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Elucidation of the Signaling Pathway of MERTK

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

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Abstract

Mer tyrosine kinase (MERTK) is a receptor tyrosine kinase which mediates efferocytosis-the recognition and uptake of apoptotic cells. MERTK serves as the predominant efferocytic receptor in several tissues, including in the heart, with single nucleotide polymorphisms associated with up to a 75% increased risk of atherosclerosis in humans. MERTK facilitates the internalization of apoptotic cells via a MERTK/integrin pathway that involves, in part, PI-3-kinase signaling and signaling via Src-family kinases. While these elements of the MERTK signaling pathway have been identified, much of MERTK's signaling mechanisms remain to be elucidated. Pharmacological inhibitors were used to block signaling through canonical phagocytic signaling pathways, and through signalling molecules identified as part of the MERTK signalosome by mass spectrometry, and the efferocytosis of MERTK-specific targets assessed using a human macrophage cell line. These experiments identified PI3K, Src, Syk, ERK, and ILK as regulators of MERTK efferocytic function. To identify non-canonical pathways, we began the development of a CRISPR-tagged endogenous MERTK allele which can be selectively activated in a human macrophage cell line without inadvertent activation of other phagocytic receptors; using this cell line and phosphoprotein mass spectrometry, future studies will be able to identify non-canonical tyrosine-kinase dependent pathways mediating MERTK efferocytic function. Identification of these pathways will elucidate the signaling pathway utilized by MERTK, and potentially may identify signaling pathways which drive aberrant MERTK function during diseases such as atherosclerosis.

Keywords

Cell Signalling, Cross-link, CRISPR, Cas9, Efferocytosis, Fluorescence Microscopy, Growth arrest-specific 6, Macrophage, MER tyrosine kinase (MERTK), Phagocyte, Phagocytosis, Immunoprecipitation.

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Table of Contents

Abstract	i
Acknowledgments.....	ii
Table of Figures	v
Table of Abbreviations.....	vi
Chapter 1	1
1 Introduction.....	1
1.1 Apoptosis and Efferocytosis	1
1.2 TAM Receptors	2
1.3 MERTK Signalling and Efferocytosis.....	6
1.4 Clinical Relevance of MERTK.....	8
Chapter 2	11
2 Materials and Methods.....	11
2.1 Materials	11
2.2 THP-1 Culture and Differentiation.....	12
2.3 HA-MERTK-Cos7 Culture.....	12
2.4 Efferocytosis Assays.....	12
2.5 Immunoblot	13
2.6 Immunoprecipitation	14
2.7 CRISPR-Cas9	14
2.8 Statistical Analysis	15
Chapter 3	16
3 Results.....	16
3.1 Canonical Phagocytic Pathways	16
3.2 MERTK Signaling Molecule Identification	21
3.3 Moving Towards a Better MERTK Experimental System.....	26
Chapter 4	30
4 Discussion	30

4.1	Role of Canonical Phagocytic/Efferocytic Pathways in MERTK-Mediated Efferocytosis	31
4.2	Clinical Implications of Deficiencies in MERTK-Mediated Efferocytosis	32
4.3	Limitations of the MS/MS Model	33
4.4	Moving Towards an Endogenous MERTK Expression System	34
	References	35
	Curriculum Vitae	41

Table of Figures

Figure 1. MERTK Domains and Clustering	5
Figure 2. Identification of Elements of the MERTK Signalosome using ReCLIP.....	8
Figure 3. Role of Src and Syk Kinases in MERTK-Mediated Efferocytosis	17
Figure 4. Role of PI3-kinases in Fc-Mediated Phagocytosis	18
Figure 5. Role of PI3-kinases in MERTK-Mediated Efferocytosis.....	19
Figure 6. Role of Erk and ILK in MERTK-Mediated Efferocytosis	20
Figure 7. Structure of the HA-MERTK construct showing the location of the inserted HA-tag .	21
Figure 8. Expression of Stably Integrated HA-MERTK on the cell surface of Cos7 cells	22
Figure 9. Schematic of the method for identifying MERTK-activated signaling molecules	23
Figure 10. Detection of phosphotyrosine signaling following MERTK cross-linking.....	24
Figure 11. Loss of proteins during 4G10 immunoprecipitation	25
Figure 12. CRISPR-Cas9 mediated integration of an HA-tag at the THP-1 MERTK locus.....	27
Figure 13. Flow cytometry sorting of CRISPR-edited THP-1 cells	28

Table of Abbreviations

Akt	Protein kinase B
AXL	AXL receptor tyrosine kinase
BAI	Brain-Specific Angiogenesis Inhibitor
BSA	Bovine Serum Albumin
Cas9	CRISPR associated protein 9
CD14	Cluster of differentiation 14
CD36	Cluster of differentiation 36
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
cRNA	Complimentary RNA
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
GAP	GTPase-activating proteins
GAS6	Growth arrest-specific 6
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte monocyte colony-stimulating factor
HA	Hemagglutinin
IBD	Inflammatory bowel disease
IFNAR	Type I interferon receptor
Ig	Immunoglobulin
ILK	Integrin-linked kinase

IL-1 β	Interleukin 1-beta
IL-4	Interleukin 4
IL-10	Interleukin-10
INF γ	Interferon gamma
IRF5	Interferon regulatory factor 5
JAK	Janus kinase
kDA	Kilodalton
LDLR	Low-density lipoprotein receptor
LPA	Lysophosphatidic acid
MERTK	Mer tyrosine kinase
MFGE8	Milk fat globule-EGF factor 8 protein
NaF	Sodium fluoride
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
oxLDL	Oxidized low-density lipoprotein
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-3 kinase
PIP ₂	Phosphatidylinositol-4,5-bisphosphate
PIP ₃	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PMA	Phorbol 12-Myristate 13-Acetate
PMSF	Phenylmethylsulfonyl fluoride
PPAR γ	Peroxisome proliferator-activated receptor gamma
PtdSer	Phosphatidylserine
RA	Rheumatoid arthritis
Rac1	Ras-related C3 botulinum toxin substrate 1

Rap1	Ras-related protein 1
ReCLIP	Reversible cross-link immunoprecipitation
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation assay
RPE	Retinal pigment epithelium
RPMI	Rosewell park memorial institute
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
SOCS	Suppressor of cytokine signaling
Syk	Spleen Tyrosine kinase
TAM	Tyro 3 Axl Mer
TGF β	Transforming growth factor beta
TIM	T cell/transmembrane, immunoglobulin, and mucin
TKD	Tyrosine Kinase Domain
TNF α	Tumor necrosis factor alpha
tracrRNA	Trans-activating crRNA
TYRO3	Tyrosine-protein kinase TYRO3

Chapter 1

1 Introduction

1.1 Apoptosis and Efferocytosis

Apoptosis, or programmed cell death, is a highly regulated physiological process that is essential for the development and maintenance of homeostasis in multicellular organisms¹. It is a prerequisite for the removal of infected or damaged cells from host tissues. Tens of billions of cells undergo apoptosis on a daily basis within the human body, a phenomenon that is usually counterbalanced by efferocytosis - the phagocytic clearance of apoptotic cells¹. Together, apoptosis and efferocytosis facilitate the removal of apoptotic cell debris from healthy individuals in order to maintain homeostasis¹. If efferocytosis is impaired, however, uncleared apoptotic cells accumulate, lyse through secondary necrosis, and release their intracellular contents into the extracellular milieu. These newly-released intracellular molecules are proinflammatory and can induce pathological conditions such as autoimmunity and chronic inflammatory disease¹. Apoptotic cells are recognized by efferocytic cells via “eat-me” signals such as phosphatidylserine (PtdSer) exposed on the surface of apoptotic cells. PtdSer is the primary eat-me signal for efferocytosis. Normally, PtdSer is confined to the inner portion of the plasma membrane, but in cell undergoing apoptosis, it accumulates on the outer leaflet of the membrane because of inactivation of flippase proteins which are responsible for maintaining membrane lipid asymmetry, coupled with activation of scramblases that disrupt asymmetry². Exposed PtdSer is recognized by an array of efferocytic receptors expressed by both professional phagocytes such as macrophages and dendritic cells, and by non-professional phagocytes such as epithelial cells which then proceed to engulf and degrade the apoptotic cell³⁻⁶.

Efferocytosis is an inherently anti-inflammatory process that is associated with the release of anti-inflammatory and pro-resolving cytokines such as interleukin-10 (IL-10) and transforming growth factor beta (TGF β)⁷. These factors not only inhibit the inflammatory response, but also promote increased tissue regeneration and wound healing⁸. Macrophages exhibit an inherent

phenotypic plasticity in response to the specific types of pro- or anti-inflammatory cytokines and chemokines present in the extracellular milieu. Stimulation by cytokines such as granulocyte-monocyte colony-stimulating factor (GM-CSF), and interferon gamma (IFN γ), or activation of toll-like receptor (TLR) and ligands such as lipopolysaccharide (LPS), results in the polarization of macrophages into a pro-inflammatory M1 phenotype⁹. M1 macrophages are primarily characterized by upregulated pro-inflammatory cytokine production, reactive oxygen species (ROS) production, and phagocytic activity⁹, but are impaired in their ability to mediate efferocytosis due to decreased peroxisome proliferator-activated receptor gamma (PPAR γ) activity¹⁰. PPAR γ acts as a transcription factor that interferes with inflammatory signaling pathways such as the NF- κ B pathway, which upregulates the activity of known pro-inflammatory cytokines TNF α and IL-1 β ¹⁰. In addition to repression of PPAR γ signaling, M1 macrophages increase expression of IRF5, a transcription factor known to inhibit expression of CD36 and CD14 efferocytic receptors while simultaneously inducing expression of pro-inflammatory cytokines¹¹. Alternatively, macrophages can also be polarized towards the anti-inflammatory M2 phenotype by exposure to increased levels of anti-inflammatory cytokines such as IL-4, IL-3, IL-10, TGF β , macrophage colony-stimulating factor (M-CSF), and by hormones such as glucocorticoids⁹. M2 macrophages have been observed to exhibit enhanced anti-inflammatory and efferocytic activity coupled with downregulated production of pro-inflammatory cytokines and reactive oxygen species (ROS)⁹. These are mediated, in part, by an increase in PPAR γ expression and activity facilitated by upregulated IL-4 signaling to this receptor¹². Direct recognition of apoptotic cells by phagocytes also appears to induce an increase in PPAR γ activation which, in turn, is associated with M2 polarization and subsequent increases in the expression of certain efferocytic receptors^{13,14}.

1.2 TAM Receptors

One family of efferocytic receptors is the TAM (Tyro3, Axl, and Mertk) family of receptor tyrosine kinases¹⁵. These receptors are known to play key roles in the maintenance of homeostasis in fully-differentiated adult tissue¹⁵. Studies involving TAM-knockout mice that are either lacking these receptors entirely or possess receptors with defective TKDs have shown that

loss-of-function mutants, while initially viable and fertile, go on to develop debilitating phenotypes later in life that are associated with inefficient clearance of apoptotic cells^{16–19}. In murine models, loss of the TAM-facilitated efferocytosis of apoptotic germ cells by Sertoli cells during spermatogenesis results in the eventual death of all sperm progenitor cells, eventually rendering the afflicted mouse infertile¹⁶. In the retina, retinal pigment epithelium (RPE) cells must intermittently remove the outer, photo-damaged ends of photoreceptor cells through MERTK or TYRO-3 mediated phagocytosis in order prevent the progression of blindness brought about by photoreceptor death¹⁸. Disruptions in TAM signaling have been linked to the onset of broad-spectrum autoimmune disease in mouse models¹⁷. This disease is characterized by the clinical symptoms of both rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) and develops as a result of an unabated inflammatory response caused by high levels of uncleared apoptotic cell debris¹⁷. A prolonged inflammatory response caused by deficiencies in TAM-mediated apoptotic cell clearance has also been reported to play an important role in the pathology of inflammatory bowel disease (IBD)¹⁹. In nearly all of these aforementioned cases, mutations in MERTK are associated with more severe symptoms than mutations in TYRO3 or AXL, highlighting the importance of this receptor in the maintenance of homeostasis through efferocytosis¹⁷. Indeed, MERTK deficiency alone is sufficient to cause blindness due to a failure in RPE efferocytosis, and MERTK deficiency leads to autoimmunity, albeit, with later onset than in triple-TAM knockout animals¹⁸.

Single nucleotide polymorphisms MERTK are similarly linked to human inflammatory and autoimmune diseases. Indeed, SNPs in MERTK have strong associations with atherosclerosis, multiple sclerosis²⁰, lupus²¹ and rheumatoid arthritis²², while human MERTK deficiency is one cause of inherited retinitis pigmentosa²³. The TAM receptors do not recognize apoptotic cells directly, and instead bind to apoptotic cells via soluble opsonins such as GAS6. As expected, mutations in these opsonins are also associated with disease^{24–27}. It is important to note that not all associations between TAMs and disease are due to defects in efferocytosis. For example, TAM receptor and Gas6 overexpression has been linked with tumor metastasis^{28,29}. This association is unlikely to be due to enhanced efferocytosis, and instead is thought to stem from activation of various cell proliferation, survival, and motility signaling pathways via TAM-dependent activation of Protein kinase B (Akt) and extracellular signal-regulated kinase (ERK)

^{28,29}.

Despite the importance of MERTK and other TAM receptors in many clinical conditions, relatively little is known about their signaling pathways. Structurally, all TAM receptors are composed of an intracellular tyrosine kinase domain (TKD), a transmembrane domain, and an extracellular domain composed of tandem fibronectin type III repeat domains and tandem immunoglobulin-related (Ig) domains¹⁵ (Fig 1A). MERTK is about 150 kDA-170 kDA in size and is composed of 999 amino acids, making it significantly larger than the other two TAM receptors Axl and Tyro3, which are both around 120 kDA and 890 and 894 amino acids in size respectively¹⁵. Unlike other efferocytic receptors such as those of the TIM³⁻⁴, BAI⁵, and Stabilin⁶ families, TAM receptors require Gas6 or Protein S (ProS) to act as “bridging molecules” (opsonins) which recognize exposed phosphatidylserine (PtdSer) on the surface of apoptotic cells^{15,30}. Both Gas6 and ProS are 80kDA in size and are paralogs³¹ with 44% amino acid similarity¹⁵. The TAM Ig domains bind to the carboxy-terminal ends of these opsonins, which are secreted into the circulation by cells such as vascular endothelial cells and hepatocytes³². ProS is present at a concentration of around 300 nM³⁰ in human serum while Gas6 is present at about 0.2 nM, with much of the Gas6 bound to a soluble Axl ectodomain^{33,34}. Binding of these opsonins results in the dimerization of the Ig domains and the subsequent activation of the TAM kinase domain¹⁵. Gas6 has been observed to activate all three TAM receptors, while ProS is only known to activate Axl and MERTK. While Axl has been shown to have the highest affinity for Gas6, MERTK is more readily activated when exposed to Gas6 in the presence of PtdSer, indicating that it has an important role in apoptotic cell clearance¹⁵.

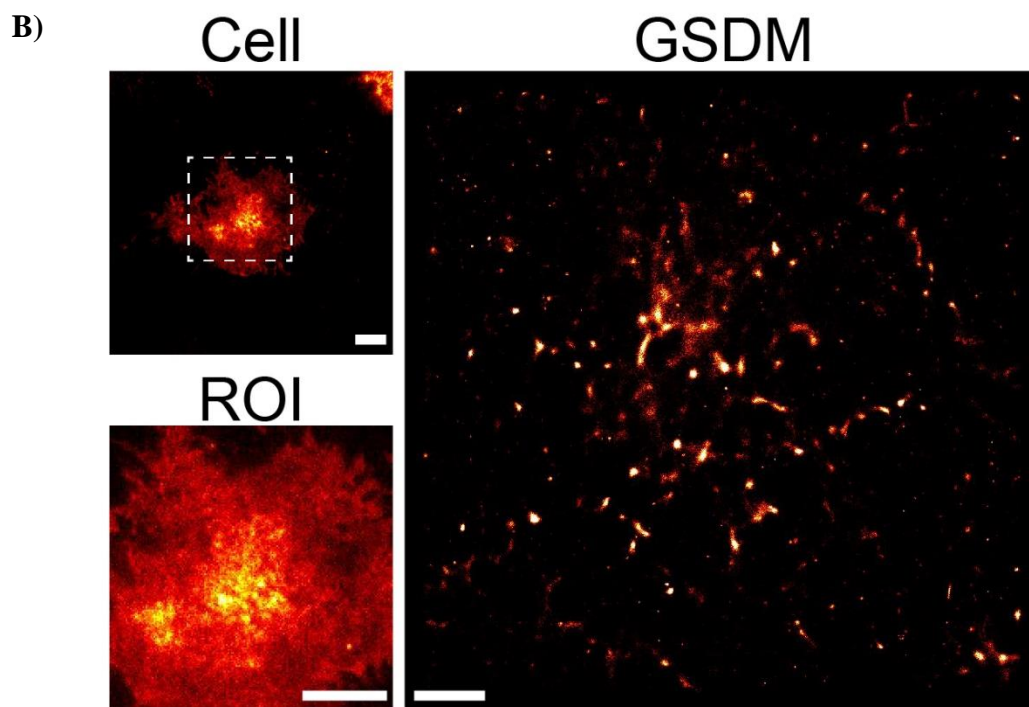
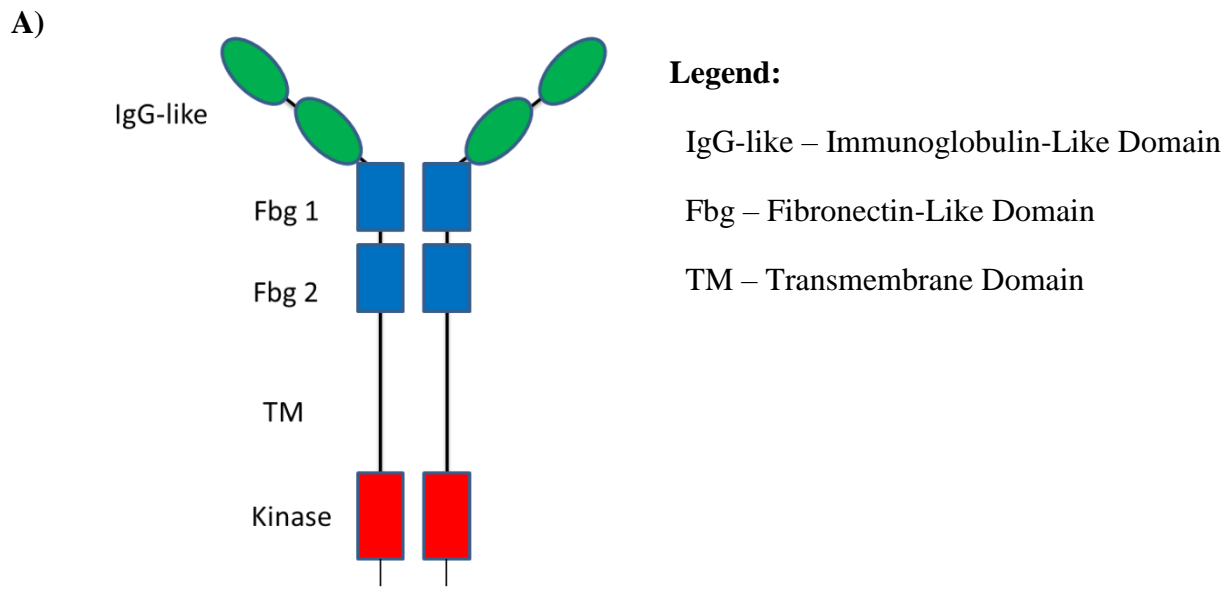


Figure 1. MERTK Domains and Clustering. **A)** MERTK domains. **B)** Clustering of MERTK as quantified by super-resolution ground-state depletion microscopy (GSDM). ‘Cell’ shows the cell being imaged, ROI shows the area imaged at super-resolution. GSDM shows MERTK clustering with ~20 nm resolution. Scale bars are 2 μ m, image is shown as a heat-map. (Image courtesy of Amanda Evans).

Previous studies have already elucidated part of the overall TAM receptor signaling pathway. Phosphorylation of the TKD domain of TAM receptors has been shown to result in activation of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway^{32,35}. After activation of the TAM receptor, the SH2 domain of growth factor receptor-bound protein 2 (Grb2) binds to the tyrosine residue immediately downstream of the TKD. Grb2 subsequently recruits the p85 and p110 subunits of PI3K into a complex^{34,35}. This complex then goes on to phosphorylate the lipid phosphatidylinositol-4,5-bisphosphate (PIP₂) to form phosphatidylinositol-3,4,5-trisphosphate (PIP₃), which in turn recruits and activates Akt^{34,35}. Akt-associated substrates are known to mediate a variety of important cellular processes including survival, proliferation, angiogenesis, and autophagy³⁶. TAM signaling has also been implicated in the resolution of inflammation via inhibition of the Janus kinase (JAK)/ Signal transducer and activator of transcription (STAT) pathway³⁷⁻³⁹. When dimerized, JAKs autophosphorylate and activate STAT proteins which act as transcription factors for several genes involved in inflammatory responses³⁷⁻³⁹. TAM receptors couple with Type I interferon receptor (IFNAR), with activation of the TAM-IFNAR complex by Gas6 inducing the expression of suppressors of cytokine signaling-1 (SOCS1) and -3 (SOCS3) which disrupt JAK-STAT interactions and reduce type I IFN activation⁴⁰. It is unlikely this pathway inhibits all forms of pro-inflammatory signalling known to be inhibited by TAM receptors, as not all of these pro-inflammatory pathways require JAK-STAT signaling or signal via IFNAR.

1.3 MERTK Signalling and Efferocytosis

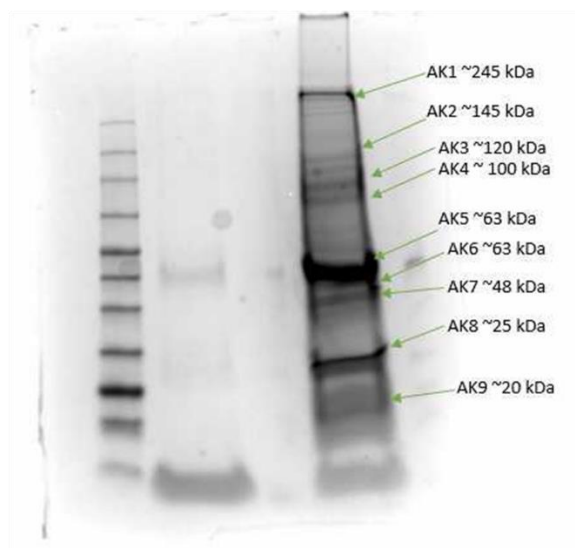
In the context of efferocytosis, MERTK-mediated engulfment of apoptotic cells appears to require the activation of integrin co-receptors such as $\alpha v \beta 5$ integrin^{32,41,42}. Integrins are transmembrane receptors that conventionally mediate attachment of cells to the extracellular matrix. However, some integrins are co-opted for processes such as phagocytosis and efferocytosis. Efferocytic integrins have been shown to interact with exposed PtdSer via the opsonin MFG-E8^{43,44}. MERTK and integrin co-activation has been proposed to involve the

recruitment of Src proteins via autophosphorylation of the Tyr-867 residue which induces the Src-mediated phosphorylation of focal adhesion kinase (FAK)³². Phosphorylated FAK is subsequently recruited to the integrin where it induces formation of the p130cas/CrKII/Dock180 complex which stimulates efferocytosis-associated cytoskeletal remodelling via Ras-related C3 botulinum toxin substrate 1 (Rac1) GTPase activity^{32,41}. Rac1 facilitates this remodeling by promoting membrane ruffling and engulfment of the apoptotic cell via actin polymerization and remodeling^{35,41}. Another protein that may be involved in the activation of integrins during efferocytosis is Ras-related protein 1 (Rap1), which has been observed to be associated with cell adhesion and junction formation signaling pathways⁴⁵. Various Rap1 effectors such as RAPL, Riam⁴⁵, and Krit1⁴⁶, are known to mediate integrin activity. Rap1 has also been shown to affect Rac1-linked cell spreading and adhesion through interactions with Rac1 guanine nucleotide exchange factors (GEF)⁴⁷. Binding of Rap1 to these GEFs allows for the localization of Rac1 activity to areas of active cell spreading⁴⁷. Additionally, Rap1 may also modulate the activity of another GTPase called Ras homolog gene family, member A (RhoA) which is required for efferocytosis⁴⁸. Activation of RhoA can be induced by exposure to TNF α ⁴⁹ or lysophosphatidic acid (LPA)⁵⁰. Binding of Rap1 to RhoA GTPase-activating proteins (GAP) appears to increase the activity of RhoA⁵¹. Thus, Rap1 may play an important role in regulating the balance between the Rac1 and RhoA-dependent mediation of integrin-associated engulfment of apoptotic cells⁴⁸. A specific association between MERTK and integrins, however, has not yet been observed in phagocytic cell lines, and MERTK has even been reported to induce efferocytosis in the absence of an integrin co-receptor in some cases^{32,42}.

Our group demonstrated that MERTK is pre-structured in large complexes on the cell surface, with recent evolution increasing the cluster size of human MERTK compared to our recent ape and primate common ancestors (Fig. 1B). In this study we demonstrated that MERTK clustering increases its affinity, which occurred concurrently with a downregulation of MERTK expression - likely an adaptation to limit viral binding to MERTK without adversely affecting MERTK function⁵². In recent work (Fig. 2), we have used reversible Cross-Link Immunoprecipitation (ReCLIP) of HA-tagged MERTK expressed on Cos7 cells, followed by mass spectrometry, to identify the contents of these MERTK clusters. This revealed potential elements of the MERTK signalosome (Fig. 2A), including PI3K^{32,35}, which is involved in large particle internalization, and ERK^{28,29} and FAK³², which are involved in phagocytosis-associated cytoskeletal

remodelling (Fig. 2B). In addition, Integrin $\beta 1$ and ILK were also found in this analysis, indicating the potential presence integrins and integrin-activating signalling molecules within these clusters^{32,41,42} (Fig. 2B).

A)



B)

Sample	Protein
AK2	Phosphoinositide 3-kinase (PI3K) regulatory subunit 4, Mitogen-activated protein kinase 4 (MAPK4), Protein Kinase C (PKC) binding protein 1
AK3	Focal Adhesion Kinase (FAK)
AK4	Integrin $\beta 1$
AK6	Integrin-linked kinase (ILK)
AK7	Mitogen-activated protein kinase 1 (MAPK1/ERK2)

Figure 2. Identification of Elements of the MERTK Signalosome using ReCLIP. **A)** Cos7 cells expressing MERTK with an extracellular HA tag were treated with the ReCLIP protein cross-linking reagent, and the MERTK signalosome precipitated from the resulting cell lysates with anti-HA antibody. When resolved on a gradient SDS-PAGE gel multiple protein bands were visible, and the indicated bands were sent for mass spectrometry to identify the MERTK-associated proteins. **B)** Putative MERTK interacting proteins identified from the corresponding protein bands in panel A. Data courtesy of Angela Vrieze.

1.4 Clinical Relevance of MERTK

MERTK-facilitated efferocytosis notably appears to play a critical role in the development of atherosclerosis, the most common form of cardiovascular disease⁷⁴. Atherosclerosis is characterized by the buildup of plaque in the sub-arterial space. This plaque is composed primarily of cholesterol and lipid-filled apoptotic cells, and necrotic debris⁵³. Plaque growth

results in the stretching and weakening of the arterial wall, culminating in plaque rupture and prompting the onset of thrombosis⁵⁴. Acute thrombus can occlude blood vessels causing a myocardial infarction (heart attack) or an ischemic stroke if the thrombus lodges in the heart, brain or other tissues respectively. Cardiovascular disease is currently the second leading cause of mortality in Canada after cancer⁵⁵.

The primary process driving atherosclerotic plaque progression is the accumulation of cholesterol-laden foam cells within the vascular intima. Foam cells are generated from macrophages which take up large amounts lipoprotein-bound cholesterol from lipoproteins deposited in the sub-vascular space. Foam cells begin as normal monocytes in the circulation; modifications of lipoproteins in the vascular intima via processes such as oxidation and enzymatic cleavage induce an immune response which facilitates the recruitment of these circulating monocytes⁵⁴. Cytokines and chemokines secreted by vascular endothelial cells then promote adhesion of the monocytes to the vascular wall and their subsequent extravasation into the subendothelial space⁵⁴. These monocytes proceed to differentiate into macrophages which take up oxidized low-density lipoprotein (oxLDL)-bound cholesterol molecules; the modifications made to these lipids and cholesterol prevent their normal processes sing, leading to accumulation in the macrophages. These lipids and cholesterol accumulate until the macrophages until they are converted into foam cells. In addition to secreting molecular factors that promote inflammation and degradation of the extracellular matrix, foam cells also undergo apoptosis due to elevated endopasmic reticulum stress caused by cholesterol loading^{56,57}. Normally efferocytosis should clear these debris, but for unknown reasons this does not occur in the plaque, leading to the accumulation of apoptotic cells, secondary necrosis, and eventually the formation of a highly unstable necrotic core⁵⁴.

While the exact reasons behind this ineffective clearance of apoptotic cells are still largely unclear, deficiencies in MERTK function have been found to be associated with the buildup of apoptotic cells and subsequent plaque necrosis in murine models. Apolipoprotein E-deficient (Apoe^{-/-}) mice expressing defective MERTK protein containing a truncated TKD that were fed a high cholesterol diet, for example, exhibit larger atherosclerotic lesions composed of higher amounts of uncleared apoptotic cells relative to control mice⁵⁸. Decreased expression of MFG-E8, which acts as an opsonin for MERTK's integrin co-receptors, has also been linked to

increased atherosclerosis severity⁵⁹. In addition, low-density lipoprotein receptor deficient (LDLR^{-/-}) mice reconstituted with bone marrow from MFG-E8^{-/-} mice seem to possess larger amounts of plaque necrosis, apoptotic cell accumulation, and IFN γ expression levels relative to healthy controls⁶⁰. In humans, SNPs in MERTK are associated with atherosclerosis, and moreover, MERTK appears to be the main efferocytic receptor expressed by macrophages in the heart^{61,62}.

Despite MERTK's well-established clinical importance, little is understood of the signaling pathways utilized by MERTK to activate co-receptors, engulf apoptotic cells and mediate the subsequent degradation of engulfed apoptotic cells. As such *it is the goal of my thesis project to elucidate MERTK signaling under homeostatic conditions and to identify MERTK-associated signaling molecules involved in efferocytosis* **I hypothesize that the MERTK signaling pathway will require integrins as co-receptors, and engage canonical phagocytic pathways to mediate apoptotic cell internalization.**

For the first objective of this project, signaling molecules of interest identified in the literature and in Fig. 2 will be inhibited with pharmacological inhibitors in order to assess each molecule's role in the MERTK-mediated efferocytosis. The second objective of this project will be to identify non-canonical intracellular signaling pathways utilized by MERTK in macrophages. The data produced by this project will characterize MERTK-associated signalling pathways and molecules and clarify the possible roles that they play in efferocytosis.

Chapter 2

2 Materials and Methods

2.1 Materials

HA-MERTK construct was generated by Amanda Evans at our laboratory. Briefly, an extracellular HA tag was added by linearizing our lab's pEGFP-N1 MERTK vector using the HA-FWD (5' Phos-GGCGT AGTCA GGCAC GTCGT AAGGA TAGTC GGTCT GCAGG CTTCC G), HA-REV 5' (5' Phos-CACAC ACCAC TGCTG TCACT G) PCR primers. PCR was then conducted using Phusion DNA polymerase at 36 cycles with a 63°C annealing temperature and an 8 minute elongation at 72°C. The resulting product was then treated with DpnI for 1 hour to degrade the template and gel purified. The purified linearized construct was then recircularized with T4 DNA ligase before being transformed into DH5α *E. coli*. Viable clones were then selected for on agar plates containing ampicillin. Cos7 stably expressing our HA-MERTK construct (HA-MERTK-Cos7) were generated by Angela Vrieze by subcloning the HA-MERTK-Cos7 construct into a pLVX vector containing a selectable resistance marker for G418 (Geneticin), transfecting the vector into Cos7 cells using Genjet, and selecting for viable cells via G418 mediated selection. THP-1 cells were obtained from ATCC. Rosewell Park Memorial Institute (RPMI) media was purchased from ThermoFisher Scientific. Dulbecco's Modified Eagle Medium (DMEM), and Fetal Bovine Serum (FBS) were purchased from Wisent. Trypsin-EDTA and antibiotic/antimycotic was purchased were purchased from Corning. 16% Paraformaldehyde (PFA) and #1.5mm thickness coverslips were purchased from Electron Microscopy Supplies (Hatfield, Pennsylvania). Rat high-affinity anti-HA antibody clone 3F10 was purchased from Sigma-Aldrich (Oakville, Canada). Anti-phosphotyrosine (4G10) antibody was purchased from EMD-millipore. PP1, Syk Inhibitor, Ly294002, Wortmannin, Cpd22, and U0126 inhibitors were purchased from Cayman Chemical. Phorbol 12-Myristate 13-Acetate (PMA) was purchased from Bioshop Canada. Polystyrene-DVB beads were purchased from Bangs Laboratories. Mouse IgG antibody was purchased from Invitrogen. Recombinant human Gas6 (rhGas6) was purchased from R&D Biosystems. Guide RNA, donor DNA, and Cas9

protein were purchased from Sigma-Aldrich. Prism software was purchased from GraphPad. ImageJ was downloaded from www.imagej.nih.gov/ij/ respectively.

2.2 THP-1 Culture and Differentiation

THP-1 cells were maintained in RPMI containing 10% FBS in T25 flasks at 37°C and 5% CO₂. Cells were passaged upon reaching a density of 2,000,000 cells/ml. Prior to passage, cells were counted using a hemocytometer and a volume of media containing 1,000,000 cells was removed and placed in a 15 mL Falcon tube. The cells were then washed twice with 1 ml of phosphate-buffered saline (PBS) by centrifuging cells and discarding the supernatant. Cells were then resuspended in 5 ml of RPMI media pre-warmed to 37°C and placed in a new T25 flask for a final density of 200,000/ml. For experiments, #1.5 thickness 18 mm diameter circular coverslips were placed in wells of a 12 well plate and a volume of media containing 200,000 cells was aliquoted into each well containing a coverslip. Pre-warmed RPMI media was added to each well if necessary to bring the total volume of media in each well to 1 ml. PMA was then added to each well at a concentration of 100 ng. Cells were then allowed to differentiate into a macrophage-like phenotype for 3 days prior to the beginning of the experiment.

2.3 HA-MERTK-Cos7 Culture

HA-MERTK-Cos7 cells were maintained in DMEM containing 10% FBS in T25 flasks at 37°C. Cells were subculture upon reaching 90% confluency by washing with PBS before being incubated for 5 mins with trypsin-EDTA followed by suspension in DMEM + 10% FBS. #1.5 18 mm coverslips were placed in the wells of a tissue culture plate, 1 mL of DMEM + 10% FBS added to each well, and then 100 µl of cell suspension added dropwise to each well. Cells were cultured for 24 hours at 37°C prior to the beginning of the experiment.

2.4 Efferocytosis Assays

THP-1 cells were prepared and differentiated as indicated in section 2.2 3 days prior to the beginning of the experiment. 10 µl of polystyrene beads were suspended in PBS in a 1.5 ml falcon tube and opsonized with 10 µl of either IgG or Gas6 for 24 hrs at 4°C on a rotating

platform prior to the beginning of the experiment. On the day of the experiment, the opsonized beads are blocked with blocking buffer (5% BSA + PBS) for 20 mins, washed and resuspended in PBS. The cells were then incubated with a few crystals (~0.05 mg) of biotin for another 20 mins, washed with PBS, and resuspended in 100 μ l PBS. THP-1s were incubated at 37°C with the indicated concentration of the pharmacological inhibitors for at least 20 mins before the beginning of the experiment. 5 μ l of opsonized beads are then added to the wells containing cells and the well plate is centrifuged at 1500 rpm for 1 min to allow the cells to come into contact with the beads. The cells were then incubated at 37°C to allow for internalization of the beads. After incubation, cells were washed 3x with PBS before being stained with 1:20,000 Hoescht 33342 and streptavidin for 10 mins. Cells were then washed 3x with PBS once more before being fixed in 4% paraformaldehyde (PFA) and mounted on coverslips using Permafloor mounting reagent. Imaging of cells was carried out using a Leica DMI6000 widefield microscope equipped with 63 \times /1.40NA and 100 \times /1.40NA objectives, a heated/CO₂ perfused stage, Chroma Sedat Quad filters, a Photometrics Evolve 2 Em-CCD camera, operated by Leica LAS-X software. Images were captured in which light (DIC) and in the indicated fluorescence channels, exported to ImageJ and % efferocytic efficiency was calculated by dividing the total amount of macrophages internalizing at least 1 (unstained) bead divided by the total number of macrophages multiplied by 100.

2.5 Immunoblot

HA-MERTK-Cos7 cells were prepared as outlined in section 2.3 and seeded onto 6-well plates. Cells were serum-starved for 5 hours prior to the beginning of the experiment. Cells were then cross-linked with 1:1000 high affinity rat anti-HA antibody for 20 mins before being cross-linked with 1:10,000 goat anti-rat Cy3 secondary antibody and 1:10,000 rabbit anti-goat Cy3 tertiary antibody for 20 mins each. Cells were lysed with Lamelli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8) plus protease/phosphatase inhibitor cocktail, PMSF, NaF, and sodium orthovanadate before being run on a 10% SDS PAGE gel at 100 V for 2 hours. Samples were then transferred to nitrocellulose membranes at 4 degrees Celsius for 2 hours at 80 V. The membrane was then blocked for 2 hrs with 5% BSA in TBST before being treated with 1:1000 4G10 anti-phosphotyrosine primary

antibody for 2 hours, washed 3X with TBST, incubated with 1:10,000 IR700 anti-mouse secondary antibody, and washed another 3X with TBST. Blots were imaged using a LI-COR Odyssey GelDoc.

2.6 Immunoprecipitation

HA-MERTK-Cos7 cells were prepared as outlined in section 2.3 and seeded onto 100 mm x 20 μ M tissue culture dish. Cells were serum-starved for 3 hours prior to the beginning of the experiment. Cells were then cross-linked with 1:1000 high affinity rat anti-HA antibody for 20 mins before being cross-linked with 1:10,000 goat anti-rat Cy3 secondary antibody and 1:10,000 rabbit anti-goat Cy3 tertiary antibody for 20 mins each. Cells were then lysed with RIPA buffer plus protease/phosphatase inhibitor cocktail, PMSF, NaF, and sodium orthovanadate. Lysates were subsequently incubated for 4 hours with agarose phosphotyrosine beads before being washed 3x with wash buffer and 2x with PBS. Supernatant was then removed, and beads were resuspended in 70 μ l of phenyl phosphate. Beads were then centrifuged, and the resulting supernatant was removed. DTT and Lammeli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8) was then added to the supernatant and the solution was run on a 10% SDS-PAGE gel at 100V for 2 hours. The gel was then fixed with a solution containing 50% methanol and 10% acetic acid and subsequently stained with Coomassie Blue staining solution for 2 hours. The gel was then submerged in water to allow it to destain for 24 hours. Gels were then imaged using a BioRad GelDoc EZ Imager.

2.7 CRISPR-Cas9

cRNA, tracrRNA, Cas9 protein, and a donor DNA template containing the HA-tag were electroporated into THP-1 cells using the Neon electroporation system. 48 hours after electroporation, cells were immunostained with an anti-HA antibody and a Cy3 secondary antibody in order to assess surface expression of the HA-tag. Cells were then sorted using a FACSI Aria Cell Sorter and any HA-expressing cells identified were cultured. The cRNA and donor DNA target sequences are described in the results section.

2.8 Statistical Analysis

Unless other wise noted, one-way ANOVA with Tukey correction was used for all analyses. Data associated with a p-value < 0.05 was considered significant. All statistical analyses were conducted using GraphPad Prism software.

Chapter 3

3 Results

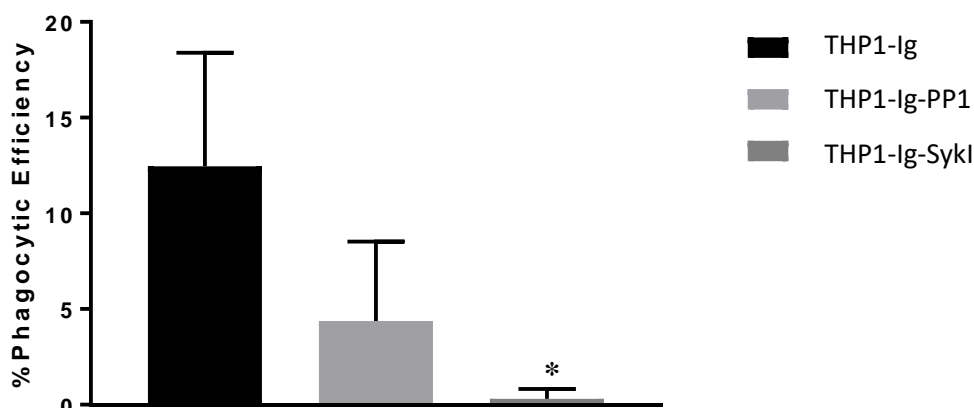
3.1 Canonical Phagocytic Pathways

Efferocytosis assays were performed on PMA-differentiated THP-1 macrophages using our laboratory's established efferocytosis assay protocols⁶³ with or without inhibitors against canonical efferocytic/phagocytic signaling molecules. This included inhibitors against proteins proposed to be involved in MERTK's signaling pathway such as Src^{53,64}, PI3K^{64,65}, and Syk⁵³, as well as non-canonical signaling molecules that have been identified by our lab (Fig. 2) to be associated with the MERTK signalosome such as ILK and ERK. For these experiments, THP-1-derived macrophages were used, as these cells are known to exhibit substantially increased MERTK expression upon PMA-differentiation and do not express any other members of the TAM receptor family⁷⁵, thus allowing us to use Gas6-coated beads as MERTK-specific targets. As a positive control, to demonstrate the inhibitors were functional, the phagocytic uptake of IgG-coated beads was assessed. IgG-mediated phagocytosis is known to be blocked by the inhibitors used in these experiments⁶⁶. For all inhibitors, phagocytosis/efferocytosis was measured as phagocytic or efferocytic efficiency (% of Mφ (macrophages) with ≥ 1 bead internalized).

Syk inhibition was found to significantly decrease phagocytic efficiency for both IgG and Gas6-coated beads, while unexpectedly, Src inhibition was found to decrease phagocytic efficiency only for macrophages exposed to Gas6 beads (Fig. 3). It is unclear why the Src inhibitor did not block phagocytosis, as previous studies have found phagocytosis to be sensitive to the Src inhibitor PP1⁶⁷. This difference may be due to PP1's cross-reactivity with some serine/threonine kinases; a cross-reactivity not reported for the SykI inhibitor used in this study. PI3K inhibition via both LY294002 and Wortmannin was found to decrease phagocytic efficiency in macrophages incubated with both IgG (Fig. 4) and Gas6 beads (Fig. 5). LY294002 specifically inhibits the formation of PI(3,4,5)P₃ at the cell membrane by Class I PI3K's, whereas wortmannin inhibits Class I PI3K's, as well as Class II/III PI3K's which are responsible for producing PI(3)P and PI(3,4)P₂, predominantly at endomembranes. The identical inhibitory

response observed between LY294002 and wortmannin suggests that Class I PI3K activity is required for efferocytosis via MERTK, consistent with previous studies³². Inhibition of ILK was found to reduce efficiency in macrophages incubated with both IgG and Gas6 (Fig. 6) beads while ERK inhibition was only found to decrease efficiency in macrophages incubated with Gas6 beads (Fig. 6). These data indicate that MERTK-mediated efferocytosis utilizes many of the same signalling pathways as Fc-mediated phagocytosis but does require some unique signalling pathways not utilized for phagocytosis.

A)



B)

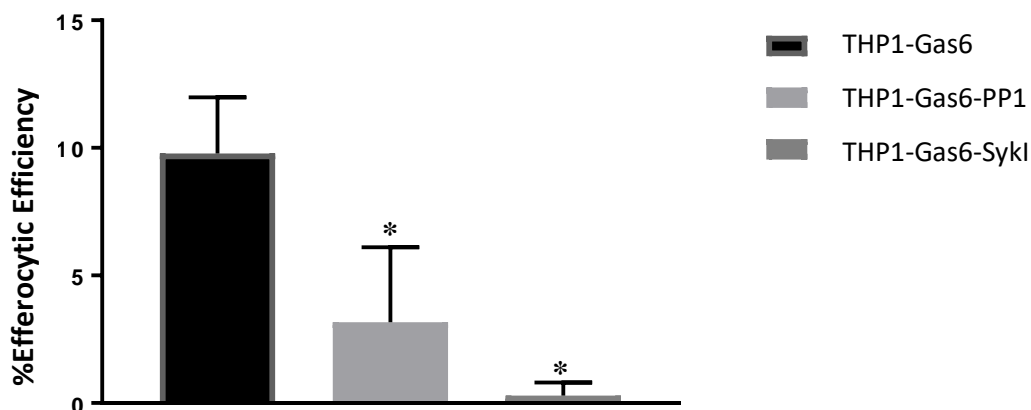


Figure 3. Role of Src and Syk Kinases in MERTK-Mediated Efferocytosis. THP-1 monocytes were PMA-differentiated for 3 days prior to being subjected to an efferocytosis assay. Uptake of **A)** IgG-opsonized, or **B)** Gas6-opsonized beads. Src was inhibited with PP1 (20mM) and Syk with Syk-inhibitor I (SyKI, 20mM) for 20 minutes before efferocytosis/phagocytosis

assays were performed. $n = 3$. n.s. = $P > 0.05$, * = $P < 0.05$, compared with ANOVA with Tukey correction.

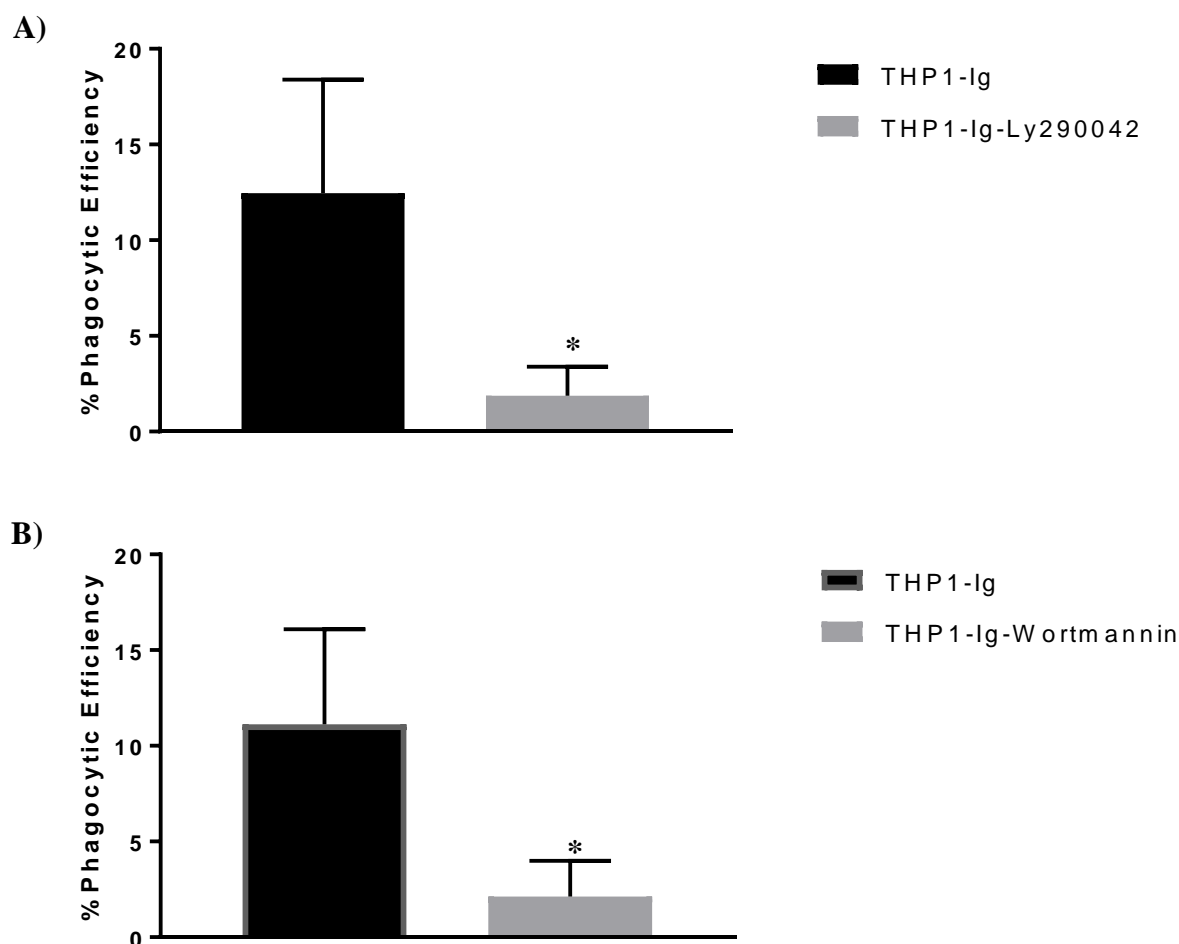


Figure 4. Role of PI3-kinases in Fc-Mediated Phagocytosis. PMA-differentiated THP-1 cells were incubated with or without inhibitors of **A)** Class I PI3K (LY294002, 20mM) or **B)** Class I, II and III PI3K (Wortmannin, 20mM). Inhibitors were added 20 minutes before phagocytosis assays with IgG-coated beads were performed. $n = 3$. n.s. = $P > 0.05$, * = $P < 0.05$, compared with ANOVA with Tukey correction.

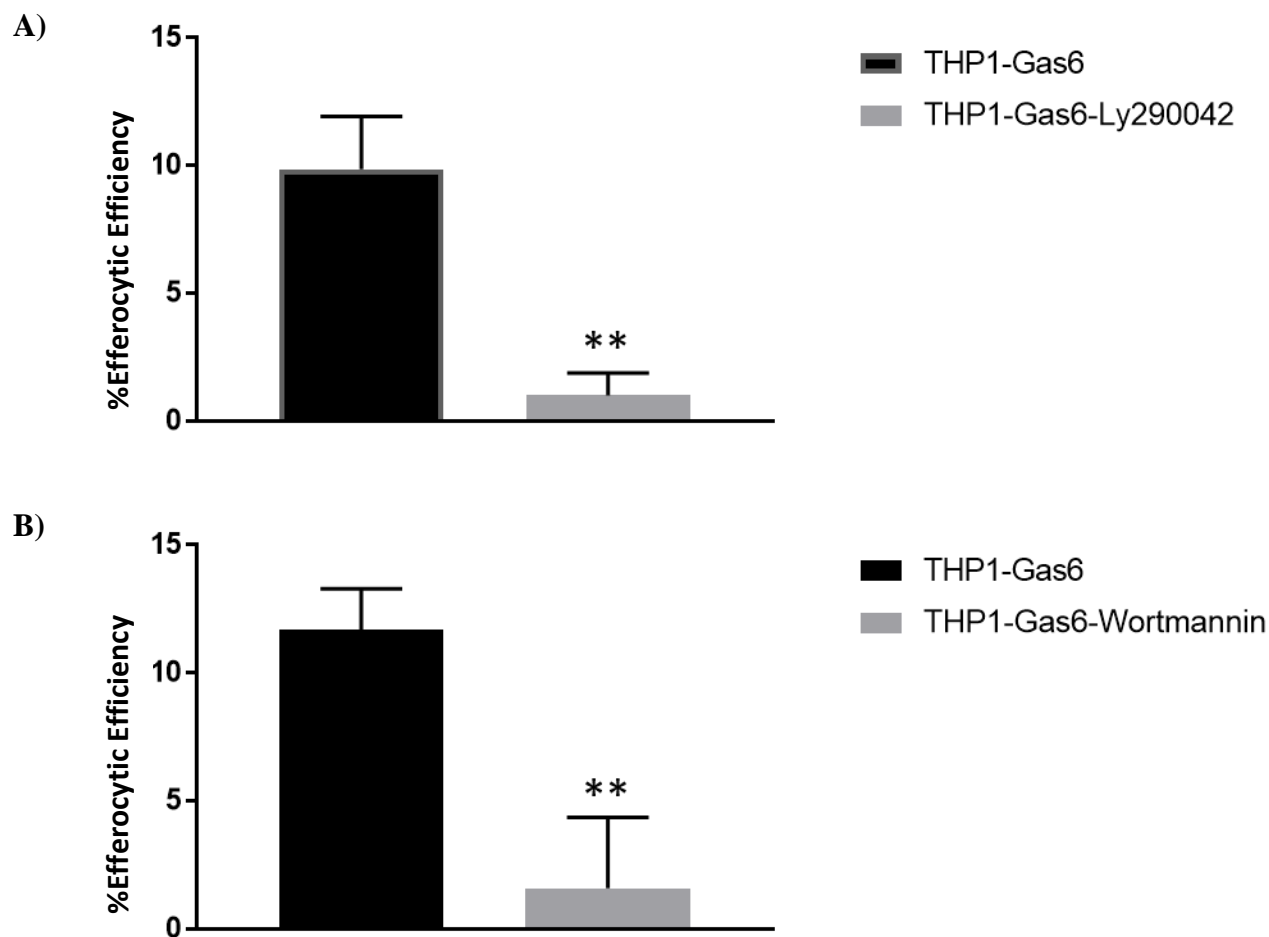


Figure 5. Role of PI3-kinases in MERTK-Mediated Efferocytosis. PMA-differentiated THP-1 cells were incubated with or without inhibitors of **A)** Class I PI3K (LY294002, 20 μ M) or **B)** Class I, II and III PI3K (Wortmannin, 20 μ M). Inhibitors were added 20 minutes before efferocytosis assays with Gas6-coated beads were performed. $n = 3$. n.s. = $P > 0.05$, * = $P < 0.05$, compared with ANOVA with Tukey correction.

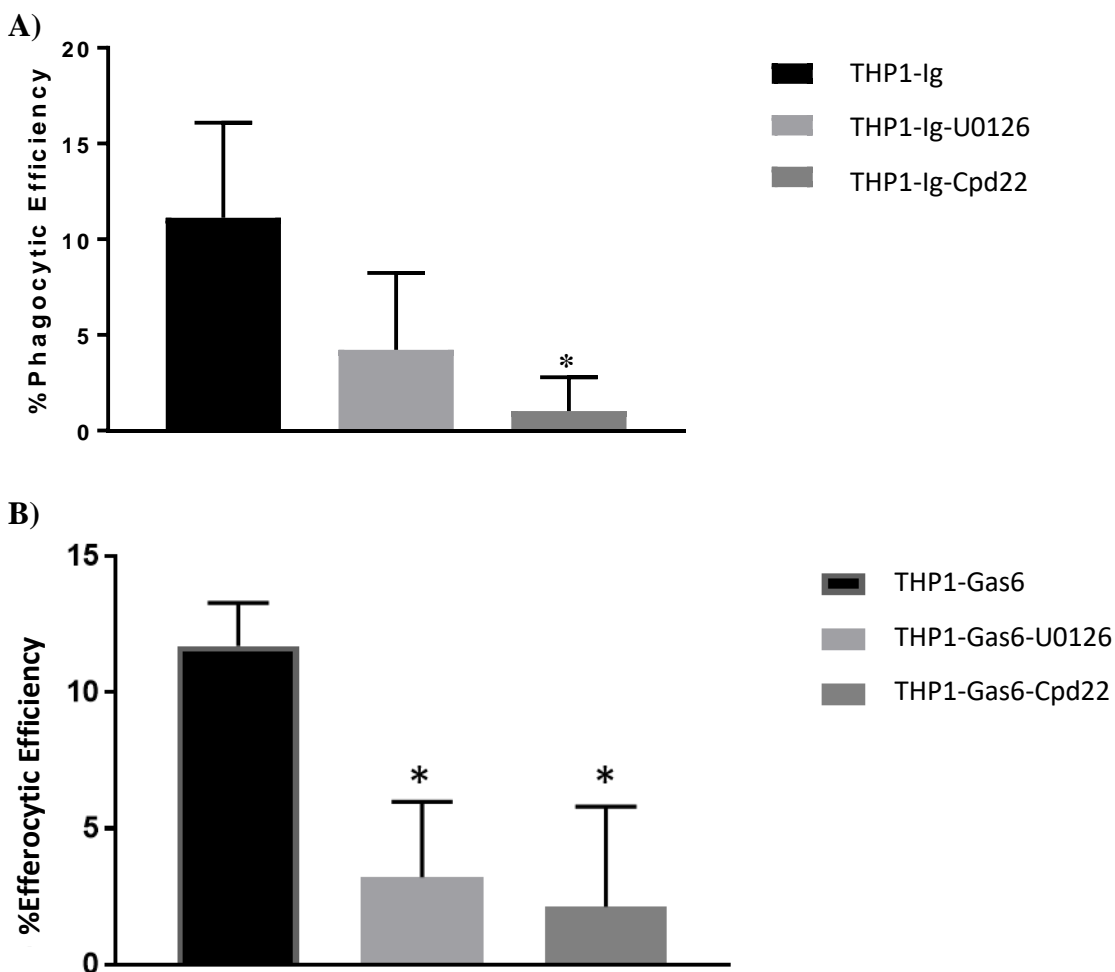


Figure 6. Role of Erk and ILK in MERTK-Mediated Efferocytosis. PMA-differentiated THP-1 were incubated with or without inhibitors of Erk (U0126, 20 μ M) or ILK (Cpd22, 20 μ M) and **A**) phagocytic index of IgG coated beads, or **B**) efferocytic index of Gas6-coated beads determined. Inhibitors were added 20 minutes before phagocytosis/efferocytosis assays. $n = 3$. n.s. = $P > 0.05$, * = $P < 0.05$, compared with ANOVA with Tukey correction.

3.2 MERTK Signaling Molecule Identification

Antibody cross-linking assays were used to identify specific elements of the MERTK signaling pathway. Usage of MER-specific antibodies to activate signaling pathways is preferable to the use of apoptotic cells, as these cells will be simultaneously recognized by all efferocytic receptors on the responding cell, thus obscuring the MERTK-derived signals. However, of the many commercial anti-human MERTK antibodies we have tested, only one which binds the intracellular kinase domain of MERTK has been found to be functional (data not shown), making this antibody unsuitable for cross-linking experiments. To address this problem, our lab previously developed an HA-MERTK construct wherein an HA tag was inserted in a non-conserved domain located after the MERTK signal peptide (Fig. 7).

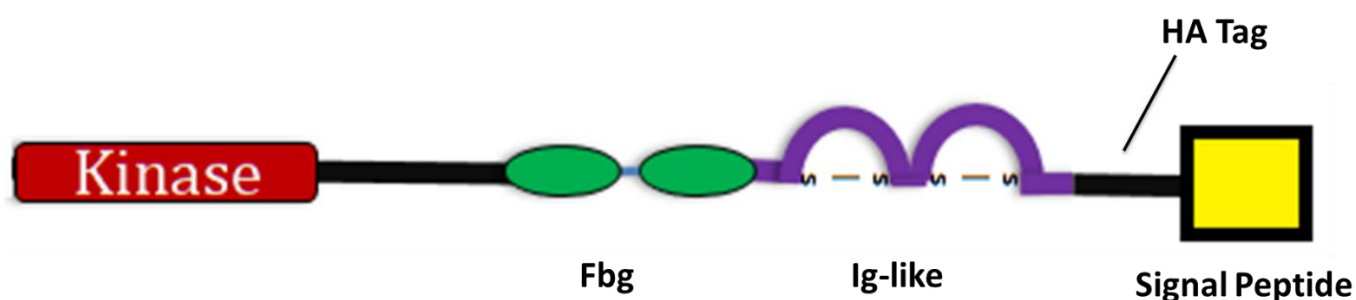


Figure 7. Structure of the HA-MERTK construct showing the location of the inserted HA-tag. The HA tag was inserted in an evolutionarily non-conserved region between the N-terminal signal peptide (Yellow) and Ig-like repeats (purple). Fbg/green = fibrinogen-like repeats; the transmembrane domain is located between the Fbg and intracellular, c-terminal kinase domain (red).

This HA-MERTK construct was then transfected into the Cos7 cell line, and expression confirmed by anti-HA immunostaining (Fig. 8). Cos7 cells were used because, while not natively phagocytic/efferocytic, they carry the molecular machinery required to perform such functions. As reported previously, human MERTK was observed to form large clusters, some of which were large enough to be resolved in these conventional microscopy images.

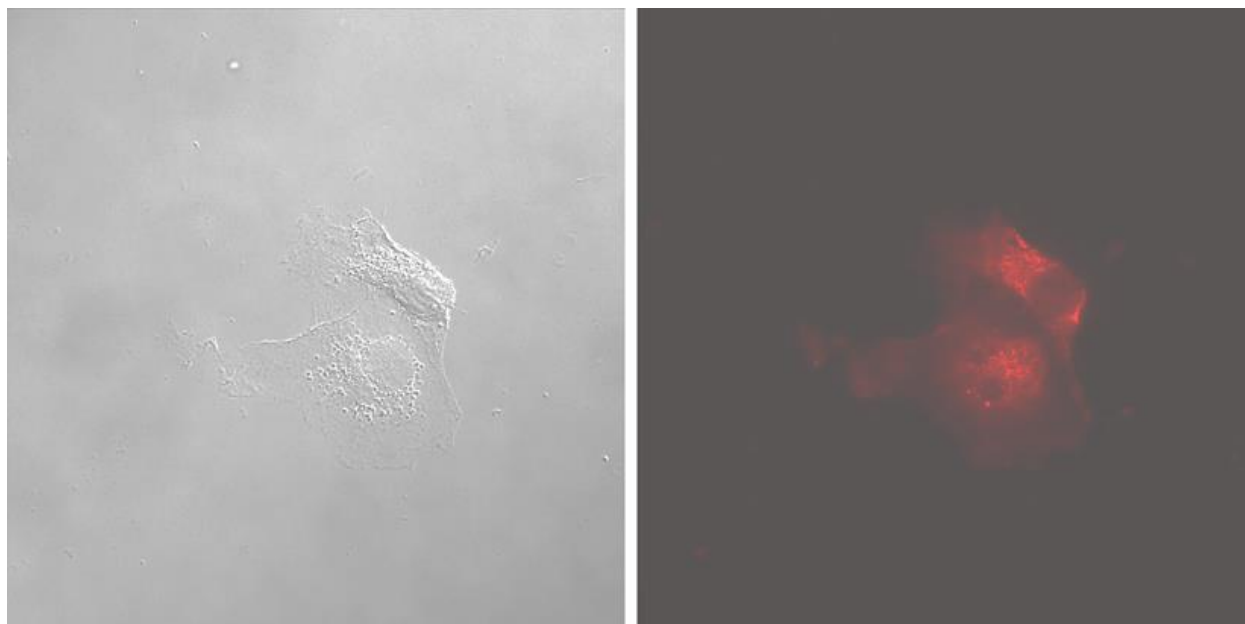


Figure 8. Expression of stably integrated HA-MERTK on the cell surface of Cos7 cells.

Fixed but non-permeabilized Cos7 cells were transfected with HA-MERTK and immunostained with anti-HA and a Cy3-labeled secondary antibody. Left: DIC image, Right: anti-HA immunofluorescence. Images are representative of 30 images taken over 2 independent experiments.

Once MERTK expression was confirmed, another lab member prepared Cos7 cells stably expressing this HA-MERTK construct (Fig. 8). Cross-linking and immunoprecipitation experiments on this stable HA-MERTK-Cos7 cell line was carried out using full-length high affinity anti-HA antibody, crosslinked by the addition of F(ab')₂ secondary and tertiary antibodies (Fig. 9). F(ab')₂ antibodies are used for this cross-linking protocol because they lack an Fc-portions and thus will be unable to bind to Fc-receptors on target cells, thereby preventing the activation of non-MERTK specific signaling pathways. Prior to the cross-linking experiment, cells are serum-starved for 3 hours in order to reduce basal signalling through growth factor receptors. After antibody-mediated cross-linking was completed, the cells were lysed and tyrosine-phosphorylated proteins precipitated using (4G10) anti-phosphotyrosine agarose beads, followed by resolution of the immunoprecipitated using SDS-PAGE and Coomassie Blue staining.

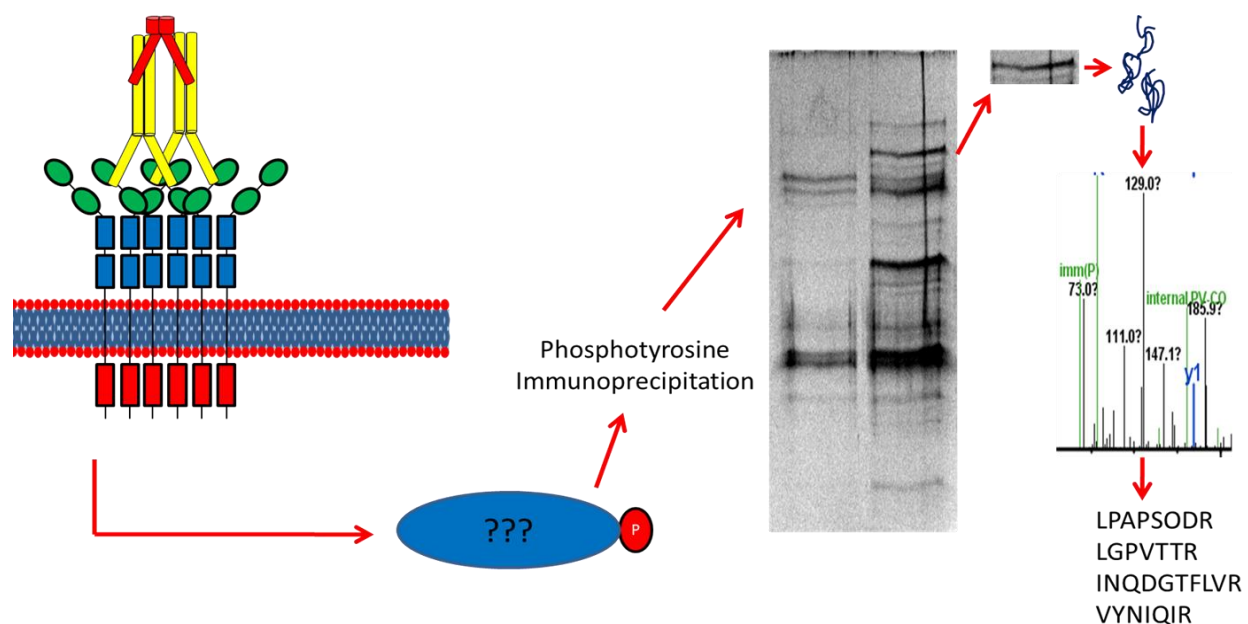


Figure 9. Schematic of the method for identifying MERTK-activated signaling molecules.

HA-MERTK is activated by crosslinking with an anti-HA antibody and cross-linking secondary/tertiary F(ab')₂ antibodies. Tyrosine phosphorylated proteins are immunoprecipitated, resolved with SDS-PAGE and identified by mass spectrometry.

Cross-linking of HA-MERTK on these cells induced a strong and reproducible pattern of protein phosphorylation, which could readily be detected by immunoblotting with the anti-phosphotyrosine 4G10 antibody (Fig 10). However, we were unable to immunoprecipitate these phosphorylated proteins using the same 4G10 antibody conjugated to agarose beads (Fig. 11). Coomassie staining of gels containing cell lysates and bead wash supernatants suggest that the precipitated proteins are either being degraded soon after incubation with the 4G10 beads or are being lost during the wash steps (Fig. 11).

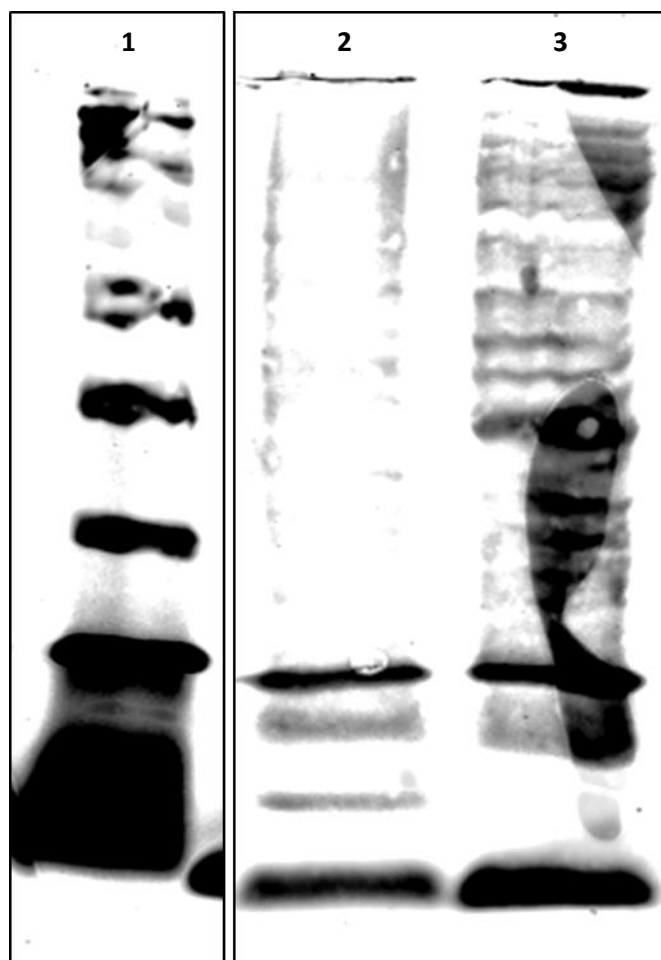


Figure 10. Detection of phosphotyrosine signaling following MERTK cross-linking. Anti-phosphotyrosine (4G10) immunoblot of HA-MERTK-Cos7 cells that are either non-crosslinked (lane 2) or cross-linked with an anti-HA antibody and F(ab')₂ secondary and tertiary antibodies (lane 3). Lane 1 is the protein ladder. Gel is representative of 3 independent experiments.

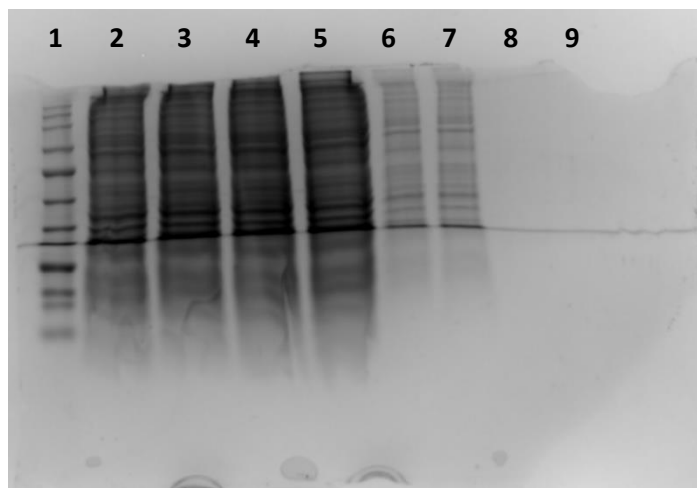


Figure 11. Loss of proteins during 4G10 immunoprecipitation. Proteins precipitated from cross-linked HA-MERTK-Cos7 cells were tracked during 4G10 immunoprecipitation by SDS-PAGE and coomassie staining. Lane 1: Ladder, Lane 2: non cross-linked HA-MERTK-Cos7 (total cell lysate), Lane 3: cross-linked HA-MERTK-Cos7 (total cell lysate), Lane 4: post-immunoprecipitation cell lysate (including 4G10 beads) from non cross-linked HA-MERTK-Cos7 cells, Lane 5: post-immunoprecipitation cell lysate (including 4G10 beads) from cross-linked HA-MERTK-Cos7 cells, Lane 6: wash buffer after first wash of non cross-linked HA-MERTK-Cos7 4G10 precipitates., Lane 7: wash buffer after first wash of cross-linked HA-MERTK-Cos7 4G10 precipitates, Lane 8: proteins precipitated from non cross-linked HA-MERTK-Cos7 after 1 wash, and Lane 9: proteins precipitated from cross-linked HA-MERTK-Cos7 after 4 washes. Image is representative of 12 independent experiments.

3.3 Moving Towards a Better MERTK Experimental System

While Cos7 and THP-1 monocytes have been used for our analyses, both cell types have drawbacks. Cos7 cells are non-human cells which are not intrinsically efferocytic, and therefore may form aberrant MERTK clusters that differ in their composition from what is found on human cells. Similarly, while THP-1's express MERTK, the absence of quality antibodies recognizing the extracellular portion of human MERTK and the presence of other efferocytic receptors, limits our ability to selectively engage and isolate MERTK in these cells. We have had great difficulty in expressing HA-MERTK ectopically in THP-1 cells, and moreover, our previous study of MERTK clustering⁵² suggests that MERTK clustering – and therefore signalosome composition – may be highly dependent on the expression level of MERTK, thus making over-expression systems inappropriate for studies of the MERTK signalosome. We are therefore currently attempting to use the CRISPR-Cas9 genome editing platform to generate a stably-transfected THP-1 cell line that constitutively expresses HA-MERTK. Briefly, this technology involves the generation of a CRISPR guide RNA containing a sequence matching that of the target DNA sequence on the genome of interest. Cas9 endonuclease then recognizes this sequence on the CRISPR guide RNA and begins cutting all matching sequences on the genome of interest. A donor DNA containing the sequence that is to be inserted at the cut sites can subsequently be introduced and used as a template for repair, effectively inserting a new sequence at the genetic loci of the original. Our first approach aims to induce a double-stranded break in the MERTK loci near the region where the HA-tag is introduced in our complex; a repair template, containing the HA tag and ~60 bp of homology surrounding the cut site, is introduced at the same time as the Cas9 riboprotein complex, in the hopes that homologous DNA repair will use this template to fix the Cas9-induced DNA break (Fig. 12A). To improve our chances of success we have designed two different donor DNAs (single and partially-double stranded versions) containing the HA-tag, as well as two guide RNAs (gRNAs) designed by Sigma-Aldrich which we complex with Cas9 and tracrRNA to form functional riboprotein. The donor DNAs and riboprotein complexes were electroporated into the THP-1 cells using the Neon electroporation system and HA-MERTK expressing cells are sorted via flow cytometry. Our early attempts at these experiments produced a small number of cells which appeared to be positive for HA-MERTK by immunofluorescence microscopy (Fig. 12B), and while a similar

population can be separated by FACS sorting, subsequent testing failed to identify HA-MERTK in these cells (Fig. 13).

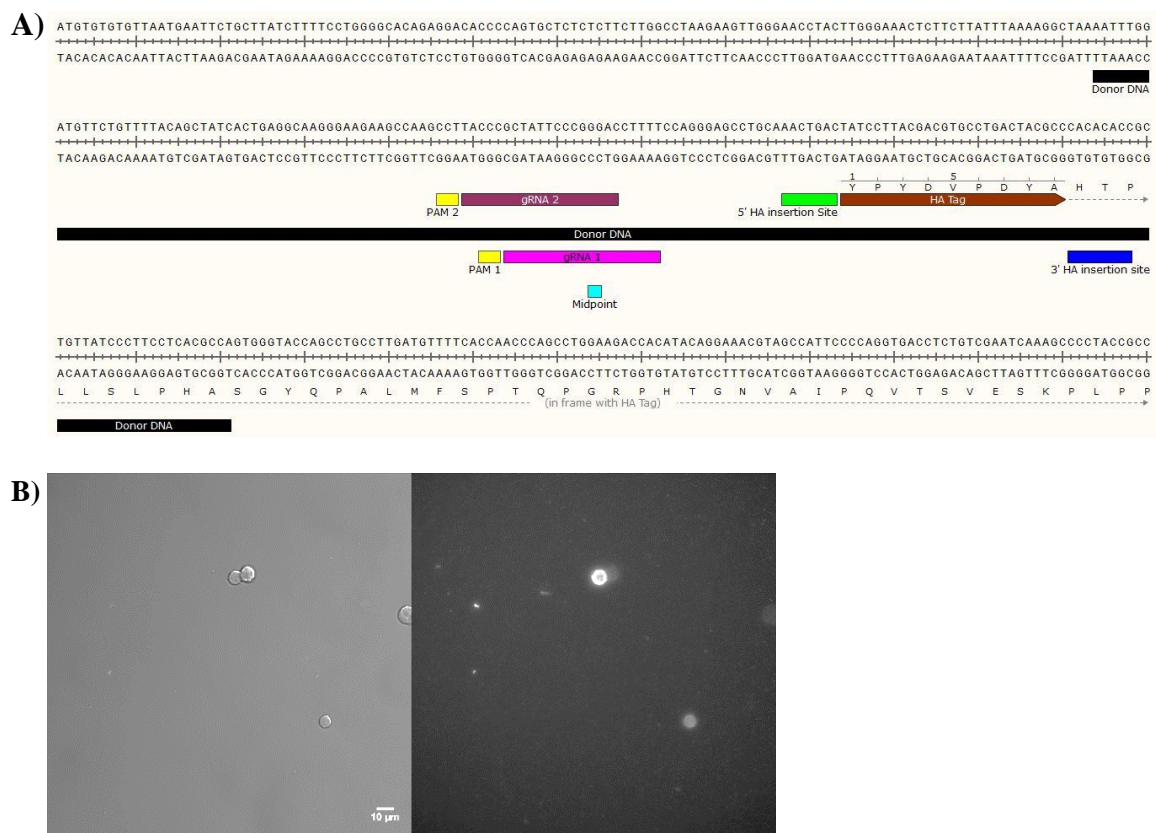


Figure 12. CRISPR-Cas9 mediated integration of an HA-tag at the THP-1 MERTK locus.

A) The two guide RNAs (gRNA 1 & 2) bind 5' of the desired HA insertion site (green). The Cas9-RNA ribonuclear complexes should cut the DNA within the PAM site at the 5' end of each gRNA (yellow). Following homologous DNA repair, using a donor DNA template containing the HA tag flanked on either side by 30 bp of either single or double-stranded DNA, the HA tag should be permanently incorporated into the endogenous MERTK locus. B) crRNA, tracrRNA, Cas9 protein, and a donor DNA template containing the HA-MERTK-Cos7 sequence were electroporated into THP-1 cells using the Neon electroporation system. 48 hours after electroporation, cells were immunostained with an anti-HA antibody and a Cy3 secondary antibody in order to assess surface expression of the HA-tag. The cells in this image were transfected using gRNA 1 and donor DNA 1. Images were captured using an HKD Leica Widefield Microscope. Scale bar = 10 μ m.

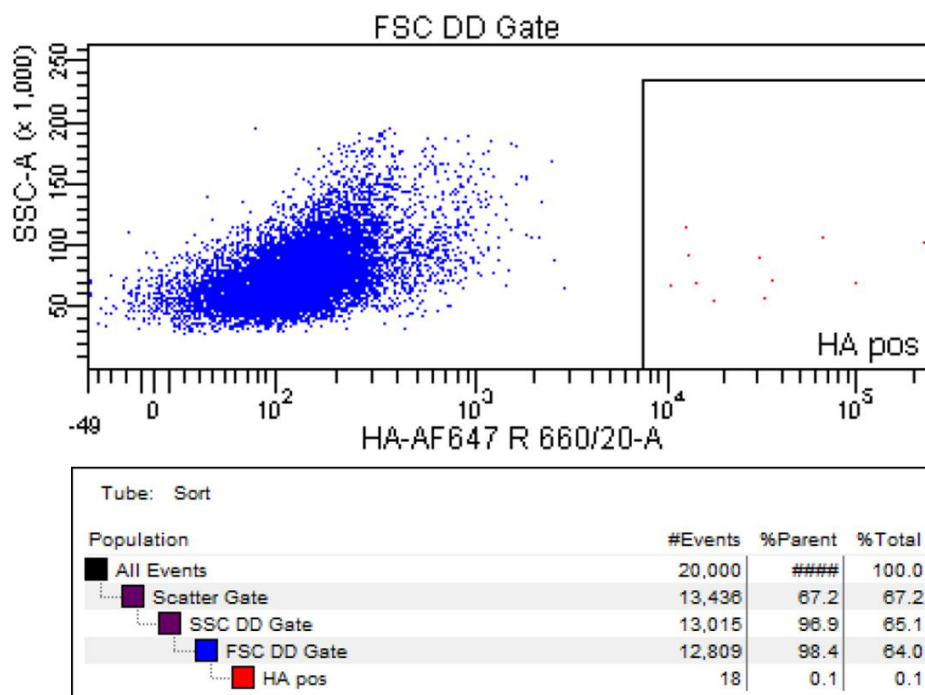


Figure 13. Flow cytometry sorting of CRISPR-edited THP-1 cells. THP-1 cells, edited with CRISPR to add an HA tag to the extracellular domain of MERTK, were immunolabeled with anti-HA and sorted by FACS. While a small portion of cells were positive for HA-MERTK (HA pos/red), these cells did not remain viable after recovery.

To resolve the issues associated with the CRISPR-Cas9 mediated transfection of THP-1 monocytes with our HA-MERTK construct, our laboratory is investigating the viability of the CRISPaint variation of this system⁶⁸. CRISPaint uses non-homologous end-joining to promote the insertion of a gene at a specific locus and has been successfully used to modify THP-1 cells. CRISPaint relies on the transfection of three plasmids; the first plasmid expresses the guide RNA and Cas9, the second plasmid is the donor vector and contains the sequence to be inserted into the genome, while the third plasmid contains a gRNA sequence that allows Cas9 to linearize the donor vector such that the inserted sequences is in-frame⁶⁸. Aside from the fact that this method takes advantage of non-homologous end-joining machinery to promote precise DNA template insertion and repair at the genetic loci of interest, the 3 aforementioned DNA/gRNA plasmids employ minicircle technology, which allows for the removal of unnecessary prokaryotic elements from the donor plasmid resulting in a reduction in overall plasmid size and

subsequently improves plasmid uptake by cells⁶⁸. At the time of this thesis we have identified a gRNA which cleaves 3 base-pairs before the MERTK start codon (AATTACAGATCCGCAGCCCCGGG) and have begun cloning HA-MERTK into the donor DNA vector. Once complete, this vector set should allow for us to insert our HA-MERTK construct into the translational start site of the endogenous MERTK locus, thus producing HA-MERTK-THP-1 cells expressing MERTK under control of the endogenous promotor.

Chapter 4

4 Discussion

In this thesis I tested the hypothesis that MERTK mediates efferocytosis through canonical phagocytic signaling pathways. I successfully identified several signaling pathways required for efferocytosis through MERTK. These included the canonical phagocytosis signaling molecules Src-family kinases, Syk and PI3K, but also included the non-canonical signaling molecules Erk and ILK, indicating that MERTK-mediated efferocytosis occurs via a unique signalling pathway that is only partially shared by phagocytosis. I also developed a cross-linking based assay that was able to specifically induce phosphotyrosine signaling following MERTK cross-linking. While I was not able to identify any of the activated signaling molecules, this platform can be used in the future to determine whether Rap1 and/or SOCS1/3 are activated downstream of MERTK. Lastly, I have begun the development of a new cell-based system for studying MERTK function. This new system offers several advantages over the model system used to-date; specifically, it allows for the selective activation of MERTK via HA-mediated cross-linking, in a relevant human efferocytic cell type that normally expresses MERTK. Combined, my thesis has provided a preliminary assessment of the signalling lying downstream of MERTK activation and has laid the groundwork for future mass spectrometry and cell signaling studies into MERTK function.

4.1 Role of Canonical Phagocytic/Efferocytic Pathways in MERTK-Mediated Efferocytosis

As expected, inhibition of PI3K prevented both MERTK-efferocytosis and phagocytosis, as PI3K is well-known to be involved in large particle internalization by phagocytes^{64,65}. Specifically, PI3K enables the large-scale actin reorganization required for large-particle internalization. In phagocytic studies, small (<1 μm) particles do not require PI3K activity for their uptake; it is unclear at this time whether this is true for MERTK as I only assessed efferocytosis using large (5 μm) particles whose uptake was predicted to require PI3K. Src and Syk are known membrane-proximal regulators of phagocytic signaling, with phagocytic receptors directly activating Src, which in-turn recruits and activates Syk to mediate actin cytoskeleton polymerization through proteins such as VAV1^{53,64}. Specifically, the Src/Syk signaling pathway signals via immunoreceptor tyrosine-based activation motifs (ITAMs) which are found in most phagocytic receptors, and which must be phosphorylated to initiate the phagocytic process⁶⁴. Curiously, while both Src and Syk inhibition were shown to decrease efferocytic efficiency in THP-1 cells, a greater decrease was observed for Syk inhibition as opposed to Src inhibition, despite the fact that Syk is activated by Src. This may have been due to better inhibition of Syk than of Src; indeed, we used a newly developed and high affinity inhibitor for Syk, versus a lower-affinity and reversible agent for Src inhibition. Phagocytosis – both of antibody and complement opsonized targets – also requires ILK, with my data indicating that MERTK likewise requires this signaling molecule. During phagocytosis, ILK mediates the activity of integrins, which are required to exclude inhibitory signalling molecules during Fc-mediated phagocytosis and which can independently mediate the uptake of complement-opsonized targets⁶⁹. Consistent with this, we observed a decrease in phagocytic efficiency that accompanies ILK inhibition. It is unclear what ILK is doing in our system, as no integrin ligands were present on our Gas6 beads. Some studies have proposed that ILK independently promotes cytoskeletal remodelling through by activating Rac1, and this may account for the role of ILK in our model system⁷⁰; alternatively, Gas6 may be recognized by a promiscuous integrin such as Mac1. Combined, these inhibitor studies demonstrate that the PI3K, Src, Syk and ILK pathways are required for MERTK-mediated uptake, indicating that MERTK employs much of the same molecular machinery for target internalization as does canonical Fc-mediated phagocytosis. This

is unsurprising as the uptake of large particulates requires large-scale, complex reorganisation of the actin cytoskeleton. The signaling mediating this reorganization during phagocytosis is deeply conserved, having first evolved in single celled amoebas, with the more recent evolution of efferocytosis apparently coopting this previously evolved pathway.

While most of the pathways I identified as mediators of MERTK efferocytosis, one non-canonical signaling molecule – ERK – was identified as indispensable for MERTK function. ERK has a less defined role in phagocytosis, with some studies showing a weak but significant reduction in FcγR-mediated phagocytosis in ERK-inhibited neutrophils and macrophages⁷¹, while other studies and this thesis have demonstrated ERK to be dispensable for phagocytosis. Like ILK, ERK has also been shown to be involved in Rac1-mediated cytoskeletal remodeling, although it is unclear if these pathways activate Rac1 via the same signaling pathways⁷¹. Critically, in this thesis, I found that ERK inhibition profoundly blocked MERTK-mediated efferocytosis, but had no effect on Fc-mediated phagocytosis, indicating that MERTK has evolved some unique signaling pathways not utilized by phagocytic receptors. This further validates our goal of fully elucidating the MERTK signalling pathway, as the presence of non-canonical signaling pathways offers the opportunity to selectively target efferocytosis without significantly altering other internalization processes. One confounding issue in these assays was a lower-than-expected uptake of particles under control conditions; an issue which may be addressed in future experiments through increasing the target:macrophage ratio and/or increasing the experiment time. Additionally, off-target effects associated with the inhibitors used in these experiments may have played a role in the observed discrepancy in particle uptake, an issue that could be investigated by performing further efferocytosis assays using siRNAs directed against the same molecules targeted by the inhibitors.

4.2 Clinical Implications of Deficiencies in MERTK-Mediated Efferocytosis

Future studies regarding the signaling pathways associated with MERTK-mediated efferocytosis will not only likely be aimed at identifying novel signaling molecules but also their roles in

clinical conditions such as atherosclerosis. Experiments aimed at investigating MERTK's role in this disease could involve culture of macrophages in atherosclerotic conditions (i.e. high oxLDL), followed by assessment of the activation of previously identified MERTK-associated signaling molecules, to determine the source of MERTK defects in atherosclerosis. If the expression or phosphorylation levels of these signaling molecules are found to be dysregulated under atherosclerotic conditions, normal levels could be restored and their effects on the efferocytic ability of the macrophages measured. The data produced such studies will characterize MERTK-associated signalling pathways and molecules and clarify the possible roles that they play in the mediation of efferocytosis. Additionally, the results of such data may also increase understanding of MERTK's role in the development of atherosclerosis and allow for identification of putative targets for treatment of this disease.

4.3 Limitations of the MS/MS Model

Optimization of the immunoprecipitation-mass spectrometry method of identifying potential MERTK-associated signaling proteins presents another significant challenge, as activated signaling molecules either become degraded or are lost when the lysates are washed. The source of this issue remains unclear, as indeed, our lab has successfully used this approach previously^{72,73}. There are two likely causes of this issue; first, the commercially purchased 4G10 agarose may have been non-functional. This seems unlikely as we tested two lots of this reagent, with both failing to precipitate any proteins. However, direct (chemical) antibody conjugation to beads has the potential to inactivate antibodies, and thus may account for our failure to precipitate proteins. To address this issue, we will use protein A/G beads to precipitate 4G10 in the future; while this makes subsequent detection of proteins more difficult (due to the presence of the antibody in the precipitate), it would resolve any issues with antibody inactivation during crosslinking to beads. Alternatively, we may have failed to fully inhibit cellular phosphatases, and thus lost the 4G10 target motif (phosphotyrosine). We are in the process of testing phosphatase inhibitor cocktails from other companies, in the hopes of identifying a better reagent.

4.4 Moving Towards an Endogenous MERTK Expression System

Moving forward, the optimal system in which to study MERTK signaling would be in a human cell line that expresses our HA-MERTK construct under control of the endogenous MERTK locus. Given that human MERTK has been shown to cluster on the cell surface differently than its primate counterparts, any study of signaling based around using human-based cell lineages would provide more biologically relevant results as opposed to those obtained from primate cell lineages such as Cos7. In addition, endogenous expression of the receptor would allow for expression levels similar to those observed physiologically, with transgene-mediated overexpression promoting aberrant clustering and potentially altered function of MERTK. Thus, CRISPR-mediated gene editing of the endogenous MERTK locus in THP-1 cells represents a better model system. As outlined in Section 3.3 however, CRISPR-Cas9 mediated insertion of HA-MERTK into the genome of the human monocyte cell line THP-1 has so far been unsuccessful as flow cytometry sorts of edited samples do not return a significant number of HA-positive cells. The use of the CRISPaint variation of the CRISPR-Cas9 technique however, may yet yield more favorable results, as its usage of minicircle technology and non-homologous end-joining DNA repair may improve both delivery of the gRNA/DNA plasmids into the cell and the insertion of the DNA template sequence into the genome respectively.

In conclusion, in this thesis I determined that MERTK mediated efferocytosis is dependent on the canonical Src-Syk-PI3K-ILK phagocytic signaling pathway but has also evolved to require the non-canonical ERK signalling pathway. This work provides the first study of the role of these pathways in mediating the internalization of efferocytic targets by MERTK and lays the groundwork for continuing investigation into the signaling pathways regulating MERTK-mediated efferocytosis and the downstream cellular events which follow efferocytosis.

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Curriculum Vitae

Education

Masters of Science in of Microbiology and Immunology, Western University, 2018

Honours Bachelor of Science with Specialization in Biology (Cellular/Molecular option), University of Ottawa, 2016

Research Experience

2016 – 2018

Masters Student • Western University • London, ON, CA

Conducted a two-year graduate thesis project in the immunology laboratory of Dr. Bryan Heit at the University of Western Ontario involving the identification of signaling molecules activated downstream of Mer Tyrosine Kinase (MERTK), a receptor expressed on phagocytes which is involved in the uptake of apoptotic cells.

- Identified the signaling molecules PI3K, Src, Syk, ERK, and ILK as potential regulators of MERTK efferocytic function.
- Worked on the design and development of a CRISPR-Cas9 based system of inserting a MERTK DNA construct into the genome of phagocytic cell lines.
- Authored two scientific publications, one of which was a first-author publication.

2015 – 2016

Undergraduate Research Student/Laboratory Assistant • Agriculture and Agri-Food Canada, Ottawa, ON, CA

Conducted a two-semester undergraduate honours research project in the microbiology laboratory of Dr. James Tambong at Agriculture Canada's Ottawa Research and Development Centre involving the *de novo* assembly and analysis of the type strain draft genomes of three plant pathogenic bacteria: *Pantoea anthophila*, *Pantoea deleyi*, and *Pantoea eucalypti*.

- Assembled and analyzed draft genomes from DNA sequence data derived from cultured bacterial cells using both the command-line based user-friendly software.

Publications

Taruc K, Yin C, Wootton G, Heit B. Quantification of Efferocytosis by Single-Cell Fluorescence Microscopy. *J Vis Exp*. 2018.

Evans AL, Blackburn JWD, Taruc K, et al. Antagonistic Coevolution of MER Tyrosine Kinase Expression and Function. *Mol Biol Evol*. 2017.

Lab Skills

Mammalian cell culture

Efferocytosis assays

Immunostaining

Transfection using commercial transfection reagent and electroporation protocols

Gel electrophoresis (polyacrylamide/agarose)

de novo Genome assembly using both the command-line based user-friendly software packages

Genome analysis using the command-line based CMG Biotools software package

Liquid and petri dish bacterial culture preparation

DNA extraction and purification

PCR sample preparation and operation of Biometra TProfessional thermocyclers

Relevant Coursework

Animal Physiology I (BIO 3303)

Genetics (BIO 2133)

Animal Physiology II (BIO 3302)

Genomics (BPS 3101)

Bioinformatics Laboratory (BPS 4104)

Honours Project for Biology Students
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Cell Biology (BIO 3153)

Molecular Biology (BIO 3170)

Computational Tools for Biological Sciences (BIO 3360)
3151)

Molecular Biology Laboratory (BIO

Conservation Biology (BIO 3115)

Molecular Evolution (BIO 3102)

Ecology (BIO 2129)

Molecular Genetics (BIO 4115)

Environmental Microbiology (EVS 3120)

Seminar-Cell and Molecular (BIO 4900)

General Microbiology (BIO 3124)

Vertebrate Zoology (BIO 3158)

Conference Attendance

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Western Research Forum 2017 – Poster Presentation

London Health Research Day 2017 – Poster Presentation

Infection and Immunology Research Forum 2017 – Poster Presentation

London Health Research Day 2018 – Poster Presentation

Canadian Society of Immunology Scientific Meeting 2018 – Poster Presentation

Volunteer Positions

Let's Talk Science Outreach Volunteer October 2016 – May 2018

Western Foot Patrol Volunteer January 2017 – April 2017

Western International Peer Guide September 2017 – May 2018

Extracurricular Activities

Western Dragonboat Team September 2016 – July 2018

Western Judo Club September 2016 – December 2017