4-21-2014 12:00 AM

The Role of KIM-1 Mediated Efferocytosis by Cancer Cells in Blocking the Immunogenicity of Tumor Cell Death

Sahra Nathoo
The University of Western Ontario

Supervisor
Dr. Gunaratnam
The University of Western Ontario

Graduate Program in Microbiology and Immunology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

THE ROLE OF KIM-1 MEDIATED EFFEROCYTOSIS BY CANCER CELLS IN BLOCKING THE IMMUNOGENICITY OF TUMOUR CELL DEATH

(Thesis format: Monograph)

By

Sahra Nathoo

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies The University of Western Ontario
London, Ontario, Canada

_____

Abstract

The phagocytic clearance of apoptotic cells –efferocytosis- is essential for maintaining immune homeostasis. Uncleared apoptotic cells can undergo secondary necrosis releasing endogenous danger signals such as high mobility group box protein 1 (HMGB1) into the extracellular milieu, triggering the innate immune system. Kidney Injury Molecule -1 (KIM-1) is a phosphatidylserine (PS) receptor that has been shown to confer on proximal tubular epithelial cells (PTECs) the ability to clear apoptotic cells during acute kidney injury. KIM-1 is overexpressed by various human tumours including renal clear cell carcinoma (RCC), though the impact of this on tumour progression is not known. Importantly, RCC tumours are highly resistant to chemotherapies and radiotherapies that are known to concurrently induce tumour cell apoptosis and trigger an immune response to the dying cancer cells. In this thesis I show, for the first time, that endogenous KIM-1 expressed by human RCC cell lines enables them to become semi-professional phagocytes and efficiently engulf apoptotic and necrotic cells. Using siRNA-mediated knockdown of KIM-1 expression in RCC cells, we show that KIM-1-dependent phagocytosis by RCC cells significantly reduced the leakage of HMGB1 from apoptotic cells undergoing secondary necrosis or necrotic cells. In addition, we demonstrate that the failure to clear dying cells by RCC cells was associated with enhanced activation of primary dendritic cells when they were exposed to the conditioned medium from RCC cells fed apoptotic or necrotic cells. Therefore, we propose that the upregulation of KIM-1 expression by cancers may allow them to evade the immune system and immunogenic cell death by chemotherapy thereby making KIM-1 a potential therapeutic target.

Key Words: KIM-1, cancer, HMGB1, danger signals, efferocytosis, immune surveillance, renal cell carcinoma, RT-PCR, apoptosis, Western Blot, Flow cytometry

Dedications

I dedicate this thesis to my parents who through all the ups and downs in the past three years have served as my motivators, advisors and strongest supporters. Without you both I never would have made it this far, learned the value of failure and the importance of cherishing the successes. You taught me to aim as high as I could imagine but never to lose sight of what I was aiming for.

I also dedicate this to my sister whose wisdom, beyond her years, reminded me that this was only one chapter of many to come. From a young age you taught me how to stand up for myself, to not hide from my potential and as you always said "the power of positive thinking". You are my rock and for that I am eternally grateful.

"Character cannot be developed in ease and quiet. Only through experience of trial and suffering can the soul be strengthened, ambition inspired, and success achieved."

- Helen Keller

Acknowledgements

I would like to express my gratitude and appreciation towards my supervisor Dr. Gunaratnam for his support and guidance over the last three years. His willingness to teach in conjunction with his dedication has taught me to think analytically, fostering a genuine appreciation for science.

I would also like to thank Dr. Bhagirath Singh and Dr. Bryan Heit for sitting on my advisory committee and for steering me in the right direction as needed.

I would like to thank all the members, past and present, of the Gunaratnam lab for their support and encouragement. I would like to say a special thanks to Dr. Xizhong Zhang for teaching me how to do cell culture and for appreciating that patience is a virtue. I am also indebted to Ola Ismail for the training she provided me upon my arrival to the laboratory and to this date. Finally the undergraduates, new graduates, and medical students I interacted with over the course of my Masters, I look forward to witnessing your successes in the future.

Lastly, I would like to acknowledge the many friends and peers I have met here at the University of Western Ontario, you made this experience a truly memorable one.

Table of Contents

List of Tables

List of Figures

List of Appendices

Abbreviations

Ab	Antibody
AKI	Acute Kidney Injury
AC	Apoptotic Cells
ANOVA	Analysis Of Variance
Bcl-2	B cell lymphoma-2
BrdU	Bromodeoxyuridine
ccRCC	clear cell Renal Carcinomas
cDNA	Complementary DNA
CFSE	Carboxyfluorescein Succinimidyl Ester
DCs	Dendritic Cells
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Essential Medium
EMEM	Eagles Modified Essential Medium
FACS	Fluorescence Assisted Cell Sorting
GAPDH	Glyceraldehyde 3-phosphate Dehydrogenase
GOH	Geraniol
G418	Geneticin
HAVCR1	Hepatitis A Virus Cellular Receptor 1
HRP	HorseRadish Peroxidase
IFN-γ	Interferon gamma

IL-10	Interleukin-10
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IRI	Ischemia Reperfusion Injury
KIM-1	Kidney Injury Molecule-1
KIM-1 PK1/WT-KIM-1	LLC-KIM-1-PK1
LKB1	Liver Kinase B1
LPC	Lysophosphatidylcholine
MCP-1	Monocyte Chemoattractant Protein-1
MDSC	Myeloid Derived Suppressor Cells
MFG-E8	Milk-fat-globule-EGF factor 8
NEAA	Non-Essential Amino Acids
NC	Necrotic Cells
PBS	Phosphate Buffered Saline
pCDNA-PK1	LLC pcDNA-PK1
PFA	Paraformaldehyde
PMN	Polymorphonuclear leukocytes
P/S	Penicillin Streptomycin
PS	Phosphatidylserine
RCF	Relative Centrifugal Force
RB	Retinoblastoma
RNA	Ribonucleic Acid
RCC	Renal Cell Carcinoma

SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of Mean
SIR	Sterile Inflammatory Response
SIRPα	Signal regulatory protein α
qRT-PCR	Quantitative Real Time Reverse Transcription-Polymerase Chain Reaction
TAM	Tumour Associated Macrophage
TCE	Tricholoethylene
TGF-β	Transforming Growth Factor Beta
TIL	Tumour Infiltrating Lymphocytes
TSP-1	Thrombospondin-1
TNF-α	Tumour Necrosis Factor Alpha
Tx/Thy	Thymocytes
uKIM-1	Urinary KIM-1
UV	Ultraviolet
VEGF-A	Vascular Endothelial Growth Factor-A

Chapter 1:Introduction

1.1.0 The Hallmarks of Cancer

The simplest descriptor of a tumour is a grouping of transformed host cells with an accumulation of genetic and epigenetic aberrations[1]. Beyond that, the simplicity of the definition is replaced by the true and complex nature of tumour formation, sustenance and survival. Within the same host, tumour cell populations display a high degree of heterogeneity[2]. Studies have shown that biopsy samples taken from the time of primary specimen collection to metastasis were highly dissimilar. Additionally a study by the Sanger group on the profile of renal carcinoma sites demonstrated that some molecular transformations were only present at primary and metastatic sites and not uniformly throughout all tumour colonies[3]. While the inherent complexity of tumour masses became more apparent, researchers noticed fundamental characteristics that enabled tumours to progress.

In 2000, Hanahan and Weinberg introduced and coined these collective traits, “the hallmarks of cancer”. The hallmarks served as a characteristic framework for neoplastic diseases. The six hallmarks of cancer; sustained proliferative signalling, avoiding growth suppressors, resisting cell death, angiogenesis, activating invasion and metastasis and enabling replicative immortality, were considered independent yet complementary, and form the foundation for cancer development. In 2011 Hanahan and Weinberg amended their hallmarks to include two burgeoning characteristics that reflected the progress in cancer research over the last decade. The six hallmarks introduced culminated in the recognition that the tumour microenvironment played a significant role in successful cancer establishment.

1.1.1 Sustained Proliferative Signalling

The ability to deregulate the cell cycle is considered the most fundamental attribute of cancer cells. This process allows for the development of neoplasms and ultimately

metastases due to their rapid cellular proliferation[4]. The origin of the growth signals that serve to sustain proliferation are under-characterized to date. Studies have highlighted the ability for both the tumour and nearby non-tumour cells to supply cancers with growth signals[5, 6]. Characteristics for increased growth signalling include the upregulation of growth receptors on tumour cells, constitutive activation of growth pathways[7] and paracrine signalling[8]. Research has also shown the propensity of cancers to disrupt negative feedback loops that attenuate proliferation[9, 10] and the induction of cell senescence to avoid detection by the immune system of erratic growth patterns[11].

1.1.2 Avoiding Growth Suppressors

Part in parcel with sustaining a tumours' proliferative capabilities, exists the need to avoid growth suppressors. Through various gain or loss of function experiments in mice, proteins whose sole functions are to suppress tumours have been identified. The tumour suppressor protein p53 is perhaps the most well researched protein in cancer[12]. It determines if cells should become apoptotic, proliferate or become senescent[13]. The absence of p53 is prevalent across many human cancers and permits persistent cell proliferation[12, 14]. Further support for the necessity to evade growth suppressors stems from the ability of cancers to overcome the effects of contact inhibition. Contact inhibition occurs when cell division is halted as a result of high cellular density[15]. Under normal conditions, cells will stop proliferating and form a monolayer. Contact inhibition has been shown to be an anticancer mechanism mediated by cell surface proteins[16]. Inactivation of proteins involved in cell-structure[17, 18] result in multilayered three-dimensional structures characteristic of tumours[19, 20]. One example is liver kinase B1 (LKB1), a serine threonine kinase that is characterized as a tumour suppressor gene and is inactivated in certain human malignancies[21, 22]. LKB1 inactivation enhances STAT3 signalling, which results in tumour cell proliferation, in both lung and thyroid cancers [23, 24].

1.1.3 Resisting Cell Death

Apoptosis serves as the primary means to limit tumourigenesis, as established by studies examining the death pathways in cancers[25]. When cancer cells undergo stress, such as chemotherapy, various pro-apoptotic or anti-apoptotic genes are upregulated to induce cancer cell apoptosis [26, 27]. Therefore tumours have developed a way to circumvent apoptotic death in order to allow for the development of less immunogenic tumours. One highly manipulated apoptotic pathway in cancer is the signal B cell lymphoma-2 (Bcl-2) protein family. In the presence of cellular damage or stress, the pro-survival signal Bcl-2 becomes inactivated. Downstream effectors such as Bax and Bak then make the outer mitochondrial membrane permeable, releasing cytochrome c. As a result of cytochrome c release, caspase 9, as well as other caspases, become activated resulting in cell apoptosis[28]. The overexpression of Bcl-2 protein has also been associated with increased resistance to chemotherapy in various human cancers[29]. As a result, the inhibition of Bcl-2's pro-survival function is being used a potential chemotherapeutic target[30].

Autophagy is a mechanism that has come to light as a potential tumour enabler. For the most part autophagy occurs basally at low levels and is induced in instances of cell stress such as nutrient deficiencies[31]. This process, mediated by autophagosomes, allows for the breakdown of cellular organelles in order to recycle catabolites for energy metabolism and biosynthesis. The process is characterized by the degradation of cytoplasmic macromolecules and organelles[32-35]. Studies have shown a correlation between autophagic activation and cancer progression in the early stages of development[36]. However, various groups have noted either pro-cancer or anti-cancer outcomes of autophagy, leaving its influence up for debate[37]. Radiotherapy and nutrient starvation in the form of chemotherapeutic treatments have been shown to have a cytoprotective role in cancer cells and even allow cancer cells to regress to a state of reversible dormancy due to autophagy.

1.1.4 Angiogenesis

Solid tumours require oxygen and nutrients to survive. Tumours also need to effectively remove waste products, toxins and carbon dioxide that could potentially impede growth. Angiogenesis is the process whereby new tumour blood vessels spring from established vasculature. [38]. One well-known angiogenesis promoting factor is vascular endothelial growth factor (VEGF-A). VEGF-A expression is upregulated in the early stages of cancer formation[38-41]. As tumours grow and nutrient sources become limited, the inner mass becomes the most isolated from nutrients and oxygen resulting in tumour hypoxia. The induction of hypoxic signalling in the inner mass via hypoxia-inducible factors results in VEGF-A production[42, 43]. VEFG-A expression has been shown to increase tumour survival by allowing for the migration of tumours to new sites abundant in resources[44-46]. The importance of angiogenesis to solid tumour progression is underscored by the myriad of anti-angiogenic therapies that have been developed and are being tested in patients[47, 48].

1.1.5 Activating invasion and metastasis

One nearly universal feature of human solid tumour cells is the loss of E-cadherin expression. E-cadherin helps to form junctions with adjacent epithelial cells, resulting in fixed monolayers without migratory capabilities. The high frequency of E-cadherin gene inactivation or protein downregulation alluded to it being a key contributor to cancer survival[49, 50]. As a result this process was termed the "invasion-metastasis cascade"[51] based on a schema of discrete steps that results in multiple macroscopic tumours throughout the body[52]. Other pro-invasion and metastatic genes manipulated by cancers include the transcription factor involved in cell proliferation and differentiation HOXB9, matrix metalloproteases MMP-2 and MMP-8[53] and breast tumour kinase (BRK) which is a cell migratory factor, to name a few[54].

1.1.6 Enabling Replicative Immortality

In order to grow, it is understood that cancer cells require unlimited replicative capacity[55, 56]. One agent implicated in immortalizing cells is telomerase. Telomerase is a specialized DNA polymerase that adds telomere repeats to the end of DNA to prolong life span. The necessity for telomerase in cancers has been demonstrated by its silencing, resulting in cancer cells becoming senescent as opposed to developing into functional cancer cells [57]. Recent research has detailed an interesting role for telomerase, where its expression in premalignant lesions is not observed, yet its function is enhanced in established tumours[58, 59]. Researchers hypothesize that delayed acquisition of telomerase expression serves as a means for tumours to generate tumour-promoting mutations and once established, enhanced telomerase activation stabilizes the mutated genome to allow for unlimited replication of cancer cells [4]. As a result, tumours have developed a calculated mechanism to ensure their survival.

1.1.7 Enabling Characteristic: Genome Instability and Mutation

The acquisition of many of the hallmarks discussed thus far is dependent on genomic instability and the acquisition of mutations in various genes and pathways to produce the tumourigenic phenotype. A substantial number of mutations are not only heritable but epigenetic, meaning methylations and histone rearrangements can induce mutagenesis[60]. In the process of acquiring the series of mutant genes needed to orchestrate tumourigenesis, cancer cells often increase their rates of mutation[61, 62] so that they can transform at an accelerated rate. The ability to increase mutation rates, is often a result of tumour cells being sensitive to mutagenic agents as a result of these heritable changes, and so the cyclical nature of mutation acquisition influencing mutational rate increases until the tumour has outcompeted the immune system.

1.1.8 Enabling Characteristic: Tumour Promoting Inflammation

In the last decade, a plethora of data was generated supporting the functionally important tumour-promoting effects that immune cells—largely of the innate immune system—have on tumour progression[63-66]. This was first proposed by the finding in the early 1970s that tumour growth could be promoted by macrophages found within the tumour environment[67]. Inflammation can contribute to tumour growth by; supplying growth survival factors that limit cell death, delivering extracellular matrix-modifying enzymes that promote angiogenesis, releasing factors that sustain proliferative signalling, and transporting pro-angiogenic, invasion and metastatic factors directly to the tumour. One specific example is the finding that tumour localized macrophages tend to have a M2 profile as opposed to an M1 profile[65]. M1 macrophages are associated with interferon gamma (IFN-γ), interleukin- 6 (IL-6) and interleukin -1β (IL-1β) production, and have been associated with tumour destruction[68]. In contrast M2 macrophages are associated with interleukin-4 (IL-4) and interleukin-10 (IL-10) production that drive tissue repair and remodelling after tissue injury through cellular division. It is postulated that M2 macrophages can drive tumour progression[69, 70]. Furthermore, M2 macrophages promote the development of regulatory T-cells (T_{regs}) that are critical to the neutralization of effector cell-dependent killing of tumour cells (ex. $CD8^+$ T-cell function) [71]. The identification of additional tumour promoting agents such as a myeloid derived suppressor cells (MDSCs) that can induce T-cell anergy and dendritic cell immaturity, brought attention to the role of inflammation in cancer survival.

1.1.9 An Emerging Hallmark: Reprogramming Energy Metabolism

A very under characterized hallmark that has not been explored much to this date is the role of reprogramming energy metabolism to sustain the growth and proliferation patterns seen within cancer cells. In 1930 Otto Warburg observed the switch cancers make to glycolysis as their primary metabolic process, even in the presence of oxygen[66]. The upregulation of various glucose transporters, such as GLU1, has been demonstrated in numerous cancers[72, 73]. Because of the relatively low ATP production associated with glycolysis relative to oxidative phosphorylation, it is elusive as to why cancers would

prefer this method.

1.1.10 An Emerging Hallmark: Evasion from Immune Destruction

The successful establishment of tumours in the presence of an ever-alert immune system implies that some tumour variants have successfully evaded immune destruction. The necessity for an intact and strong immune system to prevent tumour establishment was demonstrated in studies with patients who were immunodeficient. Though many of the cancers observed in these patients are caused by viruses such as lymphomas (Epstein-Barr virus), Kaposi's sarcoma (herpes viruses), and cervical cancer (human papilloma viruses), they are also at greater risk for malignancies where there is not a viral etiology such as cancers of the colon, lung, pancreas, kidney, head and neck, and non-melanoma skin cancers[74]. Patients with AIDS and recipients of solid organ transplants are at an increased risk of developing cancer due to their immunosuppressed status[75]. For example as a result of transplantation, patients are given immunosuppressive drugs to reduce the chances of organ rejection and increase the lifespan of organ function[76]. A review by the Cincinnati Transplant Tumour Registry from 1968 to 1995 found transplant patients had a two-fold greater risk of developing melanomas compared to healthy people[77]. Moreover, the University of Pittsburgh between 1980 and 1993 demonstrated that transplant patients were 25 times more likely to develop lung tumours than their healthy counterparts[78]. In combination with the plethora of data from studies in immunodeficient animal models, it is generally accepted that immunosuppressed or immunocompromised individuals have a higher propensity to develop cancers.

1.2.0 The cancer immunosurveillence theory

The concept of cancer immunosurveillence was first developed in the early 1900s by Paul Ehlrich who proposed that the body could suppress an "overwhelming frequency" of carcinomas[79]. For the most part of the twentieth century however, due to the infancy of the field of immunology, this concept was not appreciated. Approximately 50 years later, the discovery of tumour antigens served as the cornerstone of the immune surveillance hypothesis established by Sir Macfarlane Burnet and Lewis Thomas in 1957[80]. The immune surveillance theory is defined below:

"In large, long-lived animals, … inheritable genetic changes must be common in somatic cells and a pro- portion of these changes will represent a step toward malignancy. It is an evolutionary necessity that there should be some mechanism for eliminating or inactivating such potentially dangerous mutant cells and it is postulated that this mechanism is of immunological character." Sir Macfarlane Burnet and Lewis Thomas ([80])

The immediate corollary of the immunosurveillence theory was that immunodeficient individuals would have a higher incidence of spontaneous induced tumours, and that the agent of recognition and elimination were lymphocytes. However, initially numerous studies determined that there was no difference in tumour formation or latency period after injection with the various carcinogens such as methycholanthrene (MCA) into mice. [81-85]. As a result of the lack of appreciation of NK T-cells (NK) in nude mice profiles [86, 87], and the infancy of the field of immunology (ex. poorly designed studies), the immune surveillance theory was not fully appreciated until the early 2000s [88, 89].

During the late 1990s, as the potential and reach of immunology grew, two key findings relit the interest in the concept of cancer immunosurveillence. The first was the role of interferon gamma (IFN-γ) in the protection of the host against transplanted and chemically induced tumours[90]. The second was the discovery that C57BL/6 mice lacking perforin were more prone to tumour formation than their wild type counterparts[91, 92]. As a result, attention was redirected back towards the differences between immune competent and immune deficient hosts in tumour development.

The importance of NK, T-cell and B-cell function in tumour establishment was demonstrated using recombination activating gene-1 (RAG-1) and RAG-2 knockout mice. Mice lacking RAG-1 or RAG-2 cannot undergo rearrangement of lymphocyte antigen receptors, resulting in a lack of B-cells and T-cells[93, 94] and xenogenic tumours grown in these mice experienced prolonged tumour survival.

1.3.0 From immune surveillance to immune editing

In 2001, seminal work by Schreiber and colleagues led to the realization that the immune system controls not only tumour quantity but also tumour quality (immunogenicity) prompting a major revision of the cancer immune surveillance hypothesis. Shankaran *et al.*, found that tumours formed in immunodeficient mice, as a group, were more immunogenic than similar tumours derived from immune competent hosts ([95]). It was clear from the above studies that tumours grown in immunocompetent mice were qualitatively different from tumours grown in immunodeficient mice. The anti-cancer immune response thus functions as an effective extrinsic tumour-suppressor system. This observation led to the formulation of the cancer immunoediting hypothesis by Schreiber who later coined the term "cancer immunoediting"[96]. The notion that the immune system not only protects the host against tumour formation but also shapes tumour immunogenicity is the basis of the cancer immunoediting hypothesis, which stresses the dual host-protective and tumour-promoting actions of immunity on developing tumours.

1.4.0 The three phases of cancer surveillance

The cancer immunoediting theory is broken down into three components; elimination, equilibrium and escape[97]. The elimination phase encompasses the active component of the immune surveillance theory, whereby highly immunogenic tumours are eliminated without progressing to subsequent phases. Throughout the process, the immune system attempts to eliminate tumours through IFN-γ secretion and lymphocyte recruitment [98, 99]. As solid tumours grow, their growth induces disruptions in the surrounding tissue resulting in the release of various inflammatory signals. The result is the recruitment of γδ T-cells, macrophages and dendritic cells into the site[100-102]. As a result, IFN-γ is produced, inducing cell apoptosis and direct tumour destruction[103]. Additionally chemokines (ex. IP-10, MIG, and I-TAC) produce anti-angiogenic effects leading to tumour starvation and elimination[104]. The third phase of the elimination process involves the recruitment of NK cells, macrophages, and tumour antigen specific $CD4^+$ and $CD8^+$ T-cells to the tumour site to successfully eliminate the most resistant components of the tumour[101, 105, 106]. During the elimination phase, innate and

adaptive immune cells have to not only discern between mutated and healthy cells, but also know where to find them. One way to discern and navigate is through the use of situational and chemotactic markers that can attract or repel both professional and non-professional phagocytes. Theses cues are referred to as “eat-me”, “don’t- eat-me” and “come-get-me” or “find-me” signals which are discussed further at a later point.

The equilibrium phase is reached when there is a balance between the immune response to the tumour and tumour survival. During this period, a form of Darwinian selection occurs, where immunogenic variants are eliminated and through the process of mutation, rare non-immunogenic forms are selected. If tumour cell destruction goes to completion, the elimination phase represents an endpoint of the cancer immunoediting process[98]. If, however, rare tumour cell variants survive the elimination phase, they enter the equilibrium phase in which the adaptive immune system prevents tumour cell outgrowth and also sculpts the immunogenicity of tumour cells. The equilibrium stage is considered the longest and may occur over a period of years where tumour may lay dormant while any outgrowth of occult tumours is specifically controlled by immunity[107]. The tumour may reside in patients for decades before being detected as either recurrence of the primary tumour or distant metastases.

Finally, the escape phase results when the strength of the surviving tumour variants exceeds the capacity of the immune system to contain them and expand rapidly[107, 108]. Conceivably, the selective pressure that promotes outgrowth of tumour cells is the result of acquired immunoevasive mutations or epigenetic changes. Potential mechanisms of immune evasion include increased resistance to the cytotoxic effects of immunity and down-regulation of major histocompatibility complex (MHC) class I proteins that result in the loss of antigen processing function[108]. At this point the tumour becomes clinically observable and may result in metastasis and the death of the host if untreated.

1.5.0 Efferocytosis

Apoptosis, or programmed cell death, is a mechanism for eliminating damaged and dying cells and is crucial for maintaining tissue homeostasis in multi-cellular organisms[109]. The process of removing apoptotic cells through phagocytosis is referred to as efferocytosis[110]. Apoptosis occurs as a consequence of the turnover of cells following natural death, infection, tissue injury, and chemotherapy or radiotherapy of tumours. The specific recognition and ingestion of apoptotic cells is a highly conserved process throughout the body[111]. If not cleared rapidly, apoptotic cells can undergo secondary necrosis and release their pro-inflammatory intracellular contents into the surrounding tissue[112]. When released into the extracellular milieu, intracellular proteins such as high mobility group box 1 (HMGB1) can serve as danger associated molecular patterns (DAMPs) to trigger the innate immune response by signalling via pattern recognition receptors (e.g. Toll-like receptors) on cells of the innate immune system. DAMP molecules are normal cell- or extracellular matrix- components that are released by cellular injury or action of proteases at the site of tissue damage respectively[113]. Defects in apoptotic cell clearance mechanisms have been linked to autoimmunity and chronic inflammation[114]. While the removal of apoptotic cells is usually assigned to "professional" phagocytes such as macrophages and dendritic cells[115], efferocytosis is now known to occur in a large number of "non-professional" phagocytes including fibroblasts, endothelial cells and proximal renal tubular epithelial cells (PTECs)[116, 117].

1.6.0. Immunogenic cell death

Apoptosis is largely regarded as an "immunologically silent" means of ridding the body of dying cells. The systemic injection of apoptotic cells has been reported by many to induce peripheral tolerance[118]. However, apoptotic cells are not always tolerogenic and can be immunogenic or "pro-inflammatory" for instance when virus-infected cells undergo apoptosis[119] or when some tumours are subjected to chemotherapy or radiotherapy[120, 121]. Therefore, a number of factors can determine if dying cells promote immunity or tolerance. Given that dendritic cells (DCs) play a central role in the

uptake of apoptotic cells and in the initiation of an immune response [122], it is not surprising that factors produced by dying cells can influence how dendritic cells promote tolerance or immunity[123]. Ideally, immunogenic cell death should be directed toward tumour cells and infected cells, whereas tolerogenic cell death should be associated with preventing unwanted immune responses to self.

1.7.0 Adjuvant role for cell death during chemotherapy of cancer

Emerging evidence suggests that cytotoxic chemotherapeutic agents such as anthracyclines work not by killing all cancer cells within a tumour but by eliciting an anticancer T-cell response triggered by chemotherapy-induced immunogenic cancer cell death[121, 124]. One requirement for dying cancer cells to elicit an immune response is the release of DAMPs, that mediate the maturation of DCs that have taken up the dead cells [123]. Certain forms of chemotherapy[120] and radiotherapy[124] can trigger immunogenic tumour cell death by causing dying cancer cells to release HMGB1, which interacts with Toll-like receptor 4 (TLR4) expressed by DCs[121, 124]. While the engulfed apoptotic cancer cell serves as a source of tumour antigen, the passive release of HMGB1 from dying cells is required for immunostimulatory presentation of antigen to anti-tumour $CD8^+$ T-cells by dendritic cells (DCs). During chemotherapy or radiotherapy, DCs require signalling through TLR4 and its adaptor MyD88 for efficient processing and cross-presentation of antigen from dying tumour cells. Furthermore, chemotherapy likely produces more apoptotic tumour cells than the DC-clearance program can cope with, and thus are expected to proceed to secondary necrosis that can further result in HMGB1 release. The delayed clearance of apoptotic cancer cells may allow for enhanced cross-presentation of intracellular antigens by mature dendritic cells [125]. Cancer cells, however, have evolved intricate mechanisms to evade T-cell-mediated killing such as MHC downregulation and induction of T-cell anergy.

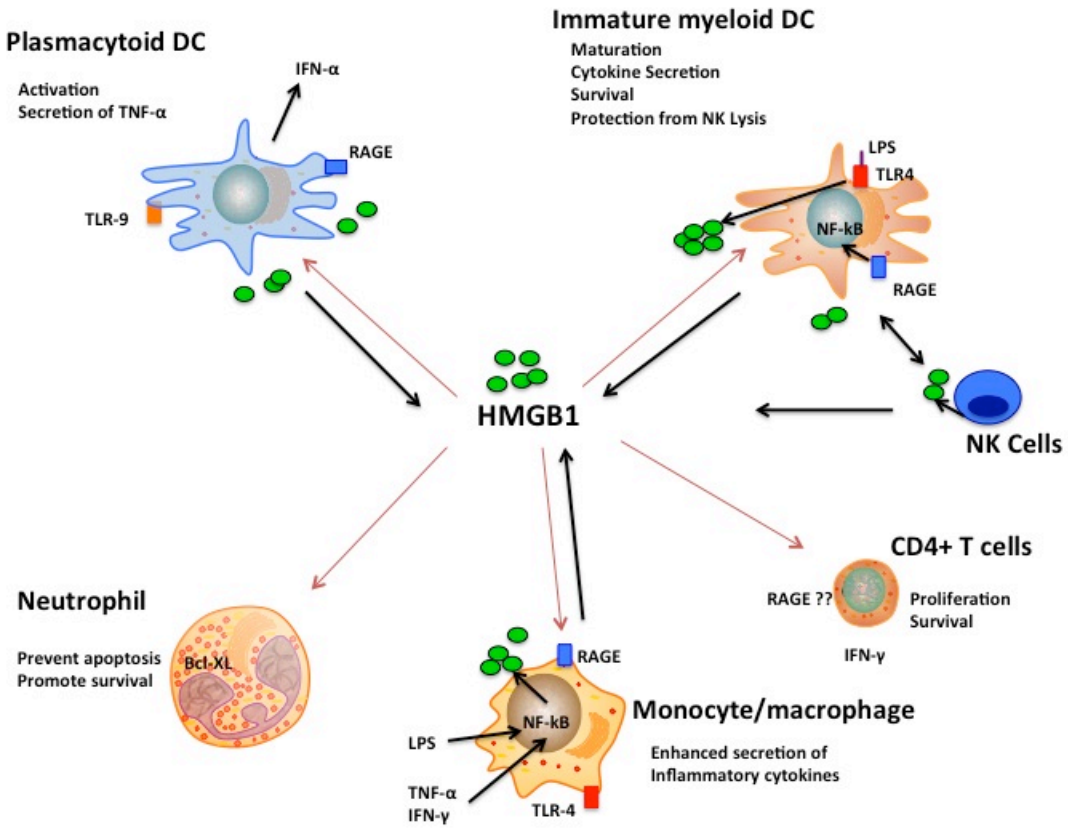
1.8.0 Dying cells trigger sterile inflammation

Injured or dying cells stimulate a sterile inflammatory response (SIR)[126] through collateral tissue damage from the inflammation[127-130]. This may be triggered in part by endogenous DAMPs that are released from cells that are distressed, damaged, or dying an abnormal death in order to alert the immune system [102, 130, 131]. Innate immune cells sensing and transducing inflammatory signals from injured cells initiate tissue damage by secreting pro-inflammatory mediators such as interleukin-1α (IL-1α)[130]. Mice deficient in certain Toll-like receptors or the IL-1R1 receptor (IL-1R), for instance, exhibit a markedly reduced SIR response and secondary tissue damage[130].

1.9.0 High Mobility Group Box-1 (HMGB1)

HMGB1 is a 215 amino acid amphoterin, which is ubiquitously expressed and highly conserved through various cell types[132]. HMGB1 is a non-histone chromatin binding protein that serves to influence nuclear transcription and stabilize the genome[133, 134]. The release of HMGB1 from DNA can be done passively or actively following cellular injury and is therefore regarded to as a DAMP. The passive release of HMGB1 from necrotic cells has been characterized, though if the passive release of HMGB1 from apoptotic cells can occur is still highly debated[135, 136]. The active release of HMGB1 mostly by monocytes and macrophages is used to amplify already existing pro-inflammatory signals through its interaction with receptors such as the receptor for advanced glycation of end products (RAGE), TLR2 and TLR4[135, 137-139]. Interactions between HMGB1 and TLR4 have been demonstrated as necessary to induce a pro-inflammatory response, therefore characterizing HMGB1 as an alarmin[140]. Additionally HMGB1 release can be done in complex with nucleic acids which can also induce TLR signalling on macrophages and dendritic cells[141] The diverse effects of HMGB1 release are summarized in **Figure 1.1**.

Figure 1.1 *The pro-inflammatory effects and results of HMGB1 release*. HMGB1 is a highly pro-inflammatory molecule, resulting in the activation and recruitment of macrophages, dendritic cells, T-cells and neutrophils to name a few. HMGB1 can be released either passively or actively from apoptotic and necrotic cells



Adapted from Ulloa et al., 2006[142]

Additionally, studies have shown the neutralization of HMGB1 results in decreased neutrophil and macrophage recruitment and increased damage in cardiovascular tissues[143]. As HMGB1 is ubiquitously expressed throughout the body it will act as a danger signal not only in specialized organs, but also throughout the body. In fact, increases in plasma/serum levels of HMGB1 have been reported in a wide spectrum of diseases including cerebral ischemia (stroke), appendicitis, arthritis, liver injury, and kidney injury[144-147].

The relationship between HMGB1 and cancer prognosis is still being elucidated. Studies have shown the integral role HMGB1 plays in inciting immune responses necessary for tumour destruction[148] whereas contrasting evidence exists for the role of HMGB1 in the metastasis and survival of various cancers. HMGB1 has been linked to the polarization of tumour associated macrophages (TAMs) to a M2-like cancer promoting phenotype, and has been correlated to lymph node metastasis in cervical squamous cell carcinomas (CSCC) indicating a potential tumour pro-survival role for HMGB1[149-151].

1.10.0 Professional vs. Non-professional Phagocytes

Phagocytosis is an actin polymerization dependent uptake of large particles by cells known as phagocytes[152-154]. Molecules on the particles surface interact with receptors on the phagocyte either via direct or indirect (ex. opsonins) mechanisms[155]. Once eaten the cargo is degraded by the acidic and hydrolytically rich vesicles within the cell and processed by cellular machinery. Phagocytosis is the mechanism by which microorganisms and senescent cells are degraded, therefore being central to the hosts immune detection system[156, 157]. Professional phagocytes are primarily polymorphonuclear granulocytes (PMNs), monocytes, dendritic cells, primary macrophages or monocyte-macrophage like cell lines. Unlike non-professional phagocytes, professional phagocytes constitutively express phagocytic receptors, for example Fc receptors that bind to antibody coated particles, to trigger phagocytosis[158]. They move easily and quickly in response to chemotactic factors and can phagocytose

cargo at a rapid rate. Additionally they are strategically positioned within tissues or organs that filter blood and lymph, allowing for rapid movement and access to damaged sites in order to facilitate a quick immune response[159]. They also secrete mediators like radicals of oxygen and nitrogen, enzymes that degrade the extracellular matrix, cytokines, products stimulated by LPS, lipid mediators of inflammation and other agents that can modify the behaviour of phagocytes[160].

Non-professional phagocytes on the other hand include epithelial cells and fibroblasts[154, 161]. The distinction between non-professional and professional phagocytes beyond the lack of Fc receptors expression is the strengthened phagocytic response from professional cells. Unlike professional phagocytes, studies show that non-professional cells take a significantly longer time to clear dying cells. However in the absence of professional phagocytes, non-professional cells are as effective as professional cells in clearing apoptotic and necrotic debris[162]. Furthermore, the range of particles which non-professional cells can consume is smaller than that of professionals due to their lack of diverse receptors. Non-professional cells cannot secrete microbial oxygen or nitrogen products and may not secrete cytokines beyond IFN-γ seen with fibroblasts[160].

1.11.0 The Post-Phagocytic Response

The post-phagocytic response is characterized by numerous steps including but not limited to; the processing and presentation of antigen, the induction of tolerance or immune activation, and paracrine and downstream signalling [163]. Of particular importance is the alteration in the surrounding microenvironment and what this may mean in the context of cancers. Phagocytosis of apoptotic cells is coupled to the resolution of inflammation not only by limiting the release of noxious cellular contents, but also because apoptotic cell recognition or engulfment activates signals that suppress pro-inflammatory responses in the phagocyte[116, 118, 164-166]. Apoptotic cell recognition and/or engulfment by macrophages can promote release of IL-10, TGF-β, PAF, and PGE2 as well as the inhibition of TNF-α, CSF, IL-12, IL-1β, and IL-18

production[118, 167]. This anti-inflammatory response to apoptotic cell is not exclusive to professional phagocytes and may extend to epithelial cells [110, 168, 169].

1.12.0 The Three Signals of Tumour Targeting

While the adaptive immune response is recognized to play an important role in anti-tumour immunity, the innate immune system, specifically phagocytes such as macrophages and DCs, are also critical for regulating tumour growth. In addition to antigen presentation and cytokine production, Chao *et al.,* recently showed that macrophages can also regulate cancer cell survival by programmed cell removal or phagocytosis[170]. Programmed cell removal is a key mechanism that links programmed cell death to the removal of the dying cell[170]. Under physiologic conditions, programmed cell removal of damaged and dying cells by macrophages is thought to be crucial for maintaining immune homeostasis[171]. Uncleared apoptotic and necrotic cells can incite inflammation through the release of toxic intracellular contents that can passively leak from cell corpses [172]. Regardless of whether phagocytes are engulfing dying cells or tumour cells, it is imperative that they be able to discern between their targets and healthy cells and also know where to find them. Phagocytic engulfment depends on the relative expression of pro-phagocytic and anti-phagocytic signals on the target cell as well as localization signals. Theses cues are referred to as “eat-me”, “don’t-eat-me” and “come-get-me” or “find-me” signals[173].

1.12.1 “Eat-Me Signals”

“Eat-me” signals serve as markers for phagocytes to identify and engulf dying or damaged cells. These signals are often newly upregulated or modified existing molecules that can either appear on or change the charge of the cell surface[164, 174-176]. Professional phagocytes and semi-professional phagocytes employ cell-surface receptors to directly and indirectly bind to these “eat-me” signals to induce phagocytosis. There are numerous classes of phagocytic receptors including scavenger receptors (ex. CD36),

integrins ($\alpha_v\beta_3$ integrin receptor), lectins, and tyrosine kinases (ex. MerTK) to name a few[177-179].

The most common and best-characterized "eat-me" signal is the membrane phospholipid, phosphatidylserine (PS). PS exposure occurs very early during the apoptotic process and is almost universal in its requirement for engulfment[112, 180-183]. PS is a phospholipid that is bound to the inner leaflet of the cellular membrane in healthy cells. Upon initiation of the cell death program, inhibition of a scramblase that normally confines PS to the inner leaflet, allows for the translocation of PS to the outer plasma membrane. In all cells capable of engulfment a PS receptor is universally expressed.

After engagement of PS, the resulting phagocytic process can result in changes in the cellular microenvironment. For example PS mediated engulfment promotes the release of TGF-β while inhibiting TNF-α production, promoting an anti-inflammatory response by the phagocyte[167]. PS can bind to receptors directly in the case of KIM-1 or via bridging molecules such as milk-fat-globule-EGF factor 8 (MFG-E8), serum protein S growth arrest-specific 6 (GAS 6) and B2 glycoprotein 1 (B2GP1)[184]. PS bound to MFG-E8 on apoptotic cells can be recognized by $\alpha_v\beta_3$ integrins on macrophages and dendritic cells[185, 186]. Knockout studies of a phosphatidylserine receptor by M. Li *et al.,* demonstrated the necessity of PS in the phagocytosis of apoptotic cells[187]. PS exposure however, is not sufficient. Studies have shown that in healthy erythrocytes coated with PS, PS expression alone was not sufficient to induce adhesion or internalization of live red blood cells[188].

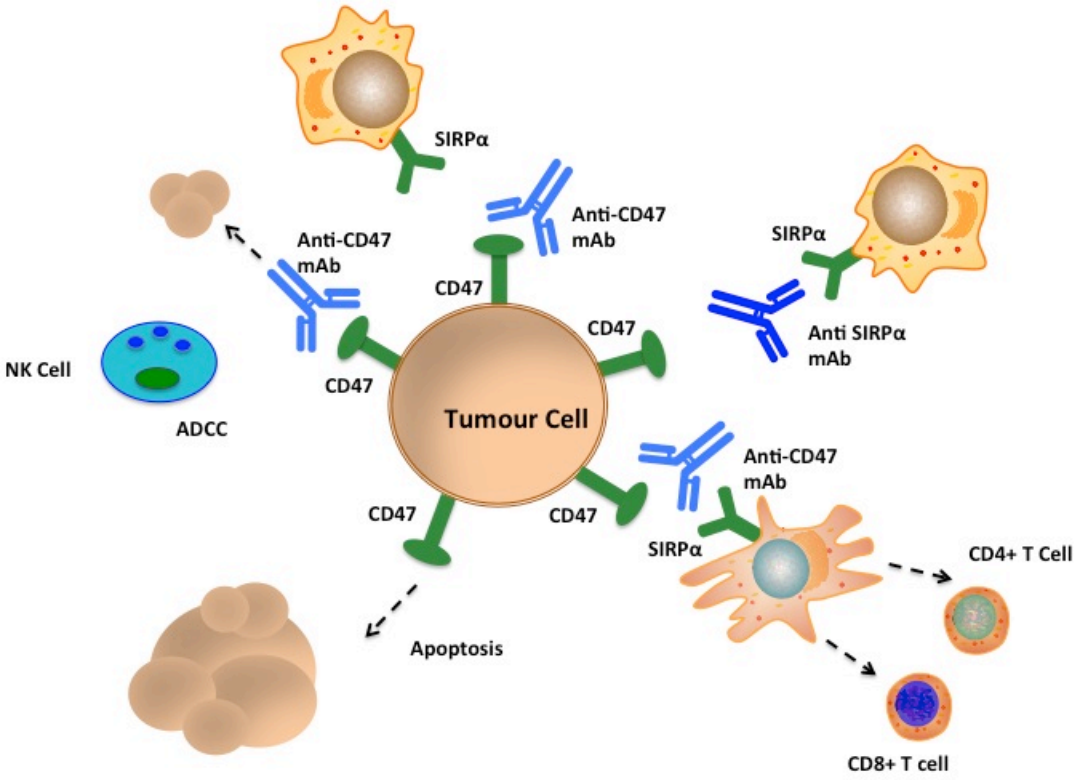
1.12.2 "Don't-Eat-Me Signals"

During the surveillance process, phagocytes will encounter live cells that do not need to be destroyed. Therefore there must be a mechanism that allows immune and non-immune cells to differentiate between healthy and apoptotic cells. Studies by Brown *et al*., highlighted that the interaction between CD31 molecules on live leukocytes and macrophages facilitated the detachment of leukocytes from the macrophages[189].

Additionally, Brown also demonstrated that during apoptosis this detachment process between leukocytes and macrophages was inhibited. Thus CD31 functioned as a "don't-eat-me" signal.

The idea of "don't-eat-me" signals or other repulsive signals coincides with the theory that 'markers of self' must also exist to allow the immune system to discern between foreign and potentially dangerous, and self and potentially harmless. For example CD47 expressed on red blood cells sends negative engulfment signals via signal regulatory protein α (SIRPα) to splenic macrophages to not eat them[190]. CD47 is expressed on numerous cell types and binds to SIRPα on macrophages to prevent the accumulation of myosin-11A at the phagocytic synapse [191]. In the context of cancers, recent efforts have been directed towards exploiting the expression of "don't-eat-me" signals on various cancer forms. Wessman and colleagues have revealed that tumours can evade phagocytosis of macrophages by expressing "don't-eat-me" signals such as CD47[170]. CD47 expression has been characterized on seven major types of cancer and is therefore being examined as a means to manipulate tumour survival[192]. The SIRPα receptor is expressed on macrophages, T-cells and dendritic cells to negatively regulate phagocytosis [190, 191, 193-195]. Therefore tumours can evade phagocytosis and immune detection by all of the above-mentioned pathways, ensuring their survival. **Figure 1.2** represents the influence of "don't-eat-me signals" on immune cells.

Figure 1.2 *The result of CD47 expression on tumour cell survival.* The expression of CD47 by tumour cells may serve as a way to evade immune detection by numerous immune cells including T-cell, macrophages and dendritic cells. The characterization of CD47 on seven major cancer forms has prompted research into the neutralization of CD47 as a means for tumour detection.



Adapted from Chao et al., 2012[170]

1.12.3 "Come-and-Get-Me Signals"

"Don't-eat-me" and "eat-me" signals serve as critical mediators of phagocytosis. However these signals are deemed sufficient for efferocytosis only when phagocytes are in close proximity with their apoptotic targets. When the phagocyte is not within the immediate environment, it has no way of knowing if apoptotic cells are in need of clearance before they become necrotic. As a result, attraction signals must exist that induce the rapid migration of phagocytes to the site of interest in order to prevent necrosis[196] and autoimmunity. Lauber *et al.,* demonstrated using an *in vitro* transwell migration assay, that the supernatants of apoptotic cells contained various chemoattractants for monocytes and primary macrophages[197]. The authors show that apoptotic cells, and not non-apoptotic cells, released chemotactic factors via caspase-3 mediated Ca^{2+} release that would draw phagocytes to them. Documented "come-and-get-me" signals include lysophosphatidylcholine (LPC), S19 ribosomal dimers, endothelial monocyte-activating peptide and CX3CL1 [111, 172, 198]. Studies have shown that dying cells can actively release "come-and-get-me " signals recruiting macrophages and monocytes to remove apoptotic cells[173, 199]. Similar findings were demonstrated in MCF7 breast cancer cells, where post induction of apoptosis, apoptotic breast cancer cells would release LPC, attracting human acute monocytic leukemia (THP-1) monocytes *in vitro*[197]. As a result of "come-and-get-me" or "find-me" signalling, phagocytes can effectively recruit semi-professional and professional macrophages that will engulf "eat-me" signal expressing apoptotic cargo.

1.13.0 Kidney Injury Molecule-1

Kidney Injury Molecule-1 (KIM-1) was first discovered as the hepatitis A virus cellular receptor 1 (HAVCR1) [200]. It was then observed in the kidney epithelium where the structural similarities became known [201] and then again in T-cells[202]. As part of its characterization in T-cells, KIM-1 is also referred to as T-cell immunoglobulin mucin-1 (TIM-1) and is thought to function as a co-stimulatory molecule in T-cell activation[203]. Human TIM-1/KIM-1, TIM-3 and TIM-4 belong to the T-cell immunoglobulin mucin

(TIM) gene family that have been characterized as influencing factors on a variety of cell types including T-cells [204-207], dendritic cells[208-210], B-cells [211], NK cells[212, 213] and monocytes/macrophages[214-218]. Each of the TIM-1 gene family members is a type 1 transmembrane glycoprotein. The structure of KIM-1 is characterized by an extracellular immunoglobulin (Ig) like domain, followed by mucin domain containing O-glycosylation and N-glycosylation sites and finally a short intracellular and transmembrane domain. A cartoon diagram depicting the structure of human KIM-1 is in Appendix 1.1. The molecular weight of KIM-1 is approximately 100kDa and can undergo spontaneous extracellular cleavage resulting in a 90kDa soluble fragment and a 15 kDa transmembrane component, with the ectodomain portion released into the lumen of the tubule[219, 220].

In 2008 Ichimura *et al.,* demonstrated the ability of KIM-1 to bind to the 'eat-me' signal phosphatidylserine (PS) on the surface of apoptotic and necrotic cells, resulting in their engulfment[117]. The same is true of TIM-1 and TIM-4, with the strongest mediator being TIM-1[117]. Structural interactions are mediated by the hydrophobic head of PS inserting itself into the metal ion binding side in the Ig domain of KIM-1. KIM-1 can bind to other proteins such as oxidized LDL, necrotic debris, phosphatidylethanolamine (PE) and phosphatidylcholine (PC). Therefore, KIM-1 is also regarded as a scavenger receptor but its highest affinity is to PS[219, 221, 222]. The cytoplasmic domain has various cell signalling capacities through the MAP kinase stress pathway and JAK/STAT signalling families[223, 224].

In relation to the kidney, KIM-1 was identified as part of an mRNA screen for genes upregulated on proximal tubule epithelial cells 24 to 48 hours after kidney injury in rats subjected to ischemia reperfusion injury (IRI)[225], a form of injury that mimics acute kidney injury (AKI) in humans and IRI during transplantation of solid organs[201]. KIM-1 is unique in that it is the only characterized molecule on epithelial cells that transforms cells into semi-professional phagocytes[226]. KIM-1 expressing cells are characterized by an increase in 5-bromodeoxyuridine (BrdU), which is a marker for proliferation and vimetin, a marker for dedifferentiation[227]. In fact multiple studies exist linking the

dedifferentiation of kidney epithelial cells to KIM-1 expression[228-230]

Beyond conferring the ability to induce proximal tubular epithelial cells to become semi-professional phagocytes, KIM-1 is being tested as a strong biomarker for kidney damage[227, 230, 231]. The absence of KIM-1 expression in the normal kidney, its rapid and significant expression in the proximal tubule post-injury, its persistence until full recovery and the fact that robust and stable ectodomain cleavage is correlated to damage degree, make KIM-1 a potentially strong biomarker[225]. A recent study by Tonomura *et al.,* tested thirteen highly used biomarkers for injury and concluded that KIM-1 was not only the most sensitive but the most accurate predictor of damage, even outperforming serum creatinine[232-234]. These data suggest that KIM-1 is upregulated in kidney in an injury-specific fashion to mediate clearance of apoptotic cells by tubular epithelial cells.

1.14.0 Renal Cell Carcinoma

The prevalence of kidney cancers throughout the world presents the need for rapid and effective curative and preventative solutions. Kidney cancer is among the ten most frequent cancers in the Western world[235] with renal cell carcinoma (RCC) representing 90% of those cases[236]. Despite advances in imaging techniques and diagnoses tools, 20-30% of diagnosed cases exhibit metastases[237] and 95% of those are fatal due to late stage identification[238]. Additionally, beyond nephrectomy or chemotherapy, prognosis for RCC patients is extremely poor. The only FDA approved treatment for RCC is an IL-2 based treatment, approved over twenty years ago[239]. The complexities with developing novel treatments lies within the inherent toxic nature of the molecules used. For example various groups have highlighted that with the current regimens used for IL-2 treatment, even in small molecule minimally toxic therapies, the systemic effects can be taxing, rendering IL-2 somewhat ineffective[240]. In RCC clinical therapies, 15-20% of patients on average withdraw from treatment and 30-40% required a reduced dose of the drug[241-246]. Standard treatment for RCC is complete resection by either a radical or partial nephrectomy and remains the mainstay of curative treatment[247]. Thus, treatment is usually limited to surgical nephrectomy, which is a significant risk factor for the

development of chronic kidney disease[248]. Furthermore the resistive nature of RCC to chemotherapy necessitates alternative forms of therapies that can increase patient survival[249].

1.15.0 KIM-1 is expressed by many human cancers

Beyond the kidney, others have demonstrated the expression of KIM-1 protein in approximately 40% of ccRCCs [250]. In a screen done for human KIM-1 mRNA levels in various cancers, Lin and colleagues found detectable levels of KIM-1 mRNA in non-clear cell lung carcinomas (NSCLC) and ovarian and renal carcinomas[251]. The significance of KIM-1 expression in cancers however, remains unclear. A study done in 2004 using the 769P renal cell carcinoma (RCC) cell line demonstrated the role of KIM-1 in blocking the expression of differentiation markers in epithelial cells and contributing to the metastasis of tumours[252]. A second epidemiological study looked at trichloroethylene (TCE) and renal cancer. In this study patients exposed to critical levels of TCE ultimately developed renal cancer and all had elevated levels of KIM-1[253]. In contrast a study using geraniol (GOH), a chemoprotective agent, showed that GOH treatment reduced the levels of KIM-1 expression in rat renal tumours [254]. The area of KIM-1 expression as a direct influencing factor as opposed to predictive factor of tumour development is highly understudied. Furthermore the signalling pathways of KIM-1 in cancers have yet to be examined.

The role of shed KIM-1 as a predictive biomarker for RCC and other kidney injuries has been highly characterized[231]. A retrospective study done by Cuadros and colleagues in 2013 correlated higher urinary (uKIM-1) and cytosolic levels of KIM-1 to a more aggressive and invasive phenotype in RCC patients[250]. Another group examining urinary neutrophil geletinase-associated lipocalin (uNGAL) and uKIM-1 found uKIM-1 in conjunction with uNGAL was predictive in 91.6% of RCC patients of disease status[255]. A third group examining urine KIM-1 levels post-nephrectomy in a KIM-1 positive population found uKIM-1 levels fell in accordance with the removal of renal masses, indicating a relationship between mass presence and KIM-1 level[238]. An

additional study showed a linear correlation between uKIM-1 and tumour size pre-nephrectomy for RCC[256]. A follow-up study done in 2014 demonstrated that the shedding of KIM-1 activates the IL-16/STAT-3/HIF-1α pathway that is connected to angiogenesis and tumour growth pathways[257]. The large amount of evidence highlighting the potential role of KIM-1 in influencing RCC tumour outcome warrants further investigation into potential mechanisms by which KIM-1 expression may confer an advantage to RCC tumours.

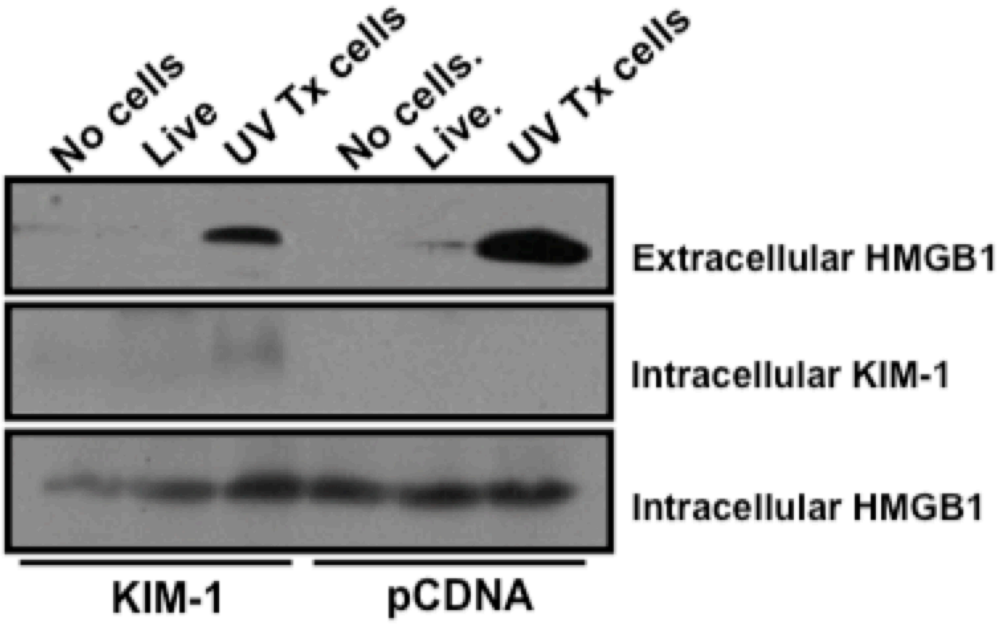
1.16.0 Foundational Work and Thesis Rationale

1.16.1 Foundational Work

Preliminary work done by Dr. Gunaratnam, suggested that KIM-1 expressing kidney tubular epithelial cells might regulate extracellular DAMPs (HMGB1) through efferocytosis, as seen in **Figure 1.3**. Here, he added apoptotic cells (at an effector to target ratio of 1:2) to cultures of renal epithelial cells expressing (WT-PK1) and not expressing KIM-1 (pcDNA-PK1). After a 24 hour incubation period, the resultant conditioned cell media and lysates were analyzed by Western blot for the presence of HMGB1. Compared to that of control cells not expressing KIM-1, he observed significantly more intracellular HMGB1 in the lysates and less HMGB1 in the conditioned medium of KIM-1-expressing cells when exposed to thymocytes. This clearly demonstrated a role for KIM-1 in limiting HMGB1 release. It is based on this work that our lab decided to look at how KIM-1 might regulate HMGB1 release from dying cells.

Figure 1.3 *KIM-1 expression prevents the leakage of HMGB1 from apoptotic cells.* Pig tubular cells were transfected with pcDNA-KIM-1 or pcDNA alone and fed apoptotic thymocytes (Tx). The leakage of HMGB1 was measured in the media and a difference in HMGB1 release as a by-product of KIM-1 expression was observed.

Adapted from Gunaratnam, unpublished


No cells
Live
UV Tx cells
No cells.
Live.
UV Tx cells
Extracellular HMGB1
Intracellular KIM-1
Intracellular HMGB1
KIM-1
pCDNA

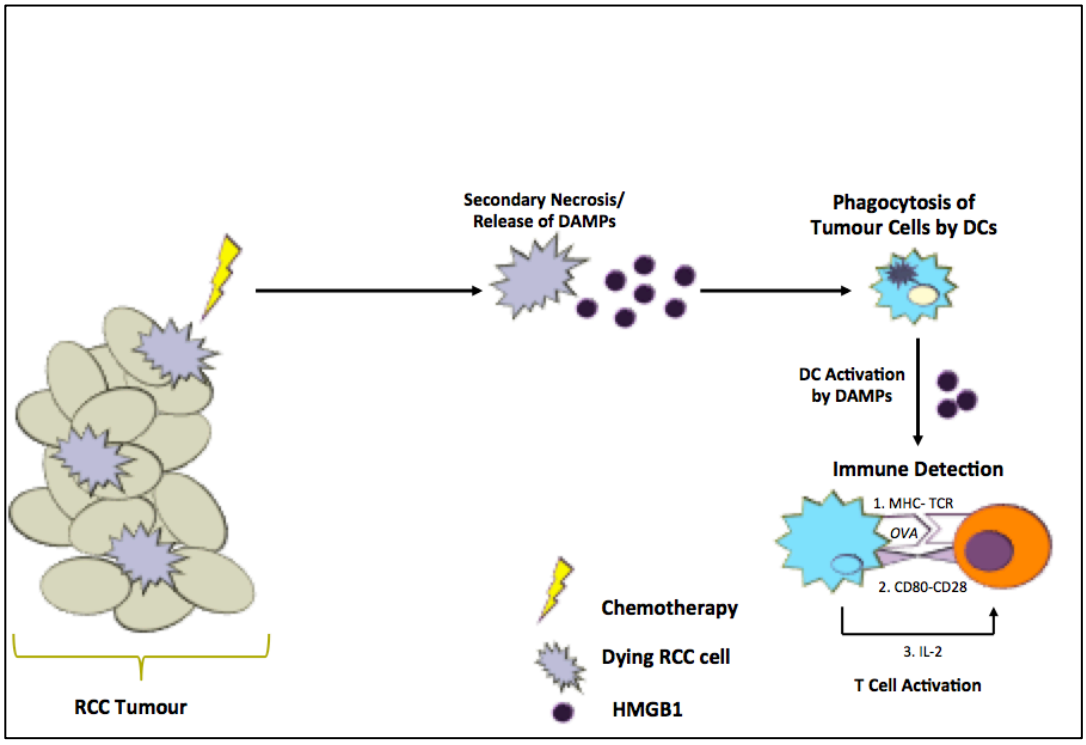
1.16.2 Thesis Rationale

RCC accounts for approximately 3% of all new cancer cases and the incidence rates for all stages have been rising steadily over the last three decades [258]. It is the most common and deadly form of urogenital cancer in humans[258]. Unfortunately, RCC is relatively resistant to chemotherapy and radiotherapy making surgery the mainstay of therapy for patients. KIM-1 is a cell-surface receptor for PS that is widely expressed in RCC tumours[231, 251, 259]. KIM-1 overexpression in a variety of cells including epithelial cells converts them into avid phagocytes for apoptotic[117, 221] and necrotic[117] cells. Work done by Lin and Vila demonstrated HAVCR-1/KIM-1 mRNA and protein expression in numerous other types of cancers[251, 252]. Moreover, RCC cell lines established from patient tumours constitutively express KIM-1[117, 221]). The above data together with the possibility that KIM-1 expression and shedding by RCC tumours may be a predictive biomarker of tumour progression[259] led us to explore further the role of KIM-1 in RCC tumours. The biological relevance of KIM-1 expression to the pathogenesis of cancer is unknown. Phagocytes that ingest apoptotic cells actively suppress local inflammation while preventing the release of immunogenic contents (e.g. HMGB1) as they undergo secondary necrosis. When released into the extracellular milieu, intracellular proteins such as HMGB1 can serve as DAMPs to trigger the innate immune response. By contrast, cancer cell death elicited by certain chemotherapeutic agents such as anthracyclines is immunogenic [123]. Immunogenic death involves exposure of “eat-me” signals on the cell surface, as well as the release of soluble immunogenic signals or alarmins such as HMGB1 that serve to license DCs and macrophages to activate anti-tumour immunity [123]. The preliminary work done by Dr. Gunaratnam in Figure 1.3 suggested that KIM-1-expressing epithelial cells could limit the passive release of HMGB1 from apoptotic cells undergoing secondary necrosis and necrotic cells by rapidly clearing them from the extracellular milieu. Thus, we argued that the ability of RCC cells to alter the immunogenic tumour microenvironment (free HMGB1) resulting from cancer cell death (e.g. due to chemotherapy) might serve as a novel tumour evasion mechanism. Therefore, **we hypothesized that the constitutive expression of KIM-1 by RCC cells would enable cancer cells to prevent the**

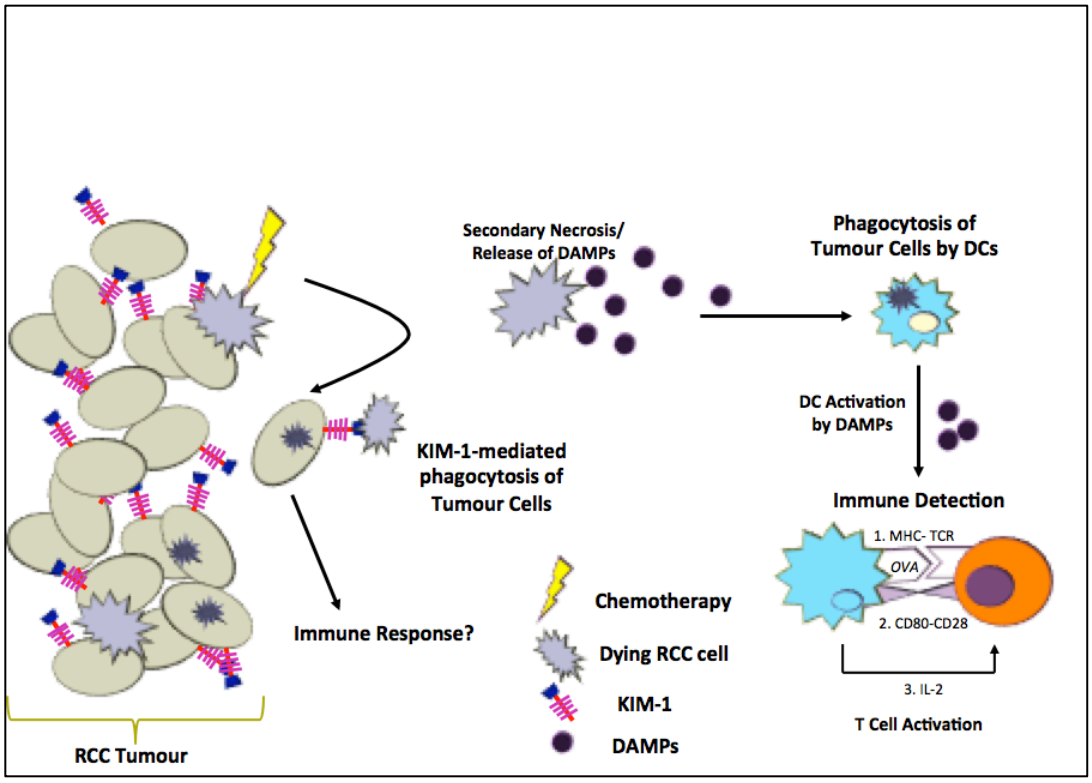
activation of the innate immune system (DCs) by the passive release of alarmins (HMGB1) from uncleared apoptotic and necrotic cells in their tumour microenvironment. A working model is seen in **Figure 1.4.**

Figure 1.4 *Proposed model for KIM-1 mediated sequestration of DAMPs*. (A) In the absence of danger signals, the release of HMGB1 from primary and secondary necrotic cells would result in immune activation. (B) The expression of KIM-1 would result in the phagocytosis of surrounding dying tumour cells and limit the release of HMGB1 and downstream activation

(A)


Secondary Necrosis/
Release of DAMPs
Phagocytosis of
Tumour Cells by DCs
DC Activation
by DAMPs
Immune Detection
1. MHC- TCR
OVA
2. CD80-CD28
3. IL-2
T Cell Activation
RCC Tumour
Chemotherapy
Dying RCC cell
HMGB1

(B)


Secondary Necrosis/
Release of DAMPs
Phagocytosis of
Tumour Cells by DCs
DC Activation
by DAMPs
KIM-1-mediated
phagocytosis of
Tumour Cells
Immune Detection
1. MHC- TCR
OVA
2. CD80-CD28
3. IL-2
T Cell Activation
Immune Response?
RCC Tumour
Chemotherapy
Dying RCC cell
KIM-1
DAMPs

Chapter 2: Materials and Methods

2.1.0 Cell Culture

The origins of the cell lines used in this thesis are outlined in **Table 2.1.** All cells were cultured at 37ºC and 5% CO_2. Cells were grown in Roswell Park Memorial Institute (RPMI) media, Dulbecco's Modified Eagle's Medium (DMEM) or Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS). FBS components were purchased from Lonza (Basel, Switzerland). Penicillin Streptomycin (P/S) was purchased from Wisent Inc. Geneticin (G418) was purchased from Santa Cruz Biotechnology (Dallas, TX). Cells that were not donated were purchased from ATCC.

Table 2.1 List of cell lines

Cell Line	Host	Type	Growth Conditions	Source
A549	Human	Lung carcinoma	RPM1 +10% FBS	Dr. John DiGuglielmo
OCC-1	Human	Ovarian carcinoma	RPMI + 10% FBS + 1% P/S	Dr. DiMattia
769P	Human	Renal carcinoma	RPMI + 10% FBS + 1% P/S	ATCC
786-0	Human	Renal adenocarcinoma	RPMI + 10% FBS + 1% P/S	ATCC
SKOV3	Human	Ovarian adenocarcinoma	RPMI + 10% FBS + 1% P/S	Dr. Gabriel DiMattia
PC3	Human	Prostate adenocarcinoma	RPMI + 10% FBS + 1% P/S	Dr. Paula Foster
ACHN	Human	Renal cell adenocarcinoma	EMEM + 10% FBS +1% P/S	Dr. Alp Sener
HeLa	Human	Cervical epithelial adenocarcinoma	EMEM + 10% FBS	ATCC
LLC-KIM-1-PK1	Pig	Kidney epithelial	DMEM + 10% FBS + G418 (400ug/ml)	Self established
LLC-PK1	Pig	Kidney epithelial	DMEM + 10% FBS + G418 (400ug/ml)	ATCC

2.2.0 Plasmid Transfection

LLC-PK1 cells are pig kidney epithelial cells that were used to establish the LLC-KIM-1-PK1 (WT-PK1) cell line. One million LLC-PK1 cells were plated 24 hours prior to transfection on a $10cm^2$ dish (BD Biosciences Franklin Lakes, NJ). When cells reached 70-85% confluence they were transfected using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) with either an empty pcDNA3 plasmid or pcDNA3 encoding full length KIM-1 cDNA. G418-resistant (Santa Cruz Biotechnology Inc.) stable cell lines were selected from positive colonies grown in complete medium containing 400 ng/µl of G418 as described elsewhere [117, 201, 260].

2.3.0 Thymocyte Culture and Induction of Apoptosis or Necrosis

Single-cell suspensions of primary thymocytes were taken from the thymi of C57BL/6 mice ranging from 6-8 weeks of age by grinding them over cell strainers (BD Biosciences) using a 10µl syringe plunger (BD Biosciences). To remove red blood cells, the thymocyte suspension was resuspended in ACK lysis buffer (Lonza) for 5 minutes. Thymocytes were washed three times in ice-cold PBS and resuspended in RPMI media containing 10% FBS and 1% P/S and incubated at 37ºC and 5% CO_2 to keep them alive.

For induction of apoptosis, cells were counted and placed under ultraviolet (UV) radiation (254nm) for 3 minutes. For phagocytosis assays, cells were first labeled with carboxyfluorescein succinimidyl ester (CFSE), (Invitrogen, Carlsbad, CA) at a dilution of 1µl/ml for 5 minutes, washed with complete medium to remove any excess CFSE and then left for a minimum of 8 hours prior to use at 37ºC and 5% CO_2.

For inducing necrosis, cells were counted using a hemocytometer (VWR, Radnor, PA) and placed in a water bath at 56ºC for 15 minutes in 1.5 ml eppendorf microcentrifuge tubes (Eppendorf, Hamburg, Germany). Cells were used immediately after heat shock.

2.4.0 RNA Extraction

For total RNA isolation from cells cultured in 6-well tissue culture plates, 1ml of Trizol (Life Technologies) was added per well and left to incubate for 2-3 minutes at room temperature. Total RNA was isolated according to the protocol established by Chomczynski & Sacchi, in 1987[261]. Samples were then resuspended in 30 µl of diethylpyrocarbonate (DEPC) treated water. The concentration and purity of each RNA sample was determined by measuring the absorbance at 260nm as well as the 260/280 and 260/230 nm ratios using a Nanodrop 1000 spectrophotometer (Nanodrop Products, Wilmington, DE). Samples were then stored at -80ºC until used for cDNA synthesis.

2.5.0 Complementary DNA (cDNA) synthesis

As part of first strand synthesis, 1µg of total RNA was combined with a 40nM Oligo-dT primer (New England Biolabs, Ipswich, MA), 10nM dNTP mix (Quantas, Gaithersburg, MD) and DEPC water in a total reaction volume of 10 µL. The samples were heated for 5 minutes at 80ºC using a Mastercycler thermocycler (Eppendorf, Hamburg, Germany). Next 10xRT buffer, M-MuLV Reverse Transcriptase and RNAse Inhibitor at 10U/µl (New England Biolabs, Ipswich, MA) were added to a final volume of 20µl. Cycling parameters were as follows: 42ºC for 60 minutes, 90ºC for 10 minutes and 4ºC until ready for use. Samples were stored at -20ºC.

2.6.0 Quantitative Real Time PCR (qRT-PCR)

For qRT-PCR, 100ng of the cDNA stock was amplified using the Perfecta SYBR Green Fastmix with ROX (Quantas, Gaithersburg, MD). Primers were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and designed by myself, previous lab members or taken from former labs. Primers were added at 250nM concentrations and are listed in **Table 2.2.** Amplification was done using the StepOne Plus thermocycler (Life Technologies) and analyzed with the corresponding software. The parameters for the run included one cycle of 95ºC for 30 seconds, followed by 40 cycles at a temperature between 0-5ºC lower then the primer melting temperatures (Tm) and finally a melt curve dissociation step. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization[262].

Table 2.2 Human PCR primers

Gene Target	Forward Primer (5'→3')	Reverse Primer (5"→ 3')	Product Size
KIM-1	GAA GTG GCT ACT GGT TCA TGG	ACG ACT GTT CGA ACG AGC AC	260
GAPDH	CTC TTC TGC TCC TCC TGT TCG AC	TGA GCA ATG TGG CTC GGC T	162

2.7.0 KIM-1 siRNA Knockdown

Transfection of KIM-1 siRNA was performed in Opti-MEM Media (Life Technologies) in conjunction with Lipofectamine 2000 (Life Technologies) using the Life Technologies protocol. 24 hours prior to transfection, cells were plated at between 60-75% confluence in 2 mls per well of RPMI media containing 10% FBS and 1% P/S. Two hours prior to transfection, the medium was exchanged with 1 ml of Opti-MEM media in each well of the 6-well dish. Commercially available KIM-1 siRNA (sc-61691) or control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology and transfected at a final concentration of 10nM into cells plated in 6-well plates containing 500ul of Opti-MEM media per well. Cells were given an additional 1.5 µl of RPM1 containing 10% FBS media to ensure adequate survival for 24 hours until apoptotic or necrotic cells were added.

2.8.0 Western Blot Procedure

After the appropriate treatment, cells were harvested at approximately 90-95% confluence and lysed using a lysis buffer containing 4% sodium dodecyl sulfate (SDS) in PBS[7]. Lysate samples were then scraped using cell scrapers (Mandel, Guelph, ON) and sheared with a 26-gauge syringe (BD Sciences). Samples were then boiled for 5 minutes at 98ºC and quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Typically, 60µg of total protein was loaded in each well for SDS-PAGE. For conditioned media samples used for detection of soluble HMGB1, samples were centrifuged at 7.6 x relative centrifugal force (RCF) for 5 minutes to remove any cellular material. Approximately 80% of the sample was collected to ensure cellular debris was left behind. All lysate and media samples were diluted in water to total volume of 55µl containing 9µl of 6x gel loading dye (New England Biolabs). A total volume of 50µl was loaded in each well of a 10-well 10% SDS page gel using the Bio-Rad Mini Trans-Blot system (BioRad, Mississauga, ON). The samples were then transferred onto a PVDF membrane (EMD Millipore, Darmstadt, Germany) and blocked with 5% skim milk powder (Carnation Inc., Switzerland) in PBS and .2% Tween-20 for 30 minutes to one hour. The appropriate primary antibody was then applied at the concentration listed below and incubated overnight at 4^{o}C on a rocker (VWR Canada). A secondary antibody

conjugated to horse radish peroxidase (HRP) was applied at varying dilutions for an hour to two hours and then developed using HRP-specific Luminata Forte (EMD Millipore) and imaged with the Licor Imaging system (Mandel Scientific). The images were digitally visualized and quantified by densitometry using the ImageJ Software. A list of all the primary and secondary antibodies used in our studies is listed below in **Table 2.3**

Table 2.3 Primary and secondary antibodies for western blot

Specificity	Description and predicted MW of band	Usage	Dilution Factor from Stock	Source
KIM-1	Intracellular KIM-1 binding Size: 100kDa	Primary	1: 1500	Ichimura Lab (Harvard, MA)
HMGB1	Chromosomal protein Size: 29kDa	Primary	1:1000	Sigma-Aldrich
AKG-3	Extracellular KIM-1 Size: 95kDa	Primary	1:3	Bonventre Lab (Harvard, MA)
Actin	Beta-actin loading control Size: 40kDa	Primary	1:1500	Novus Biologicals
Goat IgG	Goat specific HRP conjugate	Secondary	1:20,000	Jackson Research Labs
Mouse IgG	Goat specific HRP conjugate	Secondary	1:30,000	Jackson Research Labs
Rabbit IgG	Goat specific HRP conjugate	Secondary	1:30,000	Jackson Research Labs

Note: All primary and secondary antibodies are diluted in 5% skim milk powder in 0.2% PBS tween and added to the membrane

2.9.0 Dendritic Cell Culture

Primary bone marrow-derived dendritic cells were isolated from the bone marrow of 6-8 week-old C57BL/6 mice and cultured in IL-4 and GM-CSF (Santa Cruz) for a span of one week. The bone marrow was first isolated from mice and washed multiple times with a syringe and PBS. Cells were then spun down at 500 x *g* and resuspended in RPMI and 10% FBS with non-essential amino acids (NEAA), and sodium pyruvate. IL-4 and GM-CSF was added in a 1µl/ml concentration and the media was changed every 2 days with new IL-4 and GM-CSF added. Cells were then plated the night prior to usage.

2.8.0 Dendritic Cell Marker Staining

After the associated experiments were complete, DCs were washed with PBS and transferred into fluorescence assisted cell sorting (FACS) tubes (BD Sciences). The samples were spun down at 350 x *g* for 5 minutes using a centrifuge (Eppendorf) and then incubated with a mixture of the following markers; an isotype control, CD11c, CD40, CD86 (Santa Cruz) and unstained solutions all resuspended in FACS buffer. 2 mls of PBS was then added to each tube, spun down, and resuspended in 1% paraformaldehyde (PFA) FACS buffer (Gunaratnam Lab).

2.10.0 KIM-1 Surface Staining

Cells were harvested in a single cell suspension containing cell staining buffer (Biolegend). Cells were then centrifuged at 350 x *g* for five minutes followed by resuspension in staining buffer containing PE-anti-KIM-1 antibody (Biolegend) at 1 µg/ml and incubated for 15-20 minutes on ice in the dark. Cells were then washed twice with staining buffer and spun at 350 x *g* for 5 minutes prior to analysis on the LSR II flow cytometer at the Robarts Research Institute.

2.11.0 Annexin V/PI staining and Analysis

Determination of live, apoptotic and necrotic status for thymocytes was done using Annexin V and Propidium Iodide (PI) (Biolegend) staining. Protocol used was optimized by Dr. Xizhong Zhang in our laboratory. Briefly, single-cell suspensions of live or killed thymocytes were incubated with 40uM of Annexin-V-FITC for 30 minutes after which

10mM PI was added. After staining each group, samples were analyzed on a FACS Calibur cytometer (BD Sciences) equipped with 488 and 633 nm lasers, and emission filters for FITC, PI, APC and AlexaFluor633 at Robarts Research Institute. Data was analyzed with FlowJo Mac software (Tree Star Inc.). Percentage of maximum intensity was calculated by dividing the MFI of each sample by that of the maximally affected sample, (i.e. percent max = MFI_{sample}/ MFI_{max}*100).

For phagocytosis assays, apoptotic cells were labeled with CFSE at a concentration of 1µl/1 million cells and a minimum of 8 hours. Thymocytes were added to target cells and left for 24 hours to allow for sufficient phagocytosis. Percent positive CFSE cells were run using FACS Calibur (BD Sciences). All analysis of flow data was done using FlowJo (Treestar, Ashland, OR). Forward-scatter/side-scatter (FSC/SSC) ratio was used to exclude apoptotic cell debris.

A summary of all Flow Cytometry antibodies used during the course of this project can be found in **Table 2.4**

Table 2.4 Antibodies for flow cytometry

Antibody	Species	Fluorochrome	Manufacturer
CD11c	Mouse	APC	Biolegend
CD40	Mouse	FITC	Biolegend
CD86	Mouse	Brilliant Violet 421	Biolegend
CFSE	Mouse	FITC	Biolegend
Annexin V	Mouse	FITC	Biolegend
Propidium Iodide	Mouse	PI	Biolegend
KIM-1	Human	PE	Biolegend

2.12.0 Animals

Six to eight week-old male C57BL/6 mice were purchased from Charles River Laboratories and housed at the animal facility at Western University (London, ON, Canada). Experiments were conducted by following established guidelines for animal care approved by the University Council on Animal Care at Western University.

2.13.0 Statistical Analyses

All statistical analysis was calculated using GraphPad Prism 5.01 (La Jolla, CA). Error bars among samples reflect the standard error of the mean (SEM). Significant differences between samples were determined using an unpaired t-test with $p<0.05$ considered significant for all experiments. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$. For grouped analyses a two-way ANOVA test with a Bonferroni post-test correction was used. $P<0.05$ is considered significant for all experiments. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$

Chapter 3.0 Results

3.1 Characterizing the expression of KIM-1 in human cancers

In order to evaluate the role of KIM-1 as an immune evasion mechanism in cancer, we first characterized the expression levels of KIM-1 on a diverse group of cancer cell lines. Established cell lines from patients with prostate cancer, lung cancer, renal cancer, ovarian cancer and cervical cancer were grown *in vitro*. Total RNA was isolated from each of these cell types after culturing them in 6-well plates for 24 hours. Samples were then reverse transcribed and amplified using quantitative PCR **(Figure 3.1)**. As shown below, KIM-1 mRNA was detected in all the tested cancer cell lines except for HeLa and OCC-1. KIM-1 gene expression was significantly higher in the renal cancer cells tested and is in keeping with what has been reported[263]. Nonetheless, KIM-1 mRNA was observed in the prostate and lung cancer cell lines as well. Because 769P and 786-0 cells expressed the highest levels of KIM-1, these were selected for further study. Next, the surface expression of KIM-1 protein was examined in both 769P and 786-0 cells by flow cytometry. For both 769P and 786-0 cells, LLC-PK1 cells that do not express human KIM-1 were used as a negative control. The lack of KIM-1 in LLC-PK1 cells was characterized previously[264]. Approximately 96% and 92% of the 769P cells and 786-0 cells stained positive for KIM-1 respectively relative to the negative control (3.71%).

Figure 3.1 *Comparison of KIM-1 expression in various cancer cell lines.* HeLa (cervical), OCC-1 (ovarian), SKOV3 (ovarian), PC3 (prostate), A549 (lung), ACHN (renal), 786-0 (renal) and 769P (renal) cancer cells were grown in culture and once confluent processed with Trizol to make total RNA. RNA samples were then quantified, synthesized into cDNA and then amplified using qRT-PCR. GAPDH was used as a reference gene and ROX was used as a normalizer dye. Data represents three independent experiments. Y-axis represents fold change in gene expression relative to HeLa cells.

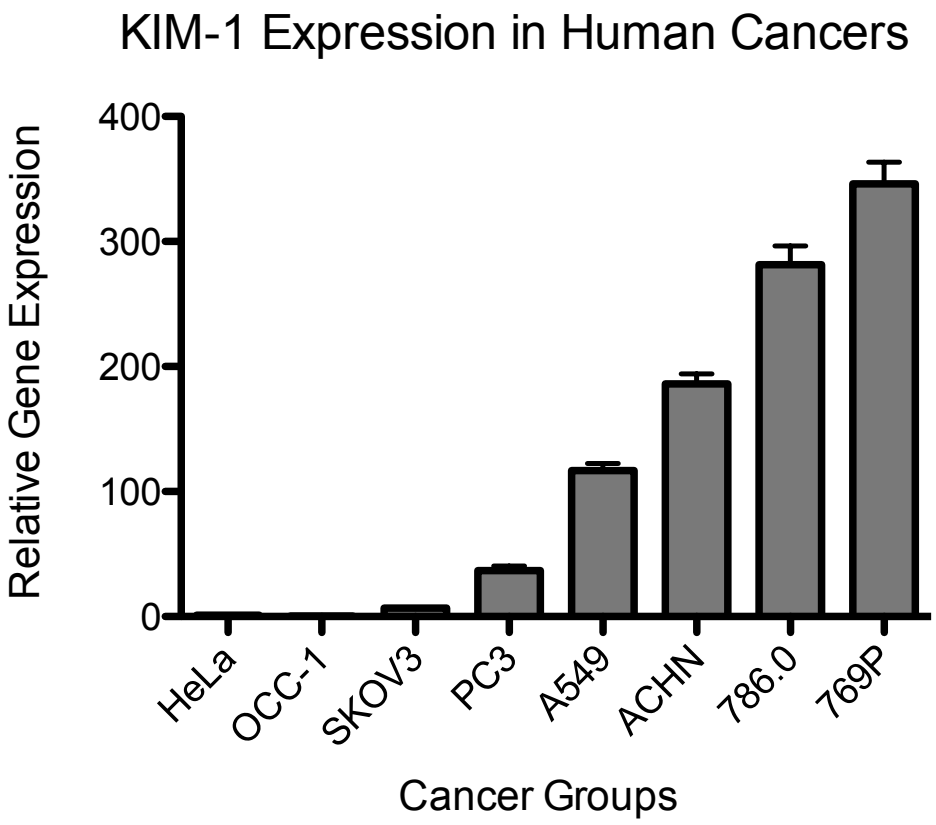
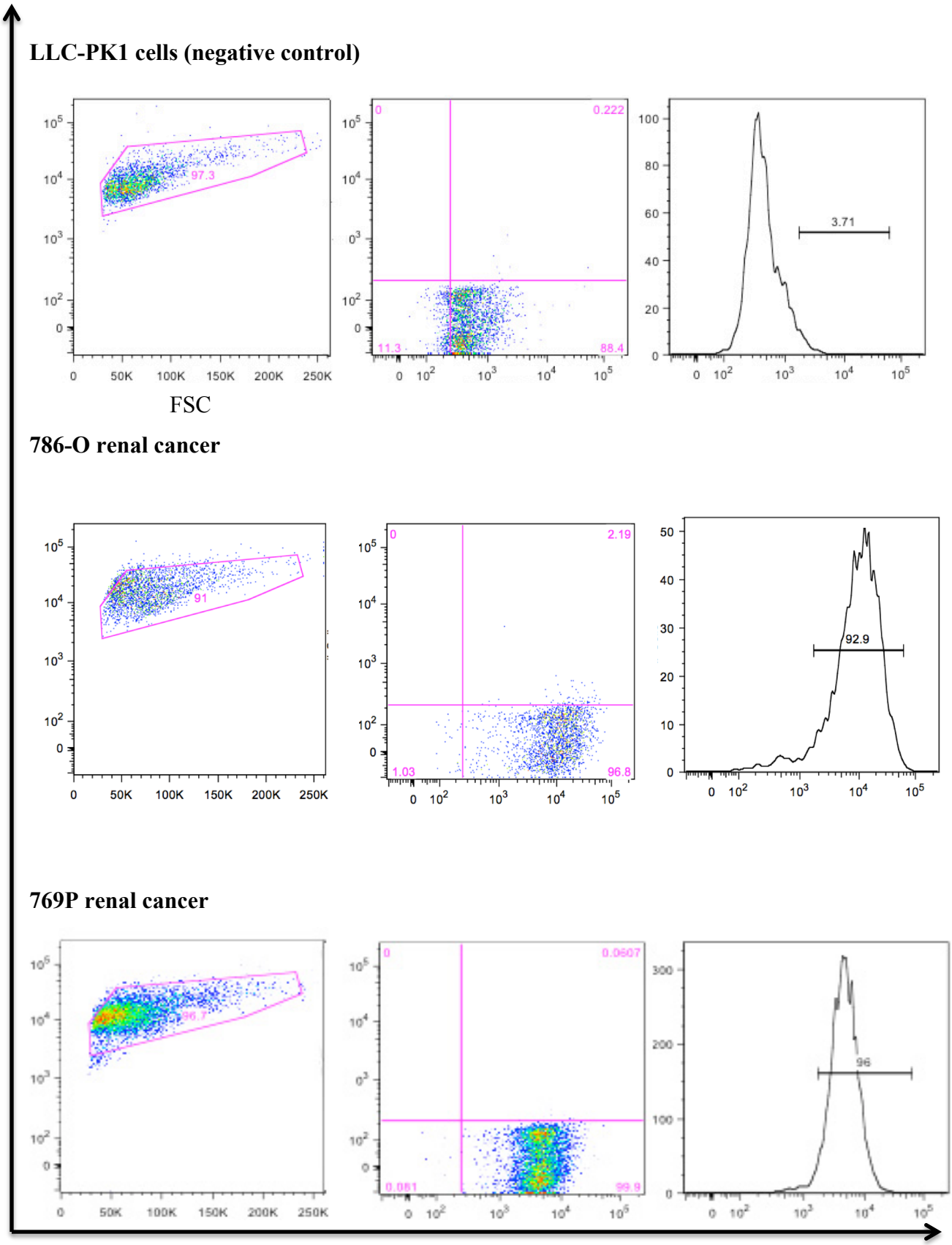

KIM-1 Expression in Human Cancers
Relative Gene Expression
400
300
200
100
0
HeLa
OCC-1
SKOV3
PC3
A549
ACHN
786.0
769P
Cancer Groups

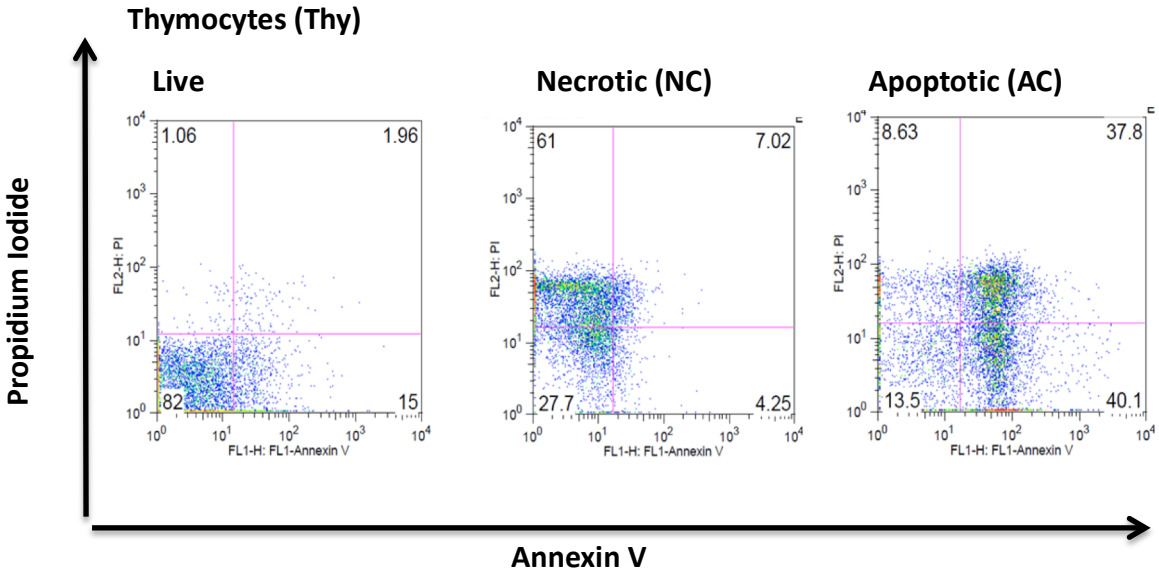
Figure 3.2 *Surface staining for KIM-1 on 769P and 786-0 cells*. Cells were grown until confluent, scraped from the well and stained with PE conjugated hKIM-1 antibody. Cells were resuspended in cell-staining buffer and incubated in the dark for 15 minutes at 1μg/ml concentrations. Cells were then analyzed using LSR II and fixed with PFA. 769P and 786-0 cells were 96% and 92.9% positive for surface KIM-1 expression represented by the far right column. 10,000 events were collected. Surface staining in Figure 3.2 is representative of one of three experiments done to confirm KIM-1 surface expression. Error bars represent standard deviation.


LLC-PK1 cells (negative control)
97.3
0
0.222
11.3
88.4
3.71
FSC
786-O renal cancer
91
0
2.19
1.03
96.8
92.9
769P renal cancer
96.7
0
0.0607
0.081
99.9
96
Side Scatter
hKIM-1

3.2 KIM-1 expression is required for phagocytosis

Based on the expression profile of KIM-1 mRNA and protein, we chose the 769P cells as our model system to test our hypothesis within tumours. In order to determine the impact of KIM-1 mediated phagocytosis of apoptotic and necrotic cells in cancers we used an already established porcine kidney tubular epithelial cell line (LLC-PK1) that overexpresses human KIM-1[117, 265]. Our lab has previously confirmed that KIM-1 expression in LLC-PK1 cells is sufficient to transform these cells into semi-professional phagocytes for apoptotic and necrotic cells[117](Gandhi *et al.,* submitted). As a result the LLC-PK1 model served as a positive control for the role of KIM-1 in our cancer model. LLC-PK1 pig tubular cells stably transfected with a pcDNA3 plasmid with (WT-KIM-1) or without KIM-1 (pcDNA-PK1) were characterized for their capacity to engulf apoptotic or necrotic thymocytes. First, primary mouse thymocytes were kept alive or killed by inducing apoptosis or necrosis and stained with Annexin V-FITC and PI to confirm cell death by necrotic or apoptotic pathways **(Figure 3.3).** Annexin V binds to PS on the outer membrane of cells once they become apoptotic. PI binds to exposed DNA fragments as a result of a loss of membrane integrity seen with necrosis. Live cells were established by keeping thymocytes in serum-supplemented media in an incubator for the course of the experiment. Apoptotic cells were produced by 3 minutes of UV-exposure followed by incubation for minimum 8 hours[266]. Lastly necrotic cells were generated by heat-shock in a water bath for 15 minutes at 56°C[267]. Live cells stained negative for both Annexin V and PI, whereas approximately 40% of apoptotic cells were positive for Annexin V with approximately 37 % double positive for both Annexin V and PI. While the Annexin V single-positive thymocytes were considered early apoptotic, the double-positive population was likely late apoptotic[268]. Lastly, the necrotic thymocytes were approximately 61% PI positive indicative of loss of membrane integrity typical of necrotic cells.

Figure 3.3 *Characterization of live, apoptotic and necrotic thymocytes.* Thymocytes were isolated from the thymic lobes of C57BL/6 mice and were cultured as outlined in the materials and methods section. Cells were then kept alive, made apoptotic through UV exposure or turned necrotic through heat shock. Cell status was confirmed through Annexin V and PI staining. Live cells stained Annexin V and PI negative. Apoptotic cells were Annexin V positive and late apoptotic cells were both Annexin V and PI positive, and necrotic cells were predominantly PI positive. Data is representative of one independent experiment and was analyzed via flow cytometry. Results have been verified more than three independent times.


Thymocytes (Thy)
Live
Necrotic (NC)
Apoptotic (AC)
Propidium Iodide
Annexin V
1.06
1.96
82
15
61
7.02
27.7
4.25
8.63
37.8
13.5
40.1
FL2-H: PI
FL1-H: FL1-Annexin V

Although the phagocytic capacity of KIM-1-PK1 and pcDNA-PK1 cells had been previously established [117], the long-term kinetics of apoptotic and necrotic cell-uptake had not been addressed. The presence and absence of cell-surface human KIM-1 expression in WT-PK1 and pcDNA-PK1 cells is seen below **(Figure 3.4)**. 44% of WT-PK1 cells stained positive for surface KIM-1 expression whereas 3.71% of the pcDNA-KIM-1 cells stained positive. Next, phagocytosis was quantified using flow cytometry after feeding either pcDNA-PK1 or WT-PK1 cells fluorescently-labeled thymocytes that had been rendered apoptotic or necrotic as described above. The percent of pcDNA-PK1 **(Figure 3.5A)** and WT-PK1 cells **(Figure 3.5B)** engulfing either apoptotic or necrotic thymocytes is shown below. A representative gating strategy of one replicate can be seen in Appendix II. Lastly at the 24-hour time point, we did a comparison between the phagocytosis of live, apoptotic and necrotic cells by pcDNA vs. WT-PK1 cells. From this we decided to focus specifically on apoptotic and necrotic cells, as the phagocytosis of live cells was only minimal and yielded no difference between the two groups **(Figure 3.6)**.

One shortfall to our method of evaluating phagocytosis is that a shift in fluorescence does not necessarily indicate internalization but instead may represent binding to the outside of the cell. Therefore, an alternative cell tracer, PHrodo dye (Invitrogen), was used instead of CFSE to label the apoptotic and necrotic cells before quantifying phagocytosis. PHrodo dye is a pH sensitive dye whose fluorescence intensity increases at an acidic pH. Apoptotic and necrotic cells labeled with PHrodo were expected to generate a more intense signal when delivered to the acidic environment of phagolysosomes indicating that the corpse was indeed inside the cell and not outside it. Phagocytosis experiments done using PHrodo verified our results.

Results shown in **Figure 3.6** demonstrate that at 24-hours, WT-KIM-1 cells engulfed significantly more apoptotic and necrotic cells compared to pcDNA-PK1 cells. Moreover, WT-PK1 cells were on average 2-3 times more efficient at engulfing necrotic compared to apoptotic cells. Because the induction of necrosis often results in the disintegration of cells into debris that can confound the findings from our phagocytosis experiments, we compared the mean fluorescent intensity of the WT-PK1 cells that had engulfed either apoptotic or necrotic cells. The MFI findings (Appendix III) mirrored the percent phagocytosis data shown (**Figure 3.6)**. Therefore WT-PK1 cells were more adept at internalizing necrotic cells than apoptotic cells. Importantly, the phagocytosis of live cells was significantly lower than that of apoptotic or necrotic cells at between 5- 7%. Also live cell-uptake was independent of KIM-1 expression suggesting that an alternative mechanism of their uptake by LLC-PK1 cells.

Figure 3.4. *Surface staining for pig epithelial LLC-PK1 cells stably expressing human KIM-1.* To confirm KIM-1 had been incorporated successfully into PK1 cells WT-PK1 cells were surface stained for KIM-1 expression using a human PE conjugated anti-KIM-1 antibody at 1µl/ml using the same method as in Figure 3.2. Using flow cytometry ten thousand events were collected and gated on KIM-1 positive cells. The panel on the left represents control LLC-PK1 cells and on the left represents WT-KIM-1 cells. Surface staining represents one of three independent staining experiments. Analysis was completed using FlowJo Software.

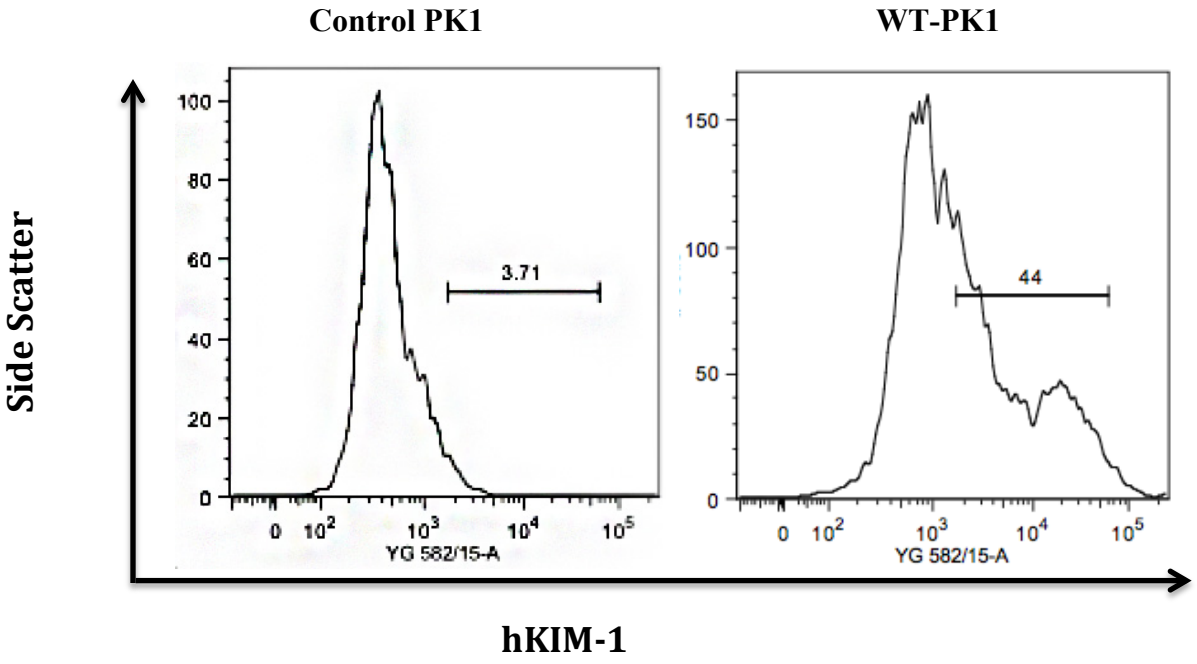

Control PK1
WT-PK1
Side Scatter
100
80
60
40
20
0
3.71
0 10^2 10^3 10^4 10^5
YG 582/15-A
150
100
50
0
44
0 10^2 10^3 10^4 10^5
YG 582/15-A
hKIM-1

Figure 3.5. *KIM-1 expression enhances the phagocytosis of apoptotic and necrotic cells.* pcDNA-PK1 (A) and WT-PK1 (B) cells were fed CFSE stained five million apoptotic and necrotic thymocytes respectively for 24 hours. Samples were then spun down, resuspended in FACS buffer and processed using a FACS Calibur. Ten thousand events were collected and gated against LLC-PK1 cells at WT-KIM-1 cells alone as a negative control respectively. The percent change in fluorescent intensity compared to the negative controls was used to establish a change in percent phagocytosis at each time point. Antibody concentrations were used at 1 µl/ml and staining procedure is listed in the materials and methods. Data represents three independent experiments. *** $P<0.001$

Figure 3.5A

Control PK1

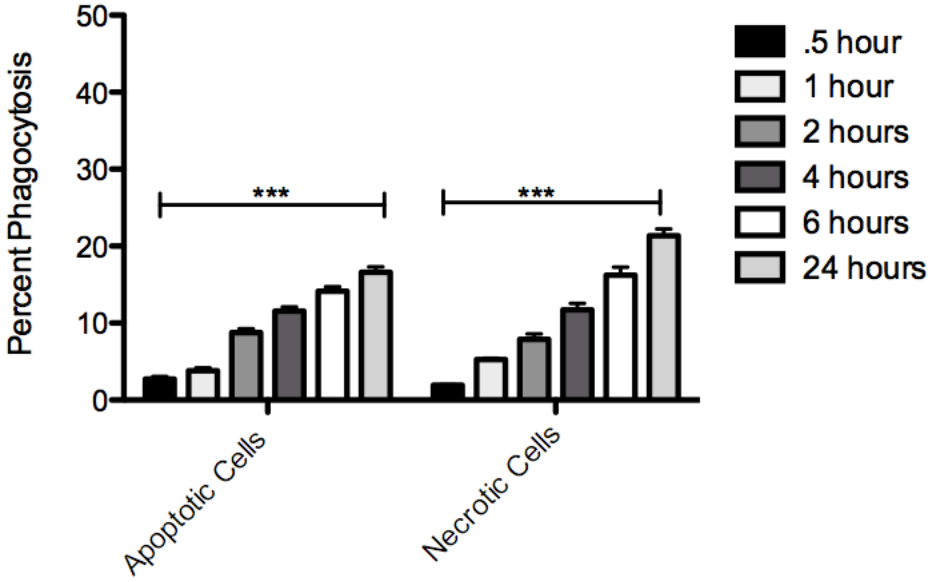


Figure 3.5B

WT-PK1

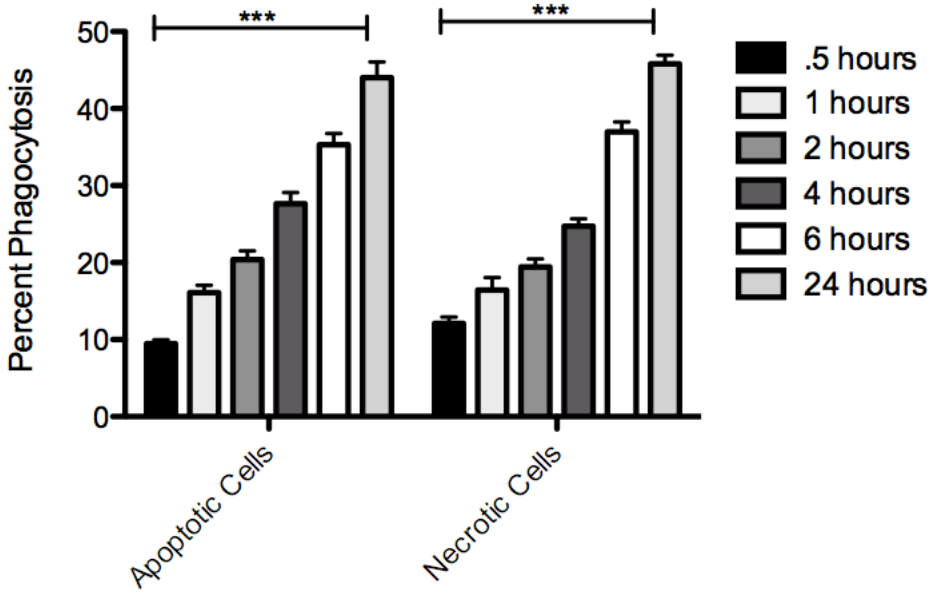
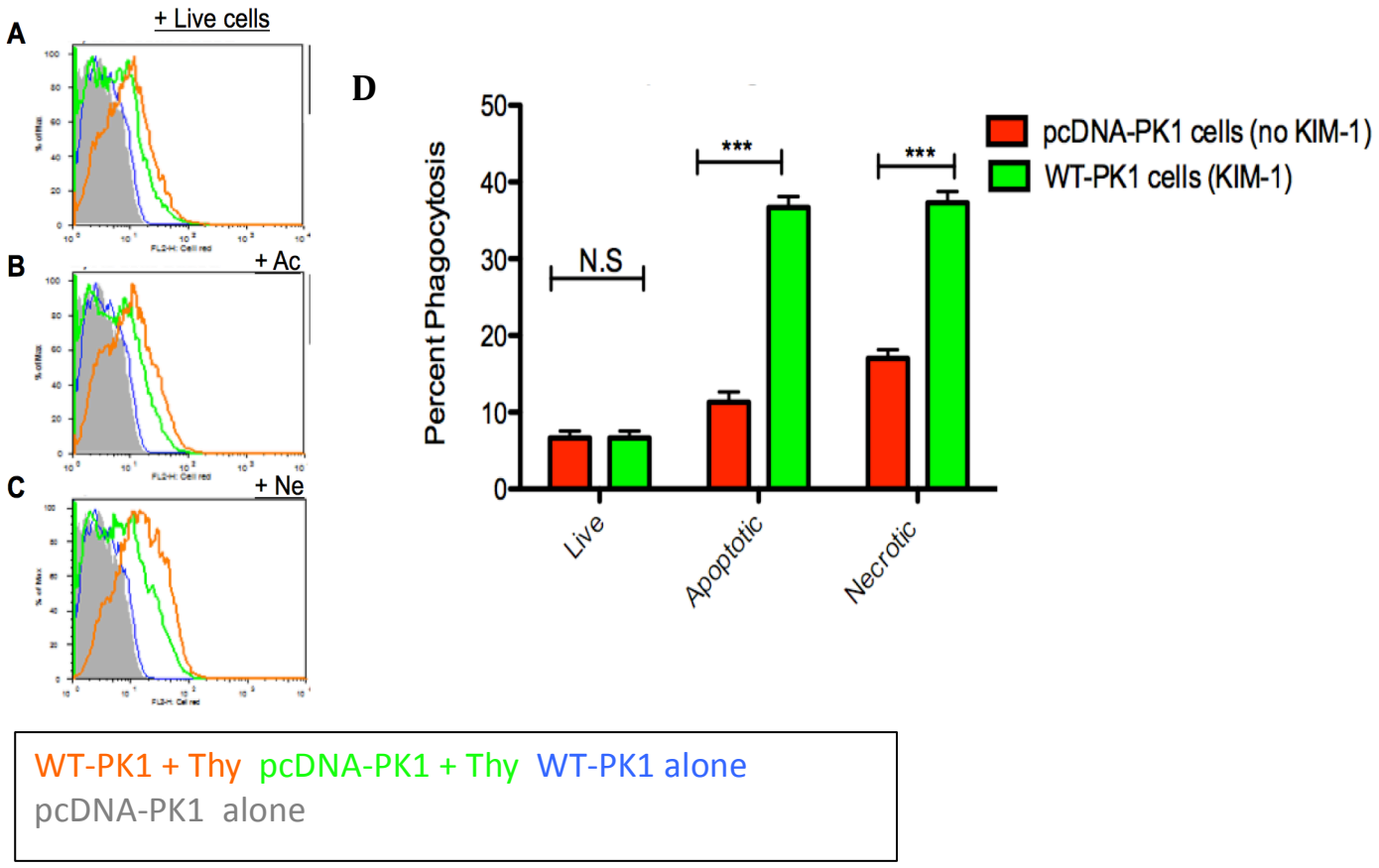


Figure 3.6 *Representative trend in the phagocytosis of live, apoptotic and necrotic cells by KIM-1 and non-KIM-1 LLC-PK1 expressing cells.* Representative phagocytosis of live (A), apoptotic cells (B), and necrotic cells (C) by KIM-1 and non-KIM-1 expressing cells using flow cytometry at 24 hours. A-C represent one experiment. Panel D is a comparison of phagocytosis data from three independent experiments from panels A-C. *** $P<0.001$.



WT-PK1 + Thy pcDNA-PK1 + Thy WT-PK1 alone
pcDNA-PK1 alone

3.3 KIM-1 is required for RCC- phagocytosis of apoptotic and necrotic cells

A central premise to our hypothesis is that RCC cells possess the ability to engulf apoptotic and necrotic cells. Therefore, the phagocytic capacity of 769P cells was measured using flow cytometry. 769P cells were fed CFSE-labeled apoptotic or necrotic thymocytes for different periods of time and the engulfment of apoptotic **(Figure 3.7A)** and necrotic cells **(Figure 3.7B)** was quantified over a period of 24 hours to characterize phagocytosis within the 24 hour time frame our experiments were to be performed. Once again, similar experiments performed by Ola Ismail (Gunaratnam Lab) using PHrodo dye to label the dead cells helped confirm that we were not merely measuring surface binding but rather phagocytosis in our experimental results (data not shown). Fluorescent thymocytes were fed to 769P cells at different time points and all cells were harvested at the same time before analyzing the results by flow cytometry. The percent uptake of apoptotic cells was increased gradually over the 24 hour period tested. At 24 hours, the percent uptake of apoptotic and necrotic cells was 37.5% and 45.5% respectively. These data demonstrated that 769P phagocytosis occurred in as little as 30 minutes and did not plateau up even at 24 hours.

Having established that 769P cells could phagocytose both apoptotic and necrotic cells, we determined if KIM-1 was necessary for phagocytosis. 769P cells were either left untreated, transiently transfected with siRNA targeting human KIM-1 or non-specific control siRNA, which does bind KIM-1, using Lipofectamine 2000. As shown, 24 hours after transfection, KIM-1 siRNA appreciably inhibited KIM-1 protein expression in 769P cells as determined by Western blot **(Figure 3.8A).** As a result of siRNA treatment, KIM-1 protein expression was reduced approximately 60% and was significantly lower than control siRNA transfected cells **(Figure 3.8B**). We then characterized the change in cell-surface expression KIM-1 in 769P cells after siRNA knockdown via flow cytometry **(Figure 3.8C)**. There was a 55% decrease in surface expression of KIM-1 in 769P cells treated with KIM-1 siRNA. In a different series of experiments, the effect of siRNA-knockdown of KIM-1 was compared at 24 hours, 48 hours and 72 hours of incubation

(data not shown) and the 24-hour time point was determined to be optimal for further studies.

Next, the effect of KIM-1 silencing on phagocytosis of both apoptotic **(Figure 3.9A)** and necrotic cells by 769P cells was determined **(Figure 3.9B)**. As a result of KIM-1 down-regulation, phagocytosis of both apoptotic and necrotic cells was reduced by 60% in comparison to untreated controls. There was also no significant difference between the control siRNA transfected and untreated groups.

Figure 3.7 *769P cells engulf both apoptotic and necrotic cells*. 769P cells were fed CFSE labeled apoptotic (A) or necrotic (B) thymocytes at various time intervals to profile the optimal time point for phagocytosis. Samples were then collected and analyzed. Identification of CFSE positive cells was based on the 769P cell alone gate (negative control). Results are based on ten thousand events collected and represent three independent experiments via flow cytometry. *** $P<0.001$.

Figure 3.7A

Apoptotic cells

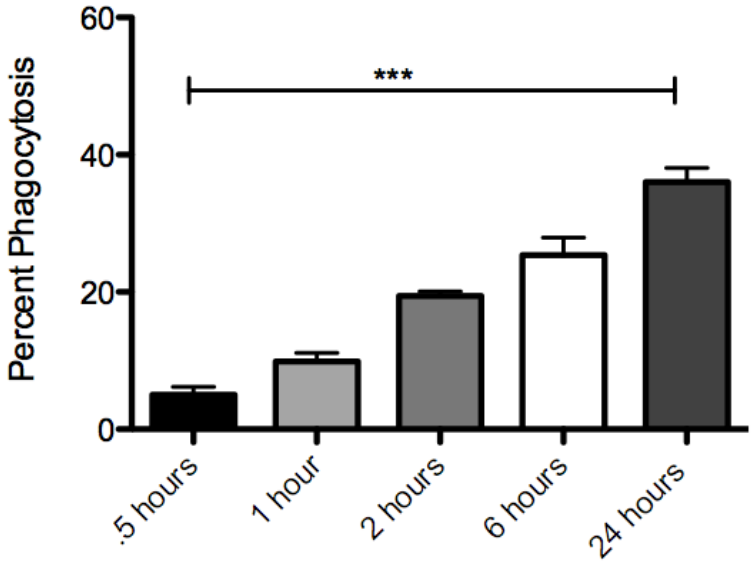


Figure 3.7B

Necrotic cells

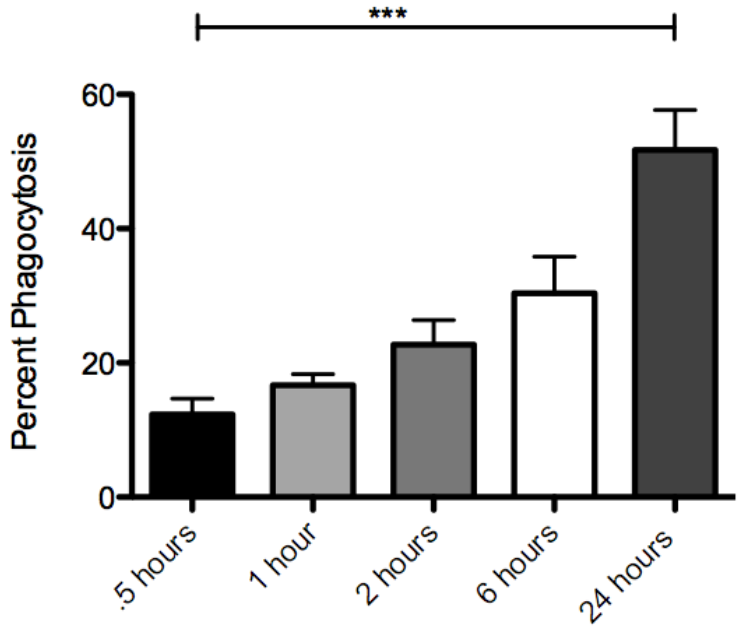


Figure 3.8 *Knockdown of KIM-1 in 769P cancer cells.* 769P renal cancer cells were transiently transfected with a human KIM-1 siRNA using OPTI-MEM media and Lipofectamine 2000. Final concentrations of control and KIM-1 siRNA were 10nM and untreated cells contained only OPTI-MEM media. Transfection was performed at 24 hours and is represented here in triplicate. Panel (A) is representative of three blots acquired in three independent experiments. Panel (B) is a quantification of these using densitometry where knockdown was determined as the percent reduction in density from 769P untreated cells. (C) Represents the change in surface staining of 769P cells in shRNA KIM-1 stable cells by FACS. ** $p<0.01$, *** $P<0.001$.

Figure 3.8A

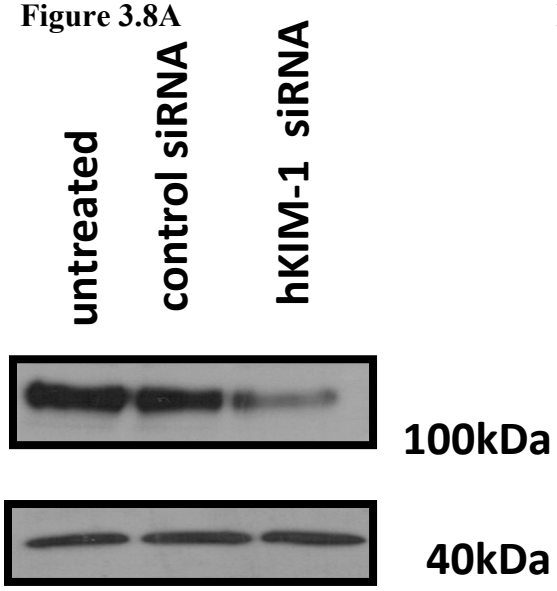


Figure 3.8B

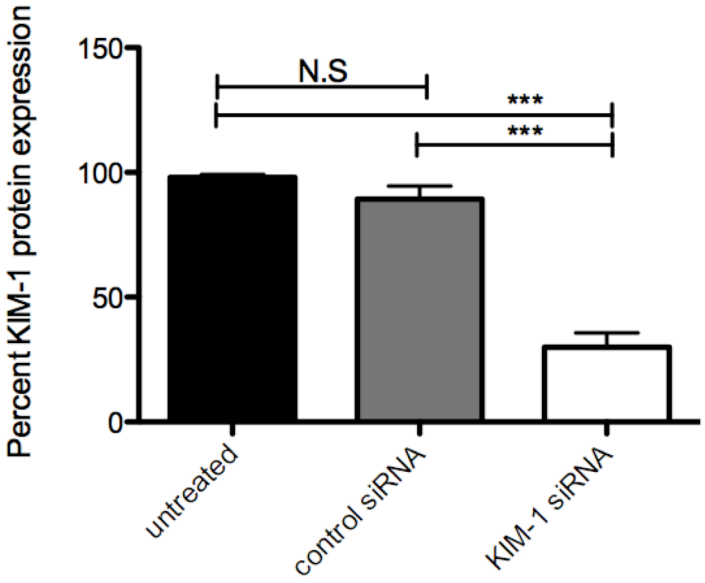


Figure 3.8C

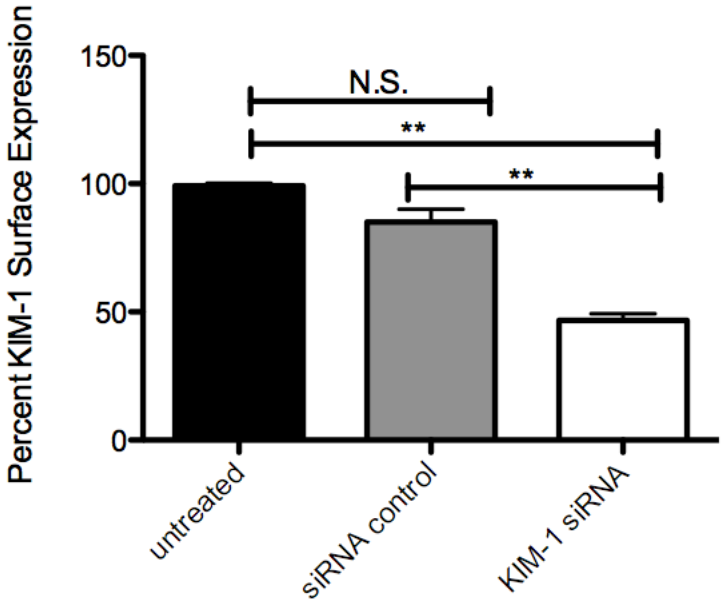


Figure 3.9 *Change in phagocytosis of apoptotic and necrotic cells in KIM-1 siRNA treated cells*. 769P cells were transfected with hKIM-1 siRNA or control siRNA for 24 hours and then fed CFSE apoptotic or necrotic thymocytes. Change in phagocytosis as a result of KIM-1 expression was measured via flow cytometry and gated relative to untreated cell phagocytosis. Panels A and B graphs represent triplicate experiments *** representing $P<0.001$. (A) Apoptotic cell phagocytosis and (B) Necrotic cell phagocytosis.

Figure 3.9A

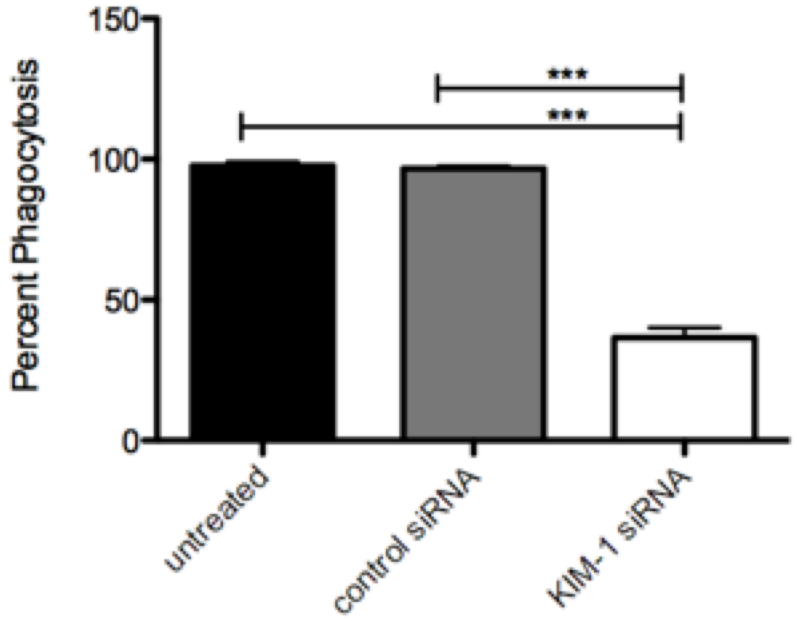
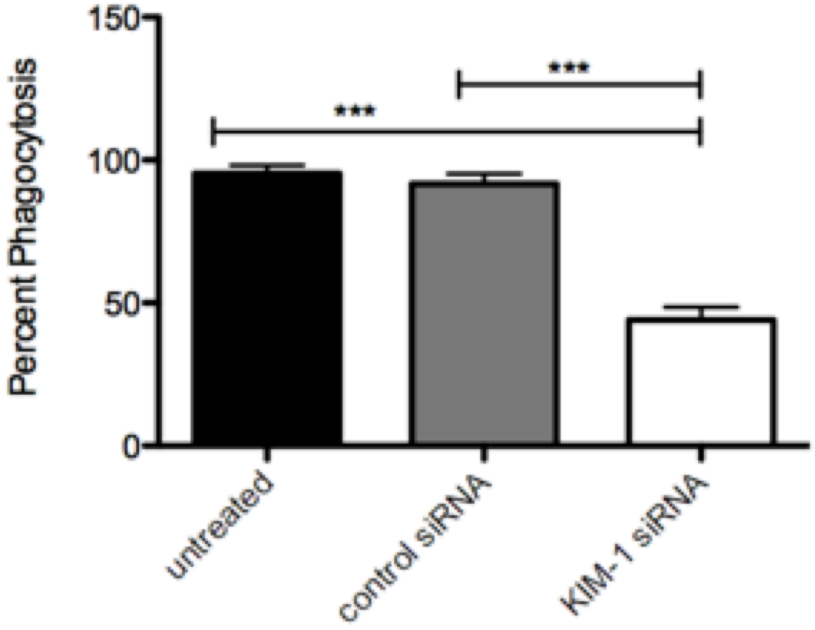


Figure 3.9B



3.4 HMGB1 is released from apoptotic and necrotic cells

HMGB1 is a nuclear structural protein that serves as a DAMP when released into the extracellular milieu[144, 269]. While HMGB1 has been shown to be released passively from necrotic cells due to loss of membrane integrity, apoptotic cells are believed to sequester HMGB1 in the nucleus [122]. We therefore characterized the release of HMGB1 from live, apoptotic and necrotic thymocytes. Equal numbers of thymocytes were untreated (kept alive) or treated as described previously (Materials and Methods section 2.3.0) to induce apoptosis or necrosis. Cells were then harvested at various time points after induction of cell death and the respective conditioned medium was analyzed by Western Blot for HMGB1 expression **(Figure 3.10A).** The experiment was completed in triplicate and the media samples were analyzed for relative HMGB1 expression **(Figure 3.10B).**

During the 72-hour time period, necrotic cells exhibited a persistent release of HMGB1 into the conditioned medium. While live thymocytes began to slowly leak HMGB1 between 24 and 48 hours, apoptotic cells began releasing HMGB1 as soon as 8 hours of induction of cell death. At 72 hours, both live and dead thymocytes exhibited large amounts of HMGB1 into the extracellular medium. The leakage of HMGB1 from live cells appeared surprising, but it is known that live thymocytes do undergo spontaneous cell death under these conditions[270]. At 24 hours nearly 50% of the cellular HMGB1 content had been released into the surrounding environment. Necrotic thymocytes however, began to release HMGB1 immediately into the surrounding media, demonstrated by the 15% leakage of HMGB1 at the zero hour time point. HMGB1 leakage continued to increase steadily up to 72 hours and by 24 hours, had released approximately 75% of its cellular HMGB1 into the surrounding environment. Samples are graphed relative to HMGB1 release by necrotic cells at 72 hours using densitometry.

Figure 3.10 *Passive release of intracellular of HMGB1 from live, apoptotic and necrotic thymocytes into conditioned media.* Cells were induced to undergo necrosis, apoptosis or remain alive and were plated in equal numbers into 6 well plates after CFSE labeling. At each time point, cells were collected, spun down to remove cellular debris and the conditioned media was analyzed via Western Blot (A). The change in HMGB1 levels was quantified (B) using 72-hour necrotic cells as the reference sample using densitometry. Panel A is representative of three blots acquired in three independent experiments. Panel B is quantification of these blots. Data from three independent experiments, ***$P<0.001$.

Figure 3.10A

Conditioned Media

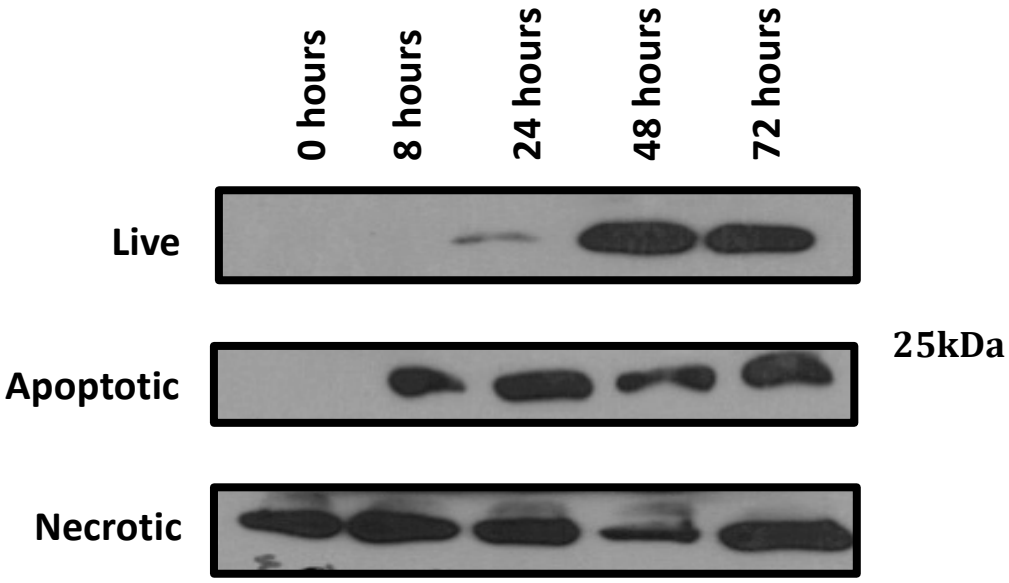
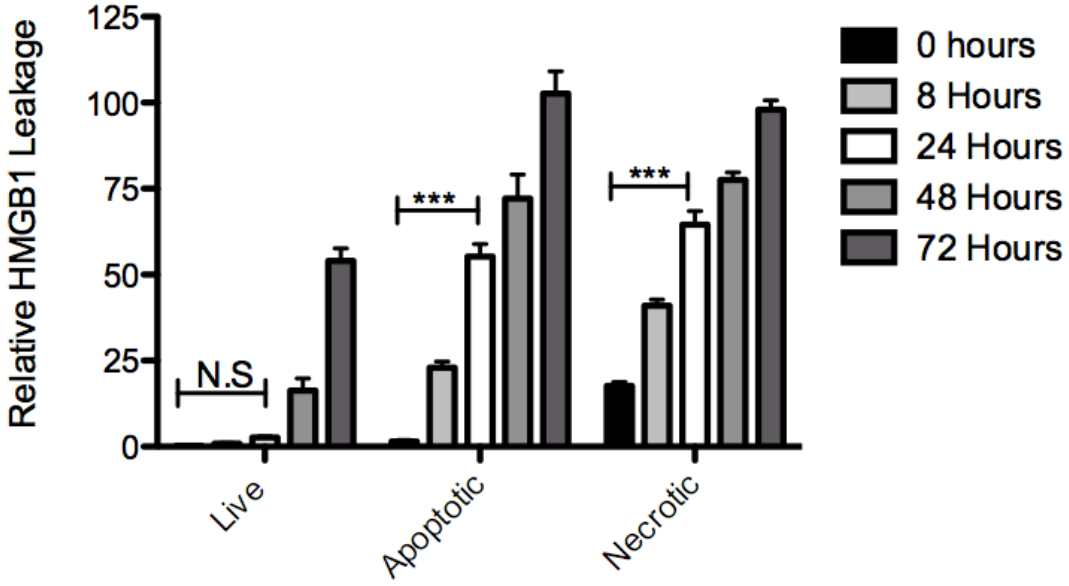


Figure 3.10B



3.5 Assessing the status of HMGB1 in the media post phagocytosis

Having established that HMGB1 is released from dying cells passively over time, we postulated that engulfment of both apoptotic and necrotic cells by phagocytic 769P cells might enable 769P cells to regulate alarmin levels within its extracellular milieu[271]. Correspondingly, we expected that 769P cells that engulf dying cells would have increased intracellular content of HMGB1. This has never been shown to our knowledge. First, we used KIM-1-PK1 cells and its control pcDNA-PK1 cells to test this hypothesis and confirm the preliminary data provided by Dr. Gunaratnam (**Figure 1.3**). WT-KIM-1 and pcDNA-PK1 cells were fed live, apoptotic or necrotic thymocytes for 24 hours to allow for efficient uptake keeping in mind that KIM-1 phagocytic capacity was not saturated during this time period (**Figure 3.6)**. The conditioned medium and cell lysates from each cell type fed either apoptotic or necrotic cells were then harvested and analyzed by Western Blot for HMGB1 release **(Figure 3.11A)** and the triplicates were analyzed using densitometry **(Figure 3.11B)**. In keeping with our hypothesis, HMGB1 levels were higher in the conditioned media of non-KIM-1 expressing cells in comparison to that of KIM-1 expressing cells. This correlated with a nearly 50% increase in sequestered HMGB1 from apoptotic cells in the WT-KIM-1 lysates and a 100% increase in the HMGB1 levels from necrotic cells in the same group. Correspondingly, HMGB1 levels were higher in the media and lower in the lysate of non-KIM-1 expressing cells in comparison to KIM-1 expressing cells. Overall, HMGB1 levels from necrotic cells were significantly higher compared to apoptotic cells in both the media and the lysate regardless of KIM-1 expression. Live cells were not included due to their negligible phagocytosis by KIM-1-PK1 cells discussed previously. Importantly, WT-PK1 and pcDNA-PK1 cells alone did not secrete significant levels of HMGB1 into their respective conditioned media and had lower cellular levels of HMGB1 than their counterparts fed either apoptotic or necrotic cells.

The same experiment was repeated in 769P cells pre-treated with siRNA targeting KIM-1 or control siRNA. First, 769P cells were either untreated or transfected with control siRNA or KIM-1 siRNA and incubated overnight. After overnight incubation, equal

numbers of apoptotic or necrotic thymocytes were added to the siRNA-treated 769P cells for another 24 hours. Thereafter, the conditioned media was analyzed using Western Blot for detection of HMGB1. The levels of HMGB1 seen in the conditioned medium of siRNA KIM-1 cells fed apoptotic cells **(Figure 3.12A)** and necrotic cells **(Figure 3.12B)** was significantly higher than what was measured in both the untreated and control siRNA treated 769P cells. The levels of HMGB1 seen in the conditioned medium of untreated or control siRNA treated 769P cells fed apoptotic cells were comparable. A duplicate experiment was done using necrotic cells **(Figure 3.12 C and D)** and showed that silencing of KIM-1 in 769P cells abrogated their ability to limit HGMB1 leak from necrotic cells. Once again, there was no statistically significant difference in HMGB1 release in the conditioned media of either untreated or control siRNA treated 769P cells, demonstrating that the findings were KIM-1 specific and not due to non-specific effects of siRNA or the transfection reagent. Importantly, there was no detectable HMGB1 in the conditioned medium of 769P cells that were not fed any dead thymocytes, confirming that any HMGB1 released into the medium was from the dying cells. Also, necrotic cells released approximately twice the amount of HMGB1 into the surrounding media compared to apoptotic cells.

Figure 3.11 *Comparison in the leakage of HMGB1 from dying cells after incubation with LLC-PK1 cells expressing or not expressing KIM-1*. Approximately $1x10^6$ WT-KIM-1 and pcDNA-KIM-1 cells were plated 24 hours prior and then fed either apoptotic or necrotic thymocytes. 24 hours later the wells were harvested and both the conditioned media (right panel) and the lysate (left panel) and were analyzed by western blot for HMGB1 expression (A). Results are normalized to the actin of WT-KIM-1 and pcDNA-KIM-1 cells alone. Panel A is representative of three blots acquired in three independent experiments. Panel B is quantification of HMGB1 within lysate blots

*** $P < 0.001$

Figure 3.11A

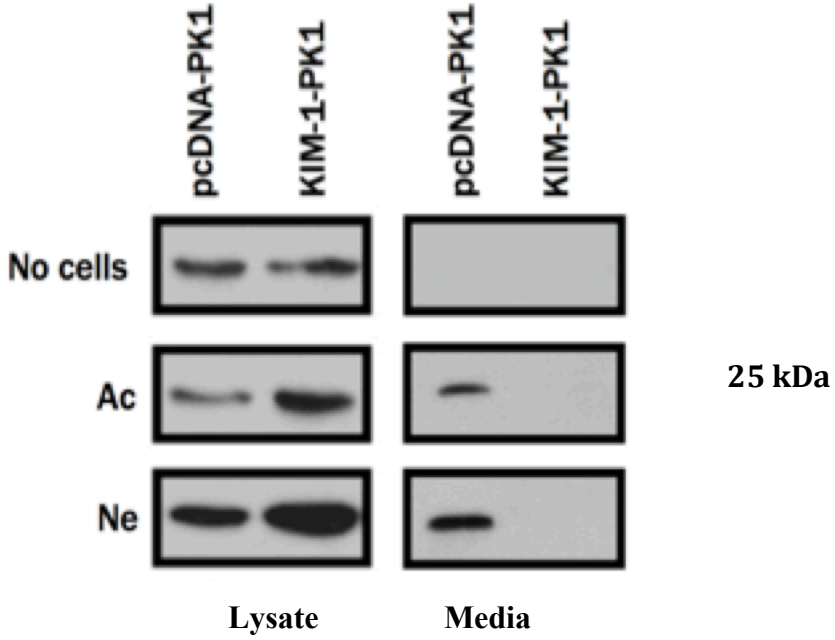


Figure 3.11B

Lysate

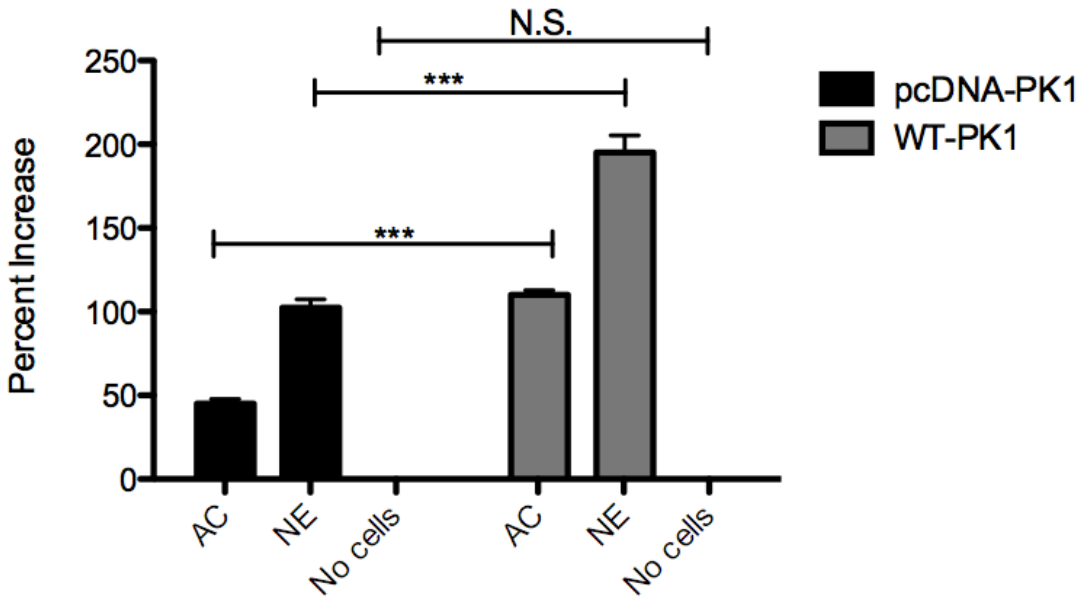
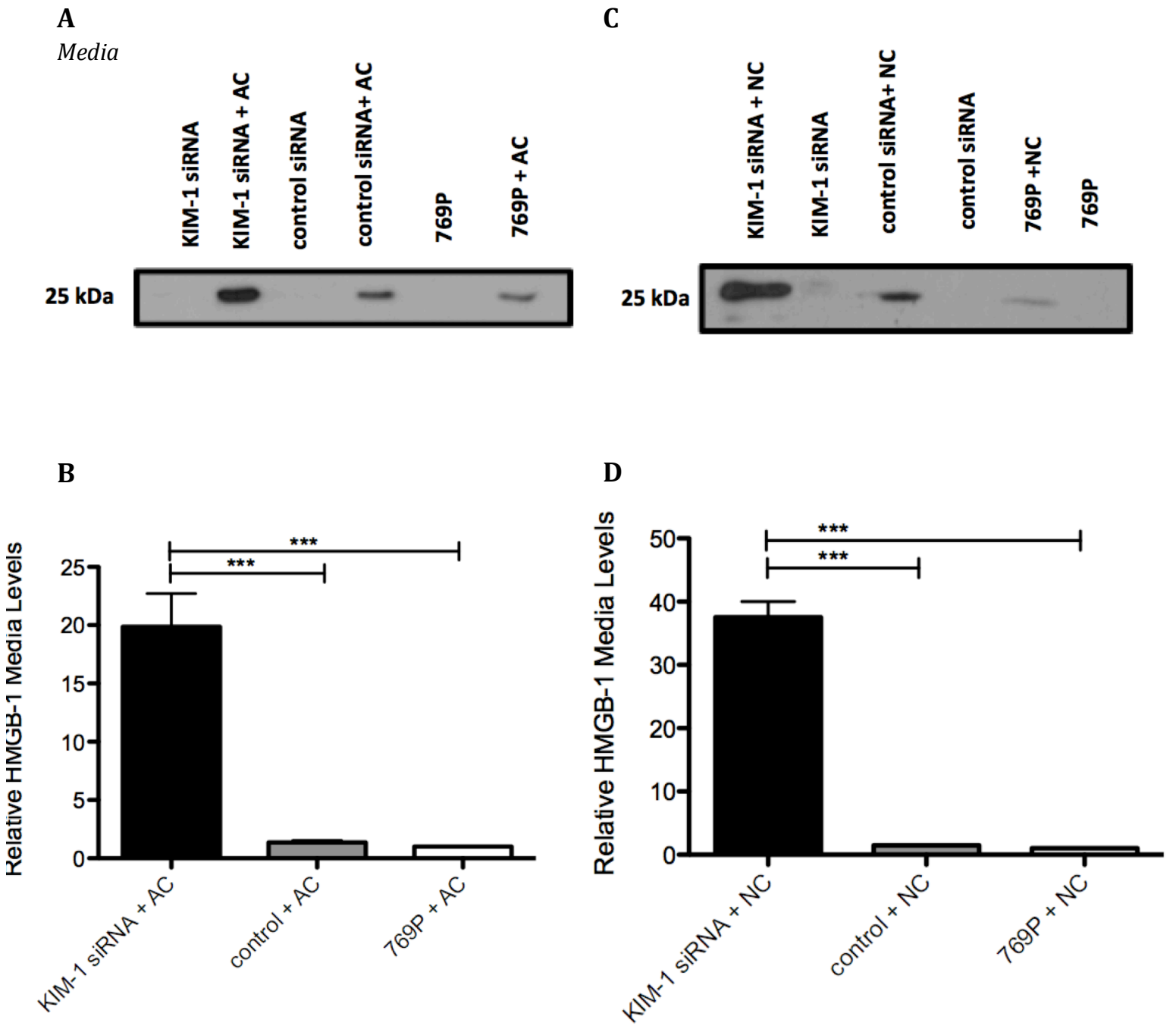


Figure 3.12 *Inhibition of HMGB1 release from dying cells into the media as a result of KIM-1 expression.* The same experiment in Figure 3.11 was repeated in Figure 3.12 except with 769P that were left untreated or treated with control siRNA or KIM-1 siRNA. After 24-hour incubation with apoptotic or necrotic cells, media samples were analyzed via Western Blot for apoptotic cells (A) or necrotic cells (C) Panel A and C are representative of three blots acquired in three independent experiments for apoptotic and necrotic cells respectively. Panel B and D are quantifications of these blots. ***$P<0.001$.


A
Media
KIM-1 siRNA
KIM-1 siRNA + AC
control siRNA
control siRNA+ AC
769P
769P + AC
25 kDa
C
KIM-1 siRNA + NC
KIM-1 siRNA
control siRNA+ NC
control siRNA
769P +NC
769P
25 kDa
B
Relative HMGB-1 Media Levels
***
***
KIM-1 siRNA + AC
control + AC
769P + AC
D
Relative HMGB-1 Media Levels
***
***
KIM-1 siRNA + NC
control + NC
769P + NC

3.6 KIM-1 inhibits dendritic cell activation by apoptotic cells

The pro-inflammatory nature of HMGB1 has been well characterized[136]. The pleotropic effects of HMGB1 on the innate immune system was outlined in Figure 1.2. Based on its ability to induce a strong immune response, it would stand to reason that residual HMGB1 in the extracellular milieu resulting from tumours exposed to chemotherapy might activate innate immune pathways including dendritic cells. The ability to limit such inflammation, for instance via KIM-1 expression (**Figure 3.12**), would be expected to promote cancer survival through enhanced immune evasion. We postulated that as a result of the phagocytic clearance of apoptotic cells by KIM-1 expressing cells, the activation of DCs by HMGB1 released from cells undergoing secondary necrosis would be limited[272]. This was tested by feeding WT-PK1 and pcDNA-PK1 cells either apoptotic or necrotic thymocytes for 24 hours, and thereafter transferring the conditioned medium containing any soluble HMGB1 to activate primary DCs [122]. The activation of primary DCs was confirmed by LPS-treatment (10 ng/ml) for the same period of time as apoptotic cells and by measuring the percent of DCs that expressed CD40 and CD86 on their surface by flow cytometry. Compared to pcDNA-PK1, WT-PK1 cells were able to reduce apoptotic cell- and necrotic cell-dependent activation of DCs by approximately 30% **(Figure 3.13).** This experiment was only performed once and needs to be reproduced.

A similar experiment was conducted with media from 769P cells transfected with KIM-1 siRNA, control siRNA, or left untransfected and transferred to dendritic cells. Both untransfected or control siRNA transfected 769P cells limited DC activation compared to KIM-1- siRNA transfected cells **(Figure 3.14)**. LPS was used as a positive control for DC activation as before. Interestingly, DC-activation was increased in the KIM-1 siRNA treated population by approximately 30% in comparison to untransfected cells.

One possibility for both 769P and WT-PK1 experiments with dendritic cells is that HMGB1 may not be the predominant or sole factor inducing a dendritic cell response. As mentioned earlier, the release of HMGB1 can be coupled with nucleic acids or other

cellular components that can induce a TLR response in innate immune cells[141]. A way to confirm if HMGB1 is a significant player would be to use an HGMB1 neutralizing antibody in media samples and see if the same response in dendritic cells to both 769P and WT-PK1 cells can be produced.

Figure 3.13 *KIM-1 expression is sufficient to inhibit the activation of dendritic cells by media transfer from uncleared apoptotic and necrotic cells in LLC-PK1 cells.* WT-PK1 and pcDNA-PK1 expressing cells were fed apoptotic or necrotic thymocytes for 24 hours. The spun-down supernatants were then transferred to primary dendritic cells overnight to stimulate their activation. Dendritic cells were then stained with fluorescently-conjugated antibodies to detect CD11c, CD40 and CD86. Samples were then analyzed via flow cytometry and double positive cells were plotted relative to LPS double positive cells. N=1.

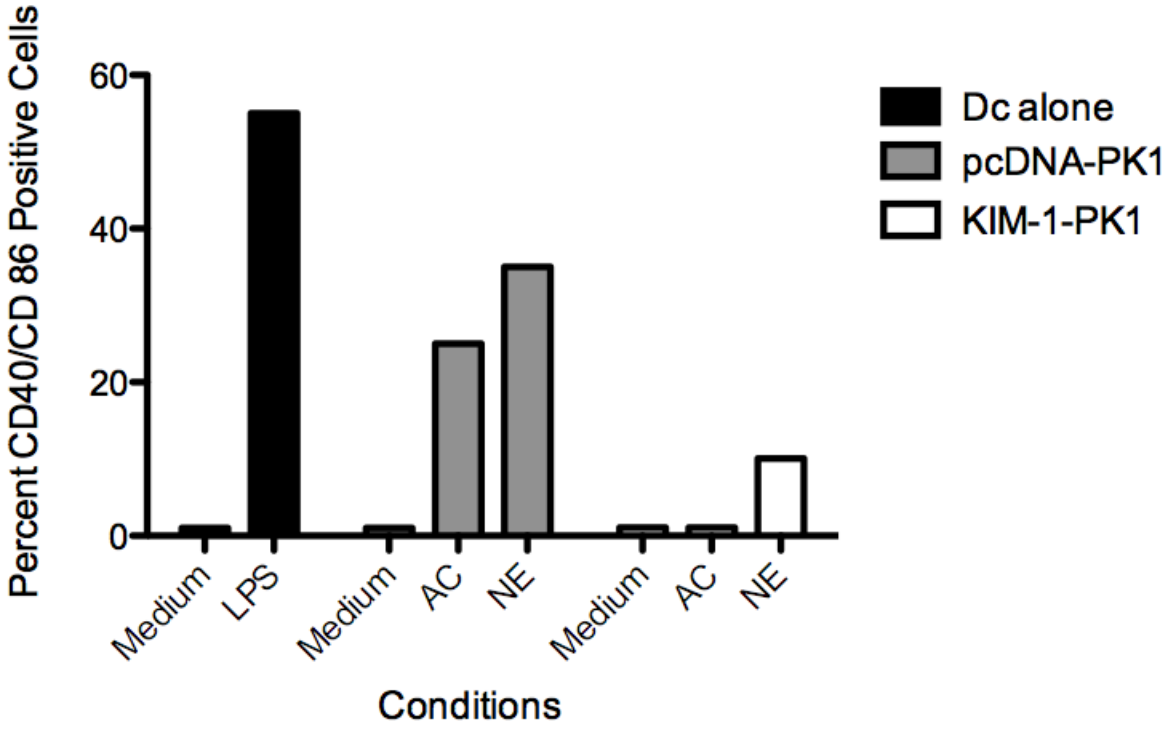
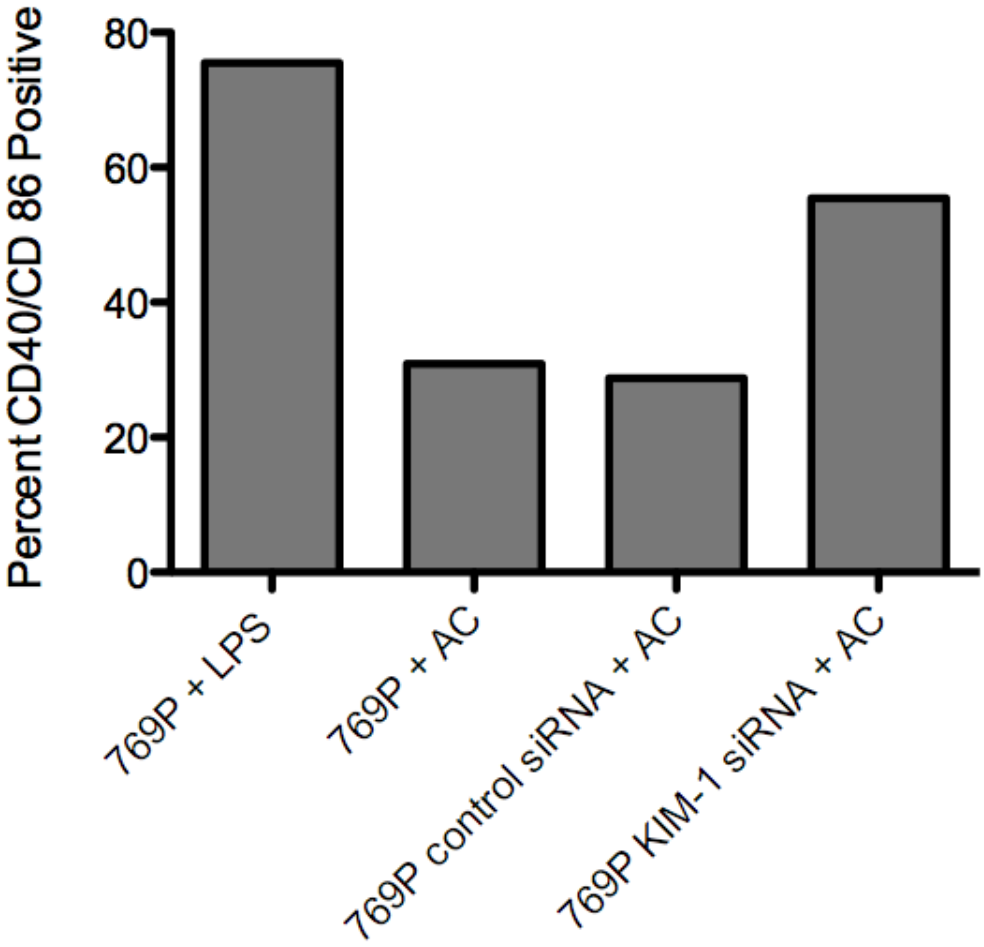

Percent CD40/CD 86 Positive Cells
60
40
20
0
Medium
LPS
Medium
AC
NE
Medium
AC
NE
Conditions
Dc alone
pcDNA-PK1
KIM-1-PK1

Figure 3.14 *KIM-1 expression is required to limit the activation of dendritic cells by uncleared apoptotic and necrotic cells in 769P cells.* After siRNA treatment for 24 hours, KIM-1 and non-KIM-1 expressing cells were fed apoptotic thymocytes for 24 hours. Conditioned media post-phagocytosis was then transferred to dendritic cells overnight and samples were then stained for CD11c, CD40 and CD86. Samples were then analyzed via flow cytometry and double positive cells were plotted relative to LPS double positive cells. Figure 3.14 is N=1

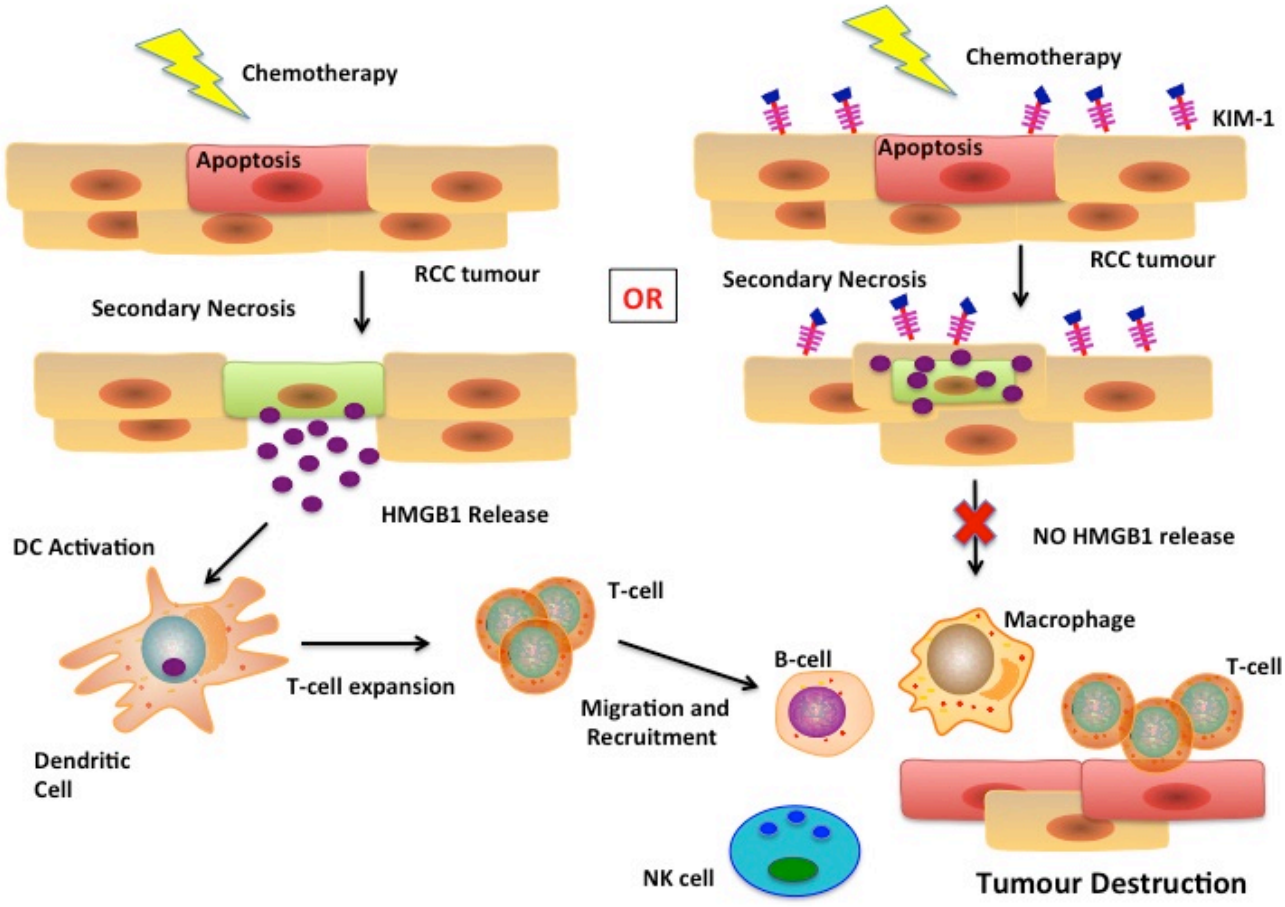

Percent CD40/CD 86 Positive
80
60
40
20
0
769P + LPS
769P + AC
769P control siRNA + AC
769P KIM-1 siRNA + AC

Chapter 4: Discussion

4.1.0 Overview

The interplay between the host immune response and the tumour microenvironment profoundly influences the clinical outcome of malignancies [4, 108]. Tumours have however, developed a variety of intriguing strategies to evade immune surveillance[107]. Understanding such mechanisms may lead to novel therapies for human cancer. Recent studies have shown that certain cytotoxic chemotherapies induce a form of immunogenic cell death in which the release of DAMPs such as HMGB1 from dying tumour cells serves as an endogenous adjuvant[124]. Meanwhile, the phagocytic clearance of cell corpses has been shown to be immunosuppressive. Here we identified that the PS receptor KIM-1, normally found in the injured kidney, is overexpressed by a variety human cancers, enabling them to mediate phagocytosis of apoptotic and necrotic cells. Moreover, we demonstrated that through expression of KIM-1, human RCC cancer cells reduced the release of the DAMP HMGB1 and circumvented the immunogenic effects of cell death on DC activation. A model illustrating the potential affects KIM-1 expression may have on tumour survival can be seen in **Figure 4.1.**

Figure 4.1 *Potential effects of KIM-1 expression on tumour survival.* Based on our findings that KIM-1 expression can limit the release of HMGB1 from apoptotic and necrotic cells, as well as potentially reduce dendritic cell activation, it would stand to reason this would influence tumour survival. A decrease in HMGB1 release as a result of KIM-1 expression would result in a reduction or abrogation of dendritic cell activation. As a result T-cell expansion and recruitment to the site of tumour existence would be limited or potentially non-existent. Therefore tumour elimination would be dampened or lost, resulting in tumour survival as opposed to destruction. A lack of KIM-1 expression would coincide with dendritic cell activation and ultimately tumour destruction.


Chemotherapy
Apoptosis
RCC tumour
Secondary Necrosis
HMGB1 Release
DC Activation
Dendritic Cell
T-cell expansion
T-cell
Migration and Recruitment
B-cell
NK cell
OR
Chemotherapy
KIM-1
Apoptosis
RCC tumour
Secondary Necrosis
NO HMGB1 release
Macrophage
T-cell
Tumour Destruction

In the first part of the results section we began identifying the expression of KIM-1 on a variety of cancer cell lines of lung, renal, prostate, ovarian and kidney origin. Using RCC 769P cells as a model and porcine epithelial cells expressing KIM-1 as controls, we showed that KIM-1 expression in RCC cells converts them into semi-professional phagocytes for apoptotic and necrotic cells (Figure 3.9). Next we characterized the release of the prototypical alarmin, HMGB1, from live, apoptotic and necrotic thymocytes (Figure 3.10). We then examined the ability for KIM-1 expressing cells to prevent the passive release of HMGB1 from late apoptotic and necrotic cells. Lastly, we revealed that KIM-1 modulated the innate immune response to cell death as it inhibited DC activation (Figure 3.14).

4.2.0 Identification of KIM-1 in human cancers

KIM-1 has been largely investigated as a biomarker for kidney injury associated diseases such as AKI, lupus nephritis and cardiac dysfunction[273-275]. The high sensitivity and consistency of KIM-1 expression- even above traditional markers[276]- has brought KIM-1 to the forefront of kidney injury detection and treatment. Although KIM-1-deficient mice have been generated and have no renal phenotype at baseline [277], their response to acute kidney injury has not been reported. Mice expressing a KIM-1 mucin domain deletion mutant, rendering it phagocytosis-defective, are normal at baseline [278], but have been reported to exhibit severe inflammation and tissue injury after being subjected to renal ischemia-reperfusion injury [279].

KIM-1 is overexpressed by a number of cancers including renal cell carcinomas[251]. KIM-1 undergoes ectodomain shedding[231, 265] and soluble KIM-1 has been proposed to be a urinary marker of renal cell carcinoma[231, 250]. In contrast, constitutive KIM-1 expression in RCC has been examined as a susceptibility gene as well as high levels of KIM-1 shedding has been correlated to invasiveness and aggressiveness of tumours [250]. Lastly a recent study correlated the expression of KIM-1 to an increase in the IL-

6/STAT3 growth pathway that may result in growth and angiogenesis of the tumour [280]. The association of KIM-1 being either positive or negative with cancer outcome is unknown, as most studies are epidemiological in nature. The implication of KIM-1 in the pathogenesis of cancer prompted us to investigate a potential role for KIM-1 in the regulation of innate immune responses via its phagocytic function.

Since phagocytosis could only be studied *in vitro*, we first characterized expression for KIM-1 in a variety of cancer cell lines that were available to us. To date, KIM-1 gene expression has been reported in renal clear cell carcinomas and ovarian clear cell carcinomas, testing negative for numerous other cancers including lung and prostate groupings[251]. As a result of our research, we identified high levels of KIM-1 mRNA in renal cancer lines (ex. ACHN, 769P, 786-0), a prostate cancer cell line (ex. PC3) and a lung cancer cell line (ex. A549) and lower yet significant levels in an ovarian cancer cell line (ex. SKOV3) (Figure 3.1). What was interesting to note was that OCC-1 clear renal cell carcinoma tested negative for KIM-1, even after studies have shown that tissue samples of ovarian clear cell cancers stained 94% positive for KIM-1[251]. Upon researching this further, there were as many ovarian clear cell lines that tested positive for KIM-1 as there were negative [281, 282]. The discrepancy in KIM-1 expression between tumour tissues vs. cell lines may be explained by a tumour microenvironment-dependent induction of KIM-1 expression. For example there are many groups who have reported both the expression and lack thereof of KIM-1 in cell culture and observed contrasting results in clinical samples[252] [250].

Ideally, we would have liked to have more thoroughly characterized KIM-1 expression by including colorectal cancer cells and some of the ovarian cancers that have been confirmed to express KIM-1 to make our applications more exhaustive. In addition to recently beginning to characterize the surface expression of KIM-1 in these cancers, determining protein levels of KIM-1 in both the lysate and shed into the media would be an interesting element to identify further trends between KIM-1 surface expression and shedding. We have replicated a substantial amount of our experimental data using 786-0 cells, but it would be interesting to look at repeating our studies using PC3 or ACHN cells that express significantly lower levels of KIM-1 mRNA. Also, we have now

established stable cell lines lacking KIM-1 expression in 769P, 786-0 and ACHN cells using lentivirus to deliver shRNA-targeting KIM-1, and hope to use these cells in *in vivo* studies in mice to test if the lack of KIM-1 expression affects the ability of these cells to form tumours in the presence of an immune system. In order to truly evaluate the implications of KIM-1 to the immune evasion of cancers, we need to move toward an *in vivo* model such as the B16 melanoma model in C57BL/6 mice [124]. B16 melanoma cells do not endogenously express KIM-1 (Gunaratnam, unpublished observations). To this end, we have begun to generate B16 cells overexpressing murine KIM-1 using stable transfection. This model has been used effectively to study the immune response to cytotoxic chemotherapy-induced cell death[124, 208].

4.3.0 KIM-1 expression confers on RCC cells the ability to phagocytose apoptotic and necrotic cells.

A variety of non-professional phagocytes have been shown to possess the ability to engulf apoptotic cells including epithelial cells, fibroblasts, hepatocytes and Sertoli cells [283, 284]. In fact, epithelial bladder tumours have been shown to phagocytose and process damaged red blood cells[285]. In comparison to professional cell phagocytosis, non-professional phagocytosis is often regarded to as the secondary or lesser form of the two, only playing a noticeable role in the absence or defective nature of professional phagocytes[160, 286]. Kidney epithelial cells however are avid phagocytes of apoptotic cells[117]. Our goal here was to characterize the capacity for renal cancer cells to phagocytose either apoptotic or necrotic cells in a KIM-1 dependent fashion. KIM-PK1 cells provided a positive control to test our hypothesis as a lack of a response in 769P cells may have been indicative of a lack of the phagocytic signalling machinery in RCC cells. Accordingly, the LLC-PK1 cells expressing KIM-1 and its counterpart, pcDNA-PK1 cells, exhibited expected results in our tumour cell model. The strong and comparable phagocytic capacity between WT-PK1 and 769P cancer cells demonstrates effective expression and signalling of KIM-1 in 769P cells (Figure 3.7). KIM-1 expression in WT-PK1 cells resulted in more than twice an increase in phagocytosis of both apoptotic and necrotic cells. Furthermore, the lack of KIM-1 expression in both the

pcDNA cells and 769P cells with KIM-1 knockdown demonstrated the necessity of KIM-1 expression for phagocytosis of dying cells. Admittedly, the inhibition of phagocytosis was not complete with siRNA treatment and likely reflected the incomplete knockdown of KIM-1 in these cells. Yet, the near 60% reduction in phagocytic uptake of both necrotic and apoptotic cells in 769P cells was mirrored by the 60% inhibition of KIM-1 expression with siRNA treatment. Taken together, these data showed that KIM-1 phagocytosis plays a significant role in the ability for renal cells to phagocytose. However, without complete inhibition of KIM-1 expression in these cells we cannot rule out whether 769P cells employ mechanisms independent of KIM-1 (e.g. other PS receptors) to mediate efferocytosis.

In contrast, what was interesting to note was that only 44% of WT-PK1 cells stained positive for KIM-1 where 96% of 769P cells stained positive for KIM-1. This resulted in nearly identical capacities for phagocytosis of the same number of cells by both PK1 and 769P cells. Considering both of these cell types are of epithelial cell origin[287, 288], this would imply that the WT-PK1 cells are more efficient at phagocytosing apoptotic or necrotic cells. To address this, we are currently testing 769P and WT-PK1 cells for their ability to engulf PHrodo stained apoptotic and necrotic cells in a time- and dose-dependent manner to test their maximum capacity for phagocytosis of apoptotic and necrotic cells

One of the limitations of our data is that we used dying thymocytes, rather than dying cancer cells as phagocytic targets. The rationale for this was that we had established assays for this. To fully characterize the phagocytosis by RCC cells in the context of tumours, ideally we should repeat the described experiments using necrotic or apoptotic renal cancer cells. This might be important given that RCC cells are larger than thymocytes, and therefore might be less amenable to phagocytic uptake. KIM-1 has been shown to effectively phagocytose viruses, gram-positive and gram-negative bacteria on epithelial cells accounting for size diversity [289, 290]. Additionally TIM-4, which belongs to the same family of genes as KIM-1, is a PS receptor expressed on dendritic cells and has been shown to phagocytose apoptotic and necrotic cancer cells [222]. As a result of the diverse cells known to be phagocytosed via KIM-1 mediated PS interactions,

the phagocytosis of larger cancer cells should not present as an issue.

Another model that our lab has been exploring is the mouse B16 melanoma model for in vivo work. This model can activate both BimS and FADD death pathways to result in apoptotic and necrotic cell death via tetracycline[291]. A potential application of this model would be to inject mice with either B16 or KIM-1 B16 tumours. Using tetracycline to induce either apoptotic or necrotic death, we would then measure the ability for mice to clear tumours. By comparing attributes such as the percent of mice that establish tumours, the size of the established tumours, the percent of tumours that are cleared and the overall health status of these mice, we can infer a role of KIM-1 in tumour progression and survival.

4.4.0 KIM-1-expressing cancer cells sequester HMGB1 release by apoptotic and necrotic cells via phagocytosis

The role of HMGB1 in the context of cancers has been identified as both a tumour promoting and tumour-limiting agent. One group examining patient samples of individuals with hepatocellular carcinoma found higher HMGB1 expression levels being correlated with lower 5-year survival rates in patients[292, 293]. Further studies have identified an interaction between HMGB1 and tumour suppressor protein retinoblastoma (Rb) limits the growth of breast tumours in vivo[294]. HMGB1 has also been linked to an increase in genome stability through telomere lengthening [295, 296] resulting in decreased genetic mutations and lower tumour establishment rates.

In favour of HMGB1 expression as a tumour promoting mechanism, studies have shown HMGB1 plays a role in fulfilling the high-energy requirements seen in tumours. HMGB1 expression has been linked to increased ATP production and pancreatic tumour proliferation[297]. Furthermore mitochondrial HMGB1 interaction with the RAGE receptor has also been shown to increase ATP production and loss of RAGE expression has been shown to prevent tumourigenesis in the skin pancreas and intestine [298-300]. HMGB1 secretion has also been linked to enhancing anti-tumour immunity, through the promotion of T_{reg} mediated IL-10 production[301]. Additionally inhibition of RAGE-HMGB1 interactions has been seen to suppress invasion and metastasis[302] and the downstream signalling of RAGE-HMGB1 has been linked to VEGF production as well as the production of other pro-inflammatory cytokines to induce angiogenesis[303]. Therefore the production and secretion of HMGB1 may have either direct or indirect advantageous or disadvantageous roles in the survival of tumours through its effect on the immune environment.

HMGB1 is a DNA-binding nuclear protein released actively by macrophages and monocytes[304, 305] or passively during cell necrosis[272]. It has important roles in models of non-infectious inflammation, such as autoimmunity, cancer, trauma, and ischemia reperfusion injury[306]. Immunogenic cell death-mediated HMGB1 release activates dendritic cells and promotes their functional maturation [123, 124, 307] and anti-tumour immunity. Tumour cell evasion of immunogenic cell death represents a

potential mechanism for avoiding immune surveillance[208, 308, 309].

Unlike necrosis or cell death caused by certain cytotoxic agents, the passive release of HMGB1 from apoptotic cells is highly debated [122, 310, 311]. As demonstrated in our studies, live, apoptotic and necrotic cells all were capable of releasing HMGB1, albeit in a time dependent manner. The release of HMGB1 from live cells was deemed insignificant for the purpose of our study, since most of our end points were 24 hours or less when live thymocytes undergo spontaneous apoptosis as determined by positive staining with Annexin V (data not shown). We presume that once thymocytes are removed from a viable host they immediately, though at a significantly slower rate, begin to transition to an apoptotic and necrotic cell state, shown by the eventual release of HMGB1 from into their environment. Apoptotic cells, however, began leaking HMGB1 as early as 8 hours post UV exposure and continued to do so at an increasing rate as time progressed. Over time apoptotic cells can transition from apoptosis to secondary necrosis, which is characterized by a lack of membrane integrity not found in apoptotic cells[148, 272]. This is supported by the Annexin V and PI staining profile of late apoptotic cells[312]. Accordingly, we observed a rapid increase in HMGB1 release from 8 hours to 24 hours, ultimately matching HMGB1 release by necrotic cells at 72 hours. In contrast, HMGB1 release from necrotic cells was immediate and persistent over 72 hours. It remains to be tested if HMGB1 release from apoptotic and necrotic 769P RCC cells is comparable to that of thymocytes. Delayed release or sequestration of HMGB1 from dying tumour cells might serve as a novel mechanism of immune evasion and resistance to chemo/radio-therapy[123].

Having characterized the release of HMGB1 from dying cells we determined if KIM-1 expression by RCC cells would circumvent the passive release of HMGB1 from late apoptotic and necrotic cells. We found that HMGB1 levels in the conditioned media of KIM-1-PK1 cells was significantly lower than pcDNA-PK1 cells fed apoptotic or necrotic cells. A corresponding increase in HMGB1 levels was present in the lysates of cells expressing KIM-1 suggesting that it was sequestered there. Because only extracellular HMGB1 is immunologically active[272], we examined the conditioned media for HMGB1 release in 769P cells fed apoptotic or necrotic cells (Figure 3.12). A

paralleled response in HMGB1 release in 769P cells confirmed our prediction that 769P cell circumvented HMGB1 release from dying cells. Moreover, the recovery of HMGB1 in the extracellular compartment after silencing KIM-1 expression suggested a causative role for KIM-1 in this process. Despite concluding that KIM-1 expression is necessary for preventing the release of HMGB1 from dying cells, we are unable to confirm if this was mediated by corpse engulfment. We can merely associate KIM-1-dependent phagocytosis of apoptotic and necrotic cells by 769P cells with their ability to limit HMGB1 release into the surrounding environment. The absence of HMGB1 in the conditioned medium of 769P cells cultured in the absence of dying cells ruled out the possibility that the observed HMGB1 in the media was released by 769P cells. The reversal of HMGB1 sequestration by 769P cells by pre-treating the cells with cytochalasin D that inhibits actin polymerization (and phagocytosis) would argue that corpse engulfment was the mechanism limiting HMGB1 levels in the extracellular milieu by 769P cells. An alternative possibility is that HMGB1 could be released by dying cells into the surrounding media and that KIM-1 could bind to and phagocytose HMGB1 directly. There is no support in the literature however, that KIM-1 can interact with HMGB1 either directly or indirectly. To confirm one way or another, we could do a co-immunoprecipitation for HMGB1 and KIM-1 to determine if any interaction between the molecules exist.

It would be interesting to determine if as a consequence of phagocytosis of dying tumour cells, cancer cells that express KIM-1 also sequester tumour antigens from reaching antigen-presenting cells. As a result, KIM-1-expressing tumours would not only limit HMGB1-mediated activation of innate immunity, but also activation of tumour antigen-specific T-cells by antigen presenting cells [131].

4.5.0 KIM-1 expression may help cancer cells evade the innate immune response to apoptotic and necrotic cancer cells

Having established that KIM-1 expression on RCC cells is both sufficient and necessary for phagocytosis of apoptotic and necrotic cells, and determining that KIM-1 is necessary in repressing alarmin release from dying cells, we evaluated whether KIM-1-expressing cells could alter the immune response to immunogenic cells death (i.e. cell death resulting in HMGB1 release). The induction of inflammatory and immune responses by HMGB1 is mediated by expression of TLRs and various pattern recognition receptors expressed for instance by DCs. We evaluated the ability for KIM-1 expressing 769P cells to prevent DC activation by late apoptotic and necrotic cells. KIM-1 expression in 769P cells resulted in impaired expression of the activation markers CD40 and CD86 on CD11c-positive primary mouse dendritic cells compared to control siRNA-treated cells or LPS control treated cells. These results are, however, premature and require confirmation. If confirmed, these data would imply that as a result of phagocytic clearance of apoptotic and necrotic cells, cancer cells could limit the immunogenicity of dying cancer cells mediated by HMGB1 release into the tumour microenvironment.

4.6.0 Future Directions

The next step for this project is to move the project in the direction of an *in vivo* study. We have yet to identify mouse cell lines that express KIM-1 endogenously and as a result we would have to establish stable KIM-1 mouse cancers. During the course of this project I used CT26 mouse colon cancers and transiently transfected them with mouse KIM-1. We observed a substantial increase in the phagocytosis of both apoptotic and necrotic cells. We did encounter difficulty however, in establishing and characterizing stable or high expressers of mouse KIM-1 via western blot. A lab member has been working for the past year on the same model and has found the incorporation of KIM-1 in CT26 cells to be at extremely low levels. As a result using cell lines that have been shown to establish tumours well and transfect easily, and be used frequently *in vivo*- like the E0771 breast cancer cell line.

4.7.0 Implications

Kidney cancer represents the eighth most common type of cancer in the world, with over 90% of cases being of RCC origin. As mentioned previously RCC is highly resistant to chemotherapy, resulting in a 95% fatality rate in metastatic conditions[294]. Drug based approaches that induce direct tumour cell killing seem to be ineffective begging the question if RCC cells are resistant to chemotherapeutic cell death. In the last decade or so, the paradigm surrounding cancer treatment has shifted from direct killing to using the immune system activation to kill tumours. This principle is founded on the idea that the immune system is the most capable agent in tumour killing, as it does it on a daily basis as part of its immune surveillance function. Examples of this include the ideas of tumour vaccines or activating dendritic cells towards tumour epitopes and then reintroducing them back into the affected host.

The role for anti-KIM-1 therapies in tumour eradication would be used as a means to "unmask" the tumour to dendritic cells and the innate immune system so that they can effectively remove cancer cells. Knowing that KIM-1 can sequester danger signals and potentially antigens, knocking down KIM-1 expression would unveil tumours before they are able to outrun the immune system. Therefore KIM-1 may be a potential therapeutic target that can help circumvent the resistivity of renal cancers to chemotherapy by potentially enhancing tumour immunogenicity, and potentially improve patient survival rates.

References

1. Shipitsin, M., et al., *Molecular definition of breast tumor heterogeneity.* Cancer Cell, 2007. **11**(3): p. 259-73.
2. Ogino, S., et al., *Cancer immunology--analysis of host and tumor factors for personalized medicine.* Nat Rev Clin Oncol, 2011. **8**(12): p. 711-9.
3. Gerlinger, M., et al., *Intratumor heterogeneity and branched evolution revealed by multiregion sequencing.* N Engl J Med, 2012. **366**(10): p. 883-92.
4. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
5. Cheng, N., et al., *Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion.* Mol Cancer Res, 2008. **6**(10): p. 1521-33.
6. Bhowmick, N.A., E.G. Neilson, and H.L. Moses, *Stromal fibroblasts in cancer initiation and progression.* Nature, 2004. **432**(7015): p. 332-7.
7. Gunaratnam, L., et al., *Hypoxia inducible factor activates the transforming growth factor-alpha/epidermal growth factor receptor growth stimulatory pathway in VHL(-/-) renal cell carcinoma cells.* J Biol Chem, 2003. **278**(45): p. 44966-74.
8. Davies, M.A. and Y. Samuels, *Analysis of the genome to personalize therapy for melanoma.* Oncogene, 2010. **29**(41): p. 5545-55.
9. Sudarsanam, S. and D.E. Johnson, *Functional consequences of mTOR inhibition.* Curr Opin Drug Discov Devel, 2010. **13**(1): p. 31-40.
10. O'Reilly, K.E., et al., *mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt.* Cancer Res, 2006. **66**(3): p. 1500-8.
11. Collado, M. and M. Serrano, *Senescence in tumours: evidence from mice and humans.* Nat Rev Cancer, 2010. **10**(1): p. 51-7.
12. Burkhart, D.L. and J. Sage, *Cellular mechanisms of tumour suppression by the retinoblastoma gene.* Nat Rev Cancer, 2008. **8**(9): p. 671-82.
13. Sherr, C.J. and F. McCormick, *The RB and p53 pathways in cancer.* Cancer Cell, 2002. **2**(2): p. 103-12.
14. Deshpande, A., P. Sicinski, and P.W. Hinds, *Cyclins and cdks in development and cancer: a perspective.* Oncogene, 2005. **24**(17): p. 2909-15.
15. Seluanov, A., et al., *Hypersensitivity to contact inhibition provides a clue to cancer resistance of naked mole-rat.* Proc Natl Acad Sci U S A, 2009. **106**(46): p. 19352-7.
16. Abercrombie, M., *Contact inhibition and malignancy.* Nature, 1979. **281**(5729): p. 259-62.
17. Partanen, J.I., A.I. Nieminen, and J. Klefstrom, *3D view to tumor suppression: Lkb1, polarity and the arrest of oncogenic c-Myc.* Cell Cycle, 2009. **8**(5): p. 716-24.
18. Hezel, A.F. and N. Bardeesy, *LKB1; linking cell structure and tumor suppression.* Oncogene, 2008. **27**(55): p. 6908-19.

19. Curto, M., et al., *Contact-dependent inhibition of EGFR signaling by Nf2/Merlin.* J Cell Biol, 2007. **177**(5): p. 893-903.
20. Okada, T., M. Lopez-Lago, and F.G. Giancotti, *Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane.* J Cell Biol, 2005. **171**(2): p. 361-71.
21. Shaw, R.J., *Tumor suppression by LKB1: SIK-ness prevents metastasis.* Sci Signal, 2009. **2**(86): p. pe55.
22. Wijnhoven, B.P., W.N. Dinjens, and M. Pignatelli, *E-cadherin-catenin cell-cell adhesion complex and human cancer.* Br J Surg, 2000. **87**(8): p. 992-1005.
23. Shliankevich, M.A., et al., *[Cultivation of heterokaryons in diffusion chambers].* Vopr Onkol, 1972. **18**(12): p. 55-9.
24. Kim, D.W., et al., *Tumor suppressor LKB1 inhibits activation of signal transducer and activator of transcription 3 (STAT3) by thyroid oncogenic tyrosine kinase rearranged in transformation (RET)/papillary thyroid carcinoma (PTC).* Mol Endocrinol, 2007. **21**(12): p. 3039-49.
25. Evan, G. and T. Littlewood, *A matter of life and cell death.* Science, 1998. **281**(5381): p. 1317-22.
26. Adams, J.M. and S. Cory, *The Bcl-2 apoptotic switch in cancer development and therapy.* Oncogene, 2007. **26**(9): p. 1324-37.
27. Lowe, S.W., E. Cepero, and G. Evan, *Intrinsic tumour suppression.* Nature, 2004. **432**(7015): p. 307-15.
28. Cory, S. and J.M. Adams, *The Bcl2 family: regulators of the cellular life-or-death switch.* Nat Rev Cancer, 2002. **2**(9): p. 647-56.
29. Yoshino, T., et al., *Bcl-2 expression as a predictive marker of hormone-refractory prostate cancer treated with taxane-based chemotherapy.* Clin Cancer Res, 2006. **12**(20 Pt 1): p. 6116-24.
30. Kang, M.H. and C.P. Reynolds, *Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy.* Clin Cancer Res, 2009. **15**(4): p. 1126-32.
31. He, C. and D.J. Klionsky, *Regulation mechanisms and signaling pathways of autophagy.* Annu Rev Genet, 2009. **43**: p. 67-93.
32. Liu, J.J., et al., *Targeting apoptotic and autophagic pathways for cancer therapeutics.* Cancer Lett, 2011. **300**(2): p. 105-14.
33. Huett, A., G. Goel, and R.J. Xavier, *A systems biology viewpoint on autophagy in health and disease.* Curr Opin Gastroenterol, 2010. **26**(4): p. 302-9.
34. Chen, S., et al., *Autophagy is a therapeutic target in anticancer drug resistance.* Biochim Biophys Acta, 2010. **1806**(2): p. 220-9.
35. Li, Z.Y., et al., *Mitochondrial ROS generation for regulation of autophagic pathways in cancer.* Biochem Biophys Res Commun, 2011. **414**(1): p. 5-8.
36. Kundu, M. and C.B. Thompson, *Autophagy: basic principles and relevance to disease.* Annu Rev Pathol, 2008. **3**: p. 427-55.
37. Ouyang, L., et al., *Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis.* Cell Prolif, 2012. **45**(6): p. 487-98.
38. Hanahan, D. and J. Folkman, *Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis.* Cell, 1996. **86**(3): p. 353-64.

39. Ferrara, N., *Pathways mediating VEGF-independent tumor angiogenesis.* Cytokine Growth Factor Rev, 2010. **21**(1): p. 21-6.
40. Carmeliet, P., *VEGF as a key mediator of angiogenesis in cancer.* Oncology, 2005. **69 Suppl 3**: p. 4-10.
41. Raica, M., A.M. Cimpean, and D. Ribatti, *Angiogenesis in pre-malignant conditions.* Eur J Cancer, 2009. **45**(11): p. 1924-34.
42. Semenza, G., *Signal transduction to hypoxia-inducible factor 1.* Biochem Pharmacol, 2002. **64**(5-6): p. 993-8.
43. Ferrara, N., H.P. Gerber, and J. LeCouter, *The biology of VEGF and its receptors.* Nat Med, 2003. **9**(6): p. 669-76.
44. Goel, H.L. and A.M. Mercurio, *VEGF targets the tumour cell.* Nat Rev Cancer, 2013. **13**(12): p. 871-82.
45. Ribatti, D., *Endogenous inhibitors of angiogenesis: a historical review.* Leuk Res, 2009. **33**(5): p. 638-44.
46. Nyberg, P., L. Xie, and R. Kalluri, *Endogenous inhibitors of angiogenesis.* Cancer Res, 2005. **65**(10): p. 3967-79.
47. Wei, X.W., Z.R. Zhang, and Y.Q. Wei, *Anti-angiogenic drugs currently in Phase II clinical trials for gynecological cancer treatment.* Expert Opin Investig Drugs, 2013. **22**(9): p. 1181-92.
48. Cabebe, E. and H. Wakelee, *Role of anti-angiogenesis agents in treating NSCLC: focus on bevacizumab and VEGFR tyrosine kinase inhibitors.* Curr Treat Options Oncol, 2007. **8**(1): p. 15-27.
49. Berx, G. and F. van Roy, *Involvement of members of the cadherin superfamily in cancer.* Cold Spring Harb Perspect Biol, 2009. **1**(6): p. a003129.
50. Cavallaro, U. and G. Christofori, *Cell adhesion and signalling by cadherins and Ig-CAMs in cancer.* Nat Rev Cancer, 2004. **4**(2): p. 118-32.
51. Fidler, I.J., *The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited.* Nat Rev Cancer, 2003. **3**(6): p. 453-8.
52. Ribatti, D., G. Mangialardi, and A. Vacca, *Stephen Paget and the 'seed and soil' theory of metastatic dissemination.* Clin Exp Med, 2006. **6**(4): p. 145-9.
53. Kessenbrock, K., V. Plaks, and Z. Werb, *Matrix metalloproteinases: regulators of the tumor microenvironment.* Cell, 2010. **141**(1): p. 52-67.
54. Regan Anderson, T.M., et al., *Breast tumor kinase (Brk/PTK6) is a mediator of hypoxia-associated breast cancer progression.* Cancer Res, 2013. **73**(18): p. 5810-20.
55. Blasco, M.A., *Telomeres and human disease: ageing, cancer and beyond.* Nat Rev Genet, 2005. **6**(8): p. 611-22.
56. Shay, J.W. and W.E. Wright, *Hayflick, his limit, and cellular ageing.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 72-6.
57. Artandi, S.E. and R.A. DePinho, *Mice without telomerase: what can they teach us about human cancer?* Nat Med, 2000. **6**(8): p. 852-5.
58. Raynaud, C.M., et al., *DNA damage repair and telomere length in normal breast, preneoplastic lesions, and invasive cancer.* Am J Clin Oncol, 2010. **33**(4): p. 341-5.
59. Chin, K., et al., *In situ analyses of genome instability in breast cancer.* Nat Genet, 2004. **36**(9): p. 984-8.

60. Berdasco, M. and M. Esteller, *Aberrant epigenetic landscape in cancer: how cellular identity goes awry.* Dev Cell, 2010. **19**(5): p. 698-711.
61. Negrini, S., V.G. Gorgoulis, and T.D. Halazonetis, *Genomic instability--an evolving hallmark of cancer.* Nat Rev Mol Cell Biol, 2010. **11**(3): p. 220-8.
62. Salk, J.J., E.J. Fox, and L.A. Loeb, *Mutational heterogeneity in human cancers: origin and consequences.* Annu Rev Pathol, 2010. **5**: p. 51-75.
63. DeNardo, D.G., P. Andreu, and L.M. Coussens, *Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity.* Cancer Metastasis Rev, 2010. **29**(2): p. 309-16.
64. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
65. Qian, B.Z. and J.W. Pollard, *Macrophage diversity enhances tumor progression and metastasis.* Cell, 2010. **141**(1): p. 39-51.
66. Karnoub, A.E. and R.A. Weinberg, *Chemokine networks and breast cancer metastasis.* Breast Dis, 2006. **26**: p. 75-85.
67. Mantovani, A., et al., *The origin and function of tumor-associated macrophages.* Immunol Today, 1992. **13**(7): p. 265-70.
68. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**: p. 13.
69. Mantovani, A. and A. Sica, *Macrophages, innate immunity and cancer: balance, tolerance, and diversity.* Curr Opin Immunol, 2010. **22**(2): p. 231-7.
70. Mantovani, A., et al., *Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes.* Trends Immunol, 2002. **23**(11): p. 549-55.
71. Mougiakakos, D., et al., *Regulatory T cells in cancer.* Adv Cancer Res, 2010. **107**: p. 57-117.
72. Jones, R.G. and C.B. Thompson, *Tumor suppressors and cell metabolism: a recipe for cancer growth.* Genes Dev, 2009. **23**(5): p. 537-48.
73. Hsu, P.P. and D.M. Sabatini, *Cancer cell metabolism: Warburg and beyond.* Cell, 2008. **134**(5): p. 703-7.
74. Morath, C., et al., *Malignancy in renal transplantation.* J Am Soc Nephrol, 2004. **15**(6): p. 1582-8.
75. Rama, I. and J.M. Grinyo, *Malignancy after renal transplantation: the role of immunosuppression.* Nat Rev Nephrol, 2010. **6**(9): p. 511-9.
76. Ensor, C.R., et al., *Generic maintenance immunosuppression in solid organ transplant recipients.* Pharmacotherapy, 2011. **31**(11): p. 1111-29.
77. Penn, I., *Malignant melanoma in organ allograft recipients.* Transplantation, 1996. **61**(2): p. 274-8.
78. Pham, S.M., et al., *Solid tumors after heart transplantation: lethality of lung cancer.* Ann Thorac Surg, 1995. **60**(6): p. 1623-6.
79. Old, L.J. and E.A. Boyse, *Immunology of Experimental Tumors.* Annu Rev Med, 1964. **15**: p. 167-86.
80. Chouaib, S., *[At the crossroads of cancer].* Bull Cancer, 2013. **100**(6): p. 569-74.
81. Stutman, O., *Immunodepression and malignancy.* Adv Cancer Res, 1975. **22**: p. 261-422.

82. Stutman, *Tumor development after polyoma infection in athymic nude mice.* J Immunol, 1975. **114**(4): p. 1213-7.
83. Grant, G.A. and J.F. Miller, *Effect of neonatal thymectomy on the induction of sarcomata in C57 BL mice.* Nature, 1965. **205**(976): p. 1124-5.
84. Trainin, N., et al., *Enhancement of lung adenoma formation by neonatal thymectomy in mice treated with 7,12-dimethylbenz(a)anthracene or urethan.* Int J Cancer, 1967. **2**(4): p. 326-36.
85. Burstein, N.A. and L.W. Law, *Neonatal thymectomy and non-viral mammary tumours in mice.* Nature, 1971. **231**(5303): p. 450-2.
86. Hayday, A.C., *[gamma][delta] cells: a right time and a right place for a conserved third way of protection.* Annu Rev Immunol, 2000. **18**: p. 975-1026.
87. Maleckar, J.R. and L.A. Sherman, *The composition of the T cell receptor repertoire in nude mice.* J Immunol, 1987. **138**(11): p. 3873-6.
88. Rygaard, J. and C.O. Povlsen, *The mouse mutant nude does not develop spontaneous tumours. An argument against immunological surveillance.* Acta Pathol Microbiol Scand B Microbiol Immunol, 1974. **82**(1): p. 99-106.
89. Outzen, H.C., et al., *Spontaneous and induced tumor incidence in germfree "nude" mice.* J Reticuloendothel Soc, 1975. **17**(1): p. 1-9.
90. Street, S.E., E. Cretney, and M.J. Smyth, *Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis.* Blood, 2001. **97**(1): p. 192-7.
91. van den Broek, M.E., et al., *Decreased tumor surveillance in perforin-deficient mice.* J Exp Med, 1996. **184**(5): p. 1781-90.
92. Smyth, M.J., et al., *Differential tumor surveillance by natural killer (NK) and NKT cells.* J Exp Med, 2000. **191**(4): p. 661-8.
93. Shinkai, Y., et al., *RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement.* Cell, 1992. **68**(5): p. 855-67.
94. Mombaerts, P., et al., *RAG-1-deficient mice have no mature B and T lymphocytes.* Cell, 1992. **68**(5): p. 869-77.
95. Shankaran, V., et al., *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity.* Nature, 2001. **410**(6832): p. 1107-11.
96. Schreiber, R.D., L.J. Old, and M.J. Smyth, *Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion.* Science, 2011. **331**(6024): p. 1565-70.
97. Mittal, D., et al., *New insights into cancer immunoediting and its three component phases-elimination, equilibrium and escape.* Curr Opin Immunol, 2014. **27C**: p. 16-25.
98. Ikeda, H., L.J. Old, and R.D. Schreiber, *The roles of IFN gamma in protection against tumor development and cancer immunoediting.* Cytokine Growth Factor Rev, 2002. **13**(2): p. 95-109.
99. Smyth, M.J., N.Y. Crowe, and D.I. Godfrey, *NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma.* Int Immunol, 2001. **13**(4): p. 459-63.
100. Girardi, M., et al., *Regulation of cutaneous malignancy by gammadelta T cells.* Science, 2001. **294**(5542): p. 605-9.

101. Smyth, M.J., D.I. Godfrey, and J.A. Trapani, *A fresh look at tumor immunosurveillance and immunotherapy.* Nat Immunol, 2001. **2**(4): p. 293-9.
102. Matzinger, P., *Tolerance, danger, and the extended family.* Annu Rev Immunol, 1994. **12**: p. 991-1045.
103. Bromberg, J.F., et al., *Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon alpha and interferon gamma.* Proc Natl Acad Sci U S A, 1996. **93**(15): p. 7673-8.
104. Gollob, J.A., et al., *Gene expression changes and signaling events associated with the direct antimelanoma effect of IFN-gamma.* Cancer Res, 2005. **65**(19): p. 8869-77.
105. Swann, J.B., et al., *CD1-restricted T cells and tumor immunity.* Curr Top Microbiol Immunol, 2007. **314**: p. 293-323.
106. Qin, Z., et al., *A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells.* Cancer Res, 2003. **63**(14): p. 4095-100.
107. Kim, R., et al., *Tumor-driven evolution of immunosuppressive networks during malignant progression.* Cancer Res, 2006. **66**(11): p. 5527-36.
108. Dunn, G.P., et al., *Cancer immunoediting: from immunosurveillance to tumor escape.* Nat Immunol, 2002. **3**(11): p. 991-8.
109. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics.* Br J Cancer, 1972. **26**(4): p. 239-57.
110. Medeiros, A.I., et al., *Efferocytosis impairs pulmonary macrophage and lung antibacterial function via PGE2/EP2 signaling.* J Exp Med, 2009. **206**(1): p. 61-8.
111. Lauber, K., et al., *Clearance of apoptotic cells: getting rid of the corpses.* Mol Cell, 2004. **14**(3): p. 277-87.
112. Fadok, V.A., et al., *The role of phosphatidylserine in recognition of apoptotic cells by phagocytes.* Cell Death Differ, 1998. **5**(7): p. 551-62.
113. Walker, P.D., G.P. Kaushal, and S.V. Shah, *Meprin A, the major matrix degrading enzyme in renal tubules, produces a novel nidogen fragment in vitro and in vivo.* Kidney Int, 1998. **53**(6): p. 1673-80.
114. Potter, P.K., et al., *Lupus-prone mice have an abnormal response to thioglycolate and an impaired clearance of apoptotic cells.* J Immunol, 2003. **170**(6): p. 3223-32.
115. Nakayama, M., et al., *Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation.* Blood, 2009. **113**(16): p. 3821-30.
116. Fadok, V.A., *Clearance: the last and often forgotten stage of apoptosis.* J Mammary Gland Biol Neoplasia, 1999. **4**(2): p. 203-11.
117. Ichimura, T., et al., *Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells.* J Clin Invest, 2008. **118**(5): p. 1657-68.
118. Voll, R.E., et al., *Immunosuppressive effects of apoptotic cells.* Nature, 1997. **390**(6658): p. 350-1.
119. Albert, M.L., B. Sauter, and N. Bhardwaj, *Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs.* Nature, 1998. **392**(6671): p. 86-9.

120. Casares, N., et al., *Caspase-dependent immunogenicity of doxorubicin-induced tumor cell death.* J Exp Med, 2005. **202**(12): p. 1691-701.
121. Obeid, M., et al., *Calreticulin exposure dictates the immunogenicity of cancer cell death.* Nat Med, 2007. **13**(1): p. 54-61.
122. Gallucci, S., M. Lolkema, and P. Matzinger, *Natural adjuvants: endogenous activators of dendritic cells.* Nat Med, 1999. **5**(11): p. 1249-55.
123. Tesniere, A., et al., *Immunogenic cancer cell death: a key-lock paradigm.* Curr Opin Immunol, 2008. **20**(5): p. 504-11.
124. Apetoh, L., et al., *Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy.* Nat Med, 2007. **13**(9): p. 1050-9.
125. Rovere, P., et al., *Delayed clearance of apoptotic lymphoma cells allows cross-presentation of intracellular antigens by mature dendritic cells.* J Leukoc Biol, 1999. **66**(2): p. 345-9.
126. Rock, K.L., et al., *The sterile inflammatory response.* Annu Rev Immunol, 2010. **28**: p. 321-42.
127. Bolisetty, S. and A. Agarwal, *Neutrophils in acute kidney injury: not neutral any more.* Kidney Int, 2009. **75**(7): p. 674-6.
128. Kinsey, G.R., L. Li, and M.D. Okusa, *Inflammation in acute kidney injury.* Nephron Exp Nephrol, 2008. **109**(4): p. e102-7.
129. Majno, G., M. La Gattuta, and T.E. Thompson, *Cellular death and necrosis: chemical, physical and morphologic changes in rat liver.* Virchows Arch Pathol Anat Physiol Klin Med, 1960. **333**: p. 421-65.
130. Chen, C.J., et al., *Identification of a key pathway required for the sterile inflammatory response triggered by dying cells.* Nat Med, 2007. **13**(7): p. 851-6.
131. Apetoh, L., et al., *The interaction between HMGB1 and TLR4 dictates the outcome of anticancer chemotherapy and radiotherapy.* Immunol Rev, 2007. **220**: p. 47-59.
132. Peter, K. and A. Bobik, *HMGB1 signals danger in acute coronary syndrome: emergence of a new risk marker for cardiovascular death?* Atherosclerosis, 2012. **221**(2): p. 317-8.
133. Bianchi, M.E., M. Beltrame, and G. Paonessa, *Specific recognition of cruciform DNA by nuclear protein HMG1.* Science, 1989. **243**(4894 Pt 1): p. 1056-9.
134. Einck, L. and M. Bustin, *The intracellular distribution and function of the high mobility group chromosomal proteins.* Exp Cell Res, 1985. **156**(2): p. 295-310.
135. Basta, G., *Receptor for advanced glycation endproducts and atherosclerosis: From basic mechanisms to clinical implications.* Atherosclerosis, 2008. **196**(1): p. 9-21.
136. Li, G., X. Liang, and M.T. Lotze, *HMGB1: The Central Cytokine for All Lymphoid Cells.* Front Immunol, 2013. **4**: p. 68.
137. Park, J.S., et al., *High mobility group box 1 protein interacts with multiple Toll-like receptors.* Am J Physiol Cell Physiol, 2006. **290**(3): p. C917-24.
138. Park, J.S., et al., *Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein.* J Biol Chem, 2004. **279**(9): p. 7370-7.

139. Dumitriu, I.E., et al., *Requirement of HMGB1 and RAGE for the maturation of human plasmacytoid dendritic cells.* Eur J Immunol, 2005. **35**(7): p. 2184-90.
140. Zhang, W., J. Tian, and Q. Hao, *HMGB1 combining with tumor-associated macrophages enhanced lymphangiogenesis in human epithelial ovarian cancer.* Tumour Biol, 2013.
141. Blasius, A.L. and B. Beutler, *Intracellular toll-like receptors.* Immunity, 2010. **32**(3): p. 305-15.
142. Ulloa, L. and D. Messmer, *High-mobility group box 1 (HMGB1) protein: friend and foe.* Cytokine Growth Factor Rev, 2006. **17**(3): p. 189-201.
143. Ding, H.S., et al., *High mobility group [corrected] box 1 mediates neutrophil recruitment in myocardial ischemia-reperfusion injury through toll like receptor 4-related pathway.* Gene, 2012. **509**(1): p. 149-53.
144. Andersson, U. and K.J. Tracey, *HMGB1 is a therapeutic target for sterile inflammation and infection.* Annu Rev Immunol, 2011. **29**: p. 139-62.
145. Yang, Q.W., et al., *High-mobility group protein box-1 and its relevance to cerebral ischemia.* J Cereb Blood Flow Metab, 2010. **30**(2): p. 243-54.
146. Albayrak, Y., et al., *High mobility group box protein-1 (HMGB-1) as a new diagnostic marker in patients with acute appendicitis.* Scand J Trauma Resusc Emerg Med, 2011. **19**: p. 27.
147. de Grauw, J.C., et al., *Rapid release of high mobility group box protein-1 (HMGB-1) in transient arthritis.* Clin Exp Rheumatol, 2010. **28**(2): p. 292-3.
148. Sims, G.P., et al., *HMGB1 and RAGE in inflammation and cancer.* Annu Rev Immunol, 2010. **28**: p. 367-88.
149. Hao, Q., et al., *[Expression and clinical significance of HMGB1 and RAGE in cervical squamous cell carcinoma].* Zhonghua Zhong Liu Za Zhi, 2008. **30**(4): p. 292-5.
150. Hagemann, T., et al., *Ovarian cancer cells polarize macrophages toward a tumor-associated phenotype.* J Immunol, 2006. **176**(8): p. 5023-32.
151. Duluc, D., et al., *Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells.* Blood, 2007. **110**(13): p. 4319-30.
152. Yeo, J.C., et al., *High-throughput quantification of early stages of phagocytosis.* Biotechniques, 2013. **55**(3): p. 115-24.
153. Bettencourt, P., et al., *Actin-binding protein regulation by microRNAs as a novel microbial strategy to modulate phagocytosis by host cells: the case of N-Wasp and miR-142-3p.* Front Cell Infect Microbiol, 2013. **3**: p. 19.
154. Tanaka, M. and Y. Miyake, *Apoptotic cell clearance and autoimmune disorder.* Curr Med Chem, 2007. **14**(27): p. 2892-7.
155. Kang, Y., et al., *Structural study of TTR-52 reveals the mechanism by which a bridging molecule mediates apoptotic cell engulfment.* Genes Dev, 2012. **26**(12): p. 1339-50.
156. Moulder, J.W., *Comparative biology of intracellular parasitism.* Microbiol Rev, 1985. **49**(3): p. 298-337.
157. Falkow, S., R.R. Isberg, and D.A. Portnoy, *The interaction of bacteria with mammalian cells.* Annu Rev Cell Biol, 1992. **8**: p. 333-63.

158. Joiner, K.A., et al., *Toxoplasma gondii: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts.* Science, 1990. **249**(4969): p. 641-6.
159. Schoenberg, D.A. and T.C. Cheng, *Concanavalin A-mediated phagocytosis of yeast by Biomphalaria glabrata hemocytes in vitro: effects of temperature and lectin concentration.* J Invertebr Pathol, 1982. **39**(3): p. 314-22.
160. Rabinovitch, M., *Professional and non-professional phagocytes: an introduction.* Trends Cell Biol, 1995. **5**(3): p. 85-7.
161. Rabinovitch, M. and M.J. De Stefano, *Interactions of red cells with phagocytes of the wax-mouth (Galleria mellonella, L.) and mouse.* Exp Cell Res, 1970. **59**(2): p. 272-82.
162. Herrera, E.M., et al., *Mediation of Trypanosoma cruzi invasion by heparan sulfate receptors on host cells and penetrin counter-receptors on the trypanosomes.* Mol Biochem Parasitol, 1994. **65**(1): p. 73-83.
163. Bouvier, G., et al., *Relationship between phagosome acidification, phagosome-lysosome fusion, and mechanism of particle ingestion.* J Leukoc Biol, 1994. **55**(6): p. 729-34.
164. Savill, J., et al., *A blast from the past: clearance of apoptotic cells regulates immune responses.* Nat Rev Immunol, 2002. **2**(12): p. 965-75.
165. Meagher, L.C., et al., *Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B2.* J Leukoc Biol, 1992. **52**(3): p. 269-73.
166. Cocco, R.E. and D.S. Ucker, *Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure.* Mol Biol Cell, 2001. **12**(4): p. 919-30.
167. Fadok, V.A., et al., *Macrophages that have ingested apoptotic cells in vitro inhibit pro-inflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF.* J Clin Invest, 1998. **101**(4): p. 890-8.
168. Patel, V.A., et al., *Recognition of apoptotic cells by epithelial cells: conserved versus tissue-specific signaling responses.* J Biol Chem, 2010. **285**(3): p. 1829-40.
169. Matute-Bello, G. and T.R. Martin, *Science review: apoptosis in acute lung injury.* Crit Care, 2003. **7**(5): p. 355-8.
170. Chao, M.P., R. Majeti, and I.L. Weissman, *Programmed cell removal: a new obstacle in the road to developing cancer.* Nat Rev Cancer, 2012. **12**(1): p. 58-67.
171. Gal, A., et al., *Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa.* Nat Genet, 2000. **26**(3): p. 270-1.
172. Ravichandran, K.S., *Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways.* Immunity, 2011. **35**(4): p. 445-55.
173. Chekeni, F.B., et al., *Pannexin 1 channels mediate 'find-me' signal release and membrane permeability during apoptosis.* Nature, 2010. **467**(7317): p. 863-7.
174. Fadok, V.A., D.L. Bratton, and P.M. Henson, *Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences.* J Clin Invest, 2001. **108**(7): p. 957-62.

175. Erwig, L.P. and P.M. Henson, *Clearance of apoptotic cells by phagocytes.* Cell Death Differ, 2008. **15**(2): p. 243-50.
176. Franc, N.C., *Phagocytosis of apoptotic cells in mammals, caenorhabditis elegans and Drosophila melanogaster: molecular mechanisms and physiological consequences.* Front Biosci, 2002. **7**: p. d1298-313.
177. Hanayama, R., et al., *Identification of a factor that links apoptotic cells to phagocytes.* Nature, 2002. **417**(6885): p. 182-7.
178. Ogden, C.A., et al., *C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells.* J Exp Med, 2001. **194**(6): p. 781-95.
179. Vandivier, R.W., et al., *Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex.* J Immunol, 2002. **169**(7): p. 3978-86.
180. Schlegel, R.A. and P. Williamson, *Phosphatidylserine, a death knell.* Cell Death Differ, 2001. **8**(6): p. 551-63.
181. Fadok, V.A., et al., *Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages.* J Immunol, 1992. **148**(7): p. 2207-16.
182. Fadok, V.A., et al., *Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts.* J Biol Chem, 2001. **276**(2): p. 1071-7.
183. Williamson, P. and R.A. Schlegel, *Transbilayer phospholipid movement and the clearance of apoptotic cells.* Biochim Biophys Acta, 2002. **1585**(2-3): p. 53-63.
184. Nagata, K., et al., *Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases.* J Biol Chem, 1996. **271**(47): p. 30022-7.
185. Fadok, V.A., D. Xue, and P. Henson, *If phosphatidylserine is the death knell, a new phosphatidylserine-specific receptor is the bellringer.* Cell Death Differ, 2001. **8**(6): p. 582-7.
186. Arur, S., et al., *Annexin I is an endogenous ligand that mediates apoptotic cell engulfment.* Dev Cell, 2003. **4**(4): p. 587-98.
187. Bitto, E., et al., *Mechanism of annexin I-mediated membrane aggregation.* Biochemistry, 2000. **39**(44): p. 13469-77.
188. Hoffmann, P.R., et al., *Phosphatidylserine (PS) induces PS receptor-mediated macropinocytosis and promotes clearance of apoptotic cells.* J Cell Biol, 2001. **155**(4): p. 649-59.
189. Brown, S., et al., *Apoptosis disables CD31-mediated cell detachment from phagocytes promoting binding and engulfment.* Nature, 2002. **418**(6894): p. 200-3.
190. Oldenborg, P.A., et al., *Role of CD47 as a marker of self on red blood cells.* Science, 2000. **288**(5473): p. 2051-4.
191. Brown, E.J. and W.A. Frazier, *Integrin-associated protein (CD47) and its ligands.* Trends Cell Biol, 2001. **11**(3): p. 130-5.
192. Chao, M.P., et al., *Anti-CD47 antibody synergizes with rituximab to promote phagocytosis and eradicate non-Hodgkin lymphoma.* Cell, 2010. **142**(5): p. 699-713.

193. Jiang, P., C.F. Lagenaur, and V. Narayanan, *Integrin-associated protein is a ligand for the P84 neural adhesion molecule.* J Biol Chem, 1999. **274**(2): p. 559-62.
194. Blazar, B.R., et al., *CD47 (integrin-associated protein) engagement of dendritic cell and macrophage counterreceptors is required to prevent the clearance of donor lymphohematopoietic cells.* J Exp Med, 2001. **194**(4): p. 541-9.
195. Reinhold, M.I., et al., *In vivo expression of alternatively spliced forms of integrin-associated protein (CD47).* J Cell Sci, 1995. **108 (Pt 11)**: p. 3419-25.
196. Quinn, M.T., N. Kondratenko, and S. Parthasarathy, *Analysis of the monocyte chemotactic response to lysophosphatidylcholine: role of lysophospholipase C.* Biochim Biophys Acta, 1991. **1082**(3): p. 293-302.
197. Lauber, K., et al., *Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal.* Cell, 2003. **113**(6): p. 717-30.
198. Gregory, C.D. and J.D. Pound, *Cell death in the neighbourhood: direct microenvironmental effects of apoptosis in normal and neoplastic tissues.* J Pathol, 2011. **223**(2): p. 177-94.
199. Elliott, M.R., et al., *Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance.* Nature, 2009. **461**(7261): p. 282-6.
200. Kaplan, G., et al., *Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus.* EMBO J, 1996. **15**(16): p. 4282-96.
201. Ichimura, T., et al., *Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury.* J Biol Chem, 1998. **273**(7): p. 4135-42.
202. McIntire, J.J., et al., *Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family.* Nat Immunol, 2001. **2**(12): p. 1109-16.
203. de Souza, A.J., et al., *T cell Ig and mucin 1 (TIM-1) is expressed on in vivo-activated T cells and provides a costimulatory signal for T cell activation.* Proc Natl Acad Sci U S A, 2005. **102**(47): p. 17113-8.
204. Liberal, R., et al., *The impaired immune regulation of autoimmune hepatitis is linked to a defective galectin-9/tim-3 pathway.* Hepatology, 2012. **56**(2): p. 677-86.
205. Wu, W., et al., *Blockade of Tim-3 signaling restores the virus-specific CD8(+) T-cell response in patients with chronic hepatitis B.* Eur J Immunol, 2012. **42**(5): p. 1180-91.
206. Anderson, A.C., *Tim-3, a negative regulator of anti-tumor immunity.* Curr Opin Immunol, 2012. **24**(2): p. 213-6.
207. Nozaki, Y., et al., *Endogenous Tim-1 (Kim-1) promotes T-cell responses and cell-mediated injury in experimental crescentic glomerulonephritis.* Kidney Int, 2012. **81**(9): p. 844-55.
208. Chiba, S., et al., *Tumor-infiltrating DCs suppress nucleic acid-mediated innate immune responses through interactions between the receptor TIM-3 and the alarmin HMGB1.* Nat Immunol, 2012. **13**(9): p. 832-42.
209. Patel, J., E.N. Bozeman, and P. Selvaraj, *Taming dendritic cells with TIM-3: another immunosuppressive strategy used by tumors.* Immunotherapy, 2012. **4**(12): p. 1795-8.

210. Anderson, A.C., et al., *Promotion of tissue inflammation by the immune receptor Tim-3 expressed on innate immune cells.* Science, 2007. **318**(5853): p. 1141-3.
211. Ding, Q., et al., *Regulatory B cells are identified by expression of TIM-1 and can be induced through TIM-1 ligation to promote tolerance in mice.* J Clin Invest, 2011. **121**(9): p. 3645-56.
212. Ndhlovu, L.C., et al., *Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity.* Blood, 2012. **119**(16): p. 3734-43.
213. Lee, H.H., et al., *Apoptotic cells activate NKT cells through T cell Ig-like mucin-like-1 resulting in airway hyperreactivity.* J Immunol, 2010. **185**(9): p. 5225-35.
214. Monney, L., et al., *Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease.* Nature, 2002. **415**(6871): p. 536-41.
215. Frisancho-Kiss, S., et al., *Cutting edge: T cell Ig mucin-3 reduces inflammatory heart disease by increasing CTLA-4 during innate immunity.* J Immunol, 2006. **176**(11): p. 6411-5.
216. Yang, X., et al., *T cell Ig mucin-3 promotes homeostasis of sepsis by negatively regulating the TLR response.* J Immunol, 2013. **190**(5): p. 2068-79.
217. Zhang, Y., et al., *Tim-3 regulates pro- and anti-inflammatory cytokine expression in human CD14+ monocytes.* J Leukoc Biol, 2012. **91**(2): p. 189-96.
218. Zhang, Y., et al., *Tim-3 negatively regulates IL-12 expression by monocytes in HCV infection.* PLoS One, 2011. **6**(5): p. e19664.
219. Santiago, C., et al., *Structures of T cell immunoglobulin mucin protein 4 show a metal-Ion-dependent ligand binding site where phosphatidylserine binds.* Immunity, 2007. **27**(6): p. 941-51.
220. Guo, L., et al., *Shedding of kidney injury molecule-1 by membrane-type 1 matrix metalloproteinase.* J Biochem, 2012. **152**(5): p. 425-32.
221. Kobayashi, N., et al., *TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells.* Immunity, 2007. **27**(6): p. 927-40.
222. Freeman, G.J., et al., *TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity.* Immunol Rev, 2010. **235**(1): p. 172-89.
223. Ajay, A.K., et al., *A bioinformatics approach identifies signal transducer and activator of transcription-3 and checkpoint kinase 1 as upstream regulators of kidney injury molecule-1 after kidney injury.* J Am Soc Nephrol, 2014. **25**(1): p. 105-18.
224. Cao, W., et al., *Tim-4 inhibition of T-cell activation and T helper type 17 differentiation requires both the immunoglobulin V and mucin domains and occurs via the mitogen-activated protein kinase pathway.* Immunology, 2011. **133**(2): p. 179-89.
225. Yang, L., et al., *Epithelial cell cycle arrest in G2/M mediates kidney fibrosis after injury.* Nat Med, 2010. **16**(5): p. 535-43, 1p following 143.
226. Forbes, J.M., et al., *Ischemic acute renal failure: long-term histology of cell and matrix changes in the rat.* Kidney Int, 2000. **57**(6): p. 2375-85.

227. Han, W.K., et al., *Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury.* Kidney Int, 2002. **62**(1): p. 237-44.
228. Bonventre, J.V., *Dedifferentiation and proliferation of surviving epithelial cells in acute renal failure.* J Am Soc Nephrol, 2003. **14 Suppl 1**: p. S55-61.
229. Ichimura, T., et al., *Kidney injury molecule-1: a tissue and urinary biomarker for nephrotoxicant-induced renal injury.* Am J Physiol Renal Physiol, 2004. **286**(3): p. F552-63.
230. Vaidya, V.S., et al., *Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury.* Am J Physiol Renal Physiol, 2006. **290**(2): p. F517-29.
231. Han, W.K., et al., *Human kidney injury molecule-1 is a tissue and urinary tumor marker of renal cell carcinoma.* J Am Soc Nephrol, 2005. **16**(4): p. 1126-34.
232. Tonomura, Y., et al., *Evaluation of the usefulness of urinary biomarkers for nephrotoxicity in rats.* Toxicology, 2010. **273**(1-3): p. 53-9.
233. Zhou, Y., et al., *Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium.* Toxicol Sci, 2008. **101**(1): p. 159-70.
234. Sinha, V., L.M. Vence, and A.K. Salahudeen, *Urinary tubular protein-based biomarkers in the rodent model of cisplatin nephrotoxicity: a comparative analysis of serum creatinine, renal histology, and urinary KIM-1, NGAL, and NAG in the initiation, maintenance, and recovery phases of acute kidney injury.* J Investig Med, 2013. **61**(3): p. 564-8.
235. Ljungberg, B., et al., *The epidemiology of renal cell carcinoma.* Eur Urol, 2011. **60**(4): p. 615-21.
236. Gupta, K., et al., *Epidemiologic and socioeconomic burden of metastatic renal cell carcinoma (mRCC): a literature review.* Cancer Treat Rev, 2008. **34**(3): p. 193-205.
237. Athar, U. and T.C. Gentile, *Treatment options for metastatic renal cell carcinoma: a review.* Can J Urol, 2008. **15**(2): p. 3954-66.
238. Zhang, P.L., et al., *Urine kidney injury molecule-1: a potential non-invasive biomarker for patients with renal cell carcinoma.* Int Urol Nephrol, 2014. **46**(2): p. 379-88.
239. Acquavella, N. and T. Fojo, *Renal cell carcinoma: trying but failing to improve the only curative therapy.* J Immunother, 2013. **36**(9): p. 459-61.
240. Escudier, B., et al., *Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial.* Lancet, 2007. **370**(9605): p. 2103-11.
241. Escudier, B., et al., *Sorafenib in advanced clear-cell renal-cell carcinoma.* N Engl J Med, 2007. **356**(2): p. 125-34.
242. Motzer, R.J., et al., *Sunitinib versus interferon alfa in metastatic renal-cell carcinoma.* N Engl J Med, 2007. **356**(2): p. 115-24.
243. Sternberg, C.N., et al., *Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial.* J Clin Oncol, 2010. **28**(6): p. 1061-8.
244. Hudes, G., et al., *Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma.* N Engl J Med, 2007. **356**(22): p. 2271-81.

245. Motzer, R.J., et al., *Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial.* Lancet, 2008. **372**(9637): p. 449-56.
246. Rini, B.I., et al., *Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial.* Lancet, 2011. **378**(9807): p. 1931-9.
247. Mourad, W.F., J. Dutcher, and R.D. Ennis, *State-of-the-Art Management of Renal Cell Carcinoma.* Am J Clin Oncol, 2012.
248. Huang, W.C., et al., *Chronic kidney disease after nephrectomy in patients with renal cortical tumours: a retrospective cohort study.* Lancet Oncol, 2006. **7**(9): p. 735-40.
249. Focan, C., *Renal cell carcinoma (RCC). Place of chemotherapy either alone or in combination regimens.* Acta Urol Belg, 1996. **64**(2): p. 9-10.
250. Cuadros, T., et al., *Hepatitis A virus cellular receptor 1/kidney injury molecule-1 is a susceptibility gene for clear cell renal cell carcinoma and hepatitis A virus cellular receptor/kidney injury molecule-1 ectodomain shedding a predictive biomarker of tumour progression.* Eur J Cancer, 2013. **49**(8): p. 2034-47.
251. Lin, F., et al., *Human kidney injury molecule-1 (hKIM-1): a useful immunohistochemical marker for diagnosing renal cell carcinoma and ovarian clear cell carcinoma.* Am J Surg Pathol, 2007. **31**(3): p. 371-81.
252. Vila, M.R., et al., *Hepatitis A virus receptor blocks cell differentiation and is overexpressed in clear cell renal cell carcinoma.* Kidney Int, 2004. **65**(5): p. 1761-73.
253. Vermeulen, R., et al., *Elevated urinary levels of kidney injury molecule-1 among Chinese factory workers exposed to trichloroethylene.* Carcinogenesis, 2012. **33**(8): p. 1538-41.
254. Ahmad, S.T., et al., *Preclinical renal cancer chemopreventive efficacy of geraniol by modulation of multiple molecular pathways.* Toxicology, 2011. **290**(1): p. 69-81.
255. Shalabi, A., et al., *Urinary NGAL and KIM-1: potential association with histopathologic features in patients with renal cell carcinoma.* World J Urol, 2013. **31**(6): p. 1541-5.
256. Morrissey, J.J., et al., *Sensitivity and specificity of urinary neutrophil gelatinase-associated lipocalin and kidney injury molecule-1 for the diagnosis of renal cell carcinoma.* Am J Nephrol, 2011. **34**(5): p. 391-8.
257. Cuadros, T., et al., *HAVCR/KIM-1 Activates the IL-6/STAT-3 Pathway in Clear Cell Renal Cell Carcinoma and Determines Tumor Progression and Patient Outcome.* Cancer Res, 2014.
258. Chow, W.H., et al., *Rising incidence of renal cell cancer in the United States.* JAMA, 1999. **281**(17): p. 1628-31.
259. Cuadros, T., et al., *Hepatitis A virus cellular receptor 1/kidney injury molecule-1 is a susceptibility gene for clear cell renal cell carcinoma and hepatitis A virus cellular receptor/kidney injury molecule-1 ectodomain shedding a predictive biomarker of tumour progression.* Eur J Cancer, 2013.

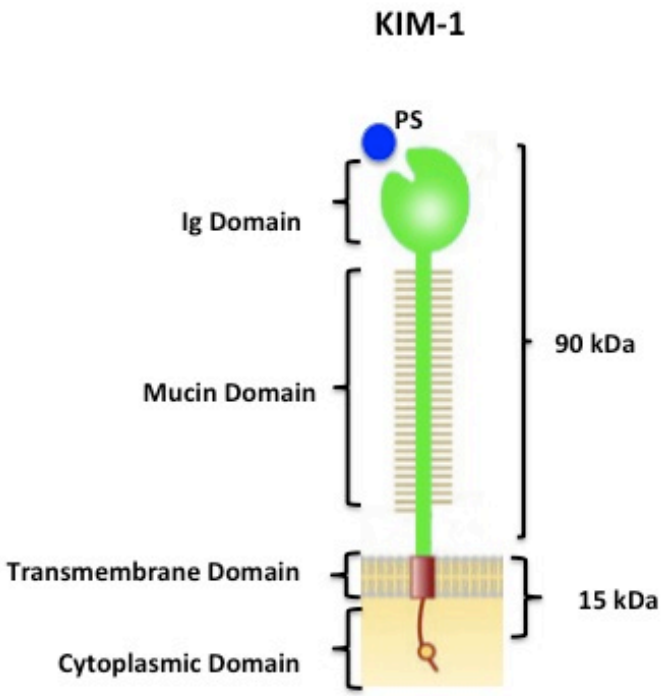
260. Bailly, V., et al., *Shedding of kidney injury molecule-1, a putative adhesion protein involved in renal regeneration.* J Biol Chem, 2002. **277**(42): p. 39739-48.
261. Chomczynski, P. and N. Sacchi, *Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.* Anal Biochem, 1987. **162**(1): p. 156-9.
262. Mori, R., et al., *Both beta-actin and GAPDH are useful reference genes for normalization of quantitative RT-PCR in human FFPE tissue samples of prostate cancer.* Prostate, 2008. **68**(14): p. 1555-60.
263. Dong, Y.C., et al., *[Expression and clinical significance of kidney injury molecule-1 in renal epithelial neoplasms].* Zhonghua Bing Li Xue Za Zhi, 2010. **39**(1): p. 35-9.
264. Ichimura, T. and M. Shan, *Kidney Injury Molecule-1 in acute kidney injury and renal repair- a review.* 2008.
265. Zhang, Z., B.D. Humphreys, and J.V. Bonventre, *Shedding of the urinary biomarker kidney injury molecule-1 (KIM-1) is regulated by MAP kinases and juxtamembrane region.* J Am Soc Nephrol, 2007. **18**(10): p. 2704-14.
266. Yao, J., et al., *Ultraviolet (UV) and Hydrogen Peroxide Activate Ceramide-ER Stress-AMPK Signaling Axis to Promote Retinal Pigment Epithelium (RPE) Cell Apoptosis.* Int J Mol Sci, 2013. **14**(5): p. 10355-68.
267. Song, A.S., A. Najjar, and K.R. Diller, *Thermally Induced Apoptosis, Necrosis, and Heat Shock Protein Expression in 3D Culture.* J Biomech Eng, 2014.
268. Chen, S., et al., *Detection of apoptosis induced by new type gosling viral enteritis virus in vitro through fluorescein annexin V-FITC/PI double labeling.* World J Gastroenterol, 2008. **14**(14): p. 2174-8.
269. Dumitriu, I.E., et al., *HMGB1: guiding immunity from within.* Trends Immunol, 2005. **26**(7): p. 381-7.
270. Caricchio, R., L. D'Adamio, and P.L. Cohen, *Fas, ceramide and serum withdrawal induce apoptosis via a common pathway in a type II Jurkat cell line.* Cell Death Differ, 2002. **9**(5): p. 574-80.
271. Diener, K.R., et al., *The multifunctional alarmin HMGB1 with roles in the pathophysiology of sepsis and cancer.* Immunol Cell Biol, 2013. **91**(7): p. 443-50.
272. Scaffidi, P., T. Misteli, and M.E. Bianchi, *Release of chromatin protein HMGB1 by necrotic cells triggers inflammation.* Nature, 2002. **418**(6894): p. 191-5.
273. Nozaki, Y., et al., *Estimation of kidney injury molecule-1 (Kim-1) in patients with lupus nephritis.* Lupus, 2014.
274. Charlton, J.R., D. Portilla, and M.D. Okusa, *A basic science view of acute kidney injury biomarkers.* Nephrol Dial Transplant, 2014.
275. Coca, S.G., et al., *Urinary Biomarkers of AKI and Mortality 3 Years after Cardiac Surgery.* J Am Soc Nephrol, 2013.
276. Damman, K., et al., *Tubular damage and worsening renal function in chronic heart failure.* JACC Heart Fail, 2013. **1**(5): p. 417-24.
277. Wong, S.H., et al., *Tim-1 is induced on germinal centre B cells through B-cell receptor signalling but is not essential for the germinal centre response.* Immunology, 2010. **131**(1): p. 77-88.

278. Xiao, S., et al., *Defect in regulatory B-cell function and development of systemic autoimmunity in T-cell Ig mucin 1 (Tim-1) mucin domain-mutant mice.* Proc Natl Acad Sci U S A, 2012. **109**(30): p. 12105-10.
279. Lan, Y.F., et al., *MicroRNA-494 reduces ATF3 expression and promotes AKI.* J Am Soc Nephrol, 2012. **23**(12): p. 2012-23.
280. Cuadros, T., et al., *HAVCR/KIM-1 Activates the IL-6/STAT-3 Pathway in Clear Cell Renal Cell Carcinoma and Determines Tumor Progression and Patient Outcome.* Cancer Res, 2014. **74**(5): p. 1416-28.
281. Lu, K.H., et al., *Selection of potential markers for epithelial ovarian cancer with gene expression arrays and recursive descent partition analysis.* Clin Cancer Res, 2004. **10**(10): p. 3291-300.
282. Hendrix, N.D., et al., *Fibroblast growth factor 9 has oncogenic activity and is a downstream target of Wnt signaling in ovarian endometrioid adenocarcinomas.* Cancer Res, 2006. **66**(3): p. 1354-62.
283. Conlan, J.W. and R.J. North, *Early pathogenesis of infection in the liver with the facultative intracellular bacteria Listeria monocytogenes, Francisella tularensis, and Salmonella typhimurium involves lysis of infected hepatocytes by leukocytes.* Infect Immun, 1992. **60**(12): p. 5164-71.
284. Elliott, M.R., et al., *Unexpected requirement for ELMO1 in clearance of apoptotic germ cells in vivo.* Nature, 2010. **467**(7313): p. 333-7.
285. Wakefield, J.S. and R.M. Hicks, *Erythrophagocytosis by the epithelial cells of the bladder.* J Cell Sci, 1974. **15**(3): p. 555-73.
286. Parnaik, R., M.C. Raff, and J. Scholes, *Differences between the clearance of apoptotic cells by professional and non-professional phagocytes.* Curr Biol, 2000. **10**(14): p. 857-60.
287. Huang, J., et al., *miR-199a-3p inhibits hepatocyte growth factor/c-Met signaling in renal cancer carcinoma.* Tumour Biol, 2014.
288. Othman, E.M., et al., *Insulin-mediated oxidative stress and DNA damage in LLC-PK1 pig kidney cell line, female rat primary kidney cells, and male ZDF rat kidneys in vivo.* Endocrinology, 2013. **154**(4): p. 1434-43.
289. Morizono, K. and I.S. Chen, *The role of phosphatidylserine receptors in enveloped virus infection.* J Virol, 2014.
290. Ichimura, T., C.R. Brooks, and J.V. Bonventre, *Kim-1/Tim-1 and immune cells: shifting sands.* Kidney Int, 2012. **81**(9): p. 809-11.
291. Lohmann, C., et al., *Induction of tumor cell apoptosis or necrosis by conditional expression of cell death proteins: analysis of cell death pathways and in vitro immune stimulatory potential.* J Immunol, 2009. **182**(8): p. 4538-46.
292. Xiao, J., et al., *The Association of HMGB1 Gene with the Prognosis of HCC.* PLoS One, 2014. **9**(2): p. e89097.
293. Liu, F., et al., *High expression of high mobility group box 1 (hmgb1) predicts poor prognosis for hepatocellular carcinoma after curative hepatectomy.* J Transl Med, 2012. **10**: p. 135.
294. Jiao, Y., H.C. Wang, and S.J. Fan, *Growth suppression and radiosensitivity increase by HMGB1 in breast cancer.* Acta Pharmacol Sin, 2007. **28**(12): p. 1957-67.

295. Giavara, S., et al., *Yeast Nhp6A/B and mammalian Hmgb1 facilitate the maintenance of genome stability.* Curr Biol, 2005. **15**(1): p. 68-72.
296. Polanska, E., et al., *HMGB1 gene knockout in mouse embryonic fibroblasts results in reduced telomerase activity and telomere dysfunction.* Chromosoma, 2012. **121**(4): p. 419-31.
297. Kang, R., et al., *The HMGB1/RAGE inflammatory pathway promotes pancreatic tumor growth by regulating mitochondrial bioenergetics.* Oncogene, 2014. **33**(5): p. 567-77.
298. Tang, D., et al., *High-mobility group box 1, oxidative stress, and disease.* Antioxid Redox Signal, 2011. **14**(7): p. 1315-35.
299. Gebhardt, C., et al., *RAGE signaling sustains inflammation and promotes tumor development.* J Exp Med, 2008. **205**(2): p. 275-85.
300. Heijmans, J., et al., *Rage signalling promotes intestinal tumourigenesis.* Oncogene, 2013. **32**(9): p. 1202-6.
301. Liu, Z., L.D. Falo, Jr., and Z. You, *Knockdown of HMGB1 in tumor cells attenuates their ability to induce regulatory T cells and uncovers naturally acquired CD8 T cell-dependent antitumor immunity.* J Immunol, 2011. **187**(1): p. 118-25.
302. Kuniyasu, H., et al., *Expression of receptors for advanced glycation end-products (RAGE) is closely associated with the invasive and metastatic activity of gastric cancer.* J Pathol, 2002. **196**(2): p. 163-70.
303. van Beijnum, J.R., et al., *Tumor angiogenesis is enforced by autocrine regulation of high-mobility group box 1.* Oncogene, 2013. **32**(3): p. 363-74.
304. Andersson, U. and K.J. Tracey, *HMGB1 in sepsis.* Scand J Infect Dis, 2003. **35**(9): p. 577-84.
305. Wang, H., et al., *HMG-1 as a late mediator of endotoxin lethality in mice.* Science, 1999. **285**(5425): p. 248-51.
306. Klune, J.R., et al., *HMGB1: endogenous danger signaling.* Mol Med, 2008. **14**(7-8): p. 476-84.
307. Tesniere, A., et al., *Immunogenic death of colon cancer cells treated with oxaliplatin.* Oncogene, 2010. **29**(4): p. 482-91.
308. Miyanishi, M., et al., *Identification of Tim4 as a phosphatidylserine receptor.* Nature, 2007. **450**(7168): p. 435-9.
309. Baghdadi, M., et al., *TIM-4 glycoprotein-mediated degradation of dying tumor cells by autophagy leads to reduced antigen presentation and increased immune tolerance.* Immunity, 2013. **39**(6): p. 1070-81.
310. Cardinal, J., et al., *Cisplatin prevents high mobility group box 1 release and is protective in a murine model of hepatic ischemia/reperfusion injury.* Hepatology, 2009. **50**(2): p. 565-74.
311. Gauley, J. and D.S. Pisetsky, *The translocation of HMGB1 during cell activation and cell death.* Autoimmunity, 2009. **42**(4): p. 299-301.
312. Lee, J.E., et al., *Induction of apoptosis with diallyl disulfide in AGS gastric cancer cell line.* J Korean Surg Soc, 2011. **81**(2): p. 85-95.

Appendices

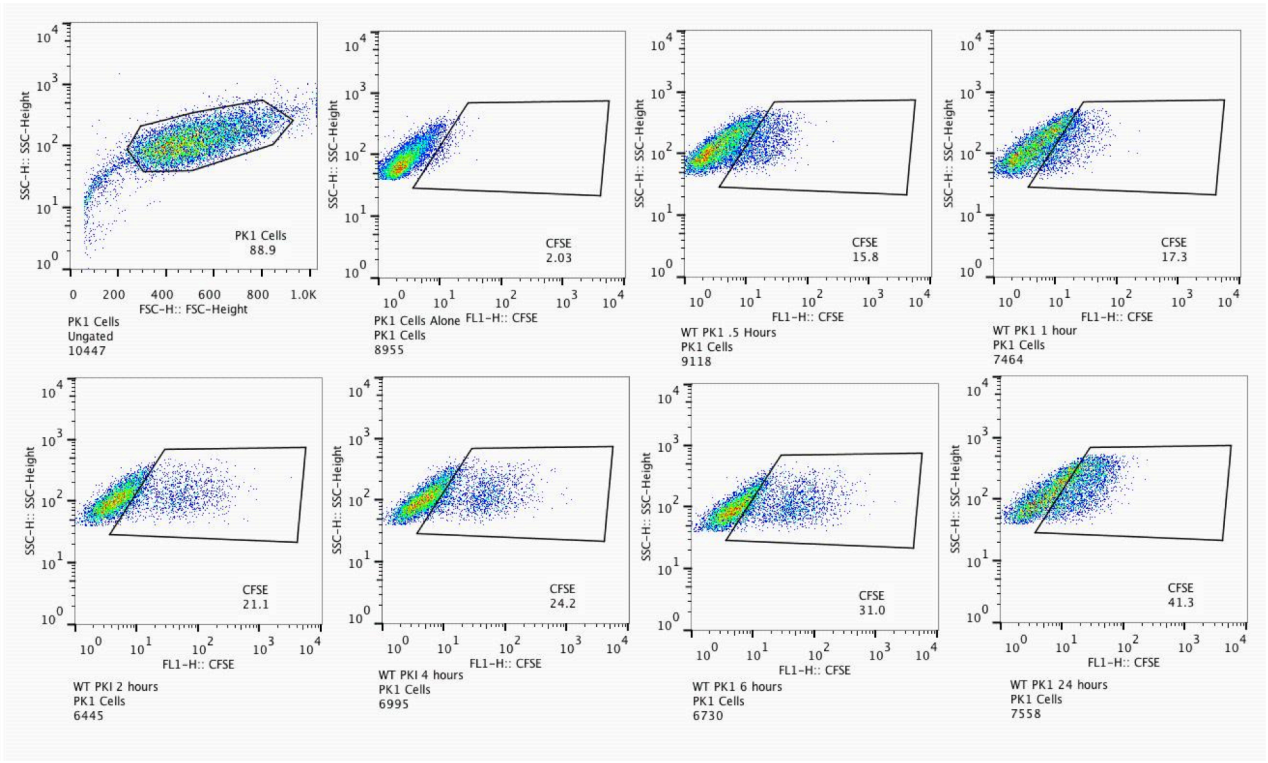
Appendix I: *Structure of hKIM-1.* Animation of human KIM-1 including the PS binding domain located within the Ig head. Structural features include an O-linked glycosylated mucin domain as well as a transmembrane and cytoplasmic domain. The blue arrow represents the potential cleavage site for soluble KIM-1.



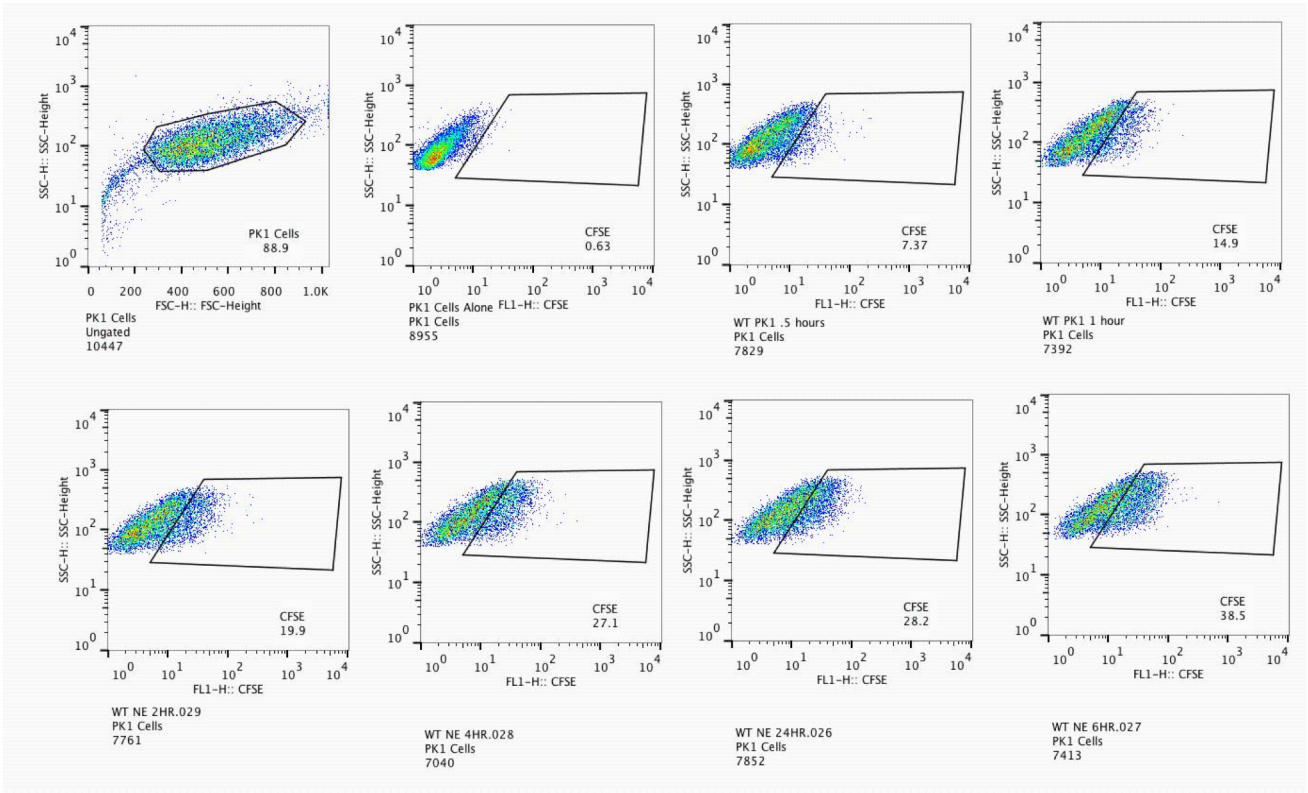
Adapted from Freeman et al., 2010[222]

Appendix II: *Gating strategy for WT-PK1 and pcDNA-PK1 cells.* WT-PK1 and pcDNA-PK1 cells were fed CFSE stained apoptotic or necrotic thymocytes and allowed to phagocytose for .5 hours, 1 hour, 2hours, 4 hours, 6 hours and 24 hours. 10,000 events were collected and below represent the applied gating strategy for (A) WT-PK1 cells fed apoptotic thymocytes, (B) WT-PK1 cells fed necrotic thymocytes (C) pcDNA-PK1 cells fed apoptotic thymocytes and (D) pcDNA-PK1 cells fed necrotic thymocytes.

(A)



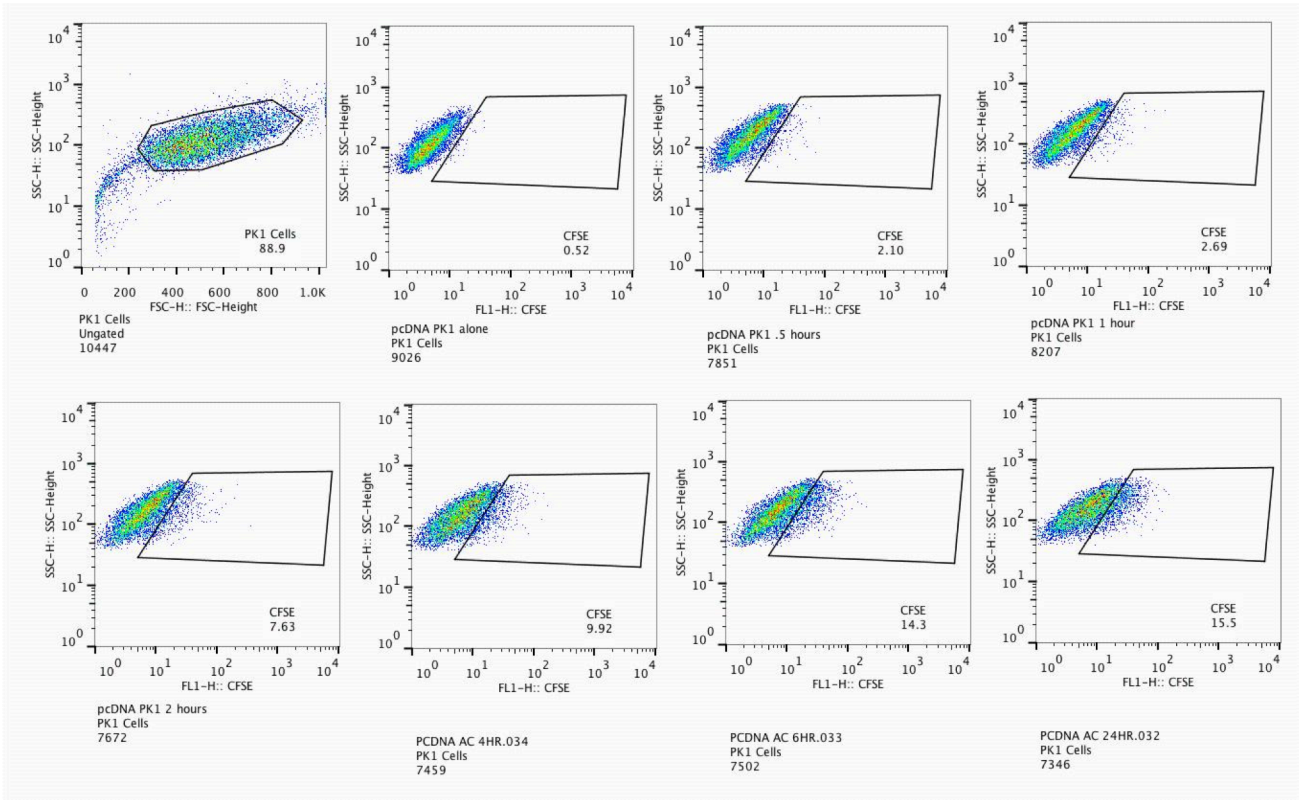
(B)



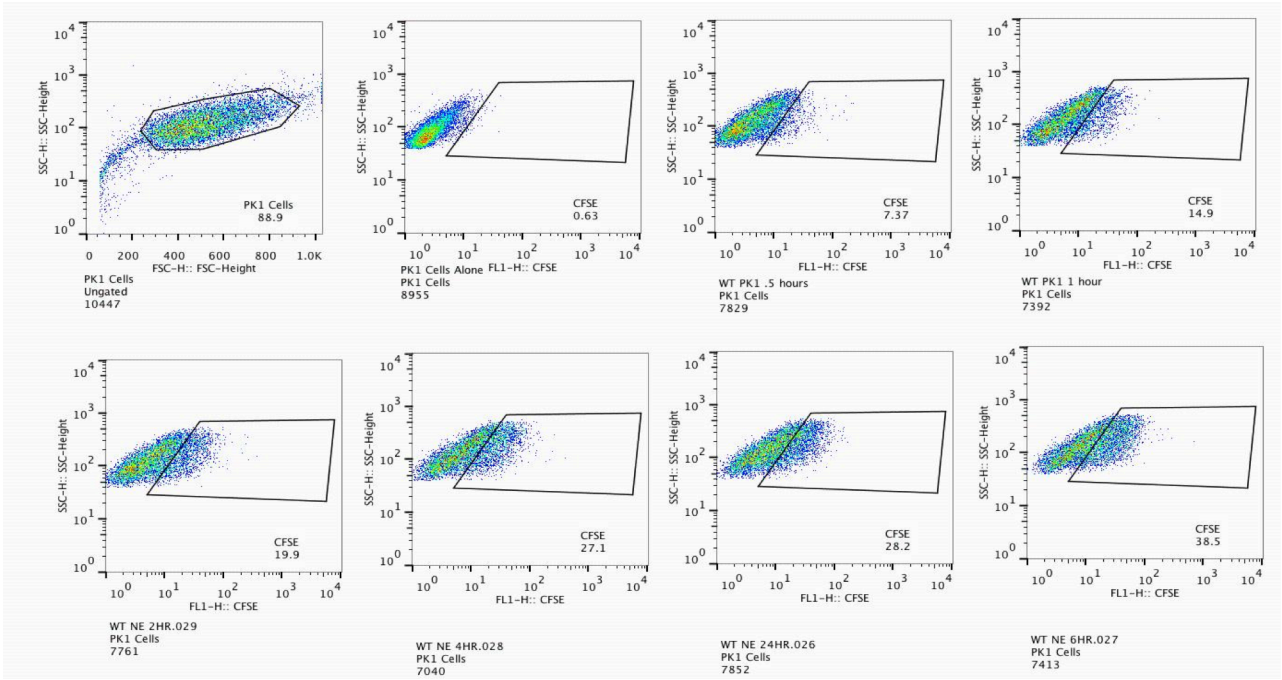
Side Scatter

CFSE

(C)



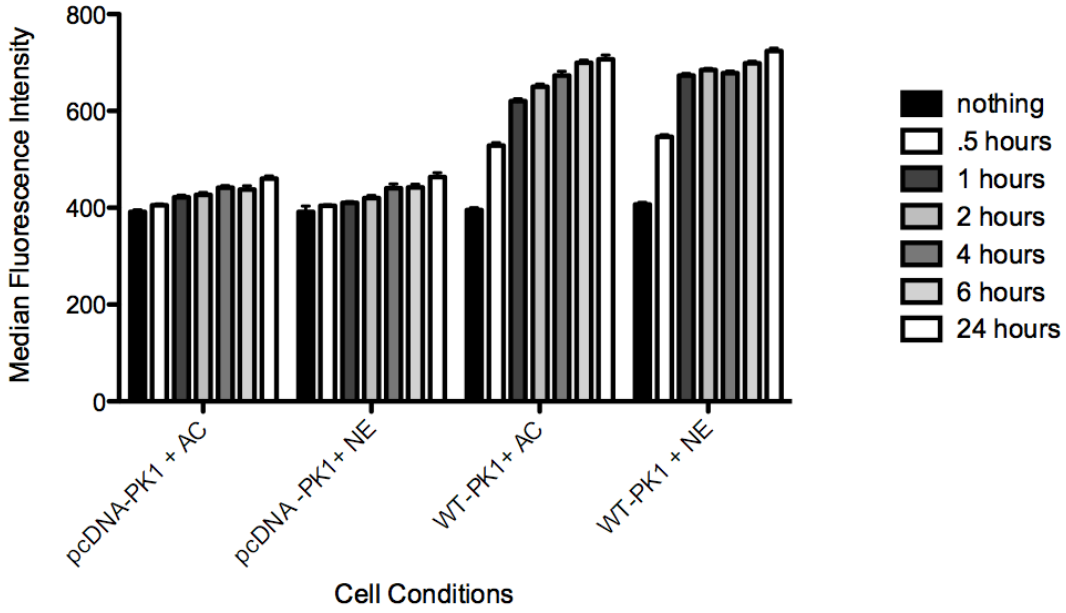
(D)



Side Scatter

CFSE

Appendix III: *MFI for WT-PK1 vs. pcDNA-PK1 phagocytosis of apoptotic and necrotic cells.* Representative Median Fluorescence Intensity (MFI) of the phagocytosis of CFSE labeled apoptotic and necrotic thymocytes by WT-PK1 and pcDNA-PK1 tubular cells via flow cytometry. Error bars calculated based on three replicates and calculations done by Flow Jo software


800
600
400
200
0
Median Fluorescence Intensity
pcDNA-PK1 + AC
pcDNA -PK1+ NE
WT-PK1+ AC
WT-PK1 + NE
Cell Conditions
nothing
.5 hours
1 hours
2 hours
4 hours
6 hours
24 hours

Curriculum Vitae

Name:	Sahra Nathoo
Place of Birth:	Toronto, Canada
Post-secondary Education and Degrees:	The University of Western Ontario London, Ontario, Canada M.Sc. Candidate Microbiology and Immunology 2011-2013
	McMaster University Hamilton, Ontario, Canada Honors Life Science, minor in Business 2007-2011 B.Sc.
Honours and Awards:	Lawson Studentship Award 2012-2013
	Western Graduate Research Scholarship 2011-2013
Related Work Experience:	Advanced Mouse Training Animal Care and Veterinary Services London, Ontario, Canada