Characterization of Tie2 signalling during acute inflammation

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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Abstract

The inflammatory response is essential for the eradication of lipopolysaccharide (LPS) presenting microbial invaders. However, resolution is also important to prevent detrimental vascular inflammation to the host. Endothelial cells play active roles in inflammation by detecting LPS using Toll-Like Receptor 4 (TLR4), leading to the activation of Nf-κB, but also participate in the resolution of inflammation, in part, through the actions of the receptor tyrosine kinase, Tie2. The process by which Tie2 can attenuate LPS-TLR4 driven inflammation is poorly understood. The work herein centers upon the characterization of Tie2 signalling during acute inflammation.

The effects of Tie2 activation using the Tie2 agonist, Vasculotide (VT), was monitored in an animal model of endotoxin induced inflammation. Activation of Tie2 resulted in reductions in vascular permeability, levels of circulating inflammatory cytokines, and the number of leukocytes at the site of inflammation following LPS stimulation, confirming the anti-inflammatory effect of Tie2 signalling following LPS-TLR4 driven acute inflammation.
To investigate the crosstalk between Tie2 and TLR4, Nf-κB activation was monitored in cells expressing Tie2 mutants harboring tyrosine (Y) to phenylalanine mutations in the carboxy-terminal tyrosine residues essential for downstream Tie2 signalling. Tie2 signalling reduced LPS induced Nf-κB activation in a Y1100 and Erk1/2 dependent manner. Tie2 signalling decreased the expression of the TLR4 signalling proteins, TRAF6 and IRAK1 and preserved the expression of the Nf-κB inhibitor, IκBα, though Y1100 initiated Erk1/2 signalling. The ability of Tie2 to increase the expression of miRNA-146b-5p was investigated and found to be dependent on functioning of all three carboxy-terminal tyrosine residues. Our results show that Y1100 initiated Erk1/2 signalling is essential for the anti-inflammatory effect of Tie2 on TLR4 driven Nf-κB activation.

Characterization of the role of Tie2 signalling during an inflammatory cascade will further our understanding of the biology of Tie2 and ultimately contribute to our understanding of the regulation of vascular inflammation.
This thesis is dedicated to Dr. Daniel Dumont.
Dan was an uncommonly patient, kind, and supportive mentor who left an indelible mark on my life and career. For that, I will always be grateful. He was a tireless and innovative scientist, who always put his family first. The example he set as a scientist and as a deeply caring person will truly be a constant inspiration in my life.
Acknowledgments

One of the most fortunate events of my life was Dr. Dan Dumont taking me on as his student - I don’t know where I would be if it wasn’t for Dan’s belief in me. Dan took me under his wing and embarked on the daunting task of turning me into a decent scientist. I could not have found a more supportive and encouraging mentor.

Dan also made sure to surrounded me with a cast of strong scientist role models. Particularly, Dan gave me the opportunity to put Dr. Jane McGlade on my supervisory committee. From the beginning Jane was an invaluable source of advice and suggestions, helping to shape my project. When the Dumont lab closed, Jane invited me to join her lab and showed me unwavering kindness and patience. She could not have been more helpful and encouraging, making my transition to her lab easy. Jane has been a constant source of inspiration, setting an example for the kind of person/scientist I hope to be.

I am also indebted to my committee members, Dr. Philippe Poussier, and Dr. Stan Liu. This thesis would not be possible without their expertise, patience, and mentorship. Their feedback has been an integral part of my work and experimental design.

Working in two labs has introduced me to so many amazing people who have contributed to my PhD experience. Harold, Paul, Annie, and Maribelle were tasked with unofficially mentoring me. My experience at Sunnybrook would not have been the same without the fun and friendship from all the Dumont lab crew. For the second leg of my PhD I was fortunate to gain another source of advice and encouragement. For that, I would like to thank everyone in the McGlade lab.

Of course, there are many people outside the lab without whom this journey would not have been possible. Thank you to my Mom and Dad, and Sarah-Girl for your unwearied love and for putting up with a frequently stressed out daughter/sister. To Caffy, Mark, and Gabby for supporting and harboring me. A special thank you to my best buddy Lee, who is always there to bounce ideas off and is a constant source of fun and hilarity, even from a distance.
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List of Abbreviations

A  Alanine
Ang Angiopoietin
CC  Coiled-Coil Domain
CLP Cecal-Ligation Puncture
DAMP Damage Associated Molecular Pattern
DMSO Dimethyl sulfoxide
Dok2 / DokR Downstream of Tyrosine Kinases 2
EGF Epidermal Growth Factor
EGR Early Growth Response
F  Phenylalanine
FBS Fetal Bovine Serum
FGF Fibroblast Growth Factor
FLD Fibrinogen Like Domain
FNIII Fibronectin Type III Repeats
Grb Growth Factor Receptor-Bound
HMVEC Human Microvascular Endothelial Cells
HRP Horseradish Peroxidase
hrs Hours
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intracellular Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB Kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRAK1</td>
<td>IL-1 Receptor-Associated Kinase 1</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of κB</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>KD</td>
<td>Kinase Dead</td>
</tr>
<tr>
<td>LBP</td>
<td>LPS-Binding Protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemotactic Protein 1</td>
</tr>
<tr>
<td>mg</td>
<td>Miligram</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid Differentiation Factor 88</td>
</tr>
<tr>
<td>NEMO</td>
<td>Nf-κB Essential Modulator</td>
</tr>
<tr>
<td>Nf-κB</td>
<td>Nuclear Factor of κB</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>Pre-miRNA</td>
<td>Precursor miRNA</td>
</tr>
<tr>
<td>Pri-miRNA</td>
<td>Primary microRNA</td>
</tr>
<tr>
<td>PTB</td>
<td>Phosphotyrosine Binding</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radioimmunoprecipitation Assay Buffer</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>SCD</td>
<td>Superclustering Domain</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic Hedgehog</td>
</tr>
<tr>
<td>SOCS2</td>
<td>Suppressor of Cytokine Signalling 2</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of Sevenless</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-Buffered Saline-Tween</td>
</tr>
<tr>
<td>Tie</td>
<td>Tyrosine Kinase with Immunoglobulin and Epidermal Growth Factor Homology Domains</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 Receptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>-----------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF Receptor-Associated Factor 6</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule 1</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular Endothelial Cadherin</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VE-PTP</td>
<td>Vascular Endothelial Protein Tyrosine Phosphatase</td>
</tr>
<tr>
<td>VT</td>
<td>Vasculotide</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>Y1100</td>
<td>Tie2 tyrosine residue 1100</td>
</tr>
<tr>
<td>Y1106</td>
<td>Tie2 Tyrosine Residue 1106</td>
</tr>
<tr>
<td>Y1111</td>
<td>Tie2 Tyrosine Residue 1111</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μl</td>
<td>Mircolitre</td>
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Chapter 1
Introduction
1 Literature Review

1.1 Endothelium

The endothelium, first described in 1865 (Aird, 2007), was initially regarded as the inert barrier separating circulating blood from the underlying tissues. It wasn’t until 1973 that researchers began culturing endothelial cells, which make up the endothelium, and isolated human umbilical vein endothelial cells (HUVECs) (Jaffe, Hoyer, & Nachman, 1973). As a result, scientists were able to conduct controlled investigations of endothelial cells for the first time. In the 1980s, research revealed the complexity of endothelial cells as well as their response to inflammation or endothelial “activation”, whereby inflammatory stimulation cues a phenotypic change in endothelial cells prompting differential receptor expression and increased leukocyte adherence (Gamble, Harlan, Klebanoff, & Vadas, 1985; Pober & Gimbrone, 1982; Schleimer & Rutledge, 1986). Through subsequent investigation, the endothelium is now regarded as an important and biologically active compartment involved in the regulation and propagation of a variety of pathologies.

The field of endothelial cell biology experienced a critical breakthrough in 1992, when Dr. Dan Dumont cloned a new member of the receptor tyrosine kinase family, Tek (tunica interna endothelial cell kinase), later renamed Tie2 (tyrosine kinase with immunoglobulin-like loops and epidermal growth factor homology domains), while working in Dr. Martin Breitman’s group at the University of Toronto (Dumont, Yamaguchi, Conlon, Rossant, & Breitman, 1992). Subsequent knockout studies
performed in mice demonstrated a critical role for Tie2 in cardiovascular development and showed enrichment of the Tie2 receptor in the endothelium (Dumont et al., 1994). Dr. Dan Dumont’s subsequent research was dedicated to the study of the functions of Tie2 and its ligands, Ang1 and Ang2, in blood and lymphatic vessels. We owe a tremendous amount of our understanding of Tie2 signalling and its role in endothelial cell biology and embryonic development to the pioneering work of Dr. Dumont.

1.1.1 Embryonic Development of the Vasculature

The cardiovascular system is the first to develop during embryonic development, giving rise to the vasculature through the processes of vasculogenesis and angiogenesis. Vasculogenesis involves de novo generation of endothelial cells from mesodermal precursors and the establishment of the yolk sac vasculature and the primary vascular plexus. During vasculogenesis, angioblasts differentiate and assemble into immature blood vessels forming a primitive vascular network. These immature vessels are then remodeled though the process of angiogenesis, whereby new capillaries arise from pre-existing larger vessels. This gives rise to an intricate vascular network with a hierarchy of both large and small blood vessels including arteries, veins, arterioles, and capillaries, each with specific functions (De Val & Black, 2009; Risau, 1997). Endothelial cells recruit and interact with support cells such as pericytes (for small vessels) and smooth muscle cells (for larger vessels), these interactions are essential for the stability and function of mature blood vessels (Carmeliet, 2003).
1.1.2 Endothelial Cells

Endothelial cells, which make up the endothelium, line the inner lumen of blood and lymphatic vessels in a monolayer providing a semi-permeable interface between blood and the underlying tissue. Endothelial cells constitute a large population of cells. It has been estimated that there are roughly $10^{13}$ endothelial cells in the human body (Aird, 2003). Endothelial cells are flattened epithelial cells ranging in thickness from less than 100 nm in microvessels, such as capillaries, to 1 µm in macrovessels like arteries (Pries, Secomb, & Gaehtgens, 2000). In culture, endothelial cells form a monolayer tightly grouped together in a characteristic cobblestone pattern. This thesis is concerned specifically with the endothelial cells of the body’s blood vessels.

Endothelial cells are connected to each other through a series of junctional proteins, which allows for the selective flux of fluids and macromolecules between the blood and interstitium. Contact inhibition, whereby cell proliferation halts when cells come into contact with each other, is part of the process responsible for the characteristic tight monolayer of endothelial cells. An endothelial cell specific protein, VE-cadherin (discussed in further detail below) was shown to be responsible for this characteristic (Grazia Lampugnani et al., 2003).

On the apical or luminal facing side, endothelial cells display a series of glycoproteins and proteoglycans, forming a structure known as the glycocalyx (Pries et al., 2000). By immobilizing inflammatory chemokines released from immune cells within their glycocalyx endothelial cells increase the local concentration of these inflammatory mediators (Bao et al., 2010). Endothelial cells directly interact with components of the
blood including circulating immune cells, cytokines and chemokines, and also respond to blood flow patterns and integrate this input to direct endothelial activation.

The basal surface of endothelial cells interacts with mural cells that include smooth muscle cells and pericytes depending on the vascular bed. Pericytes are mural cells that wrap around capillaries and venules forming a discontinuous sheath. Pericytes interact with endothelial cells in arterioles, capillaries, and venules. The number of pericytes associated with the endothelium depends on the vascular bed. Pericyte density can reach approximately 50% coverage of endothelial cells (Shepro & Morel, 1993). Vascular smooth muscle cells are found in larger vessels such as arteries and veins, contracting when necessary to regulate vessel radius and local blood pressure (Gaengel, Genove, Armulik, & Betsholtz, 2009). Interactions with other cell types are critical for the proper functioning of endothelial cells.

Adding to the complexity of the endothelium, associations with structures external to endothelial cells assist in the proper functioning of the endothelium. Endothelial cells work in cooperation with structures such as glycocalyx on the luminal side, and the extracellular membrane on the apical side to maintain its proper function. Glycocalyx is thought to contribute to the ability of endothelial cells to integrate external biomechanical signals such as transluminal pressure and blood flow patterns (Dull et al., 2012; Mochizuki et al., 2003). It is also known to regulate the binding of leukocytes by blocking adhesion sites and serving as a binding site for chemokines where they are presented to leukocytes (Constantinescu, Vink, & Spaan, 2003; Schmidt et al., 2012). Glycocalyx also assists in the barrier function of the endothelium (van Haaren, VanBavel, Vink, & Spaan, 2005; Yang & Schmidt, 2013).
Endothelial cells are situated on a basement membrane that forms part of the extracellular matrix (Pries et al., 2000) where endothelial cells interact with the basement membrane via focal adhesions (G. E. Davis & Senger, 2005). Focal adhesions are concentrated areas of cytoskeletal proteins that are linked to the extracellular matrix via integrins (Schwarz & Gardel, 2012). The basement membrane of blood vessels consists of a tightly linked network of proteins including laminins and type IV collagen among several glycoproteins. This basement membrane functions as a scaffolding structure for the organization of endothelial cells during angiogenesis (G. E. Davis & Senger, 2005). Pericytes are integrated into the basement membrane where they communicate with endothelial cells via focal contacts (Armulik, Abramsson, & Betsholtz, 2005). Endothelial cells coordinate input from neighboring cells, structures, and the cellular milieu and adapt to their local environment giving rise to their broad spectrum of response to external stimuli and their function.

1.1.3 Homeostasis of the Endothelium

The endothelium maintains homeostasis of the vasculature by regulating vascular tone and endothelial barrier function, and providing a non-thrombogenic, non-reactive surface that, in quiescent state, has very little interaction with circulating leukocytes (Ley & Reutershan, 2006).

Endothelial cells regulate vasomotor tone by constitutively producing nitric oxide (NO) and prostacyclin (PGI2), which both cause relaxation of the surrounding smooth muscle cells. Endothelial cells regulate production of NO as a result of biomechanical signals such as shear stress from blood flow as well as biochemical signals including
acetylcholine and histamine (Alderton, Cooper, & Knowles, 2001; Palmer, Ferrige, & Moncada, 1987).

Endothelial cells in a quiescent state are anticoagulant and antithrombogenic. Endothelial cells express tissue factor pathway inhibitors that prevent the initiation of coagulation (Pober & Sessa, 2007). Additionally, thrombomodulin produced by endothelial cells activates protein C. Activated protein C complexes with protein S resulting in the inactivation of the clotting factors VIIIa and Va, preventing formation of blood clots and maintaining blood in a fluid state (Aird, 2007). Endothelial cells also release tissue-type plasminogen activator (tPA), which is involved in the degradation of clots (Aird, 2001). These endothelial cell dependent mechanisms maintain the non-thrombogenic state of vessel homeostasis.

1.1.4 Endothelial Heterogeneity

The endothelium is known for its heterogeneity. Remarkable variability in structure and molecular markers exists between the various organs and tissue beds, segments of the vascular tree, between blood vessels and lymphatic vessels, as well as the cellular microenvironment through interactions with other cell types or chemical mediators (Bevilacqua et al., 1986; Fukuhara et al., 2008; Page, Rose, Yacoub, & Pigott, 1992; Saharinen et al., 2008). Endothelial cells generally adopt a flattened morphology; however, the shape can vary across the vasculature as does the thickness of the endothelium. The endothelium also varies by the organization of its endothelial cell connections. The endothelium may be “continuous”, with tight endothelial cell to endothelial cell connections surrounded by a continuous basement membrane. This is
typical of the endothelium found in the arteries, veins, and capillaries of the brain and heart. The endothelium may also be “continuous and fenestrated”, possessing several holes or fenestrae which span the endothelial layer. This organization is found in locations that are characterized by increased filtration functions as in the capillaries of intestinal mucosa and renal tubules. The endothelium may also be “discontinuous”, with more numerous and larger fenestrae and large gaps between cells and a poorly formed underlying basement membrane. Discontinuous endothelium is ideal for two-way solute filtration and is located in organs such as the liver and spleen (Aird, 2007).

Endothelial cells are responsive to their microenvironment and exhibit a great capacity for plasticity. These cells pick up cues from the biochemical and biomechanical milieu, and modify their phenotype accordingly. When exposed to different external stimuli such as changes in blood flow pattern, transmural pressure, or oxygen levels, endothelial cells are able to enact structural changes as well as changes in the gene expression, protein localization, and intracellular signalling events (Dai et al., 2004; Tsukurov et al., 2000). A number of the vascular bed specific proteins are lost in endothelial cells when cultured and passaged in vitro (Chi et al., 2003; Lacorre et al., 2004), however several genes persist (Chi et al., 2003). Additionally, the location of Tie2 within the cell, and level of cell confluency is another source of phenotypic difference in endothelial cell function and downstream signalling (Fukuhara et al., 2008; Saharinen et al., 2008). These inherent differences add a layer of complexity to interpreting data obtained from endothelial cells. These caveats imply that findings from endothelial cells in vitro cannot be automatically extrapolated to in vivo systems.
Due to frictional forces from circulating blood, vascular endothelial cells are continuously exposed to shear stress. Certain geometries of blood vessels such as branch points, bifurcations, or curves create disturbed or turbulent blood flow patterns, whereas in straight blood vessels blood flow precedes in a unidirectional, laminar pattern. These hemodynamic forces have been shown to initiate functional changes in endothelial cell phenotypes. Endothelial cells sense shear forces applied by blood passing the luminal surface and respond by modulating intracellular signalling, leading to changes in shape and cytoskeletal organization. Endothelial cells in regions of normal laminar flow express lower levels of anti-inflammatory adhesion molecules and are less atheroprone than areas of turbulent flow (Hahn & Schwartz, 2009). As well, when grafted into the arterial circulation, endothelial cells collected from the venous circulation were shown to display an arterial function (Tsukurov et al., 2000). Further, endothelial cells collected from the veins were shown to affect an arterial phenotype when subjected to flow patterns typically found in arteries (Dai et al., 2004). In addition to responding to signals from other cell type, endothelial cells also integrate signals from blood flow patterns further underscoring the amazing plasticity and broad heterogeneity of endothelial cells.

1.1.5 Endothelial Cell – Cell Contacts

A fundamental function of the endothelium is to act as a gatekeeper or barrier while allowing for the controlled transport of fluids and solutes into and out of the blood and tissue. Paramount to this ability is the presence of junctions connecting endothelial cells to one another. Endothelial cells accomplish barrier function via several adherens junctions and less numerous tight junctions. The location of the endothelial cells within
the vascular bed dictates the tightness of these cell-cell junctions. Impermeable regions of the vasculature, such as the blood brain barrier, possess a high concentration of tight junctions (Dejana, 2004). Vascular endothelial cadherin (VE)-cadherin, an endothelial specific member of the cadherin family, is the major protein component of endothelial adherens junctions (Dejana, 2004). The major constituents of tight junctions are from the claudin family of proteins (Steed, Balda, & Matter, 2010).

VE-cadherin, the major factor in the maintenance and control endothelial cell-cell contacts, bridges endothelial cells together with homotypic interactions to form dimers with the VE-cadherin of adjacent cells (Turowski et al., 2008). Regulation of its activity and localization at cell contacts is essential for control of the permeability of the blood vessel wall. Tyrosine phosphorylation and dephosphorylation events regulate the internalization and recycling of VE-cadherin. VE-cadherin is constitutively associated with the endothelial cell specific phosphatase vascular endothelial protein tyrosine phosphatase (VE-PTP). VE-PTP maintains VE-cadherin in an unphosphorylated state and its localization at adherens junctions (Vestweber, 2012). VE-cadherin is linked to the cytoskeleton of endothelial cells through the association of the cytoplasmic tail of VE-cadherin with proteins of the catenin family (Vestweber, 2015). During leukocyte occupancy, VE-PTP dissociates from VE-cadherin, promoting phosphorylation and relocalization of VE-cadherin away from endothelial cell junctions and its internalization (Broermann et al., 2011; Vockel & Vestweber, 2013).
1.2 Receptor Tyrosine Kinases

The receptor tyrosine kinase (RTK) family are transmembrane proteins that possess an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic domain with tyrosine kinase catalytic activity (Hubbard & Till, 2000). RTK comprise a large family of receptors; in humans there are 58 members which are further organized into 20 subfamilies based on the sequence of their kinase domains (Robinson, Wu, & Lin, 2000). Binding of ligand to the extracellular domain of an RTK results in the clustering and oligomerization of several receptors units. Oligomerization brings the kinase domains of the receptors into proximity, which triggers the activation of each receptors’ intrinsic tyrosine kinase activity, allowing the activated cytoplasmic domains to transphosphorylate each other. The phosphorylated tyrosine residues are now able to serve as docking sites for signalling intermediates, which specifically recognize phosphorylated tyrosine residues via Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains leading to downstream intracellular signalling events involved in a wide range of biological responses (Schlessinger, 2000; van der Geer, Hunter, & Lindberg, 1994).
1.3 Tie2

Originally named Tek (Dumont et al., 1992), the receptor tyrosine kinase Tie2, and closely related receptor Tie1 (Partanen et al., 1992), belong to the Tie (tyrosine kinase with immunoglobulin and epidermal growth factor homology domains) family (Schnurch & Risau, 1993). Tie2 is expressed largely on endothelial cells but has also been shown to be expressed on a subset of leukocytes referred to as Tie2 expressing leukocytes (TEL), which includes a subset of monocytic cells (De Palma et al., 2005), neutrophils (Sturn et al., 2005), and eosinophils (Feistritzer et al., 2004).

1.3.1 Tie2 Function

Tie2 plays a critical role in vascular development and angiogenesis. Signalling from Tie2 is essential for embryonic development; previous research has demonstrated that Tie2 null mouse embryos die before embryonic day E12.5 due to insufficient sprouting of the primary capillary plexus, diffuse oedema, multifocal hemorrhages, and severe heart defects (Dumont et al., 1994). These mice also exhibit a dramatic decrease in total endothelial cell numbers, which was the original indication of a role for Tie2 in endothelial cell survival (Dumont et al., 1994).

In the adult, angiogenesis is less common, occurring only in the female reproductive system, during wound healing processes, and in pathological states (Carmeliet, 2003). In addition to angiogenesis, it is now recognized that Tie2 signalling in the adult is important for mediating endothelial survival, activation, integrity, and vascular inflammation (Barton et al., 2006). Tie2 is not only expressed in non-angiogenic mature vessels but is
also constitutively phosphorylated and activated (Sato et al., 1995; A. L. Wong et al., 1997), which is thought to stabilize the vasculature.

Tie2 is critically involved in the interactions between endothelial cells and underlying mural cells. When Tie2 is knocked out in blood vessels those vessels experience a dramatic decline in the number of connected mural cells (Patan, 1998). Additionally, mice lacking the endogenous Tie2 agonist Ang1 display a weak association of endothelial cells with the surrounding mesenchymal cells and matrix (Suri et al., 1996). Thus, Tie2 signalling is essential for the interactions of endothelial cells with neighboring cells and is critical for the stabilization and general well-being of the vasculature.
1.4 Tie2 Ligands

1.4.1 Angiopoietin

The Tie2 ligand angiopoietin-1 (Ang1) was initially suggested as the natural Tie2 agonist with the discovery that Ang1 deficient embryos share a common embryonic lethal phenotype with the Tie2 deficient embryos (Suri et al., 1996). Structural evidence later confirmed angiopoietin-Tie2 binding and angiopoietin dependent Tie2 activation (Barton et al., 2006; Maisonpierre et al., 1997). Conversely, overexpression of the Tie2 antagonist, Ang2, induces a similar vascular defect as observed in Tie2 and Ang1 deficient animals indicating that Ang2 functions as a Tie2 antagonist (Maisonpierre et al., 1997). Interestingly, Ang2 deficiency itself is not lethal during embryonic development, however, mice develop normally but die within two weeks as a consequence of lymphatic system defects including chylous ascites (Gale et al., 2002).

The angiopoietin family is made up of four members, Ang1-4 (S. Davis et al., 1996; Maisonpierre et al., 1997; Valenzuela et al., 1999). Ang1 and 4 are considered agonists of Tie2 activation, while Ang2 and Ang3 act as context dependent antagonists (Maisonpierre et al., 1997). The angiopoietins are glycoproteins containing a signal sequence that directs secretion from cells at the amino-terminus. They also possess a super-clustering domain (SCD), a coiled-coil domain (CC), and a fibrinogen-like domain (FLD). The SCD and CCD mediate multimerization of Angiopoietin monomers, while the FLD is responsible for the binding specificity of angiopoietins to Tie2 (S. Davis et al., 2003; K. T. Kim et al., 2005). Of the angiopoietins, Ang1 and Ang2 are the best characterized (Figure 1.1)
The amino acid homology between Ang1 and Ang2 is quite high (60%) yet they have distinct functional roles (Maisonpierre et al., 1997; Yu et al., 2013). Ang1 and Ang2 bind to the same site in the extracellular domain of Tie2 and with a similar affinity (Maisonpierre et al., 1997). Ang2 acts as a context competitive antagonist by blocking the ability of Ang1 to activate Tie2 in endothelial cells (Maisonpierre et al., 1997). Much of the difference in biology is thought to reside in how Tie2 is clustered by these closely related ligands. Ang2 exists as a dimer and Ang1 predominantly exists as a tetramer or a higher order multimer. A tetramer is the smallest cluster size able to effectively activate Tie2 (S. Davis et al., 2003; K. T. Kim et al., 2005; Procopio, Pelavin, Lee, & Yeilding, 1999). Interestingly, evidence indicates that higher order angiopoietin oligomers act as Tie2 agonists while engineered Ang1 dimers act as antagonists (S. Davis et al., 2003), providing a plausible explanation for the divergent functions of Ang1 and Ang2 (Figure 1.2).

Ang1 is primarily produced by pericytes with a small contribution from smooth muscle cells and platelets, suggesting a paracrine function (S. Davis et al., 1996; Sundberg, Kowanetz, Brown, Detmar, & Dvorak, 2002). In the adult vasculature Ang1 is widely expressed, consistent with its vasculature stabilizing functions. In contrast, Ang2 expression is associated mainly with sites of vascular remodeling, consistent with its angiogenic initiation and inflammatory functions (Maisonpierre et al., 1997). Pericytes constitutively produce Ang1, but expression can be upregulated by Sonic hedgehog (Shh) and downregulated by Fibroblast Growth Factor-2 (FGF-2) signalling (Fujii & Kuwano, 2010). Ang2 is produced by endothelial cells and is stored in Weibel-Palade Bodies.
(WPB) for rapid release through exocytosis in response to various stimuli including inflammatory signals suggesting autocrine functions (Fiedler et al., 2004).
Figure 1.1. Structure of clustered angiopoietin-1. Protein domains of angiopoietin-1 (Ang1). The super-clustering and coiled-coil domains collectively mediate multimerization of Angiopoietin monomers, while the fibrinogen-like domain is responsible for binding to Tie2 receptors. Adapted from (Augustin, Koh, Thurston, & Alitalo, 2009; H. Huang, Bhat, Woodnutt, & Lappe, 2010).
Figure 1.2. Tie2 is activated when multimeric Angiopoietin binds Tie2 and clusters the receptor. Clustering of Tie2 puts the tyrosine kinase domains of individual Tie2 receptors close together. Adjacent kinase domains phosphorylate each other, activating the receptors. Adapted from (Barton et al., 2006).
1.4.2 Vasculotide

Use of recombinant Ang1 in a clinical setting or larger experimental models is unrealistic. Large scale production of recombinant Ang1 is costly and is hindered by the insolubility of the protein. Ang1 is known to aggregate when injected into animals and has a short half-life. It is quickly absorbed by the vessel lining due to its high affinity for extracellular matrix (Saharinen et al., 2008). To circumvent these issues, a rationally designed Ang1 peptidomimetic was engineered by Dr. Dumont’s lab. This mimetic, termed Vasculotide (VT), is composed of four units of a 7-amino acid peptide known to specifically bind Tie2 (His-His-His-Arg-His-Ser-Phe (Tournaire et al., 2004)) covalently conjugated to a four-armed branched polyethylene glycol moiety (Kumpers et al., 2011) (Figure 1.3). Vasculotide activates Tie2 and has been shown to activate the known downstream targets of Tie2 (Bourdeau et al., 2016; David, Ghosh, et al., 2011; Korpela et al., 2014; Kumpers et al., 2011; Rubig et al., 2016; Sugiyama et al., 2015; Thamm et al., 2016; Trieu et al., 2017; Van Slyke et al., 2009). Recently, through use of tryptophan fluorescence spectroscopy, VT was shown to directly bind to the murine Tie2 receptor (Gutbier et al., 2017). The obligate tetramer formation of VT results in more reproducible activation of Tie2, compared to recombinant Ang1 which can form a variety of cluster sizes with variable activity. Unlike Ang1, VT has a low cost of production and is chemically stable.

In addition, while Ang1 has been shown to bind several different integrins including α2β1, α5β1, αvβ3, and αvβ5 (Cascone, Napione, Maniero, Serini, & Bussolino, 2005; Dallabrida, Ismail, Oberle, Himes, & Rupnick, 2005; C. C. Weber et al., 2005) while VT does not. The specificity of VT for Tie2 allows for the separation of integrin involvement from Tie2 activation.
Figure 1.3. Schematic of the structure of Vasculotide. Four monomers of a peptide known to specifically bind Tie2 (Histidine-Histidine-Histidine-Arginine-Histidine-Serine-Phenylalanine) (Tournaire et al., 2004) are covalently attached to a four-arm branched polyethylene glycol-maleimide moiety which acts as a scaffold to cluster the peptides into a tetrameric compound.
1.5 Tie2 Structure

Tie2 is a 140 kDa glycoprotein with an extracellular ligand binding domain, a single-pass transmembrane domain, and an intracellular tyrosine kinase domain (Dumont, Gradwohl, Fong, Auerbach, & Breitman, 1993). The extracellular ligand binding domain of Tie2 is composed of 3 immunoglobulin (Ig) domains, 3 epidermal growth factor (EGF) repeats, and 3 fibronectin type III (FNIII) repeats (Barton et al., 2006; Dumont et al., 1993; Jones, Iljin, Dumont, & Alitalo, 2001). The intracellular portion of Tie2 contains the juxtamembrane region, the catalytic domain divided by a kinase insert, and a carboxy-terminal tail that contains phosphorylation and protein interaction sites (Barton et al., 2006; Dumont et al., 1993; Macdonald et al., 2006; Schnurch & Risau, 1993) (Figure 1.4). In a non-activated state, the carboxy-terminal region of the receptor physically interferes with substrate binding through interaction with the kinase domain (Shewchuk et al., 2000). Tie family members, Tie2 and Tie1, display structural similarity. The cytoplasmic domains are highly conserved displaying ≈ 75% identity, while the extracellular domains are ≈ 30% similar (Schnurch & Risau, 1993).

Of the two Tie members, Tie2 is the best characterized. Tie1 is considered an orphan receptor as no Tie1 ligands have been identified (Leppanen, Saharinen, & Alitalo, 2017). This has hindered the full characterization of the Tie1 receptor. It has been reported however, that Tie1 and Tie2 can form heterodimers and in doing so Tie1 is able to regulate the activation of Tie2 in a mechanism that remains to be fully characterized (Leppanen et al., 2017). Tie1 does appear to play a strong role in the developing embryo as Tie1 deficient mice develop lymphatic vascular abnormalities, edema, microvessel rupture, and hemorrhaging. As a result embryos die between 13.5 and 14.5 of gestation
(D'Amico et al., 2010; Puri, Rossant, Alitalo, Bernstein, & Partanen, 1995). The biology and signalling events of Tie2 is the focus of this thesis.
Figure 1.4. **Domain structure of Tie2. Schematic of the protein domains of the Tie2 receptor.** The extracellular domain of the Tie2 receptor contains three immunoglobulin-like (Ig) domains with three epidermal growth factor (EGF) domains in between the second and third Ig domains, followed by three fibronectin type III repeats. Tie2 possesses a single-pass transmembrane domain, crossing the endothelial cell plasma membrane (EC). The intracellular domain possesses a juxtamembrane region, a split tyrosine kinase domain, and a carboxyl tail containing (Y) tyrosine residues important for the initiation of downstream signalling. Adapted from (Augustin et al., 2009; H. Huang et al., 2010).
1.6 Tie2 Activation and Signalling

A receptor tetramer is the smallest cluster able to activate Tie2 signalling (Eklund & Olsen, 2006; Hubbard & Till, 2000; Jones et al., 2001; Jones et al., 1999). The Tie2 activation loop contains one tyrosine (Y) residue at position Y992 (Jones et al., 1999). This residue is the first to become phosphorylated upon Tie2 receptor activation. Subsequent autophosphorylation provides high-affinity binding sites for the docking of adaptors. The cytoplasmic domain of Tie2 possesses 19 intracellular tyrosine residues. Of these residues, Y1100, Y1106, and Y1111 of the carboxy-terminal tail are phosphorylated and required for downstream Tie2 signalling by acting as docking sites for adapter proteins (Jones et al., 1999) (Figure 1.5).

The p85 subunit of PI3K is recruited to activated RTK by its SH2 domains. Binding of p85 to phosphorylated tyrosine confers a conformational change in p85. This relieves the inhibitory action of p85 on the p110 catalytic subunit and brings p110 into proximity with the plasma membrane where PI3K converts PtdIns (4,5)P\(_2\) into PtdIns (3,4,5)P\(_3\). Production of PtdIns(3,4,5)P\(_3\) results in the recruitment and activation of the serine-threonine kinase Akt, subsequently resulting in downstream signalling that affects various cellular processes such as growth, survival, and proliferation (Vivanco & Sawyers, 2002). Tyrosine residue Y1100 on Tie2 has been shown to interact with the p85 subunit of PI3K (Jones et al., 1999). This Tie2 dependent activation of PI3K/Akt signalling is essential for the endothelial cell survival effects of Tie2 activation (Fujikawa et al., 1999; Jones et al., 1999; I. Kim et al., 2000; Kontos et al., 1998; Kwak et al., 2000; Papapetropoulos et al., 2000). PI3K is also partly responsible for the cell migration effects of Ang1 on
endothelial cells (Jones et al., 1999; I. Kim et al., 2000; Moon et al., 2015; Witzenbichler, Maisonpierre, Jones, Yancopoulos, & Isner, 1998).

Phosphorylation of Y1100 also allows for the SH2 domain of Grb2 to bind activated Tie2 (Jones et al., 1999). Grb2 is an adapter protein that signals through the Ras-MEK-ERK1/2 pathway (Qu et al., 2014). Grb2 is found in a complex with the guanine nucleotide exchange factor son of sevenless (SOS) via interactions of the SH3 domain of Grb2 with the proline rich regions of SOS. Interaction of the Grb2-SOS complex with receptors such as Tie2 recruits the complex to the plasma membrane bringing it into proximity with Ras, located at the cell membrane. SOS subsequently converts Ras from its inactive GDP-bound state to its active GTP-bound state. Activated Ras can then interact with Raf, a serine kinase, which in turn catalyzes the activation of Mek1/2 resulting in the phosphorylation and activation of Erk1/2 (Giubellino, Burke, & Bottaro, 2008). The tyrosine residue Y1100 on Tie2 has also been shown to interact with Grb7 (Jones et al., 1999). Overexpression studies indicate that Grb7 becomes tyrosine phosphorylated by the Tie2 receptor (Jones et al., 1999). The consequence of Grb7 activation following Tie2 activation is not understood.

The tyrosine residue Y1100 on Tie2 has been shown to act as a multidocking site for several SH2 domain containing adapter molecules (Jones et al., 1999). Little is known about potential recruitment preferences of one signalling molecule over the other as well as the temporal dynamics of recruitment.

Phosphorylated Y1106 of Tie2 has been shown to bind the PTB domain of Downstream of Tyrosine Kinases 2 (Dok2, formerly DokR) resulting in the phosphorylation of Dok2
on multiple tyrosine residues (Jones et al., 2003). This association and phosphorylation of Dok2 creates a binding site for the adaptor protein Nck, which in cooperation with the p21 activating kinase (PAK), has been implicated in cell motility and actin rearrangement. This association has been implicated in Ang1 dependent cell motility (Jones et al., 2003; Master et al., 2001). Phosphorylated Y1111 has been shown to bind the SH2 domains of the ubiquitously expressed tyrosine phosphatase Shp2 (L. Huang, Turck, Rao, & Peters, 1995; Jones et al., 1999). Shp2 does not become phosphorylated when co-expressed with activated Tie2 (Jones et al., 1999). Overexpression of Shp2 in HEK293T cells did not reduce the phosphorylation of the Tie2 receptor (Sturk, Kim, Jones, & Dumont, 2010) suggesting that Shp2 does not regulate Tie2 phosphorylation. The effect of Shp2 on Tie2 activation remain unclear.
**Figure 1.5. Tie2 mediated signal transduction.** Schematic representation of select signal transduction events that occur upon Tie2 activation. Following activation, tyrosine (Y) residues Y1100, Y1106, and Y1111 become phosphorylated and act as docking sites for adapter proteins. Y1100 serves as a multi-docking site for the PI3K/Akt subunit p85, Grb2, and Grb7. Y1106 is the docking site for DokR/Dok2 resulting in Nck/PAK signalling, and Y1111 associates with Shp2. Adapted from (Jones et al., 1999).
1.7 Tie2 Localization

Context is a principal factor in the outcome of Tie2 signalling. Endothelial cells are known to have vascular bed and tissue specific heterogeneity (Aird, 2007). Phenotypic differences are common between endothelial cells derived from lymphatic vessels and blood vessels (Nguyen et al., 2007). Endothelial cells have unique molecular signatures that distinguish those derived from lymphatic or blood vessels.

In sparsely confluent cultured endothelial cells, Tie2 clusters at the basal plasma membrane with extracellular matrix bound Ang1. In this context, activated Tie2 signals preferentially to the ERK pathway resulting in increased cell migration. In more densely confluent cells, Ang1 causes Tie2 to translocate to cell-cell contacts, where Ang1 bridges Tie2 receptors across neighboring cells conferring vessel stability (Fukuhara et al., 2008; Saharinen et al., 2008). Due to these inherent differences it is essential for researchers to keep the cellular context as consistent as possible when working with endothelial cells.
1.8 Regulation of Tie2

As a factor involved in cellular survival and angiogenesis, the Tie2 signalling axis requires exquisite control. Tie2 activation is controlled by a natural agonist and a competitive antagonist, Ang1 and Ang2, respectively. Additionally, receptor internalization and degradation downregulates Tie2 signalling. Binding of Ang1 was shown to induce Tie2 internalization and degradation (Bogdanovic, Nguyen, & Dumont, 2006). The Angiopoietin ligand is released and not internalized (Bogdanovic et al., 2006). It was later demonstrated that Tie2 activation results in ubiquitylation of the Tie2 receptor by the E3 ubiquitin ligase, c-Cbl, leading to its internalization and degradation (Wehrle, Van Slyke, & Dumont, 2009).

The physical structure of Tie2 also provides a level of control. Unlike many RTK, the activation loop of Tie2 adopts an ‘active like’ conformation even in the absence of phosphorylation. The crystal structure of Tie2 indicates that the carboxy-terminal tail of Tie2 is in position to physically interfere with the activation of Tie2 by blocking the substrate binding site (Shewchuk et al., 2000). Carboxy-terminal tyrosine residue Y1111 appears to play an important role in this negative regulation as the Y1111F mutant displays increased receptor phosphorylation and kinase activity (Sturk et al., 2010). Additionally, deletion of carboxy-terminal residues 1108-1123 was shown to increase Tie2 receptor kinase activity and signalling (Niu, Peters, & Kontos, 2002).

Removal of phosphate groups by tyrosine phosphatases are a common means of RTK control. Treatment of cells with the phosphatase inhibitor sodium orthovanadate results in an increase in Tie2 phosphorylation (Jones et al., 1999), which indirectly suggests that phosphatases do in fact play a functional role in the regulation of Tie2 activation. One
phosphatase shown to reduce Tie2 activation is the protein tyrosine phosphatase VE-PTP (Fachinger, Deutsch, & Risau, 1999). Tie2 also interacts with the phosphatase Shp2 (Jones et al., 1999), however, an effect on Tie2 phosphorylation has not been demonstrated. Overexpression of Shp2 in HEK293T cells does not reduce the phosphorylation of the Tie2 receptor (Sturk et al., 2010).

Additionally, the extracellular ligand-binding domain of Tie2 undergoes proteolytic cleavage producing a soluble Tie2 fragment in response to vascular endothelial growth factor (VEGF) treatment, and has been observed in blood from healthy adults (Findley, Cudmore, Ahmed, & Kontos, 2007; Onimaru, Yonemitsu, Suzuki, Fujii, & Sueishi, 2010; Reusch et al., 2001). Cleavage of Tie2 dampens Tie2 signalling by reducing the number of receptors that Ang1 can bind, and by producing a soluble fragment that can bind to and sequester Ang1. This prevents the activation of other Tie2 receptors (Findley et al., 2007), suggesting that cleavage of the Tie2 ectodomain may be an important mechanism of Tie2 regulation.
1.9 Pathological Angiogenesis and the Involvement of Tie2

Angiogenesis is essential for development of the embryonic vasculature and is required postnatally during growth and wound healing, but it can also enact deleterious effects. Inappropriate angiogenesis has been implicated in a variety of pathologies including tumorigenesis, psoriasis, arthritis, atherosclerosis, endometriosis, and diabetic retinopathy (Capobianco et al., 2011; Carmeliet, 2003). As an integral factor in the angiogenic process, Tie2 is involved in these processes. Indeed, overexpression of Tie2 in the skin can cause a psoriasis-like phenotype in mice (Voskas et al., 2005). In rare cases, venous malformations have been linked to an autosomal dominant mutation causing an arginine to tryptophan substitution (R849W) in the kinase domain of Tie2, rendering the receptor constitutively active and increased endothelial cell survival via the PI3K/Akt pathway (Calvert et al., 1999; Morris et al., 2005; Vikkula et al., 1996). Thus, aberrant angiogenesis caused by deregulated Tie2 signalling can have detrimental effects.
1.10 Inflammation, Immunity, and Clinical Impact

The cardinal signs of inflammation: rubor (redness), calor (tissue warmth), tumour (swelling), and dolor (pain) are all tightly coupled to the response of the vasculature during the inflammatory response. An increase in blood flow, as a result of dilation of blood vessels, results in local redness and warmth at the site of inflammation. The swelling is caused by an increase in endothelial permeability of the blood vessels, recruitment and activation of leukocytes, and the leakage of protein rich fluid known as exudate. Exudate provides a provisional matrix for the extravasation of leukocytes to the site of inflammation. Following recruitment and activation the leukocytes release chemical mediators that act on sensory nerve fibers causing the sensation of pain (Pober & Sessa, 2007). These responses highlight the ability of endothelial cells to dynamically modify their phenotypes during the inflammatory process and coordinate the response to inflammatory stimuli.

1.10.1 Vascular Inflammation

Inflammation is an essential event for the elimination of pathogenic microbes to protect the host from infection. The endothelium is an active participant in this response. TLR4-Nf-κB signalling leads to endothelial activation, increased expression of inflammatory cytokines and luminal adhesion molecules, as well as an overall decrease in endothelial barrier function (Dauphinee & Karsan, 2006). Cell adhesion molecules allow the leukocytes to adhere to endothelial cells promoting their extravasation from the circulation through endothelium to the site of infection (Parikh, 2013). If inflammation is not resolved following pathogen clearance it can also paradoxically cause detrimental
effects to the host. This process is fundamental to the vascular component of several pathologic inflammatory states ranging from acute lung and kidney injury to sepsis (David, Kumpers, van Slyke, & Parikh, 2013).

1.10.2 Endothelial Response to Endotoxin

Endothelial cells play an active role in inflammatory responses. Lipopolysaccharide (LPS) is a critical glycolipid constituent of the cell wall of gram-negative bacteria, such as *E. coli*. LPS is recognized on endothelial cells by toll-like receptor 4 (TLR4). LPS-binding protein (LBP) assists TLR4 by binding to LPS and forming a complex with CD14, a co-receptor involved in the recognition of LPS by TLR4 (Dunzendorfer, Lee, Soldau, & Tobias, 2004; Schumann et al., 1990). TLR4 is found in a complex with another co-receptor, MD2. This complex receives the LPS from the LBP-CD14 complex resulting in initiation of the TLR4 signalling cascade (da Silva Correia, Soldau, Christen, Tobias, & Ulevitch, 2001; Wright, 1995), which ultimately gives rise to the activation of the transcription factor Nf-κB (Akira, Uematsu, & Takeuchi, 2006; Alexander & Rietschel, 2001). In endothelial cells this can result in the upregulation of several proinflammatory mediators including adhesion molecules, inflammatory cytokines, and chemokines. It can also result in the loss of endothelial barrier function causing a permeable and pro-coagulant vasculature, and allowing the recruitment of immune cells (Dauphinee & Karsan, 2006).
1.10.3 Extravasation of Leukocytes Across the Endothelial Barrier

In order for an immune reaction to successfully eradicate microbial invaders, leukocytes circulating in the blood must be able to move from the blood to the site of the eliciting stimulus. Vascular permeability and leukocyte extravasation are tightly controlled events that occur during inflammation. Inflammatory stimulation leads to the activation of endothelial cells that support the extravasation of leukocytes. During endothelial activation a leukocyte adhesion cascade is initiated (Figure 1.6). The translocation of leukocytes across the endothelium is a complex multistep process that includes capturing, slow rolling, firm adhesion, intraluminal crawling to sites of exit, paracellular or transcellular migration, and finally migration through the basement membrane (Ley, Laudanna, Cybulsky, & Nourshargh, 2007; Rao, Yang, Garcia-Cardena, & Luscinskas, 2007). This process is supported by the upregulation of specific adhesion molecules on endothelial cells following activation. Selectins and integrins are essential for the initial capturing and rolling steps. Activated endothelial cells present E-selectin and P-selectin on the cell surface while leukocytes express L-selectin (Dauphinee & Karsan, 2006). Selectins are crucial for the endothelial cells to capture leukocytes from flowing blood. Inflammatory cytokines released by endothelial cells via exocytosis of intracellular granules such as Weibel-Palade bodies or deposited on the endothelial surface by other cell types, trigger leukocyte activation and arrest on the endothelium (Kinashi, 2005).

Activation of leukocytes situated on the endothelium leads to the activation of the integrins expressed on leukocytes, which bind to adhesion molecules induced on the cell surface of endothelial cells following inflammatory stimulation. These adhesion proteins
are members of the endothelial immunoglobulin superfamily including vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule 1 (ICAM-1) (Ley et al., 2007). The activation of integrins involves a change in the conformation of the integrin proteins from the resting low-affinity compact integrin structure to a high-affinity conformation where the ligand binding site is exposed (Luo, Carman, & Springer, 2007). Once firmly adhered to the endothelium the leukocytes move along the endothelial cell surface until suitable sites for transmigration are located (Phillipson et al., 2006). Leukocytes preferentially transmigrate in the junctions between adjacent endothelial cells. These junctions are transiently opened by endothelial-mediated signalling triggered by the adhesion of leukocytes, which increases the permeability of the endothelial barrier (Vestweber, 2015).

For leukocytes to translocate from the circulating blood to the eliciting stimulus, leukocytes must cross though the endothelium, pericytes, and the extracellular matrix. Leukocytes will most often cross between adjacent endothelial cells (paracellular) through the protein rich cell-cell junctions, but can also cross through the endothelial cells themselves (transcellular) (Ley et al., 2007). Intracellular signalling triggered by leukocytes results in the transient opening or widening of endothelial cell to endothelial cell junctions (Vestweber, 2015). The docking of leukocytes on endothelial cells results in transient changes in Ca^{2+} levels leading to contractions in an actomyosin-dependent fashion, resulting in the endothelial cells being pulled apart from one another, as well as the re-localization and internalization of VE-Cadherin (Nourshargh & Alon, 2014). VE-cadherin is regarded as the major junctional protein preventing the extravasation of leukocytes. Binding of leukocytes to ICAM-1 and VCAM-1 results in the
phosphorylation of VE-cadherin (Vestweber, 2015) Phosphorylation of VE-cadherin causes its redistribution from cell contacts and its dissociation from homotypic interactions with adjacent cells resulting in weakened adherens junctions, increased permeability, and promotion of leukocyte emigration (Turowski et al., 2008). Several additional endothelial cell proteins, including platelet endothelial cell adhesion molecule 1 (PECAM-1), junctional adhesion molecules (JAMs), endothelial cell selective adhesion molecule (ESAM) (Nourshargh & Alon, 2014), among others, are involved in assisting in the extravasation of leukocytes and are located at endothelial cell junctions (Carman & Springer, 2004). It takes a leukocyte approximately 2-3 minutes to traverse the endothelial cell layer.

After penetrating the endothelial cell barrier, leukocytes must then cross the underlying vascular basement membrane and associated pericytes through a process that generally takes about 15-20 minutes (Vestweber, 2015). How leukocytes are able to breach the basement membrane is not completely understood, however, research has identified regions of the vascular basement membrane that have fewer matrix proteins, making the region more permeable (Voisin, Probstl, & Nourshargh, 2010). These weaker regions of the basement membrane are located in the spaces between adjacent pericytes and act as gates for leukocyte transmigration (Voisin & Nourshargh, 2013; Voisin et al., 2010; Voisin, Woodfin, & Nourshargh, 2009). Additionally, emigrating leukocytes express matrix metalloproteinase enzymes that have the capacity to degrade extracellular matrix proteins, however, the contribution of proteolytic cleavage by emigrating leukocytes to the transmigration process is unknown and not agreed upon (Rowe & Weiss, 2008).
Figure 1.6. Leukocyte migration cascade. Schematic representation of the leukocyte adhesion and transmigration process through a blood vessel and the underlying basement membrane and pericytes. Adapted from (Vestweber, 2015).
1.11 Inflammatory Response

The immune response is the body’s orchestrated effort to sequester and eliminate invading pathogens and disease. The immune system is divided into two distinct categories; the innate and adaptive immune system. The innate immune system is the body’s first line of defense following infection and tissue damage. Inflammation begins when cells of the innate immune system detect an invader. These immune cells include macrophages, neutrophils, eosinophils, basophils, dendritic cells, and natural killer cells. Innate immune cells possess pattern recognition receptors (PRR) that recognize pathogen or damage associated molecular patterns (PAMP and DAMP respectively). PAMP are highly conserved, essential features of a microbe (D'Elia, Harrison, Oyston, Lukaszewski, & Clark, 2013), while DAMP are host-specific molecules released following cell stress such as ischemia, trauma, or necrotic death. Binding of PAMP to PRR results in receptor activation and the initiation of an inflammatory response. PRRs include C-type lectin receptors, Nod-like receptors (NLR), Mannose binding lectin, Retinoic acid-inducible gene like receptors (RLR), and Toll-like receptors (TLR) (D'Elia et al., 2013).

1.11.1 TLR

There are 11 identified TLRs in humans (O'Neill, 2008). TLRs are type 1 transmembrane proteins containing a cytosolic Toll/interleukin-1 receptor (IL-1R) (TIR) homology domain that activates downstream signalling cascades (Kawai & Akira, 2011). TLRs are highly expressed in antigen-presenting cells including macrophages and dendritic cells, and are also expressed in other cell types. TLR4, the best characterized TLR member, has
been identified in endothelial cells (Dauphinee & Karsan, 2006), adipocytes (Vitseva et al., 2008), and myocytes (Frantz et al., 1999) in addition to leukocytes.

TLR and IL-1R (interleukin-1 receptor) signalling cascades share common intermediates including Tumor necrosis factor receptor-associated factor 6 (TRAF6), IL-1 receptor-associated kinase 1 (IRAK1), IRAK4, and myeloid differentiation factor 88 (MyD88) (Muroi & Tanamoto, 2012). TLR4 is the primary sensor of gram-negative bacteria, is a sensor for host derived damage associated molecular patterns released following cellular stress (Poltorak et al., 1998), and is expressed on endothelial cells. These facts implicate TLR4 as an indispensable regulator of vascular inflammation.

1.11.2 TLR4

TLR4 recognizes PAMP which includes molecular structures from pathogens including LPS (O’Neill et al., 2003), and DAMP which includes High Mobility Group Box 1 protein, a DNA binding protein released from the nucleus as a result of tissue injury and inflammation (Nogueira-Machado, Volpe, Veloso, & Chaves, 2011). Ligand binding induces the dimerization of the TLR receptors at their respective TIR domains (Weber, Moncrieffe, Gangloff, Imler, & Gay, 2005). Dimerization of TLR4 results in a conformational change in the TLR cytoplasmic domain, which in turn acts to recruit adaptor molecules. TLR4 binds the adaptor protein myeloid differentiation factor 88 (MyD88), or a combination of TIR-domain-containing adapter-inducing interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) in the MyD88 independent pathway.

In the canonical MyD88-dependent pathway (Fig. 1.7), MyD88 is recruited to TLR4 upon ligand binding through TLR4 and MyD88’s TIR domains. Subsequently, MyD88
recruits, via its death domain (DD), the serine/threonine kinases IRAK1, and IRAK4 (O’Neill et al., 2003). Within the receptor complex, IRAK4 phosphorylates IRAK1, which causes autophosphorylation and activation of IRAK1 itself (Cao, Xiong, Takeuchi, Kurama, & Goeddel, 1996; S. Li, Strelow, Fontana, & Wesche, 2002; Wesche, Henzel, Shillinglaw, Li, & Cao, 1997). Phosphorylated IRAK1 loses affinity for MyD88 and this allows for interaction with TRAF6, leading to oligomerization of TRAF6 (Cao et al., 1996; Wesche et al., 1997). The complex of IRAK1 and TRAF6 dissociates from the receptor to form a complex at the membrane with TAK1 and the adaptor molecules, TAB1 and TAB2. TAB2 links TAK1 to TRAF6 and TAB1 activates TAK1, leading to the ubiquitination of TRAF6 (Jiang, Ninomiya-Tsuji, Qian, Matsumoto, & Li, 2002) (Kishida, Sanjo, Akira, Matsumoto, & Ninomiya-Tsuji, 2005; Takaesu et al., 2000). TRAF6 auto-ubiquitinates itself, forming Lys$^{63}$ linked polyubiquitin chains (Deng et al., 2000; Jiang et al., 2002). NEMO, a regulatory component of the IκB kinase (IKK) complex, binds Lys$^{63}$ linked polyubiquitin chains on TRAF6. This results in activation of the IKK complex leading to the phosphorylation and degradation of the Inhibitor of κB (IκB) inhibitory proteins. The IκB inhibitory proteins sequester Nf-κB in the cytoplasm in an inactive state, however, following degradation of the IκB complex Nf-κB becomes activated and is now able to translocate to the nucleus (discussed in further detail below) (Arthur & Ley, 2013; Deng et al., 2000; Wang et al., 2001).

### 1.11.3 Nf-κB

Nf-κB (nuclear factor of κB) was first identified as a molecule able to bind to the enhancer regions of immunoglobulin light chain genes (Sen & Baltimore, 1986). Since then the Nf-
κB family of transcription factors has been shown to play a vital role in innate immunity and associated inflammation.

1.11.3.1 Nf-κB Activation and Signalling

In unstimulated cells Nf-κB is held in an inactive state by being sequestered in the cytoplasm by Inhibitor of κB (IκB) inhibitory proteins (Chen et al., 1995). There are three typical IκB molecules that are known to interact with Nf-κB in the cytosol: IκBα, IκBβ, and IκBε (Perkins, 2007). Nf-κB family member subunits, p50, p52, RelA/p65, cRel, and RelB, assemble into homo or heterodimers and bind DNA elements, known as κB sites, in the enhancers/promoters of target genes. All members possess a Rel homology domain, which allows Nf-κB to dimerize and bind to DNA. The dimer of p50/p65 is the predominant Nf-κB in endothelial cells, and preferentially associates with IκBα (Ghosh et al., 1990).

IκBα is the prototypical member of the IκB family (Hayden & Ghosh, 2004). In endothelial cells, binding of IκBα specifically masks the nuclear localization sequence on Nf-κB and inhibits its import into the nucleus (Malek, Chen, Huxford, & Ghosh, 2001; Zen, Karsan, Eunson, Yee, & Harlan, 1998). Following phosphorylation of the IκB proteins by the IκB kinase (IKK) complex, the inhibitory IκB proteins are targeted for degradation. This results in the Nf-κB dimer being freed from the inhibitory complex allowing it to translocate to the nucleus where it binds to specific sequences in the promotor or enhancer regions of target genes (Malek et al., 2001).

The IKK complex is composed of three subunits: IKKα, IKKβ and the regulatory subunit IKKγ or NEMO (Nf-κB essential modulator) (Yamamoto & Gaynor, 2004). NEMO, in
complex with IKKα and IKKβ, is recruited to K63 polyubiquitin linked TRAF6, facilitating TAK1-mediated phosphorylation of the IKK complex (Arthur & Ley, 2013). IKK phosphorylate IκB proteins on two serine residues which leads to their polyubiquitination and degradation by the 26S proteasome, freeing Nf-κB for nuclear import (Zen et al., 1998).

The Nf-κB subunit p65 is subject to a variety of post-translational modifications that affect the duration and magnitude of Nf-κB activation. p65 has been shown to be phosphorylated on serine and threonine residues and acetylated or methylated on lysine residues (B. Huang, Yang, Lamb, & Chen, 2010).

1.11.3.2 Nf-κB Target Genes

Nf-κB induces the transcription of numerous target genes that encode proteins involved in immune and inflammatory responses of the vasculature and the endothelium. In endothelial cells these proteins include inflammatory cytokines such as Interleukin 6 (IL-6) (Libermann & Baltimore, 1990), IL-8 (Kunsch & Rosen, 1993), monocyte chemotactic protein 1 (MCP-1) (Ueda et al., 1994), and cell adhesion molecules including E-Selectin (Schindler & Baichwal, 1994; Whelan et al., 1991), vascular cell adhesion molecule (VCAM-1) (Iademarco, McQuillan, Rosen, & Dean, 1992), and intracellular cell adhesion molecule 1 (ICAM-1) (Bunting et al., 2007; van de Stolpe et al., 1994).

1.11.3.3 Control of Nf-κB Activation

To avoid chronic inflammation, the level of Nf-κB activation is tightly controlled. Several molecular events are in place to regulate the intensity and duration of an inflammatory
response. For example, activation of Nf-κB induces the production of IκB inhibitory proteins. Unbound IκBα contains a nuclear localization sequence and is imported into the nucleus where it associates with Nf-κB. This enables IκBα to transport Nf-κB out of the nucleus since IκBα also contains a nuclear export sequence that allows the exportin machinery to transport the IκBα and bound Nf-κB back to the cytoplasm (Arenzana-Seisdedos et al., 1995).

The degradation of signalling proteins allows the magnitude and length of cellular signals to be controlled. Phosphorylation of IRAK1 by IRAK4 leads to the degradation of IRAK1 (Kubo-Murai et al., 2008). Although the exact mechanism is not fully understood, this is likely a proteasome dependent mechanism because non-specific proteasome inhibitors have been shown to inhibit the degradation of IRAK1 (Yamin & Miller, 1997). Following TLR stimulation, TRAF6 is associated with Lys48-ubiquitinated IRAK1, leading to the degradation of both IRAK1 and TRAF6 by the proteasome (Muroi & Tanamoto, 2012). Additionally, TRAF6 was shown to be Lys48 poly-ubiquitinated, in a Suppressor of Cytokine Signalling 2 (SOCS2) dependent mechanism, and subsequently degraded by the proteasome (McBerry, Gonzalez, Shryock, Dias, & Aliberti, 2012). Proteasomal degradation of TLR4 signalling intermediates allows for the negative regulation of signalling.

An additional means of regulating the inflammatory response is through RNA silencing and post-transcriptional regulation of inflammatory gene expression by microRNA (miRNA). miRNA are small (20-22 nucleotides), non-coding RNA molecules that function through base pairing with complementary sequences within the 3’ untranslated regions of mRNA molecules. miRNA silence mRNA molecules by cleaving the mRNA
transcript, destabilizing the mRNA through its poly(A) tail, or causing less efficient ribosomal translation of the mRNA into proteins (Bartel, 2009; Geraldo, Yamashita, & Kimura, 2012; O'Neill et al., 2003; Sevignani, Calin, Siracusa, & Croce, 2006). miRNAs are transcribed from genomic DNA to primary miRNA (pri-miRNA) transcripts that are then processed by the ribonuclease, Drosha, into precursor miRNA (pre-miRNA). Pre-miRNA are then exported from the nucleus into the cytoplasm and finally processed by the ribonuclease, Dicer, into mature miRNAs (Sevignani et al., 2006).

Insights into the role of miRNA as powerful regulators of inflammation is beginning to emerge and several miRNA have been implicated in the endothelial cell response to inflammatory signalling. miRNA-126, miRNA-31, miRNA-17-3p, and miRNA-146b-5p inhibit the expression of VCAM-1 (Harris, Yamakuchi, Ferlito, Mendell, & Lowenstein, 2008), E-selectin (Suarez, Wang, Manes, & Pober, 2010), ICAM-1 (Suarez et al., 2010), IRAK1 and TRAF6 (Taganov, Boldin, Chang, & Baltimore, 2006), respectively. Emerging evidence continually implicates the miRNA network in the control of inflammation.
Figure 1.7. Binding of LPS to TLR4 results in the activation of the transcription factor Nf-κB. Activation of Toll-like receptor 4 (TLR4) results in the recruitment of myeloid differentiation primary-response protein 88 (MyD88), which in turn recruits IL-1 receptor-associated kinases 1 and 4 (IRAK1 and IRAK4). This induces a complex with TNF receptor-associated factor 6 (TRAF6) leading to the formation of K$_{63}$-linked polyubiquitin chains (Ub) on TRAF6. Nf-κB essential modulator (NEMO), complexed with IKKα and IKKβ, binds to polyubiquitin linked TRAF6 facilitating the TGFβ-activated kinase 1 (TAK1)-mediated phosphorylation of IKKβ. This induces the phosphorylation and subsequent degradation of the IκB inhibitory complex allowing for the nuclear translocation of nuclear factor-κB (Nf-κB) subunits. Adapted from (Arthur & Ley, 2013).
1.12 Anti-Inflammatory Effects of Tie2

1.12.1 Endothelial Inflammation

The endothelium was once thought to simply function as a passive cell lining of the blood vessels, but it is now accepted that the endothelial cells making up the endothelium are actually very metabolically active and involved in the initiation and resolution of innate immune responses. One way that the endothelium mediates inflammation is through the Tie2 signalling axis.

The discovery that Tie2 was expressed and substantially phosphorylated in the mature, non-angiogenic adult vasculature, suggested that Tie2, in addition to its established role in vascular development and angiogenesis, is also important for maintenance functions in the mature endothelium (A. L. Wong et al., 1997). In landmark studies Thurston et al. reported that mice overexpressing Ang1, Tie2’s natural agonist, were resistant to VEGF-, serotonin-, and mustard oil- induced vascular leakage (Thurston et al., 2000; Thurston et al., 1999). Since then Tie2 has been shown to abrogate vascular permeability induced by bradykinin, thrombin, Tumor necrosis factor (TNF), and LPS (Fiedler et al., 2006; Gamble et al., 2000; I. Kim, Moon, Park, Chae, & Koh, 2001). Studies using viral overexpression of Ang1 (Witzenbichler, Westermann, Kneippel, Schultheiss, & Tschope, 2005), direct administration of recombinant Ang1 protein (David, Park, et al., 2011), and the synthetic Tie2 agonist VT (David, Ghosh, et al., 2011; Kumpers et al., 2011) have implicated Ang1/Tie2 signalling as an important means of vascular regulation following endotoxin exposure.
As an antagonist of Tie2, Ang2 is considered an inflammatory agent. Ang2 is produced and stored in Weibel-Palade bodies within endothelial cells themselves to be rapidly released following inflammatory stimulation (Fiedler et al., 2004). This ability to rapidly induce circulating Ang2 suggests an important function for Ang2 beyond blood vessel remodeling. Ang2 results in increased vascular permeability and promotes endothelial activation and neoangiogenesis (Benest et al., 2013; Fiedler et al., 2006; Le et al., 2015; Oliner et al., 2004). Ang2 primes the endothelium to an inflammatory response prior to the exposure with the inflammatory stimulant TNF (Fiedler et al., 2006). Mice deficient in Ang2 are unable to mount an effective immune response (Fiedler et al., 2006). In a model of sepsis it was shown that Ang2 heterozygous mice developed milder kidney and lung injury, less tissue inflammation, less vascular leakage, and experienced improved survival compared to their wild-type littermates (David et al., 2012). These experimental results further implicate the Angiopoietin-Tie2 signalling axis in the regulation of vascular inflammation.

Interestingly, studies in patients with sepsis, acute lung injury, and related conditions have shown that high circulating levels of Ang1 are associated with decreased mortality in sepsis and conversely, elevated Ang2 levels correlate with disease severity and predict mortality in sepsis (David et al., 2012; Gallagher et al., 2008; Meyer et al., 2011; Orfanos et al., 2007; Parikh et al., 2006; van der Heijden et al., 2009; van der Heijden, van Nieuw Amerongen, Koolwijk, van Hinsbergh, & Groeneveld, 2008). The levels of circulating Ang1 are generally found to be around 2 fold depressed compared to baseline while the increase in Ang2 levels can be up to 20 fold (Parikh, 2013). The mechanisms driving this dysregulation are unknown, but do underscore the importance of the Tie2 signalling axis.
in vascular inflammation. The ratio of circulating Ang2/Ang1 correlates with clinical outcome, and has been suggested for use as a biomarker for sepsis prognosis (Parikh, 2013). Collectively, these studies provide a case for the clinical importance of Tie2 signalling in the regulation of vascular function in response to endotoxin exposure.

In cultured human umbilical vein endothelial cells (HUVEC), Ang1 has been shown to decrease the expression of Nf-κB responsive genes, ICAM-1, VCAM-1, and E-selectin following vascular endothelial growth factor (VEGF) stimulation (I. Kim et al., 2001). Ang1 stimulation has also been shown to ameliorate the LPS induced increase in Nf-κB luciferase reporter activity. Further, it also reduces monocyte cell adherence, and the expression of VCAM-1 and ICAM-1 (Echavarria et al., 2015).

1.12.2 Endothelial Barrier

The endothelium makes up the inner lining of the vasculature, and serves as the barrier between blood and tissue. The endothelium is a semipermeable barrier that plays a pivotal role in regulating haemostasis by regulating vascular tone, and the controlled transport of oxygen/carbon dioxide, nutrients, and metabolites, and maintains blood fluidity. Inflammatory activation converts the endothelium to a major participant in the inflammatory process (Dauphinee & Karsan, 2006). An activated endothelium leads to the recruitment of leukocytes, as well as hyperpermeability, which leads to tissue oedema. This allows for the extravasation of immune cells across the endothelium to the site of infection (Parikh, 2013). This TLR4 driven process is fundamental to the vascular component of several inflammatory states ranging from acute lung and kidney injury to
sepsis (David et al., 2013; Echavarria et al., 2015; Parikh, 2013; Pober & Sessa, 2007; Rubig et al., 2016).

Tie2 signalling reduces endothelial permeability; this is an essential mechanism by which Tie2 exerts its anti-inflammatory effects. Importantly, Tie2 signalling is able to maintain barrier function in response to gram-negative endotoxin. The presence of excess Ang1, via adenoviral transfer, reduced vascular leakage and cellular inflammation in a mouse model of endotoxemia (Witzenbichler et al., 2005).

Activation of Tie2 through VT has also been shown to exert endothelial barrier stabilizing effects. In microvascular endothelial cells stimulated with LPS and 5% septic serum, pro-inflammatory inducers of vascular permeability, VT protected against inter-endothelial gap formation (David, Ghosh, et al., 2011). In addition, VT has also been shown to prevent lung microvascular leak and improve survival in a murine model of inflammation (Kumpers et al., 2011).
1.13 Tie2’s Potential Modes of Nf-κB Regulation

While there is evidence that Tie2 signalling regulates inflammation through effects on Nf-κB activation the mechanisms by which Tie2 enacts its regulatory effects are not fully elucidated. A number of the signalling pathways activated by Tie2 have potential to impact components of Nf-κB activation pathways. For example, Tie2 residue Y1111 associates with the tyrosine phosphatase Shp2 and there is evidence to suggest that Shp2 is involved in the regulation of Nf-κB activity, as TNF stimulation of Shp2 deficient fibroblast cells resulted in a reduction of Nf-κB DNA binding activity (You, Flick, Yu, & Feng, 2001). Residue Y1106 of the Tie2 receptor has been shown to bind with Dok2, resulting in its phosphorylation and activation (Jones et al., 2003). Dok2 appears to be involved in the regulation of the response to LPS, as mice lacking Dok1 or 2 are hypersensitive to LPS as demonstrated by severe responses to doses of LPS which are sub-lethal to wild-type controls (Shinohara et al., 2005). The potential of Dok proteins to act as regulators of LPS-driven TLR4 signalling in endothelial cell remains to be evaluated. Further, the tyrosine residue Y1100 on Tie2 has been shown to act as a multidocking site, providing interactions with the p85 subunit of PI3K, growth factor receptor-bound (Grb)2, and Grb7 (Jones et al., 1999).

The role of PI3K in LPS induced TLR4 signalling is complicated. PI3K has been shown to have both activating and inhibitory effects on TLR signal transduction. In endothelial cells, LPS-driven TLR4 activation is able to activate the PI3K/Akt pathway in a MyD88, IRAK1 and TRAF6 dependent mechanism (X. Li et al., 2003; F. Wong, Hull, Zhande, Law, & Karsan, 2004). This effect was shown to be important for LPS driven Nf-κB
activation, as endothelial cells transduced with a dominant negative mutant of p85 experienced reduced IκBα degradation and Nf-κB activation. Use of a constitutively active form of Akt did not induce Nf-κB activity, which suggests that Akt activation is involved with, but not sufficient for Nf-κB activation (X. Li et al., 2003). The ability of PI3K/Akt signalling to participate in Nf-κB activation may be cell-type specific. In HEK293 cells, PI3K signalling was previously shown to induce Nf-κB DNA binding (Ozes et al., 1999). However, this was not observed in HUVEC (Madge & Pober, 2000). The expression of IKKα relative to IKKβ appears to play a role in the ability of Akt to activate Nf-κB DNA binding. Cells with a high proportion of IKKα relative to IKKβ, such as HEK293 cells, were most sensitive to PI3K inhibitors. Transient expression of IKKβ in HEK293 cells reduced their sensitivity (Gustin, Korgaonkar, Pincheira, Li, & Donner, 2006). Akt has been shown to phosphorylate and activate IKKα but not IKKβ (Ozes et al., 1999). Conversely, in response to LPS, PI3K has also been shown to negatively regulate the production of proinflammatory cytokines in monocytes (Guha & Mackman, 2002; Martin et al., 2003), and endothelial cells (Schabbauer, Tencati, Pedersen, Pawlinski, & Mackman, 2004). Together, these results demonstrate that PI3K signalling plays complex roles in the regulation of Nf-κB, which may be context specific. The role that Tie2 initiated PI3K/Akt signalling plays in the regulation of Nf-κB is not fully characterized.

Phosphorylation of Y1100 allows for the SH2 domain of Grb2 to bind to the Tie2 receptor leading to activation of the Ras-MEK-ERK1/2 pathway (Jones et al., 1999). Erk1/2 is activated following Ang1 stimulation after time periods of 15-60 minutes of stimulation (Abdel-Malak, Harfouche, & Hussain, 2007; Echavarria et al., 2015; Harfouche et al.,
The role that Tie2 induced Erk1/2 signalling plays in LPS-TLR4 driven inflammatory signalling remains unresolved.

The human miRNA-146 family comprises two miRNA genes located on different chromosomes: miRNA-146a at 5q34, and miRNA-146b at 10q24.32 (Geraldo et al., 2012). The pre-miR-146b is exported to the cytoplasm and processed to yield two mature microRNAs with 22 nucleotides each differing by only 2 nucleotides (Fish et al., 2008; Geraldo et al., 2012). Mature miRNA-146b-5p and miRNA-146b-3p are named according to whether they were processed from the 5’ or 3’ arm of the precursor hairpin (Geraldo et al., 2012).

Both miRNA-146a and miRNA-146b are enriched in endothelial cells isolated from differentiated embryonic stem cells (Fish et al., 2008). Recently, a study using long term (12 hour) treatment of HUVEC with Ang1 showed that Ang1 treatment increased the expression of miRNA-146b-5p and this correlated with a decrease in the expression of IRAK1 and TRAF6. Transfection with a specific miRNA-146b-5p mimic reduced IRAK1 and TRAF6 protein, but not mRNA expression (Echavarria et al., 2015), suggesting that the miRNA is involved in the prevention of protein translation.

A complete understanding of how Tie2 is able to crosstalk with the TLR4 signalling pathway will aid the design of therapeutics aimed at the control of vascular inflammation.
1.14 Rationale

The inflammatory response is essential for the eradication of microbial invaders, but also requires exquisite regulation. Unresolved inflammation can cause detrimental effects to the host, making negative feedback of the inflammatory response essential. Inflammatory cascade initiation occurs when cells expressing pattern recognition receptors, such as TLR4, detect an invader using molecular signals, such as lipopolysaccharide (LPS), found on bacteria. Activation of TLR4 results in a cascade that uses several adapter molecules including TRAF6 and IRAK4 to activate the pro-inflammatory transcription factor Nf-κB. Endothelial cells (EC) play an active role in the immune reaction in response to TLR4 activation by participating in the recruitment of leukocytes and increasing endothelial paracellular permeability to allow leukocytes entry to the site of infection.

One way that the endothelium resolves inflammation is through the receptor tyrosine kinase Tie2, which is found primarily on EC. Tie2 helps maintain baseline endothelial integrity and quiescence. Tie2 is constitutively phosphorylated and activated in mature vessels, and constitutive Tie2 signalling is important to stabilize the vasculature and regulate the vascular response to inflammation. The mechanisms by which Tie2 attenuates the inflammatory response is poorly understood. Activation of Tie2 results in the trans-phosphorylation of Tie2 on three key tyrosine residues, which are required for the recruitment of signalling proteins. These cascades represent potential means of crosstalk between the Tie2 and TLR4 signalling pathways. We propose that crosstalk between mediators downstream of TLR4 and Tie2 receptors allow Tie2 activation to
attenuate TLR4 driven Nf-κB signalling. By characterizing the signalling events downstream of Tie2 that interfere with LPS-TLR4 driven Nf-κB activation, we will further the understanding of the control of vascular inflammation as a whole.
1.15 Thesis Objectives

The purpose of this study was to understand the role of the receptor tyrosine kinase Tie2 during endotoxin driven inflammation. The specific research aims of this project were: (1) to determine the \textit{in vivo} effects of Tie2 activation on LPS inflammation, (2) to examine the molecular mechanisms involved in crosstalk between Tie2 and TLR4 induced Nf-\kappaB signalling, and to further investigate the role of Tie2 in the regulation of the expression of a miRNA previously implicated in the effects of Tie2 signalling on Nf-\kappaB activation.
1.16 Attributions

The data presented in Chapter 2 and Chapter 3 will be submitted for publication in the following form:

Chapter 2

Tie2 Activation Reduces LPS-TLR4 Induced Inflammation in \textit{vivo}.
Tie2 activation by Vasculotide reduces LPS-TLR4 induced inflammation \textit{in vivo}.

2.1 Abstract

Inflammation is implicated in the progression of a wide breadth of pathologies including sepsis, cancer, arthritis, and atherosclerosis. The inflammatory cascade is a complex process that ultimately leads to the recruitment of leukocytes to the site of infection. The endothelium, which lines the vasculature and lymphatics, participates in the recruitment leukocytes. Tie2, found primarily on endothelial cells has been shown to attenuate inflammation, however, how the activation of Tie2 regulates the inflammatory response of the vasculature is not fully understood. Towards examining the role of Tie2 activation following an acute inflammatory response, we have utilized the murine Air Pouch Model of inflammation and the Tie2 agonist, Vasculotide. Systemic activation of Tie2 reduced vascular permeability, the number of leukocytes at the site of inflammation, and inflammatory cytokines. Collectively, our results indicated that activation of Tie2 by Vasculotide reduces inflammation \textit{in vivo}.
2.2 Introduction

Inflammation, an inherent feature of the innate immune response, is an essential event for the elimination of pathogenic microbes to protect the host from infection. The endothelium is an active participant in this response. Normally, the endothelium, which makes up the interface between blood and tissue, maintains homeostasis of the vasculature by regulating vascular tone and endothelial barrier function as well as providing a non-thrombogenic and non-reactive surface that has very little interaction with circulating leukocytes (Ley & Reutershan, 2006).

Following inflammatory stimulation, endothelial cells change their phenotypes to support the inflammatory process. Binding of lipopolysaccharide (LPS) found on gram negative bacteria by the pattern recognition receptor TLR4 on the surface of endothelial cells results in endothelial activation and increased activity of the pro-inflammatory transcription factor Nf-κB, making the endothelium a major participant in the inflammatory process (Dauphinee & Karsan, 2006). An activated endothelium leads to the recruitment of leukocytes, as well as changes in permeability resulting in tissue oedema and leakage of plasma-protein-rich fluid (inflammatory exudate). This allows the extravasation of immune cells across the endothelium to the site of infection where they can eliminate the invading pathogen (Parikh, 2013). This TLR4 driven process is fundamental to the vascular component of several inflammatory states ranging from acute lung and kidney injury to sepsis (David et al., 2013; Echavarria et al., 2015; Parikh, 2013; Pober & Sessa, 2007; Rubig et al., 2016).
Tie2, a receptor tyrosine kinase, is a known regulator of vascular function in response to diverse inflammatory stimuli. In landmark studies Thurston et al. reported that mice overexpressing Ang1, Tie2’s natural agonist, were resistant to VEGF induced vascular leakage (Thurston et al., 2000; Thurston et al., 1999). Since then, Ang1 has been shown to prevent vascular permeability changes triggered by bradykinin, thrombin, TNF, and LPS (Fiedler et al., 2006; Gamble et al., 2000; I. Kim et al., 2001). Studies using viral overexpression of Ang1 (Witzenbichler et al., 2005) direct administration of recombinant Ang1 protein (David, Park, et al., 2011), and the synthetic Tie2 agonist Vasculotide (VT) (David, Ghosh, et al., 2011; Kumpers et al., 2011) have implicated Ang1/Tie2 signalling as an important means of vascular regulation following endotoxin exposure.

An activated endothelium is involved in the pathophysiology of diseases with a vascular inflammation component including sepsis. Tie2 is known to maintain vascular homeostasis and barrier function in response to inflammatory agents, however, how Tie2 enacts these anti-inflammatory effects is not known. Elucidating the mechanisms responsible for reducing vascular inflammation is an essential step to fully understanding the endothelium, which represents a potentially important therapeutic target.
2.3 Methods

Animals:

In vivo experiments were performed in strict accordance with protocols approved by the Sunnybrook Research Institute Animal Care Committee, accredited by the Canadian Council of Animal Care. All surgical, imaging, and euthanasia procedures were performed under inhaled isoflurane anesthesia.

Murine Air Pouch Model of Inflammation:

To study TLR4 driven inflammation we choose to use the murine air pouch model. The air pouch was created by subcutaneous injection of 2 ml of sterile air into the back of a mouse after removal of hair using depilatory cream. 48 hours later an additional 2 ml of air was injected to maintain the pouch. 48 hours after the last air injection 50 µg of ultrapure lipopolysaccharide (LPS) (O111:B4 from *E.coli* (InvivoGen)) (.5ml injection volume in sterile phosphate buffered saline (PBS)) was injected directly into the pouch to initiate an inflammatory cascade. The inflammatory exudate, which forms within the pouch, is collected by flushing the pouch with 2 ml of sterile PBS and used for measurements of inflammation.

In vivo Tie2 activation:

Vasculotide (VT), a synthetic Tie2 agonist, was used to examine the effect of in vivo Tie2 activation during an acute inflammatory response. After creation of the air pouch in FVB mice, animals received an intraperitoneal (I.P.) injection of VT (250 ng) or vehicle (PBS).
After 16 hours mice were injected with another dose of VT and LPS was immediately injected into the air pouch (Figure 2.1A).

After 24 hours of LPS stimulation the animals were sacrificed, and exudate was flushed and collected from the pouch. Lung tissue was archived for protein analysis, and blood was collected for the analysis of inflammatory cytokines. Flow cytometry was used to quantify the number of cells within the pouch exudate, using CountBright absolute counting beads, and to examine the types of cells found within the inflammatory exudate, using differential expression of CD11b and F4.80. Samples of exudate and plasma were sent to Eve Technologies Corporation for cytokine analysis using Multiplex LASER Bead Technology. This technique involves the detection and quantification of cytokines, chemokines, and growth factors using capture antibodies coupled to fluorescent beads.

**Miles assay of permeability:**

A Miles assay of was used to qualitatively measure vascular permeability following LPS induced inflammation. Evans Blue dye, which binds noncovalently to albumin, was injected into the tail vein of a subset of air pouch mice. Mice were pre-treated with VT or vehicle (PBS) and LPS was injected into the air pouch. After 6 hours, the exudate was collected from each pouch. The intensity of the dye in the exudate was measured spectrophotometrically to give a qualitative measurement of vascular permeability.

**Immunoblotting:**

Snap frozen lung tissue was homogenized in ice-cold RIPA buffer (150mM NaCl, 50mM Tris (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented
with protease inhibitors (Roche complete Protease Inhibitor Cocktail Tablet), 1 mM NaVO₄, and 1 mM NaF. Lysates were incubated at 4°C for 15 minutes, then spun at full speed for 20 minutes at 4°C in order to separate protein lysate from insoluble matter. Protein concentrations were determined using a BCA protein assay kit with bovine serum albumin as a standard (Thermo Scientific Pierce, USA).

Lysates were separated using SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes for western blotting. Membranes were blocked with 5% milk or 7% bovine serum albumin in tris-buffered saline (TBS) containing 0.05% Tween-20 then probed with specific commercially available primary antibodies. Primary antibodies included anti-Tie2 (BD, Cat#557039), anti-phospho-Tie2 (Y992) (Signalway Antibody, Cat#11756). Proteins were detected using Horseradish Peroxidase (HRP)-conjugated secondary antibodies and ECL reagents.

**Data Analysis:**

GraphPad Prism software was used for statistical analysis and figure preparation. Data are expressed as means ± standard error of mean (SEM). Statistical significance was determined by t-tests, one- or two-way ANOVA followed by Tukey's multiple comparison test. A p-value of 0.05 or less was considered statistically significant.
2.4 Results

Tie2 activation by VT reduces TLR4 mediated inflammation in vivo.

To evaluate the effect of Tie2 activation on TLR4 driven inflammation in vivo the murine air pouch model of inflammation was used. The air pouch was created by subcutaneous injection of sterile air into the back of a mouse. Forty-eight hours after creation of the air pouch animals received an intraperitoneal injection of the Tie2 activator, Vasculotide (VT), or vehicle (PBS) and an additional injection of VT or vehicle (for controls) 16 hours later at the same time of inflammation initiation. To initiate a TLR4 driven inflammatory cascade LPS was injected directly into the air pouch. 24 hours after LPS injection, the inflammatory exudate, which forms within the pouch, was removed to measure markers of inflammation, including the number of leukocytes that had migrated to the inflammatory stimulus, types of leukocytes, and the level of vascular permeability (Figure. 2.1A).

Injection of LPS caused a significant increase in the number of immune cells within the exudate compared to controls (p<0.05), while VT injection significantly reduced the increase in cellularity (p<0.05) (Figure. 2.1B). This reduction in leukocytes at the site of inflammation is indicative of a reduced inflammatory response.

Flow cytometry was used to determine the cell types present within the inflammatory exudate using differential expression of CD11b, a marker for leukocytes, and F4.80, a marker for macrophages. VT reduced the quantity of all these cell types. Cells at the site of inflammation are largely monocytes, granulocytes, and macrophages (Figure. 2.1C).
To measure vascular permeability a Mile’s assay was used. Evans Blue dye, which binds noncovalently to albumin, was injected into the tail vein of mice. After 6 hours of LPS stimulation the inflammatory exudate was collected from control mice receiving PBS (Ctrl), control mice receiving VT treatment (Ctrl + VT), mice stimulated with an LPS injection (LPS), and mice stimulated with an LPS injection as well an VT treatment (LPS + VT). The intensity of Evans Blue dye in the exudate was measured spectrophotometrically to give a qualitative measurement of permeability.

LPS injection increased permeability compared to PBS injected control groups. Tie2 activation, induced by VT injection, reduced the increase in permeability caused by LPS injection (Figure. 2.1D). Indicating that systemic Tie2 activation resulted in a decrease in vascular permeability, a feature of vascular inflammation.
Figure 2.1. Tie2 activation reduces inflammation in vivo. (A) Schematic of injection protocol. An air pouch was created by subcutaneous injection of sterile air into the back of a mouse to model an acute inflammatory response, followed by an intraperitoneal injection of the Tie2 activator, VT, or vehicle control (PBS). A second injection of VT was given 16 hours later at the time of inflammation initiation. LPS was injected directly into the air pouch of each group and the inflammatory exudate, which forms within the pouch, was removed after 24 hours of LPS stimulation. (B) Flow Cytometry was used to quantify the total cell counts per volume of exudate using count beads. Data, expressed as cells/µl of exudate, was analyzed using a one-way ANOVA followed by Tukey’s multiple comparison test. Values are means ± SEM, n=8/grp, *p<0.05, **p<0.01. (C)
Flow cytometry was used to determine the cell types present within the inflammatory exudate using differential expression of CD11B and F4.80. Representative experiment shown. (D) To measure vascular permeability Evans Blue dye was injected into the tail vein of mice and the inflammatory exudate was collected at 6 hours, and the intensity of the dye in the exudate was measured, and data, expressed as means ± SEM, was analyzed using a one-way ANOVA followed by Tukey's multiple comparison test. n=3-7 mice /grp.
**Tie2 activation reduces inflammatory cytokines.**

A key feature of the inflammatory response is the release of inflammatory cytokines which signal to resident and circulating leukocytes, as well as other cell types including endothelial cells, that an inflammatory response is required. These cytokines are present in the circulation and at the site of inflammation providing guidance cues for the propagation of an inflammatory response. Presence of inflammatory cytokines initiates a positive feedback loop, in response to inflammatory cytokines both leukocytes and endothelial cells to start releasing additional cytokines themselves (Pober & Sessa, 2007).

To measure the affect that Tie2 activation has on the presence of cytokines in the circulation and at the site of inflammation samples of plasma and exudate from the air pouch were collected from VT treated and control animals using the air pouch model of inflammation. Samples were collected, processed, and sent to Eve Technologies for analysis using Multiplex LASER Bead Technology.

LPS stimulation caused a trend of increased levels of cytokines in both plasma and exudate samples. Plasma MCP-1 was significantly increased in LPS treated animals compared to controls (p<0.05). VT mediated Tie2 activation caused a trend of reduced levels of cytokines. VT significantly reduced the levels of MCP-1 and IL-6 in plasma and MCP-1 in exudate samples (p<0.05) (Fig 2.2).

The reduction in exudate cellularity, permeability and expression of inflammatory cytokines indicated that VT treatment is associated with a reduction in the inflammatory process via Tie2 activation. The presence of inflammatory cytokines is an indicator of activated endothelial cells and leukocytes.
**Figure 2.2. Tie2 activation reduces inflammatory cytokines.** Using the air pouch model of inflammation, samples of plasma and inflammatory exudate were collected from control mice (Ctrl), mice receiving an injection of LPS (LPS), and mice receiving VT pre-treatment prior to LPS stimulation (LPS + VT). The presence of MCP-1, IL-6, and IL-8 were measured using Multiplex LASER Bead Technology. Data is expressed as means ± SEM and was analyzed using a one-way ANOVA followed by Tukey’s multiple comparison test. n=5-10/group, *p<0.05.
The anti-inflammatory effects of Vasculotide are Tie2 specific.

To confirm that the anti-inflammatory effects of VT observed in the animal model were dependent on Tie2, the lung tissue from a subset of animals was collected at the time of sacrifice for analysis of Tie2 activation. Levels of the phosphorylated fraction of Tie2 were compared in animals receiving a dose of LPS and either receiving the VT pre-treatment or vehicle (PBS). At this time point animals had not received a dose of VT for 24 hours.

The levels of phosphorylated Tie2 relative to total Tie2 levels were higher in the animals receiving VT compared to those not receiving VT (Figure. 2.3 A, B).

To further demonstrate that the anti-inflammatory effects of VT are dependent on Tie2, the air pouch model of acute inflammation was performed in Tie2 heterozygous (Het) and control Wild Type animals (WT) (Figure. 2.3 C, D). Tie2 heterozygous animals express less Tie2 than Wild Type animals (Figure. 2.3 D). Animals were given a VT pre-treatment prior to LPS stimulation.

LPS caused a significant increase in cellularity in WT (p<0.01) and in Het (p<0.05) animals. As expected, VT reduced cellularity in the WT animals (p<0.05). The anti-inflammatory effect of VT, as determined by exudate cellularity observed in WT mice, was lost in Tie2 heterozygous mice. Collectively, these results suggest that the anti-inflammatory effects of VT are at least in part, Tie2 dependent (Figure. 2.3 C).
A

B

C
Figure 2.3. The anti-inflammatory effects of Vasculotide are Tie2 dependent. (A) Western blot analysis of the phosphorylation levels of Tie2 and total Tie2 protein from the lung tissue of animals; all animals received LPS injected into the air pouch and either did (n=3) or did not receive Vasculotide (VT) supplementation (n=4). (B) Data is expressed relative to total Tie2 (t-test, p=0.0825). (C) The effects of VT supplementation on inflammation was examined in Tie2 heterozygous animals. Effects of VT in the air pouch model of inflammation in Tie2 heterozygous (Het) and Wild Type (WT) animals pre-treated with VT or vehicle control was examined. Inflammatory exudate was collected 24 hours after LPS injection, and cellularity determined by flow cytometry using CountBright absolute counting beads. Values are means ± SEM, expressed as cells/µl exudate. Data were analyzed using a one-way ANOVA followed by Tukey’s multiple comparison test n=5-7/grp. *p<0.05, **p<0.01. (D) Tie2 protein expression in lung tissue from Tie2 Het animals and WT littermates were collected at the time of sacrifice, and analyzed by a Tie2 immunoblot. Each lane represents separate animals. Tubulin was used as a loading control.
2.5 Discussion

This study demonstrates the ability of the Tie2 agonist, VT, to interfere with LPS-driven inflammation. Using the murine air pouch model, we validated the anti-inflammatory effect of pre-activating Tie2 before an inflammatory insult *in vivo*. Activation of Tie2 by VT resulted in a decrease in the number of immune cells at the site of inflammation, a reduction in inflammatory cytokines and chemokines, and a reduction in vascular permeability.

This study supports the ability of Tie2 signalling to prophylactically interfere with LPS-TLR4 driven inflammation *in vivo*. This, however, may not necessarily be true for therapeutic Tie2 activation i.e after the initiation of inflammation. Future work should focus on the ability of Tie2 to reduce Nf-κB activation in endothelial cells after the onset of inflammation to garner information about the ability of Tie2 to reduce TLR4 driven vascular inflammation in a clinical setting where the inflammatory effects of inflammation are often not predicted. Work from a cecal-ligation-puncture (CLP) model in mice showed that dosing with Vasculotide following the induction of sepsis was beneficial (Kumpers et al., 2011). Mice were injected with Vasculotide at 2, 4, and 28 hours after CLP and the authors found that mice injected with Vasculotide had a survival rate of 36% which was an improvement from the control mice whose survival rate was 0% (Kumpers et al., 2011). Although CLP is a polymicrobial model and therefore is not exclusively a LPS-TLR4 model it provides a case for the use of Tie2 agonists after the onset of inflammation. Results from other pathologies suggest that Tie2 activation after the onset of vascular stress could prove to be beneficial. Lung microvascular leak
complicates severe influenza virus infections. It was shown that VT, given as late as 72 hours after infection, was still able to reduce lung oedema and lung endothelial cell apoptosis and ultimately increased survival in a murine model of influenza (Sugiyama et al., 2015). While this is not an TLR4 driven inflammation model, it does suggest that studies examining the effect of Tie2 activation after the onset of LPS-TLR4 driven inflammation are warranted. The pre-activation design might be useful in a situation of planned inflammation such as surgery.

This study exclusively monitored LPS driven TLR4 activation and may not translate to other TLR4 ligands. In addition to PAMP, such as LPS, TLR4 can also engage endogenous triggers of sterile inflammation such as damage associated molecular patterns which are released following tissue damage (Lee, Hutchinson, & Saint, 2016). It would be interesting to examine whether the interference effect of VT-Tie2 on LPS-TLR4 inflammation holds true for other inflammatory agents.

The results presented herein demonstrate the powerful ability of Tie2 signalling to regulate vascular inflammation in vivo.
Chapter 3

LPS-TLR4 Driven Nf-κB Activation is Inhibited by Tie2 Signalling and This Inhibition is Dependent on Y1100 phosphorylation and Erk1/2 Signalling.
3 LPS-TLR4 Driven Nf-κB Activation is Inhibited by Tie2 Signalling and This Inhibition is Dependent on Y1100 Phosphorylation and Erk1/2 Signalling.

3.1 Abstract

The inflammatory response is essential for the eradication of microbial invaders. However, unresolved inflammation causes detrimental effects to the host, making negative feedback essential. Initiation of inflammation occurs when cells expressing pattern recognition receptors such as Toll-like receptor 4 (TLR4) detect an invader using molecular signals found on microbes such as lipopolysaccharide (LPS). Activation of TLR4 results in a cascade that uses several adapter molecules, including TRAF6 and IRAK4, to activate the pro-inflammatory transcription factor Nf-κB (Ley & Reutershan, 2006). Tie2 is able to interfere with Nf-κB activation, however, it is not understood how Tie2 is able to enact this effect. Here, we investigate the role that Tie2 signalling events play in the inhibition of TLR4 induced Nf-κB signalling.

To investigate crosstalk between Tie2 and TLR4 signalling, the effects of Tie2 mutants harboring tyrosine (Y) to phenylalanine mutations in the carboxy-terminal tyrosine residues whose phosphorylation is essential for downstream Tie2 signalling on Nf-κB activation was monitored. Tie2 signalling reduced LPS induced Nf-κB activation in a Y1100 and Erk1/2 dependent manner. Tie2 signalling decreased the levels of the TLR4
signalling proteins, TRAF6 and IRAK1 and preserved the Nf-κB inhibitor, IκBα, though Y1100 initiated Erk1/2 signalling. The ability of Tie2 to increase the expression of miRNA-146b-5p was found to be dependent on all three carboxy-terminal tyrosine residues. Our results show that Y1100 initiated Erk1/2 signalling is essential for the anti-inflammatory effect of Tie2 on TLR4 driven Nf-κB activation. Collectively these results further our understanding of the anti-inflammatory role and biology of Tie2.
3.2 Introduction

Endothelial cells (EC) play an active role in the immune reaction initiated by LPS through increasing endothelial permeability and promoting the extravasation of immune cells across the endothelium (Dauphinee & Karsan, 2006). EC are also involved in the resolution of inflammation and maintaining homeostasis. One way that the endothelium mediates inflammation is through the receptor tyrosine kinase Tie2 found primarily on EC. Tie2 is constitutively phosphorylated and activated in mature vessels. This tonic activation is thought to provide chronic signalling events that suppresses vascular inflammation [2,3]. The process by which Tie2 attenuates the inflammatory response is poorly understood.

Binding of Ang1 by Tie2 leads to clustering of the receptor which induces phosphorylation of the receptor on tyrosine residues providing high-affinity binding sites for signalling proteins with SH2 or PTB domains (Jones et al., 2003; Jones et al., 1999). Phosphorylation of Tie2 on three key tyrosine residues, Y1100, Y1106, Y1111, is required for activation of downstream signalling cascades (Jones et al., 2003; Jones & Dumont, 1998; Jones et al., 2001; Jones et al., 1999; Master et al., 2001).

These cascades represent potential means of crosstalk between Tie2 and inflammatory signalling from receptors such as TLR4. We propose that crosstalk between mediators downstream of the TLR4 and Tie2 receptors allow Tie2 activation to attenuate TLR4 driven Nf-κB signalling.
To investigate the effect of Tie2 signalling during LPS-TLR4 driven inflammation we examined the effects of signalling events downstream of Tie2 on levels of Nf-κB activation and the expression of TLR4 signalling intermediates. Using Tie2 mutants expressing functional tyrosine to phenylalanine mutations in the carboxy-terminal that ablate key signalling events we determined the critical signalling events required for the anti-inflammatory effects of Tie2.
3.3 Methods

Cell Culture:

HEK-Blue hTLR4 cells (InvivoGen) are HEK-293 cells stably transfected with the human TLR4 receptor gene, MD2/CD14 co-receptor genes and a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) reporter containing Nf-κB response elements. HEK-Blue hTLR4 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific Inc.) and incubated (humidified atmosphere of 5% CO2 at 37°C). The medium was supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), penicillin (50 IU/ml), streptomycin (50 mg/ml, Thermo Fisher Scientific), Normocin (100 mg/ml, InvivoGen), and a HEK-Blue selection mixture of antibiotics (2 ml per 500 ml; InvivoGen). Human microvascular endothelial cells (HMVECs) immortalized with the human telomerase reverse transcriptase catalytic subunit (hTERT), as described by Shao and Guo (2004) (Shao & Guo, 2004) provided by Dr. Shao, were grown in Endothelial Basal Medium EBM-2 (Lonza) supplemented with 10% FBS (Gibco) and 10 ng/ml EGF (Sigma-Aldrich), and 1 μg/ml hydrocortisone (Sigma-Aldrich). Before Angiopoietin-1 (Ang1) stimulation HMVEC Cells were serum starved in media containing 1% FBS for 16 hrs. Cells were stimulated for 12 hours with 300ng/ml purified recombinant human Ang1 (R&D Systems).
Tie2 mutant plasmids:

The cDNAs encoding Wild Type (WT), kinase dead (K853A), triple mutant (Y3F), single mutant (Y1100F, Y1106F, Y1111F), and double mutant (Y00/06F, Y00/11F, Y06/11F) Tie2 in pcDNA3.1 have been described previously (Jones et al., 2003; Sturk et al., 2010). Sequencing verified all mutations.

Transfection:

HEK-Blue hTLR4 cells were cultured in 10-cm cell culture plates to approximately 80% confluency and transfected with 6μg DNA using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) and according to manufactures protocol. Cells were harvested after 24 hours.

SEAP assay:

To monitor the effects of Tie2 signalling during LPS induced Nf-κB activation the HEK-Blue hTLR4 SEAP Nf-κB reporter cells were used. Following transfection with the Tie2 mutants, cells were plated into 96-well plates for 24 hours before LPS (O111:B4) stimulation. Cells were stimulated with the indicated concentrations of LPS. After 7 hours of LPS stimulation a spectrophotometer (620 nm) was used to measure Nf-κB activation. All experiments were performed in triplicate on the plate and the average of the three technical replicates were used as one data point, this was repeated in three independent experiments.
**Kinase Inhibition:**

The MEK inhibitor, UO126 (Cell Signaling Technology), and the PI3K inhibitor, LY294002, were both prepared in Dimethyl sulfoxide (DMSO) as 10mM stock solutions. To monitor the effect of kinase inhibition on Nf-κB activation, HEK-Blue hTLR4 SEAP Nf-κB reporter cells were seeded in 96-well plates for a SEAP Nf-κB reporter assay. Following a 24-hour rest period inhibitor or vehicle (DMSO) was added to cells in the indicated concentrations for 30 minutes prior to LPS stimulation.

**Immunoblotting:**

Cultured cells were washed with ice-cold PBS twice and lysed with ice-cold RIPA buffer (150mM NaCl, 50mM Tris (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Roche complete Protease Inhibitor Cocktail Tablet), 1 mM NaVO₄, and 1 mM NaF. Lysates were incubated at 4ºC for 15 minutes, then spun at full speed for 20 minutes at 4ºC in order to separate protein lysate from insoluble matter. Protein concentrations were determined using a BCA protein assay kit with bovine serum albumin as a standard (Thermo Scientific Pierce, USA).

Lysates were separated using SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes for western blotting. Membranes were blocked with 5% milk or 7% bovine serum albumin in tris-buffered saline (TBS) containing 0.05% Tween-20 then probed with specific commercially available primary antibodies. Primary antibodies included anti-Tie2 (BD, Cat#557039), anti-phospho-Tie2 (Y992) (Signalway Antibody, Cat#11756), IRAK1 (Santa Cruz, Cat# SC55530), TRAF6 (Santa Cruz, Cat# SC7221), IκBα (Santa Cruz, Cat# SC847), phospho-Akt (Cell
Signaling Technology, Cat # 9271), Akt (Cell Signaling Technology, Cat # 4691), phospho-p44/42 (Erk1/2) (Thr202/Tyr204) (Cell Signaling Technology, Cat #9101), p44/42 (Erk1/2) (NEB, Cat # 9102). β-Tubulin served as loading control (Sigma, T9026). Proteins were detected using Horseradish Peroxidase (HRP)-conjugated secondary antibodies and ECL reagents.

**miRNA Analysis:**

Total RNA was extracted using an miRNeasy Mini Kit (Qiagen), reverse transcribed using a miScript II RT Kit (Qiagen), and quantified using a miScript SYBR Green kit (Qiagen) according to the manufacturers’ instructions. Real-time PCR was used to detect expression levels of miRNA-146b-5p, using a previously described primer specific for human miRNA-146b-5p (Echavarria et al., 2015), and levels of U6, using a human U6 primer assay (Qiagen). Experiments were performed in triplicate and relative miRNA expressions were determined using the delta CT method.

**Data Analysis:**

GraphPad Prism software was used for statistical analysis and figure preparation. Data are expressed as means ± standard error of mean (SEM). T-tests determined statistical significance, one- or two-way ANOVA followed by Tukey's multiple comparison test. A p-value of 0.05 or less was considered statistically significant.
3.4 Results

**Characterizing the effect of Tie2 on Nf-κB activation using Tie2 mutants.**

To monitor the effects of Tie2 signalling on LPS induced Nf-κB activation the HEK-Blue hTLR4 cell line which expresses TLR4 and its co-receptors, CD14 and MD-2, as well as the SEAP Nf-κB reporter was used. These Tie2 null cells were transiently transfected with wild type (WT) Tie2, a kinase dead (KD) version of Tie2 which has a lysine to arginine substitution in the kinase domain rendering the receptor inactive, and Tie2 mutants with one, two, or three tyrosine (Y) to phenylalanine (F) mutations in the carboxy-terminal tail at tyrosine residues Y1100, Y1106, and Y1111 (Figure. 3.1 A).

Tie2 was constitutively active in all transfected cells except for those expressing the inactive kinase dead Tie2 mutant (Figure. 3.1 B). This is an agreement with previous reports showing that ectopic overexpression results in ligand independent activation (Jones et al., 1999; Sturk et al., 2010). Cells expressing the Tie2 mutants containing the Y1111F mutation had higher levels of Tie2 phosphorylation relative to total Tie2 compared to WT Tie2 expressing cells (Figure. 3.1 C). Previous studies have shown that the Y1111F mutation results in increased Tie2 autophosphorylation and kinase activity (Jones et al., 2003; Sturk et al., 2010). Use of the Tie2 mutants allowed for the evaluation of activated Tie2 in a ligand independent manner.
Figure 3.1. **Tie2 is constitutively activated in Tie2 transfected cells.** (A) Schematic of the Tie2 mutants used for transfection into Tie2 null HEK-Blue™ hTLR4 cells. Cells were transfected with one of nine Tie2 mutants; Wild Type (WT) Tie2, Kinase dead (KD), a triple mutant with tyrosine (Y) to phenylalanine (F) point mutations at Y1100, Y1106, and Y1111 (Y3F), single mutants Y1100F, Y1106F, Y1111F, and double mutants Y00/06F, Y00/11F, and Y06/11F. (B) HEK-Blue hTLR4 cells were transfected with the Tie2 mutants. Cell lysates were analyzed by immunoblot to detect phosphorylated Tie2. Blots were stripped and probed for total Tie2. Representative blot shown (C). Quantification of the phosphorylated fraction of Tie2 was expressed relative to total Tie2 using densitometry, n=1.
Tie2 reduces LPS induced activation of Nf-κB in a Y1100 dependent manner.

To investigate the effects of constitutively active Tie2 on LPS induced TLR4 signalling the level of Nf-κB activation was measured in a SEAP Nf-κB reporter assay. HEK-Blue hTLR4 cells were transfected with WT, KD, Y3F, Y1100F, Y1106F, Y1111F, Y00/06F, Y00/11F, or Y06/11F expression plasmids. Cells were stimulated with 100 ng/ml LPS and the level of Nf-κB activation was measured in each cell type. Expression of WT Tie2, but not the KD mutant significantly lowered levels of Nf-κB activation compared to controls. Tie2 mutants possessing tyrosine to phenylalanine mutations at Y1100 (Y3F, Y1100F, Y00/06F, Y00/11F) also had impaired ability to affect Nf-κB activation (Figure 3.2). Interestingly, Tie2 mutants lacking Y1111 or Y1106 had an effect similar to WT. These results indicate that signalling emanating from Y1100 is essential for the effect of Nf-κB inhibition exerted by Tie2.

A similar pattern of Nf-κB activation was maintained in cells stimulated with a lower level of LPS (10 ng/ml) (data not shown). Using a lower level of LPS we again found that expression WT Tie2 resulted in lower levels of Nf-κB activation compared to controls. Expression of the Tie2 mutants, except those possessing a mutation at Y1100, resulted in a decrease in Nf-κB activation compared to non-transfected controls and KD Tie2 (data not shown). This indicates that the Y1100 dependency of the anti-inflammatory effect of Tie2 is also consistent at lower levels of inflammation.
Figure 3.2. Tie2 reduces LPS induced activation of Nf-κB in a Y1100 dependent manner. HEK-Blue hTLR4 cells were transfected with Tie2 mutants and stimulated with LPS for 7 hours, and Nf-κB activation was measured by a SEAP reporter assay. Data were expressed relative to values from 0 ng/ml LPS controls for each group. The horizontal line represents relative absorbance of data points at 0ng/ml LPS for each group. Data were analyzed using a two-way ANOVA followed by Tukey’s multiple comparison test, and expressed as means ± SEM. Three technical replicates were averaged for a total of three biological replicates per group. ***p<0.001.
Tie2 reduces LPS induced activation of Nf-κB in a Y1100, and Erk1/2 dependent manner.

Y1100 has been shown to act as an important site for Grb2-Mek-Erk1/2 and PI3K signalling. To examine the involvement of these pathways signalling inhibitors were used. To characterize the role of Erk1/2 signalling, cells were treated with the Mek1/2-Erk1/2 inhibitor U0126. Initial experiments were performed to identify an optimal amount of inhibitor to use on HEK-Blue hTLR4 cells (Figure 3.3 A). Cell lysates were analyzed by immunoblot to detect phosphorylated Erk. Blots were stripped and probed for tubulin. 10 µM of UO126 was selected for subsequent experiments. In a SEAP reporter assay of Nf-κB activity, cells expressing WT, KD, Y3F, Y1100F, Y1106F, or Y1111F were pre-treated with U0126 (10 µM) or vehicle (DMSO) for 30 minutes prior to the addition of LPS.

Inhibition of Erk1/2 signalling abrogated the TLR4 interference effect of Tie2 on Nf-κB activation (Figure. 3.3 B). The fact that inhibition of Erk1/2 signalling, using UO126, is able to restore activation levels of Nf-κB implicates Erk1/2 signalling in the mechanism of Tie2 inhibition of the TLR4-Nf-κB signalling cascade. Additionally, UO126 had no impact on the levels of Nf-κB activation in cells expressing Y1100F mutants, further demonstrating that Tie2 interferes with TLR4 signalling in a Y1100F, and Erk1/2 dependent fashion.
Figure 3.3. Tie2 reduces LPS induced activation of Nf-κB in a Y1100, and Erk1/2 dependent manner. (A) HEK-Blue hTLR4 cells were treated with 0, 5, 10, 15, 20, or 30 μM UO126 for 30 minutes and levels of Erk activation were examined. Cell lysates were analyzed by immunoblot to detect phosphorylated Erk. Blots were stripped and probed for tubulin (B) HEK-Blue hTLR4 cells were pre-incubated with the Mek1/2-Erk1/2 inhibitor, UO126, or vehicle (DMSO) for 30 minutes before the addition of LPS. Line represents relative absorbance of data points at 0ng/ml LPS for each group. Data were analyzed using a two-way ANOVA followed by Tukey’s multiple comparison test. Data is expressed as means ± SEM. n=3/grp. *p<0.05, **p<0.01, ***p<0.001.

Tie2 reduces LPS induced activation of Nf-κB in a PI3K/Akt independent manner
To examine the involvement PI3K/Akt signalling, cells were treated with the selective PI3K/Akt inhibitor LY294002. Initial experiments were performed in HEK-Blue hTLR4 cells to identify an optimal dose of LY294002 that inhibits PI3K/Akt signalling in this cell type (Figure 3.4 A). Cell lysates were analyzed by immunoblot to detect phosphorylated Akt which indicates upstream activation of PI3K. Blots were stripped and probed for total Akt. 20 µM of LY294002 was selected for subsequent experiments because it achieved a complete suppression of Akt phosphorylation.

In a SEAP reporter assay of Nf-κB activity, cells expressing WT, KD, Y3F, Y1100F, Y1106F, Y1111F were pre-treated with 20 µM LY294002 or vehicle (DMSO) 30 minutes prior to the addition of LPS.

In the absence of Tie2 (non-transfected controls), inhibition of PI3K resulted in a reduction in Nf-κB activation (Figure. 3.4B). However, in WT and mutant Tie2 expressing cells inhibition of PI3K/Akt signalling, resulted in similar Nf-κB activation as vehicle treated controls indicating that inhibition of PI3K/Akt signalling in our model has no effect on the ability of Tie2 to reduce Nf-κB activation (Figure. 3.4 B). Although Nf-κB activation is reduced when PI3K signalling is inhibited in non-transfected cells, our results suggest that Tie2 interferes with LPS induced TLR4 driven Nf-κB activation via Y1100 initiated Erk1/2 signalling with little involvement from PI3K/Akt.
**Figure 3.4.** Tie2 reduces LPS induced activation of NF-κB in a PI3K/Akt independent manner. (A) HEK-Blue hTLR4 cells expressing WT Tie2 and non-transfected control cells were treated with 0, 20, or 50 μM for 30 minutes with the PI3K/Akt inhibitor LY294002 (LY) and levels of Akt activation were examined. Cell lysates were analyzed by immunoblot to detect phosphorylated Akt. Blots were stripped and probed for total Akt. (B) Cells were pre-incubated with (LY) or vehicle (DMSO) for 30 minutes prior to the addition of LPS (100ng/ml). Line represents relative absorbance of data points at 0ng/ml LPS for each group. Data were analyzed using a two-way ANOVA followed by Tukey's multiple comparison test, and expressed as means ± SEM. Three technical replicates were averaged for a total of three biological replicates per group. *p<0.05.
Tie2 decreases the levels of the TLR4 signalling protein TRAF6

TRAF6 and IRAK1 are two proteins involved in the propagation of TLR4 signalling and have been shown to be targeted by miRNA-146b-5p, preventing their translation. Tie2 has been previously linked to the regulation of IRAK1 and TRAF6 proteins in endothelial cells, as long term Ang1 stimulation increased the expression of miRNA-146b-5p (Echavarria et al., 2015). In the present study HEK-Blue hTLR4 cells were transfected with Tie2 and mutants of Tie2 to examine the effect of Tie2 activation on TRAF6 and IRAK1. Control non-transfected cells and cells expressing WT or KD Tie2, were stimulated with LPS (100ng/ml) for 0, 5, 10, and 15 minutes of stimulation.

Cells expressing WT Tie2 had lower expression of TRAF6 compared to non-transfected control cells (Ctrl) and KD expressing cells after 5 and 15 minutes of LPS stimulation (Figure. 3.5 A, B).

WT Tie2 expressing cells had lower basal levels of IRAK1 compared to Ctrl cells and cells expressing KD Tie2. IRAK1 was lower at all time points of LPS stimulation tested (Figure. 3.6 A, B). Suggesting that Tie2 signalling interferes with TLR4 signalling, in part, by reducing TRAF6 and IRAK1 protein upstream of Nf-κB activation.
Figure 3.5. Tie2 decreases the levels of TLR4 signalling protein TRAF6. Control (No DNA Ctrl), Kinase Dead Tie2 (KD), and Wild Type Tie2 expressing cells (WT) were stimulated with 100ng/ml LPS for the indicated times and protein lysates were analyzed by immunoblot with anti-TRAF6 (A, B, two biological replicates per group). Representative immunoblots are shown (A). Data is expressed relative to tubulin and analyzed using a two-way ANOVA followed by Tukey's multiple comparison test (B). Values are means ± SEM, expressed as optical densities. *p<0.05, **p<0.01.
Figure 3.6. Tie2 decreases the levels of TLR4 signalling protein IRAK1. Control (No DNA Ctrl), Kinase Dead Tie2 (KD), and Wild Type Tie2 expressing cells (WT) were stimulated with 100ng/ml LPS for the indicated times and protein lysates analyzed by immunoblot anti-IRAK1 (A, B, three biological replicates per group) antibodies. Representative immunoblots are shown. Data is expressed relative to tubulin and analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. Values are means ± SEM, expressed as optical densities. *p<0.05, **p<0.01, ***p<0.001.
**Y1100 is essential for the regulatory effects of Tie2 on IRAK1 protein levels.**

Since our previous experiments showed that Y1100 was important for inhibitory effects of Tie2 on Nf-κB signalling we tested whether this tyrosine residue was important for the effect of Tie2 signalling on IRAK1 levels. HEK-Blue hTLR4 cells were transfected with the Y1100F, Y1106F, Y1111F and WT Tie2 mutants and stimulated with LPS for 0, 10, or 15 minutes. Cell lysates were analyzed by immunoblot to detect IRAK1. Blots were stripped and probed for tubulin.

Cells expressing WT, Y1106F, or Y1111F Tie2 expressed lower basal levels of IRAK1 compared to cells expressing the Y1100F Tie2 mutant suggesting that the ability of Tie2 to lower IRAK1 depends on signalling downstream from Y1100 (Figure. 3.7 A, B).
Figure 3.7. Y1100 is essential for the regulatory effects of Tie2 on IRAK1 protein levels. Cells expressing the Tie2 mutants Y1100F, Y1106F, Y1111F, WT Tie2 were stimulated with LPS for the indicated times and protein lysate analyzed by immunoblot with anti-IRAK1 (three biological replicates per group). Representative immunoblots are shown. Data is expressed relative to tubulin and analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. Values are means ± SEM, expressed as optical densities. *p<0.05, **p<0.01, ***p<0.001.
**Tie2 signalling stabilizes the Nf-κB inhibitor, IκBα.**

The degradation of the Nf-κB inhibitor, IκBα, is an essential event upstream of Nf-κB activation. To gain a further understanding of the how Tie2 is enacting the observed effects on Nf-κB activation, the expression of IκBα was determined in Tie2 expressing cells. Control cells and cells expressing WT or KD Tie2, were stimulated with LPS (100ng/ml) for 0, 5, 10, and 15 minutes. Cell lysates were analyzed by immunoblot to quantify IκBα. Blots were stripped and probed for tubulin.

HEK-Blue hTLR4 cells expressing WT Tie2 had higher levels of IκBα at baseline (0 minutes of LPS stimulation) as well as after 10 minutes of LPS stimulation compared to KD Tie2 expressing cells and non-transfected controls (Figure. 3.8 A, B). Suggesting that Tie2 signalling stabilizes IκBα, and inhibition of Nf-κB.

**Tie2 signalling stabilizes the Nf-κB inhibitor, IκBα, via signalling emanating from Y1100.**

To determine the individual importance of the three carboxy-terminal tyrosine residues involved in preserving IκBα protein, cells were transfected with one of the Tie2 single mutants (Y1100F, Y1106F, Y1111F) or WT Tie2 and stimulated with LPS for 0, 10, or 15 minutes.

After 10 minutes of LPS stimulation cells expressing the Y1100F mutation had significantly lower levels of IκBα protein compared to cells expressing the Y1106F, and Y1111F mutants as well as WT Tie2 expressing cells (Figure. 3.9 A, B). Indicating that the ability of Tie2 signalling to preserve IκBα protein depends on Y1100 signalling.
Figure 3.8. Tie2 signalling stabilizes the Nf-κB inhibitor, IκBα. HEK-Blue hTLR4 cells were transfected with Wild Type (WT) or Kinase Dead (KD) Tie2 and stimulated with LPS for 0, 5, 10, or 15 minutes. Cell lysates were analyzed by immunoblot with anti-IκBα (A) and expressed relative to tubulin (B) (four biological replicates per group. Representative immunoblots are shown. Values are means ± SEM, expressed as optical densities). Data were analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001.
Figure 3.9. Tie2 signalling stabilizes the Nf-κB inhibitor, IκBα, via signalling emanating from Y1100. To determine the individual contributions of the carboxy-terminal tyrosine (Y) residues on the Tie2 receptor, cells expressing the Tie2 mutants Y1100F, Y1106F, Y1111F, and WT were stimulated with LPS for the indicated times and the lysate analyzed by immunoblot with anti-IκBα (A) and expressed relative to tubulin (B) (five biological replicates per group). Representative immunoblots are shown. Values are means ± SEM, expressed as optical densities). Data were analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001.
**Tie2 signalling stabilizes the Nf-κB inhibitor, IκBα, in part through Erk1/2 signalling emanating from Y1100.**

In our Nf-κB activation experiments, we found Erk1/2 signalling to be essential for the anti-inflammatory effects of Tie2. To determine if Y1100 activation of Erk1/2 is important for the effects on IκBα, the MEK1/2-Erk1/2 inhibitor, UO126, was used to inhibit Erk1/2 signalling. Control cells and cells expressing WT or KD Tie2 were pre-treated with U0126 (10 µM) or vehicle (DMSO) for 30 minutes prior to the addition of LPS for the indicated times. Cell lysates were analyzed by immunoblot with anti-IκBα and expressed relative to tubulin.

Treatment of cells with UO126 resulted in a reduction in the expression of IκBα protein in Wild Type Tie2 expressing cells (Figure. 3.10 A, B). The ability of Wild Type Tie2 to preserve the levels of the Nf-κB inhibitor IκBα is lost when Erk1/2 signalling is inhibited. These results in HEK-Blue hTLR4 cells suggest that Y1100 and Erk1/2 signalling is essential for the IκBα stabilization effects of Tie2 signalling.
Figure 3.10. Tie2 signalling stabilizes the Nf-κB inhibitor, IκBα, in part through Erk1/2 signalling. To evaluate the role of Erk1/2 signalling in the effect of Tie2 on IκBα protein, control cells (No DNA Ctrl), KD Tie2, and WT Tie2 expressing cells were pre-incubated with the Mek1/2-Erk1/2 inhibitor UO126 (10 μM) or vehicle (DMSO) for 30 mins prior to the addition of LPS. Cell lysates were analyzed by immunoblot with anti-IκBα (A) and expressed relative to tubulin (B) (four biological replicates per group). Representative immunoblots are shown. Values are means ± SEM, expressed as optical densities. Data were analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. ***p<0.001.
Ang1 stimulation of Human Microvascular Endothelial Cells results in stabilization of the Nf-κB inhibitor protein IκBα protein.

To examine the mechanisms by which Tie2 effects LPS driven TLR4 signalling in endothelial cells we utilized human dermal microvascular endothelial cells (HMVEC) which express endogenous Tie2. HMVEC were incubated for 12 hours with the Tie2 agonist, Ang1 (300 ng/ml). Cells were then stimulated with LPS for 0, 15, 30, or 45 minutes. Cell lysates were analyzed by immunoblot with anti-IκBα and expressed relative to tubulin.

Cells receiving Ang1 treatment expressed slightly higher basal levels of IκBα, and significantly higher levels of IκBα compared to PBS control cells after 15 minutes of LPS stimulation (Figure. 3.11 A, B).

Ang1 stimulation of Human Microvascular Endothelial Cells results in stabilization of the Nf-κB inhibitor protein IκBα protein, partially through Erk1/2 signalling.

To determine the importance of Erk1/2 signalling in Tie2 dependent IκBα preservation, HMVEC were stimulated with Ang1 for 12 hours, and treated with UO126 or vehicle (DMSO) then challenged with LPS. In congruence with the results found in HEK-Blue hTLR4 cells, inhibition of Erk1/2 signalling in endothelial cells resulted in a reduction of the preservation effects of Tie2 signalling on IκBα expression levels (Figure. 3.11 C, D). These results suggest that in endothelial cells Erk1/2 activity is important for the ability of Tie2 to suppress TLR4 signalling.
To demonstrate that Ang1 is able to activate Tie2 in HMVEC, cells were serum starved for 16 hours and treated with 300ng/ml of Ang1 for 0, 10, and 30 minutes of stimulation. Due to difficulty detecting total Tie2 after stripping the membrane, equal amounts of samples were loaded onto additional lanes of the same gel and the membrane was cut to simultaneously detect phosphoTie2 and total Tie2. Ang1 stimulation increased the phosphorylation of Tie2 (Figure. 3.11 E).

Collectively, these results indicate that in endothelial cells Tie2 signalling, particularly Tie2 driven Erk1/2 signalling, results in the stabilization of IκBα protein. These results in endothelial cells are in agreement with the results found in our HEK-Blue hTLR4 cell model. These results provide a plausible explanation for the reduction in Nf-κB activation levels observed in our SEAP Nf-κB reporter assays.
Figure 3.11. Ang1 stimulation of Human Microvascular Endothelial Cells results in stabilization of the Nf-κB inhibitor protein IκBα protein, partially through Erk1/2 signalling. (A, B) Human Microvascular Endothelial Cells (HMVEC) were serum starved for 16 hours and treated with 300ng/ml Ang1 or PBS for 12 hours. Cells were stimulated for the indicated times with LPS, and the cell lysates were analyzed by immunoblot with anti-IκBα and expressed relative to tubulin (three biological replicates per group). (C, D) To determine the importance Erk1/2 signalling in the effect of long term Ang1 treatment of HMVEC, cells were serum starved for 16 hours. HMVEC were treated with 300ng/ml Ang1 or PBS for 12 hours and in the last 30 minutes cells were pretreated with the Mek1/2-Erk1/2 inhibitor, UO126 or vehicle (DMSO), prior to LPS stimulation for the indicated times (0, 30, or 45 minutes). Cell lysates were analyzed by immunoblot with anti-IκBα and expressed relative to tubulin. Representative immunoblots are shown. Values are means ± SEM, expressed as optical densities. Five biological replicates per group. Values are means ± SEM, expressed as optical densities.
Data were analyzed using a two-way ANOVA followed by Tukey's multiple comparison test. *p<0.05, **p<0.01, ***p<0.001. (E) To show that Ang1 is able to activate Tie2 in HMVEC, cells were serum starved for 16 hours and treated with 300ng/ml Ang1 for the indicated times. Cell lysates were analyzed by immunoblot to detect phosphorylated Tie2 and total Tie2. Due to difficulty detecting total Tie2 after stripping the membrane, samples were loaded onto the same gel identically and the membrane was cut to simultaneously detect phosphoTie2 and total Tie2, n=1.
Tie2 regulates the expression of miRNA-146b-5p.

A previous report showed that stimulation of the macrovascular endothelial cell line, HUVEC, with Ang1 could increase the expression of miRNA-146b-5p (Echavarria et al., 2015), a miRNA shown to target TRAF6 and IRAK1 (Taganov et al., 2006). Similarly, we confirmed that stimulation of the microvascular endothelial cell line, HMVEC, with Ang1 resulted in the upregulation of miRNA-146b-5p (Figure. 3.12 A).

Further, to characterize the importance of the individual carboxy-terminal tyrosine residues the expression of miRNA-146b-5p in HEK-Blue hTLR4 cells expressing the Tie2 mutants was measured. Cells were transfected with WT, KD Tie2, or Y1100F, Y1106F, or Y1111F mutants. The presence of WT Tie2 increased the expression of miRNA-146b-5p (p=0.0505 vs. control) while presence of Kinase Dead, Y1100F, Y1106F, or Y1111F Tie2 all had no effect on miRNA-146b-5p expression (Figure. 3.12 B). This suggests that the ability of Tie2 to regulate miRNA-146b-5p expression is dependent on the function of all three carboxy-terminal tyrosine residues of Tie2.
Endothelial cells were serum starved for 16 hours and treated with Ang1 (300ng/ml) or PBS for 12 hours. Expression of miRNA146b-5p was measured by RT-PCR and expressed as fold change from non-transfected control cells. Data were analyzed using a one-tailed T-Test (*p<0.05, six biological replicates per group). (B) HEK-Blue hTLR4 cells were transfected with Kinase Dead (KD), Wild Type (WT) Tie2, or Tie2 single mutants (Y1100F, Y1106F, Y1111F). Expression of miRNA146b-5p was measured by RT-PCR and expressed as fold change from non-transfected control cells. Data, expressed as means ± SEM, was analyzed using a one-way ANOVA followed by Tukey's multiple comparison test (four biological replicates per group).
3.5 Discussion

In the present study, levels of Nf-κB activation were monitored in real time using a SEAP Nf-κB reporter assay. A reduction in Nf-κB activation was observed in the cells expressing Wild Type Tie2. This anti-inflammatory effect was lost in cells expressing an inactive kinase dead version of Tie2. Excitingly, we detected that cells expressing a tyrosine to phenylalanine mutation at Y1100 were unable to exert anti-inflammatory effects as the WT Tie2 expressing cells did. This implicated Y1100 as the key residue involved in the ability of Tie2 signalling to interfere with TLR4 driven Nf-κB. Use of the selective Mek inhibitor, UO126, allowed us to identify Erk1/2 as the signalling cascade emanating from Y1100 essential for the interference effect of Tie2 on Nf-κB activation.

In addition, we also found that Tie2 signalling lowered the protein expression of TLR4 signalling intermediates, IRAK1 and TRAF6, and that Y1100 is essential for the observed effect on IRAK1. Further, we demonstrated that Y1100 signalling is essential for the stabilization effects of Tie2 on IκBα protein. These results suggest that the interference effect of Tie2 signalling events on LPS-TLR4 induced Nf-κB activation occurs upstream of Nf-κB.

The timing of Erk1/2 and Tie2 signalling may prove to be an important variable in the observed Erk1/2 dependent TLR4 interference effect of Tie2. Our study utilized constitutively active Tie2, a model of long term Tie2 stimulation. In a previous report (Echavarria et al., 2015) it was demonstrated that only long-term stimulation with Ang1 had an anti-inflammatory effect in HUVEC, however, Erk1/2 is activated following Ang1 stimulation after short time periods 15-60 minutes of stimulation (Abdel-Malak et al.,
2007; Harfouche et al., 2003; Van Slyke et al., 2009). Additionally, LPS stimulation is also able to activate the MEK/ERK signalling pathway in endothelial cells (F. Wong et al., 2004). In one report, it was shown that that Erk1/2 was activated by LPS after 10 minutes of stimulation and was back to basal levels after 60 minutes and that Erk1/2 signalling was required for the upregulation of miRNA-146b-5p by Ang1 in HUVEC as inhibiting the pathway abrogated this effect. However, after 24 hours of Ang1 stimulation of HUVEC there was no detectable difference in the phosphorylated fraction of Erk1/2 (Echavarria et al., 2015). Future endeavors should focus on characterizing the kinetics of Erk1/2 activation following LPS and Tie2 stimulation on endothelial cells to evaluate the difference between these two methods of Erk1/2 activation.

Whether the difference observed in Nf-κB activation would translate to a reduction in Nf-κB responsive genes in endothelial cells was not tested. This study would be improved by evaluations of Nf-κB responsive genes which could include adhesion molecules such as ICAM-1 or VCAM-1. Previous studies on Ang1 stimulation of endothelial cells have shown a reduction in the levels of Nf-κB responsive genes including ICAM-1, VCAM-1, and E-selectin following VEGF (I. Kim et al., 2001) and LPS exposure (Echavarria et al., 2015).

It has been previously reported that the Tie2 agonist, Ang1, could upregulate expression of miRNA-146b-5p, an miRNA involved in the regulation of proteins involved in the TLR4 signalling cascade upstream of Nf-κB activation, in macrovascular endothelial cells (Echavarria et al., 2015). Endothelial cells have vascular bed and tissue specific heterogeneity (Aird, 2007). We built on the previous report’s findings by confirming the miRNA-146b-5p regulatory effects of Tie2 activation in a microvascular cell line,
furthering the field’s understanding of Tie2 dependent miRNA regulation. Further, we identified the docking sites essential for this effect. All three tyrosine residues are required as mutation of any of the three tyrosine residues to phenylalanine completely abolished this effect. Ang1 was shown to induce an increase in the primary transcript of miRNA-146b-5p (Echavarria et al., 2015). This suggests that the Tie2 signalling pathways increase the transcription of miRNA-146b-5p, however, effects on the processing and decay of miRNA-146b-5p are also possible. How Tie2 is able to regulate the transcription of miRNA-146b-5p and why all three carboxy-terminal tyrosine residues on Tie2 are required to increase the expression of miRNA-146b-5p remains to be determined.
Chapter 4

Discussion and Future Directions
4 Discussion and Future Directions

4.1 Discussion

This study was initiated to characterize the effects of Tie2 signal transduction during endotoxin induced acute inflammation. Specifically, we sought to characterize the outcome of Tie2 activation during acute inflammation in an animal model, and to identify the signalling events downstream of Tie2 that interfere with LPS-TLR4 induced Nf-κB activation.

In chapter two, we showed, in a mouse model of inflammation, that Tie2 signalling reduces inflammation using a synthetic Tie2 agonist, VT. The injection of VT activated Tie2 and caused a significant reduction in the number of immune cells at the site of inflammation, vascular permeability, and inflammatory cytokines, collectively indicating that Tie2 signalling reduces TLR4 driven inflammation in vivo.

One important consideration in these experiments was the degree to which VT was able to activate Tie2. To address this, lung tissue was collected to assess the level of Tie2 activation in animals pre-treated with VT compared to controls receiving vehicle (PBS). These animals had not received a dose of VT for 24 hours, yet we were still able to detect higher levels of Tie2 activation in animals receiving the VT treatment. Whether this was due to sustained phosphorylation of the receptor following the acute dose of VT, or if it is due to prolonged activation by VT which remained available due to sustained circulating or localized VT was not evaluated. An experiment to determine whether or not VT causes the same receptor downregulation dynamics reported for Ang1 (Wehrle et
al., 2009) would be one approach to address this question. Further, a study to determine how long VT remains in the circulation is warranted. The fact that VT induced a sustained level of Tie2 activation in the lung tissue of experimental animals suggest that VT is a useful tool for the characterization of chronic Tie2 activation in vivo.

Additionally, the dosing schedule of a pre-treatment followed by a second dose at the time of inflammatory stimulation was selected based on a successful pilot study. Additional studies are needed to evaluate the impact of the timing of VT treatment. For example, the contribution of VT pretreatment, or treatment concurrent with or following induction of inflammation, to the anti-inflammatory effects of Tie2 activation remains to be determined.

In chapter three, using a cell culture model, we characterized the individual contributions of the carboxy-terminal tyrosine residues of Tie2 toward the anti-inflammatory effects on Nf-κB activation in response to TLR4 signalling. Using this strategy, we identified Y1100 as the main effector of the Nf-κB inhibitory actions of Tie2, and showed that Erk1/2 signalling, which emanates from Y1100, is essential in this process. Y1100 was required for the ability of Tie2 to lower Nf-κB activation as well as the TLR4 signalling intermediate, IRAK1, and was also necessary for the stabilization effects of Tie2 on the Nf-κB inhibitor protein IκBα.

Erk1/2 signalling plays a complex, and potentially context specific, role in endotoxin induced TLR4 signalling in endothelial cells. Previous studies have demonstrated that the stimulation of endothelial cells with LPS activated the MEK/ERK signalling pathway (F. Wong et al., 2004). In one report, it was shown that Erk1/2 was activated by LPS after 10
minutes of stimulation and returned to basal levels after 60 minutes (Echavarria et al., 2015). In contrast, we found that Tie2 induced Erk1/2 signalling was essential for the reduction in LPS induced Nf-κB activation. In our model, Tie2 was constitutively active and, presumably, this resulted in sustained Erk1/2 signalling in our cells before the initiation of inflammation. These results suggest that the effects of Erk1/2 signalling on Nf-κB may have temporal and context specific dynamics and specificities.

It was previously shown that the Tie2 agonist, Ang1, could upregulate expression of miRNA-146b-5p in macrovascular endothelial cells (HUVEC) (Echavarria et al., 2015). We built on these previous findings by confirming the miRNA-146b-5p regulatory effects of Tie2 activation in a microvascular cell line (HMVEC), furthering the field’s understanding of Tie2 dependent miRNA regulation. In addition, we demonstrated that tyrosine residues Y1100, Y1106, Y1111, are all essential for the effect of Tie2 on miRNA-146b-5p expression. The finding that all three of the carboxy-terminal tyrosine residues of Tie2 examined are required to regulate the expression of miRNA-146b-5p, but only Y1100 is required for the stabilization of IκBα suggests that the effects of Tie2 signalling on the expression of miRNA-146-5p, and IκBα protein levels, are by distinct mechanisms (Figure. 4.1).

The mechanism of Ang1 induced upregulation of miRNA-146b-5p appears to be complex and remains to be fully clarified. Nf-κB has been shown to transcriptionally regulate the expression of miRNA-146a in human alveolar epithelial cells (Taganov et al., 2006), however, this was not demonstrated for miRNA146b. In human myeloid leukemia cells, the early growth response 3 transcription factor (EGR-3), induced downstream of MEK/Erk signalling, was shown to regulate transcription of miRNA-146b (Cheng et al.,
The fact that the miRNA-146 family is controlled by Nf-κB and MEK-Erk signalling suggests that the miRNA-146 family is part of the endogenous negative feedback loop used to control inflammation. However, the mechanisms enacting transcriptional regulation of miRNA-146b-5p by the Tie2 residues Y1100, Y1106, Y111F remains undiscovered.

Whether the Tie2 dependent changes in Nf-κB activation observed in our cell culture models would translate to a reduction in Nf-κB responsive genes in endothelial cells was not tested. However, we showed in vivo that Tie2 activation by VT administration was able to reduce the expression of inflammatory genes which are Nf-κB targets including IL-6 (Libermann & Baltimore, 1990), IL-8 (Kunsch & Rosen, 1993), and MCP-1 (Ueda et al., 1994). This suggests that the reduction in Nf-κB activation seen in our cell culture model would also reduce the overall effects of Nf-κB induced transcription of inflammatory mediators. The expression of Nf-κB responsive genes such as adhesion molecules like ICAM-1 or VCAM-1 could be measured following Tie2 activation in LPS treated endothelial cells to characterize the effects of Tie2 activation on Nf-κB responsive genes.

Results obtained from our animal model indicate an anti-inflammatory effect of Tie2 activation. The effects seen are likely the cumulative effect of VT-Tie2 activation on endothelial cells as well as other Tie2 expressing cells such as the subset of Tie2 expressing leukocytes that includes a fraction of monocytes and macrophages (De Palma et al., 2005), neutrophils (Sturn et al., 2005), and eosinophils (Feistritzer et al., 2004). As such, the anti-inflammatory effects observed cannot be exclusively attributed to the direct effects of Tie2 on endothelial cells, and may include secondary effects on other cell types.
However, results from our cell culture models suggest that the anti-inflammatory effects of Tie2 are largely driven by the direct effect of Tie2 activation on endothelial cells, as this effect was observed in a model devoid of leukocytes. A more comprehensive animal study, specifically targeting the endothelial cell compartment, would be required to garner a greater understanding of the specific contribution of endothelial cells to the anti-inflammatory effect of Tie2 activation in vivo.

Both our in vivo and in vitro experiments were designed to examine the effect of Tie2 activation prior to the initiation of a TLR4 driven inflammatory cascade. Future work should focus on the ability of Tie2 to reduce Nf-κB activation in endothelial cells after the onset of inflammation to garner information about the ability of Tie2 to reduce TLR4 driven vascular inflammation in a clinical setting where inflammation is often not predicted. Emerging evidence suggests that the pre-treatment design may not be necessary. For example, in a murine model of severe influenza virus infection, that is complicated by lung microvascular leakage, it was shown that VT, administered as late as 72 hours after infection, was still able to reduce lung oedema and lung endothelial cell apoptosis and ultimately increased the rate of survival (Sugiyama et al., 2015). While this is not a TLR4 driven inflammation model, it does suggest that studies examining the effect of Tie2 activation after the onset of LPS-TLR4 driven inflammation are warranted. The pre-activation design of our animal model might be useful in situations such as planned inflammation as a result of surgery.

TLR4 has also been shown to engage endogenous triggers of sterile inflammation which are released following tissue damage (Lee et al., 2016). Since our study exclusively monitored LPS driven TLR4 activation, it would be interesting to test whether Tie2 can
interfere with TLR4 signalling stimulated by other inflammatory agents or whether the effect on Nf-κB activation is ligand specific.

The present study investigated the role of *in vitro* Tie2 activation on the expression of miRNA-146b-5p. Whether Tie2 activation *in vivo* results in an increase in miRNA-146b-5p levels remains to be determined. Use of miRNA inhibitors would allow for the measurement of the importance of miRNA in the regulation of vascular inflammation in a model LPS-TLR4 driven inflammation.

We propose a model whereby Tie2 reduces inflammation using at least two different paths. LPS-TLR4 induced Nf-κB activation is reduced by Tie2, in part, through Y1100 driven Erk1/2 signalling. Y1100 driven Erk1/2 signalling acts to prevent the degradation of the Nf-κB inhibitor, IκBα. Secondly, Tie2 affects the expression of TLR4 signalling intermediates through regulation of miRNA. Tie2 signalling upregulates the expression of miRNA-146b-5p in a mechanism which requires all three of the carboxy-terminal tyrosine residues known to be essential for Tie2 signalling (Figure 4.1).
**Figure 4.1. Schematic of model.** Tie2 reduces LPS-TLR4 induced Nf-κB activation, in part, through Y1100 driven Erk1/2 signalling which prevents the degradation of the Nf-κB inhibitor, IκBα. Tie2 upregulates the expression of miRNA-146b-5p in a mechanism that requires all three of the carboxy-terminal tyrosine residues known to be essential for Tie2 signalling.
4.2 Future of the Field

The work presented herein along with work from other groups, including clinical results proposing that dysregulation of the angiopoietins could serve as a marker for clinical outcomes, indicate that the Angiopoietin–Tie2 system represents an attractive target for the development of future vascular therapies. Potential therapeutic approaches designed to target the Tie2 signalling axis for the regulation of TLR4 driven Nf-κB signalling include, directly activating Tie2 through an agonist such as Vasculotide, or indirectly activating Tie2 through the inhibition of Tie2 inhibitors such as the tyrosine phosphatase VE-PTP. Additional methods could also include the control of miRNA whose levels are affected by Tie2 activation, specifically miRNA-146b-5p.

Efforts to clinically enhance Tie2 signalling pathways should proceed with caution. The effects of long-term and short-term super-activation of Tie2 should be characterized and carefully considered. Systemic super-activation may have deleterious effects. For example, individuals with gain-of-function mutations that enhance Tie2 kinase activity experience venous malformations during development (Calvert et al., 1999; Morris et al., 2005; Vikkula et al., 1996). However, short-term Tie2 activation would be unlikely to exert vascular remodeling effects. Further, Tie2 is also known to activate endothelial nitric oxide synthase resulting in increased levels of the vasodilatory chemical nitric oxide. The effects of potential vasodilation could be harmful in some pathologies complicated by hypotension, including shock. Additionally, activation of Tie2 may have differing affects in different cells types. For example, Tie2 expressing leukocytes are highly angiogenic and have been shown to enhance tumour angiogenesis (Capobianco et
al., 2011). For these reasons, caution should be exercised when considering systemic super-activation of Tie2.

The tyrosine phosphatase, VE-PTP, has been shown to associate with Tie2, reducing the activation of the receptor (Fachinger et al., 1999). The strategy of indirectly affecting Tie2 activation through its inhibitor presents an additional means of therapeutically targeting Tie2. In fact, VE-PTP inhibitors are being tested in clinical trials for the treatment of ocular diseases (Campochiaro et al., 2015; Shen et al., 2014). Whether or not the inhibition of VE-PTP proves to be a viable strategy in the control of TLR4-NF-κB signalling by Tie2 remains to be determined.

As we learn more about the roles of specific miRNA, we will undoubtedly strive to translate that knowledge into clinical settings with the hopes of inducing or inhibiting specific miRNA in specific compartments and organs in vivo. In experimental models several miRNA have been successfully inhibited in selected tissues in vivo. For example, miRNA-712 was successfully inhibited in the endothelium of ApoE -/- mice, and inhibition decreased the development of atherosclerotic lesions (Son et al., 2013). Further work is also ongoing within the field to develop miRNA systemic delivery to specific compartments, including the use viral methods (Callegari et al., 2013) and nanoparticles (D'Abundo et al., 2017).

Collectively, miRNA play a complex role in the endothelial inflammatory process. In endothelial cells miRNA have been shown to exert both anti-inflammatory (miRNA-10a (Fang, Shi, Manduchi, Civelek, & Davies, 2010), -181b (Sun et al., 2014), 146b (Echavarria et al., 2015)) and inflammatory (miRNA-19a (Akhtar et al., 2015)) functions.
Interestingly, in ApoE/- mice, deficiency in the endothelium of the enzyme Dicer, which is responsible for the biogenesis of mature miRNA, caused an overall reduction in inflammation, with a reduction of the adhesion of monocytes, and a reduction in the development of atherosclerotic lesions (Hartmann et al., 2016). Although, a reduction in miRNA biogenesis would most likely preferentially effect miRNA with a high turnover rate, it suggests that additional work on the role of miRNA in the endothelium is warranted to assess therapeutic potential.

An additional layer of complexity in the role of miRNA in the vasculature is the regulation of miRNA expression by blood flow patterns. In the endothelium, the expression of several miRNA have been shown to be affected by blood flow patterns. The expression of miRNA-92a and miRNA-712 are increased in areas of disturbed flow and have both been shown to promote atherosclerosis (Loyer et al., 2014; Son et al., 2013). Inhibiting miRNA-92a abrogated endothelial inflammation and reduced the development of atherosclerosis in Ldlr−/− mice (Loyer et al., 2014). These emerging results underscore the importance of fully understanding the role of miRNA in order to safely design therapeutics.

When considering the control of inflammation, therapies must specifically target the desired cellular compartment to avoid off target effects. One example of the importance of target specificity comes from the differential effects of Nf-κB inhibition in distinct cell types. Inhibition of Nf-κB in endothelial cells has been shown to reduce atherosclerosis plaque formation in ApoE/- mice (Gareus et al., 2008), while inhibition in macrophages has been shown to lead to more severe atherosclerosis in LDL receptor deficient mice (Kanters et al., 2003). In addition, it is essential to fully understand what the outcome of
the control of Nf-κB would be in each specific cell type. Nf-κB targets a wide breadth of target genes, including a subset which promote cell survival under conditions of stress (Karsan, Yee, Kaushansky, & Harlan, 1996), illustrating the importance of a complete understanding of the effects of Nf-κB control in a multi-variable *in vivo* system.
4.3 Concluding Remarks

A complete understanding of the mechanisms that control vascular inflammation is essential for designing therapeutic strategies. The vascular endothelium represents an important target for the control of vascular inflammation. The endothelium clearly plays a pivotal role in the host response to inflammation. In patients with sepsis or acute lung injury, dysregulated levels of circulating Ang1 and Tie2’s antagonist, Ang2, have been observed (Gallagher et al., 2008; Orfanos et al., 2007; Parikh et al., 2006). This suggests that Tie2 is poised as a critical target for the management of vascular inflammation or could serve as marker of vascular dysregulation.

We demonstrated an anti-inflammatory effect of Tie2 signalling during acute inflammation in vivo. Further, we characterized the role of specific signalling cascades emanating from Tie2 on TLR4 driven inflammation in cell culture models. The present study contributes to our understanding of the regulatory effects of Tie2 signalling on LPS-TLR4 driven Nf-kB activation and advances the field of knowledge in the mechanisms that control vascular inflammation and the biology of Tie2 signalling.
Chapter 5: References


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