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The role of endothelial PI3 kinase activity and IQGAP1 in regulation of lymphocyte diapedesis

by

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I dedicate this work to my family, whom without their never-ending support none of this would be possible. I am forever grateful for having you.

Abstract

Leukocyte extravasation is a fundamental process of the inflammatory responses. The mechanisms that control remodelling of endothelial (EC) shape and adhesive contacts during leukocyte transendothelial migration (TEM) are not completely understood. We studied the role of EC phosphatidylinositol 3-kinase (PI3K) activity in lymphocyte TEM under shear stress conditions. Inhibition of EC PI3K activity by its pan inhibitors decreased lymphocyte diapedesis in a step after VE-cadherin opening. The importance of PI3K catalytic isoforms (p110α, p110β, p110δ and p110γ) were studied in TEM. Treatment of EC with isoform inhibitors of p110β, p110δ and p110γ did not affect lymphocyte TEM. Inhibition of p110α activity or expression reduced lymphocyte diapedesis. PI3K activity was measured in EC exposed to shear stress alone or shear stress on cells where ICAM-1 or VCAM-1 were cross-linked. The most significant effect was seen in cells cross-linked with ICAM-1 and exposed to shear stress. This suggests that cooperation of shear-induced mechanotransduction and ICAM-1 during leukocyte interaction with EC facilitates leukocyte diapedesis by inducing PI3K.

We hypothesized that Rho GTP proteins downstream of PI3K activity are involved in leukocyte TEM. We studied the role of IQGAP1, a Rac1/Cdc42 effector, during lymphocyte TEM. EC IQGAP1 knockdown decreases both microtubule (MT) tethered to the adherens junction (AJ) and lymphocyte TEM. Similarly, loss of AJ-associated MT induced by brief nocodazole (ND) treatment decreases lymphocyte TEM. Neither intervention affected leukocyte migration to the interendothelial junctions. These data indicate that IQGAP1 contributes to MT

stability at endothelial junctions and is involved in the junction remodelling required for efficient lymphocyte diapedesis. We studied a candidate Rho guanine nucleotide exchange factor named FGD5 upstream of IQGAP1. Inhibition of FGD5 expression resulted in more sensitivity to apoptotic stimuli and a higher rate of apoptosis in resting conditions. Thus, we could not study the importance of FGD5 in lymphocyte TEM. Further characterization of FGD5 knockdown cells showed that they do not respond to VEGF signalling. These results suggest that FGD5 might play an important role in growth factor–mediated EC survival.

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Table of Contents

List of Figures

List of abbreviations

ABP	Actin binding protein
AJ	Adherens junctions
BAD	Bcl-2-agonist of death
Dock180	Dedicator of cytokinesis 180
EC	Endothelial cells
ECGS	Endothelial cell growth supplement
ELMO1	Engulfment and cell motility 1
eNOS	Endothelial nitric oxide synthase
EPLIN	Epithelial protein lost in neoplasm
F-actin	Filamentous actin
FOXOs	Forkhead family of transcription factors
G-actin	Globular actin
RhoGEF	Rho Guanine nucleotide exchange factors
HEV	High endothelial venules
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IF	Immunofluorescent
ILK	Integrin-linked kinase
IQGAP1	IQ motif containing GTPase activating protein 1
JAM	Junctional adhesion molecule
LFA-1	Lymphocyte function-associated antigen 1

MLC	Myosin light chain
MT	Microtubule
MTORC2	Mammalian target of rapamycin complex 2
ND	Nocodazole
PAK	p21-activated kinases
PECAM-1	Platelet endothelial cell adhesion molecule-1
PI3K	Phosphatidylinositol-3 kinase
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PVR	Poliovirus receptor
RhoGAPs	Rho GTPase activating proteins
RhoGDIs	Rho GDP-dissociation inhibitors
TEM	Transendothelial migration
TJ	Tight junctions
TNF	Tumour necrosis factor-α
VE-cadherin	Vascular endothelial cadherin
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VE-PTP	Vascular endothelial-protein-tyrosine phosphatase
VLA-4	Very late antigen-4
WASP	Wiskott Aldrich Syndrome protein
ZO	Zona occluden

Chapter 1. Introduction

Vascular tree and endothelium

In this thesis, we sought to better understand the role of the microvascular endothelial cell (EC) in the recruitment of lymphocytes to sites of inflammation. This process involves the active participation of the EC to present adhesive and chemotactic cues to the circulating lymphocyte. Subsequent entry of the lymphocyte into the tissue compartment must require remodelling of the EC monolayer and basement membrane. This thesis will focus on the overall biology of the EC during this process.

Blood vessels consist of arteries, arterioles, capillaries, venules and veins. The morphologies of these vessels are adapted differently to cope with the unique function of the vessel: arteries have thick muscular walls and accommodate boluses of blood delivered by the contracting heart to efficiently carry the oxygenated blood to various organs; capillaries are composed of very thin walls which facilitate the exchange of nutrients and gas; veins have a thinner wall than arteries and contain valves which prevent blood from flowing backward [1]. Concordantly, the ECs covering each of these vessels have adapted a unique structure [2]. This includes variation in length, thickness, fenestration and continuity, as well as intercellular junction composition [2, 3]. For instance, in arteries, concordant with their main function of carrying the blood, ECs are continuous and have tighter junctions than in veins [4].

EC heterogeneity, and consequently its specialized function, is crucial for health. For instance, in the liver, discontinuous and large fenestrations of the endothelium are essential for a dynamic filtering of fluids, solutes and particles, and the differentiation of EC into a continuous basement membrane is associated with the occurrence of disease [5]. The endothelium lies at the interface between the blood and the tissue compartments, and plays a major role in the regulation of immune responses. Interactions with circulating immune cells outside lymphoid organs occurs predominantly at the post capillary venule, which is specialized to display chemotactic and adhesive cues to recruit leukocytes to the site of inflammation.

The first requirement of leukocyte trafficking into a target tissue is leukocyte interaction with EC, known as leukocyte transendothelial migration (TEM). This process is tightly regulated by adhesion molecules and signalling events of both ECs and leukocytes. Before describing leukocyte TEM in more detail, I will address some of the environmental conditions of ECs such as interaction with neighbouring cells, the extracellular matrix and shear stress generated by blood flow and how these conditions might affect leukocyte TEM. I will also briefly introduce different types of leukocytes and will describe examples of immune responses that require leukocyte TEM.

Endothelial cell-cell adhesion

As described earlier, ECs adopt amazingly different phenotypes in different vascular beds. This heterogeneity also includes their intercellular

junction composition [2, 6]. For instance, the endothelium in the microvessels of the brain versus venules of other organs is less permeable to micro and macro molecules, and this is attributed to ECs forming much tighter junctions, generating the blood-brain barrier [7]. Endothelial junctions consist of transmembrane proteins that are linked to cytoskeleton and signalling molecules via their cytoplasmic domains [8, 9]. These complex networks of molecules are categorized into adherens junctions (AJ), tight junctions (TJ) and adhesion molecules that do not belong to any of these.

In the vascular tree, postcapillary venules are more responsive to inflammatory stimuli and are specialized sites for leukocyte trafficking [10, 11]. The junctions of the postcapillary venules were characterized as simple and straight connections as apposed to interdigiating complexes found in EC of larger veins [12]. To study leukocyte transendothelial migration, we used human umbilical vein endothelial cells (HUVEC) because of their similar characteristics to postcapillary venules, mainly containing functional AJ and lack of organized TJ [13-16]. Thus, in this review, I will briefly introduce TJ and other components of EC junctions. AJ components, signalling and regulation, as well as junctional molecules that are depicted in leukocyte TEM will be discussed in more detail.

Tight Junctions (TJ)

TJ contain a complex molecular architecture, which are mainly known for providing a tight barrier in the monolayer. By electron microscopy, TJ appear as membranes fused together [17]. TJ transmembrane proteins include occludin,

claudins and the junctional adhesion molecule (JAM) family [14, 18, 19]. In addition to brain, JAM members (JAM-A, -B and -C) are also detected in the vasculature of a number of organs, including the liver and kidney [20]. TJ are connected to the cytoskeleton via their cytoplasmic component, the zona occludens (ZO) (Figure 1) [8, 15]. The importance of functional TJ is apparent from the phenotypes of knockout mice. For instance, claudin-5-deficient mice are defective in EC brain function and die 10 hours after birth [21].

Immunofluorescent (IF) staining of ZO and occludin is discontinuous and diffused along the cell borders of HUVECs grown in normal conditions [13, 22]. Electron microscopy of HUVEC cell borders indicate that only 16% of cell borders contain the characteristic morphology of TJ stands (fused membranes), and they tend to be localized at the apical side [13]. Conditions that increase TJ localization (astrocyte-conditioned medium) increases EC barrier function (transendothelial electrical resistance), but not the rate of neutrophil diapedesis [13]. Interestingly, conditions that decrease TJ (low growth supplements) do not reduce EC barrier function and do not affect neutrophil diapedesis [13]. These observations suggest that although the TJ components are expressed in HUVEC monolayers, they are poorly developed and unable to make functional TJ.

Adherens junctions (AJ)

AJ are essential for proper EC barrier function and the generation of outside-in signalling that regulates contact inhibition of cell growth and angiogenesis [23, 24]. In ECs, AJ are formed by the Ca^{2+} dependent glycoprotein

transmembrane adhesion protein, vascular endothelial cadherin (VE-cadherin) [9, 25]. The cytoplasmic region of VE-cadherin is highly conserved among cadherins, and interacts with several cytoplasmic proteins, the catenins (Figure 1) [9]. Early in the embryonic stage, cells start expressing VE-cadherin once committed to EC lineage [26]. In adults mice, VE-cadherin antibody injection results in lung and heart permeability and death after 24 hours [27]. During development, the homophilic VE-cadherin interactions and proper cytoplasmic interactions with catenins are essential for EC barrier function and VE-cadherin mediated signalling, as both the deletion of VE-cadherin and the expression of a truncated VE-cadherin deficient in binding β-catenin result in embryonic lethality at day 9.5 [24]. The ECs of these mice were detached from each other at the branches and gaps were formed between ECs, while apoptosis was evident in some EC. Although vasculogenesis (the formation of primitive blood vessels from angioblasts) was normal, angiogenesis (ECs branching from existing vessels and remodelling into a network of vessels) was completely abrogated [24]. Impaired survival and angiogenesis was attributed to a defect in VEGF signalling, since VEGFR2 localization to junctions, interaction with VE-cadherin and Phosphatidylinositol-3 kinase (PI3K) and Akt phosphorylation were greatly reduced by VE-cadherin deletion or truncation [24]. Since this result was reported, many other studies have illustrated the crucial role of AJ cytoplasmic components in the regulation of AJ function by the expression of recombinant VE-cadherins lacking interaction with various catenins [28, 29].

VE-cadherin interacts with β-catenin and plakoglobin (γ-catenin) via a membrane-distal site (703-784). Endothelial β-catenin expression is required for vascular integrity and survival, as its EC deletion causes embryonic death at 11.5–13.5 days [30]. β-catenin deficient EC had weaker AJs, and EC permeability was increased [30]. β-catenin interaction with VE-cadherin is important in linking VE-cadherin to actin cytoskeleton, and several pieces of evidence suggest that this anchorage is essential for AJ function and that α-catenin is involved [29, 30]. Yamada et al. (2005) showed that α-catenin cannot bind β-catenin and actin simultaneously [31]. Later, it was shown that a molecule called epithelial protein lost in neoplasm (EPLIN) facilitates an α-catenin link to actin, resulting in the simultaneous interaction of α-catenin with actin and β-catenin [32]. Whether this is the scenario in EC needs further investigation. Another function of β-catenin is in the regulation of gene expression. When unbound from VE-cadherin, β-catenin can regulate gene expression by translocating to the nucleus and interacting with transcription factors [33, 34]. Thus, it is proposed that VE-cadherin can indirectly regulate gene expression by keeping β-catenin in junctions.

VE-cadherin interacts with p120 catenin by its juxtamembrane site (621–702). In epithelial cells, p120 is recognized as a positive regulator of AJ function by stabilizing E-cadherin [35]. In EC, p120 is also required for barrier function and its inhibition significantly reduces levels of several cadherins, including VE-cadherin, along with α- and β-catenins [35-37]. Later, it was shown that p120 interaction with cadherin is required to prevent the clathrin-mediated internalization of cadherin [38]. Specific deletion of endothelial p120 in mice

resulted in embryonic death at day 11.5 [39]. These mice had reduced VE-cadherin and N-cadherin levels, reduced pericyte coverage of vessels, haemorrhages and disorganized vascular networks. Cultured primary EC lacking p120 had very little VE-cadherin expression, and strikingly, EC barrier function was not significantly affected. However, p120 deletion caused a deficiency in EC proliferation, which was rescued by exogenous VE-cadherin expression [39]. p120 was also shown to be a substrate for tyrosine kinases and in immortalized cell lines it can regulate Rho GTPases via binding to the Vav2 (a Rho GTPase guanine exchange factor that activates Rho GTPases) [40, 41].

In confluent monolayers, VE-cadherin is also associated with a transmembrane phosphatase, vascular endothelial-protein-tyrosine phosphatase (VE-PTP), which is important in AJ regulation [42, 43]. VE-PTP expression is observed to be specific to EC and it interacts and dephosphorylates the tyrosine kinase receptor, Tie-2 [44]. VE-PTP interacts with VE-cadherin via the extracellular domain of both molecules and its inhibition induces EC permeability [42, 43]. Stimuli such as vascular endothelial growth factor (VEGF) treatment and leukocyte binding prevent VE-cadherin association with VE-PTP and result in elevation of phosphorylated AJ [43]. VE-PTP inhibition increases leukocyte TEM, which will be discussed in the section on diapedesis.

Of note is that N-cadherin expression is also detected in EC. Although N-cadherin is highly expressed in EC, it does not localize to junctions when VE-cadherin is expressed [45]. N-cadherin localization at ECs that lack VE-cadherin

in knockout mice has not been studied. N-cadherin seems to be important in adhesion to pericytes and smooth muscle cells [46, 47].

AJ regulation

As will be described in detail, AJ are regulated by a variety of signalling molecules (e.g. Rho GTPases) and cytoskeleton components. These components can affect AJ distribution or stabilization by a number of mechanisms. These mechanisms include the regulation of AJ endocytosis, proper linkage of VE-cadherin to catenins and the cytoskeleton or the induction of signalling events leading to increased mechanical forces and tension at the junctions [48-50]. The state of phosphorylation of AJ is thought to be an important player in AJ regulation [43, 49, 51-54]. A detailed description of some of these mechanisms and their importance in leukocyte TEM will be given in the next sections.

Other components of EC junctions

Nectin molecules belong to the immunoglobulin superfamily [55]. The nectin family consists of nectin-1, -2, -3, -4 and the poliovirus receptor (PVR). The expression of nectin-2, nectin-4 and PVR is detected in EC [56-58]. Nectins can mediate cell-cell adhesion by both cis- and trans-dimers [56, 59, 60]. Nectins interact with the actin cytoskeleton through interaction with their cytoplasmic component, afadin (Figure 1) [60]. In epithelial cells, afadin has been shown to bind to activated Rap1 GTPase (a member of Ras family G proteins) and profilin

(an inducer of actin polymerization by activating actin monomers) [61]. In HUVECs, afadin required Rap1 to localize at the junctions and inhibition of afadin expression, or impairing its localization to junctions by Rap1 knockdown, prevented accumulation of junctional proteins to interendothelial junctions [62]. Further, afadin and Rap1 regulated VEGF mediated signalling by regulating interaction of VEGF receptor 2 (VEGFR2) with p110α catalytic subunit of PI3K [62].

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a transmembrane glycoprotein that is localized at the junctions of all confluent EC types [63]. PECAM-1 participates in EC barrier function [64], and its cytoplasmic domain is known to interact with several signalling molecules [65-67]. PECAM-1 contributes to EC barrier function by its homophilic interactions at the cell-cell levels rather than its participation at the cell signalling of junctions [68]. Although PECAM-1 knockout mice have no apparent physiological defects, EC junctions are less stable and functional during stressful conditions [69, 70].

S-endo-1 associated antigen (CD146) has been shown to localize to junctions in confluent EC monolayers, and its transfection into fibroblasts reduces their permeability, suggesting that CD146 participates in the regulation of junctions [71]. Furthermore, it is associated with the actin cytoskeleton [71].

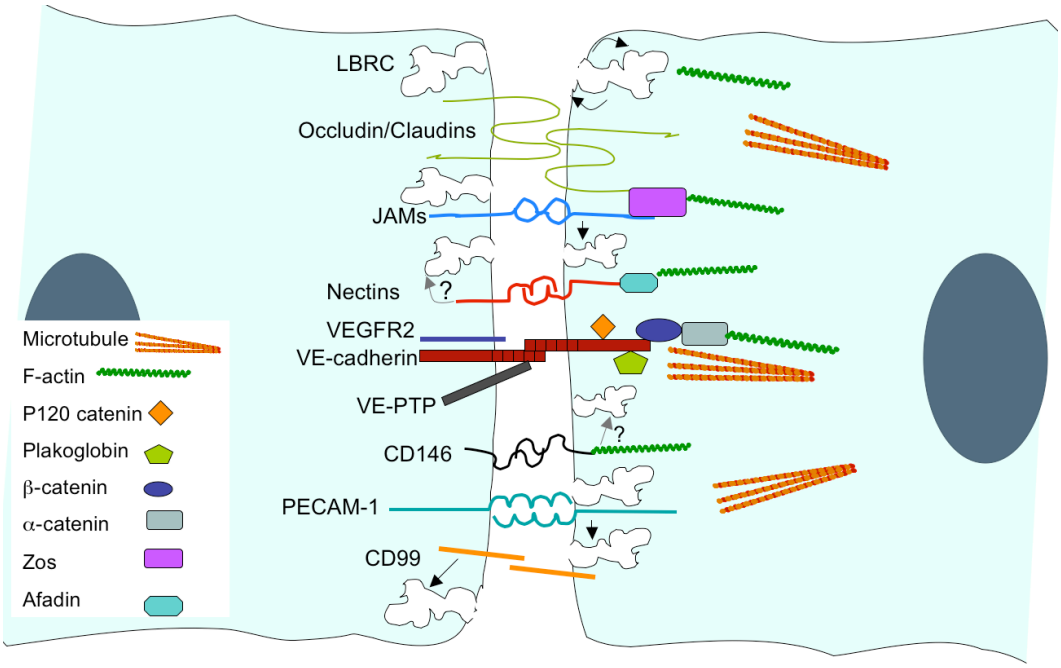

Figure 1-1. An illustration of interendothelial junctions' components. TJ are composed of transmembrane proteins occludin, claudin and JAM members, connected to actin cytoskeleton via ZO members [8]. Nectin members participate in EC adhesion [56, 59, 60]. Afadin connects the nectins to actin and is also important in recruitment of other adhesion molecules [61, 62]. AJ mediate EC adhesion via its transmembrane protein VE-cadherin, which interacts with actin via interaction with catenins [9]. VE-cadherin also interacts with VE-PTP phosphatase and VEGFR2 [24, 42, 43]. CD146 is another component of interendothelial junctions that it is regulated by actin [71]. Other transmembrane proteins localized to interendothelial junctions are PECAM-1 and CD99 [63, 72]. Membranes of EC borders are constantly recycled into vesicular structures that are named lateral border recycling compartments (LBRC) [73]. Junctional molecules PECAM-1, JAM-A and CD99, but not VE-cadherin are shown to reside in these compartments[74, 75]. Microtubules are also implicated in regulation of interendothelial junctions but the mechanisms are not clear [76, 77].

Extracellular matrix (ECM)

The ECM consists of a variety of components that are organized into a scaffold on which cells adhere. Some of the components of the basement membrane include laminins, type IV collagens, nidogen and perlecan [78, 79]. Laminin and type IV collagen can self-assemble into a scaffold in which other components are incorporated [80, 81]. Laminins are essential for the formation of basement membrane, and type IV collagens are important for function and stability, as their deletion result in embryonic lethality [82, 83]. The composition of ECM modulates cell functions (e.g. cell growth and survival) via interaction with specific integrins, which in turn activate different signalling pathways. For instance, HUVECs grown on fibronectin and vitronectin proliferate, while HUVECs grown on laminin-1 or -4 enter cell cycle arrest [84]. Furthermore, it has been shown that signals from integrin and growth factors cooperate to initiate cell growth [85]. For example, activation of integrin $\alpha 5\beta 1$ by growing HUVECs on fibronectin results in the activation of Rac, which was dependent on focal adhesion kinase (FAK), PI3K and Rac guanine exchange factor Sos [85]. Moreover, overexpression of Rac1 rescues cell cycle arrest on laminin-grown ECs [85]. The integrin-mediated adhesive sites are aggregated in the form of focal adhesions, which are highly dynamic and are linked to the cytoskeleton via their cytoplasmic interaction with a variety of signalling and cytoskeletal components, including FAK, vinculin, talin, and paxillin [86-88].

During leukocyte TEM, several components of focal adhesions such as FAK and paxillin are phosphorylated [89-91]. Furthermore, the stable adhesion of

lymphocytes with ECs induces focal adhesion remodelling, which is required for efficient lymphocyte TEM [91]. The mechanism(s) by which focal adhesions might regulate TEM is not clear.

Shear stress

ECs are constantly exposed to shear stress, the frictional force produced by blood flow. The ability of the endothelium to sense and respond to shear stress is important in many physiological (e.g. embryonic cardiovascular development) and pathophysiological conditions (e.g. atherosclerosis) [92-94]. In addition, leukocyte adhesion and lymphocyte TEM are largely regulated by shear stress [95, 96]. Cells respond to shear stress by mechanotransduction, meaning that cells are able to sense the mechanical stress to elicit a biochemical response [97-99]. For instance, ECs acquire elongated shapes and stress fibres after hours of exposure to shear stress, while in static conditions, cells contain fewer stress fibres and are rounder [100]. Integrins have been shown to be critical for modulation of these changes. One piece of evidence for this is increased expression of $\alpha_V\beta_3$ in the atherosclerotic regions of human arteries, where laminar flow is disturbed [101]. In vitro studies illustrated the activation of integrins and induction of new integrin ligands upon high shear stresses ($\geq$12Dyn/cm^2), which resulted in transient Rho inactivation [98]. Rho inactivation was required for shear-induced F-actin organization and cell alignment [98]. Moreover, integrin activation induced transient Rac activation at the beginning of shear stress, which was required for cell alignment and activation of transcription factor NF-κB and

concomitant ICAM-1 expression within 6 hours of exposure to shear stress [97]. A mechanotransduction complex consisting of PECAM-1 (which transmits the force), VE-cadherin (adaptor) and VEGFR2 (which is activated in a ligand-independent manner and activates PI3K) was identified to be upstream of shear-induced integrin activation [99]. AJs and PECAM-1, acting as mechanotransducers, were also illustrated by other investigators [102, 103]. Interestingly, unlike in wild-type mice, NF-κB activation and F-actin organization are not detected in the atherosclerotic susceptible regions of PECAM-1-deficient mice [99], suggesting a crucial role of this mechanotransduction complex in regulating EC shear responses.

It should be noted that these studies are performed under shear stresses of $\geq$12Dyn/cm^2. The shear stress, however, in postcapillary venules is about 1-4 Dyn/cm^2 [95]. Whether the described signalling events happen at these low shear stresses is not clear. Nevertheless, Cinammon and Alon (2001) have shown that lymphocyte TEM requires shear-induced signals in vitro [96].These findings emphasize the importance of environmental factors on EC function and illustrate the necessity of studying EC biological responses (such as the role in leukocyte TEM) in settings similar to physiological conditions.

Tumour necrosis factor-α (TNF)

TNF is a potent cytokine that is generated mainly by leukocytes and is known to have tumoricidal effects, as well as targeting ECs to induce

inflammatory responses [104-106]. TNF induces a number of changes in ECs that facilitate leukocyte TEM. It induces the expression of adhesion molecules that are important in leukocyte TEM, such as transient early E-selectin (4–6 hours) expression and late intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) expression (18–24 hours) [107]. In addition, TNF phosphorylation of AJ components, dispersion of tight junction components and induction of EC permeability have been reported in HUVECs and human lung ECs [108-112]. Furthermore, TNF induces stress fibre formation within 24 hours of treatment in a RhoA-dependent manner [111, 113, 114]. TNF also mediates a biphasic PI3K activity, activation at 10 minutes and then gradual increase after 6 hours [112]. PI3K activity seems to be important for TNF-induced increase in permeability, but not for stress fibre formation. PI3K-dependent Rac1 activity is also shown to partially regulate AJ remodelling in response to TNF-mediated leukocyte diapedesis [112].

TNF mediates its effect by interacting with its two receptors p75 and p55. Both of these receptors are expressed on EC [107]. While both can contribute to EC activation, signalling downstream of p55 is sufficient to induce upregulation of E-selectin, ICAM-1 and VCAM-1 [107].

Leukocytes

One of the main cell components of the immune system is leukocytes. Classically, leukocytes are categorized according to their common progenitors, either myeloid or lymphoid lineage [115], which in turn are raised from

hematopoietic stem cells of bone marrow [116]. The myeloid lineage includes granulocytes (neutrophils, eosinophils and basophils), macrophages (mature forms of monocytes), mast cells and dendritic cells [115]. These cells are crucial players of innate immunity and are also involved in development of adaptive immunity [117].

The lymphoid progenitor gives rise to T and B lymphocytes which are responsible for the function of adaptive immunity [115], as well as development of autoimmune diseases and allograft rejection. Naïve T cells (T cells that have not encountered an antigen) home to lymphoid organs by passing through high endothelial venules (HEV) of lymph nodes [118]. In the secondary lymphoid organs, lymphocytes encounter antigens that are presented to them by dendritic cells. T cells that recognize an antigen, proliferate and acquire effector functions and migrate to inflamed sites or lymph nodes depending on their surface adhesion molecules and chemokine receptors [119]. In this thesis, we study the interaction of lymphocytes with activated EC which resembles the migration of activated T cells into inflamed tissues via postcapillary venules. The next sections will focus on details of how EC participate in regulation of leukocyte TEM.

Leukocyte Trafficking

Leukocytes that are circulating in blood migrate to various tissues (extravasation) under several physiological and pathophysiological conditions. During innate immune responses, neutrophils and monocytes migrate towards the site of inflammation [120]. Lymphocyte migration into tissues, in turn, happens in

adaptive immune responses, allograft rejection and autoimmune diseases [121]. For instance, multiple sclerosis and its mouse model, experimental autoimmune encephalomyelitis, are initiated by $CD4^+$ T cells migration into central nervous system [122]. During this process, the first barrier that leukocytes encounter is endothelium of the vasculature. The interaction between EC and leukocytes, leukocyte TEM, is tightly regulated by a series of adhesion molecules and the intracellular signalling in each cell. Leukocyte TEM is classically categorized into leukocytes tethering and rolling on surface of endothelium, leukocyte activation and firm adhesion, locomotion (moving of adhered leukocytes to favourable site of diapedesis), and diapedesis (Figure 1-2) [123, 124]. There are two diapedesis routes shown in vivo and in vitro: a transcellular route in which leukocytes traverse through the body of endothelium [125-128], and a paracellular route in which leukocytes travel through the inter-endothelial junctions [120]. In vitro, the paracellular route has been shown as the main route of diapedesis in HUVECs [129, 130], and no transcellular diapedesis is observed in the TEM model that we have adopted for our studies [96, 131]. Hence, the focus of this work is on paracellular diapedesis.

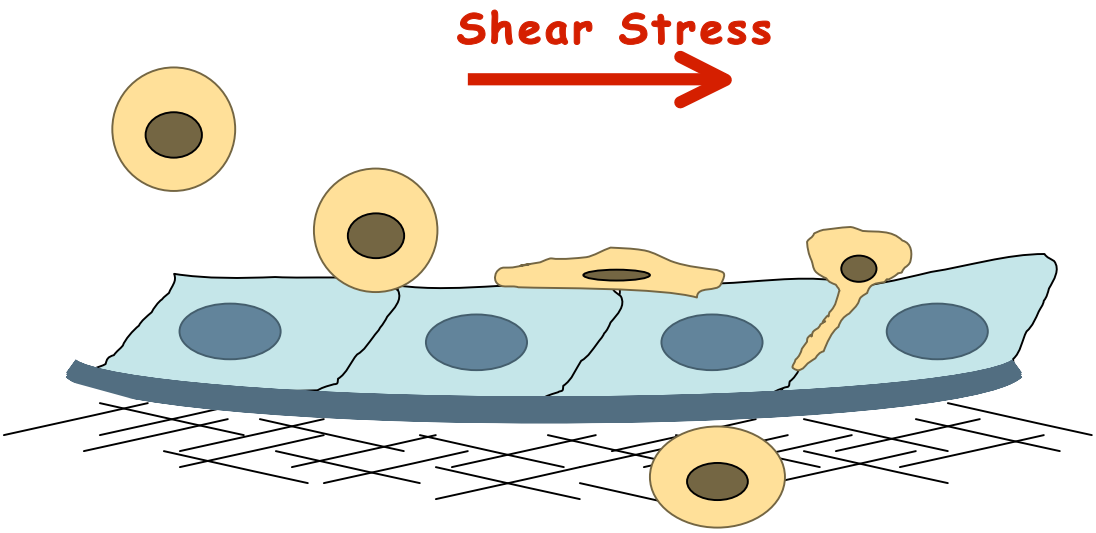

Figure 1-2. A simplified view of leukocyte TEM. Under shear stress generated by blood, leukocytes tether and role along EC, then firmly adhere and locomote on the surface of endothelium. Finally, leukocytes go under diapedesis and migrate towards the underlying tissue.

Tethering and rolling

Initial contact between leukocytes circulating in the blood and endothelium is mediated by flow of leukocytes close to the EC surface rather than in the central bloodstream [132]. This flow-mediated interaction, or leukocyte margination, is prominent in postcapillary venules and facilitates the active recruitment of leukocytes by tethering [132].

Tethering is formed mainly by mammalian lectins, selectins, and their glycoprotein ligands [133, 134]. The selectin family consists of L-selectin (constitutively expressed in leukocytes), E-selectin (highly expressed in activated

EC) and P-selectin (expressed in platelets and EC activated by thrombin, histamine or superoxide) [134-137]. These adhesion receptors are capable of rapidly engaging with ligands with high tensile strength [138]. The importance of selectin-mediated leukocyte adhesion is apparent in the immunodeficiency of patients with leukocyte adhesion deficiency type II (LADII). These patients are defective in generating fucosylated structures, including selectin ligands, and suffer from frequent, severe infections in their soft tissues [139]. In addition, the mouse model of this disease illustrates defective leukocyte homing [140]. T lymphocyte responses do not seem to be greatly affected by impaired selectin adhesion [139, 140]. This can possibly be explained by the observations that integrin adhesion molecules, VLA-4 and $\alpha 4\beta 7$, can mediate rolling of lymphocytes on EC [141-144]. For leukocytes to stop rolling and firmly adhere to ECs, their integrins must be activated by chemokines and there must be subsequent adhesion to their ligands [133].

Chemokines

Chemokines are a family of chemoattractant cytokines that are released by tissues in the early phases of infection. Chemokines are divided into four groups according to their amino terminal region structure (number and positioning of cysteine residues: C, CC, CXC and CX_3C) [145]. Chemokines can also be categorized depending on whether their expression is constitutive (e.g. Stromal cell-derived factor-1 (SDF-1), also known as CXCL12) or inducible (e.g. interleukin-8) [146]. Constitutively expressed chemokines are usually involved in

development in addition to leukocyte trafficking. SDF-1 is a chemokine that is constitutively expressed in bone marrow–derived stromal cells and ECs of the bone marrow [147-149]. Genetic deletion of either SDF-1 or its receptor CXCR4 (ubiquitously expressed) is embryonically lethal [150]. Mice deficient in SDF-1 have defects in B cell and myeloid generation from bone marrow, as well as defects in heart development [150]. Mice deficient in CXCR4 have similar defects in addition to defects in cerebellar neuronal layer formation [151]. Although these mice had no detectable deficiency in T cell generation or trafficking, previously, CXCR4 was shown to be important in T cell trafficking [152].

In the context of TEM, secreted chemokines are immobilized by binding to heparin-like glycosaminoglycans on ECs, which enables them to interact with G protein coupled receptors (GPCRs) of leukocytes [153]. The signalling in leukocytes via chemokine GPCR leading to integrin activation is termed inside-out signalling. Integrins can acquire three conformations: low affinity (closed headpiece), intermediate affinity (closed headpiece extended) and high affinity (open headpiece extended) [154]. The high affinity integrin becomes fully active once it interacts with its ligand [155].

Chemokines presented on EC and their receptors on leukocytes are localized on the microvilli of the cells, which promotes their interaction [156]. The chemokine signal from EC glycocalyx to rolling adherent leukocyte enhances binding, and promotes motility on the surface of the endothelium. This generates signalling events in leukocytes that leads to activation of integrin adhesion molecules of leukocytes in milliseconds [157]. Interestingly, soluble chemokines

can only mediate partial activation of integrins and must be presented to leukocytes by the endothelium glycocalyx in solid phase for optimal signal transduction [96, 155]. Since integrins are only fully active once they adhere to ligand, this ensures a spatial and temporal regulation of integrin activation necessary for firm adhesion of leukocytes to EC [155].

Firm adhesion (arrest) and locomotion

The main adhesion molecules involved in firm adhesion of leukocytes to EC are leukocytes integrins and their receptors on EC. The importance of integrins in inflammation is apparent in patients with leukocyte adhesion deficiency type I. The integrin chain in these patients is mutated and they suffer from a range of infections in their soft tissues including skin and mucosa [158, 159]. In the next sections, some of the in vivo and in vitro data that support importance of integrin ligands, ICAM-1 and VCAM-1 will be discussed.

Intercellular adhesion molecule (ICAM)-1

ICAM-1 is an immunoglobulin cell-surface protein with a short cytoplasmic domain that lacks intrinsic kinase activity and known protein-protein interaction domains [160]. However, ICAM-1 can interact with cytoskeletal associated proteins such as α-actinin and ezrin [161, 162], and ICAM-1 crosslinking mediates a number of signalling pathways which will be explained later. ICAM-1 upregulation in response to inflammatory molecules is observed in

vivo and in vitro [163, 164]. ICAM-1 interacts with its ligands, β_2 integrins Lymphocyte function-associated antigen (LFA)-1 ($\alpha_L\beta_2$), expressed on all leukocytes, and MAC1 ($\alpha_M\beta_2$), expressed on myeloid cells [165-167].

The leukocyte integrins, like all other integrins, are heterodimers that must be activated in order to mediate a response. While in unstimulated leukocytes, LFA-1 is predominantly in a low affinity state, upon interaction with chemokines it acquires an intermediate affinity [155]. LFA-1 is fully activated upon immediate interaction with ICAM-1 under physiological shear stress conditions [155], and this is required for TEM as blocking specific high-affinity LFA-1 reduces lymphocyte TEM by about 70% [168]. Concomitantly, LFA-1/ICAM-1 distribution changes upon this active interaction. During neutrophil TEM, evenly distributed LFA-1 on arrested leukocytes redistribute into a ring-like structure around leukocytes undergoing diapedesis [169]. LFA-1 redistribution into clusters of linear tracks associated with ICAM-1 enriched docking structures has also been shown [170]. However, in elegant experiments by Alon and colleagues, the ICAM-1/LFA-1 enriched docking structures were only inducible by addition of mn^{2+}, which artificially activates integrins [168]. Further, they found these structures to interfere with lymphocyte TEM [168]. Instead, using freshly isolated T cells and live cell imaging, they observed ICAM-1-GFP clustering underneath the crawling T cells. The fraction of LFA-1 interacting with EC surface are in high-affinity conformation and are distributed into dot-like structures [168]. Although the pattern of ICAM-1/LFA-1 distribution seems to vary depending on leukocyte subtype, chemokines and other experimental conditions [168-170], all

of these experiments suggest complex crosstalk and signalling events going on in both EC and leukocyte with the endpoint of facilitating leukocyte TEM.

Antibody blocking studies have shown that preventing ICAM-1 interaction with LFA-1 on HUVECs reduces locomotion and consequent diapedesis of monocytes and lymphocytes, but does not affect adhesion [96, 124]. However, in some TEM models leukocyte diapedesis is only greatly blocked by interfering with both ICAM-1 and VCAM-1 functions [171].

Another member of ICAM family which also interacts with LFA-1 is ICAM-2. ICAM-2 is constitutively expressed in resting cells and its expression is not inducible [172]. Further, its cytoplasmic domain does not generate any intracellular signalling such as RhoA activation or stress fibre formation [173, 174]. However, there is some evidence of ICAM-2 participation in TEM: An in vitro study looking at brain ECs isolated from knockout mice deficient in ICAM-1, ICAM-2 or both indicated while ICAM-1 deletion reduces locomotion and diapedesis of T cells to about 60% and ICAM-2 deletion does not affect locomotion and diapedesis, blockade of both ICAM-1 and ICAM-2 reduces locomotion and diapedesis of T cells by about 90% [175]. Although this study did not test the contribution of ICAM-1-generated signalling in diapedesis, others have shown that ICAM-1 cytoplasmic domain is required for leukocyte TEM [160, 176]. ICAM-1 knockout mice are viable and they have some immune deficiencies such as recruitment of neutrophils to sites of peritonitis [177]. T cell recruitment to cardiac tissue was defective in ICAM-1 knockout mice infected with a parasite [178] and also lymphocyte counts in blood were high in ICAM-1

knockout mice and had defects in generating lymphocyte allogeneic responses [177, 179]. β_2 integrin knockout mice, which are used as a model for LAD I disease, have impaired neutrophil and T cell extravasation to skin lesions [180].

Vascular cell adhesion molecules (VCAM-1)

VCAM-1 is a transmembrane glycoprotein belonging to the immunoglobulin gene superfamily [181]. Its interaction with its ligand is important for development, since VCAM-1 deficient mice and mice deficient in α_4 integrin die at the embryonic stage due to similar abnormalities—mainly cardiac abnormalities and placenta disruption [182, 183].

In ECs, VCAM-1 expression is greatly increased by cytokine stimulation, and has been shown to mediate the adhesion of all leukocytes to VCAM-1 transfected cells but neutrophils [181]. VCAM-1 interacts with leukocytes by its ligands, very late antigen-4 (VLA-4, $\alpha_4\beta_1$) and $\alpha_4\beta_7$ [184, 185]. Upon interaction with lymphoblasts, EC VCAM-1 forms cuplike structures enriched in actin, membrane-actin cytoskeleton linkers, ezrin and moesin and other actin regulating proteins such as α-actinin and VASP [186]. In contrast, neither VCAM-1 clustering nor VLA-4 redistribution was observed during the crawling of freshly isolated T cells on ECs under shear stress; instead, α_4 staining showed its enrichment at the rear of the cell [168]. It is not clear whether these different observations are due to leukocyte type, and the physiological significance of the described VLA-4 pattern is not clear.

Several studies using the antibody blocking approach have suggested that VCAM-1 does not play a significant role in leukocyte TEM [96, 171] or have a small separate roles in leukocyte adhesion [124, 175] and diapedesis [124]. However, blocking both VCAM-1 and ICAM-1 interaction with their ligands greatly reduces leukocyte adhesion and diapedesis (greater than their sum) [96, 171]. In addition, several reports have suggested that VCAM-1 crosslinking or interaction with its ligand generates distinct signalling pathways in EC, which facilitates leukocyte diapedesis (details will be discussed later) [187, 188].

Diapedesis

CD146

CD146 is expressed on ECs and lymphocytes [71, 189]. During lymphocyte TEM, CD146 seems to mediate lymphocyte rolling on EC, since its blockade by antibody inhibited lymphocyte rolling and adhesion [190]. However, recently, it was shown that CD146 expression is important in monocyte diapedesis [191]. CD146 is upregulated by up to 48 hours of TNF treatment and its localization is redistributed to EC junctions and also at the apical site. Further blockade of CD146 function with antibody or inhibition of its expression by RNA interference, did not affect adhesion of monocytes but resulted in about 40% decrease in monocyte TEM across HUVECs [191]. The exact mechanism of CD146 regulation during leukocyte TEM and also its function in vivo is not clear.

Nectin/afadin

As described, nectin/afadin adhesion molecules associate with signalling molecules, the cytoskeleton and TJ components [60, 61, 192]. These interactions suggest that nectin/afadin might be important in the remodelling of interendothelial junctions and signalling events during leukocyte diapedesis. Indeed, PVR localization at junctions plays an important role for monocyte diapedesis [58]. DNAX accessory molecule (DNAM)-1 is a member of the immunoglobulin superfamily and is expressed on T lymphocytes, natural killer lymphocytes (NK) and monocytes [193]. PVR and nectin-2 both act as ligands for DNAM-1 and these interactions signal to NK lymphocytes to stimulate cell lysis [194]. During monocyte interaction with ECs, PVR is the major ligand for DNAM-1, and blockade of either DNAM-1 or PVR using several antibodies prevented monocyte diapedesis to about 80% [58]. The blockade seems to happen before VE-cadherin gap formation [58]. DNAM-1 is a signal transduction molecule (suggested because of its ability to become tyrosine phosphorylated upon binding to its antibody), and can also interact with LFA-1 in normal lymphocytes [195]. Although the relevance of DNAM-1 phosphorylation in leukocyte diapedesis is not known, this observation might mean that ICAM-1/LFA-1 interaction during leukocyte TEM signals to DNAM-1 and facilitates leukocyte diapedesis by mediating PVR/DNAM-1 interaction. Further, DNAM-1/PVR interaction might generate signalling events in EC that facilitate leukocyte diapedesis.

Platelet-endothelial cell adhesion molecule (PECAM)-1

PECAM-1 is another member of the immunoglobulin superfamily; its involvement in neutrophil and monocyte TEM is shown in many TEM models [73, 74, 196-200]. However, PECAM-1 deficient mice do not have major defects in inflammatory responses [201], and the role of PECAM-1 in TEM seems to be dependent on the leukocyte type and cytokines, as lymphocyte TEM is not dependent on PECAM-1 [201-203]. Its deletion of its cytoplasmic domain does not block leukocyte TEM, suggesting that PECAM-1 is not involved in EC signalling that leads to leukocyte TEM [204].

PECAM-1 is one of the adhesion molecules that have been found in lateral border recycling compartments (LBRC) [73]. These compartments are below the plasma membranes of the cell borders, are not sealed vesicles, and are constitutively recycled to the junctions [73]. Adhesion molecules PECAM-1, CD99 and JAM-A, but not VE-cadherin, are located in these compartments [73, 75]. During diapedesis, these compartments are specifically targeted to the leukocyte migration channel in a Src-, kinesin- and microtubule-dependent manner, and are required for both transcellular and paracellular TEM [73-75, 205]. Interestingly, constitutive membrane recycling was not found to be blocked by microtubule inhibition; instead this seems to be actin dependent [74]. The fact that the targeted membrane recycling around migration channel regulation differs with the constitutive membrane recycling is a great example of meticulous signalling and active participation of the endothelium in diapedesis. How exactly LBRC facilitates diapedesis is not clear. It is suggested that these compartments

might mediate more homophilic adhesion bonds by bringing unbound adhesion molecules to the surface, and/or they might increase the plasma membrane at the junctions, which might also contribute to VE-cadherin separation [73, 74].

CD99

CD99 is a heavily glycosylated protein that is expressed in leukocytes [206] and ECs [72]. CD99 endothelial expression and localization at junctions was first described by Muller and colleagues (2002) [72]. Using function-blocking antibodies, they demonstrated that endothelial CD99 is greatly involved in diapedesis but not adhesion, as its blockade inhibited about 90% of monocyte TEM across HUVECs. Later, other in vivo and in vitro studies confirmed CD99's importance in monocyte, neutrophil and lymphocyte TEM [207-209]. CD99 mediates homophilic interactions with leukocytes during TEM, and its blockage seems to trap leukocytes at the interendothelial junctions in a step distal to PECAM-1 and independent of PECAM-1 [72].

Junction adhesion molecules (JAMs)

As described earlier, JAMs are transmembrane proteins found at TJs. The function-blocking antibody to JAM-A inhibits monocyte TEM across mouse lung and heart ECs in vitro, and reduces monocyte and neutrophil accumulation to the brain and lung [14, 210]. Later, it was shown that JAM-A can interact with LFA-1 and regulate lymphocyte and neutrophil TEM across HUVECs [211]. In addition,

JAM-B interacts with the integrin VLA-4; however, the physiological significance of this interaction has not been tested [212]. JAM-C has also been implicated in leukocyte TEM in vivo and in vitro [213-215]. JAM proteins' importance in TEM seems to be dependent on inflammatory conditions, as well as on other unidentified conditions, since JAMs' involvement in TEM was not detected by all investigators [124, 169, 216]. One proposed condition is compensatory effects from other JAM members [217]. For instance, Corada and colleagues (2005) studied the importance of endothelial and polymorphonuclear leukocytes JAM-A in leukocyte recruitment into inflamed peritoneum or in the heart after ischemia reperfusion injury using bone marrow transplanted JAM-A knockout mice [217]. Endothelial JAM-A was not required for leukocyte TEM, while JAM-A-deficient leukocytes were not able to transmigrate [217]. However, another study using JAM-A knockout mice and mice with specific deletion of endothelial JAM-A in a model of post-ischemic injury of the liver showed that endothelial JAM-A is crucial for neutrophil TEM but has a moderate effect on lymphocyte TEM [218]. It was proposed that this discrepancy might be due to a low expression of JAM-B and JAM-C in the liver, and therefore a lack of compensation by other JAM members [217].

Endothelial morphological and signalling changes during TEM

Interaction between ECs and leukocytes has been investigated for more than 100 years; in the last 50 years, the focus has been on the morphological changes of leukocytes. Active participation of ECs in leukocyte TEM and the

great structural and signalling endothelial changes are just being appreciated. Originally, EC changes were hinted at by crude staining of both ECs and leukocytes, which illustrated actin enrichment and α-catenin enrichment around the migration channel [219]. Later studies illustrated that VCAM-1 and ICAM1 crosslinking results in the formation of cuplike structures enriched in actin, membrane-actin cytoskeleton linkers, ezrin and moesin and other actin regulating proteins such as α-actinin and VASP [186]. Another study showed that the ICAM-1-mediated docking structure formation requires intact microtubules, actin and cytosolic free Ca^{2+}, and these structures are highly associated with diapedesis [129, 170]. Further, it was shown that upon ICAM-1 crosslinking, a guanine exchange factor for RhoG is recruited to the cup structures [220]. RhoG is activated after ICAM-1 crosslinking and inhibition of its activity reduces cup formation and leukocyte (an immortalized cell line) TEM without affecting leukocyte adhesion [220]. The function of these structures is not clearly known. In addition to their association with diapedesis, it is also shown that they are involved in leukocyte firm adhesion under high shear stresses [221]. Although enrichment of several adhesion molecules during TEM has been reported by others, these three-dimensional docking structures are not observed in all TEM models [74, 169]. In any case, these observations suggested formation of a signalling platform that might facilitate leukocyte diapedesis by initiating necessary changes in endothelium.

Studies looking at endothelial signalling during TEM started in the early 1990s. The original experiments illustrated ICAM-1 binding and localization to F-

actin, as well as association with α-actinin [162, 222]. In more physiological settings, it was shown that ICAM-1 crosslinking of brain endothelium mediates the phosphorylation of several proteins, including cortactin, an actin binding protein, as well as focal adhesion kinase (FAK) and paxillin, which is dependent on Rho activity [89, 223]. Further, Rho activity and intact F-actin of brain endothelium was shown to be important in diapedesis but not for adhesion of T lymphocytes to EC [224]. An attempt to identify a mechanism for opening of the EC junction during TEM led to identifying myosin light chain (MLC) phosphorylation, actin stress fibre formation and changes in myosin II patterns during the adhesion of neutrophils to HUVECs [225]. These events and neutrophil TEM were dependent on cytoplasmic Ca^{2+}, calmodulin and MLC kinase (MLCK) activity [225]. Earlier studies also showed that neutrophil and lymphocyte interactions with cytokine-activated HUVECs greatly increase cytosolic Ca^{2+}, which is essential for diapedesis but not adhesion of leukocytes to ECs [226, 227].

Recently, endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide production downstream of ICAM-1 clustering was shown [228]. ICAM-1 crosslinking activates eNOS via an AMP-activated protein kinase (AMPK), and PI3K is not activated by ICAM-1 crosslinking in static conditions [228]. eNOS signalling was shown to be required for VE-cadherin tyrosine phosphorylation and lymphocyte diapedesis across immortalized mouse brain ECs [228]. A recent study has shown that elevated nitric oxide can mediate nitration of a Rho GTPase-activating protein, p190RhoGAP-A [229]. This inactivates the RhoGAP, which causes elevation of RhoA activation and increased junction

permeability [229]. This suggests that eNOS activation during leukocyte TEM might mediate the opening of EC junctions by affecting Rho GTPase activities.

The importance of AJ remodelling and whether the disappearance of VE-cadherin is a passive or active phenomenon have been tested by many investigators. Injection of function-blocking antibodies against VE-cadherin in mice increases the rate of neutrophils migrating to an inflamed peritoneum [230]. Early in vitro studies showed an overall reduction of AJ triggered by neutrophil adhesion to HUVECs [231, 232], but not T lymphocyte adhesion [231]. These observations were possibly due to a nonspecific function of proteases produced during PMN preparation. Indeed, later studies illustrated a specific and transient loss of VE-cadherin during transmigration, but not the locomotion of leukocytes [233, 234]. The mechanism(s) that govern this and the true nature of VE-cadherin disappearance are not clear. Phosphorylation of AJ components has been proposed as one mechanism for AJ remodelling during diapedesis. Adhesion of leukocytes or specific ICAM-1 crosslinking induces activity of tyrosine kinases Src and PYk2 and phosphorylation of VE-cadherin at Y658 and Y731 in HUVECs [53, 235]. Mutation of these sites prevented their phosphorylation and reduced leukocyte TEM. In rat brain ECs, ICAM-1 crosslinking also induces VE-cadherin phosphorylation, albeit in a Src-independent manner; furthermore, mutation of Y658 did not affect lymphocyte TEM [54]. A recent study indicated that PI3K catalytic subunit p110α is required for PYk2 activity and phosphorylation of VE-cadherin at Y731 [112]. However, inhibition of Pyk2 expression by itself does not affect lymphocyte TEM [112], suggesting that other

mechanisms in addition to VE-cadherin phosphorylation are involved in migration channel formation. These authors also reported Rac activation downstream of PI3K class IA activation during leukocyte TEM [112].

There are several proposals for how VE-cadherin phosphorylation would facilitate diapedesis. Phosphorylation might reduce the binding of VE-cadherin to catenins and consequently the cytoskeleton, thus causing weak junctions. This is based on the induction of AJ phosphorylation by other factors that induce permeability and direct observations of junction destability by VE-cadherin phosphorylation [49, 51, 52]. However, ICAM-1 crosslinking did not mediate less association of VE-cadherin with any of the catenins [54]. Another possibility is that VE-cadherin phosphorylation at the p120 site might destabilize AJ by inducing internalization of VE-cadherin during diapedesis. In fact, overexpression of p120 greatly increased VE-cadherin levels and reduced TEM [53]. Unexpectedly, the authors could not detect any endocytic event during TEM. Phosphorylation of plakoglobin is also observed during neutrophil or lymphocyte adhesion to ECs [43]. Plakoglobin phosphorylation is associated with VE-PTP dissociation from VE-cadherin and this seems to be important in leukocyte TEM, since the inhibition of VE-PTP increases leukocyte TEM [43]. It is not clear how exactly plakoglobin phosphorylation facilitates leukocyte diapedesis.

It is also proposed that AJ opening is regulated by actomyosin-based cytoskeletal contractility induced by RhoA [89, 224, 236]. Myosin light chain phosphorylation and induced permeability are also observed downstream of Rac GTPase [50].

Another proposed mechanism for AJ opening during TEM involves reactive oxygen species (ROS, such as H_2O_2) [52]. VCAM-1 crosslinking leads to Rho and Rac activation [188]; VCAM-1 mediated Rac activation induces ROS in HUVECs, which is important for gap formation but not stress fibre formation [188]. ROS production downstream of NADPH oxidase is also observed in VCAM-1 crosslinking of high endothelial venules [187].

Another mechanism of VE-cadherin opening might relate to the function of LBRC. The extra plasma membrane that is targeted to the migration channel might dilute out the adherens junctions' components, since they are not present in these vesicles [75].

Phosphatidylinositol-3 kinase (PI3K)

Phosphatidylinositol lipids

Understanding how the extracellular environment regulates cell activities has been an area attracting major interest in biology. Phosphatidylinositol lipids (PtdIns; also referred to as phosphoinositides) have been identified as major components of the signal transduction pathways that transfer signalling cues from membrane to cytoplasm [237]. PtdIns consists of inositol-1-phosphate attached to diacylglycerol via its phosphate group. There are three hydroxyl sites on the inositol head group that can be potentially phosphorylated in cells. PI3K phosphorylates the 3-hydroxyl group on three substrates: PtdIns, PtdIns4P and PtdIns(4,5)P_2 [238-240]. PI3K are categorized into three classes: the PI3KI-

favoured substrate in cells is PtdIns(4,5)P_2 generating PtdIns(3,4,5)P_3 (PIP3); PI3KII uses PtdIns4P to make PtdIns(3,4)P_2; and PI3KIII's substrate is PtdIns, and it generates PtdIns3P [238, 239, 241]. Regulation and function of each of these PI3Ks is greatly complex and varies in different cell types and cell context. In this review, I will focus on PI3KI's structure and regulation, mainly in EC functions related to leukocyte TEM.

PI3K class I

Originally, class I PI3K heterodimers were divided into classes IA (catalytic subunits p110α, p110β and p110δ) and IB (catalytic subunit p110γ). Class IA catalytic subunits interact with five different p85 regulatory isoforms (p85α, p55α, p50α, p55γ and p85β) downstream of a tyrosine kinase receptor or a cytoplasmic tyrosine kinase [242-246]. Class IB interacts with p101 or p84 (also called $p87^{PIKAP}$) regulatory subunit downstream of GPCRs [247-250]. Recently, this classification has been challenged by observations that implicate p110β downstream of GPCR [251-254] and indirect mechanisms such as activation of Ras by GPCRs can potentially induce the indirect activation of class IA subunits by GPCRs [255-257]. PI3K is negatively regulated by phosphatases. One important phosphatase for PIP3 is the phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [258, 259]. PTEN-deficient mice die at embryonic day 9.5. PTEN-deficient cells are less sensitive to apoptotic stimuli and have high PI3K activity [259].

PI3K class IB structure and function in leukocyte trafficking

There are two regulatory subunits that interact with the p110γ catalytic isoform: p101 and p84 [247, 249, 260]. p101 contains a Gβγ binding site and a p110γ binding site. In vivo, p101 mediates p110γ activation via Gβγ by recruiting p110γ to membrane [261]. The other class IB regulatory subunit, p84, is differentially expressed in relation to p101. For instance, in the heart, p84 expression is five times higher than that of p101 [249]. p101/p110γ have been shown to induce much greater PI3K activity than the p101/p84 heterodimer [249]. Whether this scenario applies to different cell events and cell types and expression of p84 in EC is not clear.

During leukocyte TEM, chemokines induce PI3K activity in leukocytes [262]. p110γ was found to be important in leukocyte recruitment since leukocytes from p110γ knockout mice do not respond to chemokines evident by lack of PIP3 production and Akt phosphorylation [263-265]. In addition, they have impaired migration towards a chemotactic stimulus and recruitment to infection sites [263-265]. Later, p110γ was detected in EC and a part of defect in neutrophil recruitment to site of inflammation was attributed to EC [266]. It turned out that EC in mice chimeric for p110γ activity (p110γ -/- TNFα stimulated EC interacting with wild-type neutrophils) are defective in efficient capturing of circulating neutrophils via E-selectins [266, 267]. Further, in response to a viral or antigen challenge, p110γ null mice do not develop footpad swelling as severe as control mice, suggesting p110γ importance in T cell mediated responses [265]. Since lymphocytes do not require selectins for TEM [143, 268], this T cell

response defect might be due to separate mechanisms, and the importance of endothelial p110γ in this model was not tested.

PI3K class IA structure

PIK3R1 (encodes p85α, p55α and p50α which are produced by mRNA transcribed from alternative promoters) and PIK3R2 (encoding p85β) are widely expressed while PIK3R3 (encodes p55γ) is more restricted [269, 270]. The regulatory subunit consists of several domains. All p85 isoforms contain two Src-homology-2 (SH2) domains which both have affinity for doubly phosphorylated tyrosines on tyrosine kinase receptors or cytoplasmic tyrosine kinases, hence engage p85 to tyrosine kinase signalling [242, 243, 271]. The N-terminal SH2 domain of p85α can also interact with the helical region of p110α which is suggested to be importance in negative regulation of p110α [272]. The domain between the two SH2 domains (intervening domain, iSH2) interacts with catalytic [270] subunits and mediates stable dimer formation [273]. p85α and p85β contain a domain called BH domain (also referred to as RBD domain) with homology to GTPase-activating proteins (GAPs) [274]. This domain interacts with Rac-GTP and Cdc42-GTP without causing their GTP hydrolysis [275, 276]. This interaction can explain how PI3K can be an effector of Rac1/Cdc42 [277, 278].

All p110 catalytic subunits contain adaptor binding domain (ABD), the Ras-binding domain (RBD), membrane binding domain (C2), a helical domain and the catalytic domains [272].

Regulatory subunits modulate the activity of PI3K in several ways. First, they stabilize the catalytic isoforms, which is exemplified by the rapid thermal degradation of p110α in the absence of a regulatory subunit [279]. Second, they keep the catalytic subunit in a low basal activity state [272, 279]. Once PI3K interacts with pYXXM motifs, this inactive conformation switches to the fully activated enzyme.

PI3K IA tissue distribution and function

In rodents, p85α protein is expressed ubiquitously, while its splice variants p50α and p55α have a restricted tissue distribution being enriched in the kidney, lung and brain [269, 270]. p85β is expressed at low levels in most cells [270]. p55γ is expressed in the embryonic tissues and in adult mice it is expressed at high levels in brain, lung, kidney and testis [270]. The expressions of catalytic isoforms p110α and p110β are broad, while p110δ is expressed in hematopoietic and nervous systems and in ECs [267, 280]. p110γ is also present mostly in leukocytes and detected in endothelium [266, 281].

A number of transgenic mice have been generated to study the role of each PI3K subunit. However, some of these knockouts led to altered expression of other subunits of PI3K and unexpected increased PIP3 generation. For instance, knockout of p85α leads to increased expression of p50α and p55α along with increased PIP3 production [282]. In addition, deletion of p85α, p50α and p55α result in a great reduction of expression of PI3K IA catalytic subunits [283-285].

Interpreting observations with mice lacking p110α is also difficult, since it was associated with p85 upregulation, which might act in a dominant negative fashion [286]. However, in elegant experiments, Graupera et al. inactivated global p110α by replacing endogenous p110α with a kinase-dead allele. These mice had growth retardation and although they had normal heartbeats and blood flow in central vessels, they were defective in angiogenic remodelling, which resulted in embryonic lethality at day 12.5 [251]. Remarkably, p110α inactivation in ECs alone was sufficient to cause lethality at the same age. Specific deletion of endothelial p110α resulted in defective angiogenesis, as was made evident by poorly remodelled vessels in the head and trunk and the absence of angiogenic sprouts [251]. Further, in vitro studies indicated that p110α activity in resting EC is highest among all p110 isoforms and also VEGF stimulated PI3K activity and cell events such as in vitro angiogenesis and migration is almost entirely dependent on p110α [251]. Additionally, RhoA activity under basal and serum stimulated conditions was reduced by p110α deletion or pharmacological inactivation to a great extent and Rac1 activity was decreased modestly [251].

P110β knockout embryos die at day 3.5. The stoichiometry of PI3K was not assessed in these mice [287]. Mice with specific P110β deficiency in EC were viable and fertile and did not have any apparent vascular defects, consistent with dominant role of p110α activity in EC [251].

P110δ [288, 289] and p110γ [263, 265] knockout mice are viable. Also, specific deletion of P110δ in EC did not affect mice viability or vascular functions [251]

Endothelial p110δ and p110γ activity are both required for neutrophil trafficking by mediating transition from tethering and rolling of neutrophils to firm adhesion [266, 267]. Recently, however, Ridley and colleagues (2010) showed that endothelial p110α activity is required for efficient T lymphoblast and monocyte diapedesis [112].

PI3K effectors

PI3K is implicated in a great variety of cell functions. This can be explained by the vast number of proteins that interact with PIP3 and are considered as PI3K effectors. PI3K regulation of the cytoskeleton is mediated mostly via Akt and activation of Rho GTPases, which will be the focus of this review.

Akt kinases (Akt)

Akt (also known as protein kinase B, PKB) serine/threonine kinases are well-known downstream effectors of PI3K [290, 291]. There are three isoforms of Akt: Akt1 (PKBα) is ubiquitously expressed; Akt2 (PKBβ) is mostly found in insulin-sensitive tissues; and Akt3 (PKBγ) is mostly expressed in the testis and brain. These isoforms are encoded by three different genes but have similar structural domains, including a PH domain that mediates translocation to membrane and interaction with PIP3, the product of PI3K [290, 292].

Full activation of Akt is associated with its phosphorylation at two sites: threonine 308 and serine 473. T308 phosphorylation seems to induce the catalytic active conformation and phosphorylation at S473 stabilizes Akt to expose the catalytic domain to downstream substrates [293]. S473 phosphorylation also seems to enhance T308 phosphorylation [294]. PI3K regulate Akt phosphorylation and activation as follows. Upon PIP3 generation at the membrane, a PH domain containing protein called PDK1 (PIP3-dependent protein kinase-1) is recruited to PIP3 [295], and Akt is recruited to PIP3 via its PH domain. Akt recruitment to PIP3 causes a conformational change in Akt [296], which along with Akt close proximity to PDK1 mediates Akt phosphorylation at T308 and S473 by another kinase. The regulation and kinases that mediate phosphorylation of S473 are not as clear. Mammalian target of rapamycin complex 2 (mTORC2) is one of the enzymes that have been shown to phosphorylate S473 [294, 297, 298]. There is evidence that mTORC2 requirement for S473 is cell-type and stage-specific: While during embryogenesis, mTORC2 is essential for survival and S473 phosphorylation, in adult skeletal muscles, mTORC2 is not required for S473 phosphorylation [299, 300]. During stress, DNA-dependent protein kinase seems to be involved in S473 phosphorylation [301-303]. It is also shown that integrin-linked kinase (ILK) can phosphorylate S473 [304].

Once fully phosphorylated, Akt regulates many cellular pathways by phosphorylating a variety of substrates (about 50 putative Akt substrates have been identified so far) [305]. Akt participates in cell survival by inactivating pro-

apoptotic pathways, including Bcl-2-agonist of death (BAD) and phosphorylation of the Forkhead family of transcription factors (FoxOs) [306, 307]. Akt is negatively regulated by PTEN, and this regulation is physiologically significant since mutations in PTEN leads to hyperactive PI3K/Akt, decreased sensitivity to apoptosis and increased tumour formation [259, 308-311].

Akt activation is also implicated in F-actin remodelling. mTORC2 signalling is shown to mediate cell spreading and actin polymerization in a fibroblast cell line [312], and Hela cells via protein kinase C (PKCα)-dependent pathway [313].

Rho GTPases

Rho GTPases belong to the Ras superfamily of GTPases and are implicated in many cellular functions such as cell division and survival, changes in cell shape, cell motility and regulation of cell-cell and cell-matrix interactions [86, 314-316]. These small monomeric GTPases are molecular switches that regulate many cellular functions by cycling between GTP- and GDP-bound states [317]. When bound to GTP they bind to their downstream effectors and forward an upstream signalling response hence they are considered in their active state and once the GTP is hydrolyzed to GDP they become inactive [317]. This “on” and “off” state is tightly regulated by three main classes of molecules: Guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Rho guanosine nucleotide dissociation inhibitors (GDIs) [318-320]. So far, at least 20

members of Rho family proteins have been identified in humans; among them, RhoA, Rac1 and Cdc42 are the best-characterized members.

Rho Guanine nucleotide exchange factors (RhoGEFs)

The human genome contains at least 83 RhoGEFs [321]. RhoGEFs activate Rho GTPases by facilitating the exchange of GDP to GTP [318]. One family of RhoGEFs is diffuse B cell lymphoma (dbl)-family [318]. The dbl family members contain a dbl homology (DH) domain, which mediates the RhoGEF activity, and a pleckstrin homology (PH) domain which interacts with phospholipids [322, 323]. Members of this family, Tiam and βPIX, are involved in the EC barrier protective effects of an oxidized lipid, OxPAPC, upstream of Rac1 and Cdc42 [324]. In addition, Tiam-1 is implicated in VE-cadherin regulation of Rac1 [325].

Members of a subgroup of Dbl family contain the G protein signalling domain (RGS), which mediates the interaction of GEF with the Gα subunit of the heterotrimeric G proteins [326, 327].

An atypical RhoGEF is Dock180 (dedicator of cytokinesis 180), which acts as a RhoGEF for Rac1 by forming a complex with ELMO1 (engulfment and cell motility 1) [328, 329]. DOCK180 lacks DH and PH domain but can interact with PIP3 by two DHR (DOCK homology region) domains [330, 331]. Global Dock180 deletion or mutation in mice is lethal upon birth due to skeletal muscle defects and cardiovascular defects [332, 333]. Dock180 is involved in cell motility and phagocytosis via Rac1 regulation [334]. Its functions in motility and

vascular development have also been reported in the ECs of zebra fish and mice [333, 335].

GEF activity is mediated by subcellular localization, which mainly happens by recruitment via PH domains or DHR domains for Dock180 to membrane-bound PIP3 [322, 331]. An example of this is that Rac1 activation, after serum, requires Tiam-1 localization to the membrane [336]. GEF phosphorylation is also implicated in GEF activation. For instance, only phosphorylated Vav-1 can induce Rac activation in vitro and in COS-7 cells [337]. Phosphorylation (tyrosine residue) seems to relieve an autoinhibition in the structure, and exposes the DH domain [338]. PIP3 increases Vav activation by enhancing Vav phosphorylation and also recruiting Vav to the membrane [339, 340].

Another interesting mechanism of regulation that is observed in RGS containing GEFs is regulation by the Gα subunit of heterotrimeric G proteins. p115RhoGEF binds to $G\alpha_{12}$ and $G\alpha_{13}$, which induces GTPase activity of Gα subunits, while the GEF activity towards RhoA is increased [326, 327]. This mode of regulation is important in EC function since p115RhoGEF is shown to regulate thrombin-induced EC barrier dysfunction and microtubule disassembly [76]. p115RhoGEF is also implicated in TNF-induced activation of Rho and EC permeability in immortalized mouse brain cell line [341].

Rho GTPase activating proteins (RhoGAPs)

RhoGAPs are a large family of molecules that regulate Rho GTPases by stimulating their intrinsic GTPase activity which causes inactivation of Rho GTPases [320]. An example of RhoGAP importance in Rho GTPase function is seen in EC that p73 RhoGAP is knocked down. p73 was identified as a specifically expressed vascular GAP (smooth muscles and EC) [342]. The knockdown of p73 results in high Rho (but not Rac or Cdc42) activity and high stress fibres. Furthermore, inhibition of p73 resulted in defective angiogenesis due to defects in proliferation and migration [342]. RhoGAPs are also implicated in EC barrier function regulation. For instance, p190RhoGAP is localized to EC junctions after treatment with the barrier-stabilizing compound oxidized phospholipids, OxPAPC, in a Rac-dependent manner [343]. p190 RhoGAP knockdown in vitro and in vivo attenuated barrier protective effects of OxPAPC. These observations indicate the importance of negative regulation of Rho GTPases in physiological conditions. They also show orchestrated function of Rho GTPAses: In response to a stimulus, Rac is activated [344], which mediates barrier protective effects, and RhoA activity is downregulated to further enhance these effects. Recently, p190RhoGAP was shown to become inactivated, resulting in elevated RhoA activity in caveolin-1 knockout mice [229]. These mice had impaired barrier function, which was associated with upregulated eNOS activity because of lack of caveolin-1, resulting in elevated nitric oxide, nitrated and inactivated p190RhoGAP [229].

Rho GDP-dissociation inhibitors (RhoGDIs)

RhoGDIs prevent spontaneous GTP binding by interacting with the switch domains of Rho GTPases, locking them in the inactive form and also by shielding their membrane tag [319]. So far, only three GDIs members are discovered. While GDIα is ubiquitously expressed, GDIβ expression is restricted to haematopoietic cells and GDIγ is seen in the brain, pancreas, lung, kidney and testis [345-348]. During treatment of ECs with reagents that impair EC barrier function, such as thrombin, Rho activation is associated with RhoGDI phosphorylation, allowing the dissociation of RhoGDI from Rho and making it available for activation by GEFs [349].

Rho family

Rho family consists of three isoforms: RhoA, RhoB and RhoC. Most original studies on Rho have used clostridial enzyme C3 transferase, which inhibits all Rho isoforms. In ECs, similar to fibroblasts, RhoA activation induces stress fibre formation [114, 314]. RhoB is implicated mostly in endocytic trafficking. In EC, inhibition of RhoB results in apoptosis during angiogenesis in vitro and in vivo, which seems to be due to a defect in Akt nuclear trafficking [350]. RhoC in ECs seems to regulate cell migration because the specific inhibition of RhoC in human ECs moderately reduces EC motility and in vivo inhibition of RhoC results in reduced angiogenesis [351]. Although RhoA knockout mice have not been reported, RhoA has been characterized more extensively, and we will focus on the RhoA regulation of the EC barrier below.

Rac Family

Based on sequence similarity, Rac1, Rac2, Rac3 and RhoG are placed in the Rac family [352]. While Rac1 and RhoG are ubiquitously expressed, Rac2 and Rac3 expressions are specific to haematopoietic and brain cells, respectively [353]. The physiological significance of Rac1 activity is apparent, since the global knockout of Rac1 results in embryonic death at day 9.5 [354]. These mice had great defects in the formation of all germ layers and their epiblasts grown in culture had deficiency in terms of adhesion to matrix, lamellipodia formation and migration; furthermore, they exhibited a great amount of apoptosis [354].

The importance of endothelial Rac1 in mediating EC responses, such as in regulation of EC cytoskeleton and barrier function, downstream of growth factors and cytokines such as VEGF, TNF-α, histamine and thrombin has been shown by many studies [48, 114, 315, 355, 356]. The importance of Rac1 in EC function is confirmed by mice knockout studies. Specific endothelial deletion of Rac1 results in embryonic death at day 9.5 in mice [357]. Endothelial Rac1 deletion resulted in defects in the development of major vessels and the complete lack of small vascular branches. In vitro culturing of these EC showed no effect on viability or proliferation; however, Rac1 knockdown ECs were completely insensitive to VEGF- and sphingosine-1-phosphate-stimulated migration, tubulogenesis and adhesion [357]. In addition, increases in monolayer permeability by VEGF and sphingosine-1-phosphate were blocked by Rac1 deletion [357]. The importance of

EC Rac1 activity in junction remodelling is also confirmed by many investigators, as discussed later [355, 358, 359].

Cdc42

Cdc42 is well known for its role in formation of the filopodia, actin-rich, finger-like protrusions of membrane that might be important in sensing the environment [86]. Another well-established function of Cdc42 is in cell polarization [360]. Cdc42 has an indispensable role during development since the global knockout of Cdc42 in mice leads to embryonic lethality at day 5.5 [361]. The embryonic stem cells derived from blastocysts were not defective in proliferation, viability and signalling downstream of MAP kinases. However, cells developed a round shape and the actin cytoskeleton had a diffused and disorganized pattern while the wild-type cells could develop stress fibres, lamellipodia and filopodia. These cells were defective in adhesion and migration and were not able to generate de novo actin filaments in vitro in response to PIP2 [361]. Transgenic mice with specific Cdc42 deletion in ECs are not reported. The specific role of Cdc42 in EC junction regulation will be discussed below [362, 363].

It is of note that several reports have pointed to the sequential activation of Rho GTPases. In fibroblasts, the microinjection of constitutively active Cdc42 results in Rac1 activation [86]. Rac activation also leads to Rho activation [86, 315]. These observations suggest that Cdc42 might regulate some Rho GTPase-dependent pathways, indirectly, by regulation of Rac and Rho proteins.

Rho GTPases' Regulation of Cell Junctions

In epithelial cells, inhibition of the global Rho family by C3 transferase, or the dominant negative expression of RhoA, Rac1 and Cdc42, results in deficient E-cadherin localization to junctions [364, 365]. Although inhibition of these Rho GTPases in ECs did not affect VE-cadherin localization to junctions [366], there are a number of reports that point to the crucial role of Rho GTPases in the EC barrier function, as follows. RhoA is generally considered a negative regulator of EC [367, 368]; however, a basal level of Rho seems to be required for intact EC, as the long-term inhibition of Rho kinase reduces EC barrier function and causes fragmentation of VE-cadherin localized at the junction [369]. Rho is also essential for the function of TNF, thrombin and histamine on cell permeability as shown by inhibition of Rho family by toxins, specific inhibition of RhoA or inhibition of the downstream effector Rho kinase [111, 114, 355, 367].

Many growth factors and cytokines, such as VEGF, TNF, histamine and thrombin also require activation of Rac1 for remodelling the EC junctions and inducing EC permeability [48, 50, 114, 355, 356]. Interestingly, Rac1 activation seems to both enhance and reduce endothelial barrier function depending on the stimuli: Dominant negative expression of Rac1 and Cdc42, or inhibition of their expression by RNAi reduces the effects of the barrier-stabilizing compound OxPAPc on permeability, possibly by perturbing cortical actin rim formation [324] and/or by downregulation of RhoA by phosphorylating and activating p190RhoGAP [343]. In addition, Rac1 can enhance VE-cadherin stabilization and

actin rim structures at the junctions downstream of agonists that induce cAMP levels [29]. On the other hand, Rac1 inhibition or inhibition of its downstream activator, PAK, prevents VEGF-induced VE-cadherin internalization and permeability [48]. Here, Rac/Cdc42 downstream of VEGFR2 activates PAK, which leads to phosphorylation of VE-cadherin. Phosphorylated VE-cadherin acts as a docking site to recruit β-arrestin and induce clathrin-dependent VE-cadherin internalization [48]. Work in Schwartz's laboratory have shown that PAK reduces the barrier function mainly by mediating tension at the junctions via inducing myosin light chain phosphorylation [50, 356, 370]. These observations implicate complex Rho GTPases' roles depending on cell context.

Cdc42 seems to play an important role in the stabilization and restoration of endothelial barrier function. This is supported by in vitro and in vivo studies indicating that late Cdc42 activation after thrombin stimulation associates with the recovery of ECs, and the dominant negative expression of Cdc42 delays the reannealing of EC junctions [362]. Conversely, Broman et al. (2006) used a model of inducing junction destabilization by transfecting ECs with a cytoplasmic domain of VE-cadherin in vivo and in vitro [363]. This fragmented VE-cadherin induced gap formation and permeability by competing with endogenous VE-cadherin to bind α and β catenins. Cdc42 was activated in these cells and dominant negative expression of Cdc42 reduced junction destabilization. Cdc42 seemed to regulate F-actin interaction with AJs by preventing association of α and β catenin [363]. The authors suggest that Cdc42 regulation of junctions might be different depending on cellular context and the downstream engaged effectors.

Nevertheless, activation of Cdc42 when AJ are disrupted might initiate regulatory pathways that induce the annealing of EC junctions.

Rho GTPases are also implicated in leukocyte TEM, as described in the leukocyte trafficking chapter.

Rho GTPase Effectors

Activated Rho GTPases mediate biological responses via their effectors. Based on in vitro, in vivo and proteomic analysis, there are about 70 molecules that are known/proposed (based on structure) as Rho GTPases' effectors [371]. Regulating localization, mediating conformational changes or influencing effector interaction with other proteins are all methods that Rho GTPases use to regulate effectors' functions, and consequently, downstream signalling pathways [371]. p21-activated kinases (PAKs, serine/threonine kinases) acting downstream of Rac1 and Cdc42 are well established [320, 372, 373]. In ECs, PAK mediates actin rearrangement and focal adhesion remodelling induced by VE-cadherin ligation [325]. IQGAP1 is another Rac1/Cdc42 effector that is shown to act in ECs, and which will be described in detail.

Actin Cytoskeleton

Actin Structure and Regulation

Dynamic actin cytoskeleton remodelling is the underlying process of many cellular events such as regulation of cell-cell/cell-matrix interactions and cell motility, which in turn is important for biological responses, including angiogenesis, wound healing and leukocyte trafficking [325, 374-377]. In cells, actin exists in globular (G-actin) and filamentous (F-actin) forms, and its dynamics is regulated by controlling the balance between these two in response to stimuli [314, 315]. In resting ECs, filamentous actin is reported to organize as dense peripheral bands (more pronounced in aortic EC than HUVEC) and fine stress fibres along the cells [378-380]. Actin stress fibres are bundles of actin and non-muscle myosin that generate isometric forces and are connected to focal adhesions and possibly adherens junctions [381, 382].

Actin can bind to ATP, and ATP-bound G-actin binds to F-actin via the barbed end (the fast growing end), and once the ATP is hydrolysed, the monomer leaves the filament [383]. Cells use a great number of proteins (actin binding proteins, ABPs) to tightly regulate de novo F-actin generation. ABPs control actin dynamics at several stages: nucleation (formation of small actin oligomers), elongation, severing filaments, capping/uncapping barbed ends, and sequestering actin monomers [383-385].

Original experiments (using inhibitors, dominant negative and constitutively active expression) found Rho GTPases downstream of stimuli such

as growth factors and cytokines to have an essential role in actin cytoskeleton remodelling [86, 314-316, 386]. Later, Rho GTPase activation was directly linked to actin remodelling by showing the Cdc42-dependent N-WASP regulation of actin polymerization [387]. One of the main nucleating factors that mediates branched F-actin is Arp2/3 complex [388]. However, it has been shown that by itself Arp2/3 is a weak nucleating factor and, for full activity, requires interaction with nucleating factor proteins such as Wiskott Aldrich Syndrome protein (WASP) superfamily proteins [389, 390]. In resting cells, N-WASP is autoinhibited by intramolecular interactions. Binding of activated Cdc42 mediates a conformational change in N-WASP that leads to actin polymerization via Arp2/3 complex [387, 391].

Another family of nucleating factor proteins are formins, comprising diaphanous formins, mDia1, mDia2 and mDia3. mDia proteins are shown to colocalize with activated Rho and profilin in the membrane ruffles of motile cells and induce fine linear actin filaments [384]. Similar to WASP proteins, most members of formins are autoinhibited and become active by activated Rac, Cdc42 and Rho [392-394]. Formins are involved in processes such as stress fibres and filopodia formation [384, 395]. mDia acts downstream of Rho to stabilize and orient microtubules [396]. In addition, after mDia1 activation by Rho, IQGAP1 mediates localization of mDia1 to phagocytic cups, and this localization is essential for actin polymerization and phagocytosis [397]. These data, along with the recent observation that mDia depletion by RNAi induces microtubule depolymerization [398], implicates mDia proteins as important actin/microtubule

linkers in a Rho GTPase–dependent manner. Although mDia do not seem to regulate the EC barrier function in the resting condition, they are involved in inhibition of VEGF-induced EC permeability by angiopoietin-1, an EC barrier stabilizing agent [399]. The mechanism involves angiopoietin-1 activation of its receptor, Tie-2, which results in RhoA and mDia activation. Once mDia is activated it recruits Src away from VEGFR2 and thus prevents VE-cadherin phosphorylation by VEGF [399].

Another important mechanism of actin regulation by Rho GTPases is via the regulation of myosin light chain (MLC)-dependent stress fibre formation. RhoA regulates actin remodelling via its downstream effector Rho kinase, which phosphorylates and inactivates MLC phosphatase, resulting in increased MLC phosphorylated levels and stress fibre formation [400]. RhoA effectors can also activate LIM kinase, which is an actin binding kinase that phosphorylates cofilin, an ABP that depolymerizes F-actin and inhibits its activity [401]. LIM kinase can also become activated downstream of Rac/Cdc42 and PAK activation [402].

As described earlier, endothelial F-actin cytoskeleton and localization of several actin associated proteins are greatly remodelled during leukocyte TEM. However, the exact mechanisms of actin regulation during TEM is not clear.

Microtubules (MTs)

Another component of cytoskeleton, MTs, are composed of α/β tubulin heterodimers assembled in a hollow tube. The tubulin assembly rate into filaments

is faster at one end, the plus end, than at the other end, the minus end. The minus end is associated and stabilized by microtubule organizing center (MTOC) [403].

MT plus ends generally face the cell cortex and are highly dynamic, alternating between phases of growth and shrinkage [404]. This phenomenon is called dynamic instability and is mainly regulated by MT-associated proteins (MAPs) [405]. Among MAPs, there are microtubule-plus-end tracking proteins (+TIPs) such as CLIP-170 and adenomatous polyposis coli (APC), which transiently bind to the plus end of MTs [406]. In addition to the regulation of MT dynamics, +TIPS, are involved in MT crosstalk to actin and MT capture to cortical sites [406-409].

MT motor proteins such as kinesin and dynein are a subfamily of MAPs that participate in cell function by transporting signalling molecules, vesicles and organelles [410-412]. These motors are enzymes that move on MT filaments by converting the chemical energy of ATP hydrolysis to mechanical energy [413].

The overall participation of MT in cell events is usually studied by broad MT depolymerization with MT depolymerizing agents such as nocodazole and colchicine. These studies indicated that in ECs, MT depolymerization results in myosin light chain phosphorylation, Rho activation, actin stress fibre formation and formation of gaps in junctions within 30 minutes [414-416]. This dysfunctional EC monolayer permits higher leukocyte TEM in dermal and arterial ECs [416, 417]. However, this effect seems to be dependent on cell context because MT can be depolymerized without a significant effect on the morphology of AJ [74, 170, 418]. As described earlier, heterogeneity in the structure and

regulation of different ECs and the extent of MT depolymerization might contribute to AJ sensitivity to MT depolymerization.

MT involvement in the regulation of EC responses to some agonists has also been shown. For instance, TNFα and thrombin require MT to induce pulmonary arterial EC permeability [76, 77]. MT might play a role in part through the regulation of a RhoA GEF, GEF-H1, which becomes active when released from MT [419]. It was shown that GEF-H1 depletion by RNA interference prevents EC responses to thrombin such as actin stress fibre formation and increased permeability [420].

Few publications have looked at MAPs in AJ regulation. In epithelial cells, a subpopulation of dynein directly interacts and localizes with β-catenin at the epithelial junctions in an actin-dependent manner [421]. β-catenin overexpression results in loss of MT at the cell junctions suggesting that dynein captures MT at the junctions [421]. In ECs, dynein is not localized at the junctions of resting monolayers but is transiently localized to newly formed AJ after a calcium clamp disassembly [422]. The relevance of dynein localization for AJ assembly was not tested in this study. Another means of AJ anchorage to MT is through p120 catenin. p120 catenin interacts with the MT motor, kinesin, and thus links N-cadherin to MT [423]. Further, in immortalized epithelial cells, this linkage is required for N-cadherin accumulation to newly formed AJ as expression of p120 catenin deficient in kinesin binding prevents AJ formation [423]. The exact mechanism of MT regulation of AJ still needs further work due to puzzling observations such as requirement of MT for E-cadherin clustering and

distribution in junctions, while its transport or expression does not require MT [404].

Although these observations illustrate the importance of MT for transport and localization of AJ components to junctions and provide a mechanism for the anchorage of MT to AJ, there are virtually no studies on VE-cadherin regulation by various MAPs. As described earlier, kinesin has been shown to regulate leukocyte TEM by mediating the targeted recycling of LBRC to the migration channel [74], but the function of kinesin in the basal regulation of AJ in ECs has not been studied.

Rho GTPases and MTs have been shown to be involved in the regulation of each other in many cell events. MT can regulate the activity of Rho by interaction with the GEF-H1, as described [419]. Rho GTPases can regulate MT function and structure as well. Rho and its effector mDia can interact with MTs and induce MT stabilization and orientation at leading edges of immortalized fibroblasts [396]. MTOC reorientation on the edge of a wounded fibroblast or astrocyte monolayer requires active Cdc42, dynein and dynactin [424, 425]. In addition, Rac activation is necessary for MT dynamics at the leading edge of a migrating cell, and this seems to be dependent on activation of Rac effector PAK and IQGAP1, which regulates MT dynamics in several ways [426, 427]. PAK1 can phosphorylate and inactivate an MT-destabilizing protein, Op18/stathmin [427]. PAK1 can also induce polymerization of MTs by phosphorylating a cofactor that is involved in the assembly of tubulin heterodimers into filaments, specifically tubulin cofactor B [426]. Finally, PAK1 interacts and phosphorylates

dynein light chain 1 [428], and this is important in the regulation of vesicle formation and trafficking during macropinocytosis [429]. MT capture and stabilization at the junctions can also be regulated by interaction with +TIP, CLIP-170, and an effector of Rac1/Cdc42, IQGAP1, as discussed in the following.

IQ motif containing GTPase activating protein 1 (IQGAP1)

IQGAP1 is a 189 kDa conserved protein in the eukaryotes, and contains several protein-binding domains interacting with a number of signalling molecules and the cytoskeleton [430]; the main ones are as follows. At the amino terminus, the calponin homology domain (CHD) mediates interaction with F-actin [431]. The IQ domain consists of tandem repeats of four IQ motifs which bind to calmodulin [432, 433]. The Ras GTPase–activating protein (GAP)-related domain (GRD) binds to Rac1 and Cdc42, and despite what its name implies, it lacks GAP activity and instead it enhances the activity of Cdc42 [432, 434]. The carboxy terminus contains a RasGAP domain which can mediate interaction with CLIP-170 [435], APC [406], β-catenin [436] and E-cadherin [437].

Original observations pointed to IQGAP1 interaction with active Rac1 and Cdc42, as well as calmodulin [432, 434]. While IQGAP1 localization to insulin-induced membrane ruffling areas and F-actin crosslinking was Rac1 and Cdc42 dependent, its localization to cell-cell junctions was not [431, 434]. Further,

IQGAP1 was confirmed to localize to cell-cell junctions and was shown to interact with E-cadherin and β-catenin [437]. Overexpression of IQGAP1 reduced the association of α-catenin with E-cadherin, suggesting IQGAP1 involvement in the regulation of AJ strength by controlling AJ linkage to the actin cytoskeleton [437]. Further, it was shown that activated Rac1 and Cdc42 inhibit the interaction of IQGAP1 with β-catenin, which frees β-catenin to interact with α-catenin and thus link AJ to the cytoskeleton [436].

IQGAP1 had been also implicated as an important regulator of MT dynamics. In fibroblasts, IQGAP1 partially localizes with MT at the leading edge, and this is mediated by CLIP-170 [435]. Expression of a mutant form of IQGAP1 that cannot bind CLIP-170 results in an altered MT structure [435]. IQGAP1 and CLIP-170 form a complex with Rac1 or Cdc42 and capture MT at the leading edge, thus mediating a polarized leading edge [435]. Later, APC was also found in this complex and inhibition of either molecule impaired actin enrichment and microtubule stabilization [406]. Of note, in addition to localization at the junctions and regulation of β-catenin [438, 439], APC binds and stabilizes MT [440-443]. IQGAP1 importance in MT capture and stabilization has been confirmed in some other cell types [398, 444].

In ECs, in basal conditions, IQGAP1 colocalizes with VE-cadherin and VE-cadherin localization at the junctions seems to require IQGAP1 [445]. IQGAP1 directly interacts with VEGFR2 and IQGAP1 expression is required for VEGF signalling and VEGF-induced migration and proliferation [446]. Furthermore, IQGAP1 deficiency prevents VEGF-mediated recruitment of

VEGFR2 to the AJ complex, VE-cadherin phosphorylation and capillary tube formation [445]. IQGAP1's importance in VEGF-induced angiogenesis has also been shown in vivo [447]. While in normal conditions, IQGAP1 knockout mice have no apparent defect except late gastric hyperplasia development [448]; in ischemic tissues of IQGAP1 knockout mice there is a defect in angiogenesis and tissue repair [449]. In addition, in ischemic limbs of wild-type mice, IQGAP1 expression and infiltrating macrophages were increased [445, 449]. IQGAP1 knockout mice were deficient in macrophage recruitment and ROS production [449]. Defects in macrophage infiltration in IQGAP1 knockouts were further confirmed by observing reduced macrophages in nonbacterial peritonitis sites and in vitro. Bone marrow reconstitution experiments indicated that both leukocytes and ECs are required for tissue repair and macrophage infiltration. While macrophages lacking IQGAP1 were defective in migration and adhesion, the authors did not investigate the mechanisms by which endothelial IQGAP1 participates in macrophage infiltration [449].

These observations all indicate IQGAP1 as a Rac1/Cdc42 effector and a scaffolding protein, coordinating and linking several signalling pathways or cytoskeleton components and targeting the complex to a spatially defined cellular domain, thereby generating a cell response. They also implicate IQGAP1 in dynamic regulation of the AJ.

Hypotheses

At the time of designing this project, little was known about the mechanisms behind regulation of leukocyte diapedesis by the endothelium. While the importance of the actin cytoskeleton was hinted at by the observation of actin remodelling during leukocyte TEM and also by involvement of Rho in leukocyte TEM, the role of PI3K (and specifically the role of each catalytic isoform) as an important regulator of Rho GTPases and actin remodelling was not studied [89, 223, 224]. Further, the participation of Rho GTPases in dynamic regulation of the EC cytoskeleton and AJ during leukocyte TEM was unknown. In addition, the role of MTs in leukocyte TEM had only been reported in terms of prolonged MT depolymerizing conditions, which resulted in great EC barrier dysfunction [416, 417]. This type of approach would not allow for the study of MT dynamics at the interendothelial junctions during leukocyte TEM. Finally, there were no studies on the importance of MAPs in EC junction regulation and leukocyte diapedesis. Thus, this study aimed to test the model showed in Figure 1-3. We sought to address the following hypotheses:

1. **Endothelial PI3K activity is required for lymphocyte TEM.**
2. **The activity of specific catalytic isoforms of EC PI3K class IA is important for leukocyte diapedesis.**
3. **EC PI3K activity required for lymphocyte diapedesis is induced by mechanotransduction following leukocyte interaction with EC under shear stress**.

4. **Endothelial MT-associating protein, IQGAP1, a Rho GTPase effector, regulates the EC AJ for efficient lymphocyte TEM.**
5. **Putative endothelial-enriched RhoGEF, FGD5, participates in leukocyte TEM upstream of IQGAP1.**

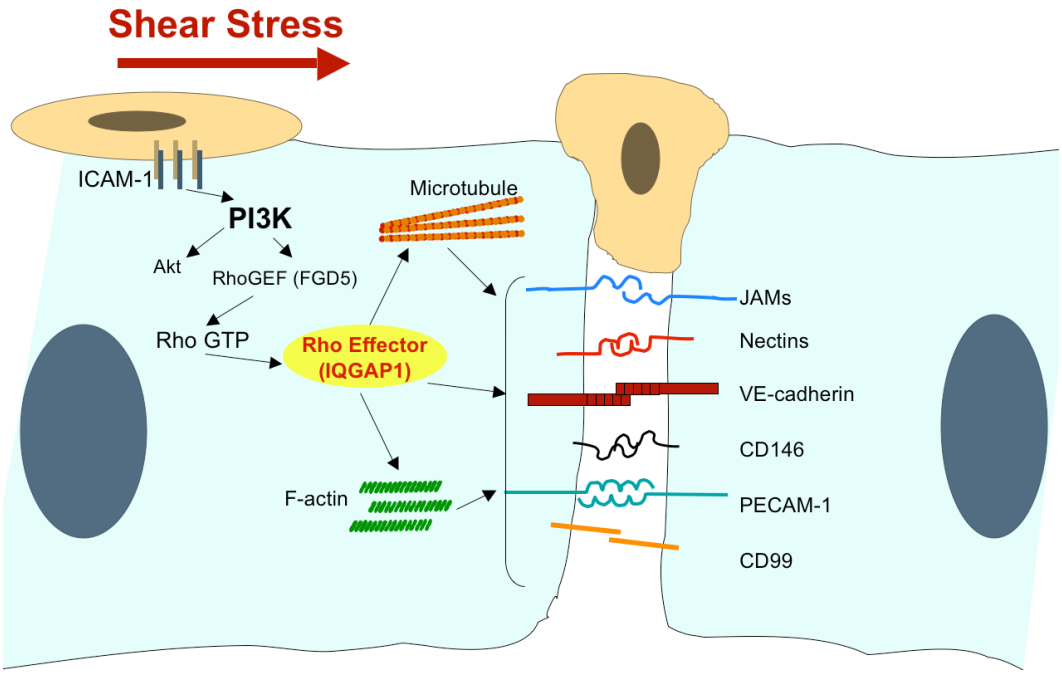

Figure 1-3. Proposed signalling events that lead to PI3K-dependent leukocyte TEM. Interaction of leukocytes with EC under shear stress mediates PI3K activation. PI3K different catalytic isoforms might participate differently in leukocyte TEM. The product of PI3K, PIP3, recruits and activates RhoGEFs leading to activation of Rho GTPases. Rho GTPases contribute to leukocyte TEM via specific functions (e.g. MT stabilization, F-actin remodelling, or direct regulation of interendothelial junction components) exerted via their effectors.

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Chapter 2. Endothelial PI3 Kinase Activity Regulates Lymphocyte Diapedesis[1]

Introduction

Traffic of lymphocytes from the blood to the tissue compartment underlies the cellular immune responses that mediate allograft rejection and many autoimmune diseases. Vascular endothelial cells (EC) at a site of inflammation provide a series of solid-phase cues to the lymphocyte that prompt tissue localization. Work by numerous investigators over the past decade has identified and characterized the role of various adhesion molecules and chemokines displayed by the EC, that provide the cues to promote the initial capture of the lymphocyte from the bloodstream and adhesion to the surface of the vascular endothelium [1]. Subsequent lymphocyte migration on the surface of the endothelium followed by diapedesis across the endothelial monolayer at the interendothelial cell junctions may be cued by different or overlapping signals [2, 3].

In addition, evidence has accumulated that leukocyte adhesion signals the EC to actively remodel both its adhesive contacts and cell shape during leukocyte diapedesis. For example, leukocyte adhesion stimulates increased rigidity of the EC cortical F-actin cytoskeleton and the development of F-actin rich projections

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to surround the leukocyte in a 'docking structure' [4, 5]. This docking structure promotes adhesion of the leukocyte to the luminal surface of the endothelium under shear stress conditions [6]. In addition, remodeling of the F-actin cytoskeleton and actin-associated interendothelial adherens junctions has been observed as the leukocyte completes the subsequent diapedesis step [7, 8]. The cues to the EC and the signaling events that mediate these late steps of leukocyte migration across the endothelial barrier remain poorly defined.

Phosphatidyl inositol 3- (PI3) kinase-dependent signal transduction pathways are candidates to mediate some of the events that underlie leukocyte diapedesis. Class I PI3 kinases are lipid kinases that use phosphatidylinositol 4,5-bisphosphate as substrate and have received particular attention in the context of the immune system. Class I PI3 kinases are subdivided into classes IA and IB that are coupled downstream of tyrosine kinase or G-protein coupled receptors by association with the p85 or p101 regulatory subunits, respectively [9]. Activation of class I PI3-kinases results in display of phosphatidylinositol-3,4,5-trisphosphate (PIP_3) on the inner leaflet of the cell membrane. PIP_3 enrichment allows membrane localization of proteins that contain plextrin homology (PH) domains, such as Akt or GTP/GDP exchange factors for Rho family GTP binding proteins [10]. In this way PI3 kinase activity is able to recruit effector molecules in a spatially restricted fashion.

PI3 kinase-dependent cell polarization enables directional actin polymerization and leukocyte motility in response to chemotactic stimuli [11]. Hence, early work identified defective polymorphonuclear leukocyte recruitment

to inflammatory sites in mice deficient in the class IA PI3 kinaseδ and the class IB PI3 kinaseγ [12, 13]. Vascular ECs share expression of PI3 kinaseδ and PI3 kinaseγ catalytic isoforms with bone marrow-derived cell lineages [14, 15]. Surprisingly, in elegant experiments that studied acute inflammatory responses in PI3 kinaseδ- or PI3 kinaseγ-deficient mice reconstituted with wild type bone marrow, PI3 kinase activity in EC was found to be required for a robust inflammatory response [15]. Intravital microscopy revealed a defect in the conversion of selectin-dependent, rolling adhesive interactions to stable, firm adhesion of the leukocyte to the endothelium. Unlike polymorphonuclear leukocytes, lymphocytes exploit immunoglobulin-superfamily adhesion molecules on the endothelium to mediate these events [16-18]. The role of endothelial PI3 kinase in lymphocyte transmigration is unknown.

In the present series of experiments we observed that inhibition of EC PI3 kinase activity decreased lymphocyte transendothelial migration through an EC monolayer in vitro. Remodeling of existing endothelial F-actin structures is required to support lymphocyte transit across an endothelial cell monolayer under physiologic shear stress. However, PI3 kinase inhibition did not block endothelial cortical F-actin remodeling or 'docking structure' formation as a consequence of EC ICAM-1 ligation. Moreover, neither inhibition of EC PI3 kinase activity nor endothelial F-actin remodeling inhibited surface migration of the lymphocytes on the luminal surface of the endothelium, but rather blocked the final diapedesis step of lymphocyte transendothelial migration.

Materials and Methods

Reagents

M199, RPMI, FBS, ECGS and HBSS were from Invitrogen (Burlington, ON). SDF-1α was from R&D (Minneapolis, MN). TNF-1α was from Biosource (Camarillo, CA). Fluorophore-conjugated antibodies against CD4 (clone RPA-T4), CD8 (clone RPA-T8), CD3 (clone UCHT1), CD49d (clone 9F10), CD14 (clone 61D3), CD19 (clone HIB19), CD54 (clone HA58), CD106 (clone STA), and control IgG were from eBioscience (San Diego, CA). Anti-CD31 (clone JC/70A) was from Dako (Mississauga, ON), anti-p85a (clone AB6) was from Upstate (Lake Placid, NY), and anti-JAM-C (clone 208206) was from R&D Systems (Minneapolis, MN). Monoclonal anti-ICAM-1 mAb (clone P2A4) was isolated from hybridoma supernatant (Developmental Studies Hybridoma Bank, Iowa City, IA) and digested to Fab by ImmunoPure Fab preparation Kit (Pierce, Rockford, IL). Rabbit polyclonal anti-VE-cadherin antibody was from Cayman (Ann Arbor, MI). Fluorophore-conjugated secondary antibody was from Jackson Immunoresearch (West Grove, PA). Celltracker Red (Molecular Probes, Eugene OR) was used to label lymphocytes as directed by the manufacturer. In vivo actin assay kit was obtained from Cytoskeleton (Denver, CO). Wortmannin, LY294002, and 1L-6-Hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (Akt inhibitor I), Y27632 were from EMD Biosciences (La

Jolla, CA). Control (cat # 1027281) and p85a-specific siRNAs (cat # SI02225405), and HiPerFect transfection reagent were from Qiagen (Mississauga, ON). Secondary anti-mouse IgG (Jackson Immunoresearch) and ECL (GE Healthcare; Buckinghamshire, UK) reagents were used for Western Blotting.FITC-phalloidin and all other reagents were from Sigma (St. Louis, MO).

Lymphocytes

Human PBLs were isolated from healthy donors under a protocol approved by the University of Alberta Health Research Ethics Board. PBL were isolated by density separation over a Ficol gradient (Lymphoprep, Axis-Shield Poc AS, Oslo, Norway). Contaminating monocyte/macrophages were depleted by incubation in 150mm plastic petri dishes (Fisher) for 1 hour at 37°C in RPMI + 10% FBS. The non-adherent PBL were washed off and resuspended in binding buffer (HBSS, 0.2% BSA, 1mM Ca^{+2}, 1mM Mg^{+2}) and adjusted to ~$1x10^7$ cells/ml. The PBL were studied by flow cytometry to characterize CD4-, CD8-, CD3-, CD49d-, CD14- and CD19-positive populations. The PBL preparations routinely contained more than 85% CD3-positive T lymphocytes, ~5% CD19-positive B lymphocytes, ~5% CD16 or CD56-positive NK cells, and <1% monocytes.

Endothelial Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described [19]. For use in an experiment, passage 4-5 HUVEC were replated at confluent density onto 35mm dishes coated with a thin layer of matrigel (1 mg/mL; Corning Inc., Corning, NY) and cultured for 48 hours. HUVEC were treated with TNF-α (200 U/mL, 20 h), then prior to assembly of the parallel plate flow chamber apparatus, HUVECs were incubated with SDF-1α at 100 ng/mL as described [20].

Where indicated, HUVEC were pre-treated with jasplakinolide (300 nM), wortmannin (10 uM) or LY294002 (30 uM) for 1 hour, Akt inhibitor I (as indicated) for 3 hours, or Y27632 (as indicated) for 30 minutes at 37°C then the EC monolayers were washed 3 times before SDF-1α was adsorbed to the EC surface. Chemical inhibitors were used at concentrations that maintained HUVEC viability >85% of mock-treated controls as assessed by the XTT assay of mitochondrial activity as described [21]. HUVEC expression of ICAM-1, VCAM-1, CD31 and JAM-C was monitored using indirect immunofluorescence and quantitated using flow cytometry. No changes were observed with the small molecule inhibitors. We detected no difference in SDF-1α adsorbed to HUVEC pretreated with DMSO or LY294002 using cell surface ELISA. The relative cellular G-actin and F-actin in DMSO or jasplakinolide-treated HUVEC was determined by Western blot using an actin quantitation kit according to the manufacturers instructions (Cytoskeleton Inc).

Endothelial Cell Transfection

HUVEC were plated at approximately 50% confluence in M199 with 2% FBS and endothelial cell growth supplement (ECGS), without antibiotics, 24 hours before transfection. Fifty nM control or p85a-specific siRNA was transfected using HiPerFect according to the manufacturers instructions. The monolayers were grown to confluence in 35 mm tissue-culture plates and used in experiments 72 hours after transfection. Endothelial p85a expression was monitored by Western Blot in each experiment using p85a-specific mAb.

Videomicroscopy Imaging

Laminar flow adhesion assays were done as described previously [22] using 35 mm tissue-culture plates as the lower surface of a parallel-plate laminar flow chamber (127μm gap; Glycotech, Rockville, MD). The chamber was mounted on the stage of an inverted phase-contrast microscope (Leica DM IRB, Leica Microsystems, Richmond Hill, ON), and lymphocyte/endothelial cell interactions were observed through a 20x objective and captured using a CCD camera (Pixelink, Vitana Corporation, Ottawa, ON) at 12 frames/sec for a 20 minute period.

Lymphocytes were perfused over the EC monolayer at low shear flow (0.5 dyne/cm^2) and allowed to accumulate on the ECs (accumulation phase). The flow rate was then increased to 1 dyne/cm^2 and was kept constant throughout the assay by perfusion of fresh binding buffer at 37°C (shear application phase). Analysis of lymphocyte motion was done manually using Quicktime Pro (Apple, Cupertino,

CA) on all accumulated cells in video fields selected to contain more than 50 adherent lymphocytes per field. Lymphocytes entering or leaving the field of view after the initial frame were not included in the analysis. Throughout the analysis period of 20 minutes, >90% of adherent PBL remained in the field of view. The movement of adherent lymphocytes was categorized into (1) locomotion: lymphocytes that migrate more than one cell body on the surface of the endothelial monolayer or (2) transmigration: lymphocytes that undergo a change from phase-bright to phase-dark appearance as described previously [20]. Lymphocyte migration across the EC monolayer was evident when the focal plane of the lymphocyte lay in the gel underlying the EC monolayer. In addition, the track of individual migrating lymphocytes was analyzed to determine if the cell migrated across an interendothelial cell junction. The data are reported as a fraction of the originally accumulated lymphocytes. In aggregate, the fraction of adherent lymphocytes under control conditions showing a motile or transmigration phenotype were 61 ± 3% and 26 ± 4% (mean ± SEM) respectively.

Fluorescence Microscopy Imaging

To characterize docking structure formation, HUVECs were grown to confluence on matrigel-coated glass coverslips and were treated with TNF-a as above. HUVECs were then pre-treated with DMSO carrier or with LY294002 as indicated. Latex beads (Bang Laboratories, Fishers, IN) adsorbed with anti-ICAM-1 mAb (clone P2A4) were allowed to attach to the EC for 20 minutes, then

the monolayer was fixed with 2% PFA in PBS/ 2 mM Ca^{+2}, permeabilized with 20 mM Hepes, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100; pH 7.4 buffer and stained with FITC-phalloidin (Sigma) for 30 minutes to visualize F-actin structures. Each adherent bead was scored by an observer blinded to the treatment group as associated with a docking structure if F-actin was present in a crescent or circle around the perimeter of the bead or immediately underneath the bead as described [5, 23].

To evaluate lymphocyte localization at interendothelial junctions and interposition between adjacent EC, lymphocytes were perfused over EC monolayers as described above and shear stress was applied for 10 minutes. The laminar flow chamber was disassembled, and gently washed with PBS/ 2 mM Ca^{+2}, then fixed with PFA as above. EC were stained with polyclonal antibody that recognizes the extracellular domain of VE-cadherin and FITC-conjugated anti-rabbit secondary antibody. DIC and fluorescence images of 3 fields in each experiment were collected using a LSM 510 confocal microscope (Zeiss Ltd, Toronto, ON) set to acquire images at 0.5 um intervals in the z-plane. Discontinuities in the linear VE-cadherin staining immediately underlying or adjacent to an adherent lymphocyte were identified. The fraction of adherent lymphocytes overlying VE-cadherin junctions and the fraction associated with VE-cadherin discontinuity of more than 1 um were scored by an observer blinded to the treatment group.

Flow Cytometry

The expression of surface molecules by HUVEC and PBL were monitored by flow cytometry [21]. Analysis was performed using a Becton Dickinson (San Jose, CA) FACScan using CellQuest software.

Statistics

The mean fraction of lymphocytes migrating on the EC surface, in contact with interendothelial cell margins, or undergoing TEM among the treatment versus control groups among several experiments was calculated and tested for statistical significance ($P<0.05$) by paired Students t-test using SPSS (SPSS, Chicago, IL). To evaluate the position of lymphocytes at the interendothelial junction, data from 4 experiments was pooled and tested for significance using Chi-square (SPSS).

Results

Endothelial cell PI3 kinase activity is required for efficient lymphocyte TEM

Loss-of-function mutations of PI3 kinase or inhibition of PI3 kinase activity has been observed to attenuate neutrophil-mediated inflammation in vivo, but the effect on other leukocyte subsets and the mechanism of the defect in recruitment has not been fully characterized [15, 24, 25]. In the first series of experiments we sought to determine if inhibition of EC PI3 kinase affected

lymphocyte transendothelial migration. We adopted the technique of Cinamon to study the migration of lymphocytes adherent to a confluent tumor necrosis factor (TNF)-treated HUVEC monolayer under laminar flow conditions in vitro [20]. Freshly isolated human PBL were perfused over the monolayer and allowed to accumulate on the surface of the EC at a low shear stress. The shear stress was then increased to 1 dyne/cm^2, and lymphocyte movement was recorded using phase-contrast videomicroscopy. Transmigration across the HUVEC monolayer was evident as a transition in the lymphocyte to a phase-dark appearance and determined at the end of the experiment by confirming that the plane of focus of the lymphocyte was in the Matrigel substratum below the EC monolayer (Figure 2-1). HUVEC were pretreated with vehicle or either of the PI3 kinase inhibitors wortmannin or LY294002 prior to assembly into the laminar flow apparatus. We observed that the fraction of adherent lymphocytes that underwent transendothelial migration across wortmannin- or LY294002-treated EC monolayers was markedly reduced, but the fraction of lymphocytes that moved on the surface of the endothelial monolayer was not inhibited (Figure 2-2A). Wortmannin pretreatment of lymphocytes did not inhibit transendothelial migration (Figure 2-2C).

Further, we used RNAi to the p85a regulatory subunit, common to the α, β, and δ catalytic isoforms of class IA PI3 kinases, to confirm the results obtained using the pharmacologic inhibitors of PI3 kinase activity. We observed marked inhibition of endothelial p85 expression (Figure 2-2B) after treatment with specific siRNA, and tested confluent p85-deficient EC monolayers for the

efficiency of lymphocyte transmigration (Figure 2-1D, 2-2A). Lymphocyte transmigration across p85-deficient monolayers, but not migration on the surface of the monolayer, was inhibited. These observations indicate that endothelial PI3 kinase activity is required during the process of transendothelial migration and is partly dependent on p85 regulation.

Endothelial cell F-actin remodeling facilitates lymphocyte transendothelial migration

PI3-kinase-mediated generation of phosphatidylinositol (3,4,5) trisphosphate (PIP_3) locally regulates the activity of a variety of actin-associated proteins to indirectly regulate F-actin turnover [26]. F-actin polymerization is thought to be important in the development of a docking structure that is associated with stable adhesion and leukocyte transendothelial migration [6, 27, 28]. Therefore we sought to determine if inhibition of EC F-actin remodelling had a similar effect on lymphocyte transendothelial migration as inhibition of endothelial PI3 kinase activity. Endothelial cells were pretreated with vehicle or jasplakinolide before assembly into the parallel-plate laminar flow apparatus to inhibit remodeling of F-actin structures in the living EC. We observed that jasplakinolide pre-treatment significantly reduced the fraction of lymphocytes able to transmigrate across the EC monolayer (Figure 2-3). However, there was no difference in lymphocyte migration on the surface of the EC monolayer.

Jasplakinolide is a macrolide marine toxin that specifically binds and inhibits the remodeling of established F-actin microfilaments [29]. To confirm

that jasplakinolide pretreatment of the HUVEC at the concentrations used in these experiments was sufficient to stabilize EC F-actin, we tested the ability of cytochalasin D to depolymerize F-actin in EC lysates in vitro. Figure 2-3B shows that HUVEC pretreated with jasplakinolide have a greater fraction of actin incorporated in F-actin structures than carrier-treated HUVEC monolayers. Moreover F-actin from the jasplakinolide-treated HUVEC was resistant to cytochalasin D-mediated depolymerization. Taken together then, these results indicate that stabilization of the HUVEC F-actin cytoskeleton impairs lymphocyte transendothelial migration.

Strong adhesion of lymphocytes to EC requires EC cytoskeletal remodeling but is independent of PI3 kinase activity

Previous work has identified an association between the formation of a 'docking structure' by the endothelium at sites of mononuclear leukocyte adhesion and subsequent leukocyte transendothelial migration [28]. In the next series of experiments we sought to determine if EC PI3 kinase activity or remodeling of the endothelial F-actin cytoskeleton was required to stabilize lymphocyte adhesion and to form the docking structure. Freshly isolated human PBL were allowed to adhere to TNF-pretreated HUVEC, then lymphocyte adhesion to the EC monolayer was determined under conditions of increasing shear stress. Figure 2-4A demonstrates that inhibition of endothelial PI3 kinase activity does not change the adhesiveness of PBL to TNF-pretreated EC, but that

jasplakinolide pretreatment of EC inhibits stable lymphocyte adhesion under high shear stress conditions.

To directly confirm that inhibition of endothelial PI3 kinase activity does not disturb F-actin remodeling required for docking structure formation, we examined the formation of F-actin rings after adhesion of latex beads to the EC surface (Figure 2-4B). These data indicate that inhibition of EC PI3 kinase does not affect formation of the docking structure or reorganization of endothelial F-actin at the site of bead adhesion. Taken together, these data indicate that inhibition of endothelial PI3 kinase does not significantly alter functional or structural characteristics of lymphocyte adhesion to TNF-pretreated EC.

Endothelial cell Rho but not Protein kinase B is required for efficient lymphocyte TEM

PI3 kinase activity to generate PIP_3 – enriched domains of plasma membrane can serve to create a docking site for the sub-cellular localization of effector molecules, such as Protein kinase B/ Akt and Rho GTP binding protein exchange factors via interaction with PH domains of the protein [30]. PI3 kinase activation of Akt has been implicated in regulation of cortical actin remodeling acting upstream of the Rac GTP binding protein [31]. However, we observed that inhibition of EC Akt had no effect on either lymphocyte surface migration or diapedesis (Figure 2-5). Therefore the effects of endothelial PI3 kinase inhibition on lymphocyte transendothelial migration appear to be independent of signaling through the Akt pathway.

The RhoA GTP binding protein is variably reported to regulate docking structure formation to promote leukocyte adhesion, or stress fiber formation and myosin contractility to generate centripetal tension that may contribute to the release of interendothelial cell adhesion during leukocyte diapedesis. Since LY294002-mediated inhibition of PI3 kinase inhibited lymphocyte transendothelial migration, we next tested the hypothesis that Rho GTP binding protein signaling was involved. We observed that inhibition of EC Rho kinase with Y27632 had a modest inhibitory effect on lymphocyte transendothelial migration under shear stress conditions (Figure 2-5), but the magnitude of the inhibition was consistently less than that seen with PI3 kinase inhibition. Nevertheless, this is consistent with the model that Rho kinase-dependent signaling occurs downstream of PI3 kinase activity to facilitate lymphocyte transendothelial migration.

Lymphocyte penetration of endothelial intercellular junctions requires endothelial PI3 kinase activity and F-actin remodeling

Leukocytes preferentially transit an endothelial monolayer at interendothelial cell junctions [3, 28, 32, 33]. Since endothelial PI3 kinase inhibition did not block docking structure formation, we determined the fraction of motile and stationary adherent lymphocytes that contacted the interendothelial cell margins of control, jasplakinolide- and LY294002-treated EC monolayers. As shown in Figure 2-6A, analysis of the videomicrographs indicates a similar fraction of lymphocytes localized to the interendothelial cell margins among

vehicle- and inhibitor-treated endothelial monolayers. Furthermore, a similar number of lymphocytes migrated along interendothelial cell margins among each group (data not shown).

To confirm these observations, we used fluorescence microscopy to analyze the fraction of lymphocytes associated with interendothelial VE-cadherin-rich adherens junctions in EC monolayers fixed after 10 minutes of interaction under shear stress conditions. In agreement with our analysis of the videomicrographs, we detected no difference in the fraction of adherent lymphocytes in contact with the endothelial adherens junctions among control or LY294002-pretreated EC monolayers (56±4 vs 51±9% respectively; p=NS).

To characterize the point diapedesis is impeded during lymphocyte transit of the interendothelial junction, we used confocal microscopy to study the junction-associated lymphocytes, fixed after 10 minutes of interaction with EC monolayers under shear stress. We grouped the lymphocytes into those that were located in the z-axis above the level of the endothelial VE-cadherin, completely below the endothelial VE-cadherin, or those that extended both above and below the adherens junction (Figure 2-7). Results from 4 independent experiments were pooled for analysis. We observed that 43 vs. 18% (n=526 lymphocytes; $p<0.001$) of lymphocytes migrating across control versus LY294002-pretreated monolayers had completed diapedesis and were completely below the level of VE-cadherin staining. A similar fraction (10 vs 11%) of lymphocytes were seen in the migration channel between adjacent EC in control and LY294002-pre treated monolayers. In contrast, 71% of lymphocytes associated with interendothelial cell

junctions among LY294002-pretreated EC monolayers were localized above the level of VE-cadherin vs. 48% among control monolayers ($p<0.001$). Of those lymphocytes adherent over endothelial adherens junctions, 22% traversing LY294002-treated monolayers were associated with a >2um gap in the VE-cadherin barrier vs 9% crossing control monolayers ($p=0.003$). Since VE-cadherin has been shown to be excluded from the developing migration pore as the leukocyte begins diapedesis [8], this suggests that the lymphocytes are able to initiate separation of the interendothelial junctions despite EC PI3 kinase inhibition, but are inefficient in extending processes to interpenetrate adjacent EC.

Finally, as a measure of the ability of the EC to accommodate lymphocyte interposition between adjacent EC, the time taken by a lymphocyte to complete transit of the EC monolayer from the point of initial interposition between adjacent LY294002- or jasplakinolide- treated endothelial cells was determined from the videomicrographs. As shown in Figure 2-6B, lymphocytes that successfully completed diapedesis across EC monolayers treated to inhibit PI3 kinase activity or F-actin remodeling transited the monolayer slower than lymphocytes transmigrating across control monolayers.

Taken together these data indicate that neither inhibition of EC PI3 kinase activity nor F-actin remodelling affected the ability of the lymphocytes to migrate on the surface of the endothelium to the favoured site of most transendothelial migration events at the interendothelial cell junction. However lymphocyte interposition between the adjacent EC to complete the diapedesis step was

impaired. These observations suggest that endothelial PI3 kinase activity is required during lymphocyte diapedesis at the interendothelial cell junction.

Discussion

Molecules that mediate leukocyte movement from the blood to the tissue compartment have been identified as potential targets for therapeutic intervention in inflammation. Our observations describe the requirement for vascular endothelial cell PI3 kinase activity, one such molecular target, to support lymphocyte diapedesis across human vascular endothelial cells. Further we demonstrate that remodeling of the existing endothelial F-actin structures is also required. We observe that inhibition of either endothelial phosphoinositide generation or F-actin remodeling impairs lymphocyte transendothelial migration without blocking surface migration toward interendothelial cell junctions. Nevertheless, careful analysis of the developing interendothelial migration channel indicates that endothelial PI3 kinase inhibition does not impair the development of a gap in the endothelial adherens junction, an early event associated with migration channel formation. Taken together, these observations indicate that endothelial PI3 kinase activity and remodeling of endothelial cortical F-actin structures is necessary at the interendothelial cell junction site of diapedesis to facilitate transendothelial migration.

We inhibited endothelial PI3 kinase activity using two pharmacologic inhibitors of all class I PI3 kinase catalytic isoforms. In addition, we used RNAi to knockdown expression of the p85a regulatory subunit in EC, commonly used

by the class IA PI3 kinase isoforms. Although both the pharmacologic and RNAi approaches inhibited lymphocyte TEM, the magnitude of the decrease was modest after p85a inhibition. This indicates that endothelial PI3 kinase activity during lymphocyte transmigration is regulated through the p85a subunit, and implicates endothelial class IA PI3 kinase activity in remodeling of the interendothelial cell junction during lymphocyte transmigration. However, redundancy with class IB PI3K activity, or among the alternate p85b, p55, or p50 regulatory subunits of class IA PI3 kinases as described earlier [34, 35] may have salvaged activity through the pathway in the absence of p85a expression.

In contrast to the effect of inhibition of endothelial PI3 kinase activity on lymphocyte transmigration, we saw little effect of lymphocyte PI3 kinase inhibition. Although earlier work identified reduced migration of lymphocytes lines in Boyden chamber assays after PI3 kinase inhibition [36, 37], under the short physiological timeframes of transmigration under shear stress, lymphocyte PI3 kinase activity appears to be dispensable [20]. Recent work identifies an alternate signal pathway to polarize lymphocytes through the atypical exchange factor Dock2 to Rho GTP binding protein activation in response to chemotactic chemokine stimulation [38, 39]. Chemokines stimulate the class IB PI3 kinaseγ isoform in lymphocytes [40], but deficiency of PI3 kinase activity in vivo has minimal effect on T cell movement [41] or homing to lymphoid organs [12, 42].

Recent work in vivo has identified an important role for class IB PI3-kinaseγ and class IA PI3-kinaseδ activity in the development of neutrophil-dependent inflammation [24, 43]. Intravital microscopy identifies impaired

conversion from a loose adhesive interaction to stable adhesion between wild type neutrophils and vascular endothelial cells deficient in PI3K-δ or -γ activity in the murine cremasteric vein model [15]. This suggests that endothelial cells use PI3 kinase to stabilize the interaction between the leukocyte and endothelium in the presence of shear forces.

F-actin-rich projections of the endothelial cell develop around adherent leukocytes in vitro and have been proposed to serve this function [6]. However, we observed no effect of endothelial PI3 kinase inhibition on either the strength of the adhesive interaction between the adherent lymphocyte and the endothelial surface under flow conditions, or on F-actin polymerization surrounding beads that crosslink the endothelial adhesion molecule, CD54. Whereas neutrophils use adhesion molecules of the selectin class and subsequently engage CD54, lymphocytes engage both CD106 and CD54 on the endothelial cell to mediate rolling and tight adhesion [17, 18]. The difference in the adhesion molecules employed by the two types of leukocyte may account for the apparent difference in the requirement for endothelial PI3 kinase activity for tight adhesion and warrants further investigation.

Leukocyte transendothelial migration across the interendothelial junctions is associated with gaps in VE-cadherin [32]. Indeed, VE-cadherin is dynamically excluded from the interendothelial cell junction underlying an adherent leukocyte as the migration channel develops [8]. We observe that lymphocytes accumulate on the surface of LY294002-treated endothelial monolayers and are associated with gaps in VE-cadherin of comparable dimensions to gaps we have observed

associated with lymphocyte migration. Interestingly, the lymphocytes are not seen to extend processes below the level of the adherens junction marker, suggesting that a LY294002-sensitive barrier exists subsequent to and independent of VE-cadherin gap formation. Our observation that the transit time to cross the EC monolayer is prolonged among lymphocytes that successfully transmigrate LY294002- or jasplakinolide-pretreated EC is consistent with a model that PI3 kinase activity and F-actin remodeling are linked.

A similar defect in transmigration, with leukocytes delayed above the interendothelial cell junction, has been reported under conditions of blockade of endothelial intercellular junction proteins JAM-C [44], CD31 [45], or the poliovirus receptor [46], whereas CD99 blockade inhibits mononuclear cell transmigration at a somewhat later step, and monocytes are observed to be trapped in the migration channel [47]. PI3 kinase activity has been indirectly linked to engagement of CD31 in endothelial cells, but truncation of the cytoplasmic domain of CD31 does not block leukocyte transmigration [48, 49]. Our observations lend support to the idea that discrete steps can be identified during leukocyte diapedesis. Further work is required to determine if engagement of endothelial junction molecules promote PI3 kinase activity in EC.

Activation of PH-domain containing proteins such as Akt or Rho GEFs is linked to PI3 kinase-dependent PIP_3 display on the plasma membrane. Activated Akt associates with the actin cytoskeleton [50] and participates in F-actin remodeling [30]. However, we observe no effect of endothelial Akt inhibition on lymphocyte transendothelial migration. In contrast, inhibition of Rho kinase, a

downstream effector molecule of the monomeric GTP binding protein RhoA, modestly inhibited lymphocyte TEM. RhoA activity is regulated indirectly by PI3 kinase activity through membrane localization of Rho-specific GTP/GDP exchange factors to membrane sites enriched in PIP_3 [51]. The coordinated activity of several Rho family members is implicated in adherens junction remodeling [52]. The effect of PI3 kinase inhibition to block lymphocyte transmigration is consistently greater than the effect of RhoA or Rho kinase inhibition, suggesting other Rho family members may also participate in remodeling the interendothelial cell junction.

In summary, endothelial PI3 kinase activity is required for efficient lymphocyte transendothelial migration. The defect in lymphocyte TEM created by inhibition of endothelial PI3 kinase is not at the formation of the docking structure. Lymphocytes migrate on the surface of the endothelium and contact interendothelial cell borders and are associated with gaps in VE-cadherin in the absence of endothelial PI3 kinase activity, but fail to complete diapedesis across the interendothelial cell junctions. This suggests that in addition to gap formation in the VE-cadherin barrier, a second rate-limiting event is involved during lymphocyte interpenetration of the adjacent EC as the EC accommodates leukocyte transmigration. Therefore our experiments have identified a role for endothelial PI3 kinase activity in lymphocyte diapedesis at a late step in the transmigration pathway.

Figures

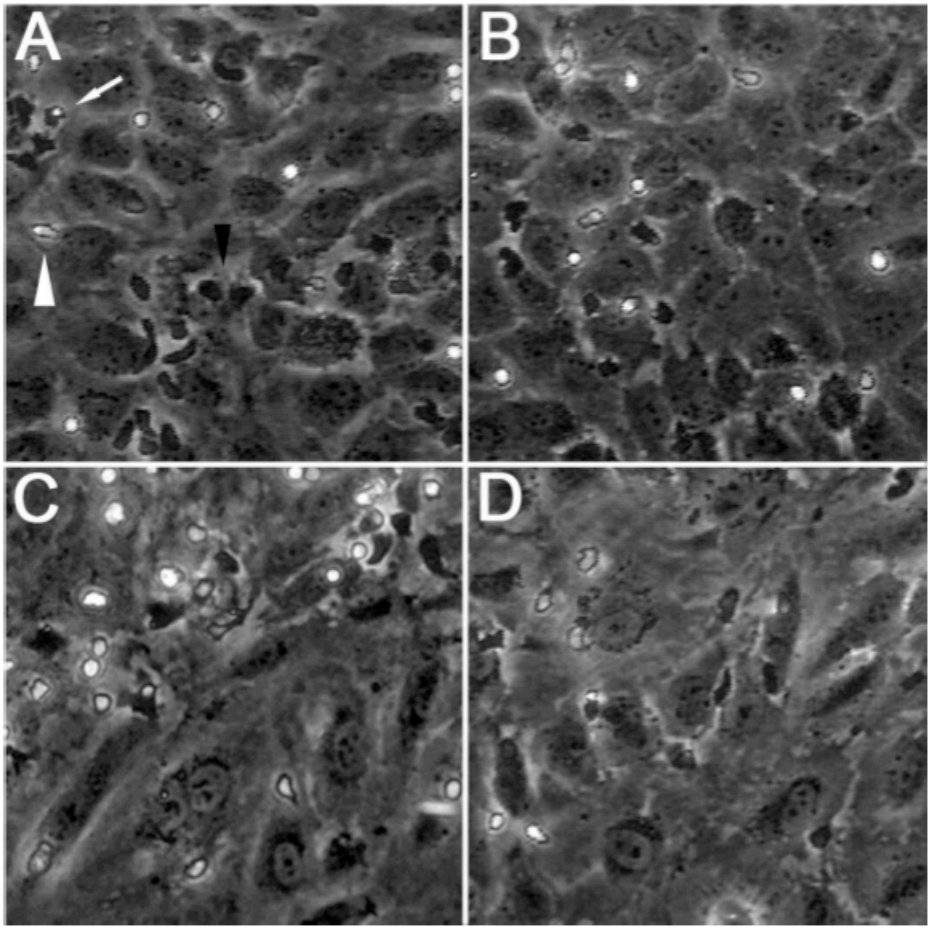

Figure 2-1. Lymphocyte adhesion and transendothelial migration across confluent endothelial cell monolayers. Representative photomicrographs of lymphocytes adherent to A) control, B) LY294002, C) jasplakinolide, or D) p85alpha specific si RNA pretreated HUVEC monolayers after the application of 1 dyne/cm2 laminar shear stress for 20 minutes as described in Methods. An example of a phase-dark lymphocyte that has migrated across and under the endothelial monolayer is indicated by the black arrowhead; an example of a surface-adherent lymphocyte is indicated by the white arrowhead. A lymphocyte captured in the migration channel is indicated by the arrow.

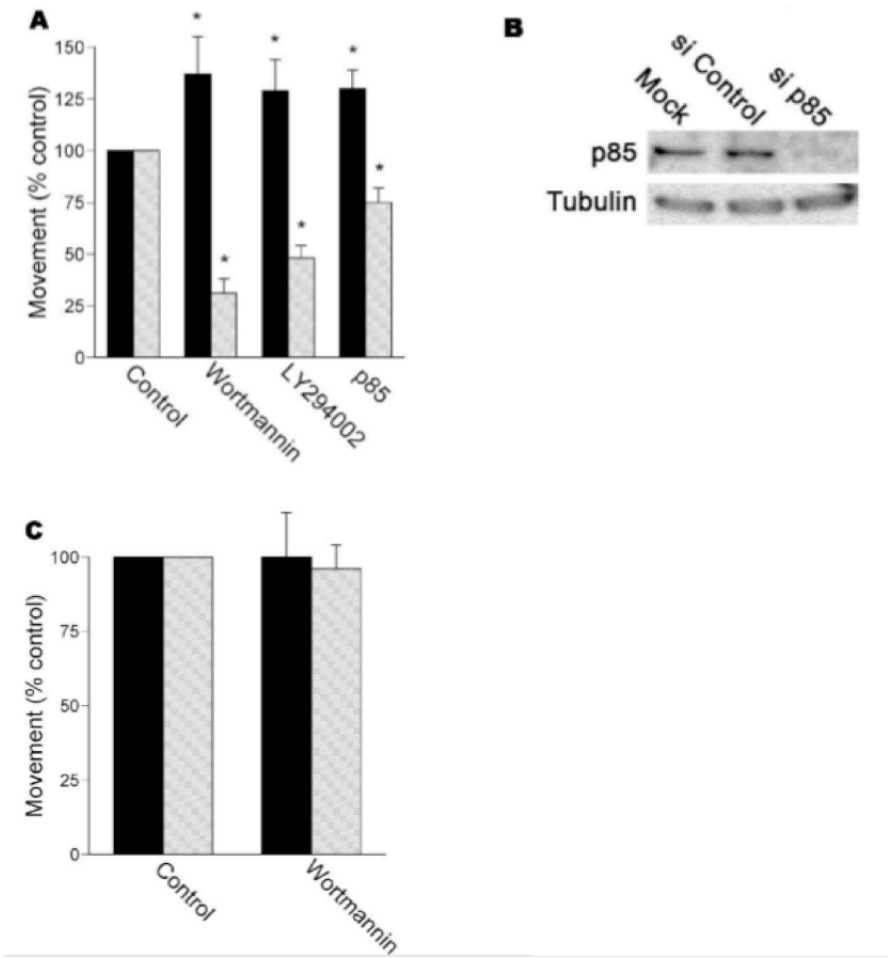

Figure 2-2. Inhibition of endothelial PI3 kinase inhibits lymphocyte transendothelial migration. A) Quantitation of the fraction of adherent lymphocytes that locomote on the luminal surface of the endothelial cell monolayer (closed) or transmigrate across the monolayer (open). Lymphocyte migration across HUVEC monolayers pretreated with wortmannin (10 uM) or LY294002 (30 uM) was performed as in Methods. Endothelial expression of the p85-alpha regulatory subunit of class IA PI3 kinases was knocked down by RNAi as indicated in Methods (mean ± SEM, n=4 experiments; * indicates $p<0.05$ vs. control). B) p85-alpha specific si RNA decreases HUVEC p85-alpha expression by Western blot as described in Methods. C) Inhibition of lymphocyte PI3 kinase activity with wortmannin does not decrease either lymphocyte locomotion or transendothelial migration (mean ± SEM, n=3 experiments).

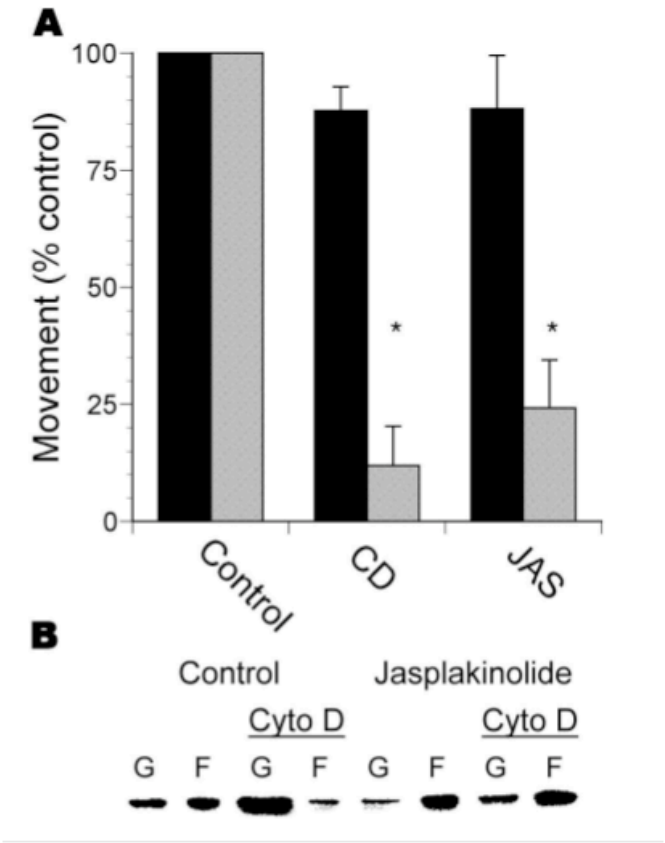

Figure 2-3. Endothelial F-actin remodeling is required for lymphocyte transendothelial migration. A) The fraction of adherent lymphocytes migrating on the surface (closed) or transmigrating (open) across carrier-, cytochalasin D-, or jasplakinolide-pretreated HUVEC monolayers under 1 dyne/cm2laminar shear stress was determined as in Methods (mean ± SEM, n=5 experiments; * indicates $p<0.05$ vs. control). B) Jasplakinolide stabilizes the endothelial F-actin cytoskeleton. HUVEC were treated with DMSO carrier or jasplakinolide 300 nM for 1 hour, then the cells were lysed, and an aliquot of each lysate was treated with cytochalasin D to depolymerize F-actin. The Globular (G) vs Filamentous (F) actin was resolved as indicated in Methods. The data are representative of 3 experiments.

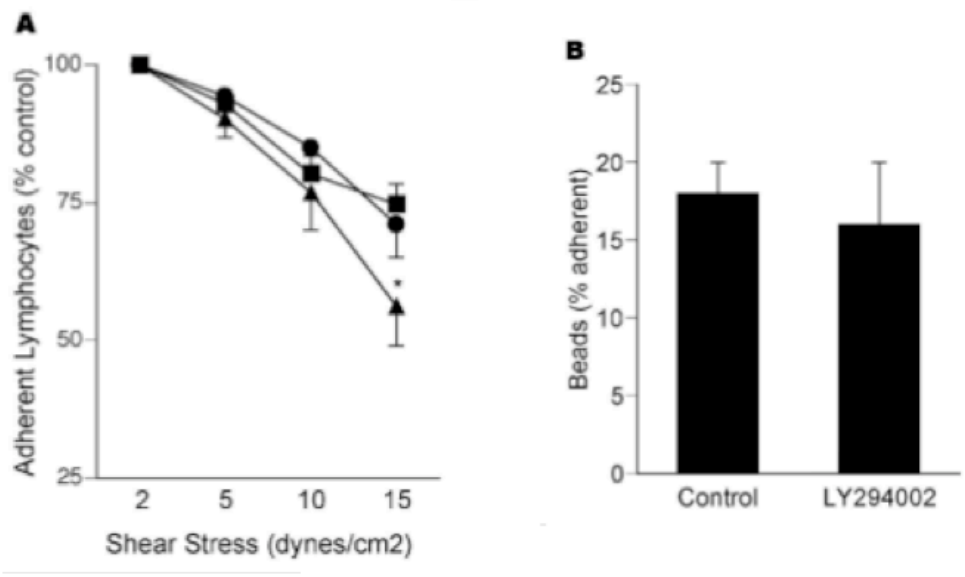

Figure 2-4. Endothelial cell F-actin stabilization but not PI 3 kinase inhibition impairs docking structure development. A) Lymphocytes adherent to TNF-pretreated EC were subjected to progressive increases in shear stress, and the fraction of remaining adherent lymphocytes was determined as indicated (mean ± SEM, n=3 experiments; * $p<0.05$). HUVEC were treated with (square) DMSO, (circle) LY294002, or (triangle) Jasplakinolide as described in Methods. B) The fraction of anti-CD54-coated latex beads bound to carrier- or LY294002-pretreated EC associated with FITC-phalloidin F-actin structures was determined (mean ± SEM, n=3 experiments; p=ns).

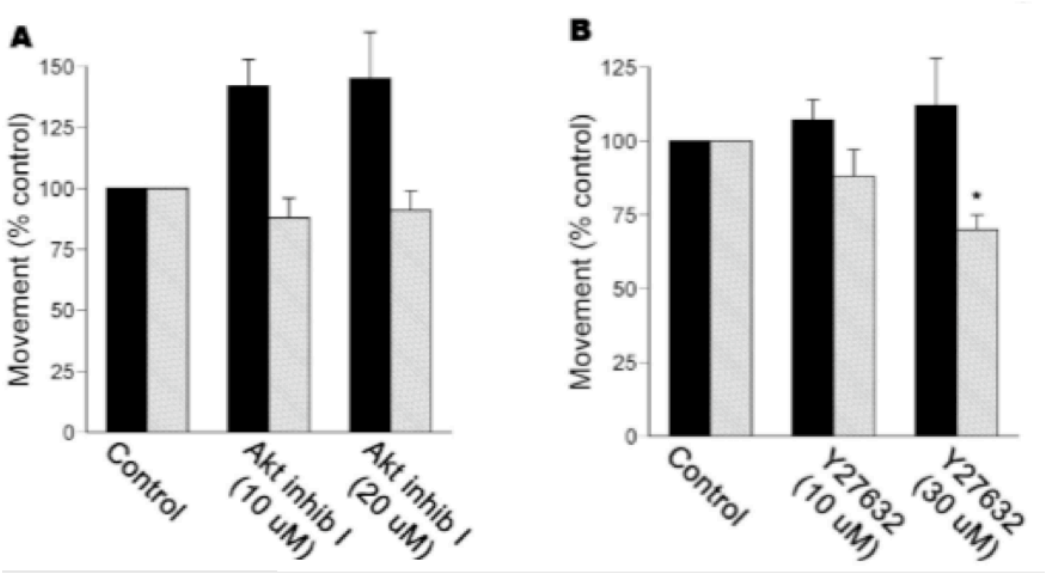

Figure 2-5. Endothelial Rho kinase but not Akt activity is required for lymphocyte transendothelial migration. Lymphocyte transendothelial migration through HUVEC monolayers pretreated as in Methods with A) Akt inhibitor I or B) Y27632 was analyzed as in Figure 2-2. The surface locomotion fraction (closed) and transmigration fraction (open) was determined (mean ± SEM, n=3 experiments; * indicates $p<0.05$ vs control).

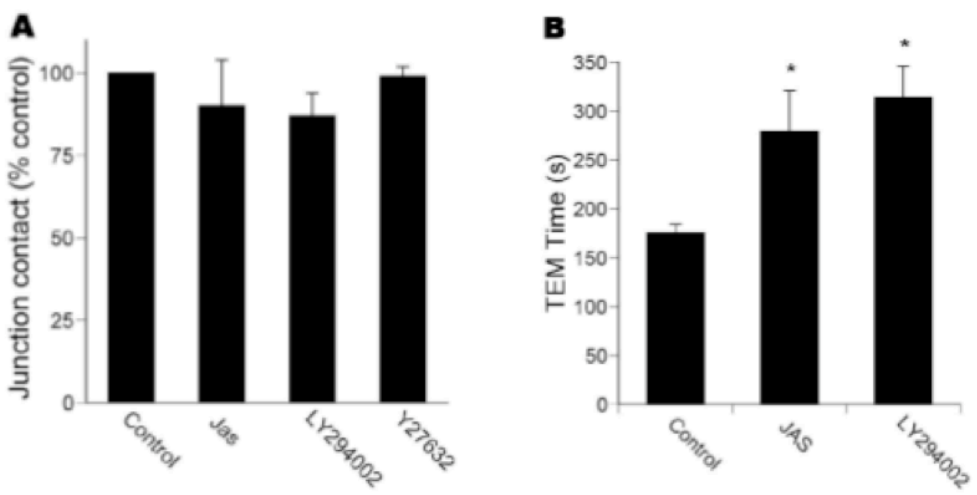

Figure 2-6. Inhibition of endothelial PI3 kinase or F-actin remodeling does not impair lymphocyte contact with the interendothelial cell margins but prolongs transit time through the migration channel. A) HUVEC monolayers were treated with carrier, jasplakinolide, LY294002 or Y27632 as in Methods, then the fraction of lymphocytes adherent overlying or migrating on the luminal surface across interendothelial junctions under laminar shear stress was determined by analysis of videomicroscopy images (mean±SEM, n= at least 3 experiments; p=NS). B) The time to complete transit across HUVEC monolayers was determined by analysis of videomicroscopy images of control, LY294002, or jasplakinolide treated HUVEC monolayers (mean±SEM, n= at least 3 experiments; * indicates $p<0.05$ vs. control).

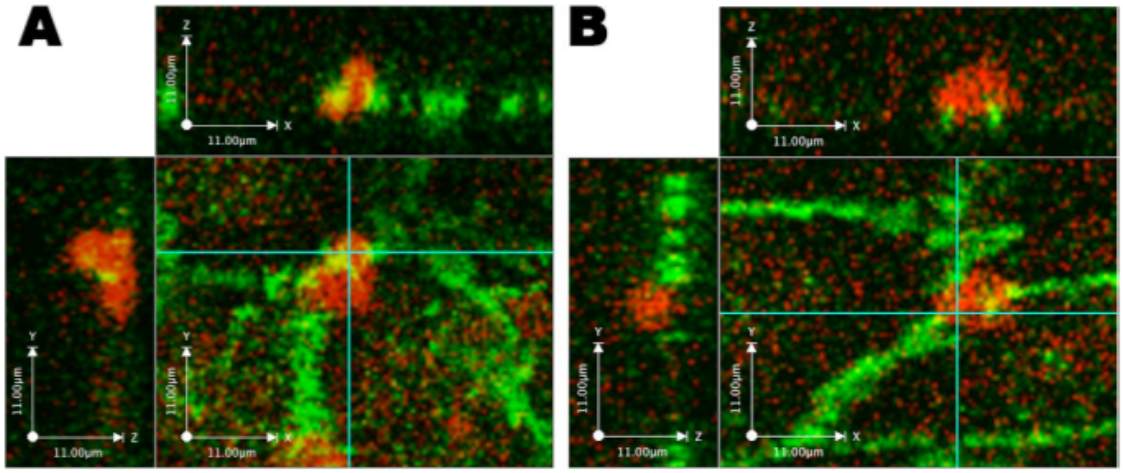

Figure 2-7. Inhibition of endothelial PI3 kinase does not prevent focal disassembly of the endothelial adherens junction. A) A lymphocyte (cytoplasm stained with CellTracker Red) migrating across a control HUVEC monolayer is seen to extend between and beneath EC adherens junctions (VE-cadherin stained green). The center panel illustrates the xy dimension, the upper panel the xz and the left panel the yz dimension respectively along a line passing through the migration channel. The basal surface of the EC monolayer in the z dimension is to the right or bottom of the yz or xz orthogonal projections respectively. B) A lymphocyte adherent to a monolayer treated with LY294002 is associated with a gap in the adherens junction but fails to extend beneath the level of endothelial VE-cadherin. Quantitation of 526 adherent lymphocytes grouped by position in the migration channel is described in the text

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Chapter 3. ICAM-1 cross-linking under shear stress generates PI3K activity and endothelial p110α catalytic isoform participates in lymphocyte diapedesis

Introduction

Lymphocyte transendothelial migration (TEM) is a central phenomenon in many immune responses and dysregulation of lymphocyte TEM contributes to autoimmune disorders and allograft rejection. As described in the introduction, the mechanisms that regulate leukocyte penetration to inter-endothelial junctions, diapedesis, are not fully understood. PI3K is identified as an important molecule in leukocyte trafficking. Several lines of evidence including our own work have shown that endothelial PI3K is required for leukocyte diapedesis [1-4]. PI3K regulate cellular events including actin cytoskeleton remodeling in a spatially restricted manner via PIP_3-meidated localization of GTP/GDP exchange factors for Rho family GTPases [5].

Class I PI3K are heterodimers that are divided into classes IA (catalytic subunits p110α, p110β and p110δ) and IB (catalytic subunit p110γ). Class IA catalytic subunits interact with five different p85 regulatory isoforms downstream of either a tyrosine kinase or a cytoplasmic tyrosine kinase, while class IB interacts with p101 or p87 regulatory subunit downstream of G protein coupled receptors. However, recent studies suggest that this is not always the case. For instance, p110β is mainly coupled to GPCR not a tyrosine kinase [6-9] also

indirect mechanisms such as activation of Ras by GPCRs can potentially induce activation of class IA subunits by GPCRs [10-12].

PI3K class IB, activated downstream of GPCRs by binding to βγ subunits of G proteins, is expressed and participates in many endothelial responses such as proliferation, migration and changes in vascular permeability in response to chemokines and it is also involved in neutrophil trafficking by firm adhesion of neutrophils on the endothelium surface [2, 8, 13, 14]

There are growing evidence that p110 isoforms selectively regulate different biological responses. The mechanism(s) that confer isoform specificity is not clear. In EC, isoform specificity has also been observed. In porcine aortic endothelial cells, p110α is required for platelet derived growth factor mediated actin cytoskeleton remodeling while p110β is involved in actin reorganization induced by insulin [15]. However, in vivo studies indicate that specific endothelial deletion of p110β and p110δ does not affect mice viability and fertility. Further, PI3K activity in immortalized cardiac EC isolated from these mice and also HUVECs indicated that these isoforms participate minimally in PI3K activity of EC [9]. p110β contributed to PI3K activity induced by SDF, downstream of GPCR [9]. On the other hand, p110α activity was essential for vascular development and its inactivation caused embryonic lethality [9]. p110α was the main isoform participating in migration (RhoA dependent) induced by VEGF signaling. In addition to great inhibition of RhoA activity, inhibition of p110α also moderately reduced Rac1 activity [9].

At the time of starting this project, little was known about the role of p110 isoforms in lymphocyte TEM. Endothelial p110δ and p110γ activity are both required for neutrophil trafficking by mediating transition from tethering and rolling of neutrophils to firm adhesion [2, 3]. Since lymphocytes can use immunoglobulin superfamily members to both tether and firmly adhere to EC, we hypothesized that p110 isoforms contribute to lymphocyte TEM differently. Further, the role of p110 isoforms in leukocyte diapedesis is not clear. Recently, Ridley and colleagues (2010) investigated the role of P110 isoforms in regulation of inter-endothelial junctions upon TNF stimulation. They showed that endothelial p110α activity is required for efficient T lymphoblst and monocyte diapedesis [4].

The aim of this study was also to look at the role of each EC p110 isoform in lymphocyte TEM. In addition, we tested the ability of ICAM-1 and VCAM-1 in the absence or presence of shear stress to induce PI3K activity. Our results indicate that endothelial p110α activity is required for efficient lymphocyte diapedesis. Further, cross-linking of ICAM-1 in activated endothelial cells in presence of shear stress induces PI3K activity.

Materials and Methods

Reagents

M199, RPMI, FBS, ECGS and HBSS were from Invitrogen (Burlington, ON). SDF-1a was from R&D (Minneapolis, MN). TNF-1a was from Biosource

(Camarillo, CA). Monoclonal anti-ICAM-1 mAb (clone P2A4) and anti-VCAM-1 (clone P3C4) were isolated from hybridoma supernatant (developed by Wanyner, E. A. from the Developmental Studies Hybridoma Bank, NICHD, Iowa City, IA). Rabbit anti mouse was from Jackson Immunoresearch (West Grove, PA). PP242 was from Sigma-Aldrich (St. Louis, MO). Protein-A coated beads (4.9 μm) was from Bang Laboratories, Fishers, IN. Non-silencing siRNA, P110α siRNA and Hiperfect were from Qiagen (Mississauga, Ont. Canada)

Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described [16]. For use in an experiment, passage 4-5 HUVEC were replated at confluent density onto 35mm dishes coated with a thin layer of matrigel (1 mg/mL; Corning Inc., Corning, NY) and cultured for 48 hours. HUVEC were treated with TNF-α (10 ng/mL, 20 h), then prior to assembly of the parallel plate flow chamber apparatus, HUVECs were incubated with SDF-1α at 100 ng/mL as described [17].

Where indicated, the following HUVEC pre-treatments were done: 3 μM PI3K-γ inhibitor (AS605240, 5-Quinoxalin-6-ylmethylene-thiazolidine-2,4-dione; Calbiochem ;Darmstadt, Germany). 1μM PIK-75, 3μM TGX-115 and 3μM IC87114 (all were gifts from Shokat laboratory, UCSF, San Francisco) for 30 minutes at 37°C then the EC monolayers were exposed to SDF-1α containing the inhibitors. Monolayers were washed 3 times and then used in TEM assays as described previously [1].

Cross-linking surface molecules

To study PI3K activity after receptor cross-linking, HUVECs were seeded on 12-well gelatin coated dishes and were starved overnight by medium containing 1%M199, 60% growth supplements, and 10ng/ml TNF-α. Then cells were washed 2 times with M199 and incubated with 10 μg/ml antibodies against CD58, ICAM-1 or VCAM-1 for 30 minutes. Cells were washed in binding buffer (Hanks buffer containing Hepes, BSA, Mg^{++} and Ca^{++}), incubated with rabbit anti mouse coated beads (~5 beads/cell were coated with 1mg/ml antibody in 4°C overnight), centrifuged immediately to ensure homogeneous binding of beads and then incubated in 37°C for indicated times and harvested immediately. To measure PI3K activity on cells exposed to shear stress or shear stress combined with receptor cross-linking, HUVECS were seeded in a contained area on gelatin coated 150 mm dishes. Cells were treated similarly as above except that beads were incubated for 90 minutes to ensure maximal binding of beads. Then cells were exposed to 1 dyne/cm^2, for indicated times using a parallel plate flow chamber (cytoshear flow chamber 75mm by 38mm, CytoDyne, La Jolla, CA). The ratio of phospho-serine 473 Akt to total Akt was measured by densitometry (Quantity One, BioRad Laboratories) and used as a read out for PI3K activity.

RNA interference

The expression of p110α was inhibited by siRNA transfection. HUVECs were seeded 24hr before transfection at 50% confluency. Cells were transfected with 50 nmol/L either non-silencing siRNA, or P110α (CTGAGTCAGTATAAGT) using Hiperfect according to manufacturers instruction on two consecutive days and used for experiments 72hr after first transfection. P110α expression was consistently decreased 85-90% of control.

Western blot

HUVEC monolayers were lysed by adding hot 2X loading buffer (24mM Tris pH 6.8, 10% glycerol,1% SDS, 6mM 2-mercaptoethanol, 0.05% Bromophenol Blue) and scraping and further boiling at 95°C for 8 minutes. About fifty micrograms of lysate was resolved on SDS-PAGE, then blotted onto nitrocellulose (Biorad) as recommended by manufacturer. For immunoprecipitation, cells were harvested with RIPA buffer (10 mM Tris, pH=7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 mM NaF, 2 mM Na_3VO_4, 0.1% SDS, 0.5% Na deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM PMSF), with added proteinase cocktail and incubated using protein G sepharose beads (Sigma).

Statistics

The mean fraction of lymphocytes migrating on the EC surface, or undergoing TEM among the treatment vs. control groups among several experiments was calculated and tested for statistical significance ($P<0.05$) by paired Students t-test. All the data are shown as mean±SEM. To evaluate PI3K activity changes by cross-linking, trends in each group were compared by linear regression analysis (Prism, GraphPad Software, Inc).

Results

Inhibition of endothelial PI3-kinase class IB activity does not perturb lymphocyte transendothelial migration

We observed in our initial experiments that p85 knockdown reduces lymphocyte TEM, but less completely than the broad-spectrum small molecule inhibitors of PI3K, LY-294002 and wortmannin. We hypothesized that endothelial p110γ might be involved in lymphocyte TEM. To determine if EC p110γ participates in lymphocyte TEM, HUVEC monolayers were treated with AS605240 (IC_{50} at 8nM), which selectively inhibits p110γ activity, and then lymphocyte TEM was studied using a real time in vitro model that allows direct observation of interactions between freshly isolated T lymphocytes and cytokine-activated HUVEC under physiological shear stress as described in Methods. Pretreatment of EC with p110γ inhibitor did not affect lymphocyte TEM (Figure

3-1; p=not significant; 3 independent experiments). This observation suggests that p110γ activity is not required for lymphocyte TEM.

Endothelial PI3-kinase class IA activity is required for efficient lymphocyte transendothelial migration

p110γ inhibition did not affect lymphocyte TEM and p85 inhibition reduced lymphocyte TEM, suggesting that PI3K IA is involved in lymphocyte TEM. To inhibit p110δ activity, cells were treated with IC87114. This compound is highly selective for p110δ (IC_{50} value of 0.1 μM) having about 100-fold selectivity between p110δ and other isoforms [18]. Lymphocyte TEM was not affected by treatment of EC with this inhibitor (Figure 3-2A).

To inhibit p110β EC were treated with TGX-115. This compound inhibits p110β activity by IC_{50} value of 0.1 μM and p110δ activity by IC_{50} value of 1 μM [18]. Since inhibition of p110δ did not affect lymphocyte TEM, any effects observed by this inhibitor, at concentration we used (3 μM), should be due to blockade of p110β activity. Treatment of EC with this inhibitor did not affect lymphocyte TEM (Figure 3-2B).

Next, we looked at importance of p110α activity in lymphocyte TEM. We used PIK-75, which has an IC_{50} value for p110α ~10 nM and has about 10 and 100-fold selectivity between p110α and p110β and p110δ, respectively [18]. Treatment of EC with PIK-75 reduced lymphocyte TEM, significantly (Figure 3-2C; 74 ± 5.5% of control; $p<0.05$) without affecting lymphocyte surface motility.

To further confirm the involvement of endothelial p110α in lymphocyte TEM, p110α expression in EC was inhibited by RNAi, as described in Methods. p110α expression was efficiently reduced as shown in Figure 3-2D. Although lymphocyte motility on the surface of endothelium was not disturbed by p110α knockdown, p110α knockdown HUVECs did not support lymphocyte TEM as efficient as EC transfected with non-silencing siRNA (Figure 3-2E; 80 ± 2.2% of control; $p<0.05$). These data indicate that endothelial PI3K IA activity through p110α is important for lymphocyte diapedesis.

Endothelial mTOR is not required for lymphocyte TEM

PI3K participates in various cellular events via different pathways. Since our previous studies suggest that PI3K regulate lymphocyte diapedesis by mediating actin remodeling and consequently remodeling of adherens junctions, we looked at PI3K downstream pathways that mediate actin remodeling. One of these pathways involves mTORC2 [19-21], mTOR is a large serine/threonine kinase that is in complex with multiple proteins. We inhibited mTOR activity using pp242, an ATP competitive inhibitor that targets both mTORC1 and mTORC2. Pretreatment of EC with pp242 resulted in lymphocytes detaching after firm adhesion (Figure 3-3A; p=not significant; 4 independent experiments), suggesting that mTOR activity is involved in stable adhesion of lymphocytes to EC. Reanalyzing data after exclusion of detached lymphocytes indicated that EC mTOR activity is not required for lymphocyte TEM (Figure 3-3B; p=not significant; 4 independent experiments).

Cross-linking ICAM-1 or VCAM-1 does not induce PI3K activity

During leukocyte TEM, the integrin adhesion molecules of leukocytes, LFA-1 and VLA-4, interact with ICAM-1 and VCAM-1, respectively, which mediates firm adhesion of leukocytes to endothelium and it initiates signaling events in EC that can facilitate leukocyte diapedesis [22-28]. Our observation that endothelial PI3K activity is required for lymphocyte diapedesis suggests that upon lymphocyte-endothelial interaction under shear stress, there would be cues from either lymphocytes or shear stress that induce specific increase in PI3K activity. To test this hypothesis, we cross-linked either ICAM-1 or VCAM-1 using beads. This model has been extensively used to mimic integrin-mediated clustering of the adhesion molecule. We also cross-linked CD58 (LFA-3) as a control since this molecule is expressed as a glycosylphosphatidylinositol (GPI)-anchored form and does not induce signaling via its cytoplasmic domain. Confluent TNF activated HUVECs were starved overnight and incubated with mouse antibody against the adhesion molecule of interest. Then cells were incubated with rabbit anti-mouse coated beads up to 30 minutes as described in Methods. Cells were harvested and phospho-Akt to total Akt levels were detected by Western blotting. CD58 or ICAM-1 cross-linking did not induce a significant increase in phospho-Akt levels. Although VCAM-1 cross-linking induced phospho-Akt levels, especially after 30 minutes of cross-linking this observation was not consistent in all experiments (Figure 3-4; p=not significant; 4 independent experiments). These results suggest

that ICAM-1 or VCAM-1 ligation by themselves do not produce a robust PI3K signalling.

Applying shear stress alone on endothelium does not increase PI3K activity

While exposure of aortic EC to high shear stress (5 dyne/cm^2 or higher) can induce a significant increase in PI3K activity [29-31], the effect of lower shear stress that microvascular EC are exposed to on PI3K activity is not clear. Cinamon (2001) reported that shear stress is essential for leukocyte diapedesis [32]. Thus, we hypothesized that lower shear stress might also induce PI3K activity in EC. Confluent TNF activated HUVECs were starved overnight and then were exposed to 1 dyne/cm^2 shear stress for the indicated times. Under these experimental settings we did not observe a significant increase in PI3K activity (Figure 3-5).

Cross-linking ICAM-1 on EC in presence of shear stress induces PI3K activity

Next, we tested the combined effect of shear stress and cross-linking of either ICAM-1 or VCAM-1 on PI3K activity. Confluent TNF activated HUVECs were starved overnight and then ICAM-1 or VCAM-1 were cross-linked followed by shear stress application for the indicated times, as described in Methods. Comparing to CD58 ligated cells, ligation of ICAM-1 induced PI3K in a time

dependent manner (Figure 3-5A; 101±7.5% vs. 143±18% after 10minutes and 110±13% vs. 177±25% after 30 minutes, data are normalized to CD58 0min; $p<0.05$; 4 independent experiments). Ligation of VCAM-1, on the other hand, failed to mediate change in PI3K activity comparing to CD58 ligation (Figure 3-5B; 101±7.5% vs. 123±8.8% after 10 minutes and 119±14.5% vs. 162±23% after 30 minutes, data are normalized to CD58 0min; p=not significant; 4 independent experiments). These data suggest that during lymphocyte TEM, interaction of LFA-1 adhesion molecule of lymphocytes with ICAM-1 mediates signaling events in EC that results in PI3K activation.

Discussion

In addition to its role in leukocyte trafficking, the PI3K signaling pathway is an important player in many other endothelial events such as growth, motility and angiogenesis. Thus, its complete inhibition for therapeutic reasons might produce many other defects in organisms. Therefore, a complete understanding of the role of each PI3K subunit and their overall regulation is a valuable step in designing more specific anti-inflammatory drugs. Previously, we showed that inhibition of expression of p85α regulatory subunit in EC reduced lymphocyte diapedesis [1]. In this study we showed that inhibition of p110α activity by a specific inhibitor reduced lymphocyte diapedesis. In addition, inhibition of p110α expression in EC also decreased lymphocyte diapedesis. All together, these data indicate that endothelial PI3K class IA through p110α isoform regulate lymphocyte diapedesis by mediating inter-endothelial junction remodeling

necessary for accommodation of lymphocyte passage. We could not detect any changes in lymphocyte TEM upon inhibition of p110β, p110δ or p110γ. During the completion of this project, Ridley and colleagues (2010) published data also showing that endothelial p110α participates in lymphocyte paracellular but not transcellular migration [4]. Cain, et al., (2010) report the defect by inhibition of p110α activity or expression to be at inter-endothelial junction remodeling and VE-cadherin phosphorylation and they propose that p110α might coordinate adherens junction-changes with actin cytoskeleton.

To obtain insights about possible mediators of PI3K activity during lymphocyte TEM, we studied PI3K activity downstream of adhesion molecules ICAM-1 and VCAM-1 in the absence and presence of shear stress. VCAM-1 cross-linking generates inconsistent results that we cannot explain at this point. Similar to our findings, Martinelli, et al., (2009) could not detect PI3K activation upon cross-linking of ICAM-1 in their static conditions [33].

In EC, a mechanotransduction complex consisting of PECAM-1, VE-cadherin and VEGFR2 was identified upstream of shear-induced integrin activation [31]. At low shear stress in our settings we were not able to detect a significant rise in PI3K, suggesting that the described mechanotransduction complex is not activated. However, ICAM-1 cross-linking in presence of shear stress significantly induces PI3K activity after 10 minutes. Shear stress might increase the cross-linking efficiency by generating extra force on beads, hence enhancing ICAM-1 signaling. However, this is not the case for VCAM-1. Another

possibility is that ICAM-1 cross-linking and shear stress both participate in induction of PI3K activity in a synergistic manner.

The importance of ICAM-1 interactions with its ligands LFA-1 and Mac-1 in inflammatory responses is apparent by recurrence of a range of infections in soft tissues and skin of LAD I patients and also in a mouse model lacking β integrin [34, 35]. Also, in vitro studies have shown that ICAM-1 mediated signaling is required for leukocyte diapedesis [22, 24, 33, 36-38]. VE-cadherin phosphorylation and/or RhoA activation are the proposed mechanisms by which ICAM-1 participate in leukocyte diapedesis [22, 24, 36, 39, 40]. ICAM-1-mediated signaling events, including PI3K activation, might also regulate opening of other junctional molecules such as nectin memebrs, which will be discussed later.

Since class IA PI3K is generally activated by tyrosine kinases, involvement of p110α in lymphocyte TEM, suggests that a tyrosine kinase becomes activated downstream of ICAM-1 which would mediate PI3K activity. A number of Tyrosine kinases have been shown to participate in leukocyte TEM including Src and FAK that are activated downstream of ICAM-1 [24, 33, 40].

In summary, endothelial PI3K IA regulates lymphocyte TEM via its p110α isoform. EC p110α inhibition of expression or activity does not affect lymphocyte surface motility but reduces their ability to undergo diapedesis, suggesting that P110α activity participates in EC junction remodeling required for passage of lymphocytes. Further, ICAM-1 ligation in the presence of shear stress

induces PI3K activity. Further work needs to be done to clarify whether the observed PI3K activity is completely p110α dependent or not.

Figures

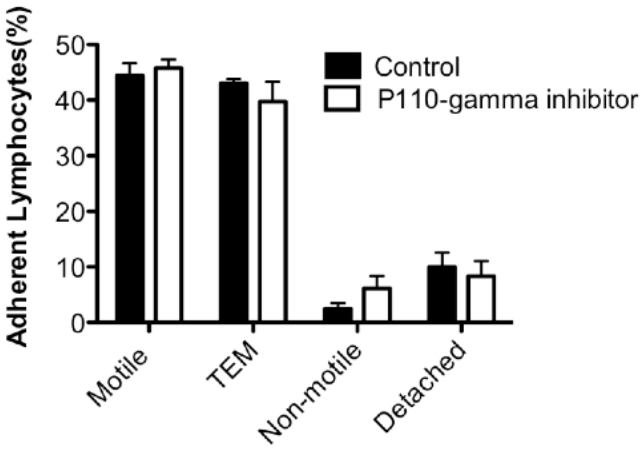

Figure 3-1. Endothelial PI3K class IB is not required for lymphocyte transendothelial migration. TNF activated HUVECs were pretreated with 3 μM P110γ inhibitor (AS605240) for 30 minutes and then lymphocyte TEM was assayed under shear stress as in Methods; (mean±SEM; p=not significant; 4 independent experiments).

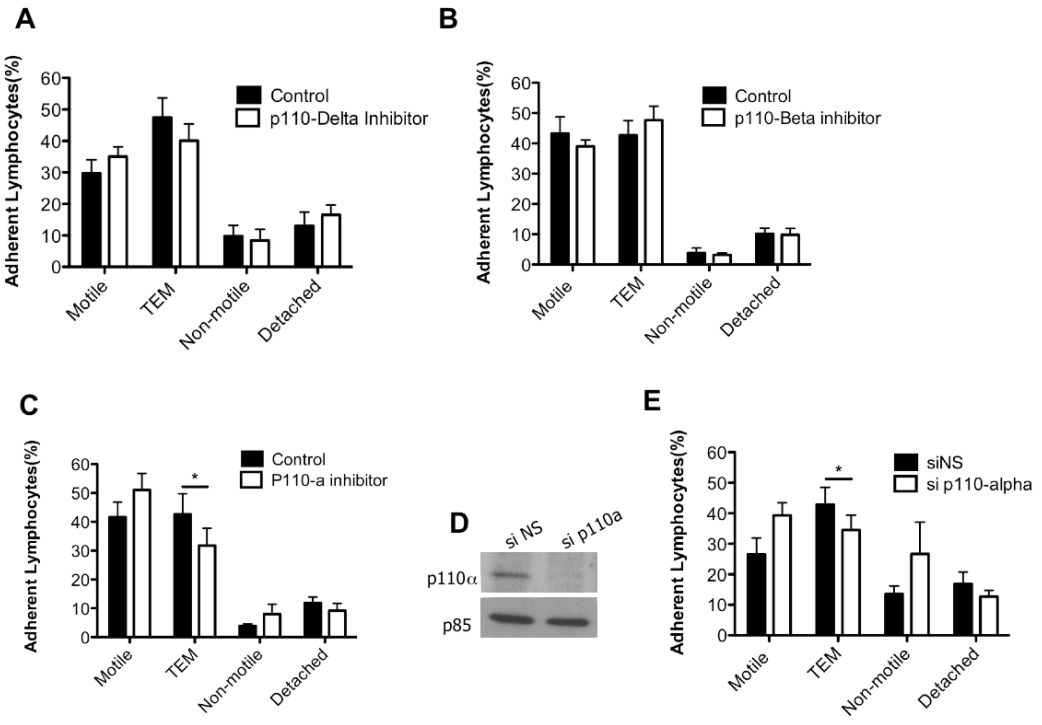

Figure 3-2. Endothelial PI3K class IA activity is required for efficient lymphocyte transendothelial migration. TNF activated HUVECs were pretreated with A) 3μM P110δ inhibitor (IC87114); B) 3μM P110β inhibitor (TGX-115); or C) 1μM p110α inhibitor (PIK-75) for 30 minutes and then used in lymphocyte TEM assays as described in Methods. D) HUVECs transfected with non-silencing siRNA or p110α siRNA were immunoprecipitated with p85 and membranes were probed for p110α. Image is a representative of 4 independent experiments. Knockdown and Immunoprecipitation was done by Qiu-Xia Zhang. E) p110α knockdown HUVECs or control were used in lymphocyte TEM assay as in Methods (mean±SEM; *=P<0.05; 4 independent experiments).

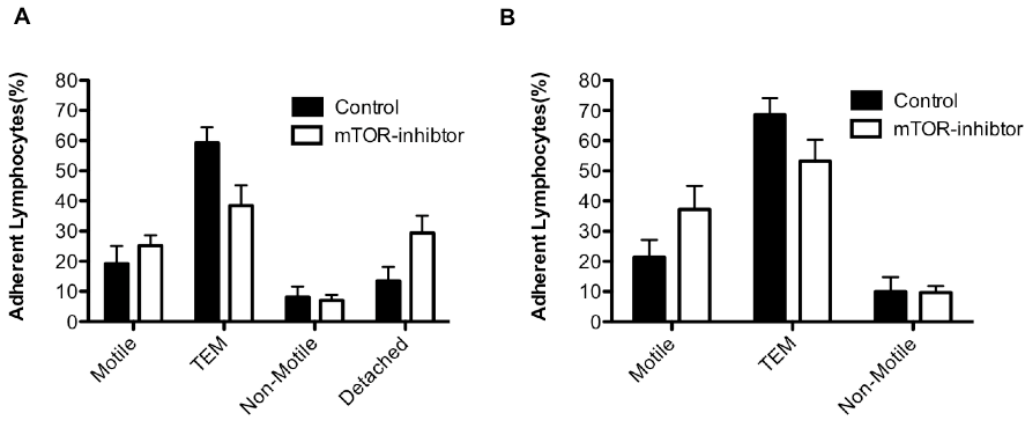

Figure 3-3. Endothelial mTOR is not required for lymphocyte TEM. TNF activated HUVECs were pretreated with 10 μM PP242 and then lymphocyte TEM was assayed as in Methods. A) Fraction of lymphocytes in each indicated group. B) Reanalaysis of experiments by excluding the detached lymphocytes (mean±SEM; P=not significant; 4 independent experiments).

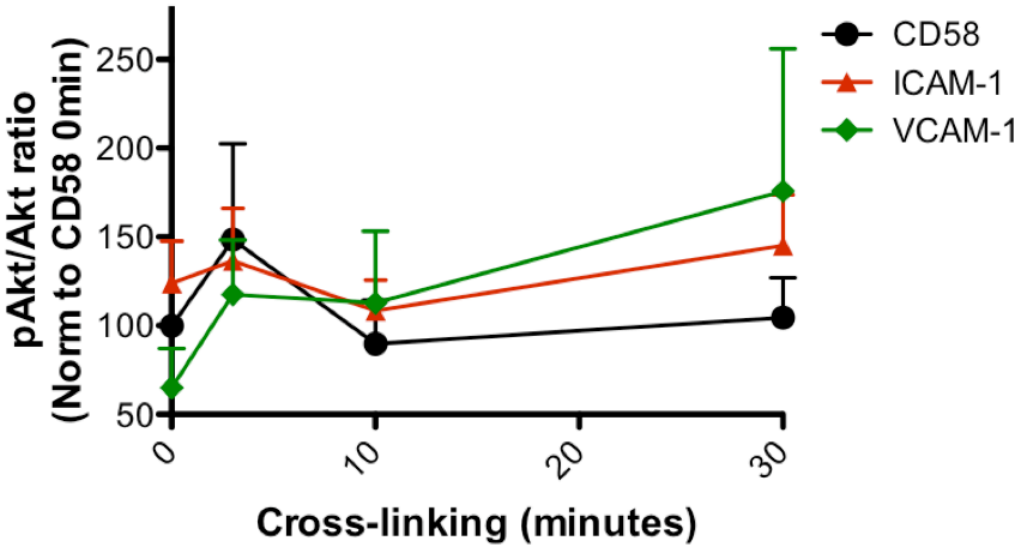

Figure 3-4. Cross-linking ICAM-1 or VCAM-1 does not induce PI3K activity. CD58, ICAM-1 or VCAM-1 on TNF activated starved HUVECs were cross-linked for the indicated times as described in Methods. Phosphorylated-Akt and total Akt were measured by densitometry and the ratio was normalized to CD58 0min values (mean±SEM; P=not significant; 4 independent experiments).

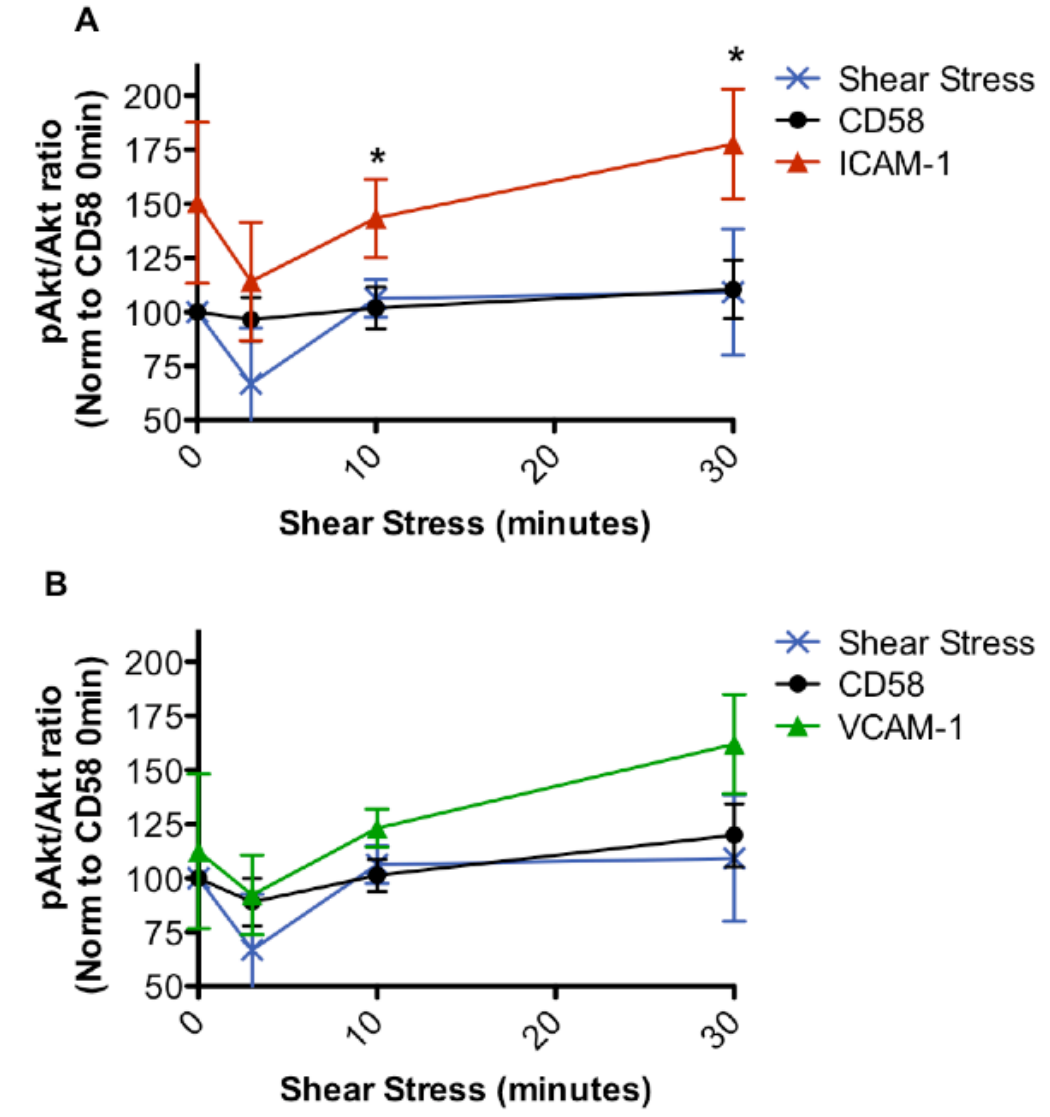

Figure 3-5. Cross-linking ICAM-1 and VCAM-1 on EC in presence of shear stress. TNF activated starved HUVECs were exposed to shear stress alone or were cross-linked with CD58, ICAM-1 (A), and VCAM-1 (B) and then cells were exposed to shear stress for the indicated times. Data were analyzed as described (mean±SEM; $P<0.05$; 4 independent experiments).

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Chapter 4. Endothelial IQGAP1 Regulates Efficient Lymphocyte Transendothelial Migration[2]

Introduction

Leukocyte extravasation is fundamental to the development of many immune responses including solid-organ allograft rejection. In this process, leukocytes leave the bloodstream and migrate into tissues through the endothelial cells (EC) that line the walls of vessels, i.e. leukocytes undergo transendothelial migration (TEM). Whereas the specific adhesion molecules, chemoattractants and possibly signaling pathways involved in TEM are unique among different subgroups of leukocytes and vascular beds, the interaction between leukocytes and EC during TEM can be generalized into a multicascade event, described in recent reviews [1-3]. EC and leukocyte adhesion molecules mediate tethering and rolling of leukocytes on EC followed by chemokine-mediated leukocyte activation, then firm adhesion to the EC. Finally, adherent leukocytes crawl on the surface of endothelium, undergo diapedesis, and enter tissues by mechanisms that are not fully understood.

Leukocyte transmigration may occur by either a transcellular, through EC, or paracellular route, between adjacent EC[4-6]. The latter is associated with structural changes in the interendothelial adhesion structures and EC

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cytoskeleton[5, 7, 8]. Cross-linking of adhesion molecules such as CD54 or CD106 is shown to mediate signals that lead to EC actin cytoskeleton remodeling[9-12]. These signaling cascades promote structural changes in interendothlelial junctions, that might be required for efficient leukocyte penetration of the endothelium, including redistribution of molecules enriched at the junction such as platelet endothelial cell adhesion molecule (PECAM-1; CD31), junctional adhesion molecule (JAM) or components of the VE-cadherin complex around the migration channel and targeted recycling of sub-plasma membrane vesicles underlying the migration pore [5, 6, 13-19]. Thus, in addition to VE-cadherin gap formation, poorly defined events that may involve remodeling of other interendothelial or endothelial-matrix adhesive contacts, the cytoskeleton of the lateral wall of the EC, or fusion of cortical vesicles with the plasma membrane likely occur to accommodate the lymphocyte during diapedesis.

IQGAP1 is a scaffolding molecule that participates in cell-cell adhesion, cell motility and polarization by interacting with both cytoskeletal and signaling molecules. IQGAP1 interacts with actin by a calponin homology domain[20], indirectly with microtubules (MT) through interaction with CLIP-170, a microtubule-Plus-End-Tracking-protein[21-23], and localizes to the adherens junctions (AJ) cadherin complex by its c-terminus domain[24-27]. IQGAP1 integrates Ca^{2+}/calmodulin with Rho GTP-binding protein signaling at spatially restricted areas of the cell[26, 28]. Functionally, recent work implicates IQGAP1 in remodeling of VE-cadherin-dependent interendothelial contacts during VEGF-stimulated angiogenesis[27].

Microtubules regulate the intercellular AJ in epithelial cells. A population of MT extend to AJ and are involved in concentrating E-cadherin at the intercellular junction[29]. Further, MT-based motors, dynein and kinesin, are shown to interact with constituent proteins of AJ complex, β-catenin and p120 catenin[30, 31], hence may also participate in dynamic regulation of AJ[19].

Remodelling of the interendothelial cell junction during TEM may involve MT. Under static conditions, MT depolymerization of dermal EC is found to promote monocyte and neutrophil TEM[32, 33]. However, under shear stress, Carman and Springer observed a 3-4 fold decrease in monocyte TEM across MT-depolymerized HUVEC, and impaired formation of a "docking structure" associated with transcellular diapedesis[4]. Recently, Mamdouh et al also observed a decrease in lymphocyte and monocyte paracellular TEM in static conditions by inducing endothelial MT depolymerization[19]. They suggested that endothelial MT are required for targeting a lateral border recycling compartment to the migration channel.

In this study, we sought to investigate the role of endothelial IQGAP1 in lymphocyte TEM under shear stress conditions that closely model the physiologic environment of lymphocyte diapedesis[34]. We analyzed the effect of IQGAP1 knockdown on actin and MT of confluent EC. The results indicate that IQGAP1 knockdown in EC monolayers decreases MT captured at the interendothelial junctions and decreases lymphocyte diapedesis. Further, drug-induced MT depolymerization decreases paracellular lymphocyte diapedesis. These results

indicate that endothelial IQGAP1 tethers MT to interendothelial junctions and participates in junction remodeling during lymphocyte transendothelial migration.

Materials and Methods

Reagents

M199, RPMI, HBSS, FBS, ECGS and Matrigel were from Invitrogen (Burlington, ON). Nocodazole and FITC-phalloidin were from Sigma (St. Louis, MO). Stromal cell derived factor-1 alpha (SDF-1α, CXCL12) and Phycoerythrin-conjugated CD144 were from R&D (Minneapolis, MN). Tumor necrosis factor-alpha (TNF-α) was from Biosource (Camarillo, CA). To isolate $CD3^+$ lymphocytes, StemSep negative selection system from StemCell Technologies Inc (Vancouver, BC) was used. Mouse anti-β-tubulin was from Biomeda and rabbit anti-VE-cadherin was from Cayman (Cedarlane laboratories, Mississauga, ON). Rabbit IQGAP1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal PECAM-1 antibody was from Endogen, Woburn, MA. Monoclonal CD99 was from MyBiosource (San Diego, CA). Monoclonal JAM-1 was from GenTex (Irvine, CA). Fluorophore-conjugated antibodies were from Jackson Immunoresearch (West Grove, PA). All secondary antibodies were tested for nonspecific binding. CellTrackers were from Molecular Probes (Eugene, OR). Hiperfect, non-silencing siRNA, IQGAP1 siRNA (sequence: AAGGAGACGTCAGAACGTGGC) and APC siRNA (sequence: CCGGTGATTGACAGTGTTTCA) were from Qiagen Inc (Mississauga, ON).

Cell culture

Human umbilical vein endothelial cells (HUVEC) and peripheral blood lymphocytes (PBL) were isolated and cultured as described previously[35]. HUVECs were grown on 35 mm dishes coated with 1mg/ml Matrigel 72 hr prior to TEM experiments, and treated with 10ng/ml TNF-α 20-24 hr before assembly of the parallel plate flow chamber apparatus. Where indicated, HUVEC were loaded with 10μmol/L nocodazole (ND) or equivalent DMSO dilution for 3 minutes and washed extensively before the experiments. Where indicated, the EC monolayer was treated with ND as above, and conditioned binding buffer was collected after 10 minutes. Lymphocytes were resuspended in this conditioned medium and used for TEM assay.

RNA interference

To inhibit IQGAP1 or APC expression, HUVEC were transfected twice on consecutive days with either 10 nmol/L non-silencing or 10 nmol/L validated IQGAP1 or APC siRNA using Hiperfect according to the manufacturer's direction. IQGAP1 and APC expression was optimally inhibited 96 hr and 72 hr after first transfection, respectively. IQGAP1or APC inhibition was tested by western blotting as described previously[36].

Analysis of lymphocyte TEM under shear stress

Lymphocyte TEM was studied by parallel-plate laminar flow adhesion assay as described previously[35]. Briefly, Lymphocytes were perfused over the EC monolayer at low shear flow (0.5 dyne/cm^2) and allowed to accumulate on the ECs. The flow rate was then increased to 1 dyne/cm^2 throughout the assay (10 or 20 minutes). The adherent lymphocytes were scored for surface motility (include both lymphocytes that migrate more than one cell body on the surface of the EC monolayer and those that transmigrate) or transmigrating lymphocytes (cells that undergo a change from phase-bright to phase-dark appearance). The data are reported as a fraction of the originally accumulated lymphocytes. Lymphocyte encounters with interendothelial junctions were determined by following the track of each lymphocyte on the videomicrographs over the characteristic phase-bright band between adjacent EC. In a second technique, lymphocytes were stained with CellTracker Orange according to the manufacturers instructions, then were made to interact with HUVEC monlayer in the parallel-plate flow chamber. After 10 minutes of shear stress application, the chamber was disassembled, and the cells were stained for VE-cadherin as described below.

To study diapedesis, the location of each lymphocyte relative to VE-cadherin staining was analysed using a LSM 510 confocal microscope (Zeiss, Toronto, ON) set to acquire images at 0.4 um intervals in the z-plane. Lymphocytes were considered to be associated with gap formation in the AJ if a break in endothelial VE-cadherin staining at least 2 μm wide was directly

superimposed on the lymphocyte footprint. Lymphocytes were scored by blinded observer for the relationship in the z-plane to the VE-cadherin signal.

To study the PECAM-1 enrichment around lymphocytes in the process of diapedesis, PECAM-1^{bright} naïve T cells (CD45RA^{+}) cells were depleted using CD45RA TAC (StemCell Technologies Inc, Vancouver, BC). The cells were stained with CellTracker Blue and were made to interact with the HUVEC monlayer in the parallel-plate flow chamber. After 10 minutes of shear stress application, the chamber was disassembled, and the cells were double stained for VE-cadherin and PECAM-1.

Immunofluorescence analysis

Confluent HUVEC monlayers seeded on Matrigel-coated glass coverlips were treated with either DMSO or ND. Cells were fixed, permeabilized and blocked as described previously[36]. The cells were then double-stained using anti-β-tubulin and anti-VE-cadherin primary and fluorophore-conjugated secondary antibodies. To determine MT and AJ morphology in cells treated with non-silencing or IQGAP1 RNAi, transfected HUVEC were trypsinized and seeded on coverslips at confluency. The monolayer was stained with either β–catenin or double-stained for MT and VE-cadherin. MT density adjacent to AJ was measured using image analysis software (OpenLab, Lexington, MA). Regions of interest were defined extending 3 μm into the cell cortex from VE-cadherin-positive junctions to quantitate MT staining intensity in at least 30 cells in each experiment. To evaluate F-actin cytoskeleton changes, confluent HUVEC

monolayers were fixed and permeabilized and F-actin was stained by FITC-phalloidin. To determine the effect of TNF-α treatment and shear stress on junction staining, HUVECs were treated with TNF-α and subjected to shear stress in conditions as described for TEM assay but with no lymphocytes. Then cells were fixed and permeabilized and stained for VE-cadherin, PECAM-1 and JAM-1. CD99 was stained without permeabilization.

Microtubule/Tubulin and G-actin/F-actin In Vivo Assay

The ratios of free vs. polymerized tubulin and G-actin vs. F-actin in EC were quantitated using kits according to the manufacture's instruction (Cytoskeleton, Denver, CO).

Statistics

The mean fraction of lymphocytes migrating on the EC surface, or undergoing TEM among the treatment vs. control groups among several experiments was calculated and tested for statistical significance ($P<0.05$) by paired Students t-test (SPSS, Chicago, IL). All the data are shown as mean±SEM. To evaluate the position of lymphocytes at the interendothelial junctions, data from four independent experiments were pooled and tested for significance using Chi square analysis (SPSS).

Results

IQGAP1 is localized to interendothelial junctions

IQGAP1 has been shown to colocalize with AJ cadherin complex and regulate cadherin-mediated cell-cell adhesion[24, 26, 27]. In EC, we observed IQGAP1 enrichment at the interendothelial junctions (Figure 4-1B). To study the role of EC IQGAP1 in lymphocyte TEM, endothelial IQGAP1 expression was inhibited by RNAi. IQGAP1 siRNA transfection of HUVEC consistently reduced IQGAP1 protein expression more than 80% (Figure 4-1A and Figure 4-1B vs. 1C). However, confluent IQGAP1-knockdown EC monolayers developed normal AJs, reflected by β-catenin (Figure 4-1E) and VE-cadherin (Figure 4-2D) localization at the junctions, similar to the control monolayers (Figure 4-1D and Figure 4-2C). Further, analysis of cell surface expression of VE-cadherin and PECAM-1 by flow cytometry identified no change in IQGAP1-knockdown vs. control cells (data not shown). Functionally, electrical impedance across an IQGAP1-knockdown vs. the control monolayer was unchanged (data not shown). Taken together, these data indicate IQGAP1 is not required for the surface expression or assembly of endothelial junction components.

Loss of IQGAP1 perturbs tethering of microtubules at adherens junctions

Next, we sought to characterize the effect of IQGAP1 knockdown on EC cytoskeletal components since IQGAP1 regulates dynamic F-actin

polymerization[23, 37, 38] and MT capture at the cell cortex[21-23]. Biochemical analysis of free and polymerized tubulin within EC determined IQGAP1 knockdown decreased the ratio of polymerized tubulin to free tubulin levels in the cytosolic extracts (Figure 4-2A and 4-2B). Further, measurements of MT density underlying junctions by immunofluorescent double-staining of VE-cadherin and tubulin indicated that tubulin fluorescence intensity per μm^2 area adjacent to the VE-cadherin band among IQGAP1 knockdown EC (Figure 4-2D vs. Figure 4-2C) decreased by ~40% (Figure 4-2E). These data indicate that IQGAP1 knockdown induced loss of polymerized MT at the interendothelial junctions.

To evaluate the effect of IQGAP1 knockdown on the actin cytoskeleton of confluent EC, the population of F-actin and G-actin in cells was measured. Quantification of results by densitometry did not show any effect in F-actin content by IQGAP1 knockdown (Figure 4-2F). Consistent with the biochemical assay, F-actin distribution did not change between IQGAP1 knockdown cells vs. control cells by immunofluorescence microscopy (Figure 4-2G vs. 4-2H).

Endothelial IQGAP1 expression is required for efficient lymphocyte TEM

To determine if EC IQGAP1 participates in lymphocyte diapedesis, lymphocyte TEM across HUVEC monolayers transfected with non-silencing or IQGAP1 siRNA was studied using a real time in vitro model that allows direct observation of interactions between lymphocytes and cytokine-activated HUVEC under physiological shear stress[34]. Freshly isolated T lymphocytes were

perfused over a TNF-α treated HUVEC monolayer as described in Methods. There were no detectable changes in AJ morphology (Supplementary figure 4-1) or in distribution of PECAM-1, JAM-1 and CD99 (Supplementary figure 4-2 and data not shown) of either IQGAP1 knockdown or control endothelium after TNF-α treatment and shear stress. Under these conditions, 50%-70% of adherent lymphocytes transmigrated across the monolayer by the paracellular route. Consistent with previous reports, we saw little transcellular migration across the activated HUVEC monolayer[39, 40]. EC IQGAP1 knockdown decreased lymphocyte TEM to about 70% of control (Figure 4-3A), while the fraction of lymphocytes that locomoted on the surface of EC monolayer was not affected by IQGAP1-knockdown (Figure 4-3A).

We hypothesized that EC IQGAP1 deficiency might alter lymphocyte locomotion to favored sites of diapedesis. We evaluated lymphocyte movement toward interendothelial junctions by two methods. First, analysis of videomicrographs indicated a similar fraction of lymphocytes encounter at least one interendothelial junction during locomotion on the surface of the EC monolayer between IQGAP1-knockdown EC and EC transfected by non-silencing siRNA (83±4% vs. 85±3% (mean±SEM); p=NS, n=6 independent experiments). Second, immunofluorescence microscopy studies of co-cultures of lymphocytes adherent to EC monolayers, fixed after 10 minutes of applied shear (pooled from 4 independent experiments including more than 200 lymphocytes) did not show any difference in the fraction of adherent lymphocytes in contact with VE-cadherin-stained junctions between control and IQGAP1 knockdown monolayers

(84% vs. 72%; p=NS). These observations suggest that EC IQGAP1 might regulate the diapedesis stage.

Knockdown of EC IQGAP1 decreases lymphocyte penetration of interendothelial junctions

To assess diapedesis in more detail, TEM through the EC monolayer was evaluated by confocal microscopy. After 10 minutes of interaction under shear stress conditions, the flow chamber was disassembled, and the co-culture of EC and pre-labeled lymphocytes was fixed and stained for VE-cadherin. Lymphocytes were classed in three groups according to the position of the lymphocyte to EC VE-cadherin: lymphocytes that were in contact with VE-cadherin were considered above the junction if no part of lymphocyte was lower than VE-cadherin staining in the z dimension (Figure 4-3B); lymphocytes that extended through a transmigration channel but still had a uropod above VE-cadherin staining were considered to be within the junction (Figure 4-3C); lymphocytes completed diapedesis if the whole lymphocyte was below the level of VE-cadherin (Figure 4-3D). Results of four independent experiments evaluating more than 200 lymphocytes associated with EC AJs were pooled for analysis. In agreement with the analysis of the videomicroscopy experiments, we observed the fraction of lymphocytes beneath IQGAP1-deficient EC was decreased to ~60% of control (Figure 4-3E, $p<0.01$). Furthermore, the fraction of lymphocytes that were in the suprajunction position was 1.6 fold higher among lymphocytes migrating across IQGAP1-knockdown vs. control endothelial

monolayers (Figure 4-3E, $p<0.01$). Taken together, these results indicate that EC IQGAP1 participates in lymphocyte diapedesis but it is not involved in lymphocyte locomotion on the surface of endothelium.

IQGAP1 is known to associate with APC at the intercellular junctions and couple MT via a complex with CLIP-170 [23, 41]. Hence we determined the effect of endothelial APC knockdown on lymphocyte TEM. Using siRNA, APC was depleted to 80-90% of control level (3 independent experiments). We observed lymphocyte TEM across APC-knockdown monolayers was decreased to 75± 2% ((mean±SEM); 3 independent experiments; $p<0.01$) versus control monolayers. Taken together with the observation that IQGAP1 knockdown decreases EC MT density, these data suggest that IQGAP1, via APC, may act to tether MT to sites at the interendothelial junctions, perhaps to facilitate junction remodeling during TEM.

Endothelial MT depolymerization decreases lymphocyte TEM

Next, we sought to directly determine whether MT depolymerization inhibits lymphocyte TEM across interendothelial junctions in a manner similar to IQGAP1 or APC knockdown. Endothelial MT were briefly depolymerized using nocodazole (ND), as described in Methods. ND treatment of the monolayer mediated depolymerization of MT as shown by assay of polymerized vs. free tubulin in EC (Figure 4-4A and Figure 4-4B). Effective MT depolymerization by ND treatment was confirmed by immunofluorescence staining of tubulin (Figure 4-4D vs. 4-4C). Unlike prolonged ND treatment which causes VE-cadherin band

fragmentation and actin stress fiber formation (supplementary figure 4-3), interendothelial junctions remained structurally intact by brief ND treatment since VE-cadherin (Figure 4-4F) and beta-catenin (not shown) staining was unchanged compared to control monolayers (Figure 4-4E). Moreover, TNF-α treatment and shear stress did not affect AJ morphology (Supplementary figure 4-4) or distribution of VE-cadherin, PECAM-1, CD99 and JAM-1 (Supplementary figure 4-5 and data not shown) of ND treated EC versus controls. Flow cytometry analysis indicated similar VE-cadherin and PECAM-1 cell surface expression in DMSO and ND treated EC (data not shown). ND treatment did not affect the content or distribution of the F-actin cytoskeleton, as assessed by G-actin/F-actin assay in EC (Figure 4-4G and Figure 4-4H) and immunofluorescence staining (Figure 4-4J vs. 4-4I), respectively.

Under these conditions, pretreatment of EC with ND decreased TEM to ~65% of control (Figure 4-5A, $p<0.01$), while the fraction of lymphocytes that locomoted on the EC surface was not affected (Figure 4-5A). To determine if residual amounts of ND remaining after washing ND-treated EC could affect lymphocyte MT to alter function, lymphocytes were resuspended in the conditioned medium as described in Methods and studied for TEM. We observed no changes in lymphocyte motility or diapedesis (Figure 4-5A).

Analysis of live-cell videomicroscopy indicated a similar fraction of lymphocytes encountered at least one interendothelial junction during movement on control or ND-treated monolayers, (83±5% vs. 87±3% (mean±SEM); p=NS, n=5 independent experiments). Further, analysis of immunofluorescence images

of co-cultures of lymphocytes adherent to EC monolayers, fixed after 10 minutes of applied shear, was consistent with the videomicroscopy results. We observed no difference in the fraction of adherent lymphocytes in contact with VE-cadherin stained junctions between control and ND treated monolayers (76±4% vs.75±5% (mean±SEM); p=NS, n=6 independent experiments). These results indicate that loss of cortical endothelial MT does not influence movement of lymphocytes to the interendothelial junction, suggesting that endothelial MTs play a role in lymphocyte interpenetration of adjacent EC.

EC microtubule depolymerization inhibits lymphocyte penetration of interendothelial junctions

The location of lymphocytes within the interendothelial junction, in EC treated with ND or vehicle reagent, was analyzed by confocal microscopy as described above. Data from lymphocytes adherent to control (n=367) or ND-treated (n=341) monolayers in 3 independent experiments was pooled. Analysis of the position of the lymphocytes revealed that the fraction of lymphocytes in a suprajunction position was 1.3 fold higher among MT-depolymerized EC monolayers vs. control (Figure 4-5B; $p<0.01$). The fraction that completed diapedesis in the ND-treated group was reduced to ~60% of the DMSO-treated group (Figure 4-5B; $p<0.01$). Thus, both videomicroscopy and confocal imaging techniques indicate that endothelial MTs are required for efficient diapedesis, but are not essential for lymphocyte locomotion on the EC surface. Further, loss of

IQGAP1 expression and MT depolymerization both cause lymphocytes to accumulate above the AJ.

EC IQGAP1 knockdown or EC MT depolymerization do not affect adherens junction gap formation during diapedesis

Leukocyte diapedesis is associated with specific and transient gap formation in AJ[13, 14, 18], hence, we investigated whether loss of EC IQGAP1 or MT depolymerization affected gap formation associated with suprajunction-localized lymphocytes. We observed 22±3% of lymphocytes adherent to control monolayers were associated with a gap>2μm in diameter. Neither IQGAP1-knockdown nor ND treatment change the fraction of lymphocytes associated with VE-cadherin gap formation (110±36% vs 98±15% of control (mean±SEM); siIQGAP1 vs ND treatment; 4 independent experiments). Further, we examined the frequency of gaps enriched in PECAM-1 distributed around transmigrating lymphocytes. In these experiments, we studied TEM of PECAM-1^{-dim} memory T cells. We observed 32±9% ((mean±SEM); 3 independent experiments) of lymphocytes migrating across control EC monolayers were associated with a VE-cadherin gap enriched in CD31 (Supplementary figure 4-6). Gaps in VE-cadherin forming in IQGAP1 knockdown EC monolayers showed similar PECAM-1 enrichment (103±10% of control; mean±SEM in 3 experiments).

These data indicate that, like IQGAP1, the endothelial MT cytoskeleton facilitates lymphocyte diapedesis, but does not appear to be critical for displacement of VE-cadherin from the nascent migration channel

Discussion

Each stage of leukocyte TEM is regulated by signaling pathways mediated in both leukocytes and endothelial cells that facilitate progress to the next stage. For instance, engagement of the adhesion molecule ICAM-1 during firm adhesion leads to signaling events that result in actin remodeling, VE-cadherin phosphorylation and subsequently, paracellular leukocyte diapedesis [13, 16, 17]. Thus, molecules localized at the interendothelial cell junctions are candidate proteins to regulate paracellular transmigration of leukocytes. In this study, we examined the involvement of endothelial IQGAP1 in this process, since this molecule localizes at the cell-cell junctions and regulates dynamic assembly of cytoskeleton components: actin filaments and microtubules.

The major observations of this study are that IQGAP1, and interendothelial junction-associated MT, regulate paracellular TEM of lymphocytes. IQGAP1 knockdown both impairs lymphocyte TEM and decreases cortical MT density underlying the AJ of HUVEC in vitro. Similarly, knockdown of APC, a component of the protein complex linking IQGAP1 and MT, decreases lymphocyte TEM. Brief treatment of EC with ND has the similar effects on both lymphocyte TEM and cortical MTs. These interventions promote accumulation of lymphocytes on the luminal surface of the EC monolayer, above the level of VE-

cadherin. Surprisingly, a similar fraction of such lymphocytes were associated with an underlying gap in the VE-cadherin band among IQGAP1-knockdown, MT-depolymerization, and control monolayers.

IQGAP1 has been implicated to participate in dynamic interendothelial junction remodeling after VEGF stimulation[27]. IQGAP1 couples VEGFR2 to the β–catenin/VE-cadherin complex to facilitate VEGF-stimulated events such as tyrosine phosphorylation of VE-cadherin. VEGF stimulation increases IQGAP1 association with VE-cadherin, and loss of IQGAP1 expression reduces the assembly of the VEGFR2/VE-cadherin complex, involved in disassembly of endothelial AJ. In contrast to this reported data, however, we did not observe any changes in the basal assembly of AJ components in IQGAP1 knockdown EC monolayers or barrier function of the IQGAP1 knockdown monolayer. In our experiments, the IQGAP1-deficient HUVEC were plated at confluence, then maintained in complete media with 20% FBS for 48 hours to promote junction maturation. Hence, in the current experiments, effects of IQGAP1 knockdown on cell migration or repopulation at subconfluent densities were minimized.

IQGAP1 is known to associate with E-cadherin, regulate actin assembly, and coordinate the tethering of MT during polarized cell migration through interaction with MT plus-end binding proteins[21, 22, 24, 37, 38]. Our data implicate the participation of MT in endothelial IQGAP1-dependent junction remodeling during lymphocyte diapedesis. First, following knockdown of IQGAP1, we observed a decrease in polymerized tubulin and MT density near AJ in cells lacking IQGAP1 expression. Although the effect of IQGAP1 knockdown

on EC MT is modest, the effect is confirmed by both biochemical and semi quantitative imaging techniques. Second, APC knockdown elicits similar effects. Third, direct pharmacologic induction of MT depolymerization mimicking the effect of IQGAP1 knockdown inhibited lymphocyte TEM. In each case, lymphocytes were seen to accumulate over the luminal surface of the nascent migration channel in a similar position. Taken together, these three lines of evidence are consistent with a model that IQGAP1 and the junction-associated MT network participates in remodeling of the EC at the interendothelial junction during leukocyte TEM.

Previous work identified that endothelial MT are critical for development of an actin-based docking structure underneath the adherent lymphocyte, that might function to promote lymphocyte adhesion under arterial shear stress and transendothelial migration[4, 42]. IQGAP1 is enriched at intercellular junctions, hence is not anticipated to participate in docking structure formation. Moreover, our data identify no defect in lymphocyte encounters with intercellular junctions. The current observations indicate that functionally, endothelial MT act to enable paracellular diapedesis of the HUVEC monolayer by adherent lymphocytes.

Previously, it has been reported MT loss produced by prolonged ND incubation of EC results in increased neutrophil and monocyte transendothelial migration associated with VE-cadherin loss, actin stress fiber formation and gap formation at interendothelial junctions[32, 33]. However, under the conditions used in these experiments, our immunofluorescence microscopy and flow cytometry results did not identify a change in VE-cadherin cell surface expression

or localization at junctions after brief ND treatment. Further, our data illustrate the structural and functional integrity of the monolayer under condition of IQGAP1 knockdown. The discordant results in TEM assays emphasizes the importance of careful evaluation of monolayer integrity with each manipulation. Similar to our observations, other groups reported intact EC monolayer and decreased monocyte or lymphocyte diapedesis under static conditions after endothelial MT depolymerization[4, 19].

In the current experiments, we report on endothelial MT function during lymphocyte diapedesis under shear stress. Our results confirm a role for endothelial MT to remodel the interendothelial cell junction under these short, physiologic timeframes. Further, our data extends these observations to indicate that the MT array associated with the interendothelial junction is not critically required for separation of the VE-cadherin junction, since neither IQGAP1 knockdown nor ND treatment altered the fraction of lymphocytes associated with nascent migration channel formation. At present the events that occur to facilitate leukocyte transendothelial migration after opening a VE-cadherin gap are unclear. These findings are reminiscent of reports of the effect of CD99 blockade [43, 44]. CD99 appears to function at a point after the development of a gap in VE-cadherin to facilitate completion of the diapedesis step. Interestingly, we identify no change in the total distribution of endothelial CD99 following either IQGAP1 knockdown or ND treatment. Mamdouh et al (2008) showed monocyte and lymphocyte diapedesis is associated with MT dependent-targeted recycling of membrane vesicles in which PECAM-1 but not VE-cadherin are components of

this membrane vesicle compartment[19]. Our data are compatible with a model that IQGAP1 is involved in the recycling of membrane vesicles that might facilitate lymphocyte diapedesis by increasing the membrane surface area or, alternatively, bringing more free junctional molecules such as CD99 to the surface. Future work will be needed to establish such a link. Our observation that VE-cadherin gap formation is not affected by loss of IQGAP1 or MT favors the model that VE-cadherin gap formation is regulated by a separate mechanism.

In our experiments we found that only about a third of lymphocytes that are associated with a VE-cadherin gap are surrounded by a ring of PECAM-1. Previously, it was reported that PECAM-1 is enriched around lymphocytes transmigrating through human microvascular endothelial cells[6]. This discrepancy might be due to the subset of lymphocytes that were analyzed. We depleted naive T cells ($CD45RA^{+}$), that have been shown to express PECAM-1, in order to be able to specifically analyze endothelial PECAM-1 enrichment [45]. Alternatively, it may be that only the fraction of PECAM-1 enriched lymphocytes in our samples are actively undergoing diapedesis. This cannot be distinguished by imaging fixed co-cultures. Nevertheless, IQGAP1 does not seem to be required for PECAM-1 enrichment around lymphocytes.

Our findings suggest a model of upstream regulation of IQGAP1 activation for interendothelial junction remodeling during lymphocyte TEM. IQGAP1 is an effector of calcium signaling, tyrosine kinases and Rho GTP binding proteins[28]. Previous work identified the participation of phosphatidylinositol 3-kinase (PI3K) activity in junction remodeling during

paracellular TEM of lymphocytes[46]. Phosphatidylinsositol-3,4,5-triphosphate, the product of PI3K activity, enables recruitment of PH domain-containing molecules such as GDP/GTP exchange factors for Rho GTP binding proteins. Future work to further define specific intermediates of this pathway will be required.

In summary, our results indicate that endothelial IQGAP1 and MT are involved in remodeling interendothelial junctions to accommodate lymphocyte diapedesis under physiologic shear stress. Conversely, lymphocyte locomotion on the EC monolayer and localization to the interendothelial junctions does not require either endothelial IQGAP1 or cortical intact MT.

Figures

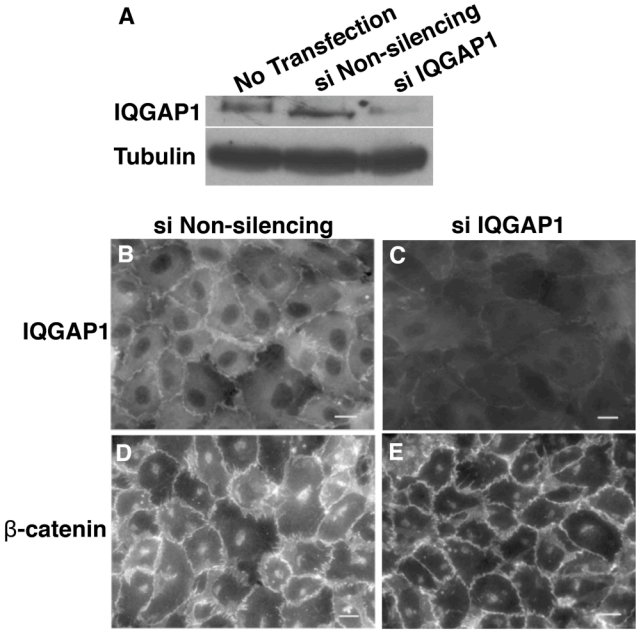


Figure 4-1. Adherens junctions remain intact after IQGAP1 knockdown. A) IQGAP1 expression in HUVEC was inhibited by RNA interference and its expression was measured by western blotting. EC transfected with non-silencing siRNA (B) and (D) or IQGAP1 siRNA (C) and (E) were replated on coverslips and stained for IQGAP1 (B) and (C) or β-catenin (D) and (E). The scale bar is 10 μm. Images are representative of 6 independent experiments.

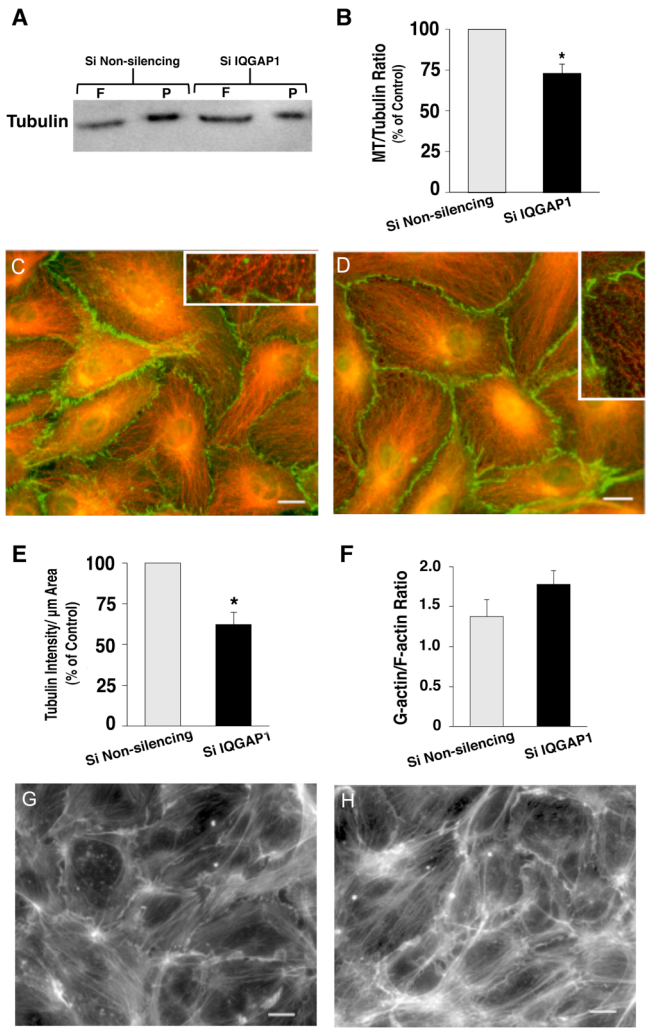


Figure 4-2. IQGAP1 inhibition reduces MT extension to the cell cortex but does not affect the actin cytoskeleton. (A) The fractions of free and polymerized tubulin in IQGAP1 knockdown EC or control cells were separated and measured as described in Methods. (B) The ratio of polymerized tubulin to free tubulin quantified by densitometry in 4 independent experiments (mean±SEM; *=$p<0.05$). MT morphology in EC transfected with non-silencing (C) or IQGAP1 siRNA (D) was assessed by immunofluorescent double staining of MT (red) and VE-cadherin (green), the insets indicate higher magnification of cortical MT adjacent to the interendothelial cell junction. (E) Quantitation of intensity of cortical MT below VE-cadherin stained junctions (3 µm thick regions) in 3 independent experiments, (mean±SEM; *=$p<0.05$). The populations of G-actin and F-actin in EC monolayers transfected with non-silencing or IQGAP1 siRNA

were measured by G-actin/F-actin in vivo assay as described in Methods; (F) illustrates the ratio of G-actin to F-actin quantitated in 3 independent experiments by densitometry (mean±SEM; p=NS). EC transfected with non-silencing siRNA (G) or IQGAP1 siRNA (H) were stained for actin. Images are representative of 3 independent experiments. The scale bar is 10 μm.

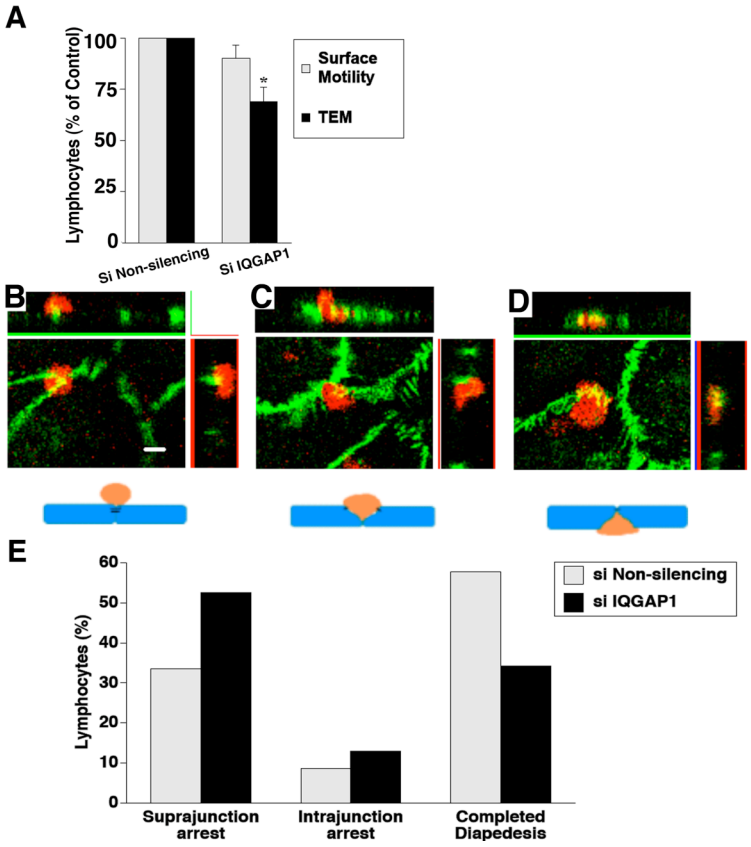


Figure 4-3. Endothelial IQGAP1 knockdown decreases lymphocyte diapedesis. (A) EC were transfected with non-silencing or IQGAP1 siRNA, then lymphocyte TEM was assayed under shear stress as in Methods; (mean±SEM; *=$p<0.05$, 6 independent experiments). EC transfected with non-silencing or IQGAP1 siRNA interacted with pre-labelled lymphocytes, then the co-culture was fixed and stained for VE-cadherin and studied by confocal microscopy as in Methods. Each panel shows stacks of confocal microscopy images in the xy (lower left), xz (top) or yz (right) dimension in a plane through the adherent lymphocyte. Lymphocyte location according to VE-cadherin was scored as (B) suprajunction, (C) intrajunction, or (D) completed diapedesis. The arrow in (C) identifies a lymphocyte protrusion extended under VE-cadherin. The scale bar is 5 µm. (E) The distribution of lymphocytes by location among control (n=240) or IQGAP1 knockdown cells (n=210) in pooled data from 4 independent experiments ($p<0.01$ by Chi square test).

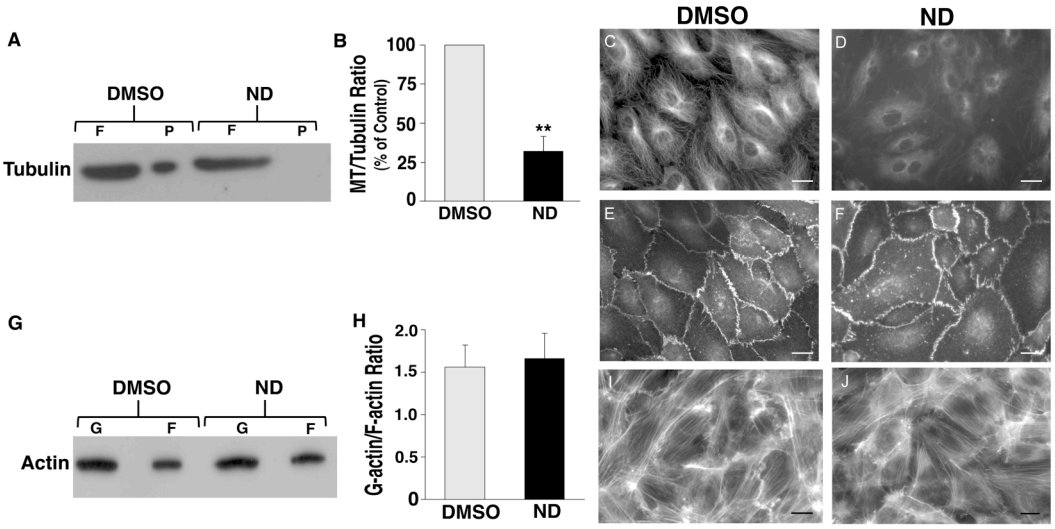


Figure 4-4. Inhibition of cortical MT extension in HUVEC by ND treatment. (A) The population of free and polymerized tubulin in DMSO or ND treated EC was separated and measured as described in Methods. (B) The ratio of polymerized tubulin to free tubulin quantitated by densitometry in 3 independent experiments (mean±SEM; **=$p<0.01$). DMSO or ND treated EC monolayers were fixed, permeabilized and stained for tubulin (C, D) or VE-cadherin (E, F) to assess MT depolymerization and AJ integrity, respectively. (G) The populations of G-actin, G, and F-actin, F, in DMSO or ND treated EC were measured by G-actin/F-actin in vivo assay as described in Methods. (H) The ratio of G-actin to F-actin quantified in 4 independent experiments by densitometry (mean±SEM; p=NS). DMSO (I) or ND (J) treated EC monolayers were stained for actin as described in Methods. Images are representative of four independent experiments. The scale bar is 10 μm.

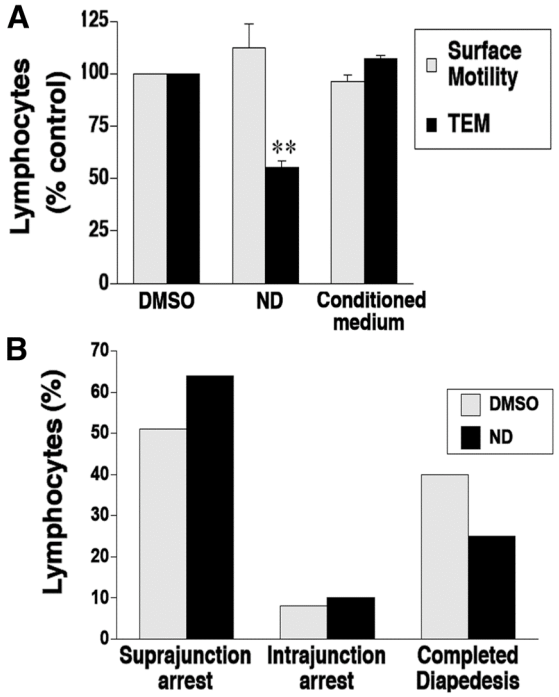
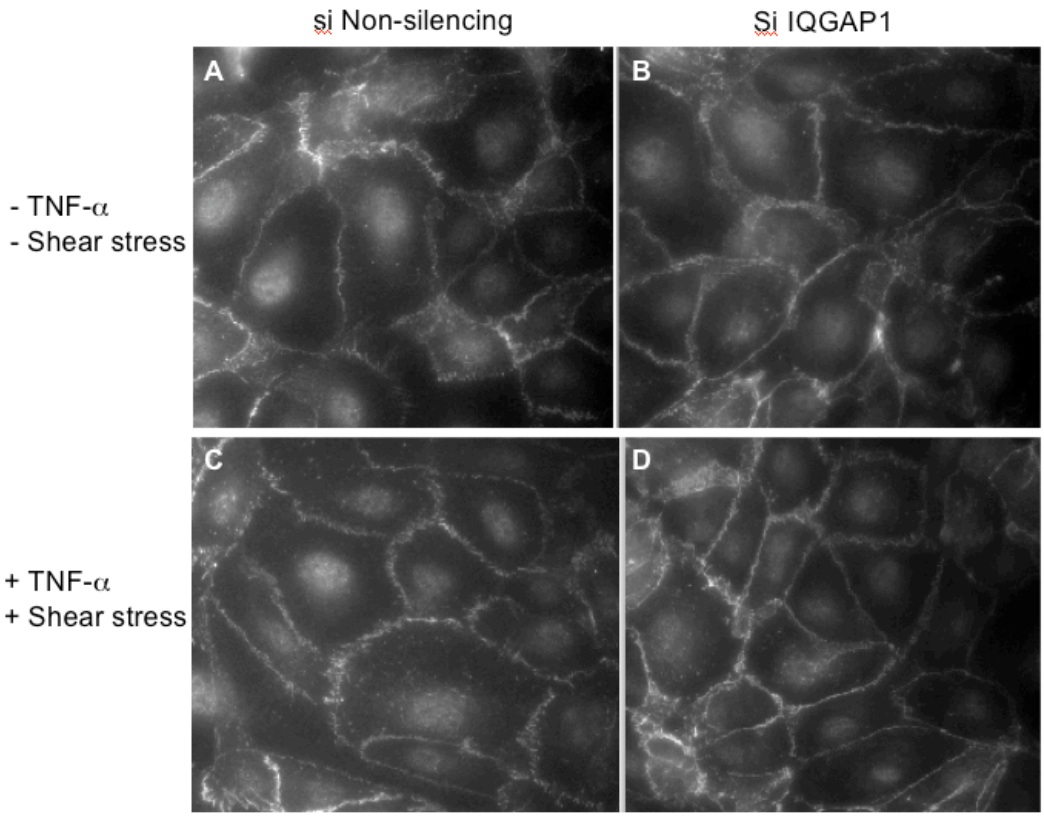
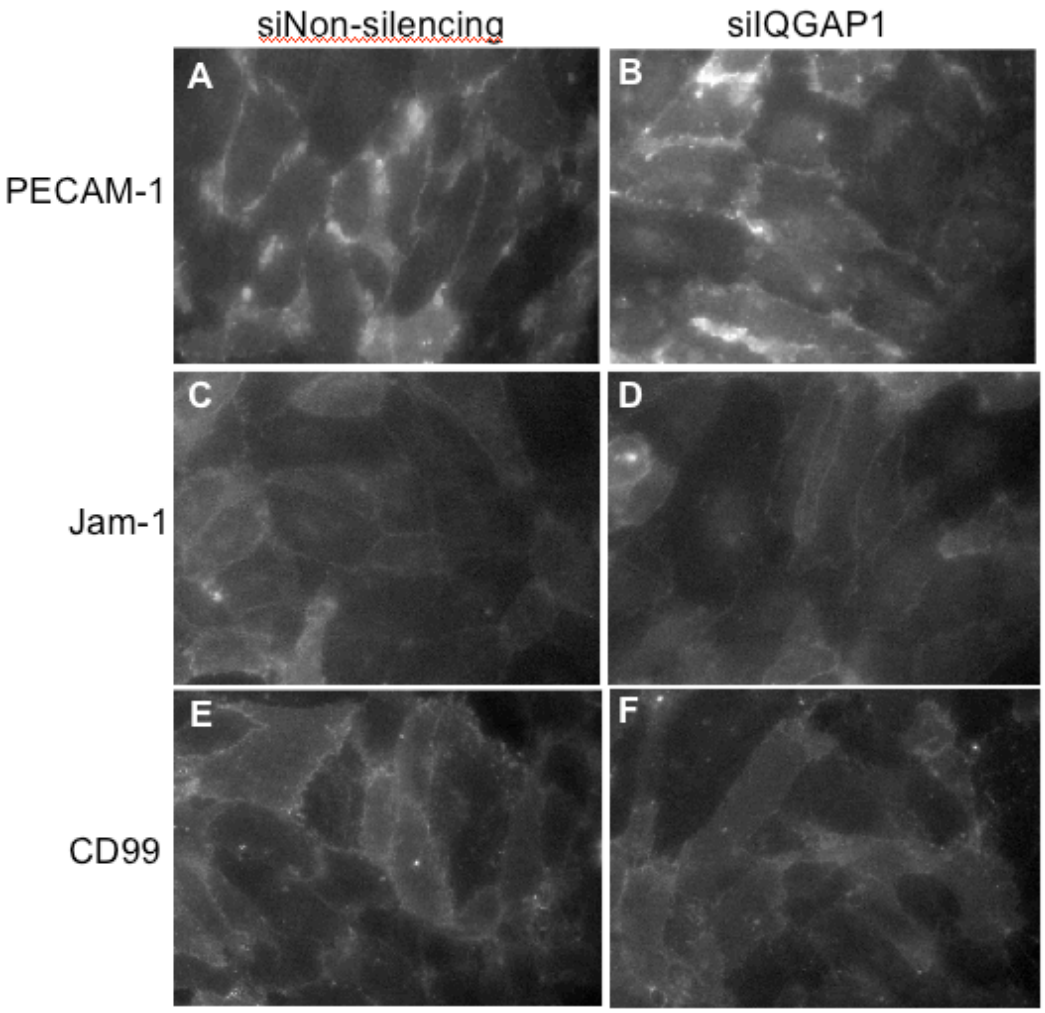


Figure 4-5. Depolymerization of cortical endothelial microtubules decreases lymphocyte TEM without affecting lymphocyte motility. (A) HUVEC were loaded with either DMSO or ND, washed extensively, then lymphocyte TEM was analyzed as in Figure 4-3. To determine the effect of ND residual amounts after washout, HUVEC were left untreated while lymphocytes were resuspended in ND-treated HUVEC conditioned medium as in Methods. The data are mean ± SEM of 5 independent experiments analyzed by video microscopy, ** = $p<0.01$. Lymphocyte TEM across ND or DMSO-treated EC monolayer was assessed by confocal microscopy as in Figure 4-3B to 4-3E. (B) illustrates the distribution of n=267 lymphocytes (control EC) vs. n=341 (ND EC) pooled from 6 independent experiments ($p<0.01$ by Chi square test).

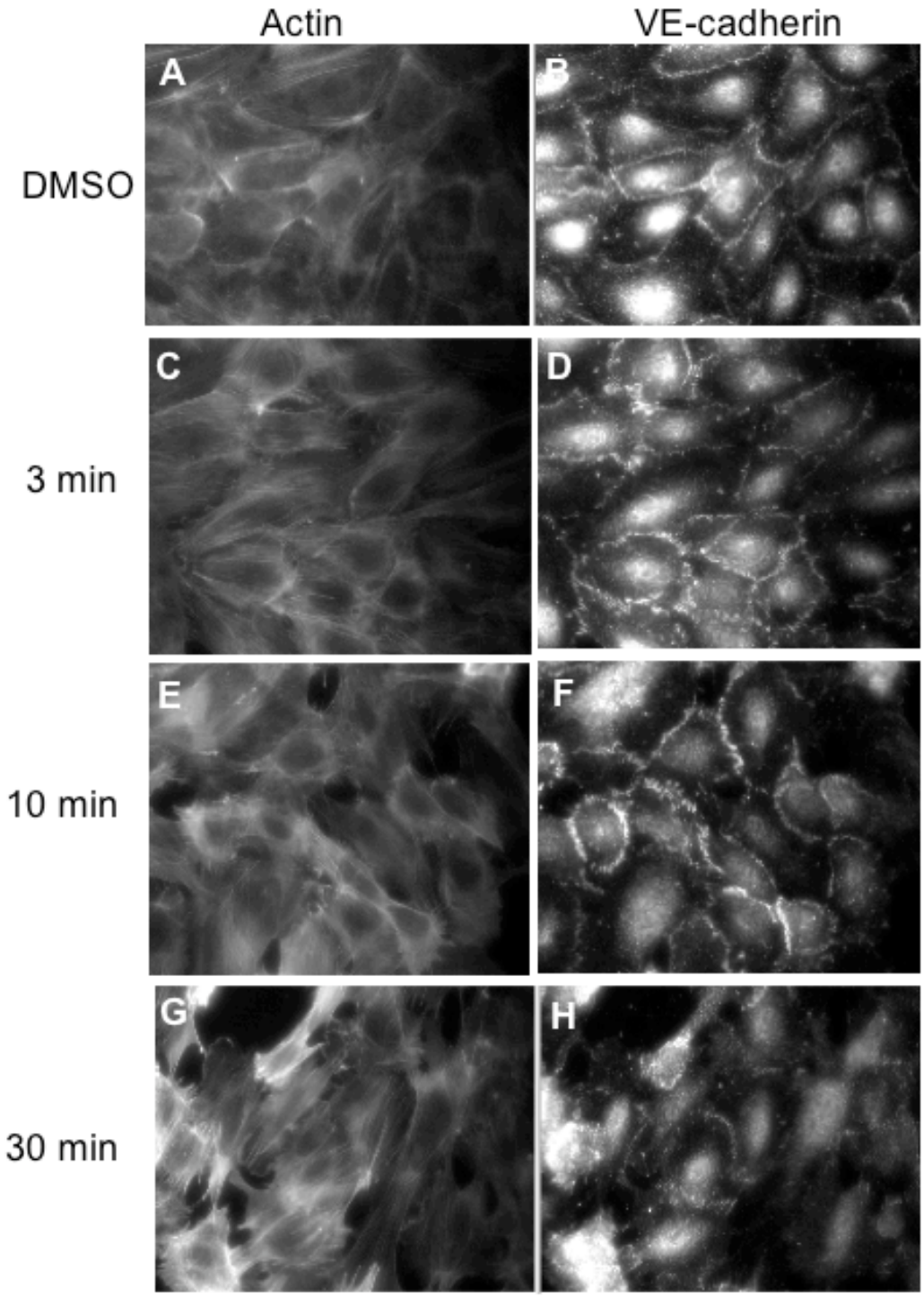
Supplementary Figures



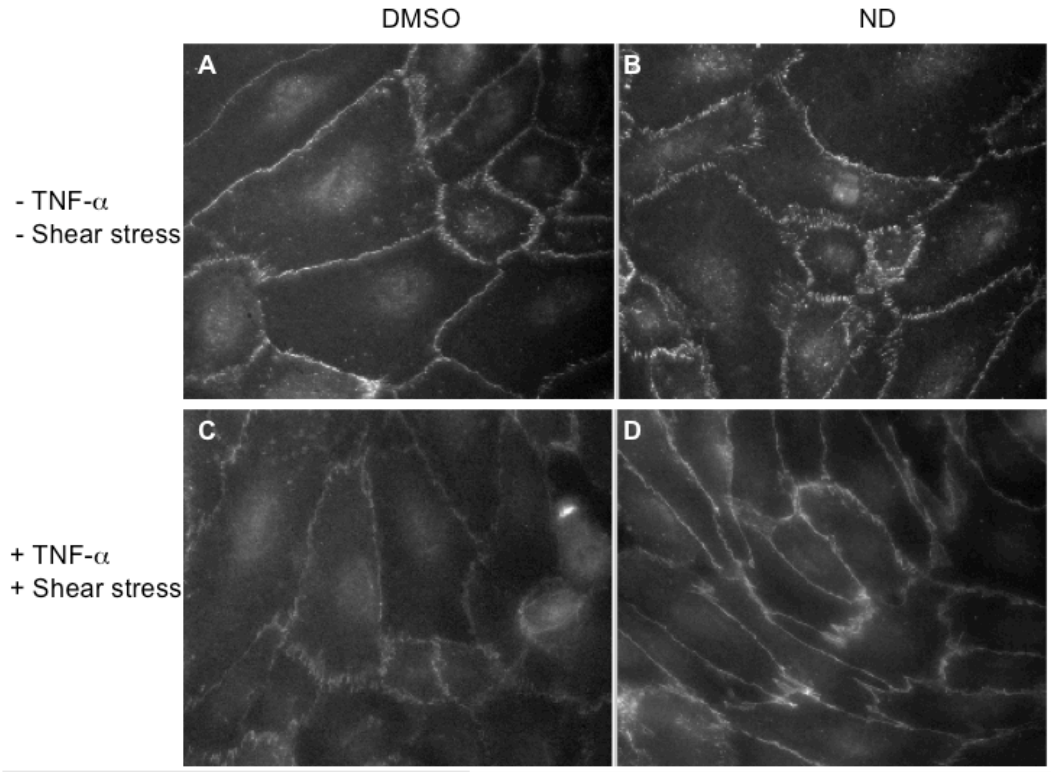
Supplementary figure 4-1. HUVEC transfected with IQGAP1 siRNA (B and D) or non-silencing siRNA (A and C) were seeded on coverslips and stained for VE-cadherin. Photomicrographs of resting monolayers (A and B) or monolayers subjected to pretreatment with TNF-α and shear stress (C and D) are shown. Images are representative of 3 independent experiments.



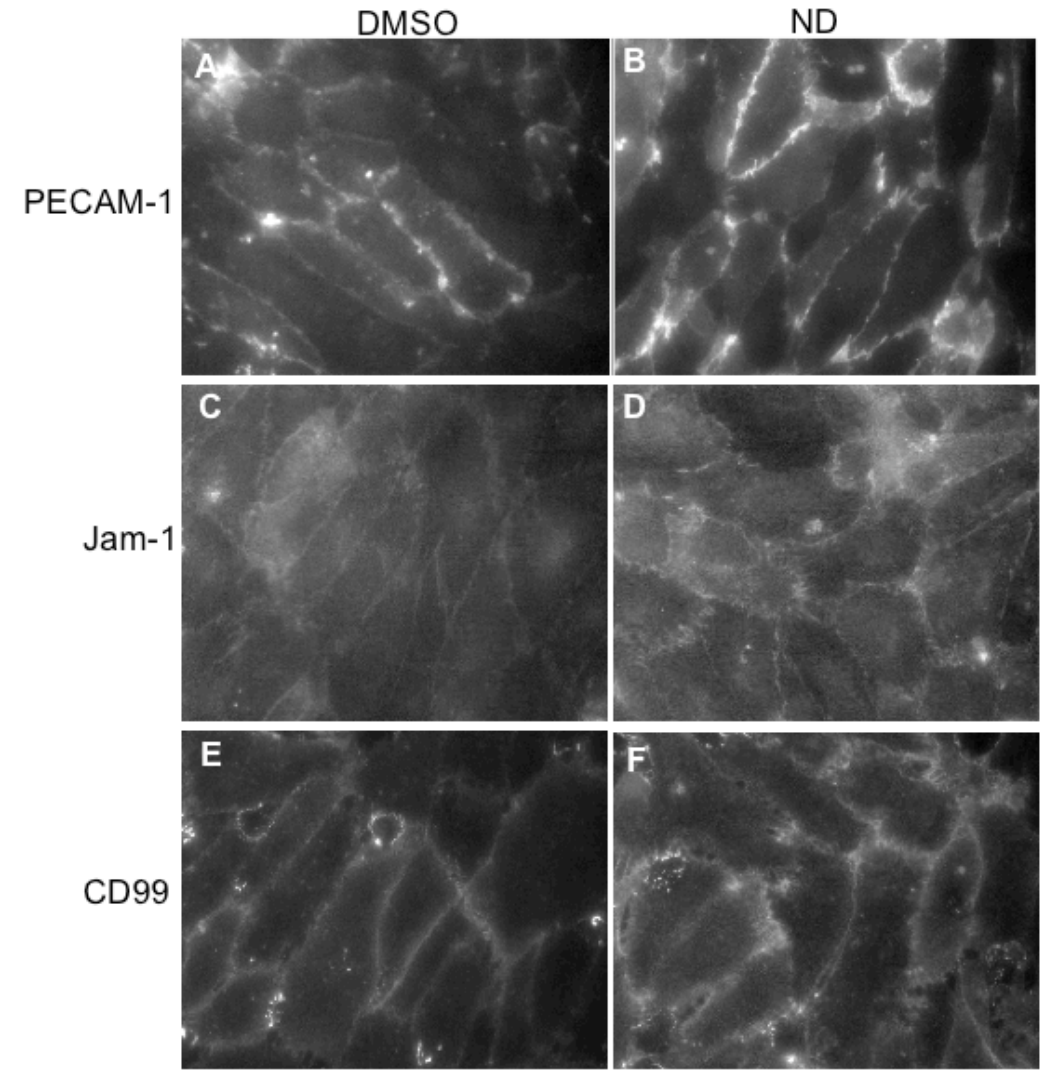
Supplementary figure 4-2. HUVEC transfected with IQGAP1 siRNA (B, D, F) or non-silencing siRNA (A, C, E) were pretreated with TNF-α and subjected to shear stress in the absence of lymphocytes, then stained for PECAM-1 (A and B), JAM-1 (C and D) or CD99 (E and F) as described in methods. Images are representative of 3 independent experiments.



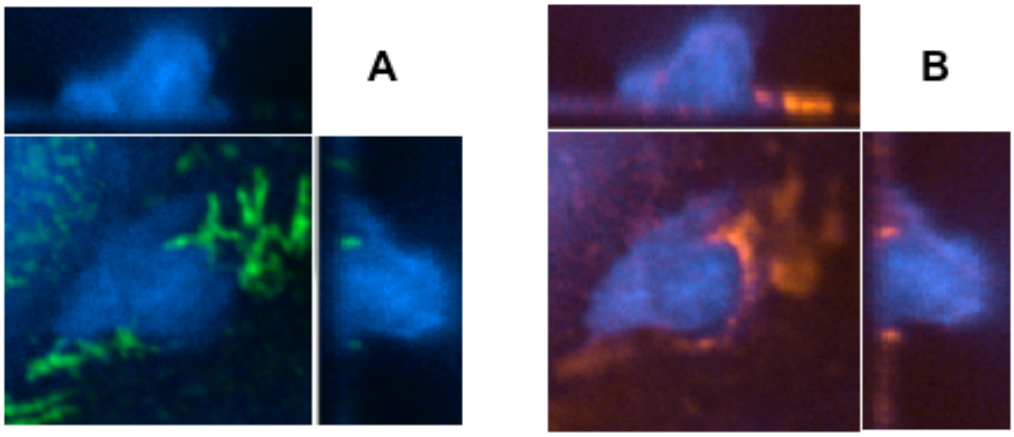
Supplementary figure 4-3. HUVEC seeded on coverslips were treated with ND (C-H) for indicated times or DMSO (30min) (A and B) and double stained for F-actin (A, C, E, G) and VE-cadherin (B, D, F, H). Images are representative of 3 independent experiments



Supplementary figure 4-4. HUVEC seeded on coverslips were treated with ND (B and D) or DMSO (A and C) and stained with VE-cadherin. Photomicrographs of resting monolayers (A and B) or monolayers pretreated with TNF-α and subjected to shear stress (C and D) are shown. Images are representative of 3 independent experiments.



Supplementary figure 4-5. TNF-α treated HUVEC were treated with ND (B, D, F) or DMSO (A, C, E) and subjected to shear stress in the absence of lymphocytes, then stained for PECAM-1 (A and B), JAM-1 (C and D) or CD99 (E and F) as described in methods. Images are representative of 3 independent experiments.



Supplementary figure 4-6. An example of a lymphocyte associated with VE-cadherin gap (more than 2μm length) that is surrounded by a ring of PECAM-1. HUVEC transfected with IQGAP1 siRNA or non-silencing siRNA then treated with TNF-α and SDF-1α were interacted under shear stress with lymphocytes stained by CellTracker Blue. The co-culture was fixed and double stained for VE-cadherin (A, green) and PECAM-1 (B, red) and studied by confocal microscopy. Image is a representative of 3 independent experiments.

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Chapter 5. The role of FGD5, an endothelial-enriched guanine nucleotide exchange factor, in cell-matrix adhesion and survival

Introduction

PI3K integrate a variety of signals from the extracellular environment that modulate endothelial function. For example the principal endothelial growth factor, VEGF, signals through PI3K to Akt to promote EC survival [1]. In parallel, cytoskeletal dynamics are regulated by PI3K through RhoGEFs [2-4]. Activated Rho GTPases are able to participate in many cellular processes such as cell morphology changes, cell division and angiogenesis, through specific effectors [5-8]. In a previous study, we identified IQGAP1, a Rac1/Cdc42 effector, as an important component of inter-endothelial junction remodeling required for efficient lymphocyte diapedesis [9]. In a search for GEFs that would mediate Rac1/Cdc42 activation and consequently IQGAP1 activation during lymphocyte diapedesis we identified FGD5 as an attractive candidate since it is highly enriched in EC [10-13]. This study aimed to characterize EC that are deficient in FGD5 expression.

Rho GTPase members have been implicated in cell survival in a number of survival pathways. One mechanism is nuclear trafficking of Akt [14]. RhoB has been shown to participate in cell survival during angiogenesis via a

mechanism distinct from VEGF [14]. Inhibition of RhoB activity reduces Akt stability and to a greater extent, its trafficking into nucleus [14]. Akt phosphorylates and promotes Forkhead family of transcription factors' (FOXOs) exit from nucleus [15, 16], hence preventing expression of pro-apoptotic proteins such as Fas ligand by FOXOs [15].

Another possible mechanism by which Rho GTPases participate in cell survival is by mediating actin remodeling. For example, TNF treatment of opossum kidney cells reduces serum starvation-induced apoptosis [17]. This was associated with PI3K dependent activation of Rac1 and Cdc42 activity, F-actin remodeling and NF-κB translocation to nucleus [17]. Transfection of cells with dominant negative Cdc42 resulted in reduced NF-κB translocation to nucleus and prevented anti-apoptotic effects of TNF [17].

A third mechanism of Rho GTPases role in cell survival is illustrated by importance of IQGAP1 in VEGF responses [18, 19]. As described in introduction, IQGAP1 induces VEGFR2 complex formation with VE-cadherin and hence mediates VEGF-dependent Akt phosphorylation, induction of cell proliferation, and cell motility [18, 19].

A fourth mechanism of Rho GTPases' contribution to cell survival is by participating in anchorage-mediated signalings. These signalings are essential for survival and are generated by integrins activation upon interaction with matrix [20]. Upon cell adhesion to matrix Cdc42 and Rac1 are transiently activated and mediate cell spreading [21, 22]. The physiological significance of integrin-mediated Rac1 signaling is apparent by embryonic lethality of a β_1 integrin

cytoplasmic mutation defective in activation of FAK [23, 24]. This mutation does not affect adhesion of embryonic fibroblasts but they are defective in transmitting the adhesion signal to cytoplasm through FAK [23]. Cells carrying this mutation were defective in proliferation and had a high rate of apoptosis [24]. Overexpression of Rac1 (downstream of FAK/PI3K) rescued survival defects while dominant negative Rac1 induced similar effects as seen in cells carrying integrin mutation [24]. Importance of Rac1-mediated cell proliferation downstream of PI3K is also shown in EC [25].

Finally, Rho GTPases might contribute to cell survival by activation of PI3K. Rac1 and Cdc42 are implicated upstream of PI3K in some cell events by interaction with p85 regulatory subunit [26-29]. Activated Rac1/Cdc42 might in turn regulate PI3K activity by a positive feedback by direct interaction with the Rho-GAP homology (BH) domain of p85 regulatory subunit of PI3K and induction of PI3K activity in vitro [27, 28]. Evidence for the potential biological significance of Rho GTPases' positive feed-forward effect on PI3K activity is demonstrated by Cdc42 acting upstream of PI3K in glucose uptake of adipocytes in response to insulin [26]. In addition, during phagocytosis, in phagocytic cups, Cdc42 contributes to partial PI3K activation and PIP3 generation [29].

Rho GTPases activity is regulated by Guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs) and Rho guanosine nucleotide dissociation inhibitors (GDIs) [30]. GEFs activate Rho GTPases by facilitating the exchange of GDP to GTP and are the best studied regulators of Rho GTPases [31]. FGD5 is a putative Cdc42 GEF that belongs to the subfamily of FGD [32].

The first member of the FGD sub-family, FGD1, was identified as the gene responsible for Faciogenital Dysplasia (FGD) (Pasteris et al., 1994). Further, FGD1 contains a Dbl homology (DH) domain and a pleksterin homology (PH) domain (found in dbl subfamily of GEFs) and was shown to have Cdc42-GEF activity [33, 34]. All FGD members also contain FYVE domain, which interacts with Phosphatidylinositol 3-phosphate and is involved in membrane trafficking and endosome mediated signaling [35, 36]. PH domains adjacent to DH domain not only are important in recruitment of protein to membrane by binding to PIP3 but also enhance GEF activity [37] .

GEF-Cdc42 activities of most members of FGD family are tested in vivo [33, 34, 38-42]. In addition, FGD4 (frabin) regulates Rac1, indirectly via Cdc42, and may have Cdc42-independent functions [32, 42, 43]. It is not clear how FGD4 mediates Cdc42-independent functions but it is suggested that the FAB domain of FGD4 (other members do not contain this domain) that mediates interaction with F-actin is involved [44].

FGD5, however, is not characterized yet. FGD5 expression was found highly enriched in endothelium of zebrafish, mice and human [10-13], suggesting that FGD5 might be important in EC specific functions such as angiogenesis. In this study, we sought to investigate FGD5 function in HUVECs as a putative regulator of Cdc42 upstream of IQGAP1. Unexpectedly, we found FGD5 expression to be important for EC survival and adhesion. Mechanistically, FGD5 seems to regulate PI3K/Akt pathway upon VEGF stimulation which may explain how FGD5 participates in cell survival.

Materials and Methods

Reagents

M199, HBSS, FBS and ECGS were from Invitrogen (Burlington, ON). Human Tumor necrosis factor-alpha (TNF) was from Cedarlane laboratories (Mississauga, ON). Cycloheximide (CHX) and propidium iodide (PI) were purchased from Sigma-Aldrich (St Louis, MO). VEGF-A was from R&D Systems (Minneapolis, MN). phospho-AKT^{S473}, phospho-FOXO1^{S319}, and FOXO1 were from Cell Signaling Technology (Danvers, MA). Tubulin-a was from Millipore Corporation (Temecula, CA). Goat FGD5 antibody and Akt were from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorophore-conjugated antibodies were from Jackson Immunoresearch (West Grove, PA). Hiperfect, non-silencing siRNA and FGD5 siRNA (sequence:TTGGATgACATGGACCATGAA) were from Qiagen Inc (Mississauga, ON).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously [45]. HUVECs under passage 6 were used for experiments. To detect VEGF-induced signals, transfected cells were starved overnight with M199 containing 1% FBS. Cells were washed twice with M199 and third wash was left on cells for 10 minutes in incubator. M199 containing vehicle control or 20 ng/ml VEGF was added to cells for indicated times and then cells were harvested as described.

RNA interference

To inhibit FGD5 expression, HUVEC were seeded at 50% confluency and transfected twice on consecutive days with either 50 nmol/L siNon-silencing (siNS) or 50 nmol/L siFGD5 using Hiperfect according to the manufacturer's direction. FGD5 expression was optimally inhibited 72 hr after first transfection. FGD5 inhibition was tested by western blotting and real-time PCR.

Real-Time PCR

RNA isolation and real-time PCR was done by Qiu-Xia Zhang. RNA was isolated by RNeasy mini kit (Qiagen, Mississauga, ON). Real time PCR was performed using Fast 7500 thermocycler (Applied Biosytems). Three mg of total RNA were reversed transcribed into cDNA using qScript synthesis kit (Quanta) and the primers were designed using PrimerExpress 3.0 (Applied Biosystems) software and purchased from IDT (Toronto, ON).

Apoptosis

Apoptosis was measured in cells in resting conditions and in response to apoptotic stimuli. To induce apoptosis, cells were incubated with a combination of cycloheximide (CHX, 3 μg/ml) and TNF (10ng/ml) for 3hours (for caspase-3 detection) or overnight (for subdipolid DNA detection) in reduced serum conditions (1% FBS). To measure activated caspase-3, Green Caspase-3 Staining kit from Promokine (Heidelberg, Germany) was used. Resting HUVECs or after

3hr CHX+TNF were incubated with the FITC-conjugated caspase-3 inhibitor, DEVD-FMK, for 30min at 37°C. Cells were trypsinized and combined with the floating cells in the medium. To measure apoptosis in suspended cells, cells were trypsinized first, suspended in complete medium in eppendorf tubes and FITC-caspase-3 inhibitor was added. After several washes cells were analyzed by flow cytometry.

To confirm apoptosis, the fraction of subdiploid DNA content in cells was measured by propidium iodide as described previously [46]. Briefly, trypsinized adherent cells and the floating cells in the medium were pulled and fixed in 70% Ethanol. Cells were resuspended in 500µl PBS+ 500µl 0.2MNaHPO4 (pH 7.8) + 0.005% Triton X-100. Then cells were stained with 40µg/ml PI + 1mg RNase and analyzed by flow cytometry.

Western blot

HUVEC monolayers were washed once with ice cold PBS and then lysed immediately in hot 2X loading buffer (60 mM Tris pH 6.8, 25% glycerol, 2% SDS, 15mM 2-mercaptoethanol and 0.1% bromophenol blue) followed by additional 7 minute boiling at 95°C. Lysates were resolved on SDS-PAGE, and then blotted onto nitrocellulose (Biorad) as described. The membranes were immunoblotted for phosphorylated proteins overnight at 4°C in 5% BSA, TBS-Tween20 blocking solution, and then proteins were visualized using ECL (GE Life Sciences, Baie d'Urfe, PQ). The membranes were stripped using Restore buffer (Thermo Scientific, Rockford, IL), and reprobed for the total protein.

Results

HUVECs express FGD5 and inhibition of expression sensitizes EC to apoptosis

FGD5 expression in several endothelial types such as EC of HUVECs, arteries and lung microvascular EC has been detected by microarray analysis and data base mining and its expression doesn't seem to vary in different types of EC [11]. We confirmed FGD5 expression in resting HUVECs using western-blotting and real-time PCR (Figure 5-1). In order to study FGD5 function in EC, we inhibited its expression using siRNA. No potential Off-target mRNAs for this siRNA was found by the siRNA distributor. Knockdown (kd) of FGD5 was confirmed by western-blotting and real-time PCR (Figure 5-1B and 5-1C). Unexpectedly, we observed FGD5 kd monolayers were less confluent than control cells, transfected with an unrelated siRNA (non-silencing, siNS) (Figure 5-2B versus Figure 5-2A). We were not able to detect FGD5 localization in EC because the available FGD5 antibodies produced immunofluoresnce stainings that did not decrease upon FGD5 kd. This suggests that the antibodies are not able to recognize the non-denatured protein.

To test the importance of FGD5 expression in resistance to apoptosis, cells were subjected to pro-apoptotic stimuli. The induction of apoptosis in EC by combination of TNF and cycloheximide (CHX) (inhibitor of protein synthesis) is well characterized [47, 48]. Thus, we treated control (Figure 5-2C) or FGD5 kd cells (Figure 5-2D) with CHX+TNF for 3 hours and measured one of the characteristic effectors of apoptosis, activated (cleaved) caspase-3. While

16±2.4% of control cells in resting conditions contain activated caspase-3, 28±4.5% of FGD5 kd cell have activated caspase-3 (Figure 5-2E; Mean ±SEM; $p<0.01$). CHX+TNF treatment induced apoptosis in control cells and to a greater extent in FGD5 kd cells (Figure 5-2E; 33±7% versus 54±3.6%; mean ±SEM; $p<0.05$). We also examined the effect of a second pro-apoptotic stress, loss of anchorage on FGD5 kd cells. While control cells were insensitive to anchorage loss (30 min), FGD5 kd cells started going under apoptosis (Figure 5-2E; 19±2.3% versus 43±4.5%; $p<0.01$).

In order to confirm FGD5 kd are more sensitive to CHX+TNF, a late event of apoptosis (DNA fragmentation) was measured by staining DNA with propidium iodide [46]. Since DNA fragmentation requires longer time than activation of caspase-3, we treated transfected cells with CHX+TNF overnight. In resting conditions 17±3% and 24±3% of control and FGD5 kd cells contained subdiploid levels of DNA, respectively (Figure 5-2F; Mean ±SEM; p=not significant). Subdiploid DNA levels increased to 29±3% and 54±4% in control and FGD5 kd cells treated with CHX+TNF overnight, respectively (Figure 5-2F; Mean ±SEM; $p<0.05$). These observations, strongly suggest that FGD5 plays an important role in protection of cells against environmental stress.

FGD5 may regulate EC adhesion to matrix

Rac1 and Cdc42, downstream of phosphorylated FAK and PI3K, are important regulators of integrin-mediated cell spreading, adhesion and signaling, which leads to cell survival [21, 22, 24, 25]. Thus, we hypothesized that FGD5, as

a putative GEF for Cdc42, might mediate cell survival by regulating cell adhesion. To test this hypothesis, cells transfected with siNS or siFGD5 were replated at equal numbers on gelatin (denatured collagen I that absorbs fibronectin from FBS and mediates adhesion via $\alpha_5\beta_1$ and $\alpha_V\beta_3$ integrins [49, 50]) and after 1 hour cells were trypsinized and counted (dead cells were excluded by trypan blue). While 90±3.7% of control cells adhered to fibronectin, only 64±3.9% of FGD5 kd cells adhered to matrix (Figure 5-3A versus 5-3B, quantified in Figure 5-3C; Mean ±SEM ; $p<0.05$). To confirm these data and determine FGD5 kd ability to specifically bind to fibronectin, cell adhesion to fibronectin was monitored by measuring changes in monolayer resistance after seeding using ECIS (Electric Cell Substrate Impedance Sensing) (Figure 5-3D). In control cells monolayer resistance increased by rate of 4.6±0.57% Ohm/min while increase in FGD5 kd monolayer resistance was 2.4 Ohm/min (Figure 5-3E; Mean ±SEM; $p<0.05$). Although these results suggest a role for FGD5 in cell adhesion, considering the observation that FGD5 kd cells start undergoing apoptosis when in suspension, reduction of adhered cells might be due to an indirect effect (reduced population of healthy cells). Thus, determining FGD5 function in adhesion was not pursued further.

Inhibition of FGD5 expression reduces EC response to VEGF stimulation

As explained, Rho GTPases can contribute to PI3K/Akt survival pathway by several mechanisms including regulation of VEGFR2/VE-cadherin/PI3K

complex [18], activation of PI3K [26-29], and nuclear transport of Akt [14]. We hypothesized that FGD5 kd increases cell sensitivity to apoptotic stimuli by impairing the PI3K/Akt pathway. We looked at the ability of FGD5 kd cells to stimulate PI3K pathway upon VEGF stimulation. In accordance with previous publications in transfected control starved cells, VEGF treatment induced robust Akt phosphorylation [1], however, FGD5 kd cells were greatly defective in inducing Akt phosphorylation (Figure 5-4A; quantified in Figure 5-4B; $P<0.01$). One mechanism by which Akt prevents apoptosis is by phosphorylation and prevention of FOXOs translocation into nucleus [15]. Similar to Akt, VEGF stimulation resulted in phosphorylation of FOXO1 in control cells, however, FGD5 kd cells were not able to induce FOXO1 phosphorylation higher than basal levels, upon VEGF stimulation (Figure 5-4A; quantified in Figure 5-4C; $P<0.05$).

To identify the blockade point in VEGF signaling pathway of FGD5 kd cells, we tested VEGF stimulation of mitogen activated protein (MAP) kinase, extracellular signal regulated-kinase (ERK). VEGF stimulation significantly increased ERK1/2 phosphorylation within 5 minutes (Figure 5-4A; $P<0.05$) as expected [51]. Interestingly, FGD5 kd cells also had a robust ERK phosphorylation (Figure 5-4A; quantified in Figure 5-4D; $P<0.05$), suggesting that FGD5 expression is not required for MAP kinase VEGF-mediated activation and FGD5 defect is specific for PI3K activation.

Discussion

Rho GTPases are implicated in regulation of a number of EC processes in response to interaction with matrix, neighbor cell or stimuli such as VEGF [22, 24, 52-54]. Rho GTPases are also shown to participate in leukocyte TEM [55-58] and our results also implicate Rac1/Cdc42 in lymphocyte diapedesis via their effector IQGAP1 [9]. We hypothesized that FGD5 mediates Rac1/Cdc42/IQGAP1 activation during leukocyte TEM. However, we were not able to study the role of FGD5 in lymphocyte TEM because EC lacking FGD5 where defective in mediating a confluent monolayer, a prerequisite for leukocyte transendothelial migration (TEM) assays. In the present study, we have shown that FGD5 inhibition sensitizes cells to apoptosis, delays adhesion to fibronectin, and prevents Akt and FOXO1 phosphorylation, but not MAPK phosphorylation, upon VEGF stimulation.

To characterize FGD5 kd cells, we exposed EC to two apoptotic stimuli, combined TNF and CHX, and also short time anchorage removal. Both stresses resulted in significant induction of apoptosis in FGD5 deficient cells. Further, FGD5 kd cells are not able to activate Akt in response to VEGF. This suggests that FGD5 depletion affects PI3K/Akt mediated survival in EC.

There are several potential mechanisms that might explain FGD5 importance in cell survival. VEGFR2 requires interaction with VE-cadherin and PI3K to mediate PI3K signaling [59] and VEGFR2 signaling to MAPK also requires interaction of VE-cadherin with VEGFR2 [60]. We did not detect any defects in MAPK signaling upon VEGF stimulation. This indicates that FGD5

does not participate in VGFR2 activation and VE-cadherin/VEGFR2/PI3K complex formation upstream of Rac1/Cdc42/IQGAP1 [18]. Another potential mechanism of FGD5 participation in PI3K/Akt signaling is via activation of Rho GTPases (RhoB) that regulate stability and nuclear trafficking of Akt [14]. However, We have not detected any significant change in total Akt levels after FGD5 kd, suggesting that Akt stability is not impaired. Another mechanism of FGD5 involvement in PI3K/Akt regulation is via activation of Rho GTPases' that further activate PI3K in a positive feed-forward mechanism. For instance Cdc42 activation of PI3K is involved in regulatation of glucose uptake in adipocytes in response to insulin [26] and phagocytosis, in phagocytic cups [29]. Thus, FGD5 might activate Cdc42 during VEGF stimulation, which further enhances PI3K activity and consequently induces Akt phosphorylation. Another mechanism for contribution of Rho GTPases to PI3K/Akt and cell survival is downstream of FAK phosphorylation upon cell adhesion to matrix [24]. Our results implicate a potential role for FGD5 in matrix adhesion. However, the FGD5 kd non-adherent cells might be a population that have started going under apoptosis during harvest. Further, FGD5 inhibition does not affect FAK expression or phosphorylation levels in resting conditions (data not shown). FGD5 expression might regulate FAK phosphorylation and consequent PI3K/Akt activation, upon a stimulus such as interaction with matrix and needs further investigation.

Whether FGD5 is a specific GEF for Cdc42 or it can activate other Rho GTPases needs further investigation. To test whether FGD5 contributes to PI3K activitation, PI3K activity needs to be directly measured by detection of PIP3

generation in control cells versus FGD5 knockdown cells. The technical difficulties we are faced in these experiments are lack of antibodies that would work for immunoprecipitation and immunofluorescent studies. In addition, in some experiments, we have difficulty in detecting FGD5 bands in our control samples. Identifying more siRNA sequences that are able to mediate FGD5 inhibition and also performing rescue experiments will help confirming our data. In rescue experiments, cells will be transfected with an siRNA sequence targeted against the 3′ untranslated region (UTR) of FGD5. Then cells will be transfected with FGD5-GFP vectors to express FGD5.

In summary, this study has started identifying FGD5 functions in endothelial cells. Identification of the Rho GTPases functions that are regulated by FGD5 is valuable for designing therapeutic drugs that will specifically target EC functions.

Figures

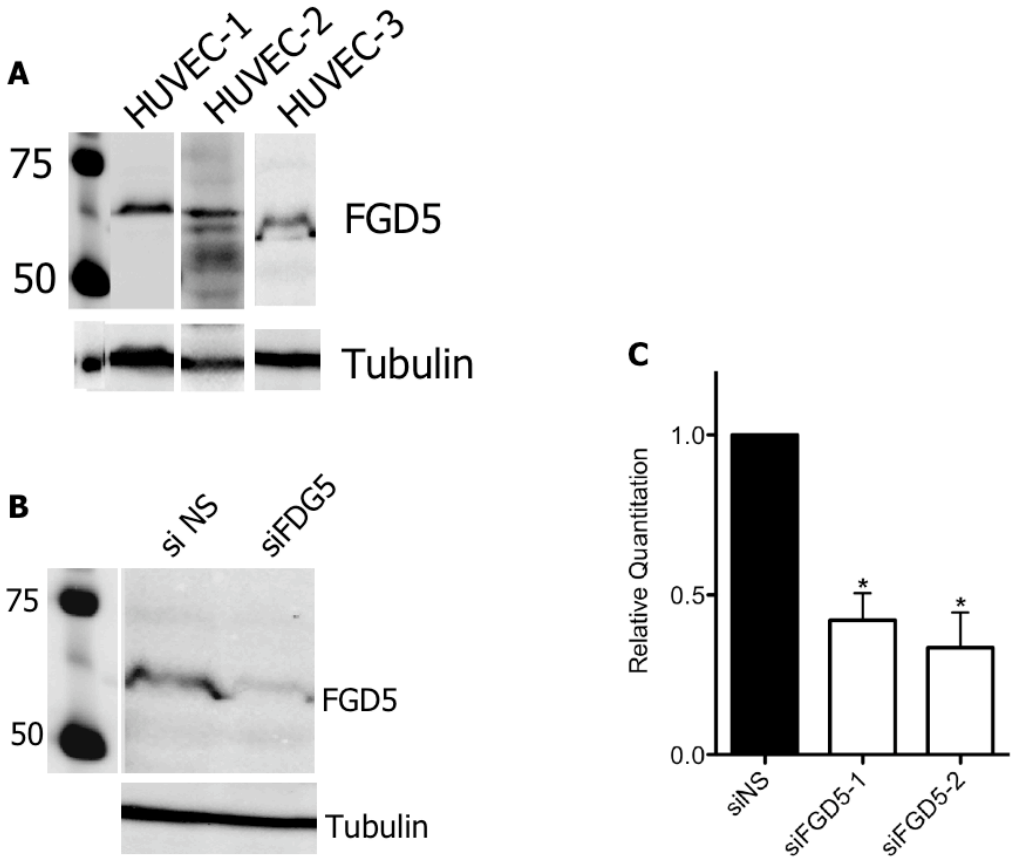

Figure 5-1. FGD5 is expressed in HUVECs and its expression is effectively inhibited by siRNA. A) FGD5 expression in cell lysates of HUVECs from different donors determined by Western blotting. B) HUVEC were transfected with siRNA against non-silencing (siNS) or FGD5 (siFGD5) and expression levels were detected by Western blotting (C) and real-time PCR (D, performed by Qiu-Xia Zhang). (A) and (B) images are representative of 6 independent experiments. (C) Data are mean±SEM of three independent experiments, normalized to siNS cDNA amounts, * indicates $p < 0.05$.

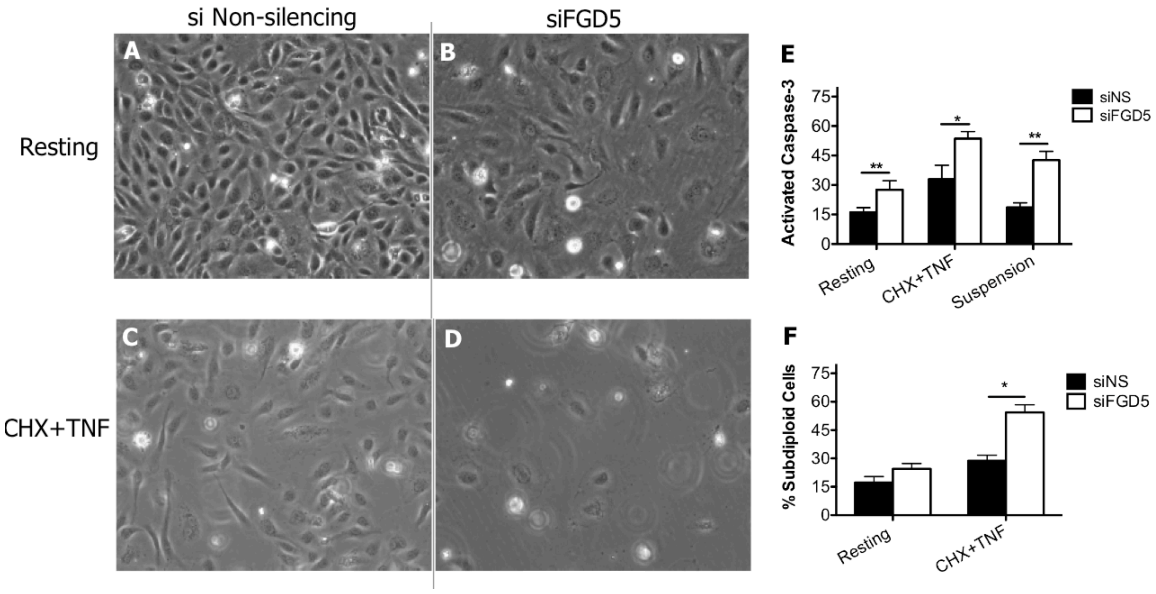

Figure 5-2. FGD5 expression protects cells from apoptosis. Phase contrast images of cells transfected with siNS (A and C) or siFGD5 (B and D) in resting conditions (A and B) and in CHX+TNF treated condition (C and D). E) Apoptosis was measured by flow cytometry detection of fluorescently labeled activated caspase-3 in resting adherent cells, cells treated with CHX+ TNF, and cells in suspension (30 min). F) Transfected cells in complete medium or in CHX+ TNF (overnight) were harvested and subdiploid DNA content was measured as described in methods. Data are means ± SEM of 4 independent experiments. * and ** indicate p values of less than 0.05 and 0.01, respectively in the indicated groups.

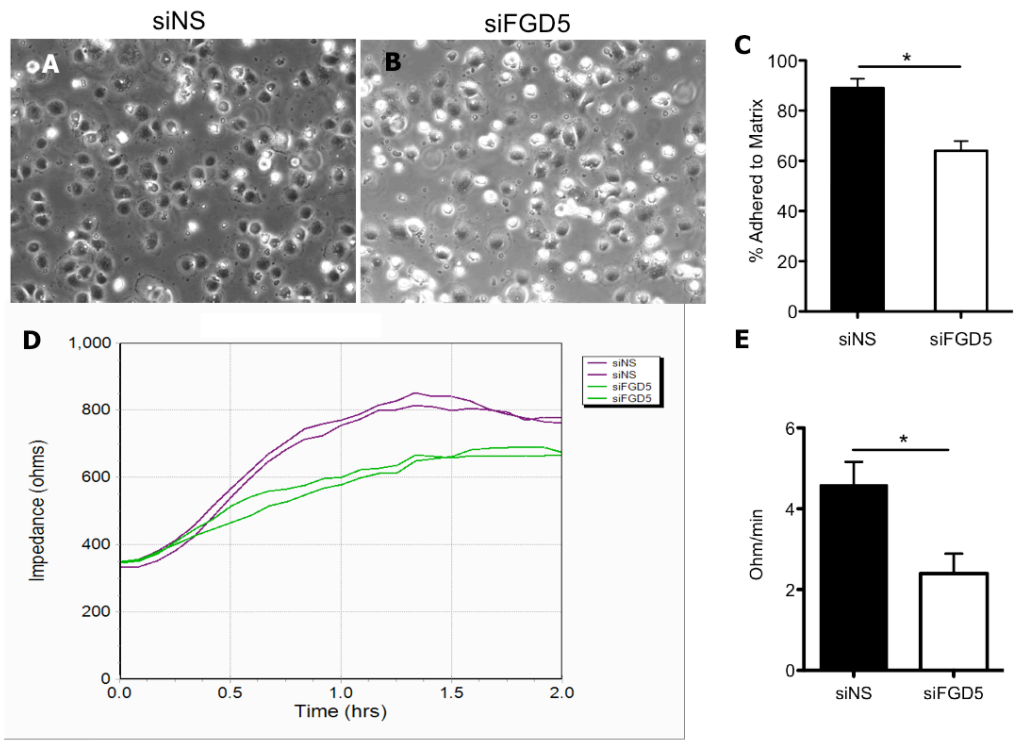

Figure 5-3. FGD5 expression is required for efficient adhesion of EC to fibronectin Matrix. siNS (A) or siFGD5 (B) transfected HUVEC were seeded at equal density on gelatin coated dishes. After 30min cells were trypsinized and counted. C) Mean fraction of cells in each group adhered to matrix. Data are mean±SEM of three independent experiments *= $p<0.05$. D) A representative experiment illustrating the tempo of cell adhesion in the first 2 hours after seeding cells transfected with siFGD5 or siNS in duplicate wells. Adhesion is quantitated by electrical impedance measurements. E) Quantification of EC adhesion in control versus FGD5 knockdown among 4 independent experiments. Data are mean±SEM, *=$p<0.05$.

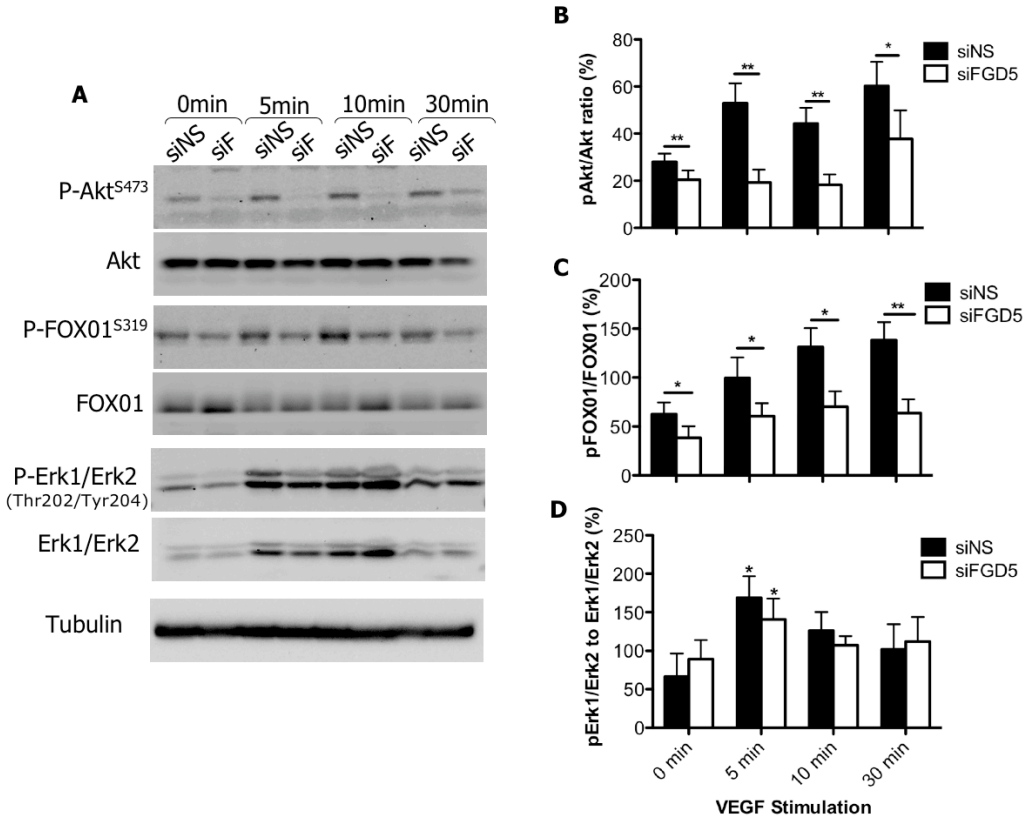

Figure 5-4. Akt but not MAPK signaling downstream of VEGF requires expression of FGD5. Serum starved transfected HUVECs (siNS or siFGD5) were stimulated with 20ng/ml VEGF for the indicated times and the levels of phosphorylated Akt and FOXO1 was determined by immunoblotting. Membranes were stripped and reprobed for the total protein. A) Micrographs are representatives of 5 and 4 independent experiments for Akt and MAPK, respectively. B), C) and D) are quantification of ratio of indicated phosphorylated protein to total protein by densitometry. Data are mean±SEM of at least 4 independent experiments. * and ** indicates $p<0.01$ and $p<0.05$, respectively.

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Chapter 6. General Discussion

Endothelium of vasculature actively participate in inflammatory responses by presenting cues to circulating leukocytes to capture them and further regulate their transmigration to underlying organ. The mechanisms that regulate EC inter-endothelial junction remodeling during leukocyte diapedesis has turned out to be very complex and the exact mechanisms for leukocyte passage at interendothelial junctions are not completely understood. In this project we sought to better understand the role of cytoskeleton and its associated proteins in remodeling of inter-endothelial junctions required for lymphocyte diapedesis. The principle findings of this thesis are as follows:

1. Endothelial F-actin remodeling, PI3K activity and RhoA activity are required for efficient lymphocyte TEM.
2. Blockade of endothelial PI3K does not affect lymphocyte motility, localization to interendothelial junctions, and opening of VE-cadherin, but lymphocytes are not able to enter the migration channel.
3. Endothelial PI3K class IA participates in lymphocyte diapedesis via activation of p110α catalytic isoform.
4. ICAM-1 cross-linking under low shear stress generates PI3K activity.
5. Depletion of Rac1/Cdc42 effector, IQGAP1, in EC impairs MT extension to interendothelial junctions and prevents lymphocyte diapedesis, but not lymphocyte motility and localization to interendothelial junctions.

6. Inhibition of MT extension by MT depolymerizing agent nocodazole, prevents leukocyte diapedesis, but not lymphocyte motility and localization to interendothelial junctions.
7. Inhibition of endothelial enriched putative RhoGEF, FGD5, sensitizes EC to apoptotic stimuli and impairs VEGF-mediated Akt activation.

We studied the role of endothelial PI3K activity and its catalytic isoforms activity during lymphocyte TEM. Pan inhibition of PI3K did not affect lymphocytes motility on EC surface but greatly reduced lymphocyte diapedesis. The observation that lymphocytes were associated with a higher number of VE-cadherin openings suggest that PI3K activity mediates leukocyte passage in a stage after VE-cadherin opening and also VE-cadherin opening does not require PI3K activity.

VE-cadherin loss during leukocyte diapedesis might be regulated by more than pathway. Several studies have associated the specific and transient loss of VE-cadherin during diapedesis with phosphorylation of AJ components after ICAM-1 cross-linking [1, 2]. However, Ridley and colleagues recently showed that PI3KIA p110α isoform mediates TNF-induced permeability and leukocyte TEM via activation of tyrosine kinase Pyk2, which mediates VE-cadherin phosphorylation and possibly VE-cadherin loss [3]. This mode of regulation argues for a global change in EC inter-endothelial junction upon TNF stimulation versus a specific and transient loss of VE-cadherin mediated by leukocyte interaction with EC, as reported previously [4, 5]. These observations suggest that PI3K- p110α activity downstream of TNF-induced signaling and EC

permeability, although important, is not the only mechanism for regulation of specific AJ remodeling during leukocyte diapedesis. Consistently, our results also show p110α as the dominant catalytic isoform during leukocyte TEM. However, our results favors the specific PI3K activation after shear stress combined with ICAM-1 cross-linking, which would lead to specific and transient changes in inter-endothelial junctions to facilitate leukocyte diapedesis.

ICAM-1 mediated interactions are important for leukocyte TEM. Impaired interaction of ICAM-1 with its ligand in β_2 integrin knockout mice, a mouse model for LAD I disease, reduces neutrophil and T cell extravasation to skin lesions [6]. Further, cytoplasmic domain of ICAM-1 is essential for generation of signaling events that are associated with increased leukocyte TEM [1, 2, 7-9]. Src tyrosine kinase activation participates in neutrophil TEM [2, 9]. Another possible tyrosine kinase that might mediate PI3K activation is FAK. FAK activation during leukocyte interaction and after ICAM-1 cross-linking has been reported previously [7, 10, 11]. Also, consistent with previous observations, our results show shear stress actively participates in leukocyte-mediated endothelial signaling and consequently leukocyte TEM [12]. Hence, we propose ICAM-1 cross-linking under shear stress might induce PI3KIA activity via activation of a cytoplasmic tyrosine kinase. Activated PI3K generates PIP3, which recruits RhoGEFs and induces activation of Rho GTPases RhoA and Rac1/Cdc42 [13-15]. Activated Rho GTPases in turn participate in remodeling of EC junctions and leukocyte diapedesis (Figure 6-1).

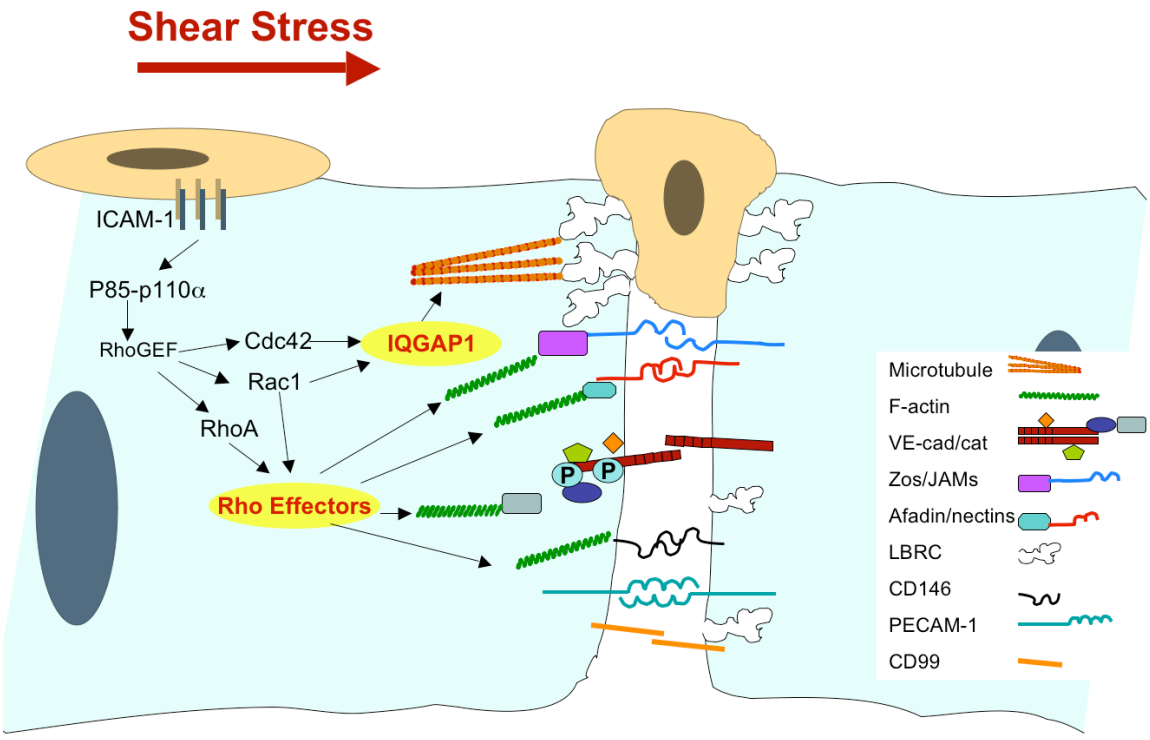

Figure 6-1. Proposed model of PI3K-dependent lymphocyte TEM. Upon lymphocyte firm adhesion to endothelium under shear stress, ICAM-1 ligation induces PI3KIA activity (mediated by p110α) in EC. PI3K product PIP3 mediates Rho GTPase activation via recruitment and activation of RhoGEFs to membrane. The Rac1/Cdc42 effector, IQGAP1, participates in lymphocyte diapedesis by capturing MT to interendothelial junctions. MT regulate leukocyte diapedesis via regulation of targeted recycling of LBRC around the migration channel. In addition, Rac1/Cdc42 and RhoA might contribute to lymphocyte diapedesis by regulation of actin remodeling and consequently destabilization of interendothelial junction components.

There are a number of lines of evidences for participation of Rho GTPases in regulation of AJ remodeling and leukocyte TEM [3, 7, 8, 16]. First, RhoA activation after ICAM-1 cross-linking has been observed, previously [7, 8]. Our results also show involvement of Rho kinase in lymphocyte diapedesis. Since the effect of global PI3K inhibition on lymphocyte diapedesis is greater than Rho kinase inhibition we propose that other Rho GTPases might be involved in regulation of leukocyte diapedesis downstream of PI3K activation. Second, Rac1 activation and generation of H_2O_2 downstream of VCAM-1 cross-linking is shown to mediate transmigration of immortalized monocytes across HUVECs [16]. However, in our system cross-linking of VCAM-1 did not generate PI3K activity, suggesting that Rac1 activation might be regulated differently in different TEM models. Third, combined inhibition of Rac1 and Pyk2 reduces leukocyte diapedesis [3]. Our results also suggest that Rac1/Cdc42 participate in leukocyte diapedesis by regulation of the activity of their effector, IQGAP1, which mediates capturing of MT to EC junctions and lymphocyte diapedesis. Consistent with our results, IQGAP1 linkage to MT via CLIP-170 and its importance in stabilization of MT tips is shown by other investigators [17-19]. In addition, Cdc42 upstream of IQGAP1 is shown to be involved in MT stabilization [20].

One possible mechanism, by which PI3K might regulate leukocyte diapedesis, is via regulation of LBRC compartments, indirectly. This is an attractive hypothesis because in our hands VE-cadherin opening does not require PI3K activity. Similarly, VE-cadherin opening and the targeted membrane insertion from the LBRC at the migration channel were two distinct pathways

[21]. This hypothesis is based on two observations. First, targeted insertion of LBRC and monocyte diapedesis was greatly decreased by broad tyrosine kinase and Src kinase inhibition [22]. Second, PI3K might regulate IQGAP1 activation via regulation of Rac1/Cdc42. IQGAP1 in turn might regulate targeted recycling of LBRC via capturing of MT at interendothelial junctions. Interestingly, IQGAP1 was shown to be able to directly bind to PIP3 [23]. However, it is not clear whether IQGAP1 can act as an effector of PIP3 independent of Rac1/Cdc42 activation. Determining the importance of PI3K, Rac1/Cdc42 and IQGAP1 in targeted insertion of LBRC will clarify the mechanisms of leukocyte diapedesis to a great extent.

Another possible mechanism by which PI3K activity might regulate lymphocyte diapedesis is by regulation of other adhesion molecules in inter-endothelial junctions such as PVR of nectin family and CD146. PVR is implicated in leukocyte diapedesis via interaction with its ligand, DNAM-1 [24]. Nectins have been shown to interact with other components of EC junctions and also actin binding protein, profilin, via their cytoplasmic component, afadin [25-28]. Also, CD146 is implicated in leukocyte diapedesis and shown to interact with actin cytoskeleton [29, 30]. Thus, future experiments will focus on the effect of p110 inhibition of expression or activity on localization of these adhesion molecules in TNF activated HUVECs. It will not be informative to examine distribution of CD146 during leukocyte diapedesis, since lymphocytes also express CD146 [31]. CD146 distribution can be studied by expression of fluorescent-tagged CD146. PVR seems to be dimly expressed in leukocytes, thus

specific changes in PVR localization during leukocyte diapedesis might be detectable via confocal imaging [24].

Involvement of IQGAP1 in lymphocyte diapedesis suggest that actin and microtubule cooperate to regulate leukocyte diapedesis. During cell motility IQGAP1 and APC form a complex with Rac1 and Cdc42, which in addition to stabilization of MT, mediate actin enrichment at the site of leading edge [32, 33]. IQGAP1 regulates actin enrichment by activation of N-WASP along with Cdc42 in a synergistic manner [34]. IQGAP1 also participates in actin enrichment around phagocytic cups by localizing Rho-induced Dia1 to phagocytic cups, which in turn induces formation of unbranched actin [33]. Our results indicate that the ratio of G-actin to F-actin is not changed in IQGAP1 knockdown EC in resting conditions. However, in response to stimuli such as leukocyte interaction, IQGAP1 might induce actin enrichment around migration channel. Consistent with our results, the importance of actin remodeling during leukocyte diapedesis and actin enrichment at the migration channel has been reported by several other investigators [8, 35, 36]. Endothelial actin enrichment at the migration channel is proposed to generate motile force necessary for leukocyte passage but the exact mechanism(s) that regulate actin enrichment and its interplay with other events at the inter-endothelial junctions such as loss of VE-cadherin is not clear.

In this project we have not shown direct requirement of PI3K activity and Rac1/Cdc42 activity upstream of IQGAP1 and is beyond the scope of this thesis. Establishing this pathway requires the following experiments. First, testing for activation of Rac1 and Cdc42 in TNF activated p110α knocked down HUVECs

that are cross-linked with ICAM-1 under shear stress will determine whether these GTPases' activities require PI3K-p110α activity. Second, F-actin visualization by immunofluorescence staining of p110α, and IQGAP1 knocked down HUVECs cross-linked with ICAM-1 under shear stress will give information about requirement of these molecules for F-actin remodeling during leukocyte TEM. Finally, to pinpoint IQGAP1 dynamics and investigate upstream regulators of IQGAP1, expression of a fluorescent-tagged IQGAP1 will help track IQGAP1 distribution during leukocyte TEM and identifying whether IQGAP1 localization is sensitive to blockade of candidate upstream molecules.

IQGAP1 can interact with a great number of signaling molecules and cytoskeleton-associating molecules [17, 23, 32, 37-41]. Identifying specific regulators and exact function of IQGAP1 in a given cell type, will enable us to gain more specificity in designing therapeutic drugs. In a search for specific Rho GTPases activators upstream of IQGAP1, we identified FGD5 as a putative Cdc42 GEF that is highly expressed in EC [42-44]. However, unexpectedly, inhibition of expression of FGD5 by RNAi resulted in increased sensitivity to apopototic stimuli and cells did not respond to VEGF stimulation, apparent by lack of Akt and FOXO-1 phosphorylation. IQGAP1 has also been implicated in VEGF signaling by binding to VEGFR2 and mediating VE-cadherin/VEGFR2 complex and angiogenesis in vitro and in vivo [45-47]. FGD5 project is work in progress and requires confirmation with another siRNA sequence. One possibility for the observed phenotype of FGD5 knockdown is FGD5 might be a RhoGEF for more than one Rho GTPase. This hypothesis can be tested by overexpression

of FGD5 in an immortalized cell line and testing activity of Rho GTPases, RhoA, Rac1 and Cdc42. Another possibility is that FGD5 is a specific GEF for Cdc42 and lack of activity of Cdc42 by FGD5 knockdown impairs activation of other Rho GTPases [48, 49]. Although depletion of Cdc42 in mice is embryonic lethal, the isolated embryonic stem cells are not defective in proliferation and are not apoptotic in resting conditions [50]. Further, they develop round shapes and are defective in developing stress fibers, lamellipodia and filopodia, consistent with possibility of impaired activity of other Rho GTPases [50]. These observations suggest that Cdc42 is essential for remodeling of actin cytoskeleton. Upon TNF treatment, remodeling of actin cytoskeleton downstream of PI3K dependent Cdc42 activation is required for anti-apoptotic effects of TNF [51]. Determining whether FGD5 mediated Cdc42 activation participates in cell survival by regulation of either actin [50] or MT dynamics [20] is another future project. Targeting FGD5 is another possible therapeutic strategy to regulate endothelial functions such as angiogenesis.

In conclusion, in this project we have identified complex regulation of the endothelium cytoskeleton in regulation of leukocyte diapedesis. We have identified a PI3K-dependent step after VE-cadherin opening that regulates lymphocyte diapedesis. We also showed that endothelial IQGAP1 expression and inter-endothelial junction associated MT are required for efficient lymphocyte diapedesis. Further, we have started to characterize FGD5, a highly endothelial enriched RhoGEF, that might be an important modulator of EC survival and function such as angiogenesis.

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