells (Figure 4.3B, C). Altogether, these results suggest that the inhibition of phagocytosis by disrupting the actin network or microtubule cytoskeleton would strengthen the interaction between KIM-1 and Tctex-1. Thus, this disruption of the KIM-1 and Tctex-1 interaction requires phagocytosis to proceed.

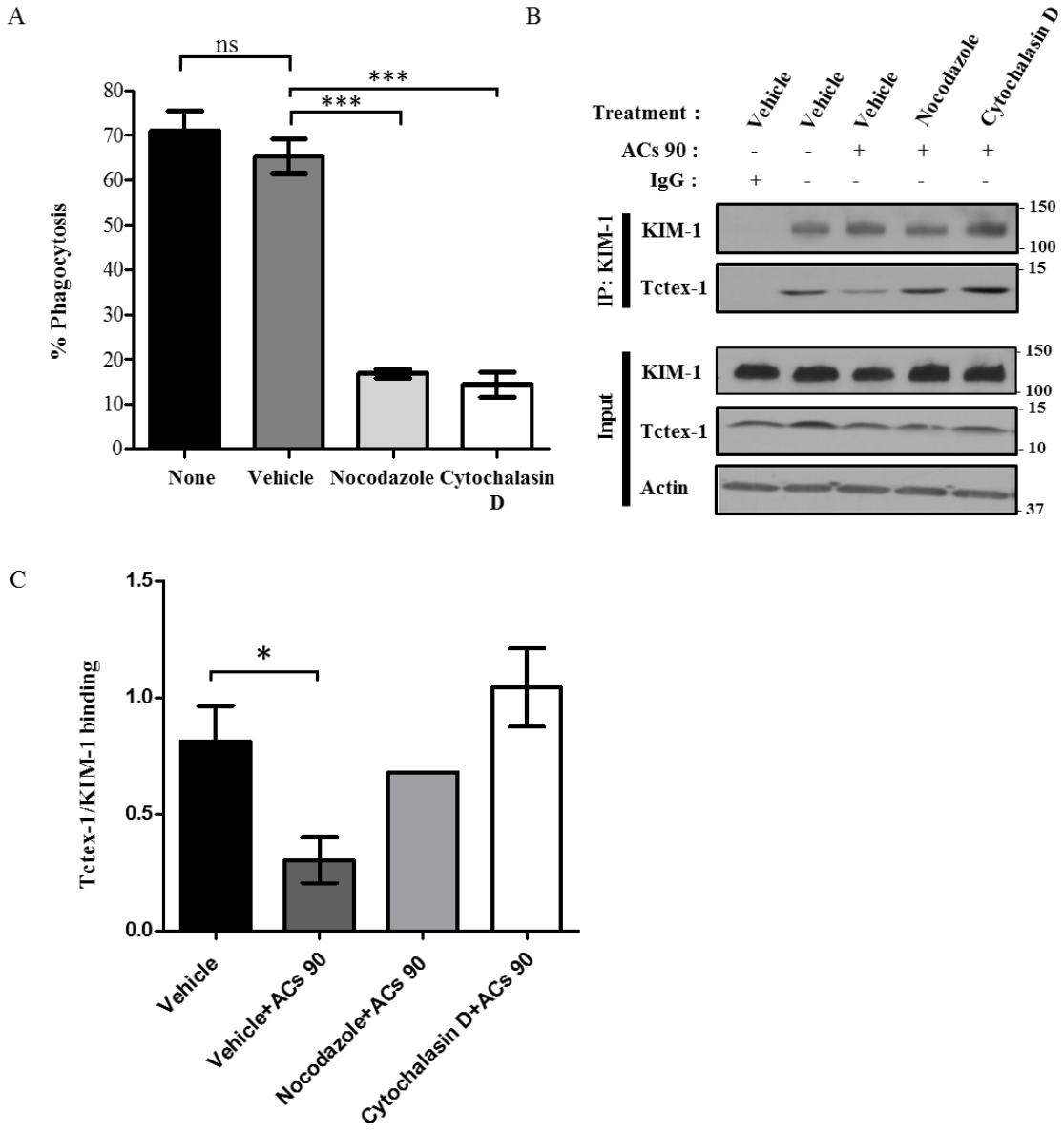


Figure 4.3. Inhibition of actin and microtubule dynamics restores the interaction between Tctex-1 and KIM-1 following apoptotic cell uptake.

A: 769-P cells were treated with vehicle (DMSO), nocodazole (10 µM) or cytochalasin D (1 µM) prior to adding fluorescently labelled apoptotic cells (pHrodo red) for 90 minutes (ACs 90). The percentage of uptake of the apoptotic cells as indicated by high pHrodo red fluorescence was measured by flow cytometry (n=3, ***p<0.001, unpaired t-test). ***B:*** Cells in A were either unstimulated or stimulated with apoptotic cells for 90 minutes and used for immunoprecipitation (IP) using KIM-1 antibody as described in the experimental procedures section. All samples were analyzed by SDS-PAGE followed by immunoblotting with antibodies against KIM-1, Tctex-1 and actin. The input lane represents 5 % of the lysate. ***C:*** Level of Tctex-1 bound to KIM-1 following different treatments was determined based on densitometric values obtained from three independent Western blotting experiments (n=3, *p<0.05, unpaired t-test).

4.4.4. Phosphomimetic mutant of Tctex-1 fails to interact with KIM-1, but does not affect KIM-1-mediated phagocytosis

Tctex-1 partakes in dynein dependent and independent functions, where the phosphorylation of Tctex-1 at Threonine-94 (Thr-94) has been shown to be a crucial step in the dissociation of Tctex-1 from the dynein light chain and consequently the microtubules (Chuang *et al.*, 2005; Song *et al.*, 2007). We sought to determine whether the phosphorylation status of Tctex-1 and its ability to bind dynein would influence its interaction with KIM-1. HEK-293 cells were transfected with KIM-1 and various Tctex-1 constructs. One of these plasmids is the phosphomimetic mutant of Tctex-1 (T94E), which fails to be incorporated into the dynein complex, resulting in a dynein-free form of Tctex-1 (Chuang *et al.*, 2005). As a control, a construct encoding an unphosphorylated mimic of Tctex-1 mutant (T94A) was used, since it has been shown to have a similar action to wild type Tctex-1 in its ability to bind to dynein complex, which is the opposite effect of T94E. We also compared the effects of these mutants to wild type Tctex-1. We then performed co-IP experiments to detect the binding of KIM-1 to the different forms of Tctex-1 either before or after stimulation with apoptotic cells for 90 minutes. The phosphomimetic mutant of Tctex-1 (T94E) showed a significant decrease in the interaction with KIM-1 regardless of whether the cells were stimulated with apoptotic cells or not (Figure 4.4A and B). On the other hand, both wild type and unphosphorylated mimic mutant (T94A) forms of Tctex-1 bound KIM-1 and showed no significant difference in their ability to interact with KIM-1. Next, we tested whether the phosphorylation status of Tctex-1 would have an effect on KIM-1-mediated phagocytosis. Following transfection of HEK-293 cells with KIM-1 and each individual Tctex-1 construct, cells were fed fluorescently-labelled apoptotic cells for 90 minutes and phagocytic ability was measured by flow cytometry (Figure 4.4C). Remarkably, no significant differences in phagocytic ability were seen between KIM-1-expressing HEK-293 cells that were transfected with either wild type, unphosphorylated mimic of Tctex-1 mutant (T94A) or the phosphomimetic mutant of Tctex-1 (T94E) (Figure 4.4D). Altogether, these results argued that the phosphorylation of Tctex-1 at Threonine-94 had an effect on the interaction between KIM-1 and Tctex-1, but do not impact KIM-1-mediated phagocytosis.

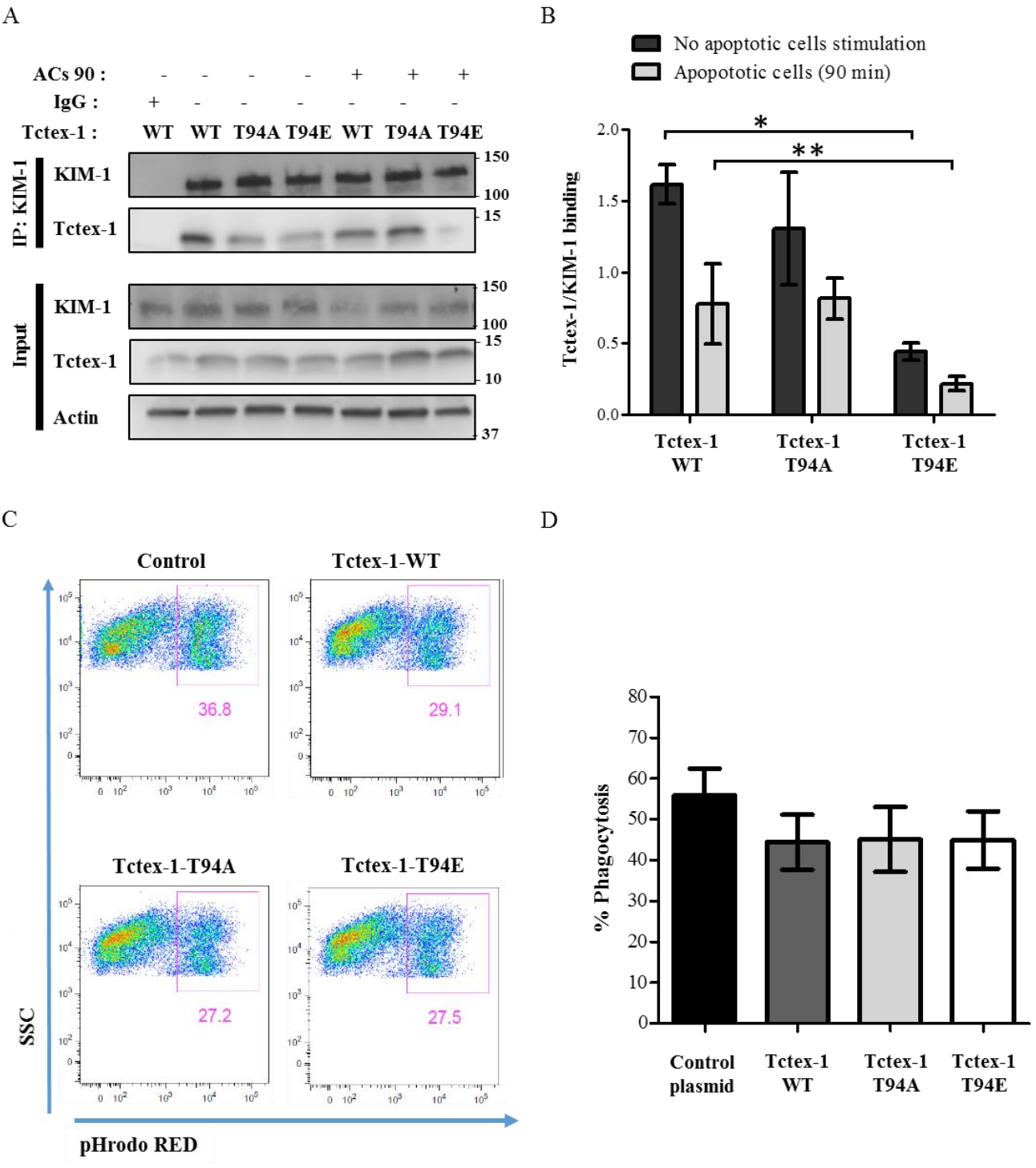
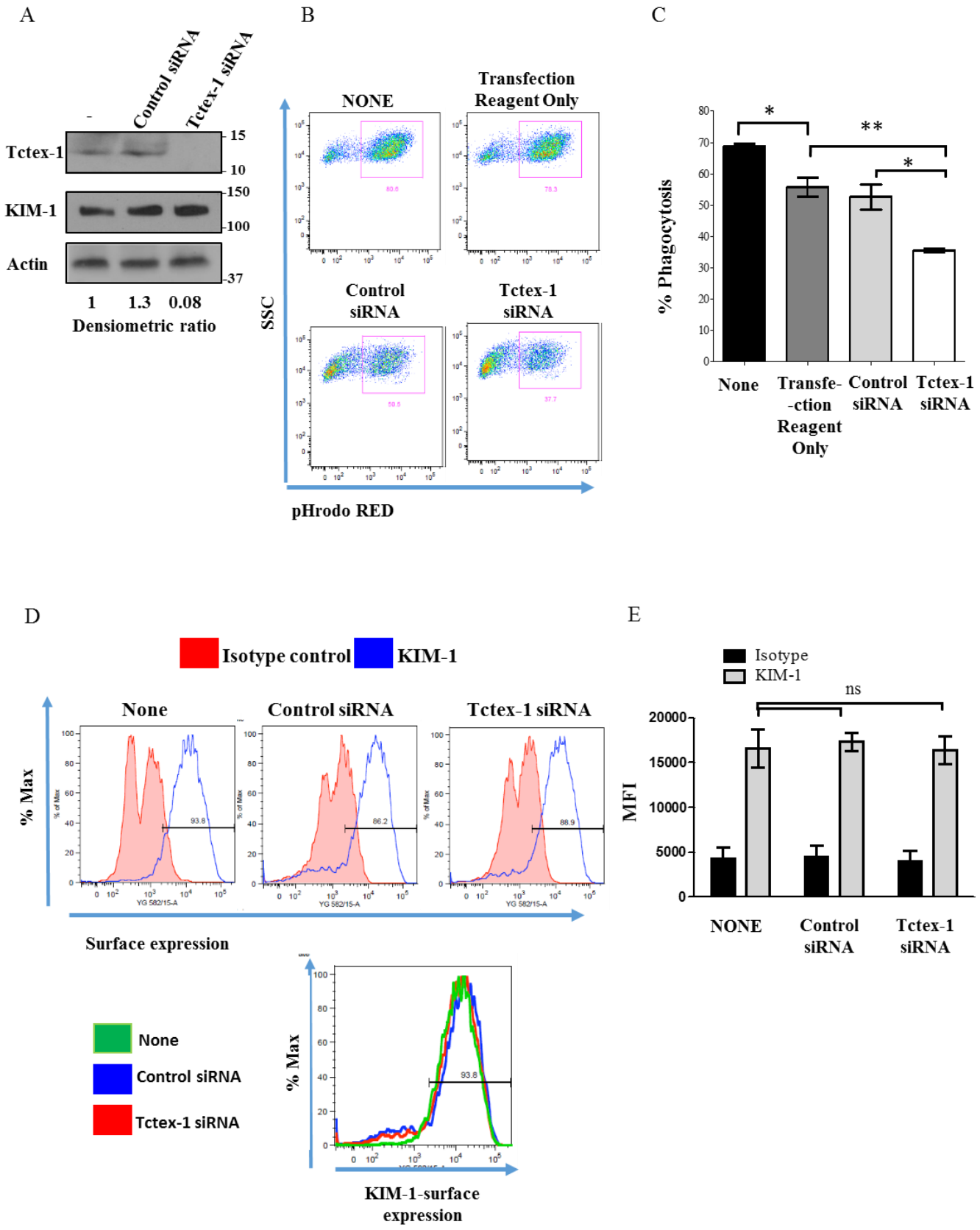

A
ACs 90 : - - - - + + +
IgG : + - - - - - -
Tctex-1 : WT WT T94A T94E WT T94A T94E
IP: KIM-1
KIM-1
Tctex-1
Input
KIM-1
Tctex-1
Actin
150
100
15
150
100
15
10
37
B
No apoptotic cells stimulation
Apopototic cells (90 min)
Tctex-1/KIM-1 binding
2.0
1.5
1.0
0.5
0.0
*
**
Tctex-1 WT
Tctex-1 T94A
Tctex-1 T94E
C
Control
Tctex-1-WT
Tctex-1-T94A
Tctex-1-T94E
36.8
29.1
27.2
27.5
SSC
pHrodo RED
D
% Phagocytosis
80
70
60
50
40
30
20
10
0
Control plasmid
Tctex-1 WT
Tctex-1 T94A
Tctex-1 T94E

Figure 4.4. Phosphomimetic mutant of Tctex-1 exhibits a reduced binding to KIM-1, but does not influence KIM-1-mediated phagocytosis.

A: HEK-293 cells were co-transfected with GFP-labelled KIM-1 and either control vector encoding flag-tag, wild type flag-tagged Tctex-1 (Tctex-1-WT), unphosphorylataed mimic of flag-tagged Tctex-1 (Tctex-1-T94A) or phosphomimetic mutant of flag-tagged Tctex-1 (Tctex-1-T94E). Cells were either unstimulated or stimulated with apoptotic cells for 90 minutes (ACs 90) and extracts were used for co-immunoprecipitation using KIM-1 antibody as described in the experimental procedures section. All samples were analyzed by immunoblotting with antibodies against KIM-1, Tctex-1 and actin. The input lane represents 5 % of the lysate. ***B:*** The level of Tctex-1 bound to KIM-1 was determined based on densitometric values acquired from Western blotting (n=3, *$p<0.05$, **$p<0.01$, unpaired t-test). ***C:*** Cells in A were fed apoptotic cells that were fluorescently labelled with pHrodo red for 90 minutes and the percent of uptake of apoptotic cells was determined by flow cytometry as shown in pHrodo red Vs. side scatter (SSC) plot. ***D:*** The percentage of phagocytosis was determined based on high pHrodo red fluorescence as measured by flow cytometry (n=4, not significant data, unpaired t-test).

4.4.5. Tctex-1 mediates phagocytosis by KIM-1

The above data characterized the interaction between KIM-1 and Tctex-1 during phagocytosis, but did not identify whether Tctex-1 was required for phagocytosis of apoptotic cells. To test this, we silenced Tctex-1 using siRNA in 769-P cells (Figure 4.5A) and carried out phagocytosis assays. When we compared the phagocytic capacity between cells left untransfected or transfected with control siRNA to the Tctex-1 siRNA treated cells, Tctex-1-silenced cells exhibited a significant decrease in the level of uptake of apoptotic cells following 90 minutes of incubation with apoptotic cells (Figure 4.5B and C). Since Tctex-1 had been shown to play a role in cellular cargo trafficking through its dynein-dependent function (Mueller *et al.*, 2002; Palmer *et al.*, 2011; Tai *et al.*, 1999), we wondered whether silencing Tctex-1 inhibited phagocytosis as a result of decreased trafficking of KIM-1 to the cell-surface. To this end, we measured KIM-1 surface expression in 769-P cells transfected with Tctex-1 siRNA and compared it to cells transfected with control siRNA (Figure 4.5D). Importantly, we did not observe any significant difference in KIM-1 surface expression between 769-P cells treated with Tctex-1 siRNA or control siRNA (Figure 4.5E). The above data suggested that Tctex-1 plays an important positive role in KIM-1-mediated phagocytosis independent of affecting surface expression of KIM-1.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-
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Tctex-1 siRNA
Tctex-1
KIM-1
Actin
15
10
150
100
37
1
1.3
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Densiometric ratio
B
NONE
Transfection Reagent Only
Control siRNA
Tctex-1 siRNA
SSC
pHrodo RED
C
% Phagocytosis
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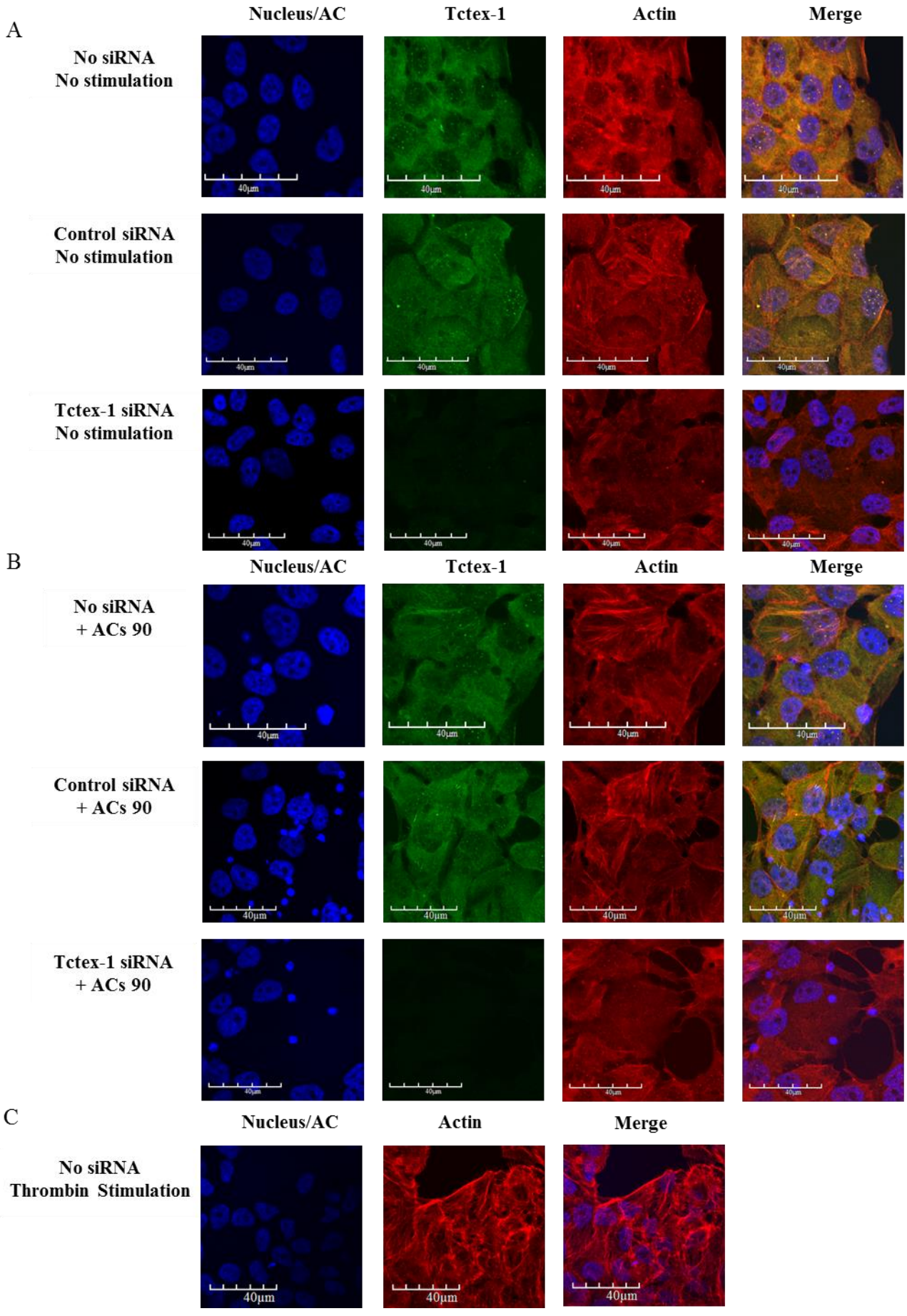
E
Isotype
KIM-1
ns
MFI
NONE
Control siRNA
Tctex-1 siRNA

Figure 4.5. Tctex-1 expression in required for phagocytosis by KIM-1, but does not affect its surface expression.

A: 769-P cells were left untransfected or transfected with either control siRNA or Tctex-1 siRNA for 24 hours. Cells were lysed and protein levels of Tctex-1, KIM-1, and actin were determined by immunoblotting. ***B:*** Cells in A and cells treated with transfection reagent only were fed fluorescently labelled apoptotic cells (pHrodo red) for 90 minutes. The percent of uptake of apoptotic cells was determined by flow cytometry as shown in the plots of side-scatter (SSC) vs. pHrodo red (apoptotic cells). ***C:*** The percentage of phagocytosis by cells in B was measured by flow cytometry and shown as representation of five independent experiments (n=5, *$p<0.05$, **$p<0.01$, unpaired t-test). ***D:*** Cells in A were stained for surface KIM-1 using PE-conjugated anti-KIM-1 antibody and its isotype control, without permeabilization, and the level of KIM-1-surface expression was determined by flow cytometry. ***E:*** Representative graph of the mean fluorescence intensity (MFI) of the flow cytometry data from D was shown as representative of four independent experiments (n=4, ns= not significant, unpaired t-test).

Since Tctex-1 had been previously shown to play a pivotal role in regulating the actin cytoskeleton in neuronal cells, we assessed whether silencing Tctex-1 would have any effect on actin cytoskeleton remodeling that happens during KIM-1-mediated phagocytosis. To this end, we silenced Tctex-1 expression with siRNA, stained the cells with rhodamine phalloidin to detect F-actin and took confocal microscopy images to look for cytoskeletal changes. The majority of cells transfected with Tctex-1 siRNA exhibited highly disorganized actin fibers with poorly bundled filaments in the center of the cells as compared to untreated cells or cells transfected with control siRNA (Figure 4.6A). The phenotype of Tctex-1-silenced cells did not change following apoptotic cell uptake (Figure 4.6B). To test the responsiveness of these cells to actin reorganization stimulus, we treated 769-P with 10 nM thrombin and found a striking reorganization of F-actin and an increase in fluorescence intensity, as previously shown (Tai *et al.*, 1999) (Figure 4.6C). All in all, this suggest that Tctex-1 might play an important role in KIM-1-mediated phagocytosis potentially though its ability to modulate actin cytoskeleton.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Actin
Merge
No siRNA
+ ACs 90
Control siRNA
+ ACs 90
Tctex-1 siRNA
+ ACs 90
C
Nucleus/AC
Actin
Merge
No siRNA
Thrombin Stimulation
40µm

Figure 4.6. Silencing Tctex-1 induces changes in the actin cytoskeleton that persist after stimulation with apoptotic cells

A: 769-P cells were transfected with either transfection reagent alone, control siRNA or Tctex-1 siRNA for 24 hours. Cells were fixed and stained for F-actin using rhodamine phalloidin (red) and immunostained for intracellular Tctex-1 using Tctex-1 antibody and secondary Alexa-488 antibody (green), as described in the experimental procedures section. Nuclei was counter-stained with DAPI (blue) (600x, bar represents 40 μm). ***B:*** Cells in A were fed apoptotic cells (blue) for 90 minutes and actin and Tctex-1 staining was performed to detect any F-actin changes following addition of apoptotic cells. ***C:*** As a control, 769-P cells were stimulated with thrombin (10 nM) and actin staining was done to confirm the cells' responsiveness to an unrelated stimulus (thrombin).

4.4.6. Tctex-1 modulates the actin cytoskeleton through a RhoGTPase-independent pathway during KIM-1-mediated uptake of apoptotic cells

Based on the above experiments, it became obvious that cells transfected with siRNA targeting Tctex-1 showed a decrease in apoptotic cell uptake and had consistently less actin structures, similar to what was previously reported (Meiri *et al.*, 2012). Tctex-1 has been shown previously to modulate the actin cytoskeleton through activation of Rac1 (Albert *et al.*, 2000; Campbell *et al.*, 1998; Chuang *et al.*, 2005) and RhoA (Conde *et al.*, 2010; McIntire *et al.*, 2001; Meiri *et al.*, 2009). In term of phagocytosis, it is well established that these Rho GTPases play important roles in actin cytoskeleton organization (Ridley, 2001; Ridley *et al.*, 1992). RhoA and Rac1 have been proposed to have opposing effects on the actin cytoskeleton (Hall *et al.*, 2000; Meyer *et al.*, 2003; Mueller *et al.*, 2002; Nakaya *et al.*, 2006). In Chapter 3, we showed that inhibition of RhoA significantly increased KIM-1-dependent uptake of apoptotic cells (Figure 3.5A and B). On the other hand, Rac1 was found to have a positive role in KIM-1-mediated phagocytosis (Figure 3.7A and B). With this in mind, the level of Rac1 activity was measured following 769-P cell incubation with apoptotic cells over the course of phagocytosis (0-90 minutes). Surprisingly, Rac1 activity seemed to increase minimally during uptake of apoptotic cells (Figure 4.7A). To test whether Tctex-1 silencing modified the actin cytoskeleton via Rac1, we measured Rac1 activation of 769-P cells with Tctex-1 siRNA or control siRNA following stimulation with apoptotic cells. The level of Rac1 activation was not significantly altered following silencing of Tctex-1 during the course of apoptotic cell engulfment (Figure 4.7B). To test the role of RhoA, we first measured the level of active RhoA during KIM-1-mediated phagocytosis. We found a steady increase in the level of active RhoA during the uptake process of apoptotic cells by 769-P cells (Figure 4.7C). This result was consistent with our previous data measuring the level of active RhoA during uptake of apoptotic cells in HEK-293 cells expressing human KIM-1 (Figure 3.6 A). Moreover, upon silencing of Tctex-1 in 769-P cells, we did not observe any change in level of active RhoA when compared to control siRNA-transfected cells (Figure 4.7D).

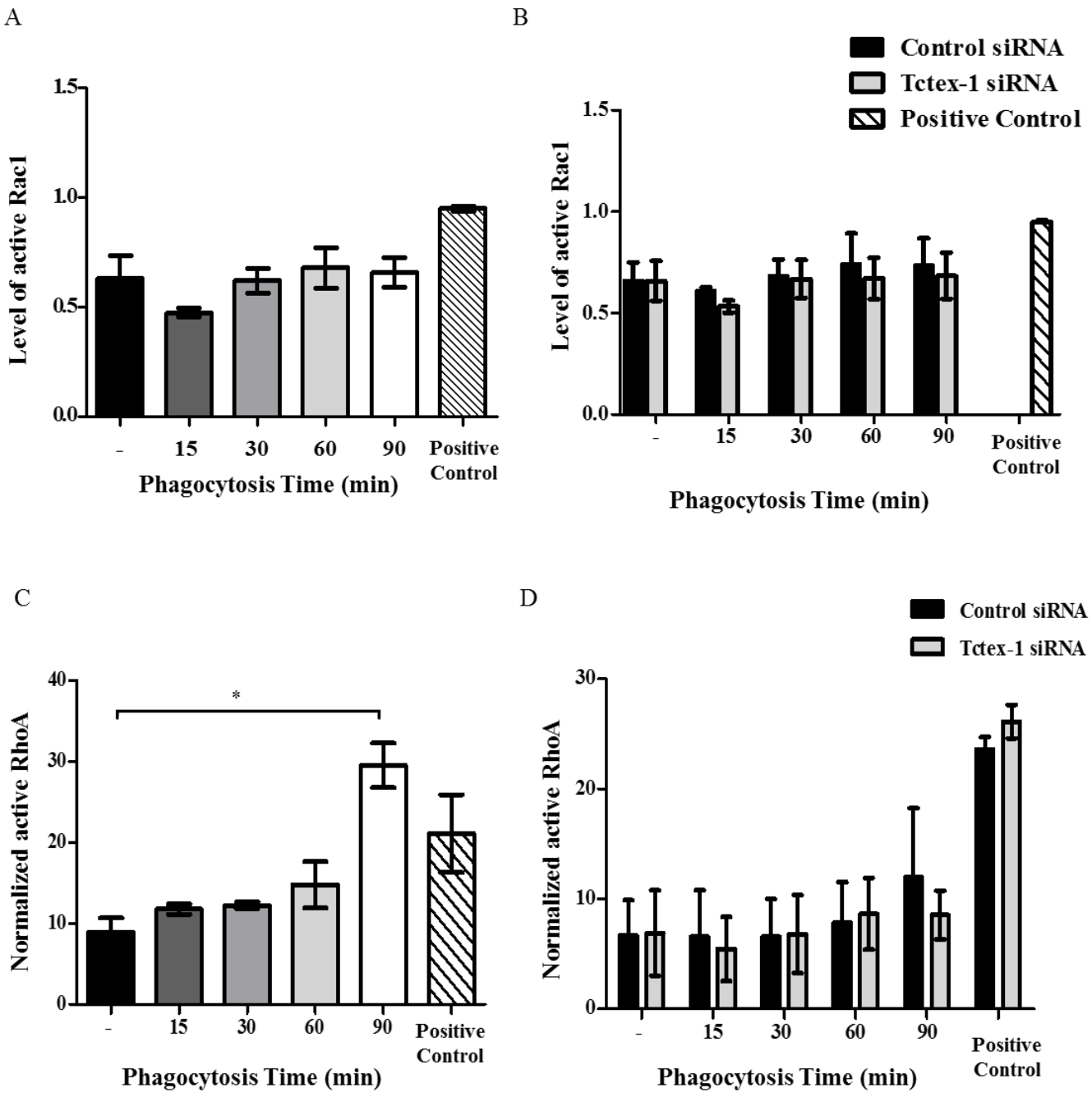
A
Level of active Rac1
1.5
1.0
0.5
0.0
-
15
30
60
90
Positive Control
Phagocytosis Time (min)
B
Control siRNA
Tctex-1 siRNA
Positive Control
Level of active Rac1
1.5
1.0
0.5
0.0
-
15
30
60
90
Positive Control
Phagocytosis Time (min)
C
Normalized active RhoA
40
30
20
10
0
*
-
15
30
60
90
Positive Control
Phagocytosis Time (min)
D
Control siRNA
Tctex-1 siRNA
Normalized active RhoA
30
20
10
0
-
15
30
60
90
Positive Control
Phagocytosis Time (min)

Figure 4.7. Tctex-1 regulates the actin cytoskeleton independent of Rho GTPases in KIM-1-mediated phagocytosis.

A: 769-P cells were fed apoptotic cells for various time periods (15-90 minutes) and the level of Rac1 activation was measured by G-LISA Rac1 activation kit (Cytoskeleton, Denver, CO). (n=4, all not significant, one-way ANOVA). ***B***: 769-P cells were transfected with either control siRNA or Tctex-1 siRNA for 24 hours. These cells were fed apoptotic cells for various time periods and the level of Rac1 activation was graphed (n=5, all not significant, unpaired t-test). Treatment of 769-P cells transfected with control siRNA with 5 % serum after serum starvation overnight served as a positive control for Rac1 activation (Curtiss *et al*., 2011). ***C***: 769-P cells fed apoptotic cells (15-90 minutes) were subjected to active RhoA and total RhoA assay measurements as determined by the active G-LISA RhoA activation assay and the total RhoA ELISA kit (Cytoskeleton, Denver, CO), respectively. Normalization of active RhoA was done according to manufacturer's recommendation and was graphed to represent data (n=3, all not significant, one-way ANOVA). ***D***: 769-P cells were transfected with either control siRNA or Tctex-1 siRNA for 24 hours. These cells were fed apoptotic cells for various time periods (minutes) and the level of normalized active RhoA was determined as in *C*. Cells treated with 10 µM Nocodazole for 15 minutes served as a positive control for RhoA activation (Martin *et al*., 2001) (n=3, all not significant, unpaired t-test).

4.5. DISCUSSION

KIM-1 is a phosphatidylserine receptor that is specifically upregulated by tubular epithelial cells (TECs) during ischemic or toxic AKI and enables TECs to clear apoptotic and necrotic cell debris (Ichimura *et al.*, 2008; Ichimura *et al.*, 2004). In the previous chapter, we showed that TECs require KIM-1 expression to mediate the clearance of apoptotic cells. This necessitated an understanding of the intracellular signalling pathway(s) downstream of KIM-1. Here we identify a novel functional interaction between the actin cytoskeleton regulatory protein Tctex-1 (Chuang *et al.*, 2005; Meiri *et al.*, 2012) and KIM-1. We have shown that the interaction between KIM-1 and Tctex-1 is a dynamic process that appears to dissipate during the later stages of apoptotic cell engulfment. Inhibition of phagocytosis by inhibiting actin and microtubule polymerization also restored KIM-1 binding to Tctex-1 during the later stages of phagocytosis. Furthermore, we have shown that phosphorylation of Tctex-1 at Threonine-94 diminishes interaction between KIM-1 and Tctex-1 regardless of whether apoptotic cells were added or not. Using siRNA against Tctex-1, we uncovered a novel positive role of Tctex-1 in KIM-1-mediated phagocytosis. Finally, we found that Tctex-1 probably mediates KIM-1 function through a non-canonical pathway that is independent of Rho GTPases.

The loss of interaction between KIM-1 and Tctex-1 during the later stages of phagocytosis is still a puzzling observation, which may suggest a role for Tctex-1 in corpse engulfment. Recently, it was reported that TIM-1 (or KIM-1) undergoes dynamic cycling through clathrin-dependent vesicles to mediate the lysosomal degradation of NUR77 (Balasubramanian, *et al.*, 2012), a nuclear receptor implicated in apoptosis and cell survival (Machacek *et al.*, 2009). Throughout our experiments, we did not find any evidence of Tctex-1 degradation during the entire period of phagocytosis (90 minutes) as determined by Western blot. Given what is known about Tctex-1 in the trafficking of various cell-surface receptors (Machado *et al.*, 2003; Mueller *et al.*, 2002; Tai *et al.*, 1998), it was surprising that Tctex-1 did not regulate KIM-1 trafficking to the cell-surface.

The phosphorylation of Tctex-1 at Threonine-94 has been shown to promote Tctex-1 dissociation from the dynein light chain complex which is required to promote dynein-independent functions (Chuang *et al.*, 2005; Li *et al.*, 2011). For example, phosphorylation of Tctex-1 has been linked to enhanced neurite outgrowth activity (Chuang *et al.*, 2005), such as cilliary disassembly, leading to cell cycle progression and S-phase entry for neural progenitor cells (Li *et al.*, 2011). The

perplexing data with regards to the lack of an effect of phosphomimetic mutant of Tctex-1 (T94E) on phagocytosis is likely reflective of the persistent expression of a low level wild type endogenous Tctex-1, which confounded the data. This can be tested by simultaneously silencing endogenous Tctex-1 with siRNA and then transfecting the mutant form Tctex-1 as described by others (Meiri *et al.*, 2012).

Cytoplasmic Tctex-1 has been shown to regulate microtubules and the actin cytoskeleton through activation of Rac1 and RhoA, respectively (Chuang *et al.*, 2005; Meiri *et al.*, 2012). Recently, it was shown that Tctex-1 sequesters a key Rho guanine exchange factor, GEF-H1 and tethers it to microtubules allowing Rac1 to execute its function unopposed by RhoA (McIntire *et al.*, 2001; Meiri *et al.*, 2012). Upon activation, Tctex-1 was shown to release GEF-H1 tethered to microtubules, allowing it to then activate RhoA by promoting GDP exchange for GTP. Because we previously demonstrated that Rac1 is required for KIM-1-dependent phagocytosis, we tested whether Tctex-1 relay its signalling through Rac1 in KIM-1-expressing cells. We failed to see any difference in the level of Rac1 activation upon stimulation with apoptotic cells or when Tctex-1 expression was silenced. Thus, we propose that Rac1 is involved in phagocytosis independently of Tctex-1. Furthermore, our preliminary data failing to show RhoA activation downstream of Tctex-1 suggests that RhoA is not involved in mediating Tctex-1 signalling during KIM-1-mediated phagocytosis. Moreover, we propose that other GTPases (e.g. cdc42) or non-GTPase pathways might be involved in KIM-1-mediated phagocytosis downstream of Tctex-1 (Conde *et al.*, 2010). Taking this into consideration, we could envision that dynein-independent Tctex-1 (or phosphorylated Tctex-1) might have a role in these KIM-1-expressing cells.

To our knowledge, this is the first study that has explored a physiological function for Tctex-1 in kidney cells, and moreover in phagocytosis. The logical next step would be to determine if Tctex-1 has any role in AKI or repair. Given that Tctex-1 has been shown to be involved in regulating cilium resorption during neurogenesis (Li *et al.*, 2011), a potential mechanism for involvement in AKI might be through regulation of the cilium of renal TECs, which act as flow sensors (Kotsis *et al.*, 2007). In fact, a number of human renal syndromes, including polycystic kidney disease, Bardet-Biedl syndrome and nephronophthisis (Balasubramanian *et al.*, 2012), have been shown to involve dysfunctional cilia, a microtubule-based organelle. Interestingly, KIM-1 is expressed on TECs during cyst development in a mouse model for autosomal dominant polycystic kidney disease (Kuehn *et al.*, 2002), and was later implicated in cilliary calcium signalling via its

interaction with the cilliary protein, polycystin 2 (TRPP2) (Kotsis *et al.*, 2007; Kuehn *et al.*, 2007). Our present work linking Tctex-1 to KIM-1-mediated phagocytosis by TECs for the first time is likely to lead to new avenues of research into the role of Tctex-1 in AKI and other chronic kidney diseases.

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Chapter 5

General Discussion

The general discussion was written by Ola Ziyad Ismail, and edited by Dr. Gunaratnam

5.1. SUMMARY AND GENERAL DISCUSSION

AKI is a clinically important disorder that is most commonly caused by an ischemic or toxic insult to the kidney (Bellomo *et al.*, 2004; Bonventre *et al.*, 2011; Lattanzio *et al.*, 2009; Mehta *et al.*, 2007). The pathophysiology of ischemic AKI is highly complicated, involving multiple processes including inflammation, tubular injury and vascular damage (Bonventre *et al.*, 2011; Togel *et al.*, 2014). Renal ischemia reperfusion injury results from the transient cessation of blood flow to an organ with immediate oxygen deprivation, followed by restoration of blood flow (reperfusion) (Bonventre *et al.*, 2011; Le Dorze *et al.*, 2009). This process leads to generation of reactive oxygen species (ROS). ROS are a major contributor to cellular injury (Oliver *et al.*, 1990), where they exhibit their pathogenic effects by causing protein oxidation, lipid peroxidation, and DNA damage (Bonventre *et al.*, 2011; Devarajan, 2005, 2006; Kaushal *et al.*, 2004). Depending on the severity of cellular injury and the resiliency of affected cells, TECs can recover from sub-lethal injury or undergo apoptosis, necrosis or necroptosis (Linkermann *et al.*, 2013; Vanden Berghe *et al.*, 2014). The failure to clear apoptotic cells can result in secondary necrosis, triggering further inflammation and secondary tissue damage (Balasubramanian *et al.*, 2012; Bonventre *et al.*, 2011; Devarajan, 2006; Yang *et al.*, 2015). KIM-1 was identified as a non-myeloid phosphatidylserine receptor that is expressed on injured renal proximal TECs that enables them to clear apoptotic and necrotic cells (Ichimura *et al.*, 2008). The aim of this thesis was to better understand the physiological function of KIM-1 in AKI *in vivo* and to uncover how KIM-1 mediates direct signalling to enable phagocytosis by TECs. To this end, we identified two novel KIM-1 interacting proteins, the alpha subunit of the heterotrimeric G12 protein (Gα12), and the dynein light chain protein Tctex-1, and characterized their respective roles in phagocytosis by TECs.

In Chapter 2, we discovered that the cytosolic domain of KIM-1 interacts with Gα12 to block its activation by blocking exchange of GDP for GTP. Previously, Yu *et al*., demonstrated that Gα12 is activated by ROS during IRI and that this promoted the disruption of junctional complexes via Src activation, thereby intensifying renal tissue damage (Yu *et al.*, 2012). This led us to uncover that TECs and kidneys from Kim-1-deficient mice exhibit exaggerated renal Gα12 and Src activation following exposure to hydrogen peroxide, a situation that mimics renal IRI *in vitro*. This resulted in worse renal dysfunction and kidney damage in Kim-1-deficient mice

subjected to renal insult compared to wild type mice. Taken together, our findings in Chapter 2 suggest that KIM-1 protects against Gα12-mediated tissue damage during ischemic AKI.

Shortly after our findings were published, Yang *et al.*, (2015) published that transgenic mice expressing a mucin-domain mutant KIM-1 (Kim-1$^{\Delta mucin}$), which is unable to recognize apoptotic cells, are unable to down regulate innate immune responses and failed to protect mice from ischemic or toxin (cisplatin) induced AKI (Yang *et al.*, 2015). This group proposed two mechanisms to explain how KIM-1 was protective in AKI: (1) via phagocytic clearance of apoptotic debris by KIM-1-expressing tubular cells and prevention of tubular obstruction; (2) via down-regulation of proinflammatory NF-κB signalling in tubular cells by phosphorylated KIM-1 upon recognition of apoptotic bodies. An important distinction between our Kim-1 knockout and the Kim-1$^{\Delta mucin}$ mice is that Kim-1$^{\Delta mucin}$ mice still express a full-length Kim-1 including its cytosolic domain (albeit lacking a portion of the mucin domain) at levels of expression comparable to wild type Kim-1 in the injured kidney (Yang *et al.*, 2015), whereas our mice completely lack the expression of Kim-1 (Wang *et al.*, 2010; Ismail *et al.*, 2015). Also, while Yang *et al.*, (2015) found that tubular epithelial cells isolated from the Kim-1$^{\Delta mucin}$ mutant mice had decreased ability to clear apoptotic cells (Yang *et al.*, 2015), tubular cells from our Kim-1-deficient mice exhibited a near complete defect in the clearance of apoptotic cells (Ismail *et al.*, 2015), likely reflecting a complete absence of the protein. As the interaction of Gα12 with Kim-1 occurs independent of apoptotic cell recognition and via the cytosolic domain, it is more than likely that the TECs from the Kim-1$^{\Delta mucin}$ mutant mice have intact Gα12-inhibitor capacity during renal IRI. However, this needs to be confirmed. We propose that the more severe phenotype of the Kim-1 deficient mice during IRI is explained by deficiencies in phagocytosis, and aberrant NF-κB and Gα12 signalling. All in all, our findings support the growing notion that KIM-1 is protective during ischemic AKI.

In Chapter 3, we investigated the role of Gα12 in KIM-1-mediated phagocytic signalling. We found that KIM-1 interacts with Gα12 and suppresses its activation to permit phagocytosis. Gα12 inhibition is required for efficient phagocytosis because it negatively regulates the phagocytic process via its known downstream mediator, RhoA. We also discovered opposing roles of RhoA and Rac1 during KIM-1-dependent uptake of apoptotic cells. Whereas RhoA seems to have an inhibitory role, Rac1 seems to have a stimulatory role on phagocytosis. This has also been shown to be the case in various professional phagocytes (Caron *et al.*, 1998; Chimini *et al.*, 2000). Our work is the first to demonstrate a role of Gα12 in phagocytosis and in KIM-1 signaling during

acute injury. Since KIM-1 expression is absolutely required for uptake of apoptotic cells by primary TECs, our findings have broad implications for understanding tubular cell behaviour after AKI.

Taken together, our findings in both Chapter 2 and 3 suggest that KIM-1 upregulation on TECs protects the kidney from ischemic AKI by at least two distinct mechanisms that involve inhibition of Gα12. First, KIM-1 blocks ROS-mediated activation of damaging Gα12 signalling via Src kinase, which may also enhance renal recovery after tissue damage as seen in Gα12 mice (Yu *et al.*, 2012). Second, KIM-1-suppression of Gα12 is required for efficient phagocytosis by tubular cells to clear cellular debris and mitigate inflammation (Yang *et al.*, 2015). These findings are consistent with the previously proposed role for KIM-1 as a regulator of immune function (Freeman *et al.*, 2010) that is upregulated during ischemic or toxin induced AKI (Ichimura *et al.*, 2008; Ichimura *et al.*, 2012). Our findings offer new insights into the down-stream signalling that takes place in tubular cells during ischemia-reperfusion injury.

Gα12 has been shown to a play a direct role in tight junction complex disassembly and thereby contributes to paracellular permeability of polarized epithelial cells (Meyer *et al.*, 2003; Meyer *et al.*, 2002; Sabath *et al.*, 2008). Once activated, Gα12 interacts with the heat shock protein (HSP) 90 chaperone to bring about phosphorylation and activation of Src kinase (Sabath *et al.*, 2008). Phosphorylation of scaffolding proteins, such as zona occludens-1(ZO-1), by activated Src is ultimately what leads to destabilization of tight junction complexes (Meyer *et al.*, 2003; Meyer *et al.*, 2002). Aberrant epithelial tight junction function occurs in several kidney diseases (Balasubramanian *et al.*, 2012), including ischemia-reperfusion injury (Balasubramanian *et al.*, 2012; Yu *et al.*, 2012). During AKI, a leaky epithelium permits the backleak of filtered or secreted electrolytes and waste products back into the circulation.

The importance of phagocytic clearance of apoptotic cells by TECs during AKI is now becoming more clear (Ismail *et al.*, 2015; Yang *et al.* 2015) and we are beginning to realize the full impact of aberrant Gα12 signalling during ischemic AKI, and the importance of KIM-1 in limiting this process (Ismail *et al.*, 2015). While Denker and colleagues have suggested that the harmful effect of ROS generated during renal IRI is at least in part mediated by activated Gα12 (Yu *et al.*, 2012), the mechanism of tissue damage caused by exaggerated Gα12 signalling is less clear. Yu *et al.* have suggested that aberrant Gα12 activation leads to impaired renal function and tissue damage during IRI as a result of Gα12 downstream effects on the disassembly and improper

reassembly of tight junction complexes (Figure 5.1A and B), the latter of which can impair renal recovery (Yu *et al.*, 2012). Whether these tight junction abnormalities affect phagocytic clearance of apoptotic cells by TECs is not known, but recent findings by Fornetti *et al.* suggest that this might be the case (Fornetti *et al.*, 2015). Whether KIM-1 protects from tissue damage by indirectly blocking the disruption of tight junctions by activated Gα12 remains to be tested.

In addition to its role in deregulating tight junction proteins, activated Gα12 is also a potent activator of RhoA signalling (Figure 5.1B) (Buhl *et al.*, 1995). Aberrant Rho signalling has been shown to be detrimental in AKI, and treatment with Rho kinase inhibitors can attenuate renal ischemia-reperfusion injury (Prakash *et al.*, 2008). The detrimental effects of activated Gα12 during AKI may be in part be caused by its effects on the actin cytoskeletal reorganization (Molitoris, 2004). Indeed, cytoskeletal dysregulation is a hallmark of AKI (J. V. Bonventre *et al.*, 2011). Our work shows that following IRI, KIM-1 upregulation on TECs allows for the suppression of Gα12 activation which inhibits Src and RhoA activation (Figure 5.1C). While Denker and colleagues showed that the Gα12 effects on tight junction disassembly was not mediated by RhoA (Yu *et al.*, 2012), our work highlights the importance of inhibiting RhoA in phagocytosis (Figure 5.1D). Given that phagocytic clearance of apoptotic cells plays a crucial protective role in renal injury and repair during AKI (Ichimura *et al.*, 2008; Yang *et al.*, 2015, Ismail *et al.*, 2015) and that activated Gα12 inhibits this process, uncovering the detailed mechanism of Gα12-inhibition of phagocytosis (i.e. via tight junctions) may help identify novel therapeutic strategies for AKI.

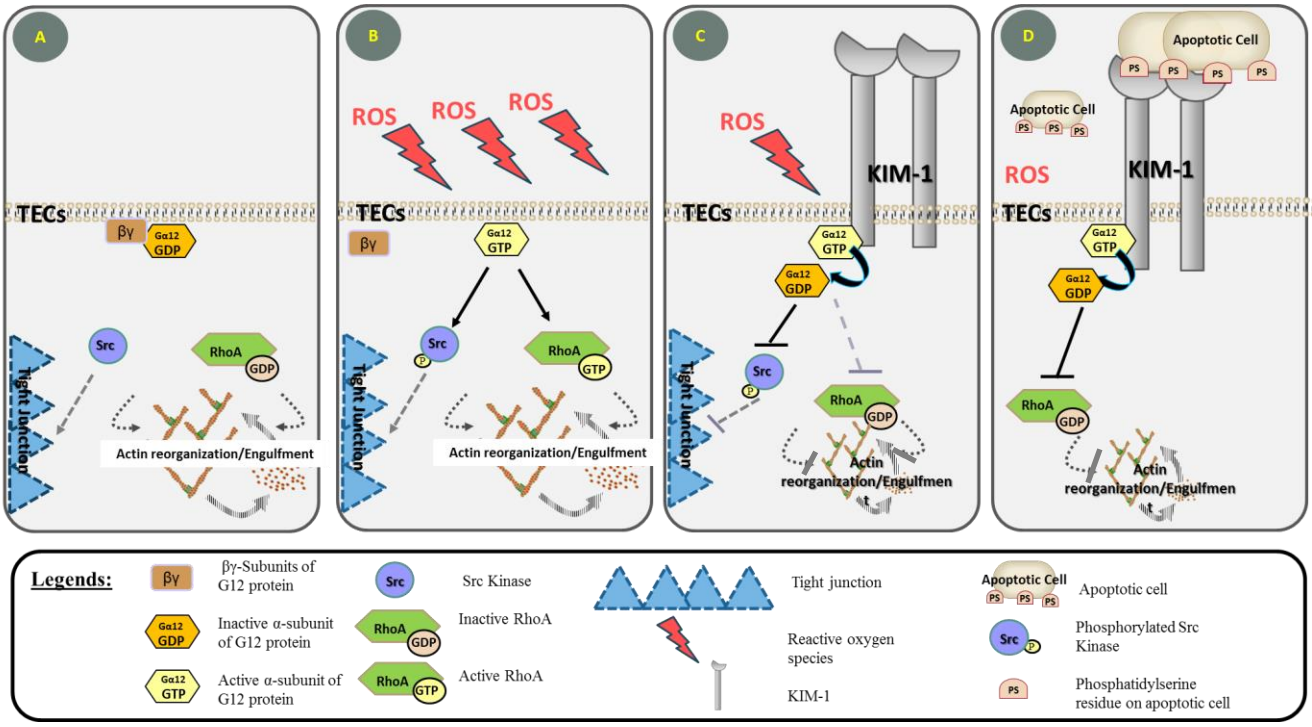

Figure 5.1. Working model for the KIM-1 and Gα12 signalling pathway in tubular epithelial cells during ischemia-reperfusion injury and phagocytosis.

***A*:** In healthy tubular epithelial cells (TECs), Gα12 is inactive and tethered to the cell inner membrane surface through its association with the Gβγ subunit of the heterotrimeric G protein family. This prevents any activation of down-stream signal mediators, such as RhoA and Src. ***B*:** Reactive oxygen species (ROS) are rapidly generated during ischemia-reperfusion injury and lead to activation of Gα12 signalling. Activated Gα12 can trigger Src activation via Src phosphorylation, which then subsequently leads to disassembly of tight junction complexes. Active Gα12 could also activate RhoA (Prakash *et al.*, 2008), which play a negative role in the uptake of apoptotic cells by TECs. ***C*:** With prolonged ischemia-reperfusion injury, KIM-1 is gradually upregulated on TECs and this lead to suppression of Gα12 activity. Suppression of Gα12 activity results in decreased Src activation and stops the disruption of tight junction complexes. KIM-1-mediated suppression of Gα12 also influences RhoA activation. ***D*:** Cells that succumb to the injury undergo apoptosis and expose phosphatidylserine (PS) on their cell-surface. PS on apoptotic cells is recognized by KIM-1 on TECs, which then rapidly engulf the apoptotic cells to prevent secondary necrosis. The persistent suppression of Gα12 activation in TECs and subsequent inhibition of RhoA stimulation is required to promote the efficient clearance of apoptotic cells. Solid lines are based on evidence presented throughout this thesis. Dotted lines represent hypothetical or predicted events based on our data and published results from other studies not related to KIM-1.

In Chapter 4, we explored the role of Tctex-1, yet another novel KIM-1-interacting protein, which was identified in a yeast-two hybrid screen. We showed that KIM-1 interacts with Tctex-1 under normal baseline conditions in TECs, but that interaction decreases during the uptake of apoptotic cells, especially at the later stages of phagocytosis (Figure 4.1 and 4.2). Silencing Tctex-1 expression impaired the uptake of apoptotic cells by TECs (Figure 4.5). In addition, we showed that phosphorylation of Tctex-1 at Threonine-94, which prevents Tctex-1 from binding to microtubules via dynein, reduced its interaction with KIM-1 (Figure 4.4). The exact mechanisms of how Tctex-1 regulates KIM-1-dependent phagocytosis is currently under investigation.

Our identification of Gα12 and Tctex-1 as KIM-1-binding proteins involved in phagocytosis using entirely different approaches (mass spectrometry and yeast two-hybrid), could suggest a possible cooperation between these proteins in mediating changes in cell behaviour required for phagocytosis (e.g. cytoskeletal reorganization). Interestingly, a link between Gα12 and Tctex-1 has been proposed by Takesono *et al.,* (1999), who were the first to show that Tctex-1 works as an activator of G-protein signalling (AGS), independent of G-protein coupled receptors (Takesono *et al.,* 1999). Tctex-1 was shown to carry out this function by binding to the Gβγ subunit of heterotrimeric G protein in order to free the alpha subunit. This leads to Gα activation and neurite outgrowth in neurons (Sachdev *et al.,* 2007).

Recent work by the Rottapel group in Toronto has eluded to a possible mechanism for this interaction by showing that Tctex-1 interacts with a microtubule-associated guanine nucleotide exchange factor, GEF-H1 (or Lfc in mice), along with dynein intermediate chain (DIC) (Conde *et al.*, 2010; Meiri *et al.*, 2009; Meiri *et al.*, 2012; Ren *et al.*, 1999). Given that GEF-H1 is an important protein that couples microtubules and actin together, Tctex-1 was shown to tether GEF-H1 to microtubules, inhibiting its action on actin filament organization via RhoA (Meiri *et al.*, 2012). Even more recently, the Rottapel group demonstrated that both the Gβγ and Gα subunits of the heterotrimeric G12 protein work together to activate or suppress GEF-H1 activity (Meiri *et al.*, 2014). This happens through the interaction of the Gβγ subunit with Tctex-1, which releases GEF-H1 from the microtubule complex with dynein. This release allows GEF-H1 to interact with Gα12 to mediate actin cytoskeletal changes, such as stress fiber formation and focal adhesion assembly via RhoA (Meiri *et al.*, 2014).

Based on the above information, we hypothesize that the interaction of both Gα12 and Tctex-1 with KIM-1 might serve to simultaneously modulate changes in actin and microtubule

structures during phagocytosis. We speculate that the interaction of KIM-1 with Tctex-1 tethers it via the dynein complex (along with GEF-H1) to the microtubule cytoskeleton, which has been shown to be important for phagocytosis (Blocker *et al.*, 1997; Blocker *et al.*, 1996; Harrison *et al.*, 2002; Newman *et al.*, 1991) (Figure 5.2A). Following from this, upon KIM-1 binding to apoptotic cells, KIM-1 maintains its interaction with Tctex-1, which in turn is still tethered to microtubules along with GEF-H1 and dynein intermediate chain proteins, to help bring the apoptotic cell cargo to the phagosome (Figure 5.2B). The continued interaction with Tctex-1 and GEF-H1 would inhibit the activation of RhoA, which we found impedes corpse uptake. Simultaneously, KIM-1 would continue to interact with and suppresses Gα12 to mediate the uptake of apoptotic cells. During later stages of phagocytosis, the loss of interaction between Kim-1 and Tctex-1 (potentially via Tctex-1 phosphorylation at Threonine-94) would then allow GEF-H1 to be released and to interact with Gα12 (Figure 5.2C). This would cause RhoA activation, which has been shown to enable phagosome formation around apoptotic targets (Ravichandran *et al.*, 2007). This hypothetical pathway could explain the consistent increase in RhoA activity we observed in our studies during the later stages of phagocytosis (Figure 3.6A and Figure 4.7C). Much exciting work lies ahead as we pursue testing this model and further study the signalling mechanisms that are triggered in TECs by IRI and that regulate phagocytosis.

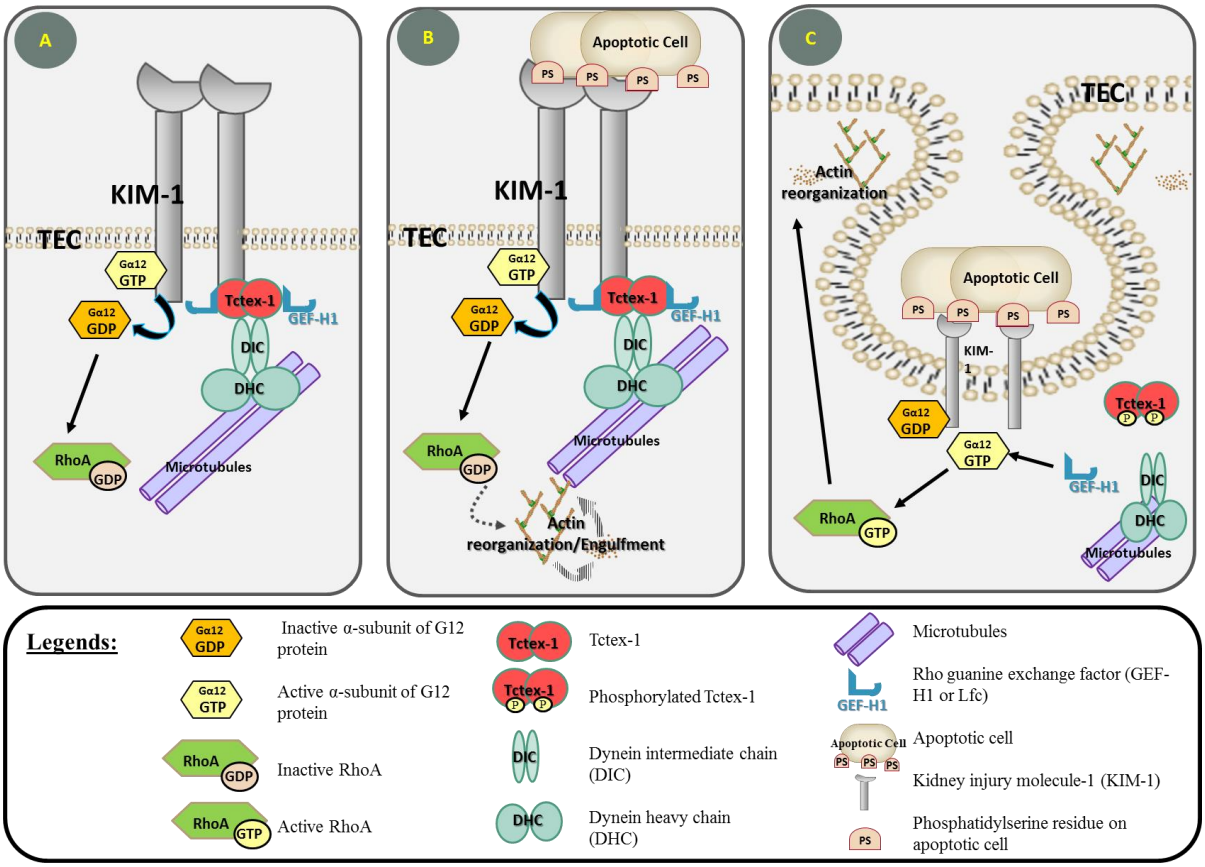

Figure 5.2. Proposed signalling pathways of KIM-1-mediated phagocytosis.

***A*:** Following injury to tubular epithelial cells (TECs), KIM-1 is expressed on the apical surface of TECs. Expression of KIM-1 suppresses Gα12 activation, which leads to the inhibition of RhoA activity. Tctex-1 is tethered to microtubules along with GEF-H1 and dynein intermediate chain (DIC) and dynein heavy chain (DHC) component of the dynein motor protein. GEF-H1 is inactive since it is bound to the Tctex-1-Dynein-Microtubule complex and hence, it further inhibits RhoA activity. ***B*:** Upon apoptotic cell-binding to KIM-1, KIM-1 still interacts with Gα12 and suppresses Gα12 activity. KIM-1 also interacts with Tctex-1 in a manner similar to conditions without binding to apoptotic cells. This situation is predicted to last for the first 60 minutes. ***C*:** During the last stages of phagocytosis, when the phagosome is formed, KIM-1 loses its interaction with Tctex-1 due to potential phosphorylation of Tctex-1 at Threonine-94. This loss of interaction with KIM-1 will potentially cause the disassembly of the Tctex-1-Dynein-GEF-H1 complex. This will allow GEF-H1 to be released, allowing it to interact with Gα12 and mediate the RhoA activation that plays a role in actin cytoskeletal reorganization (Meiri et al., 2014).

5.2. LIMITATIONS

An important limitation that hindered our Gα12 and KIM-1 studies was the lack of cell lines that expressed high levels of endogenous Gα12 and KIM-1. Throughout our experiments in Chapters 2 and 3, we used human embryonic kidney 293 (HEK-293) cells that we found to express a high level of endogenous Gα12 and which were subsequently made to stably express KIM-1. We have confirmed that these cells engulf apoptotic cells in a manner similar to cells expressing endogenous KIM-1. In addition, we have confirmed our findings in primary TECs isolated from wild type and Kim-1-deficient mice. In Chapter 4, we failed to see differences in the phagocytosis in HEK-293 cells transfected with KIM-1 and either one of the different Tctex-1 constructs (wild type Tctex-1, phosphomimetic mutant of Tctex-1 (T94E) or unphosphorylated mimic of Tctex-1 mutant (T94A)) (Figure 4.4.C and D), yet we found a reduced interaction between KIM-1 and the phosphomimetic mutant of Tctex-1 (T94E) (Figure 4.4A and B). Since the effects of phosphomimetic mutant of Tctex-1 on its interaction with KIM-1 and phagocytosis were inconsistent, this challenged our initial hypothesis which suggested that KIM-1-binding to Tctex-1 served to tether the apoptotic cargo to microtubules via dynein to allow for engulfment. This issue raises many questions that need to be addressed in future studies. For example, "What is the regulatory mechanism between KIM-1, Tctex-1, dynein and hence microtubules?", "Does Tctex-1 modulate KIM-1-mediated phagocytosis through its interaction with dynein/microtubules?", "Does the phosphorylation of Tctex-1 affects its ability to interact with KIM-1 by impairing its interaction with dynein?"

Another limitation we have struggled with is the 60 % homology between the N-terminal cysteine-rich region of human KIM-1 and murine Kim-1 (Kuchroo *et al.*, 2003; Santiago *et al.*, 2007). Even though both human and murine KIM-1 are upregulated after ischemia-reperfusion injury (Ichimura *et al.*, 2008; Ichimura *et al.*, 2004), and carry out the same function of uptake of dead cells (Ichimura *et al.*, 2008; Kobayashi *et al.*, 2007), there is a marked difference in the length of the mucin domain and the number of O-linked glycosylation sites between them (Kuchroo *et al.*, 2003). This might explain our inability to perform co-immunoprecipitation of KIM-1 and Gα12 or Tctex-1 in primary cells using the existing antibodies against mouse Kim-1 which recognize the mucin-domain of Kim-1. This limitation might suggest a role for the mucin domain of KIM-1 in carrying out its signalling function.

5.3. FUTURE RESEARCH

To date, research in AKI has primarily focused on the mechanism of apoptosis and necrosis of TECs caused by the injury (Burns *et al.*, 1998; Daemen *et al.*, 1999; Kelly *et al.*, 2001; Kelly *et al.*, 2003; Schumer *et al.*, 1992). The novel findings presented in this thesis highlight a new area of research involving the signalling mediators that are involved in phagocytic clearance of apoptotic cells during AKI. Recently, a new pathway of regulated necrosis, known as necroptosis, has emerged. Necroptosis is a receptor-interacting protein kinase-based necrotic cell death process (Linkermann *et al.*, 2012; Tait *et al.*, 2013). Blocking the pathway leading to necroptosis via chemical inhibition or knocking out RIPK3 has been shown to have a protective effect in ischemic and cisplatin-induced AKI (Linkermann *et al.*, 2013; Tristao *et al.*, 2012; L. Zhang *et al.*, 2013). It would be interesting to investigate whether KIM-1 clears necroptotic cells in a similar manner to that of apoptotic cells. In addition, newly discovered death pathways, such as mitochondrial permeability transition-mediated regulated necrosis (Vanden Berghe *et al.*, 2014) and ferroptosis (Dixon *et al.*, 2012; Linkermann *et al.*, 2014), could also play an important role in AKI and thereby engage KIM-1 signalling. Furthermore, it would be interesting to determine if the various types of dead cells display phosphatidylserine or yet undiscovered "eat me" ligands for KIM-1 that mediate clearance of these cells.

Another aspect of KIM-1 that we did not explore in this thesis work is the fact that upon upregulation on TECs, KIM-1 undergoes spontaneous membrane-proximal cleavage leading to the release of its soluble extracellular domain (90 kDa) and retention of a membrane-bound fragment (14 kDa) on cells (Bailly *et al.*, 2002). The soluble fragment is recognized as a sensitive and specific urinary biomarker for AKI (Han *et al.*, 2002; Vaidya *et al.*, 2009; Vaidya *et al.*, 2010). KIM-1 membrane-proximal cleavage was shown to be accelerated by pervanadate via activating ERK and p38 MAP Kinase (Bailly *et al.*, 2002; Z. Zhang *et al.*, 2007). Recently, our lab showed that KIM-1-cleavage is mediated by TNF-α-converting enzyme (TACE or ADAM17) and that this cleavage is accelerated by reactive oxygen species generated during injury to renal tubular epithelial cells, or by the addition of apoptotic cells (Gandhi *et al.*, 2014). Furthermore, excess KIM-1 shedding was shown to bind to apoptotic cells, thus competitively inhibiting phagocytosis by KIM-1-expressing TECs. It would be exciting, for example, to engineer a transgenic mouse expressing a cleavage-defective KIM-1 mutant to determine whether these mice exhibit different

outcomes following ischemic AKI. An equally important question is whether KIM-1 phagocytic signalling (i.e. Gα12 and Tctex-1) would be altered by KIM-1 shedding.

Another area of interest is the multiple serine and tyrosine phosphorylation sites that have been described within the intracellular region of KIM-1 (Meyers *et al.*, 2005; Santiago *et al.*, 2007). The cytoplasmic tail of human KIM-1 is relatively short and contains two putative tyrosine phosphorylation sites, at positions 350 and 356, which play a role in signalling (Binne *et al.*, 2007). For example, phosphorylation at Tyrosine-350 was shown to be required for TIM-1 (KIM-1 expressed on T cells) to mediate co-stimulatory signalling in T cells (de Souza *et al.*, 2005). This raises several important questions that need to be answered. For example, "What is the role of KIM-1 phosphorylation in ischemia-reperfusion injury or the uptake of apoptotic cells?" "Does phosphorylation of KIM-1 influence its interaction with Gα12 or Tctex-1?" "Does the phosphorylation allow KIM-1 to suppress pathogenic Gα12 activity?" Using site-directed mutagenesis of KIM-1, we could generate KIM-1 mutants where tyrosine residues are substituted with phenylalanine, which mimics unphosphorylated tyrosine, or glutamic acid, which some consider to mimic phosphorylated tyrosine. Transfecting these mutants into TECs would enable us to test KIM-1's ability to interact with Gα12 or Tctex-1 following additions of pervanadate, a protein tyrosine phosphatase inhibitor, which is known to induce KIM-1-phosphorylation (Z. Zhang *et al.*, 2007). The studies proposed above will allow us to better understand KIM-1 signalling in TECs.

In Chapter 4, we explored another KIM-1-interacting protein, Tctex-1, and its role in phagocytosis. Since Tctex-1 is part of the dynein motor protein which is involved in the trafficking of proteins via microtubules (King *et al.*, 1996), several questions remained unanswered. For example, "What is the regulatory mechanism between KIM-1, Tctex-1, dynein and, hence, microtubules?" "Does Tctex-1 modulate KIM-1-mediated phagocytosis through its interaction with dynein?" "Does the phosphorylation of Tctex-1 affect its ability to interact with KIM-1 by impairing its interaction with dynein?" These questions represent the next steps of this project. Given that Tctex-1 has been shown to be involved in regulating the cell cycle in ciliated cells (Chuang *et al.*, 2005; Conde *et al.*, 2010; Li *et al.*, 2011), it is exciting to speculate that Tctex-1 is involved in the cell cycle progression of KIM-1-expressing cells during renal regeneration after AKI. Interestingly, Tctex-1 has shown to play a protective role in hypoxia through its interaction with microtubules and voltage-dependent anion-selective channels (VDAC) on mitochondria

(Fang *et al.*, 2011; Schwarzer *et al.*, 2002). In this context, silencing Tctex-1 or phosphorylation at serine-82 has been shown to exacerbate hypoxia induced mitochondrial damage and the disruption of microtubules (Fang *et al.*, 2011; Xu *et al.*, 2013). Due to time constraints, we did not explore the role of Tctex-1 in IRI and whether IRI leads to any changes in Tctex-1 expression or modification, such as phosphorylation, that might influence its interaction with KIM-1.

5.4. SIGNIFICANCE OF FINDINGS

This thesis presented several novel signalling pathways through which KIM-1 carries out its protective effect during ischemia-reperfusion injury and in the clearance of apoptotic cells. Understanding how KIM-1 functions at a mechanistic level to clear dead cells and prevent further inflammation may allow us to identify novel therapeutic strategies to enhance its protective effects in AKI. AKI remains a serious and common medical complication in hospitalized patients for which there are no effective therapies (Bock, 1997; Lopes *et al.*, 2010; Togel *et al.*, 2014). Incomplete recovery from AKI has been shown to lead to chronic kidney disease (CKD) or fibrosis (Hsu, 2012; Wald *et al.*, 2009). Both AKI and CKD have been associated with an increased risk of death and may result in complications, such as cardiovascular disease, diminished quality of life, and the development of end-stage renal disease (Chawla *et al.*, 2014; Venkatachalam *et al.*, 2015). Understanding the signalling mechanisms of protection from AKI by KIM-1 might help elucidate important steps leading to enhanced recovery from AKI, as well as preventing subsequent progression to CKD or fibrosis. Moreover, given the scarcity of mechanistic data regarding phagocytic mechanisms in non-professional phagocytes, our work contributes significantly to advance knowledge in this growing field.

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Appendix: Animal Use Subcommittee Approval

2015-02-21, 9:03 PM

Western

AUP Number: 2010-230
PI Name: Gunaratnam, Lakshman
AUP Title: Establishing The Role Of Kidney Injury Molecule-1 (kim-1) In In Kidney Injury And Cancer
Approval Date: 10/23/2014

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Establishing The Role Of Kidney Injury Molecule-1 (kim-1) In In Kidney Injury And Cancer" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2010-230::5

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
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Curriculum Vitae

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Post-secondary Education and Degrees

Bachelor's Degree in Medical Sciences
Honors Specialization in Microbiology & Immunology with a Minor in Pharmacology & Toxicology
The University of Western Ontario
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2004-2009

Doctor of Philosophy
Microbiology and Immunology (Thesis-Based)
The University of Western Ontario
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2009- Current

Honours and Awards

Western Graduate Research Scholarship Research Award
Western University, Department of Microbiology and Immunology
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2010-present

Lawson Internal Research Fund (IRF) Competition Award
Lawson Health Research Institute
London, Ontario, Canada
2010-2011

Department of Medicine Research Day
Poster Competition award, Western University, 2014.

Graduate Student Teaching Award Nominee, Western University, 2014 and 2012.

CIHR Institute of Infection and Immunity Poster Competition award, Infection and Immunity Research Forum, Western University, 2011.

Related Work Teaching and Learning

Teaching assistant for Microbiology and Immunology 2500A/B (Biology of Infection and Immunity)
Sep-Dec 2012, Jan-Apr 2014, Jan-Apr 2015

Teaching assistant for first year Biology 1002B laboratory
Jan-Apr 2015

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Publications

Gandhi, R., Yi, J., Ha, J., Shi, H., **Ismail, O**., Nathoo, S., Bonventre, J.V., Zhang, X., and Gunaratnam, L. Accelerated Receptor Shedding Inhibits Kidney Injury Molecule-1 (KIM-1)-mediated Efferocytosis. *Am J Physiol Renal Physiol*. 14 May 2014.

Ola Z. Ismail, Xizhong Zhang, Junjun Wei, Aaron Haig, Bradley M. Denker, Rita S. Suri, Alp Sener, Lakshman Gunaratnam. Kidney Injury Molecule-1 protects against Gα12 activation and tissue damage in renal ischemia-reperfusion injury. *Am J Pathol. 2015 Mar 7. pii: S0002-9440(15)00078-4. doi: 10.1016/j.ajpath.2015.02.003. [Epub ahead of print]*

Ola Z. Ismail, Xizhong Zhang, Joseph V. Bonventre, Lakshman Gunaratnam. G protein, α 12 (Gα12) is a negative regulator of kidney injury molecule-1-mediated phagocytosis in kidney epithelial cells. *(In preparation for resubmission to the American Journal of Physiology-Renal Physiology).*

Scientific Meetings Attended

Kidney Injury Molecule-1 (Kim-1) Protects from Renal Ischemia-Reperfusion Injury (Talk).
Ola Z. Ismail, Xizhong Zhang, Junjun Wei, Giulia Michela Martone, Sahra Nathoo, Aaron R Haig, Rita Suri and Lakshman Gunaratnam. ***Canadian Society of Nephrology (CSN).*** *April 24, 2015*. Montreal, QC, Canada

Kidney Injury Molecule-1 (Kim-1) Protects from Renal Ischemia-Reperfusion Injury (Poster). Ola Ismail, Xizhong Zhang, Junjun Wei, Giulia Martone, Sahra Nathoo, Aaron R. Haig, Rita S. Suri , Lakshman Gunaratnam. ***American Society of Nephrology (ASN).*** *Nov 2014*. Philadelphia, Pennsylvania, USA

KIM-1 Interacts With Gα12 And Suppresses Its Activity To Mediate Efferocytosis (Poster). Ola Ismail, Xizhong Zhang, Lakshman Gunaratnam. ***Canadian Nephrology Society (CNS).*** *April 2014.* Vancouver, British Colombia, Canada

KIM-1 Interacts With Gα12 And Suppresses Its Activity To Mediate Efferocytosis (Talk). Ola Ismail, Xizhong Zhang, Lakshman Gunaratnam. ***American Society of Nephrology (ASN).*** *Nov 2013*. Atlanta, Georgia, USA

KIM-1 Inhibits Gα12-RhoA Signalling during KIM-1-mediated Phagocytosis of Apoptotic Cells (poster). Ola Ismail, Xizhong Zhang, Lakshman Gunaratnam. ***Canadian Nephrology Society (CNS).*** *April 2013.* Montreal, Quebec, Canada

Rho GTPases Regulate Kidney Injury Molecule-1-mediated Efferocytosis (poster). Ola Ismail, Xizhong Zhang, Lakshman Gunaratnam.***Canadian Nephrology Society (CNS).*** *April 2012*. St. John's, Newfoundland, Canada

Internal Poster Presentations/

KIM-1 Interacts With Gα12 And Suppresses Its Activity To Mediate Efferocytosis (Poster). ***Department of Medicine Research Day.*** *May 2014.* London, Ontario, Canada

KIM-1 Interacts with Gα12 and Suppresses Its Activity to Mediated Efferocytosis (poster). ***London Health Research Day***. *March 2013*. London, Ontario, Canada

KIM-1 Inhibits Gα12-RhoA Signalling During KIM-1-Mediated Efferocytosis (Poster). ***Infection and Immunity Research Forum (IIRF).*** *Nov 2013*. London, Ontario, Canada.

Role of RhoA and Rac1 GTPases in Kidney Injury Molecule-1-mediated Efferocytosis in Kidney Epithelial Cells (poster). ***London Health Research Day***. *March 2012*. London, Ontario, Canada

TACE Mediates Ectodomain Shedding of Kidney Injury Molecule-1 in Proximal Tubular Epithelial Cells (co-author, poster). ***London Health Research Day***. *March 2012*. London, Ontario, Canada

Role of RhoA and Rac1 GTPases in Kidney Injury Molecule-1-mediated Efferocytosis in Kidney Epithelial Cells (poster). ***Infection and Immunity Research Forum (IIRF)***. *November 2011*. London, Ontario, Canada