Chapter 1.0

Endothelium and Inflammation Response

The endothelium is a thin monolayer of cells (*i.e.* endothelial cells, ECs) lining the inner lumen of blood vessels. Its location at the interface between circulating blood flow and the vessel wall affords unique roles in the regulation of several important processes including vasodilation and vasoconstriction (maintaining blood pressure), thrombosis and fibrinolysis, angiogenesis, barrier function, and inflammation. In particular, inflammation, a natural process by which foreign particles are eliminated from vascular tissue, is of considerable importance due its prevalent role in a number of disease pathologies.

Endothelial response to inflammation results in the differential expression of cell adhesion molecules (CAMs, *e.g.* selectins, immunoglobulins) on the apical surface facing the bloodstream. Patterns and duration of CAM expression is critical to the balance between healthy and pathogenic inflammatory response [1-3]. Inflammation is characterized by the recruitment of white blood cells from blood circulation to the vessel wall, through the endothelial monolayer and into affected tissues. The process starts with vasodilation wherein increased flow and vessel permeability allows for leukocyte migration to the vessel wall. Leukocyte adhesion to the wall is mediated by interactions between leukocyte ligands and EC CAMs in three distinct steps: (1) tethering, (2)

adhesion, and (3) transmigration (see Figure 1.1 for a schematic). During tethering, leukocyte-expressed carbohydrate ligands (*e.g.* sialylated Lewis X) form transient bonds with EC-expressed selectins (glycoproteins, *e.g.* E-selectin). This leads to the adhesion to apical EC surfaces by means of integrin-intercellular cell adhesion molecule (ICAM)-1 interaction and eventual transmigration across the endothelial monolayer and into the inflamed tissue. Leukocytes that have extravasated to injury sites mediate the healing process by eliminating the stimulus enabling tissue recovery [4-6]. Typically, inflammation is well-regulated and is quickly resolved with introduction of anti-inflammatory factors or clearance of pro-inflammatory cells and chemicals; leukocyte recruitment abates and tissue function is restored. However, unregulated endothelial response results in the uncontrolled and continuous recruitment of leukocytes that eventually leads to an over accumulation of white blood cells. Chemicals and toxins secreted from macrophages, a phagocytic white blood cell, designed to combat foreign pathogens also begin to induce apoptosis in healthy cells causing tissue necrosis and eventually fibrous scarring and loss of function. This destructive process, or *chronic inflammation*, is due to endothelial dysfunction and leads to a number of seemingly-unrelated diseases which include neurological disorders such as dementia, epilepsy, and Alzheimer's disease, cancers and metastatic tumor growths, organ failure, and several cardiovascular diseases [7-35].

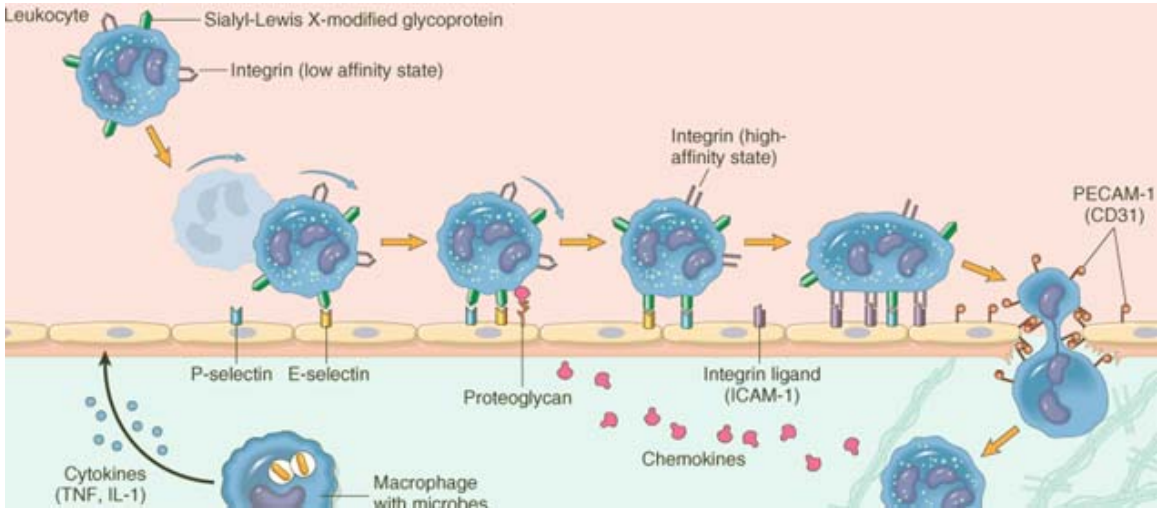

Figure 1.1. Schematic of leukocyte recruitment. *An inflamed endothelial monolayer expresses E-selectin, ICAM-1, VCAM-1, and PECAM-1 to capture leukocytes from the bloodstream in a receptor-mediated process of rolling, arrest, and transmigration ["MBBS Medicine: Inflammation." medicinembbs.blogspot.com. Feb 2011. Retrieved 2012-01-08]*

Chapter 1.1

Increasing Prevalence of Cardiovascular Diseases and Inflammation

While vascular-targeting of endothelial-expressed proteins has potential applications in several human diseases, especially cancers and metastatic tumor growth, the approach has gained recent attention in the treatment of cardiovascular (CVD)-related pathologies. The past few decades have seen increasing incidence of CVD-related pathologies. Diseases such as coronary heart disease, cerebrovascular disease (or stroke), and hypertension afflict nearly 83 million adults in the United States, accounting for 1 million deaths, or more than one-third of the annual mortality rate [36-37]; CVD-related fatalities outnumber the collective deaths caused by cancers, accidents, respiratory diseases, and diabetes [38]. The total direct (*e.g.* hospitalization, procedures, therapeutic regimens) and indirect cost (*i.e.* lost of productivity from morbidity and premature mortality) of treating CVDs in 2010 was estimated at 444 billion USD and is projected to exceed 1 trillion USD by 2030 due to an aging population and increasing prevalence of CVDs in younger demographics [39].

The most common cardiovascular disease is atherosclerosis or coronary artery disease (CAD). Blood vessels (typically arterial vasculature) afflicted with CAD are characterized by significant plaque buildup, vessel wall hardening, and are prone to developing ischemia and clots potentially leading to cerebral stroke or myocardial

infarctions – traits of chronic inflammation. Pathogenesis of CAD has been well-documented to begin with the arterial wall where dysfunction of the endothelium leads to enhanced leukocyte recruitment, accumulation of macrophages, altered hemodynamic balance, and thrombin and fibrin generation (factors associated with clot development) [40-42].

The locations of most atherosclerotic plaques are near branches and bifurcations in the vasculature at sites where blood flow patterns generate disruptive hemodynamic forces that makes endothelial cells more susceptible to developing atherosclerotic lesions (*i.e.* more atheroprone). Previous research has shown that these flow types are especially relevant to CAD and can act as pathological stimuli playing important roles in mediating endothelial dysfunction [31]. While most flow is a well-developed laminar profile and is found in non-branching, long vessels, blood flows at atheroprone sites are more disturbed encompassing one or more patterns including separation, recirculation, stagnant, reattachment, and even reverse flow [43]. The mechanical forces imparted by atypical flow elicit abnormal EC responses through over expression of cell adhesion molecules leading to abnormal accumulation of white blood cells. By restoring normal function to cell behavior (*i.e.* reducing pathological response), the overall disease can be treated and potentially eliminated.

Current treatment methods of chronic inflammation include medicines such as anticoagulant drugs that minimize clotting and embolus formation [44-45], statins that lower lipid and cholesterol levels [46-49], and vasodilators, blood thinners (*e.g.* aspirin, warfarin, heparin) or a combination thereof; however, these medicines only provide symptomatic relief and do not address the root cause of plaque and clot buildup. These

drug treatments are typically prescribed when the plaque buildup has significantly advanced to cause noticeable symptoms. This is likely due to the fact that atherosclerosis is usually not diagnosed (methods such as angiography can typically determine progression of plaque buildup but can only measure severe vessel stenosis) until the disease has progressed to the point of restricting significant blood flow. Other treatments are necessary to eliminate atherosclerotic plaques.

Angioplasty and stent placement, heart or lower extremity vessel bypass surgery, and vessel replacement procedures are common methods for treating atherosclerosis. Such coronary revascularization techniques are being improved every year [50-57]; coronary bypass and other vessel regeneration procedures have especially seen significant advances especially in development of autologous vascular grafts through tissue engineering [58-61]. Regardless, such treatments involve surgical risks that could be avoided or potentially prevented with more effective medicines as afforded by targeted drug delivery.

Chapter 1.2

Treating Chronically Inflamed Endothelium

With traditional drug treatments, the method of delivery (*e.g.* oral ingestion, intravascular injection) usually results in a systemic-wide distribution of the medicine. This not only dilutes the drug, necessitating frequent and concentrated dosages, but also elicits unwanted side effects since non-intended targets (*i.e.* healthy tissues) are also exposed to the drug. Targeted delivery not only localizes and prolongs the effects of medicines around diseased ECs, but also minimizes the side effects associated with treating healthy, non-pathogenic tissue. Additionally, the development of such selective targeting could lead to more precise imaging technology, aiding in disease diagnoses and providing alternatives to exploratory surgeries.

Development of an effective targeted drug delivery system requires evaluation of drug/vehicle parameters (*e.g.* solubility, biocompatibility, physical size and shape) [62-63]; however, the capacity to localize drug therapy to diseased vasculature while avoiding healthy vessels would also depend on a precise match between the targeting ligands on the delivery vehicle and their endothelial-based receptors and the uniqueness of the targeted endothelial-expressed receptors to the disease state. In the context of medicines, targeting E-selectin over ICAM-1 would likely enable more selective therapy due to the unique association of the former with a disease state. However, E-selectin

expression not only occurs in several disease states but is also upregulated during the natural acute inflammatory response to injury; thus E-selectin targeting alone may not isolate the disease state of choice. This realization has lead several research efforts to evaluate multiple receptor targeting since there is a greater likelihood of identifying unique protein expression patterns in a given disease state than identifying a protein unique to a disease state [64-66]. However, the realization of multiple-receptor targeting to the EC is currently limited due to the lack of existing methods for accurately measuring *in vivo* protein expression. The best attempt to obtain expression patterns rely on *in vitro* models that typically consist of ECs cultured on plastic substrates; however, these models grossly underestimate the *in vivo* environment relevant to chronic inflammation.

Chapter 1.3

Literature Review of *In Vitro* Inflammation Models

In order to treat pathogenic endothelium, the ability to distinguish disease-prone from healthy EC based on phenotypic changes during acute and chronic inflammatory response will need to be developed. Despite its prominent roles in the pathogenesis of multiple diseases, this response has yet to be fully understood. With ethical practices and government regulations precluding the use of human *in vivo* models and observed endothelial response in animal models known to not translate well to human physiology, research has focused on developing *in vitro* inflammation models. To this end, current *in vitro* models have attempted to mimic relevant EC inflammatory response under human physiological conditions; however, many have failed to simulate the actual physiological conditions under which inflammation occurs. In particular, groups have often overlooked the importance of *fluid shear stress* on endothelial response despite the well-known influence of mechanical forces on EC behavior; reports have also limited their studies to a short acute time frame over which the effects of chronic inflammation cannot be observed. Literature has also focused on ICAM-1, the adhesion molecule responsible for leukocyte firm adhesion, rather than an arguably more important identifier of inflammation, E-selectin. Additionally, a majority of reports are primarily focused on an inflammatory agonist, cytokine tumor necrosis factor α (TNFα) and have not examined inflammation under the influence of other cytokines, most notably interleukin-1β (IL-1β). A discussion of the literature will be presented in the following sections.

Inflammation Models in the Literature: Lack of Fluid Shear

Vascular endothelial cells *in vivo* are highly adherent and can maintain their monolayer integrity and function even under extreme shear stress magnitudes. Studies have identified the importance of blood fluid shear stress on regulating adaptive vessel growth and angiogenesis and even modulating signal transduction through stress-sensing mechanotransductive receptors [67-72]. Fluid shear affects ECs at both cellular and molecular levels influencing cell morphology, function, and gene expression and has also been implicated in abnormal EC behavior leading to disease pathogenesis [73]. Indeed, there is increasing evidence that prolonged exposure to high laminar fluid shear – especially found in non-branched straight lengths of the vasculature – modulates vascular endothelium toward a protective phenotype where ECs realign in the flow direction and adopt a more streamlined morphology thereby minimizing the impact of the tangential fluid shear. Disturbed flow profiles, encountered in branches, curvatures, and bifurcations where reversal flow and stagnation and low shear zones are prevalent, produces an atheroprone phenotype with cobblestone, squamous-like morphology especially relevant in diseases such as atherosclerosis and aortic stenosis [74-78].

This cobblestone-like shape is also encountered in cell culture due to the absence of shear flow in a typical stagnant *in vitro* environment. In such conditions where rigid substratum characteristic of artificial surfaces (such as those used in vascular grafts and culture flasks) also contributes to deviations from physiological norms, ECs have been observed to be less adherent, losing many of their physiological properties attributed to fluid shear preconditioning [79-80]. Despite this drawback, numerous human *in vitro*

models of inflammation have been developed over the past few decades [81] largely excluding fluid shear from their studies.

Endothelial response to inflammatory chemical stimulus has been the major focus of the literature. Whether measured by CAM synthesis or other cytokine and anti-inflammatory molecule production, response was studied in stagnant cell cultures, absent of any fluid shear despite being one of the key properties of ECs distinguishing them from other cultured cells, especially fibroblasts and smooth muscle cells. Indeed endothelial cells are characterized by their sensitive response to mechanical or physical forces, as observed in their changing morphology, induced migration [82-84], and altered survival rates [85-86]. Various groups have reported cultured ECs are susceptible to slight differences in flow type (*e.g.* pulsatile, laminar, disturbed, oscillating) and shear magnitude; through actin reorganization and activation of focal adhesion kinases, endothelial cells alter their cytoskeletal structure to adapt to interactions with its substratum, extracellular matrix, and other neighboring cells [31, 83, 87-96].

Inflammation Models in the Literature: Short Time Frame

Despite acknowledgement of the influence of physical forces, few have actually examined EC response to co-stimulation with chemicals and fluid shear. Those that have addressed shear-cytokine activation of ECs have only studied a short time frame under which chronic inflammation is largely irrelevant. Most shear-cytokine models have been limited to only the acute inflammation response; as can be seen in Figure 1.2, a survey of experiment protocols in the literature shows that a majority of past works have studied the short term, acute shear-cytokine activation in the 1-6 hr timeframe. While most of

these studies have included a long term preconditioning of ECs prior to a shear-cytokine activation period, the lack of studies in the chronic inflammation range indicates a need for further investigation to determine endothelial response relevant for disease models.

Inflammation Models in the Literature: Choice of Cell Adhesion Molecule

Leukocyte recruitment during chronic inflammation is continuous due in part to the prolonged upregulation of CAMs from basal levels. Utilizing this endothelial response, it is possible to distinguish between naïve, quiescent ECs from pathological, inflamed cells. To this end, literature has focused on EC expression patterns of a few main CAMs that are involved in leukocyte extravasation, particularly E-selectin and ICAM-1. In quiescent, healthy vascular tissue, endothelial cells do not express surface E-selectin molecules [97-98]; however, upon activation with inflammatory cytokines, E-selectin is upregulated to a significantly elevated level promoting increased leukocyte recruitment [99]. While pro-inflammatory factors also induce higher ICAM-1 levels, discrimination between non-inflamed and inflamed ECs based on ICAM-1 expression alone cannot be achieved since the molecule is constitutively expressed on both quiescent and active EC surfaces [100-102]. During acute inflammation, high E-selectin expression is only maintained for a short duration. However, in the case of chronic inflammation, selectin expression is prolonged at this high level, resulting in an excessive buildup of macrophages in the surrounding tissue. Based on these endothelial responses, E-selectin expression distinguishes pathologically inflamed endothelium (prolonged period, elevated levels) from both quiescent (no expression) and normally inflamed cells (acute period).

Literature has mainly focused on EC response through ICAM-1 expression due to observed shear-dependent response in early activation times. Studies have reported muted ICAM-1 mRNA and protein expression in shear-cytokine activated cultures relative to statically activated cultures whereas no significant response is observed on E-selectin density [103-104]. Indeed, profiling studies confirmed that the gene coding for ICAM-1 contains a shear-stress responsive element in its promoter region, while other CAMs, including E-selectin and VCAM-1, lack this genetic construct, indicating shear-independent upregulation [105]. These early studies have characterized ICAM-1 as a popular adhesion molecule to investigate; however, these reports only observed short term acute inflammatory response – and have largely ignored longer term time frames where fluid does in fact play a major role in regulating E-selectin expression, as will be shown in this thesis.

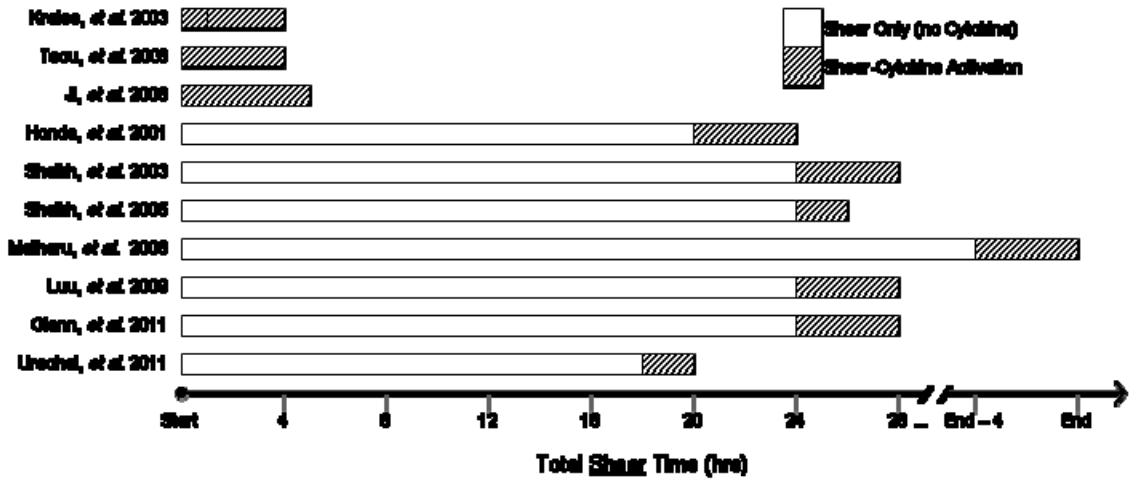

Figure 1.2. Time frame of shear-cytokine based experimental protocols. *Investigations into simultaneous stimulation of endothelial cells with cytokines in the presence of fluid shear have either been in the immediate acute response or in the long term after a prolonged shear-only preconditioning period.*

Inflammation Models in the Literature: Cytokine Choice

As previously stated, in addition to focusing on ICAM-1 and neglecting to incorporate physiological fluid shear at relevant chronic time frames into *in vitro* models, inflammation literature has primarily observed EC response to one particular cytokine, tumor necrosis factor α (TNFα) perhaps due to its role in a large number of CVDs [106-108] and its anti-cancer treatment potential in the selective targeting of tumor vasculature [109]. However, TNFα is only one of three primary pro-inflammatory factors: IL-1β has also been shown to be critical in the development of various pathologies including Alzheimer's disease [110], chronic autoimmune disorders [111], and metabolic diseases [112-113] whereas the third factor, lipopolysaccharide (LPS), an endotoxin shed from bacteria during infection, is responsible for septic shock, a large number of innate immune responses [114-115], and the secretion of additional pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, and chemoattractant cytokines from macrophages and ECs.

These three factors are considered the principal mediators of inflammation and can induce a number of responses ranging from cell activation to differentiation and apoptosis. Produced by leukocytes, the endothelium, and bacteria, the stimulants activate lymphocytes regulating immune cell response; however, their individual effects on the endothelium in regulating inflammation response are slightly different. With respect to adhesion molecules, higher E-selectin expression is reported to be elicited by IL-1β due to stronger activation of protein kinases in its signal transduction pathway, whereas ICAM-1 expression is not observed to be similarly impacted when compared with TNFα or LPS activation [116-118]. Due to the unique role of E-selectin in leukocyte adhesion

during inflammation, further exploration into its expression patterns under shear-cytokine activation is warranted.

Other common pro-inflammatory factors include IL-4, IL-6, and nitric oxide (their roles are listed in Table 1.1). Interestingly, IL-6 acts in both pro- and anti-inflammatory capacities having been shown to play prominent roles in the development of diabetes, rheumatoid arthritis, and even prostate cancer [119-121], but also in the inhibition of TNFα and IL-1β activity and induction of IL-10 production (an anti-inflammatory cytokine that has been used in clinical trials for Crohn's disease [122]). The partial list of mediators in Table 1.1 describes some functions of a few of the more common factors involved in inflammation. While understanding the effects of all pro-inflammatory factors is important, the additional complexities afforded by their overlapping signaling and activation pathways is beyond the scope of this thesis.

Table 1.1 A Partial List of Mediators of Inflammation.

Mediator	*Role/Prominence*
Lipopolysaccharides (LPS)	• Promotes secretion of IL-1, IL-6, TNFα, chemokines [114] • Leads to septic shock
Interleukin-1 (IL-1) Tumor necrosis factor (TNF)	• Stimulates and activates lymphocytes (T, B, and NK cells) • Regulates immune cell response [114]
Interleukin-6 (IL-6)	• Anti-inflammatory mediator (produced during exercise) [123] • Diabetes, rheumatoid arthritis, prostate cancer, myocardial infarction [119-121, 124] • Inhibits TNFα and IL-1 activity [125] • Induces IL-10 production [125]
Interleukin-4 (IL-4)	• Activates macrophages in tissue repair [125] • Produced during allergic response [126]
Histamine	• Increases vessel permeability leading to allergic reaction • Mediates chemotaxis of several immune cells
Nitric oxide	• Inhibits leukocyte adhesion, maintains homeostasis [127] • Overproduction contributes to reperfusion injury • Implicated in pulmonary arterial hypertension [128]
Interleukin-10 (IL-10)	• Inhibits synthesis of pro-inflammatory molecules [125]

Chapter 1.4

Thesis Aims

As described earlier, endothelial dysfunction leads to the unregulated accumulation of leukocytes and could potentially develop into chronic inflammation resulting in the pathogenesis of atherosclerosis and other disorders. A targeted delivery system could use the inflammatory pathway as a potential route for specific, localized delivery of medicines [5, 129]. If selectively localized to plaques and sites of chronic inflammation, conventional drugs used to reduce symptoms could be used more effectively in moderating and eventually eliminating the disease.

Increasing therapeutic efficacy (*i.e.* higher specificity) will open new directions and possibilities for novel treatment regimens including the development of new anti-inflammatory drugs. Natural enzymes such as matrix metalloproteinases that target pro-inflammatory cytokines for degradation or synthetic derivatives could potentially be released in the local environment around disease-prone endothelium; additionally, fast acting agents, could be delivered in concentrated doses for region-specific treatment without eliciting unwanted side effects. New drugs such as delta-9-tetrahydrocannabinol that inhibit leukocyte or neutrophil activation (and not the more delicate endothelium) could also be developed and locally delivered to inflamed regions. Numerous possibilities for novel treatment regimens, in addition to current therapeutics will provide additional

non-invasive options for treating chronic inflammatory diseases. However, selective targeting of pathological endothelium will require a thorough understanding of endothelial response to inflammation that allows for distinguishing disease-prone from healthy ECs. Unfortunately, despite the numerous existing *in vitro* models, EC biology and behavior especially under chronic inflammatory conditions – pertinent to disease pathogenesis – is still relatively unknown.

This thesis develops a more physiologically relevant *in vitro* model that addresses the aforementioned gaps in the literature. The influence of fluid shear stress on E-selectin expression due to cytokine IL-1β stimulation is investigated through simultaneous co-stimulation of naïve and preconditioned endothelial cells using a novel laminar flow apparatus designed to study the broader time frame over which chronic inflammation is relevant.

A basic *in vitro* model will initially be used to determine the individual effects of fluid shear stress stimulus and IL-1β activation (in the absence of shear). While this may not be of any particular physiological relevance, especially with the cytokine-only activation of endothelial cells, measured E-selectin expression densities will be compared with existing data in the literature in order to validate the model design. Simultaneous co-stimulation with fluid shear and IL-1β cytokine ("shear-cytokine activation") of naïve endothelial cells will then be introduced to determine selectin expression trends over a long term period; this model iteration simulates disease-prone endothelium and a discussion regarding its relevance to ischemia/reperfusion injury will be presented. Additionally, endothelial response will also be measured *via* changes in phenotype

through shape factor calculation and changes in monolayer functionality through neutrophil adhesion assays.

Increasing the physiological relevance of the *in vitro* model, further iterations will introduce fluid shear preconditioning and IL-1β cytokine redosing to develop a healthy endothelial cell phenotype prior to inflammatory stimulus and to simulate persistent chronic inflammation. The effects of shear history, specifically duration of endothelial cell exposure to low or high magnitudes of shear stress, will be explored. The shear-cytokine activation of naïve and preconditioned cells will then be tested to determine if the observed effects is cytokine-specific by comparing E-selectin expression patterns for TNFα and IL-1β activated cells. Finally, a potential applications of the *in vitro* model in testing a synthetic cannabinoid as a possible anti-inflammatory therapeutic will be explored.

Chapter 2.0

Materials and Methods

This chapter described the methods used for experiments reported in the data chapters presented in the thesis. All protocols for obtaining human tissue were approved by the University of Michigan (UM) Internal Review Board (IRB protocol numbers HUM00013973 and HUM00026898) and in line with the standards set by the Helsinki Declaration. A written informed consent approved by the UM IRB was obtained from all participants involved in the study prior to blood collection. Data collection and analyses were performed anonymously.

Chapter 2.1

Endothelial Cell Source and Cell Culture Methods

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical cords following a modified Jaffe collagenase-digestion protocol [130]. Cords were collected from C.S. Mott's Children Hospital (Ann Arbor, MI) following an Internal Review Board (IRB) exempt protocol, and HUVECs used in all assays were pooled from at least three donors.

Umbilical veins were drained and thoroughly washed with $DPBS^{++}$ (Invitrogen, Carlsbad CA) prior to a 20 minute incubation at room temperature with 3 mg/mL collagenase (Polysciences, Warrington PA). Veins were agitated to dislodge endothelial cells and the cell solution was collected by washing veins with culture media (described below). Cell solution was pelleted at 300 G for 5 minutes and resuspended in culture media. Isolated HUVECs were plated on 0.2% (w/v) gelatin (Sigma Aldrich, St. Louis, MO) surface-treated tissue culture flasks and cultured until confluent in media that consisted of Medium 199 supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, Utah), 10% (v/v) bovine calf serum (HyClone, Logan, Utah), 1% 250 μg/mL fungizone, 1% 5,000 U/mL penicillin/5,000 μg/mL streptomycin, 1% 200-mM L-glutamine, 1% 10 mg/mL heparin, 1% 1 M HEPES buffer and 50 μg/ml endothelial cell

growth supplement (BD Biosciences, Franklin Lakes, NJ). HUVECs were maintained in a 5% CO_2 humidified 37°C incubator and were passaged no later than third generation.

HUVECs were subcultured onto 30 mm #1.5 thick glass coverslips (Warner Instruments, Hamden CT) that had been coated with 1% (w/v) gelatin, cross-linked with 0.5% glutaraldehyde (Polysciences, Warrington PA), and treated with 0.1 M glycine (Invitrogen, Carlsbad CA). Coverslips were incubated for at least 36 hrs to allow for confluent monolayer development in a 5% CO_2 humidified 37°C incubator prior to experimental use.

Chapter 2.2

Parallel Plate Flow Chamber

A straight channel parallel plate flow chamber (PPFC) (GlycoTech, Gaithersburg, MD) was used for all shear studies. Briefly, for each sample, a silicon rubber gasket with a rectangular cutout was attached to an acrylic flow chamber deck with inlet and outlet flow ports. A confluent HUVEC monolayer on a glass coverslip was placed over the gasket and vacuum-sealed such that the coverslip formed the bottom plate of the assembled flow chamber. A rectangular cutout in the gasket defined the flow channel with a height of 250 μm and width and length of 1 and 2 cm, respectively (see Figure 2.1).

The PPFC was connected via 1.8 mm inner diameter Tygon tubing to inlet and outlet media reservoirs. The media height difference between reservoirs determined the flow rate through the PPFC while a programmable peristaltic pump (Ismatec, Germany) was used to recirculate media to the inlet reservoir to maintain the pressure drop across the reservoirs. Additionally, a 5% CO_2 line was fed into the inlet reservoir to maintain 7.3-7.4 pH in the media flow buffer (see Figure 2.2 for a schematic of the model setup). The entire apparatus was mounted in a humidified 37°C chamber built around a Nikon ET 2000 S inverted microscope.

Recirculation of perfusion media enabled the study of long term conditions under which chronic inflammation is relevant. One drawback of many past *in vitro* models is the use of single-pass perfusion systems, requiring prohibitively high media volumes or limited cell population. Additionally, this recirculating setup allows for the long term use

of straight channel laminar flow rather than the distinctly different flow profile generated from the use of cone-plate viscometers or orbital shakers with which a shear stress gradient applied over the entire sample population (rather than a single magnitude value) introduces an uncontrolled variable.

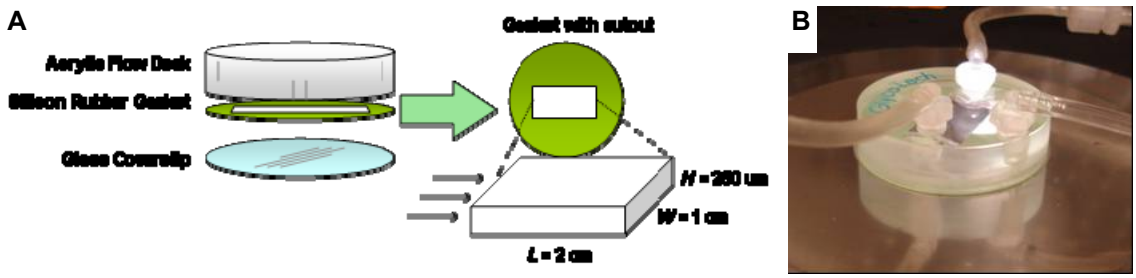

Figure 2.1. Parallel plate flow chamber (PPFC). *(A) A schematic of PPFC assembly: glass coverslip with EC monolayers is vacuum sealed against an acrylic flow deck with a silicon rubber gasket. The silicon rubber gasket is 250 μm thick and contains a rectangular cutout 1x2 cm to define a flow chamber through which media can be perfused over the cell monolayer on the glass coverslip. (B) Assembled PPFC with an inlet tube from a media reservoir, an outlet tube to a second reservoir, and a vacuum line leading to the right; the assembled device is sitting on a microscope stage.*

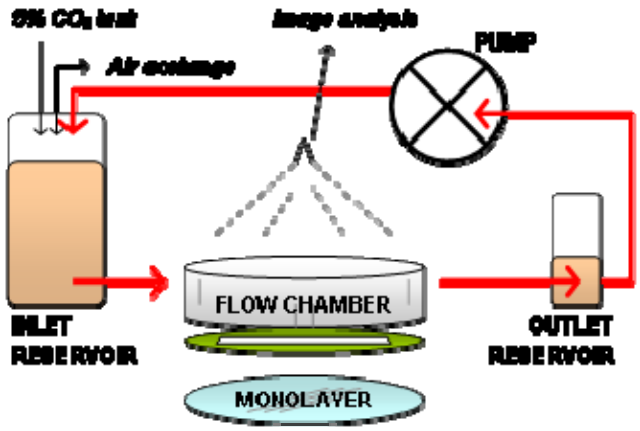

Figure 2.2. Schematic of* in vitro *setup. *Physical model of the* in vitro *system used in all shear-based experiments.*

Chapter 2.3

IL-1ß Concentration

Though endothelial cell CAM expression is dependent on cytokine concentration, only a fixed, saturating cytokine concentration was used in the experiments described in this thesis. A preliminary assay was performed on statically cultured monolayers measuring E-selectin site density at 4 hrs post IL-1ß activation for a range of concentrations. As seen in Figure 2.3, a significant increase in E-selectin expression is observed between control, non-activated cells and cells activated with 0.016 ng/mL of IL-1β. E-selectin densities for all other tested concentrations (0.016-10 ng/mL) were not significantly different, indicating that a maximum selectin expression density was reached at 0.016 ng/mL IL-1ß. Based on this data, all other experiments were treated with an order of magnitude higher concentration, 0.1 ng/mL to ensure consistency and to minimize systemic variability.

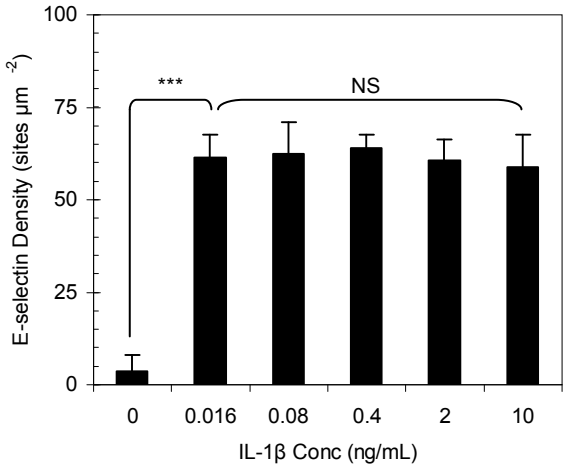

Figure 2.3. IL-1β saturating concentration. *HUVEC monolayers were treated with IL-1β for 4 hrs at various concentrations. Cells were then labeled for E-selectin molecules and analyzed via immunofluorescence microscopy. "NS" indicates no significance, "***" $p < 0.001$.*

Chapter 2.4

Calculating Shape Factor

Endothelial cells are highly responsive to fluid shear, often changing their morphology to align with their local hemodynamic flow. A way to quantify this shape change of each individual cell is to calculate their shape factor – a dimensionless number between 0 and 1 that describes the circularity of an object:

$$SF = \frac{4\pi A}{P^2}$$

where A is the cell area and P is the perimeter length; an $SF = 1$ indicates a perfect circle.

Chapter 2.5

Activation of Endothelial Cells

For static conditions, HUVEC monolayers were exposed to a single dose of 0.1 ng/mL of recombinant human interleukin-1ß (IL-1ß – Fitzgerald Industries, Concord, MA) for the desired period of time (up to 24 hr). Preliminary experiments showed maximal E-selectin expression with an IL-1ß concentration of 0.01 ng/mL at the 4 hr activation mark under static conditions.

For shear conditions, HUVEC monolayers were exposed to a fixed wall shear stress (in the range of 1-20 dyn/cm^2) in either the absence or presence of IL-1ß for a given period of time up to 24 hr. The wall shear stress (dyn/cm^2) in the flow chamber was computed using the following equation

$$\tau_w = \frac{6Q}{wh^2} \cdot \mu \text{ ; [dyn/cm}^2\text{]}$$

where Q is the fluid flow rate set by the peristaltic pump, w and h are the width and height of the flow channel, and μ is the fluid viscosity.

Chapter 2.6

E-selectin Site Density Quantification

The proposed *in vitro* model measures E-selectin protein expression on the apical surface of *intact* endothelial cell monolayers (*i.e.* confluent, bound to a substrate). Measurement by immunofluorescent microscopy is the least disruptive and provides an accurate protein density number. By maintaining a confluent EC monolayer for each sample, only E-selectin proteins that are expressed on the top side (the surface that is exposed to fluid shear) will be quantified. Selectins produced and maintained within the endothelial cell will be excluded by using a sensitive Nikon ET 2000 S microscope that allows for accurate depth-focusing on the apical surface of EC monolayers.

While the author recognizes that flow cytometry is another commonly accepted method to measure cellular protein expression, the sampling requirements (high concentration of solubilized cells) and measurement intensities (protein expression is measured in cell cytosol, bound on cell surface, and solubilized in solution) will not provide the accuracy necessitated by the *in vitro* model. (Nevertheless, flow cytometry measurements were made in order to validate the model).

Coverslips with HUVEC monolayers from static and flow assays were washed with $DPBS^{++}$ and fixed via exposure to 4% w/v paraformaldehyde for 10 min at 4°C. Cells were washed twice with $DPBS^{++}$ and incubated with 0.2 mg ml^{-1} fluorescein

isothiocyanate (FITC)-conjugated monoclonal anti-human E-selectin antibody (BBIG-E5 clone, R&D Systems, Minneapolis, MN) at 4°C for at least 1 hr to label surface-expressed protein only – no cell permeabilizing agent was used. After labeling, monolayers were affixed to flow chambers in their original orientation in order to maintain a consistent imaging depth; chambers were flushed with $DPBS^{++}$ to clear cell surface debris and unbound antibodies.

Monolayer surface E-selectin expression was imaged on a fluorescent microscope (Nikon ET 2000 S) at 485/527 nm (ex/em) and 2000 ms exposure using a Photometrics CoolSNAP EZ (Photometrics, Tucson, AZ) mounted on a Sony CCD sensor digital camera; images were captured using MetaMorph software (Downingtown, PA). A representative fluorescent image of EC E-selectin expression is shown in Figure 2.4.

Relative fluorescent intensities (RFI) were collected for at least five images per monolayer near the PPFC flow centerline at least 1 cm from the flow inlet. To convert RFI values to antibody concentration, average fluorescent intensities for 0.2, 0.1, 0.05, and 0.00 μg/mL solutions of the anti-human E-selectin antibody were measured on a calibration monolayer for a linear regression fit (see example Figure 2.5). Measured RFI values for each monolayer were then converted to E-selectin sites per μm^2 (assuming 1:1 binding ratio of antibody to receptor) with the following formula:

$$\rho_{E-sel} = RFI \cdot \alpha \cdot \frac{h \cdot N}{MW_{FITC}}; \text{[sites/}\mu m^2\text{]}$$

where α is the correcting factor from the linear regression calibration that converts *RFI* to a concentration value, h is the height of the flow chamber (250 μm), N is Avogadro's number (6.022×10^{23} molecules/mol), and MW_{FITC} is the molecular weight of the FITC-conjugated E-selectin antibody (146 kDa).

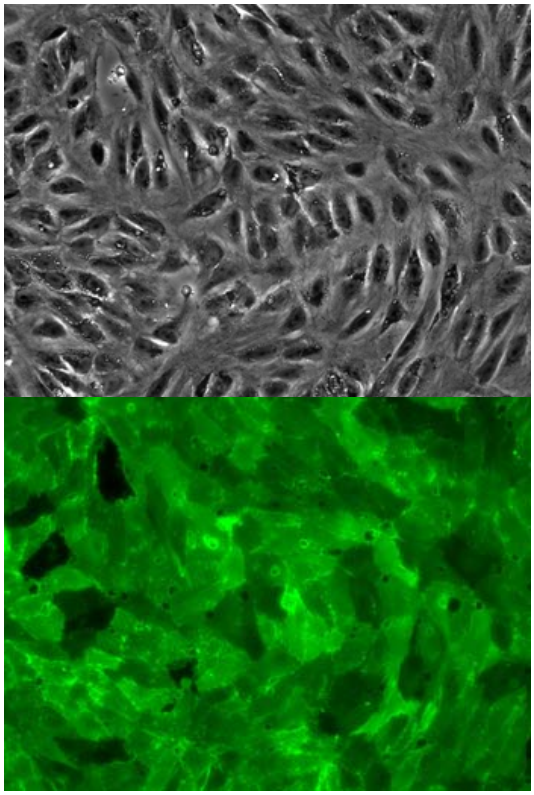

Figure 2.4. Immunofluorescence microscopy of monolayers. *Human umbilical vein endothelial cells (HUVECs) were activated with IL-1β cytokine for 4 hrs and subsequently labeled with a fluorescent antibody against human E-selectin. Images at 20x magnification of the same HUVEC monolayer of (top) brightfield and (bottom) 2000 ms exposure to UV light (false colored after image processing).*

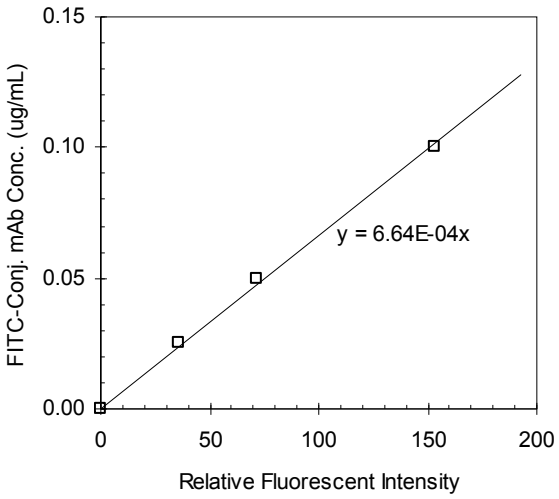

Figure 2.5. RFI calibration. *Average fluorescent intensities at 0.2, 0.1, 0.05, and 0 μg/mL solutions of fluorescein-isothiocyanate conjugated anti-human E-selectin antibody are fitted to a linear regression curve to use as a calibration for converting RFI (relative fluorescent intensities) to concentration values.*

Chapter 2.7

Neutrophil Binding Assays

Human blood was collected from donors via venipuncture into a syringe containing citrate anticoagulant (acetate-citrate-dextrose; ACD). Red blood cells (RBCs) were separated from whole blood by sedimentation using a 6% (w/v) dextran solution. Neutrophils were isolated via the standard Ficoll gradient as previously described [131]. Isolated neutrophils were washed with $DPBS^{--}$ at room temperature and used within 2 hr of isolation. Neutrophil adhesion to IL-1ß treated (up to 24 hr) HUVEC monolayers under static or shear-cytokine conditions were assessed in the PPFC described above. An activated monolayer was loaded onto the flow deck, and freshly isolated human neutrophils in 1% (w/v) human serum albumin in $DPBS^{++}$ ($1x10^6$ cells/mL) were perfused over the monolayer at 2 dyn/cm^2. Binding density was calculated as the number of adherent or rolling neutrophils per mm^2 after 2 minutes of shear.

Chapter 2.8

Treating Neutrophils with Synthetic Cannabinoid CP55,940

A range of concentrations 60 nM - 300 μM of CP55,940, a synthetic cannabinoid agonist (Tocris Inc., Ann Arbor MI) were prepared in DMSO and diluted with 1% (w/v) human serum albumin (HSA) such that the final DMSO concentration was no more than 0.01% (v/v). Donors were male and female students aged 18-27 and were not screened for exposure to cannabinoids.

For adhesion assays in buffer flow, neutrophils were isolated as described and were incubated with varying concentrations of CP55,940 for 2 hrs at 10^7 neutrophils/mL in 1 mL volumes. Incubates were kept in a 37°C incubator with 5% CO_2 and carefully mixed every 15 minutes to prevent neutrophil sedimentation. Prior to use in perfusion adhesion assay, neutrophil incubates were diluted with flow buffer to 10^6 neutrophils/mL concentration.

For adhesion assays in whole blood flow, neutrophils were *not* isolated instead whole blood was collected and stored with ACD to prevent coagulation. Appropriate concentrations of CP55,940 were added directly to blood volumes; blood vials were kept in a 37°C incubator with 5% CO_2 and carefully mixed every 15 minutes to prevent sedimentation.

Neutrophils in buffer or whole blood were perfused at shear rate 100 s^{-1} (buffer flow) or at 500 s^{-1} (whole blood) for up to 5 minutes whereupon the number of neutrophils interacting (*i.e.* rolling, firmly adhered or transmigrated, see Figure 2.6) with the monolayer were quantified and neutrophil flux was calculated (# interacting per area per time; #/mm^2/min). DMSO vehicle used to solubilize CP55,940 and non-treated, naive neutrophils were used as controls.

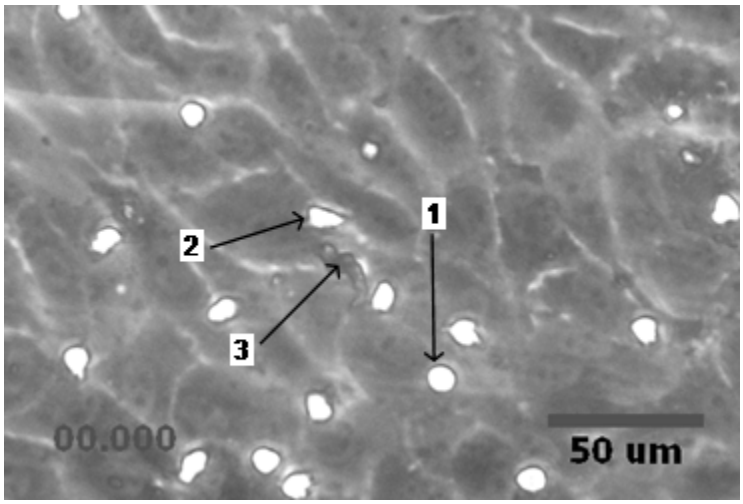

Figure 2.6. Neutrophil interaction with endothelial monolayer. *Still frame image captured from a neutrophil adhesion assay showing neutrophils rolling (1, phase bright, circular shape), firmly adhered (2, phase bright, deformed shape), and fully transmigrated (3, phase dark, deformed shape) on a HUVEC monolayer (background).*

Chapter 2.9

Statistical Methods

All experiments were performed in at least triplicate; averages were calculated and standard errors are shown on data figures unless otherwise noted. Significance was determined using one-way ANOVA with a 95% confidence interval and Bonferroni post-test using GraphPad Prism Software (San Diego, CA). Significance is indicated as needed on figures as follows: "NS" indicates no significant difference ($p > 0.05$), "*" ($p < 0.05$), "**" ($p < 0.01$), and "***" ($p < 0.001$).

Chapter 3.0

In Vitro Model of IL-1β Shear-Cytokine Activation of Naïve Endothelial Cells

In order to establish a baseline from which a more physiologically relevant *in vitro* model of inflammation can be developed, basic models of shear-only stimulus and IL-1β cytokine-only activation of naïve endothelial cells is considered so that correlations can be made with existing data in the literature. However, to reflect observed *in vivo* behavior of ECs, both of these individual stimuli are combined to co-stimulate monolayers with fluid shear and IL-1β cytokine (termed, "shear-cytokine") over a broader time frame of up to 24 hrs. In this way, this thesis presents EC response not previously measured, by E-selectin expression densities, due to chronic inflammatory stimulus under a physiological setting.

Inflammatory response is not limited to cell adhesion molecule expression as has been mentioned earlier and will be shown in greater depth in later sections. Endothelial morphology plays a key role in determining EC phenotype under fluid flow. The elongation and flow realignment in response to shear stress is a property unique to ECs that differentiates them from other vascular tissue such as smooth muscle cells – and can affect the E-selectin expression and the monolayer's functionality in attracting leukocytes from the bloodstream. This section will also explore the mechanism by which shear stress elicits a change in selectin expression profile, whether the presence of shear induces the

de novo synthesis of the adhesion molecule or prevents down regulation of already existing selectins. These initial experiments will show previously unreported observations of EC behavior under physiologically relevant simultaneous shear- IL-1β cytokine stimulation over a broader time frame and will build a base case for comparisons with more advanced iterations of the *in vitro* model in later sections.

Chapter 3.1

IL-1β Static Activation or Shear-Only Stimulation of Naive Endothelial Cells

To build a physiologically relevant *in vitro* model, a basic case is first considered. Individual contributions to E-selectin expression due to shear-only stimulus or IL-1β cytokine-only activation (in the absence of any fluid shear) is measured on HUVEC monolayers. While not physiologically relevant, this initial set of experiments identifies a baseline from which more complex models can be built.

Measured E-selectin surface expression density (sites/μm^2) on HUVECs activated by IL-1β alone and shear alone (10 dyn/cm^2) for up to 24 hrs are presented in Figure 3.1. Under static activation, maximum peak expression is observed at 4 hrs (111 ± 3.6 sites/μm^2), values that are significantly higher than the basal expression of untreated naïve cells (9 sites/um^2). Expression decreased until the 16-20 hr mark at values still significantly higher than baseline (p = 0.030). Interestingly, a slight rebound was observed at the 20-24 hr mark where E-selectin density started to recover to nearly 3.5 fold the basal expression values. Monolayers exposed to laminar shear alone displayed minimal E-selectin expression for up to 24 hrs (Figure 3.1 shows data for 10 dyn/cm^2; shear stresses from 1-20 dyn/cm^2 were tested, showing minimal, insignificant changes).

To confirm the validity of the immunofluorescent microscopy technique used to quantify E-selectin density, statically activated HUVEC monolayers were processed by

flow cytometry. E-selectin and platelet endothelial cell adhesion molecule (PECAM)-1, a molecule uniquely expressed by ECs responsible for mediating leukocyte transmigration, site densities were measured and are shown in Figure 3.2. E-selectin expression peaks at the 4-6 hr mark while PECAM-1 is constitutively expressed at all activation times, data that is also observed in the literature [132-134]. Additionally, observed EC morphology maintains a cobblestone-like shape in the statically activated samples, but (as seen in Figure 3.3) shows an elongated, flow-oriented phenotype in prolonged shear-exposed samples as has been reported in the past [135-137].

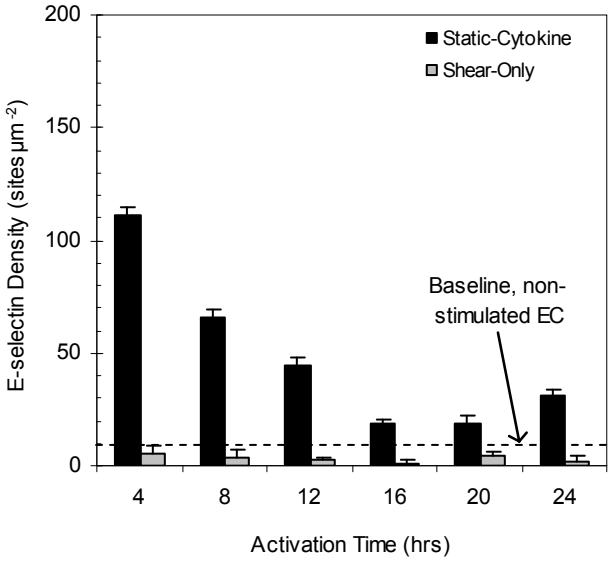

Figure 3.1. E-selectin expression density of static or shear-only activated endothelial cells. *Human umbilical vein endothelial cell monolayers were activated by IL-1β alone (solid) or shear alone (gray, 10 dyn/cm^2) for up to 24 hrs. Baseline expression of non-stimulated HUVECs are also shown (dotted line) at 9 sites/µm^2.*

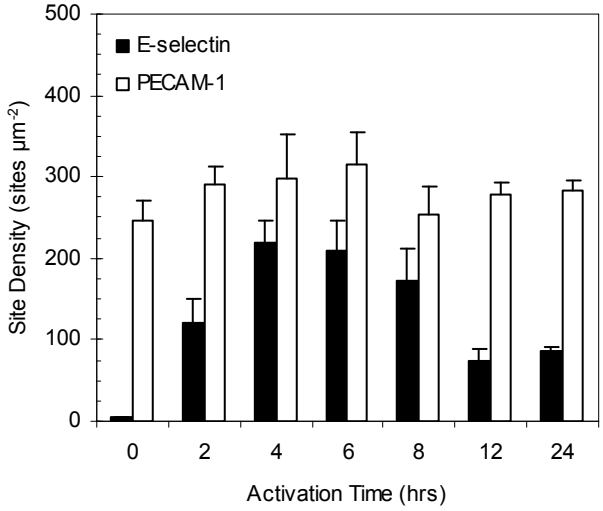

Figure 3.2. E-selectin and PECAM-1 site densities. *HUVEC samples were activated with IL-1β for up to 24 hrs under static culture. Samples were labeled for E-selectin and PECAM-1 expression via fluorescent antibodies; site densities per μm^2 cell surface area were measured by flow cytometry.*

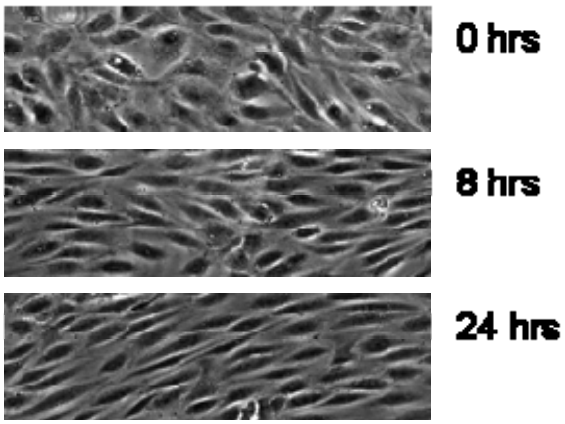

Figure 3.3. Cell morphology under shear flow. *A HUVEC monolayer was perfused under 10 dyn/cm^2 shear stress of fluid flow (left to right). Brightfield 20x magnification images were taken at 0, 8, and 24 hrs into perfusion, highlighting the elongation and realignment of individual cells.*

Chapter 3.2

IL-1β Shear-Cytokine Activation of Naïve Endothelial Cells

When IL-1ß activation was done in the simultaneous presence of laminar shear at 10 dyn/cm^2 ("shear-cytokine", hereafter), HUVEC monolayers expressed significantly higher levels of E-selectin than what was observed for static activation at all time points from 8 to 24 hr (see Figure 3.4). At 4 hrs activation, there is no significant difference between both shear-cytokine and static activated monolayers. E-selectin density on shear-cytokine activated cells is also not significantly different at 4 and 24 hr of activation time whereas the 24 hr level for static activated cells is 3.5 fold lower than their corresponding 4 hr expression.

As displayed in Figure 3.5, an extension of the fluid shear stress studied to 5 and 20 dyn/cm^2 shows that peak E-selectin expression for all shear magnitudes shifts to 8-12 hr. Shear-cytokine exposed cells have expression levels significantly higher than static-cytokine for all times greater than 4 hrs even at the 24 hr mark where expression remains 2-3 folds higher. For most activation times, a similar trend is observed even at the lower shear magnitude 1 dyn/cm^2: peak selectin expression shifts to 8-12 hr at levels comparable to that observed for 10 dyn/cm^2 and remains elevated relative to static. However, at longer activation times, the E-selectin expression values for 1 dyn/cm^2 are significantly lower than those for higher magnitudes at 16 and 24 hrs. Additionally, at the

24 hr mark, E-selectin expression for 1 dyn/cm^2 shear-cytokine activation is not statistically significant from static activated monolayers.

Shear-cytokine activation was examined in greater detail at the 24 hr activation mark. In addition to the already studied 1, 5, 10 and 20 dyn/cm^2 levels, selectin expression for magnitudes between 1 and 5 dyn/cm^2 were also explored. As shown in Figure 3.6, at magnitudes above 1.5 dyn/cm^2, selectin densities are not significant from each other; as mentioned earlier, selectin expressions at 1 dyn/cm^2 is not significantly different from static activation. This data suggests a minimum shear threshold of 1.5 dyn/cm^2 is required at the long term 24 hr activation time to induce a significantly different E-selectin expression by naïve ECs relative to static activation.

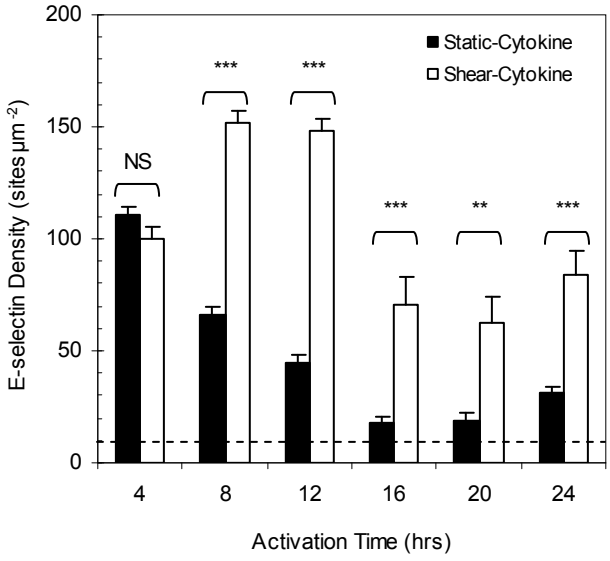

Figure 3.4. E-selectin expression on 10 dyn/cm^2 shear-cytokine activated ECs. *HUVEC monolayers were exposed to co-stimulation by IL-1ß and fluid shear flow for up to 24 hrs; corresponding E-selectin site densities were measured at given time points (clear). Measurements for statically activated (filled) and baseline non-stimulated (dotted line) HUVECs are also shown.*

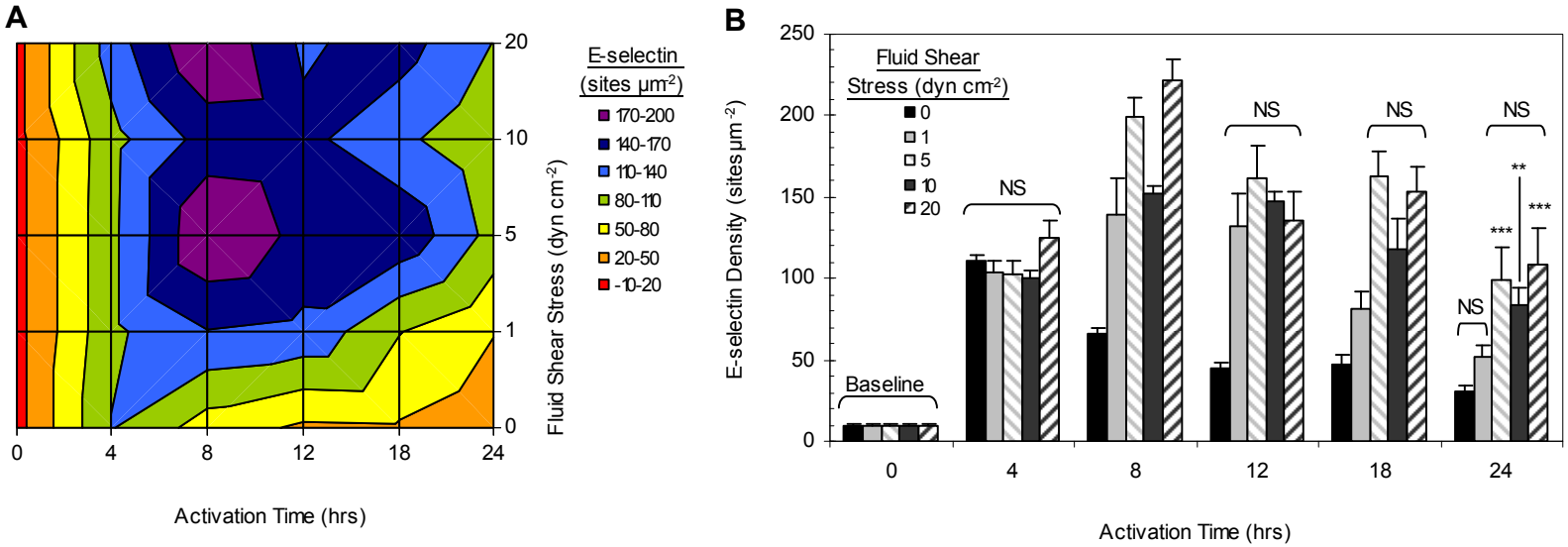

Figure 3.5. E-selectin expression on 0-20 dyn/cm^2 shear-cytokine activated monolayers. *(A) Contour plot of E-selectin expression by HUVEC monolayers activated with 0.1 ng/mL IL-1ß either under static conditions (no fluid shear) or simultaneously exposed to 1, 5, 10 or 20 dyn/cm^2 of laminar fluid shear at activation periods ranging from 0 to 24 hr. Note: Plot generated from data collected at discrete time points and shear levels as indicated on the x and y axes, respectively. (B) Bar plot of same data. "NS" indicates no significance in highlighted groups; at 24 hrs, significances from 24 hr static activation is indicated for each shear-cytokine magnitude*

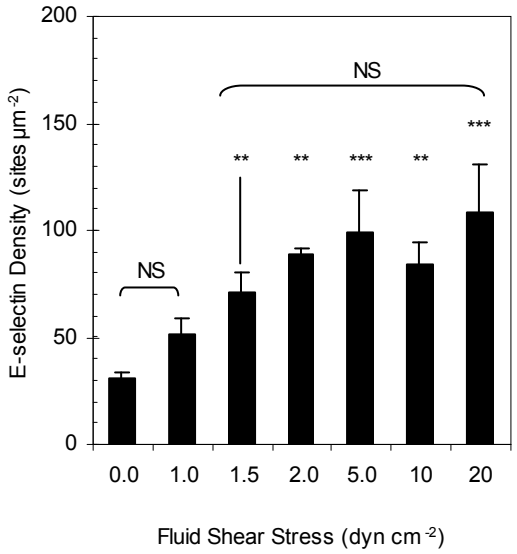

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Figure 3.6. 24 hrs comparison for shear-cytokine activated monolayers. *E-selectin expression by HUVEC monolayers activated with 0.1 ng/mL IL-1ß either under static conditions (no fluid shear) or simultaneously exposed to 1, 1.5, 2, 5, 10 or 20 dyn/cm^2 of laminar fluid shear at 24 hrs activation. Indicated individual significance notations are compared to selectin expression for static (0 dyn/cm^2) activated monolayers.*

Chapter 3.3

Mechanism of Shear-Influenced E-selectin Synthesis

To determine whether *de novo* expression of E-selectin molecules (rather than altered downregulation of previously expressed molecules) contributed to the higher site density (relative to static) observed in shear-cytokine activated ECs at the 8-12 hr mark, experiments were conducted with cycloheximide (CHX), an inhibitor of protein synthesis in eukaryotes. Specifically, a non-lethal concentration of CHX was added to the media (1.0 μg/mL) over EC monolayers that had been activating for 4 hr in static or shear conditions (thus CHX only affected *de novo* protein synthesis beyond 4 hr). Monolayers treated with CHX remained under static or shear-cytokine activation for up to 12 hr; subsequently, E-selectin levels were quantified at the 12 hr mark. As shown in Figure 3.7, the 12 hr E-selectin expression for monolayers exposed to shear-cytokine activation in the presence of CHX is significantly lower than values measured for monolayers exposed to shear-cytokine activation alone but was not significant from values measured for static activation in the presence or absence of CHX. This indicates that the increased selectin expression observed for shear-cytokine activated naïve ECs is due to contributions from newly synthesized E-selectin being upregulated to the apical surface and not due to a delayed or altered downregulation of already expressed selectins.

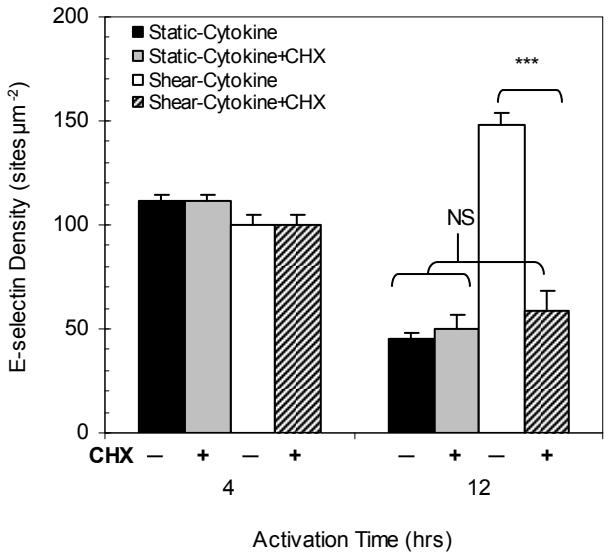

Figure 3.7. E-selectin expression on CHX-treated monolayers. *E-selectin expression on cycloheximide-treated monolayers. CHX was added to HUVEC monolayers at 4 hr activation time in both static ("Static-Cytokine+CHX," gray) and shear-cytokine (10 dyn/cm^2, "Shear-Cytokine+CHX," diagonal) conditions. Static ("Static-Cytokine," solid) and shear-cytokine ("Shear-Cytokine," clear) controls were not treated with CHX.*

Chapter 3.4

Endothelial Morphology

As discussed previously, endothelial cells are known to be highly responsive to fluid shear forces. When initially cultured *in vitro*, naïve ECs are in a squamous cobblestone-like shape, but upon perfusion onset these cells begin to realign in the direction of flow and adopt an elongated morphology. This phenotypic change can be quantified by calculating each individual cell's shape factor (*SF*, see Chapter 2.4). Figure 3.8 shows the shape factor values for ECs subjected to static activation, shear-only perfusion (10 dyn/cm^2), and shear-cytokine activation (10 dyn/cm^2) up to 24 hrs. Initially, cultured monolayers at 0 hrs display a high *SF* = 0.75-0.80, but on perfusion whether with or without IL-1β cytokine, *SF* values drop significantly to 0.53-0.57. By 8 hrs perfusion, *SF* values are significantly lower than the 4 hr values and remain unchanged up to 24 hrs perfusion. This time frame coincides with that for which naïve EC monolayers were previously observed to realign and elongate in the direction of flow in response to shear [138-140]. This observation further highlights the active role that cell cytoskeleton may have in regulating E-selectin protein expression as previously suggested by others [141-142]. The elongation and realignment in the direction of flow of individual cells has been hypothesized as a response to reduce physical stress (and

subsequent injury) and local mechanical load on cell adhesions (to basal tissue, the extracellular matrix or other neighboring cells) [143-144].

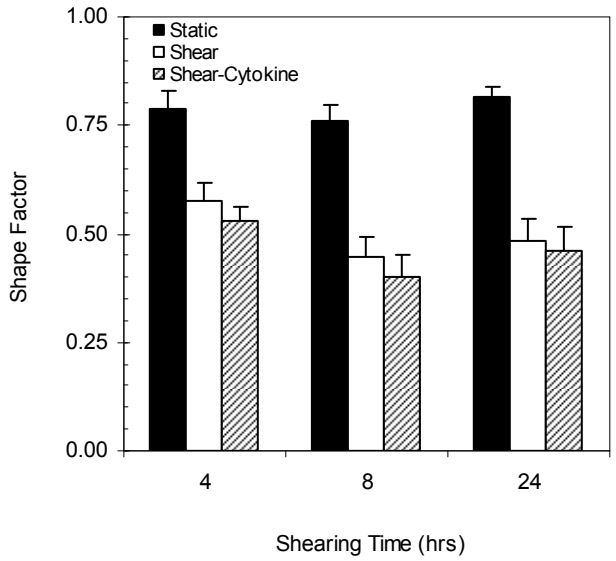

Figure 3.8. Endothelial cell shape factors. *Shape factor values were calculated and averaged for statically activated monolayers, shear-only exposed monolayers, and shear-cytokine activated monolayers at 4, 8, and 24 hrs.*

Chapter 3.5

Endothelial Functionality

Literature has also reported that E-selectin expression level on endothelial cells correlates with the level of leukocyte adhesion during inflammation response [104, 145-147]; to test the functionality of the proposed *in vitro* model, shear-cytokine activated monolayers was evaluated relative to static activated ECs via neutrophil flow adhesion assays. As shown in Figure 3.9, HUVEC monolayers subjected to shear-cytokine activation at 10 dyn/cm^2 show similar neutrophil binding densities for 4 hr treated cells (in agreement with previous protein expression data, *ref* Figure 3.4), but support significantly higher adhesion (1.6-2.4 folds higher) for 8-24 hr-treated monolayers when compared to static activated ECs. Static activated monolayers showed maximum binding for 4 hr-treated cells and nearly half that value for the 24 hr-treated monolayer – a linearly decreasing trend that is observed with protein expression data. Interestingly, neutrophil binding densities did not significantly differ for all 4-24 hr shear-cytokine treated monolayers, indicating that shear-cytokine treatment of naïve ECs yields a pro-inflammatory (*i.e.* prolonged neutrophil/white blood cell recruitment) phenotype.

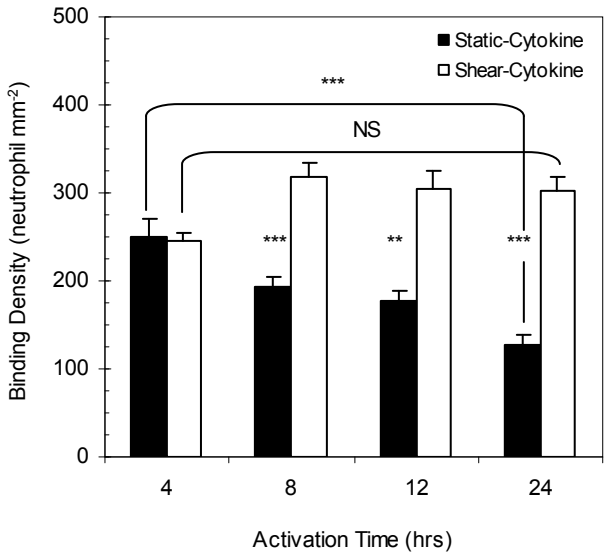

Figure 3.9. Neutrophil binding assay for activated monolayers. *Neutrophil binding density over HUVEC monolayers activated with 0.1 ng/mL IL-1ß under static culture or 10 dyn/cm^2 shear for 4, 8, 12, and 24 hr periods.*

Chapter 3.6

Discussion

The E-selectin expression trend presented in Figure 3.1 is comparable to observations reported in literature. Peak selectin expression has been documented to occur between 4-6 hrs in static activated cells; the slight increase in selectin expression at 20-24 hr mark for statically activated monolayers has also been previously reported in the literature [101, 148]. Additionally, as was observed in shear-only HUVEC treatment, E-selectin expression is typically not inducible by laminar shear alone [104-105, 149]. Morigi, *et al*. similarly observed no significant E-selectin density change from basal expression when ECs were exposed to moderate laminar shear alone, but contrary to the data reported here, they reported that E-selectin expression linearly increased with time in response to static IL-1β activation [104]. In a separate study, Sampath, *et al*. reported slightly different results, showing that E-selectin expression was downregulated within the first hour of exposure to both low and moderate laminar shear in the absence of IL-1β relative to statically cultured ECs [103].

When compared with data in the literature, results from this initial set of experiments shows that the *in vitro* model is a valid design. Monolayers consist of positively identified endothelial cells (via PECAM-1 stain and morphology observations for shear stimulated cells) and respond to IL-1β activation in producing E-selectin site densities in trends reported in other studies. Additionally, the immunofluorescent microscopy protocol used to determine selectin densities provides reproducible measurements.

The presented data shows that the co-stimulation of naïve HUVEC monolayers with flow-induced shear and IL-1β induces expression of significantly higher levels of apical E-selectin up to 24 hrs when compared with monolayers exposed to either IL-1β or laminar shear alone. The observed 8-12 hr peak shifts and elevated expression levels suggest that the *presence* of shear combined with IL-1β stimulation elicits significant change in EC behavior. Additionally, EC response via E-selectin expression for nearly all time points except for long term 16 and 24 hrs seems to be insensitive to the *magnitude* of shear imposed. At the 24 hr mark, a minimum shear magnitude threshold seems to exist that is required to elicit an elevated E-selectin density.

The aforementioned study by Morigi, *et al*. that observed a positive correlation between E-selectin density and static activation time also studied leukocyte adhesion; curiously, despite the same level of leukocyte adhesion on 6 hr statically-activated cells and shear-only exposed cells, the group reported nearly 2.5 fold increase in selectin expression for the IL-1β treated monolayers [104]. This contradicts what other studies have found, in that selectin expression positively correlates with neutrophil binding density [145-147, 150-152]. Additionally, Ji, *et al.* reported different results – ECs treated with 10 dyn/cm^2 shear for 5 hrs simultaneously with cytokine activation resulted in nearly 3 folds less neutrophil interaction when compared to statically activated monolayers [149]; however, Ji, *et al*. used a different cytokine, TNFα, which is known to effect a different signaling pathway.

Even *in vivo* studies done by Eriksson, *et al.* showed attenuated (a nearly 2 fold decrease) of leukocyte capture on inflamed mice endothelium treated with antibodies against selectin relative to untreated populations [153] A similar *in vitro* experiment

reported for IL-1β-treated HUVECs E-selectin expression profiles much akin to the observed data here (maximum at 4 hrs, decreasing to baseline values by 24 hrs) and also found that E-selectin-targeted antibodies reduced leukocyte adhesion by nearly 40% [154].

In a recent report by Tsou, *et al.*, the authors exposed an EC monolayer to a range of flow shear magnitudes (0-12 dyn/cm^2) and did not observe any noticeable differences in recruitment during a monocyte flow adhesion assay; they concluded that modification of EC morphology had "minimal impact" on adhesion [155]. However, Tsou, *et al*. had only sheared the monolayer for 4 hrs and had previously reported no significant morphological elongation – more importantly, these shearing durations were done in the absence of any cytokine and as their controls indicated, no E-selectin was produced regardless of the magnitude of shear.

Both E-selectin protein expression data and neutrophil binding assays indicate that shear-cytokine activation of naïve ECs yields strong pro-inflammatory phenotypes over an extended period of time. While this stimulation displays an E-selectin expression pattern not typically observed under normal physiological inflammation response, it has considerable relevance in the pathological inflammatory response to ischemia (a condition where blood vessel occlusion prevents adequate blood flow to continue downstream) and subsequent reperfusion.

Ischemia/reperfusion (I/R) injury is the principal cause of tissue damage in strokes, myocardial infarctions, pulmonary bypasses, and other vascular surgeries where blood supply is transiently disrupted triggering prior to reperfusion. During ischemia, endothelium downstream of the obstruction may acquire a naïve-like phenotype (a

cobblestone morphology similar to statically cultured cells) dependent on the extent of ischemia as it has been shown that elongated endothelial morphology conferred by flow is known to be reversible upon cessation of flow [136-137]. Upon ischemia relief, or blood flow reperfusion, wherein cytokine present at the blockage site is delivered *simultaneously* with flow induced-shear to downstream endothelial cells, ECs display pro-inflammatory phenotypes and an E-selectin expression pattern as observed in the presented shear-cytokine activation data (Figure 3.5). Indeed, past experiments have reported evidence of increased E-selectin expression in ischemic cerebral vasculature occurring within 4 hrs of reperfusion and persisting for up to 24 hrs [156-158]. Thus, it is possible that a hyper-inflammatory EC phenotype during flow reperfusion in the presence of IL-1β in part contributes to the high level of leukocyte recruitment that has been suggested as a cause of I/R injury [159-162]. On a therapeutic note, one study demonstrated a clinical advantage in acute anti-inflammatory treatment of post-I/R vasculature in reducing neutrophil accumulation by blocking E-selectin functionality with antibodies [158]. Other studies, though performed on canine subjects, observed similar reductions in myocardial and coronary artery reperfusion injuries when neutrophil accumulation was attenuated after treatment with antibodies against Mac1 and LFA-1 (EC-interacting ligands on leukocytes) [163-164]. Similar work focused on blocking cytokine receptors for TNFα, prevented development of pulmonary edemas in hepatic I/R injury [165].

Chapter 3.7

Conclusion

The presented *in vitro* model of co-stimulation of HUVEC monolayers with fluid induced-shear and IL-1β, results in a unique E-selectin expression profile and functional neutrophil capturing capability not seen in static-activation or shear-only stimuli. While the literature does correlate the short term acute response of static and shear-cytokine endothelial response to IL-1β activation, the observed data in this thesis broadens this time frame to a longer 24 hrs, more relevant to chronic inflammation. The prolonged, increased E-selectin expression observed in shear-cytokine activation of naïve ECs resembles that of disease-prone endothelium typical of disturbed flow regions usually found in highly branched, bifurcated areas of the vasculature where regions of stagnant and even reverse flow exist. This disease profile may be applicable for studying pathological models of inflammation, specifically ischemia/reperfusion injury which could have significant impact in identifying and distinguishing disease-prone from healthy endothelial cells.

Chapter 4.0

Increasing Physiological Relevance of the *In Vitro* Model

The previous iteration of the *in vitro* model observed *naïve* HUVEC response to simultaneous shear-cytokine activation with IL-1β and laminar fluid shear. However, naïve endothelial cells present a cobblestone-like morphology, typically encountered in disturbed, low shear regions of the vasculature or at downstream regions of prolonged ischemic vessels. This model, while representative of disease-prone endothelium, does not predict the behavior of healthy vascular ECs which typically bear a streamlined, elongated shape. In order to simulate inflammation of a healthy physiologically relevant endothelium, the *in vitro* model will have to use ECs with this elongated phenotype prior to any shear-cytokine activation. By doing so, E-selectin expression patterns can be measured for healthy ECs under inflammation and compared to the patterns observed for disease-prone ECs as studied in the previous *in vitro* model. To achieve an elongated shape, endothelial cells are preconditioned (also referred to as "presheared" or "PS") with low or high fluid shear (in the absence of any IL-1β cytokine) for a period of time. Inflammation is then simulated with shear-cytokine activation for both acute and chronic time frames and E-selectin density is measured.

While preconditioning naïve endothelium addresses the physiological relevance of the cell phenotype, cytokine concentration will need to be maintained at a high level in

order to simulate appropriate chronic inflammation conditions. The activation models presented in the previous chapters introduced only a single initial bolus of IL-1β; throughout the course of the activating period no other exogenous IL-1β is added to the system despite a likely decrease in IL-1β concentration due to consumption by endothelial cells and degradation from enzyme activity as has been reported in the literature [166-168]. In order to simulate persistent inflammation stimulus, monolayer samples will be redosed – that is, concentrated doses of IL-1β will be periodically added to the culture media. This modification to the *in vitro* model will provide a more physiological representation of *chronic* inflammation during which a persistent concentrated level of cytokine is maintained, allowing for a more accurate determination of the effects of fluid shear on EC inflammation response.

Chapter 4.1

IL-1β Activation of Preconditioned Endothelial Cells

Monolayers were preconditioned with low (1 dyn/cm^2) or high laminar fluid shear (10 dyn/cm^2) for up to 20 hr (in the absence of any inflammatory cytokine) prior to shear-cytokine activation at the same shear magnitudes. As shown in Figure 4.1A, ECs perfused for 4 hr with high shear prior to shear-cytokine activation displayed E-selectin expression levels comparable to shear-cytokine activation of naïve monolayers (clear bars, "0 PS") with maximum expression occurring at 8-12 hr during shear-cytokine activation. A significant change in selectin expression is observed when monolayers were exposed to high shear preconditioning for longer periods of 8, 12, and 16 hr prior to 4 hr shear-cytokine activation: selectin levels are 1.3-1.5 folds higher than naïve monolayers in 4 hrs static or shear-cytokine activated conditions. However, for 20 hrs preconditioned ECs, a 4 hr shear-cytokine activation period induced a low level of E-selectin expression, nearly 2 folds lower than that observed for naïve shear-cytokine activated cells (Figure 4.1C).

Beyond the 4 hr shear-cytokine activation time point, all presheared monolayers exhibited E-selectin at levels lower than their corresponding shear-cytokine control. Monolayers subjected to 8 hr PS expressed higher levels of selectin (2-2.6 folds higher) relative to static control at all shear-cytokine activation times greater than 4 hr. Relative

to 8 hrs static activation, E-selectin expression levels also remained elevated for 12 hr PS monolayers (1.4 fold higher) but not for 16 hr PS cells. E-selectin expression on both 12 and 16 hr PS monolayers dropped to levels not significant from static control at the 12 hr activation mark, but both returned to significantly different levels from static at the 16 hr mark.

Contrary to observations for high shear preconditioning, monolayers preconditioned for 8 or 12 hr at 1 dyn/cm^2 magnitude (Figure 4.1B) showed a muted response to shear-cytokine activation at the same magnitude of shear, displaying significantly lower E-selectin expression when compared to the corresponding shear-cytokine activated controls at all activation times.

Despite the different trends observed in E-selectin expression between shear-cytokine activation of high shear preconditioned (Figure 4.1A) and naïve cells (Figure 3.5B), both sets of data share a significant trend. By focusing on total shear exposure time (as depicted in Figure 4.2), in both naïve and preconditioned ECs, the maximum protein expression in response to activation occurs after cells had been perfused for at least eight hours (in the presence or absence of cytokine). For example, in naïve cells, selectin expression is at a maximum in the 8-12 hr range after the start of shear-cytokine activation, regardless of shear magnitude. For preconditioned ECs, peak expression for 4 hrs PS cells occurs after an additional 8-12 hrs shear-cytokine activation – or a total 12 hrs of perfusion; similarly, the 8-16 hrs PS cells almost all immediately express high selectin levels after 4 hr shear-cytokine activation. This phenomenon can be explained by considering fluid shear's effect on EC morphology.

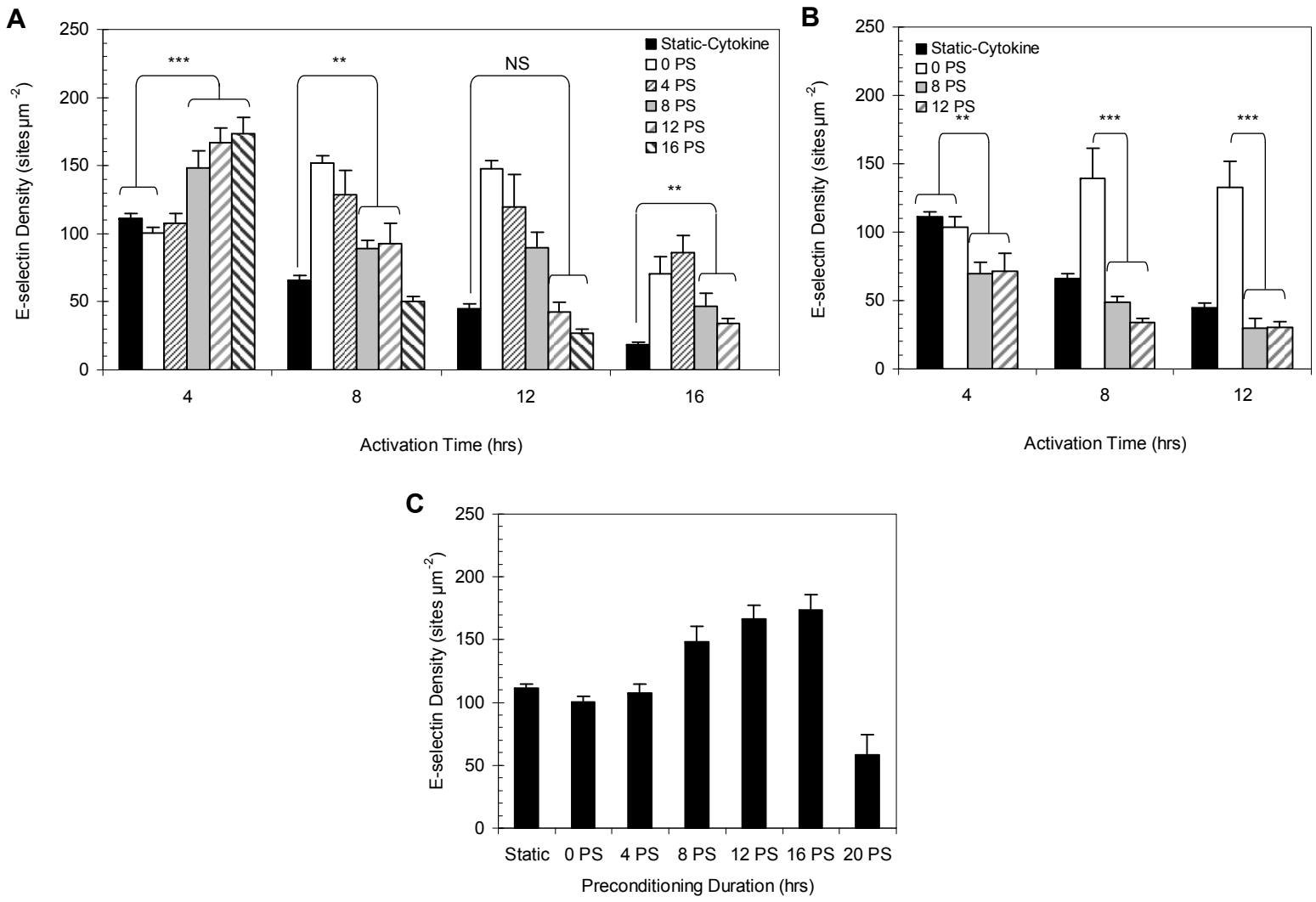

Figure 4.1. E-selectin expression on preconditioned monolayers. *Site density on HUVECs preconditioned with (A) 10 dyn/cm^2 or (B) 1 dyn/cm^2 of laminar shear for 4, 8, 12, 16 or 20 hr ("4PS," "8PS," "12PS," "16PS," and "20PS," respectively) prior to activation with 0.1 ng/mL IL-1ß under similar magnitude of shear. Filled bar = static activation controls and clear bar = "0 PS" or non-preconditioned monolayers.*

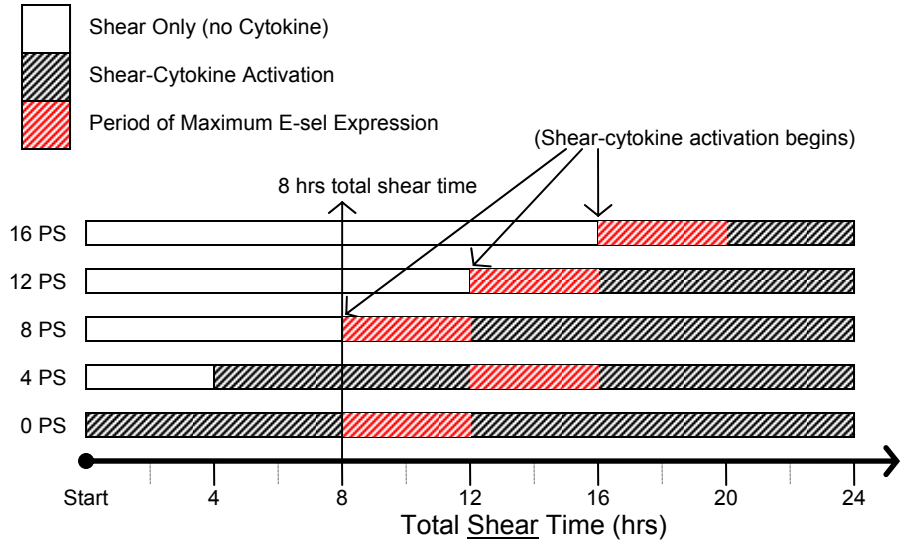

Figure 4.2. Timeline of exposure to fluid shear. *HUVEC monolayers under different experimental conditions are subjected to different periods of shear stress (perfusion), with or without cytokine activation. Clear bars indicate preconditioning – perfusion without any cytokine present; black diagonal bars indicate shear-cytokine activation; red diagonal bars indicate periods of time when E-selectin site density has peaked.*

Chapter 4.2

Fluid Shear Effects on Redosed Endothelial Cells

In order to simulate chronic inflammation (maintained exposure to a constant IL-1ß concentration), monolayers were subjected to multiple doses ("redosing," hereafter) of IL-1ß over time. Specifically, HUVEC monolayers were activated with an initial 0.1 ng/mL IL-1ß dose under static or fluid shear (10 dyn/cm^2) conditions. Every four hours, spent culture media was removed from cells and replaced with fresh media containing a fresh dose of IL-1ß.

When activated, endothelial cells produce both pro-inflammatory chemicals (among them IL-1ß) and enzymes such as matrix metalloproteinases (MMPs) that have been implicated in the degradation of IL-1β [166, 169-170]; removal of spent culture media will also remove any locally produced chemicals. To address the concern that removal of spent culture media would impact the local concentration of IL-1β, a parallel control set of monolayers was also prepared for both static and shear-cytokine activated cultures to test a different redosing method: instead of replacing spent media with fresh media, a concentrated IL-1ß dose was added directly to the present culture media. This method retains any locally synthesized cytokines such as IL-1ß and degradative enzymes (*e.g.* MMPs) in solution. As shown in Figure 4.4, no significant differences in E-selectin expression densities are observed in the two redosing methods. Since vascular endothelial

cells are exposed to fluid shear flow, endogenously synthesized chemicals are likely removed from the local environment preventing excessive buildup; to simulate this, the redosing protocol of complete media replacement is used in all activating conditions.

For static cultures, monolayers that were redosed expressed similar E-selectin densities when compared to non-redosed controls at the 4 and 8 hr marks as shown in Figure 4.3A. At 12 and 24 hrs, there was an observed significant difference in expression, with redosed samples displaying higher selectin densities (1.8 and 5.2 fold higher, $p < 0.01$). Similarly, for shear-cytokine activated cultures, monolayers that were redosed expressed similar E-selectin densities as non-redosed controls at 4 and 8 hrs (as shown in Figure 4.3B). At 12 hrs, however, there was not a significant difference between redosed and control as there was in the static cultures. A significant increase in E-selectin expression for redosed shear-cytokine activated samples was only realized at 24 hrs (1.7 fold higher, $p < 0.01$). The effect of redosing on shear preconditioned ECs was also studied (as shown in Figure 4.3C). Monolayers were exposed to 8 hr shear preconditioning at 10 dyn/cm^2 followed by shear-cytokine activation for up to 16 hr. At all time points, E-selectin expression was not significantly different between redosed samples and preconditioned controls.

In summary, redosing of high shear preconditioned ECs did not induce higher E-selectin expression even at extended shear-cytokine activation times (Figure 4.3C); in contrast, a positive effect was observed at earlier activation times for monolayers perfused for a shorter duration (shear-cytokine activated cells, Figure 4.3B) and even earlier for monolayers not exposed to any fluid shear at all (statically activated cells, Figure 4.3A). These three sets of data highlight the robust protection against chronic

inflammation conferred by prior exposure to high laminar shear, *i.e.* a sustained concentration of cytokine alone is not enough to induce pathological expression of E-selectin in healthy vasculature typically subject to high laminar shear.

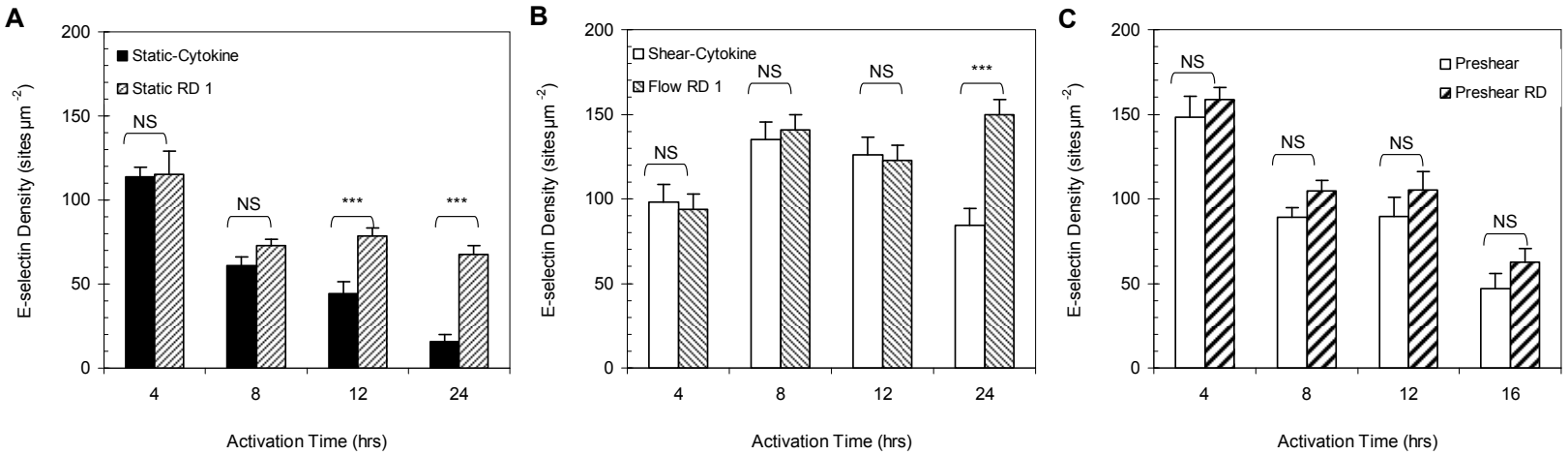

Figure 4.3. E-selectin expression on redosed monolayers. *Redosing HUVEC monolayers in (A) static-only or (B) shear-cytokine activation or (C) preconditioned cultures. Control groups (filled and clear) were given only a single initial IL-1ß dose at the start of experiment. Redosed samples (upward and downward diagonals) were given repeated doses of IL-1ß every 4 hr until immunofluorescence assay at 8, 12, or 24 hr total activation time. Indicated significance values are comparing redosing and control data within the same time point.*

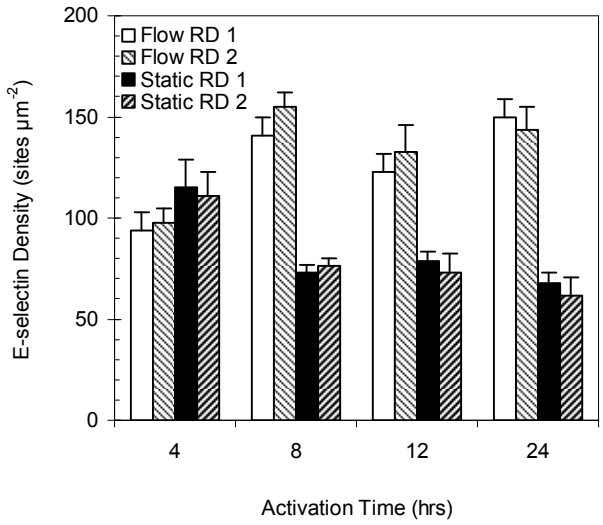

Figure 4.4. Comparing redosing methods. *Two different redosing methods were tested. For "Flow RD 1" (clear) and "Static RD 1" (filled), old culture media was replaced with fresh IL-1β containing media every four hours. For "Flow RD 2" (downward diagonal) and "Static RD 2" (upward diagonal), culture media was not replaced, but a fresh dose of IL-1β was added in a concentrated droplet every four hours.*

Chapter 4.3

Discussion

Literature has generally associated preconditioning with muted inflammatory response. Of the few existing reports, a majority has suggested this correlation and has observed that fluid shear does in fact lower E-selectin expression on presheared cells relative to statically activated endothelium. However, nearly all studies used a long duration of preconditioning followed by short-term shear-cytokine activation (see Figure 4.5 for a time frame chart indicating variations in shearing durations in the literature). For instance, Sheikh, *et al*. preconditioned HUVECs for 24 hr at low and high magnitudes followed by 4 hr shear-cytokine activation with TNFα and observed for low shear preconditioned cells the same E-selectin expression was elicited as that for a statically activated monolayer, whereas at 20 dyn/cm^2 high shear preconditioning expression dropped to baseline levels [171]. In other studies, Glen, *et al.* and Luu, *et al.* also reported similar trends using nearly the same conditions – E-selectin expression on HUVECs after 24 hrs preconditioning at 20 dyn/cm^2 were subjected to 4 hrs of TNFα shear-cytokine activation; when compared to statically activated controls, the presheared cells expressed nearly half the E-selectin density and also exhibited suppressed neutrophil recruitment [151, 172].

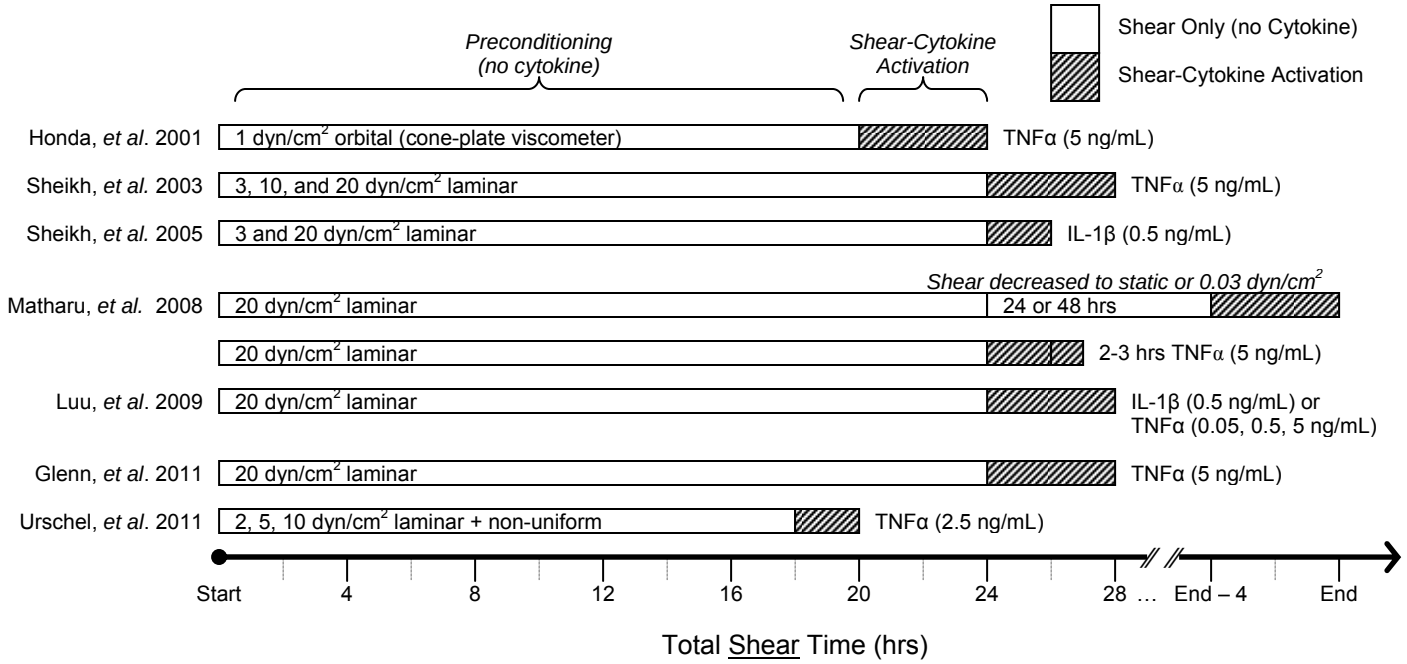

Figure 4.5. Shear-cytokine experimental protocols. *Investigations into simultaneous stimulation of endothelial cells with cytokines in the presence of fluid shear have either been in the immediate acute response or in the long term after a prolonged shear-only preconditioning period.*

However, there are some contradictions in the literature. In a separate study, Sheikh, *et al.* presheared monolayers at low and high (3 and 20 dyn/cm^2, respectively) magnitudes for 24 hrs prior to a short 2 hr shear-cytokine activation with IL-1β: no differences in E-selectin expression were reported for either low or high shear preconditioned cells relative to statically activated endothelium [173]. Additionally, Nagel, *et al*. reported that when HUVEC monolayers were exposed to 24 hrs of preconditioning at 10 dyn/cm^2 followed by a 4 hr shear-cytokine treatment with IL-1β, a marked upregulation of E-selectin from 4 hr static activation control was observed – similar to the data reported in this thesis [105]. Luu, *et al.* also investigated the effects of shear-cytokine activation with IL-1β on preconditioned monolayers and observed for the same shear conditions, E-selectin expression densities at 4 hrs of shear-cytokine were not significantly different from statically activated control monolayers [172].

These observed discrepancies in the literature in E-selectin expression can be attributed to the different methods by which expression is quantified; Luu, *et al.*, Glen, *et al.*, and Sheikh, *et al*. used quantitative polymerase chain reaction (qPCR) to measure mRNA copy, a quantity that prescribes the number of times a gene is transcripted from DNA, not necessarily a 1:1 translation to protein expression on the EC surface. While none of these studies used a direct imaging technique (such as immunofluorescence or flow cytometry), their reported data cannot be ignored; what can be concluded from their reports is that prolonged preconditioning of HUVECs at high shear magnitudes effectively prevents mRNA translation into protein molecules. In a study by Kraiss, *et al.* a similar downregulation of E-selectin on EC surfaces was observed for preconditioned monolayers. The group showed that mRNA transcription was not affected by the

presence of fluid shear, nor was shedding or degradation of E-selectin a factor in the lower expression density; instead, they attributed the decrease to translational mechanisms that they concluded to be moderated by shear [174]. As shown previously in the cycloheximide-treated HUVECs, shear-cytokine activation engenders *de novo* synthesis of new E-selectin; any downregulation of already expressed selectin is mediated by other internalization processes.

Other preconditioning studies looked at slightly shorter perfusion durations. Honda, *et al.* used an orbital cone-plate viscometer to observe the effects on monocyte adhesion of 4 hrs TNFα shear-cytokine inflammation on 20 hrs 1 dyn/cm^2 preconditioned bovine aortic endothelial cells (BAECs): presheared BAECs enhanced by nearly 3.5 fold the number of monocytes bound to the EC surface relative to perfusion-only exposed cells [175]. Urschel, *et al*. perfused HUVECs for 20 hrs at 2 and 5 dyn/cm^2 and under non-uniform shear activating with TNFα for the final 2 hrs; E-selectin densities (and monocyte adhesion) were reported at maximum for non-uniform shear exposed monolayers and nearly 2 fold higher at 2 dyn/cm^2 compared to 5 dyn/cm^2 [152].

Unfortunately, nearly all studies used long preconditioning durations (at least 20 hrs), followed by short-term shear-cytokine activation. The observed data presented in this thesis found that 4 hr shear-cytokine activation of long-term (20 hr PS) high shear preconditioned cells does in fact lower E-selectin expression to levels nearly 2 fold lower than that expressed on statically activated monolayers – in agreement with much of the literature. However, for *shorter* preconditioning durations (8-16 hr PS), it is observed that the subsequent shear-cytokine activation engenders a sharp 1.5 fold increase in selectin expression during the acute inflammation period (4 hrs). Physiologically speaking, this is

indicative of a robust, *healthy* endothelium that expresses high densities of E-selectin in order to recruit leukocytes to rapidly clear inflammatory stimulus. Additionally, during the chronic shear-cytokine activation periods, muted E-selectin response is observed in both low and high shear magnitude treated cells; this is indicative of a *protective* endothelial phenotype that prevents high selectin expression during prolonged exposure to inflammatory stimulus, thus also preventing the over accumulation of white blood cells that leads to disease pathogenesis.

Consistent with previous literature, the presented data shows that shear-conditioned endothelium is prone to a quick, acute inflammatory response and is protective against prolonged chronic inflammation as would exist in physiological occurrences of normal inflammation response [176-177]. This phenomenon is not simply attributed to cumulative shear exposure time as, for example, monolayers preconditioned for 12 hr followed by 4 hr of shear-cytokine activation (or 16 hrs total perfusion) exhibited 2.5 fold higher E-selectin expression compared to naïve cells exposed to 16 hr of shear-cytokine stimulation (166 ± 11.0 vs. 70.5 ± 12.7 sites/μm^2). Indeed, a *minimum shear stress magnitude* and *precondition duration* seems to be requisites for this response: monolayers sheared for less than 8 hrs showed a similar response as naïve monolayers subjected to shear-cytokine treatment. Additionally, monolayers preconditioned under low shear followed by shear-cytokine activation (Figure 4.1B) displayed a markedly absent E-selectin expression relative to 4 hr static activation that is present for high shear preconditioning monolayers.

The observed shear-regulated differential E-selectin expression may be cytokine-specific, as literature suggests. This discrepancy between EC response to IL-1β and

TNFα activation in shear may be due to their distinct cell surface receptors that affect somewhat unique signaling pathways [101, 178-179]. A previous report of 2 hrs TNFα activation of HUVECs following 24 hrs shear perfusion at 20 dyn/cm^2 resulted in an E-selectin expression not significantly different from that observed in static non-inflamed naïve ECs, contrary to the data reported here [171]. The same authors later showed minimal neutrophil adhesion on HUVECs preconditioned with 20 dyn/cm^2 high shear for 24 hr followed by a 3 hr TNFα activation (however, TNFα treatment of HUVECs were done under static or creeping flow conditions – 0.03 dyn/cm^2) [150]. However, Chiu, *et al.* found E-selectin expression for 4 hr TNFα shear-cytokine activated HUVECs preconditioned at 20 dyn/cm^2 for 24 hrs that was 3.5 fold lower than statically activated naïve ECs, but *nearly twice* that for non-activated EC controls [180]. Similarly in a separate study, Cicha, *et al*. also showed 2 hr TNFα shear-cytokine activated 18 hrs preconditioned HUVECs at 10 dyn/cm^2 expressed E-selectin at levels 2 fold lower than statically activated naïve ECs, but higher than non-activated controls [181]. These conflicting reports on the effect of shear preconditioning on EC response to TNFα stimulation will be explored in the next section.

Chapter 4.4

Conclusion

In summary, this data suggests human endothelial cell expression of E-selectin in response to IL-1β stimulation is dependent on the *shear history* of these cells. Naïve cells under high laminar shear perfusion in the simultaneous presence of IL-1β display strong pro-inflammatory phenotypes for an extended period whereas cells first preconditioned with laminar shear exhibit either an elevated or muted inflammatory response dependent on both the magnitude and duration of perfusion during preconditioning and on the time frame of the following shear-cytokine stimulation.

While the initial *in vitro* models exposed naïve cells to shear-cytokine IL-1β activation, simulating disease-prone endothelial response, this model iteration simulates healthy endothelial response. The recorded observations from the shear-cytokine activation of preconditioned cells and redosing experiments suggest that this *in vitro* model significantly contributes to the literature in predicting healthy endothelial behavior under physiological inflammation, especially in the long term time frame during which chronic inflammation is relevant. While literature has reported the short term acute inflammatory response to shear-cytokine IL-1β activation of highly preconditioned endothelial cells, this thesis work broadens the time frame to cover the prolonged chronic duration over which the effects of IL-1β activation can be compared. The quick and rapid

inflammatory response in the acute 4 hr period and muted chronic response in the prolonged 12+ hr range is especially observed in increasingly preconditioned cells indicating a healthy endothelial phenotype typically seen in endothelium of non-branched, well-developed flow regions of long blood vessels. This protective effect of fluid shear stress against chronic inflammation is also observed when endothelial cells are exposed to persistent inflammatory stimulus in the redosing experiments.

Chapter 5.0

Comparing TNFα and IL-1β Activation

The *in vitro* models of shear-cytokine activation of naïve and preconditioned endothelial cells with simultaneous fluid shear and IL-1β cytokine presented in previous chapters have shown that endothelial response to inflammation is dependent on shear history. Specifically, as indicated by unique E-selectin expression trends, my model showed that prolonged exposure to shear induces a strong endothelial phenotype characterized by rapid acute inflammatory response and protective against chronic, persistent stimulus for IL-1β activation. However, as has been noted in the literature, inflammation response is cytokine specific as other pro-inflammatory molecules have been implicated in many disorders [106-108, 182-183]. Reports studying E-selectin expression in EC response to shear-cytokine stimulus have primarily focused on tumor necrosis factor α (TNFα) activation as it has been observed, perhaps misguidedly, that fluid shear has no effect on EC selectin expression when activated with IL-1β. However, typical physiological inflammation occurs under the direction of multiple cytokines, not simply a single activating factor [184-186]; to address this, EC monolayers are co-stimulated with both TNFα and IL-1β to determine whether the observed effects due to

shear-IL-1β activation remains relevant even under more complex inflammatory conditions.

To determine if the observed shear effects on IL-1β activated endothelial cells is indeed cytokine-based and only unique to IL-1β, a similar comprehensive analysis was performed using the same shear-cytokine assays on TNFα-activated cells. E-selectin expression densities for statically activated naïve cells and shear-cytokine activated naïve and preconditioned cells were measured.

Chapter 5.1

TNFα Activation of Naïve Endothelial Cells

As was the case with the IL-1β experiments, a saturating concentration of TNFα was used in order to elicit maximum E-selectin response (observed at 4 hrs post activation); a brief review of literature shows other groups have used TNFα concentrations between 10 pg/mL and 100 ng/mL [187-190]. In studying physiological levels of TNFα in reperfused endothelium, Wyble, *et al.* reported a saturating concentration of 10 pg/mL *in vivo*, but showed maximum E-selectin expression of *in vitro* HUVECs at 4 hrs activation at 1 ng/mL concentration [190]. Saperstein, *et al.* noticed that TNFα was slightly toxic at the higher 100 ng/mL concentration (likely due to its function in permeabilizing and destabilizing monolayers [191-192]) and used 5 ng/mL in their *in vitro* studies, a concentration that was not observed to affect cell viability [188]. Based on the literature, for all experiments involving TNFα, a concentration of 10 ng/mL was used to elicit the same maximum E-selectin expression at 4 hrs as observed for 0.1 ng/mL IL-1β activation. This concentration was not observed to induce cell apoptosis or affect monolayer integrity at high fluid shear for long term 24 hr studies.

HUVEC monolayers were exposed to static activation with 10 ng/mL TNFα for up to 24 hrs. E-selectin site density measurements were made at 4, 8, 12, and 24 hrs to determine an expression trend. As seen in Figure 5.1A, E-selectin density is highest at 4

hrs (151.2 ± 8.9 sites/μm^2) and steadily decreases but does not return to non-activated baseline expression (9.0 sites/μm^2) by 24 hrs. At 12 hrs static activation, , E-selectin expression is observed to decrease by 2.4 fold to 63.7 sites/μm^2 relative to the maximum static-cytokine stimulated expression at 4 hrs; static-cytokine selectin density then increases, thought not significantly from 12 hrs static activation, to 85.9 ± 16.1 sites/μm^2 by 24 hrs.

Next, naïve monolayers were simultaneously activated with TNFα cytokine and 10 dyn/cm^2 fluid shear perfusion. As shown in Figure 5.1A, E-selectin expression significantly increases ($p < 0.001$) from 92.6 ± 7.0 to an overall maximum 139.6 ± 8.2 sites/μm^2 at 4 and 8 hrs of shear-cytokine activation, respectively. From 8 to 12 hrs, the expression slightly decreases ($p < 0.05$) to 108.6 ± 12.7 sites/μm^2; by 24 hrs, selectin density is at 81.8 ± 11.2 sites/μm^2. At the 4 hr activation mark, E-selectin expression on shear-cytokine activated monolayers is significantly lower ($p < 0.001$) by 1.6 fold than expression on statically activated monolayers. At 8 hrs, however, shear-cytokine activated monolayers display significantly higher selectin densities ($p < 0.05$) and by 12 hrs, values are 1.7 fold higher than those for static-cytokine activated cells. At 24 hrs, both sets of E-selectin expressions are not significantly different.

When compared to data obtained from 0.1 ng/mL IL-1β static activation of HUVECs, at all activation times (Figure 5.1B), selectin expression for TNFα activated monolayers are significantly higher (by 1.4-2.8 folds). Despite this difference, the two trends are similar – both TNFα and IL-1β-activated cells show a linear decrease in early time points (4-12 hrs), as has been reported in direct comparisons of the cytokines in the literature [101, 190, 193].

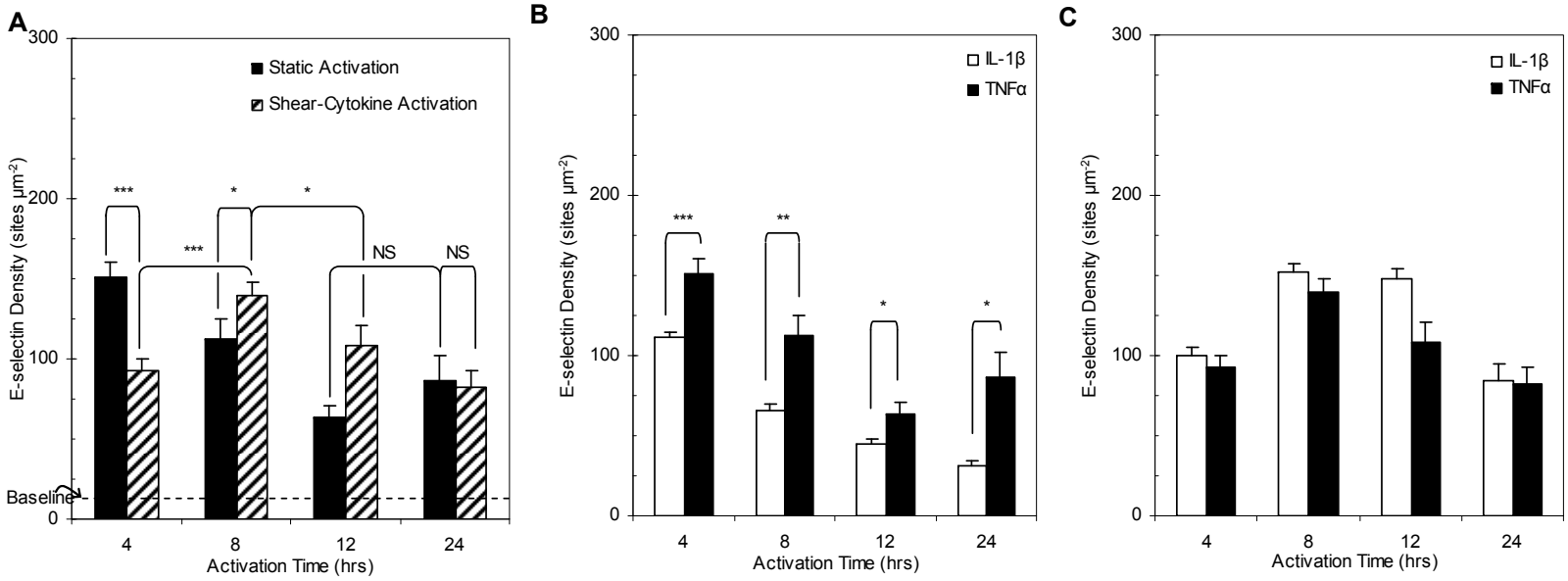

Figure 5.1. E-selectin site density of TNFα or IL-1β activation. *HUVEC monolayers were treated with 10 ng/mL TNFα (filled, diagonal stripes) or 0.1 ng/mL IL-1β (clear) for up to 24 hrs. (A) Static-cytokine and shear-cytokine activation for TNFα; comparison between IL-1β and TNFα for (B) static-cytokine; (C) shear-cytokine at 10 dyn/cm^2.*

Chapter 5.2

TNFα Activation of Preconditioned Endothelial Cells

To investigate the effects of shear-cytokine TNFα activation on preconditioned endothelium, HUVEC monolayers were subjected to 12 hrs of 10 dyn/cm^2 perfusion prior to 4-12 hrs of shear-cytokine activation. As shown in Figure 5.2, E-selectin expression densities slightly decreased (not significantly, $p = 0.07$) from 4 to 8 hrs activation after 12 hrs preconditioning; between 8 to 12 hrs, the expression level dropped more significantly ($p = 0.02$) from 57.4 to 42.5 sites/μm^2. Overall, when compared to statically activated monolayers, the preconditioned cells express a similar downward trend in E-selectin density, albeit significantly lower at each time point (2.1, 2.0, and 1.5 fold lower at the 4, 8, and 12 hrs marks, respectively). When compared with E-selectin expression of shear-cytokine activated naïve monolayers, E-selectin densities on preconditioned cells are significantly lower at 8 and 12 hr ($p < 0.001$ and $p < 0.05$, respectively), but are not significantly different at the earlier 4 hr mark.

As seen in Figure 5.2B, with increasing activation time, the fold differences between IL-1β and TNFα activation of preconditioned cells decreases from 2.3 fold higher at 4 hrs to 1.6 fold at 8 hrs and finally the same level expression is reached at 12 hrs. There is also no initial sharp increase at 4 hrs shear-cytokine activation as is observed for IL-1β activated cells indicating that physiologically, healthy umbilical vein

endothelium (*i.e.* displaying elongated, flow-aligned morphologies) display more muted response to TNFα-induced stimuli.

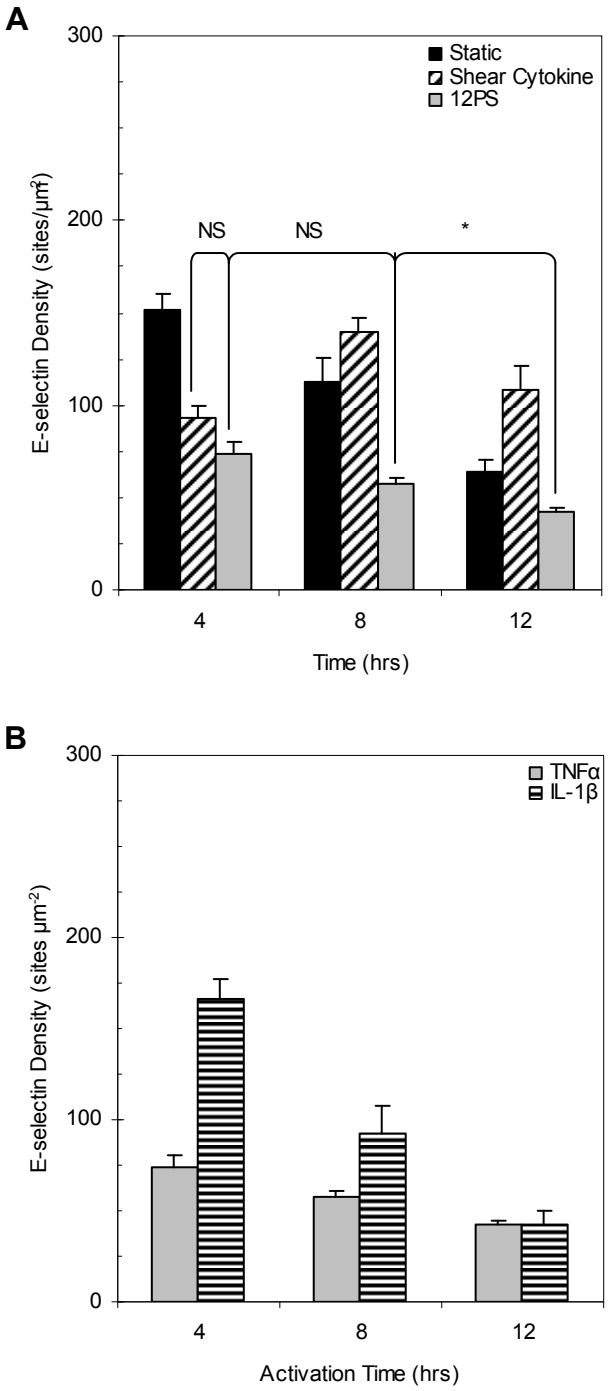

Figure 5.2. E-selectin site density of TNFα or IL-1β activation of preconditioned cells. *HUVEC monolayers were treated with 10 ng/mL TNFα or 0.1 ng/mL IL-1β for up to 12 hrs under shear-cytokine activation after 12 hours preconditioning at 10 dyn/cm^2. (A) Static-cytokine (filled), shear-cytokine (upward diagonal), and shear-cytokine activation of preconditioned monolayers (grey) for TNFα; (B) comparison between TNFα (grey) and IL-1β (horizontal stripes) shear-cytokine activated 12 hr preconditioned monolayers.*

Chapter 5.3

TNFα and IL-1β Co-Stimulation

As mentioned earlier, inflammation typically involves activation through multiple cytokines. To determine whether the observed effects of IL-1β activation remains relevant in a more complex inflammation scenario, and to also increase the *in vitro* model's physiological relevance, HUVECs were co-stimulated with both IL-1β and TNFα. For these assays, the same concentrations of cytokines are used: 10 ng/mL TNFα and 0.1 ng/mL IL-1β. Static and shear-cytokine activation were considered for naïve cells while shear-cytokine activation was considered for preconditioned cells.

As shown in Figure 5.3A, the combined stimulation of both cytokines yields a higher E-selectin expression response under each activation conditions. For statically activated monolayers, maximum E-selectin density (215.7 ± 7.5 sites/μm^2) is observed at 4 hrs; expression decreases linearly to significantly lower values of 145.1 and 90.9 sites/μm^2 at 8 and 12 hrs, respectively. This decreasing trend is similar to trends observed in monolayers individually activated by IL-1β or TNFα alone. At 4 hrs, expression in combined-cytokine activated monolayers is significantly higher than that for monolayers activated by either cytokine alone. Similar expression comparisons are evident at 8 hrs and 12 hrs maintaining an average 1.3 and 2 fold higher expressions than that for TNFα and IL-1β-treated cells, respectively.

For naïve cells simultaneously activated under fluid shear and dual-cytokines (Figure 5.3B), maximum expression occurs at 8 hrs (242.0 ± 20 sites/μm^2), significantly higher than densities at 4 and 12 hrs (149.3 and 185.8 sites/μm^2, respectively); E-selectin density at 12 hrs is also significantly higher than that at 4 hrs. Compared to the monolayers activated by either cytokine alone, at all time points, selectin expression is significantly higher by an average of 1.7 and 1.4 fold for TNFα and IL-1β-treated cells, respectively.

Finally, for 12 hrs preconditioned EC monolayers (Figure 5.3C), combined stimulation of both cytokines yields maximum E-selectin expression at 4 hrs (194.4 ± 2.3 sites/μm^2). Expression then decreases at longer activation times (to 118.9 and 73.5 sites/μm^2 at 8 and 12 hrs, respectively). Compared to preconditioned monolayers activated only by TNFα, the selectin expressions are on average 2.1 fold higher. However, relative to monolayers activated only by IL-1β, at 4 and 8 hrs, combined-cytokine activation is not significantly higher.

The observed data for co-stimulation of HUVECs with both TNFα and IL-1β, seems to correlate with selectin expression for shear-IL-1β activated monolayers. For all conditions of dual-cytokine stimulation, static activation of naïve ECs and shear-cytokine activation of both naïve and preconditioned ECs, the selectin expression trend over 24 hrs is similar to that observed for shear-IL-1β only – in fact, despite having overall higher expression densities, the more perfusion the ECs are exposed to, the less significantly different the E-selectin densities are between dual-cytokine and shear-IL-1β-only activated monolayers. This indicates that the observed selectin expression trend for shear-

IL-1β-only activated monolayers is still relevant even under more complex activation conditions.

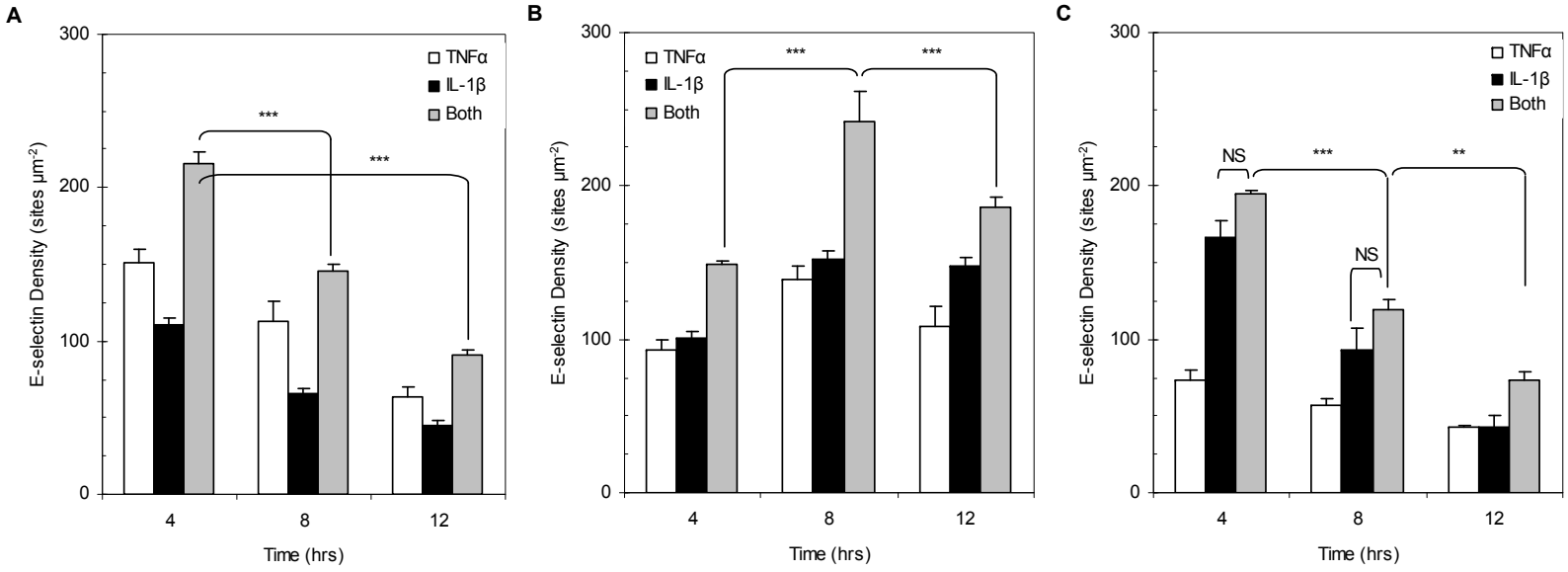

Figure 5.3. E-selectin site density of combined and individual TNFα or IL-1β activation. *HUVEC monolayers were treated with 10 ng/mL TNFα (clear), 0.1 ng/mL IL-1β (filled), or simultaneously with both cytokines at the same concentrations (grey) for up to 12 hrs under (A) static activation and (B) shear-cytokine (10 dyn/cm^2) activation of naïve cells and (C) 12 hrs preconditioned monolayers.*

Chapter 5.4

Discussion

The observed downward trend in selectin expression for statically TNFα-activated ECs is slightly different than what has been reported in literature; a majority of the reported E-selectin densities in past studies showed no significant differences between TNFα and IL-1β activated cells, especially during long term inflammation. Wyble, *et al.*, measured E-selectin *via* flow cytometry at 2, 4, 8 and 24 hrs post activation with 10 pg/mL TNFα and reported (relative to baseline expression) an initial maximum at 4 hrs but near baseline values at 24 hrs activation [190]; this is likely due to the fact that Wyble, *et al*. used a very low initial dose of TNFα – by 24 hrs, most activity would have decreased to insignificant levels. Pober, *et al.* showed that TNFα induced similar levels of E-selectin at 4 hrs static activation when compared to IL-1β-induced expression, (unlike the 1.4 fold *higher* expression observed here); the group also measured long term expression at 28 hrs and reported similar values as that for IL-1β activation, but only slightly elevated (13 fold) from the non-activated baseline controls – contrary to the observed 2.8 fold higher TNFα-induced E-selectin expression over IL-1β-activated cells. One study did observe high selectin expression during prolonged inflammation: Daxecker, *et al*. reported a nearly 130 fold increase in E-selectin expression relative to

non-stimulated monolayers when statically activating with 10 ng/mL TNFα for 16 hrs [194].

When considering shear-cytokine activation of naïve monolayers with TNFα, there are few reports in the literature of a shift in maximum E-selectin expression to 8 hrs. Most studies observed that shear-cytokine activation with TNFα attenuates E-selectin expression (and corresponding neutrophil adhesion) in the very short term and have generally accepted that fluid shear – for whatever duration – reduces CAM expression. Few have actually compared selectin densities as a function of time rather choosing to focus on the reduced responses to the presence of fluid shear or shear preconditioning. For instance, as controls in their preconditioning studies, a number of reports investigated EC monolayer exposure to only 2, 3, 4, or 5 hrs TNFα activation, with all observing reduced E-selectin or neutrophil adhesion relative to static activation [149-152, 155, 171-173, 180-181]. Kraiss, *et al.* observed muted expression at 4 hrs activation under 12 dyn/cm^2 shear-cytokine activation relative to static activation [174]. Xie, *et al.* exposed HAECs (human aortic ECs) to TNFα under 12 dyn/cm^2 for 6 hrs, but reported reduced E-selectin expression relative to static activation [195], opposite of what is observed here where shear-cytokine activated monolayers express significantly higher E-selectin ($p < 0.05$) than that for statically activated monolayers.

The widely accepted general conclusion in the literature that fluid shear attenuates CAM expression response is correlated in the presented TNFα data when considering the maximum E-selectin response between shear-cytokine and statically activated naïve cells. The observed data shows that maximum response for shear-cytokine activated monolayers is not significantly different than that observed for statically activated cells;

in fact, the addition of fluid shear actually slightly reduces selectin density. This is opposite for what was observed in the IL-1β data where maximum E-selectin expression for shear-cytokine activated monolayers is a significant 1.4 folds higher than that for statically treated cells ($p < 0.001$).

The muted response to TNFα shear-cytokine activation of naïve cells also applies to preconditioned cells and is especially correlated with numerous similar studies in the literature. Chiu, *et al.* and Cicha, *et al*. each independently showed that long term preconditioned HUVECs when shear-cytokine activated with TNFα for 4 and 2 hrs, respectively, yielded 3.5 and 2 fold lower E-selectin response than that of statically activated cells [180-181]. Urschel, *et al.* reported significantly lower selectin densities relative to 2.5 ng/mL TNFα static activation for 2 hrs shear-cytokine activation of 18 hrs preconditioned EC monolayers [152]. Partridge *et al*., similarly observed nearly 1000 fold lower E-selectin transcription in 2 hr shear-cytokine TNFα activation of 16 hr preconditioned HUVECs relative to statically activated monolayers, despite the evidence provided by Kraiss, *et al.* in that shear stress does not affect E-selectin mRNA transcription [174, 176]. A number of other studies pre-sheared ECs for at least 24 hrs – most at a high 20 dyn/cm^2 magnitude – followed by 2-4 hr shear-cytokine activation periods; measurements of E-selectin protein expression and neutrophil or monocyte adhesions were all reported to have been attenuated relative to statically activated EC controls [150-151, 171-172].

In one of these reports, Matharu, *et al.* preconditioned HUVECs at 20 dyn/cm^2 for 24 hrs. Then, to simulate EC behavior downstream of an ischemic injury (*i.e.* in a blood vessel past an occlusion site), the group exposed the cells for an additional 24-48 hrs to

very low creeping flow (0.003 dyn/cm^2) or to stagnant flow (no shear stress present) prior to 4 hrs activation with 5 ng/mL TNFα [150]. For monolayers exposed to an additional 24 hrs of stagnant flow after 24 hrs of 20 dyn/cm^2 preconditioning, neutrophil adhesion and E-selectin expression dropped nearly 2 fold relative to that measured in naïve monolayers exposed to 4 hrs of static activation; at an additional 24 hrs exposure to stagnant flow, these values recovered to nearly the same as that for naïve controls. However, for monolayers exposed to creeping flow, selectin expression and neutrophil capture were completely abolished, regardless of exposure time. These observations indicated that after initial cessation of flow, ECs grew more responsive to inflammatory activation under completely stagnant flow (physiologically, a complete vessel occlusion), but this responsiveness was inhibited under slow perfusion conditions. The presented preconditioning data suggests the same conclusion: increased perfusion duration increasingly suppresses E-selectin expression. For example, when comparing densities for shear-cytokine activation between naïve and preconditioned cells at a constant 24 hrs total perfusion time (*i.e.* 24 hrs shear-cytokine activation of naïve cells vs. 12 hrs shear-cytokine activation of 12 hrs preconditioned cells), selectin expression on preconditioned cells is nearly 2 fold *lower* than that for naïve cells (81.8 vs. 42.5 sites/μm^2).

As is observed for all times at all activating conditions, the E-selectin site densities for combined-TNFα/IL-1β activated monolayers is less than the additive sum of the densities observed for individual cytokine-activated cells (*e.g.* for 4 hrs statically activated monolayers, the observed 215.7 sites/μm^2 for combined-cytokine stimuli is less than the sum of that for TNFα-activated and IL-1β-activated cells: 151.2 + 111.1 = 262.3 sites/μm^2; see Figure 5.4). This correlates with numerous examples in the literature [118,

189, 196-200]. Kuldo, *et al*. reported synergistic, but not fully additive, upregulation of E-selectin protein on the co-stimulation of HUVECs [118]. Others have also observed similar results in *in vivo* mice studies of inflamed epithelial airways [187-188, 201]. Though the selectin expression in dual-cytokine activation may not be the fully additive value of selectin density contributions from individual cytokine-induced expression, this discrepancy is hardly significant, especially under physiological conditions (*i.e.* prolonged exposure to perfusion).

This observation is expected as activating cytokines are known to have many roles in inflammatory response. Cardell, *et al*. reported that treatment of *in vivo* mice epithelial cells with IL-1β increased transcription of genes for the TNFα receptors TNFRI and TNFRII by at least 1.7 fold from non-treated cells; additionally, pre-treatment with IL-1β upregulated expression of endogenous production of TNFα; however, unlike for the TNFα receptors, IL-1β did not increase gene transcription for the IL-1 receptor [201]. Saperstein, *et al*. explored the *in vitro* effects of static incubation of IL-1β on TNFα receptors for mice endothelial cells seeing similar increases in TNFR protein expression, but noticed this increase was only transiently upregulated for TNFRI. In fact, after the temporary increase, TNFRI expression fell to nearly baseline values seen in non-inflamed cells. However, when pre-treating the cells with TNFα itself for 6 and 24 hrs, Saperstein, *et al*. noticed "desensitization" in TNFRI and only in TNFRII in the very long term (> 30 hrs) wherein TNFα receptor surface densities dropped to baseline densities similar to quiescent, non-activated cells [187]. As shown by these studies, the effects of cytokine treatment on endothelial cells are not only observed in CAM upregulation, but also in mediating the effects of each other. In fact, under healthy conditions, activated

endothelial cells self-regulate the inflammatory process by producing anti-inflammatory cytokines such as IL-10 to initiate normal shut-down mechanisms to return the EC to a quiescent, non-inflame state [122, 202].

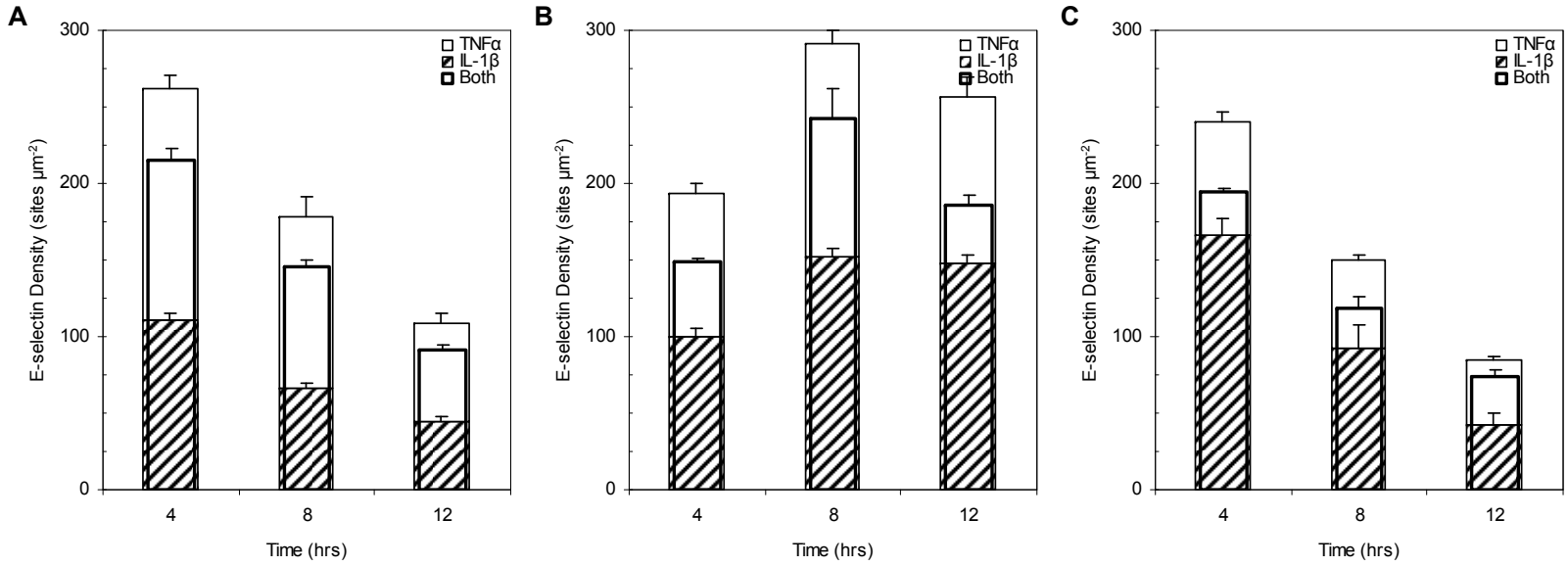

Figure 5.4. Additive E-selectin site density of combined TNFα or IL-1β activation. *HUVEC monolayers were treated with 10 ng/mL TNFα (clear), 0.1 ng/mL IL-1β (diagonal stripes), or simultaneously with both cytokines (bold outline) at the same concentrations for up to 12 hrs under (A) static and (B) shear-cytokine (10 dyn/cm^2) activation of naïve cells and (C) 12 hrs preconditioned monolayers.*

Chapter 5.5

Conclusion

The co-stimulation of HUVECs with fluid shear and cytokine is shown to be dependent not only on shear history (*i.e.* the magnitude and duration of perfusion prior to activation) and the time frame of the following shear-cytokine activation, but is also cytokine-dependent. Observed results suggest that fluid shear differently affects IL-1β and TNFα signaling pathways. While the results of this section may pique interest in examining these cytokine signaling pathways, the goal of this thesis is to investigate the *in vitro* model's ability to discriminate between healthy and pathological inflamed endothelium via E-selectin expression patterns (to develop selective targeting parameters for increased therapeutic selectivity) rather than explain the biological processes by which the adhesion molecule is upregulated.

Chapter 6.0

Potential Applications in Therapeutic Treatment

E-selectin expression by naïve endothelial cells subjected to fluid shear stress and inflammatory cytokine co-stimulation has been shown to be typical of a common chronic inflammatory disease, ischemia/reperfusion (I/R) injury wherein increased selectin density occurs within 4-10 hrs of reperfusion and remains elevated for up to 24 hrs [156, 158, 203]; this trend is indeed observed in both IL-1β and TNFα -induced shear-cytokine activation of HUVECs. It seems only prudent to investigate potential applications relevant to I/R injury with which this *in vitro* model could be purposed to study.

Ischemia/reperfusion injury is the main cause of tissue damage in the most serious cardiovascular diseases – myocardial infarctions and cerebrovascular stroke [204]. The transient disruption of blood supply caused by ischemia (due to vessel constriction or blockage caused by atherosclerosis, hypotension or thromboembolism) leads to oxygen depletion, tissue damage, and subsequent inflammatory response upon reperfusion [205-206]. Rapid endothelial inflammatory response and dysfunction leads to neutrophil and macrophage over accumulation, cell death, and eventually organ failure.

The degree to which reperfusion injury inflicts tissue damage is dependent on ischemia duration – *e.g.* studies have identified 3 hrs to be a maximum time limit (to prevent permanent damage) acute ischemic stroke can be resolved through intravenous

treatment with clot dissolving protein, tissue plasminogen activator (tPA) [207-208]. Early postischemic reperfusion does provide beneficial outcomes, but can still impair vascular function and can lead to loss of autoregulation especially with frequent I/R injuries. For decades, clinical research has explored therapies to limit I/R injury – to date an effective treatment has yet to be identified.

The *in vitro* model presented thus far has shown a correlation between E-selectin expression and functional capture of neutrophils from fluid flow. To illustrate the model's potential use as a platform for testing therapeutic treatment of pathogenically-inflamed endothelium, the effects of a synthetic cannabinoid agonist, CP55,940, on neutrophil shear dependent adhesion and migration was explored. Though other drug types (*e.g.* statins, anti-inflammatory antibodies, *etc*.) affect endothelial cells directly cannabinoids have shown promise in suppressing neutrophil respiratory burst reaction, a process that initiates the adhesion cascade responsible for neutrophil recruitment to inflamed endothelium [209-210]. The potential uses of cannabinoid agonists show significant promise as an anti-inflammatory treatment for preventing chronic buildup of macrophages, thus preventing vessel occlusions.

Chapter 6.1

Anti-Inflammatory Effects of Cannbinoids

Delta-9-tetrahydrocannabinol (Δ^9-THC), the popular phytocannabinoid active ingredient derived from *Cannabis sativa*, and its endogenous and synthetic analogues has recently shown potential in use as a therapeutic agent. Despite its recreational popularity for more than six millennia, the medical use of cannabinoids has been limited to its analgesic effects and treatments in convulsive disorders. Additionally, due to the difficulties in isolating the psychoactive and therapeutic effects and with the development of the new, effective, more reliable drugs in the modern pharmaceutical industry, interest in the pharmacological benefits of Δ^9-THC faded. However, in the mid-1970s, the identification of cannabinoid receptors (CB1R and CB2R) in the immune system and eventual revelation of endogenous production have led to studies that have discovered the anti-inflammatory benefits of cannabinoids [211]. Notable among these properties include the ability to induce vasodilation of blood vessels (via endogenous anandamide) and increase blood pressure (via synthetic agonist derivative HU-210); needless to say, these benefits play important roles in limiting damage to the heart and circulation especially during ischemia [212-218]. However, the means by which endocannabinoids, phytocannabinoids or their synthetic derivatives mediates anti-inflammatory responses is still unknown – whether cannabinoids target the endothelium or neutrophils or both is still unknown. One recent study reported anandamide inhibiting endothelial NF-κB activity (a pathway responsible for CAM upregulation) through CB1/2R-independent

pathways [219], while others have predicted the existence of a third CB receptor that may act through non-traditional receptor pathways [220].

In regards to the endogenous production of cannabinoids in the cardiovascular system, there has yet to be any conclusive studies that report significant local production especially by vascular tissue such as endothelial or smooth muscle cells. However, extensive research on their production in the nervous system shows that endocannabinoids are rapidly degraded (within minutes); their significant effects on the cardiovascular system necessitates their local production, most likely in the circulating cells in blood flow. Varga, *et al*. and Wang, *et al.* both observed limited anandamide production from platelets and monocytes in patients during endotoxic shock, but only at levels slightly higher than control [214, 221]. Despite the still unknown mechanisms of cannabinoids regulating cardiovascular response, there is undoubtedly evidence of potential therapeutic use and, considering its rapid degradation in bloodstream, targeted delivery of cannabinoids to disease-prone endothelium may play important roles in administering the drug.

Marijuana research has primarily focused on the analgesic effects of the plant and its mechanisms in psychoactive response. However, in recent years there has been a push to determine potential use in treating cardiovascular-related diseases. In fact, even as early as the 1970s, research showed that cannabinol and cannabidiol, non-psychoactive extracts from the plant, had potent implications as anti-inflammatory agents in treating paw edemas in rat models [222-225]; additionally, other early studies investigated the use of marijuana plant derivatives as hypertensive agents or its immunomodulatory effects [226-229]. Due to the lack of (or undiscovered) a cannabinoid receptor in the

cardiovascular system, most research over the years has been limited to studying systemic-wide effects of the drug, rather than its effects on specific cell mechanisms.

A better understanding of immune response and with the discovery of CB2R in the peripheral immune system, have returned attention to the anti-inflammatory and immunomodulatory effects of cannabinoids, with focus on the circulating cells of the immune system. Djeu, *et al*. and Lopez-Cepero, *et al*. first reported direct effects of cannabinoid on macrophages [209, 230] while immune regulators T- and B-cells and NK cells have been shown to be responsive towards both endogenous and synthetic cannabinoid derivatives [231-234]. However, there have been few significant *in vitro*, much less clinical studies, in determining any direct effects on neutrophils.

Being on the frontline of host defense against infection and injury, neutrophils are normally quiescent and patrol the vasculature. However, during inflammatory insult or infection, a respiratory burst reaction produces oxygen radicals within neutrophils, activating their defense reactions and upregulating adhesion molecules to its surfaces, prompting recruitment to the vessel wall for transmigration. This respiratory burst reaction and subsequent migration has been shown to be mediated by both endogenous and phytocannabinoid agonists [235-236]; in fact, Kurihara, *et al*. has even observed synthetic cannabinoids JWH015 and 2-AG suppress *in vitro* activated human neutrophils, preventing their migration [237]. The potential uses of cannabinoid agonists – not to mention the non-psychoactive properties of the synthetic analogues – shows significant promise in controlling inflammatory response and perhaps even preventing ischemia.

Chapter 6.2

CP55,940 Concentration

A range of 30 nM-300 μM CP55,940 concentration was studied. At the high end, the level of cannabinoid agonist in perfusion media is nearly 113,000 ng/mL – a value that far exceeds typical maximum Δ^9-THC concentrations measured in recreational or medical users by three orders of magnitude. As has been shown by a number of groups in the past, Δ^9-THC concentration rapidly rises during use and peaks within 5-10 minutes at values ranging between 40-231 ng/mL in blood plasma. Δ^9-THC concentration then subsequently rapidly diminishes to 10-20 ng/mL in 30 minutes to near negligible values of 1-5 ng/mL by 2 hrs (see Figure 6.1 [238]) [239-240]. The broad plasma concentration range is due to poor pharmacokinetic characterization of Δ^9-THC attributed to variable administration routes and difficulties in controlling dosages, Plasma concentrations have been shown to be dependent on %Δ^9-THC content in the administered dose, from as low as 1-5% in cigarettes (10-50 mg Δ^9-THC), 6-10% in hashish, as much as 30-60% in hash oil, and with therapeutic doses ranging from 2-25 mg [241-244]. Additionally, administration technique (*e.g.* smoke inhalation, oral delivery, and intravenous delivery) and usage history contributes towards observed variability. Another common method of introducing Δ^9-THC or its derivatives into the bloodstream is by oral ingestion; Howlett showed that oral intake of 20 mg Δ^9-THC resulted in 10-15 ng/mL circulating

concentration in blood plasma, much lower than that achieved by inhalation [245]. In a clinical trial by Chang, *et al*. patients undergoing chemotherapy treatment were given similar 25 mg concentrates of Δ^9-THC through oral delivery as an antiemetic; at blood plasma Δ^9-THC concentrations of 5, 5-10, and greater than 10 ng/mL, patients showed decreasing incidences of vomiting and nausea relative to placebos [246].

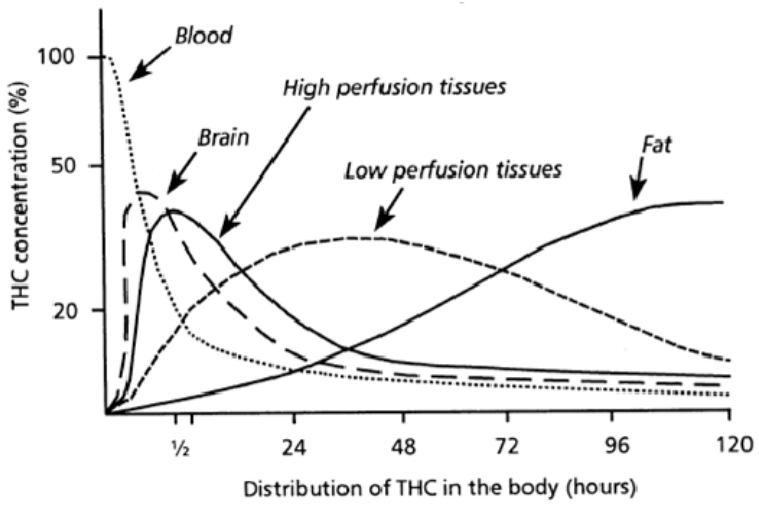

Figure 6.1. Distribution of THC in the body. *THC concentration as a percentage of initial amounts measured in marijuana cigarette prior to inhalation [238].*

Chapter 6.3

CP55,940 Effect on Neutrophils in Buffer and Whole Blood

Isolated neutrophils were treated with CP55,940 for 2 hrs in 1% (w/v) HSA buffer and subsequently perfused over a 4 hr statically activated HUVEC monolayer at a shear rate of 100 s^{-1}. The percentage of 40 ng/mL CP55,940 treated-neutrophils transmigrating across the endothelial monolayer (Figure 6.2A) is significantly lower ($p < 0.01$) than that for untreated, control neutrophils and DMSO-vehicle. At the high end 113 μg/mL CP55,940 concentration, all transmigration is abolished ($p < 0.001$), but a significant drop in total neutrophil interaction (rolling, firmly adhered, transmigrated or otherwise) is also observed ($p < 0.001$; Figure 6.2B). At all other CP55,940 treatment concentrations, no significant differences in transmigrating neutrophil fraction or total neutrophil flux is observed relative to untreated control. These results indicate that CP55,940 does have an effect on the ability of neutrophils to transmigrate (and thereby mediate inflammation) across the endothelial monolayer. The observed significant decrease in transmigration occurs at 40 ng/mL CP55,940, a concentration comparable to THC levels in blood plasma within 5-10 minutes of administration. Interestingly, at 75-110 ng/mL, transmigration seems to be reestablished to the same level as untreated neutrophils. While the results at 113 μg/mL may seem significant, the total neutrophil flux at that level is 5.2

fold lower than naïve control; nevertheless, the lack of neutrophil interaction does indicate an extreme anti-inflammatory response.

Additional shear rates were explored for the buffer-based neutrophil adhesion assay. Untreated control and 40 ng/mL-treated neutrophils were subjected to 0 s^{-1} (stagnant flow), 100 s^{-1}, and 200 s^{-1} shear rates (Figure 6.3A-D). Total neutrophil flux increases significantly from static to 100 s^{-1}, but does not change between 100 and 200 s^{-1} for both untreated control and 40 ng/mL CP55,940-treated neutrophils (Figure 6.3B, D). The fraction of neutrophils transmigrating does not significantly change for the 40 ng/mL-treated cells (Figure 6.3C); however, untreated control neutrophils show a significant increase in the fraction transmigrating from stagnant to 100 s^{-1} shear rates, but no change between 100 and 200 s^{-1} (Figure 6.3A).

Neutrophils that were retained in whole blood and treated with CP55,940 were similarly perfused over a 4 hr statically activated HUVEC monolayer at a shear rate of 500 s^{-1}. The fraction of neutrophils rolling, firmly adhering, and transmigrating during this adhesion assay in whole blood are shown in Figure 6.4A. Interestingly, DMSO-vehicle neutrophils in the control sample exhibited significantly higher transmigration than all other samples, including the naïve, untreated neutrophil control. No significant differences between the CP55,940-treated neutrophils relative to naïve, untreated control is observed except for a significant drop in percent rolling in the 10 μg/mL-treated neutrophils and increase in the 40 ng/mL-treated cells. Considering the total neutrophil flux (Figure 6.4B), there is a significant increase in neutrophil interaction for 40 ng/mL-treated neutrophils (2.9 fold, $p < 0.001$) and only slight, but not significantly higher flux for all other CP55,940 treated neutrophils. Whereas 40 ng/mL CP55,940-treatment of

neutrophils does contribute to a significant increase in neutrophil flux, the percent adhering or transmigrating does not differ from the naïve, untreated control. Percent rolling is seen to increase by 1.7 fold over control, but this could be due to secondary adhesions due to the large number of interacting neutrophils (*i.e.* neutrophil capture is increased due to neutrophil-neutrophil interaction rather than neutrophil-EC binding) and the hemodynamic flow characteristic of a central red blood cell plug excluding neutrophils from the flow centerline and pushing them towards the wall. One explanation for the observed results is that any potential effect of CP55,940 on neutrophils is likely diluted in whole blood, due to numerous other cells and factors present, compared to the relatively clean buffer used in the buffer.

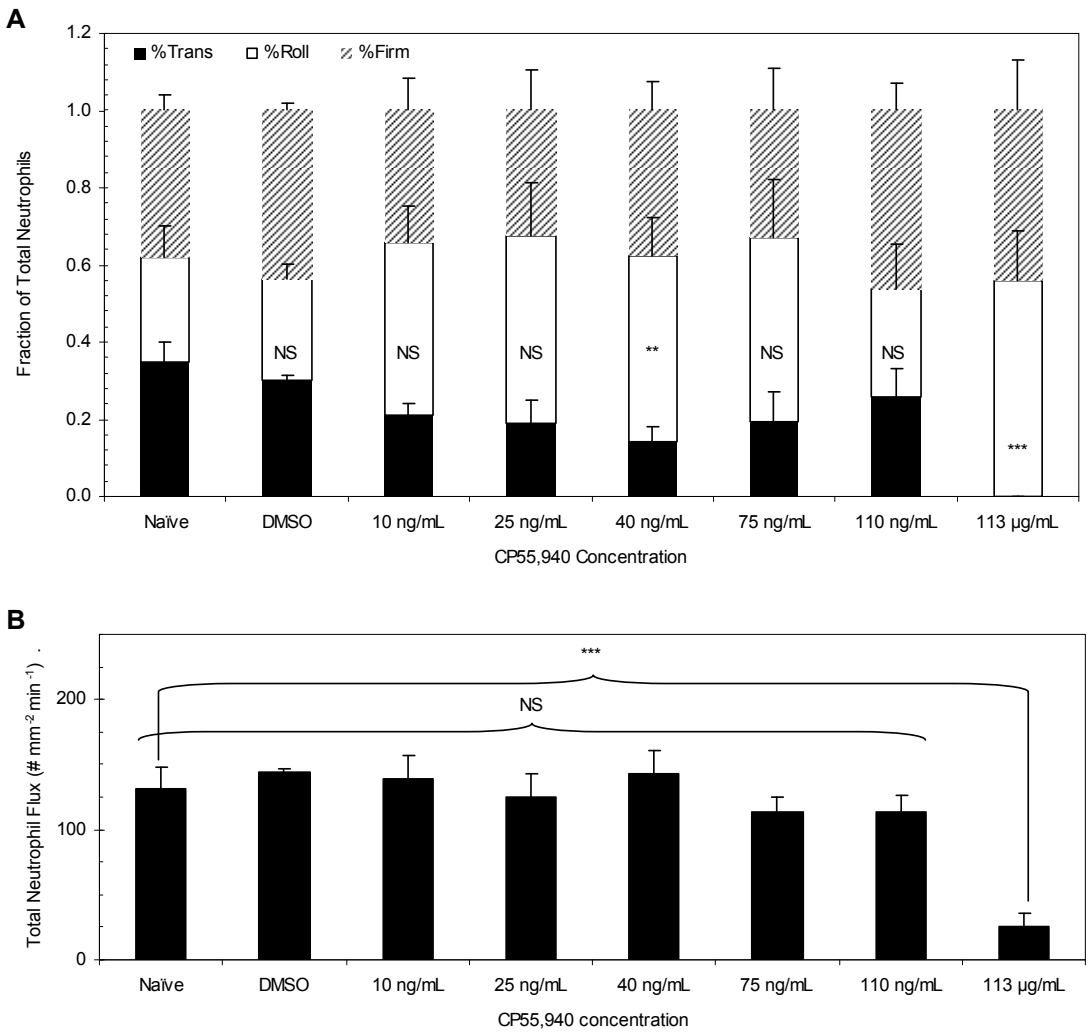

Figure 6.2. CP55,940-treated neutrophil interaction in buffer perfusion. *Neutrophils isolated from whole blood were treated for 2 hrs with varying concentrations of CP55940. Treated neutrophils were then diluted in 1% (w/v) human serum albumin and perfused over a 4 hr inflamed HUVEC monolayer for 5 minutes at 100 s^{-1}. Interactions (rolling, adherence, and transmigration) were quantified and flux was calculated. (A) Percent rolling (clear), adhered (diagonal stripes), and transmigrated (filled); (B) total neutrophil flux. Indicated significances are comparisons to naïve, untreated controls (leftmost bar)*

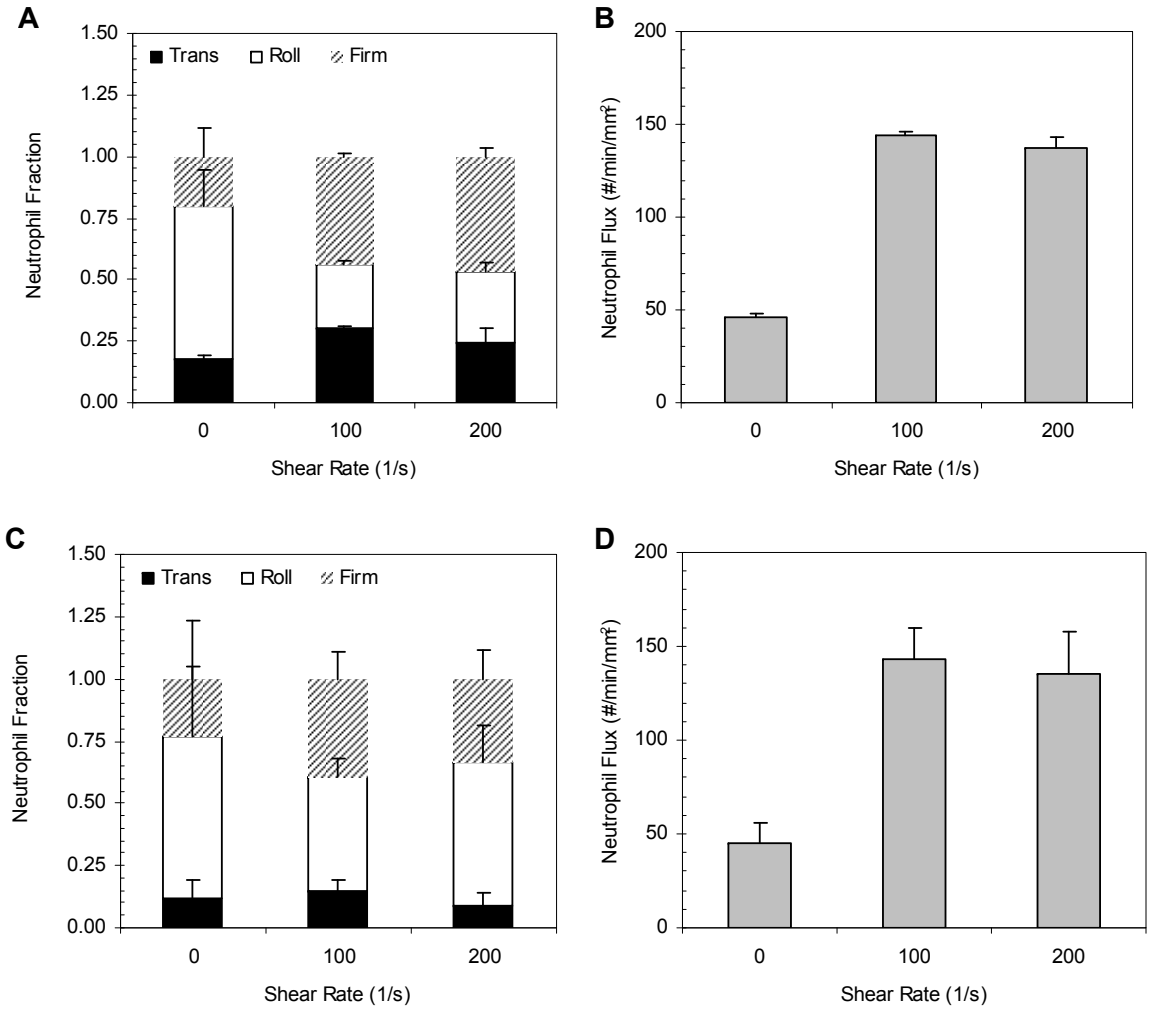

Figure 6.3. Varying shear rates in buffer perfusion. *Neutrophils isolated from whole blood were treated for 2 hrs with varying concentrations of CP55940. Treated neutrophils were then diluted in 1% (w/v) human serum albumin and perfused over a 4 hr inflamed HUVEC monolayer for 5 minutes at 0, 100, and 200 s^{-1}. Interactions (rolling, adherence, and transmigration) were quantified and flux was calculated. (A) Percent rolling (clear), adhered (diagonal stripes), and transmigrated (filled) for DMSO-control vehicle; (B) total neutrophil flux for DMSO-control vehicle; (C) neutrophil fractions rolling, adhering, and transmigrating for 40 ng/mL CP55,940-treated neutrophils; (D) total neutrophil flux for 40 ng/mL CP55,940-treated neutrophils.*

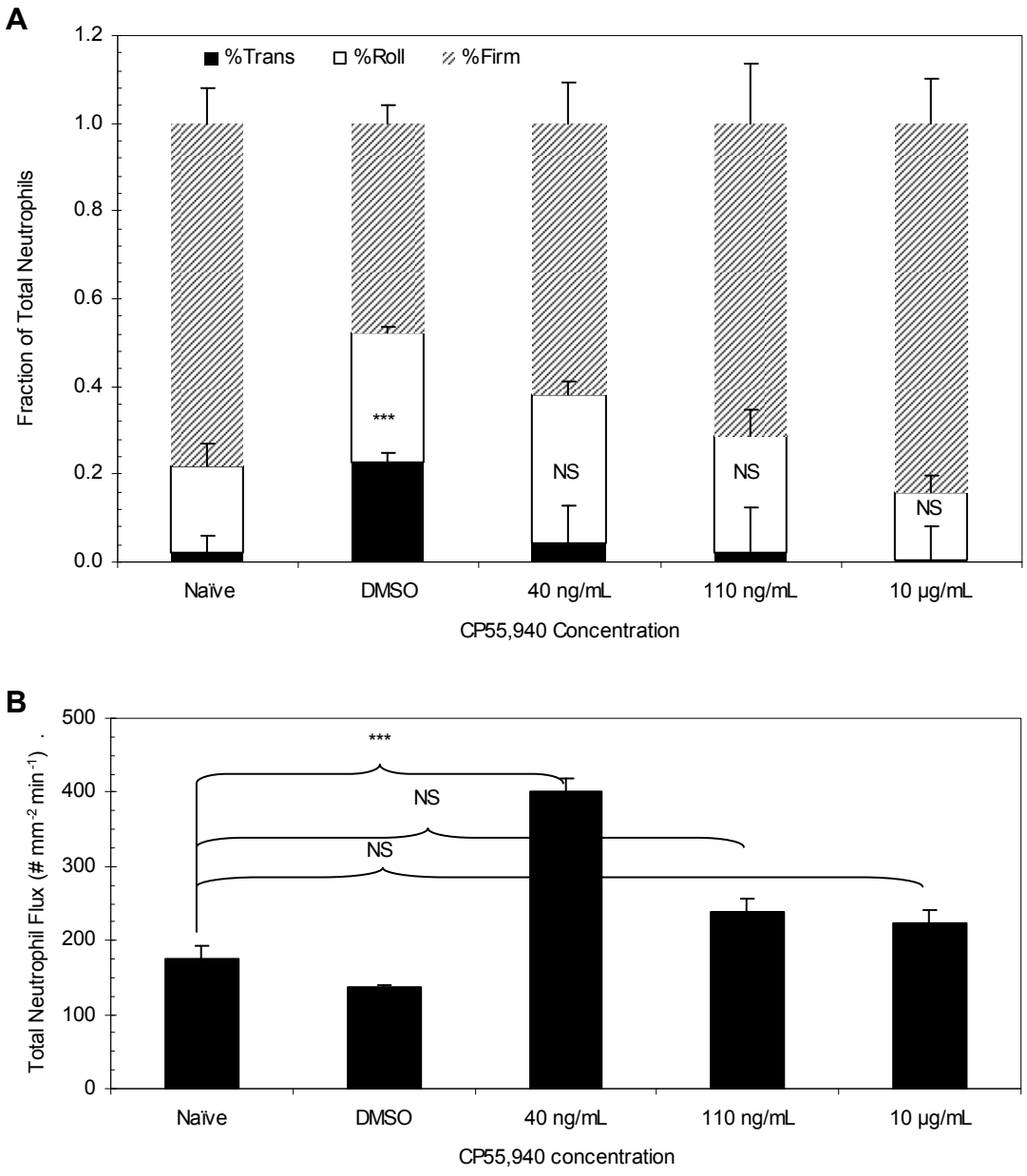

Figure 6.4. CP55,940-treated neutrophil interaction in whole blood perfusion. *Neutrophils were treated for 2 hrs with varying concentrations of CP55940 in whole blood. Treated neutrophils were then perfused over a 4 hr inflamed HUVEC monolayer for 5 minutes at 500 s^{-1}. Interactions (rolling, adherence, and transmigration) were quantified and flux was calculated. (A) Percent rolling (clear), adhered (diagonal stripes), and transmigrated (filled); (B) total neutrophil flux. Indicated significances are comparisons to naïve, untreated controls (leftmost bar)*

Chapter 6.4

Discussion

Despite the evidence of anti-inflammatory effects, there are actually few literature *in vitro* studies and even fewer clinical studies that directly observed cannabinoid actions on neutrophil regulation. For this study, the 113 μM CP55,940 concentrations at which complete neutrophil interaction was abolished, nearly 60-100 fold higher than the binding dissociation constants for CB1R and CB2R (K_i = 0.6-5.0 nM at CB1R and 0.7-2.6 nM at CB2R), nearly negates any potential practical therapeutic use for CP55,940. While neutrophil transmigration was shown to be slightly affected at the lower, more reasonable concentrations of 40 ng/mL during buffer perfusion, the 2 hr incubation time required for this effect is also of non-practical use for immediate resolution of inflammation. Clinical trials did find significant behavioral changes in patients at relatively low (5-10 ng/mL) Δ^9-THC plasma concentrations post administration of only 30 minutes [242-244]; however, it should be noted that CP55,940 has a relatively high binding dissociation constant, a few orders of magnitude higher than that of Δ^9-THC. The increased incubation time does help develop the anti-inflammatory effect, showing that in general, cannabinoids could be used as anti-inflammatory agents, although the specific synthetic analogue CP55,940 may not be an ideal candidate.

Though increased incubation time in the relatively cleaner (*i.e.* neutrophil-only solution) buffer adhesion assay did enable CP55,940 to induce an effect on neutrophil transmigration, for the whole blood assay, the 2 hr incubation time during which CP55,940 was exposed to blood factors likely degraded the cannabinoid as reported by Wang, *et al*. [221]; any effect on neutrophils would have likely been diluted as is observed in the non-significant changes in transmigration percentage.

However, the concentrations used in the buffer adhesion assay that are observed to elicit changes in neutrophil transmigration, correlate with results reported by Kraft, *et al*.; in that study, the authors compared the effects of CP55,940, endogenous anandamide, and its derivative methanandamide [210]. Neutrophils were incubated in low concentrations 0.1 nM-10 μM of CP55,940 for 10 minutes prior to induction of reactive oxidative burst reaction; at these concentrations, the reaction was not suppressed and a high level of neutrophil interaction was reported. At 100 μM concentration, however, complete suppression was observed. Additionally, by increasing incubation time to 180 minutes, Kraft, *et al*. saw similar effects at 100-fold lower concentrations (1000 nM CP55,940). In a similar study, McHugh, *et al.* treated neutrophils for 30 min with anandamide and found that increasing concentrations of the endogenous cannabinoid also inhibited stimulant-induced neutrophil transmigration, by nearly 3 fold at 100 nM concentrations [235]. While Kraft, *et al*. and McHugh, *et al.* used a synthetic stimulant to induce respiratory burst reactions in neutrophils, the adhesion assays conducted in this thesis did not use any activating chemicals. Likewise, Kurihara, *et al*. treated non-activated neutrophils with another synthetic cannabinoid JWH015 and its natural analogue 2-AG, and observed suppressed neutrophil response at therapeutic

concentrations (100 nM JWH015 or 300 nM 2-AG) [237]; though different cannabinoid analogues were used their results correlate with that observed for CP55,940 in that neutrophil chemotaxis was diminished.

Despite the potential implications suggested by these neutrophil migration-inhibition results, further research will be needed to determine methods for effective use in therapeutic applications. The mechanisms by which cannabinoids or their synthetic analogues affect macrophage or neutrophil migration is still unknown. Regardless, the novel therapeutic benefits in diminishing inflammatory response in cardiovascular diseases are promising.

Chapter 6.5

Conclusion

The developed *in vitro* model of endothelial inflammation can potentially serve as a platform for testing anti-inflammatory therapeutics. Δ^9-THC, the active ingredient of *Cannabis sativa*, and its analogues have recently shown promise as such a drug. While known for its psychoactive effects, cannabinoids have been shown to have immunomodulatory effects in suppressing neutrophil respiratory burst reactions and thereby preventing over accumulation during chronic inflammation of dysfunctional endothelium. However, despite the potential cardioprotective benefits of Δ^9-THC-based compounds, conventional administration is not advised (*i.e.* smoke inhalation) due to development of respiratory tract disorders associated with smoking other carcinogenic compounds in the administrative vehicle [241, 247-248]. Other less deleterious administrative routes such as oral ingestion or intravenous delivery is preferred, but even then, due to poor pharmacokinetic characterization of cannabinoids, care must be taken to ensure proper dosage.

This thesis studied the effects of synthetic cannabinoid agonist CP55,940 on neutrophil transmigration in buffer and in whole blood perfusion. The observed results indicate a slight anti-inflammatory role in the buffer flow system at CP55,940 concentration of 40 ng/mL, a practical concentration for potential use in therapeutic

applications; however, the 2 hr incubation time required to elicit this effect may not be therapeutically convenient if a rapid response is required. In the whole blood perfusion assays, no CP55,940 effect is observed, likely due to degradation of the drug by whole blood factors during the incubation period. Additional controls would need to be considered in order to fully comprehend the effects of this particular synthetic agonist; for instance, whole blood properties of each donor would need to be evaluated to determine the effects of CP55,940 on other types of cells and how this would affect neutrophil migratory properties.

Chapter 7.0

Conclusion

Despite decades of research, the mechanism by which chronic inflammation occurs is still disputed as evidenced by numerous conflicting scientific studies. Most *in vitro* models have focused on the effects of various pro-inflammatory chemicals, but as shown by others, hemodynamics and shear stress imparted by blood flow plays key roles in mediating endothelial behavior. Curiously, few studies have actually examined endothelial response to the combined cytokine and fluid shear conditions under which physiological inflammation occurs To that end, this thesis addresses this gap in the literature.

Despite growing cardiovascular health concerns due to chronic inflammation, there is an increasing prevalence of coronary heart disease, myocardial infarctions, cerebrovascular ischemia, and hypertension. Unfortunately, effective treatments of chronic inflammatory pathologies have yet to be developed. Though many medicines have been marketed, these pharmaceutics only provide symptomatic relief in reducing the effects of inflammation and they themselves may carry significant fatal side effects due to their systemic-wide circulation. Surgical options, such as arterial replacement, revascularization, and bypass surgeries are effective methods that eliminate clots and

plaque buildup; however, these procedures usually carry considerable risk and are preferentially avoided.

As such, there is significant impetus in determining effective treatments for chronic inflammatory diseases. Specifically, by developing a targeted drug delivery system, only diseased, chronically inflamed cells will be treated while healthy tissue is avoided; this localization and prolonged release of therapeutics eliminates most side effects associated with current medicines and provides a non-invasive alternative to surgical procedures. However, in order to develop a proper targeted drug delivery system, a viable delivery route through which medicines can be localized needs to be identified. Fortunately, the human immune system provides a natural process, inflammation, during which neutrophils and macrophages are recruited from the blood stream to the vascular wall to eliminate infections and injury and restore healthy vascular function. By exploiting this process, delivery vehicles can potentially be characterized with specific receptors mimicking white blood cells. To this end, research has focused on how inflammation progresses in the endothelium, a thin monolayer of cells lining the inner wall of vessels.

The multiple iterations of the *in vitro* model used in this thesis observed human endothelial cell expression of E-selectin in response to prolonged IL-1β stimulation relevant to chronic inflammatory stimulus and examined the differential response of endothelium to shear history. Naïve cells exposed at once to simultaneous shear-cytokine stimuli displays strong pro-inflammatory phenotypes for an extended duration whereas cells preconditioned with fluid shear, as is the case in normal physiological conditions, exhibit either an elevated or muted inflammatory response dependent on conditioning

duration and time-frame of subsequent shear-cytokine stimulation (*i.e.* its total shear history).

HUVEC monolayers co-stimulated with fluid induced-shear and IL-1β, responds through a unique E-selectin expression profile and neutrophil capturing functionality not seen in static-activation or shear-only stimuli, and has significant relevance in predicting pathological inflammatory response to ischemia/reperfusion injuries. Specifically, it was determined that the mere presence of fluid shear prolongs E-selectin expression for human umbilical vein endothelial cells and shifts the maximum peak expression from 4 to 8-12 hrs post activation. Additionally, while variations in shear magnitude did not significantly alter endothelial response in the acute timeframe, a minimum shear magnitude of at least 1.5 dyn/cm^2 was required to elicit a significant response in naïve monolayers during chronic (24 hrs) IL-1β stimuli.

Co-stimulatory shear-cytokine IL-1β treatment of preconditioned endothelium was also explored. Naïve monolayers that had been exposed to high shear magnitude perfusion in the absence of cytokines for at least 8 hrs exhibited atheroprotective phenotypes on onset of shear-cytokine inflammation. E-selectin densities increased significantly in the short term and quickly returned to baseline values indicating these preconditioned monolayers exhibit relevance towards healthy endothelium under normal acute inflammation. This 8 hr time frame was found to correspond to the time period required for naïve endothelium to elongate and realign in the direction of fluid shear – indicative of healthy endothelial response. Additionally, when subjected to persistent chronic inflammation afforded by IL-1β redosing, increasingly preconditioned

endothelium exhibited strong protection against chronic inflammation whereas non-preconditioned cells eventually upregulated prolonged E-selectin densities.

The endothelial response to shear-cytokine IL-1β activation was compared to that of TNFα activation to determine whether the observed behavior is cytokine-specific. While similar trends were exhibited by TNFα activated endothelium, a suppressed E-selectin expression profile relative to IL-1β activated cells was observed. In treating endothelial monolayers with dual-cytokine activation, it was also observed that the response attributed to IL-1β activation alone dominated endothelial response the more the endothelial cells were exposed to fluid shear, indicating a strong shear-dependent IL-1β behavior even in complex inflammatory stimulus.

Finally, this thesis also explored the potential applications of the developed *in vitro* model in studying the impact of a synthetic cannabinoid, CP55,940, on neutrophil adhesion and migration on a highly inflamed endothelial monolayer. The results presented indicate an anti-inflammatory role in suppressing neutrophil transmigration.

In summary, the developed physiologically relevant *in vitro* model of endothelial cell inflammation shows that shear history significantly affects endothelial cell response to both acute and chronic inflammatory stimulus. Prolonged preconditioning of endothelium to an elongated, healthy phenotype yields a sharp acute response and a muted chronic expression pattern of E-selectin upon shear-cytokine activation whereas activation of a naïve monolayer reveals endothelial behavior similar to that observed in disease-prone cells, especially relevant in such models as ischemia/reperfusion injury.

Chapter 7.1

Future Direction

There are numerous directions that could be followed from this point. This section will briefly summarize potential future work and their significance in order of importance.

Fluid Flow Profile

The observed data collected by this thesis was generated in a parallel plate flow chamber that recirculates perfusion media enabling long term studies. Using the same physical apparatus, in order to increase its physiological relevance to simulate effects of fluid shear flow, a non-uniform disturbed flow profile should be studied. Such a feature will mimic the blood flow pattern in large arteries near the heart (where oscillating and pulsatile flow dominates) and will also simulate the local flow environment experienced by atheroprone endothelial cells; this has particular relevance since some types of disturbed flow, such as oscillatory shear stress, have been shown to mediate endothelial response in ways not observed under laminar shear [89, 249]. Along similar lines, ischemia/reperfusion injury can also be simulated; endothelial behavior under various ischemia durations can be measured by stopping fluid flow for a variable amount of time to simulate ischemia severity. Overall, a wide range of blood flow patterns (*e.g.*

oscillatory, laminar, turbulent, disturbed or recirculating) and fluid shear magnitudes (*e.g.* creeping or stagnant flow and even reversing flow) should be studied so that a complete endothelial response profile can be developed. While the turbulent and oscillatory flow regimes may be of significant relevance especially in the larger arterial vessels, the laminar flow profile is more commonly observed in post-capillary venules where targeted therapeutics have been shown to have increased specificity and efficacy due to reduced vessel diameter and more favorable hemodynamic interactions [250-252].

Pro-Inflammatory Factors, Therapeutics, and Other Endothelial Responses

Second to investigating more complex flow profiles will be to study additional cytokines, pro-inflammatory factors, and anti-inflammatory molecules such as statins, steroids, and other therapeutics. This *in vitro* model has only used IL-1β and TNFα, there are numerous other cytokines relevant to chronic inflammation [154, 182, 253-258]. Expanding the range of chemicals could immediately be performed as the same physical apparatus could be used. Various inflammatory molecules could be featured, depending on the chronic inflammatory disease of interest (*e.g.* lipopolysaccharide, the bacterial endotoxin responsible for septic shock – over-accumulation leads to organ failures and septic shock [257, 259]; nitric oxide, an endogenously produced chemical vasodilator). Therapeutic drugs could be tested for anti-inflammatory effects as the cannabinoid agonist CP55,940 was tested in this thesis (*e.g.* anti-inflammatory antagonists, IL-10, cytokine receptor-blocking antibodies). The effects of these different chemicals on endothelial behavior and functionality will be vital in understanding endothelial response under different inflammatory conditions. Not only can cell adhesion molecule

upregulation be measured, but endothelial monolayer integrity due to effects of various chemicals can also be assessed by measuring its cell-cell interactions. This could hold significant relevance in studying endothelial blood-brain barrier dynamics or respiratory tract diseases such as acute respiratory disease syndrome.

Different Endothelial Cells

The observed selectin expression and neutrophil binding densities of shear-cytokine exposed naïve monolayers is relevant to ischemia/reperfusion injury. However, the use of HUVECs may limit the direct correlation of selectin expression to clinical relevant models; as such, potential future direction in this area will use appropriate endothelial cells from other vessel beds, such as aortic endothelial cells, that are more prone to pathologic development (*e.g.* vasculature with more disturbed flow patterns such as those typically observed in bifurcations or branching junctions) [31, 43].

Flow Chamber Geometry

As important as different fluid flow profiles is the vascular geometry. While this thesis has primarily focused on the interplay between laminar fluid shear stress and cytokine stimulation, it is recognized that cardiovascular diseases afflict larger-sized vessels where more complex flow exists [89, 249, 260-261]. The presented data focused on the low to mid-range shear stress magnitudes (0-20 dyn/cm^2) of purely laminar flow which is typical of the hemodynamic environments of the smaller diameter post-capillary venules; however, as has been discussed earlier, more atheroprone endothelial cells are located in the vascular beds where flow is more disrupted due to extensive branching

geometry, bends and curvatures, and vessel bifurcation points [1, 3, 262-263]. The current parallel plate flow chamber system used in this study can easily be modified to study different channel diameters or recirculation flow wherein a sudden expansion creates multiple flow profiles. Additional modifications would require construction of different apparatuses which could be achieved using microfabrication techniques to create microfluidic channels. Microfluidic channels can be employed to simulate vessel bifurcations and branching, curvatures, and bends; however, while the flexibility afforded by microfabrication is attractive, careful consideration will need to be taken in ensuring that endothelial cell culture is not negatively impacted by the manufacturing process (*e.g.* issues with hydrophobicity, cell viability with PDMS devices, etc.). In addition to modification of channel geometry, disruptive flow profiles can be combined with a non-linear channel to increase the physical relevance of the *in vitro* model.

Endothelial Substratum

Endothelial cells are also known to be responsive to the type of substrate they are cultured on [84, 264]; the substrate on which the endothelial monolayers are patterned can be modified to simulate the natural vessel tissue on which endothelial cells rest. Instead of using a simple gelatin-based sublayer on glass, a more complex extracellular substrate with different stiffness (*e.g.* collagen or a collagen-gelatin mixture) could be employed. Endothelium could also be grown in a co-culture system, over a smooth muscle cell layer [94]. Further, a three dimensional model in which endothelial cells are cultured inside a tube would provide another platform to study inflammation. Of course, while development of more relevant physical structures will likely provide a more

accurate representation of inflammation, current imaging techniques (*i.e.* immunofluorescent microscopy) will have to be adapted to view through thicker substrates. Confocal microscopy would likely have to be used in order to effectively image E-selectin (or other adhesion molecules) especially if three dimensional models are employed.

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