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THE INFLUENCE OF MEMBRANE CHOLESTEROL-RELATED SHEAR STRESS MECHANOSENSITIVITY ON NEUTROPHIL FLOW BEHAVIOR

Xiaoyan Zhang
University of Kentucky, zxy821224@hotmail.com

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Xiaoyan Zhang, Student

Dr. Hainsworth Y. Shin, Major Professor

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THE INFLUENCE OF MEMBRANE CHOLESTEROL-RELATED SHEAR STRESS MECHANOSENSITIVITY ON NEUTROPHIL FLOW BEHAVIOR

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctoral of Philosophy in the College of Engineering at the University of Kentucky

By

Xiaoyan Zhang

Lexington, Kentucky

Director: Dr. Hainsworth Y. Shin, Assistant professor of Biomedical Engineering

Lexington, Kentucky

2012

ABSTRACT OF DISSERTATION

THE INFLUENCE OF MEMBRANE CHOLESTEROL-RELATED SHEAR STRESS MECHANOSENSITIVITY ON NEUTROPHIL FLOW BEHAVIOR

Hypercholesterolemia is a dominant risk factor for a variety of cardiovascular diseases and involves a chronic inflammatory component in which neutrophil activity plays a critical role. Recently, fluid shear stress mechanotransduction has been established as a control mechanism that regulates the activity of neutrophils by reducing the formation of pseudopods and the surface expression of CD18 integrins, thereby rendering these cells rounded, deformable, and non-adhesive. This is critical for maintaining a healthy circulation, because chronically activated neutrophils not only release excess cytotoxic and degradative agents but also exhibit a reduced efficiency to pass through the small vessels of the microcirculation leading to increased microvascular resistance. We hypothesized that aberrant neutrophil mechanosensitivity to fluid shear stress due to the altered blood environment (i.e., excess plasma cholesterol) is a contributing factor for elevated hemodynamic resistance in the microcirculation associated with hypercholesterolemia. For this purpose, the present work firstly showed that the sensitivity of neutrophils to fluid shear stress depends on the cholesterol-dependent fluidity of the cell membrane, and that, in the face of hypercholesterolemia, the neutrophil mechanosensitivity highly correlated with the plasma levels of free cholesterol. The second part of this project demonstrated that, when subjected to shear stress fields, leukocyte suspensions exhibited transient (within 10 min of flow onset) time-dependent reductions in their apparent viscosity. Moreover, shear-induced changes in viscosity of cell suspensions were influenced by disturbances of membrane cholesterol and fluidity in a fashion similar to that for shear-induced pseudopod retraction. Finally, the third part of this work provided evidence that neutrophils played a role in hypercholesterolemia-related impairment of flow recovery response to transient ischemia. In conclusion, results of the current work provided the first evidence that cholesterol is an important component of the neutrophil mechanotransducing capacity and impaired neutrophil shear mechanotransduction may disturb the blood flow rheology, leading to elevations in the apparent viscosity as well as in the resistance. This cholesterol-linked perturbation may be a contributing factor for the pathologic microcirculation associated with hypercholesterolemia.

Keywords: Mechanotransduction; deactivation; pseudopod retraction; neutrophil-platelet binding; flow rheology.

Xiaoyan Zhang
Student's Signature

December 4, 2012
Date

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By

Xiaoyan Zhang

Hainsworth Y. Shin
Director of Dissertation

Abhijit R. Patwardhan
Director of Graduate Studies

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DEDICATION

To my husband, Zheng Cao, and my parents, Chuanbin Zhang and Daozhi Du, for their continued support, encouragement, and love.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1 INTRODUCTION

Hypercholesterolemia is a dominant risk factor for a variety of cardiovascular diseases and involves a chronic inflammatory component at both macro- and micro-circulatory levels [1, 2]. While it has long been appreciated that monocytic and lymphocytic immune cells contribute to the initiation/development of arterial diseases in hypercholesterolemia, only recently has a growing body of evidence emerged that chronically activated neutrophils also play a critical role in the hypercholesterolemia-related disorders [2-4].

Neutrophils make up the majority (approximately 60 – 75%) of the nucleated leukocytes in human blood with the remaining being monocytes and lymphocytes. As the principal gatekeepers of the acute inflammatory response of the body's immune system, neutrophils are extremely sensitive to inflammatory stimuli allowing them to rapidly (i.e., on the order of milliseconds) transition from an inactivated to an activated state. Despite the critical role of neutrophils in acute inflammation, their activity must be tightly controlled under physiological (i.e., non-inflamed, non-pathological) conditions not only because of their cytotoxic and biodegradative potential but also due to their rheological (i.e., flow) behavior particularly in the microcirculation.

Recently, fluid shear stress mechanotransduction has been established as an anti-inflammatory mechanism for neutrophils under physiological conditions [5]. Fluid shear stress deactivates neutrophils by reducing the formation of pseudopods and the surface expression of CD18 adhesive molecules. As such, this mechanism serves to maintain cells in a rounded, deformable, and non-adhesive state, ensuring their efficient transit

through the microvasculature. The importance of this control mechanism is supported by the connection between impaired shear responses by neutrophils and increased peripheral vascular resistance in cardiovascular diseases [6, 7]. Along this line, it is possible that loss of the fluid shear stress regulation of cell activity is a contributing factor for the elevated neutrophil activation and the disturbed microvascular blood flow rheology associated with hypercholesterolemia. This is reasonable particularly because the plasma membranes of neutrophils that are enriched in mechanosensors undergo significant biological and physicochemical changes in the face of hypercholesterolemia with potential effects on the cellular shear stress mechanosensitivity (i.e., the degree to which cells respond to shear stress).

Therefore, we hypothesized that *aberrant neutrophil mechanosensitivity to fluid shear stress due to the altered blood environment (i.e., excess plasma cholesterol) is a contributing factor for elevated hemodynamic resistance in the microcirculation associated with hypercholesterolemia.*

To address this hypothesis, the present study completed the following specific aims:

1) Establish a link between extracellular cholesterol levels, the cell membrane, and the shear stress mechanosensitivity of neutrophils;
2) Correlate neutrophil shear mechanosensitivity with their flow behavior;
3) Verify in vivo the connection between neutrophil shear mechanotransduction and blood flow regulation during reactive hyperemia in response to transient blood occlusion.

CHAPTER 2 BACKGROUND

2.1 Neutrophil Activation and Its Rheological Consequences

Neutrophils undergo activation in response to a wide range of stimuli including cytokines (e.g., interleukin-8), leukotrienes (e.g., leukotriene B4), platelet-activating factor (PAF), N-formyl-Met-Leu-Phe (fMLP, an exogenous peptide), and etc. [8]. Specifically, binding of activators to G-protein coupled receptors (GPCRs) on neutrophils initiates "inside-out signaling" that transitions these cells into an activated phenotype [9]. Neutrophils can also be activated by their contact with activated endothelial cells (ECs) or platelets, i.e., through the engagement of adhesive molecules (selectins, integrins) with their counter-receptors ("outside-in signaling") [9, 10].

Typically, neutrophil activation is manifested by physical changes, notably extension of pseudopods and binding to other blood borne cells (e.g., leukocytes, platelets), as well as by biochemical changes including up-regulation of adhesive integrins (e.g., CD18 integrins consisting mainly of LFA-1 and Mac-1), degranulation with release of proteolytic enzymes (e.g., myeloperoxidase or MPO), and generation of reactive oxygen species (ROS; e.g., O^{2-}, H_2O_2) [8, 9]. These features enable neutrophils to adhere to and migrate on the endothelium as well as transmigrate into target tissues to perform their immunological functions (e.g., phagocytosis, tissue repair).

Cell activation-dependent changes in the neutrophil geometry and adhesivity also have a physical effect on their flow behavior. For example, projection of F-actin enriched pseudopods leads to increases in cell size, morphological irregularity, and stiffness [8]. Moreover, activated neutrophils likely bind to other leukocytes or platelets to form larger

aggregates. All these features physically hinder the transit of neutrophils through the microcirculation and may elevate peripheral hemodynamic resistance, considering that the microvascular diameters (4 – 100 μm) are comparable to the cell dimensions [4, 8]. In the skeletal muscles, the contribution of leukocytes (mainly neutrophils) to microvascular resistance has been estimated to range from about 2% up to 20% at physiological cell counts. The leukocyte contribution can further increase to 15 – 50% of the whole organ resistance upon cell activation [11-13].

Two main mechanisms have been proposed to describe the effects of activated leukocytes on the hemodynamics of microvascular blood flow (e.g., in the skeletal muscle). In the pre-capillary arterioles (the primary resistance microvasculature), activated leukocytes, particularly neutrophils, are capable of releasing vasoactive substances that influence vascular tone via effects on the endothelial regulation of blood vessel diameter. In this regard, O^{2-} produced by activated neutrophils has been shown to annihilate endothelial-derived nitric oxide (NO) in vitro [14] and thereby promote vasoconstriction. Vasoconstriction due to leukocyte activation has been reported to increase in cardiovascular diseases [15, 16].

Another plausible mechanism is that activated neutrophils, because of their increased cell size and reduced deformability, have increased difficulty to transit through the small vessels (e.g., pre-capillary arterioles, capillaries, post-capillary venules) [8]. There are several ways by which these manifestations of neutrophil activation may influence microvascular flow resistance (Fig. 2.1). In some cases, neutrophils that are activated may disrupt the motion of red blood cells (RBCs) either by reducing their velocities in the capillaries [17] or by displacing them from axial positions and causing

more RBC collisions with a detrimental impact on the apparent viscosity of blood [18]. Both of these possibilities predictably result in elevations in local hemodynamic resistance. These disturbances of the RBC flow characteristics may be further exaggerated by the adhesive interactions between neutrophils with other leukocytes or platelets [19, 20]. Moreover, at locations where leukocyte dimensions are larger than that of the downstream lumen, activated cells cannot squeeze through such tiny tubes and will be temporarily trapped, which, if increased in numbers, will significantly raise the local resistance [8, 11, 13]. Finally, once neutrophils adhere to endothelium, e.g., in the post-capillary venules, they may dramatically increase flow resistance by reducing the effective lumen cross-sectional diameters particularly because resistance is inversely proportional to the vessel radius to the fourth power [21-23].

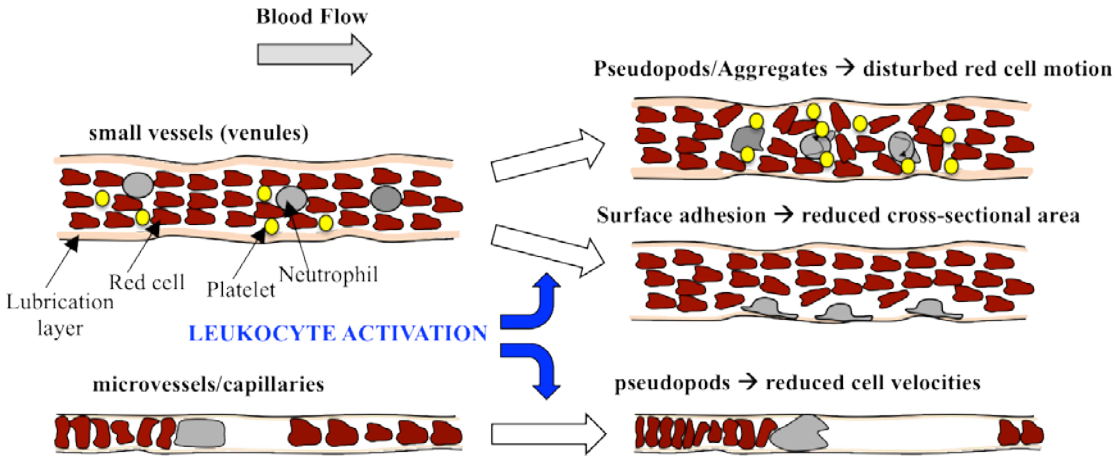

Figure 2.1 Rheological impact of leukocyte activation on the blood flow in the microcirculation.
Sustained activation, e.g., due to proinflammatory stimuli, hinders leukocyte passage through the small vessels either by promoting pseudopod projections or through enabling cell adhesion to the vascular wall. Ultimately, these may elevate peripheral resistance and contribute to microvascular dysfunctions [4].

In summary, neutrophils, in addition to their immunological function, play a critical role in determining the blood flow rheology of the microcirculation. Sustained/dysregulated neutrophil activation may underlie the disturbed or reduced microvascular blood flow associated with a variety of cardiovascular diseases such as hypercholesterolemia-related disorders.

2.2 Neutrophil Involvement in Hypercholesterolemia-Related Microvascular Dysfunctions

Hypercholesterolemia is typically viewed as a disease of the large arteries (e.g., atherosclerosis). It, however, also chronically inflames the microcirculation [2], which is thought to be linked to impaired microvascular flow regulation. Such microvascular dysfunction is particularly evident when considering a tissue's response to ischemic reactive hyperemia, a transient elevation in blood flow (i.e., a peak flow overshoot) in response to an acute period of stasis (no-flow condition) [24, 25].

During reactive hyperemia, tissue blood flow is transiently restricted and then reinstated. Downstream of the occlusive event, blood flow is momentarily stopped until release of restriction, after which blood flow overshoots to a peak value and then recovers to the original blood flow level prior to occlusion. Shear stress-sensitive endothelial events (e.g., NO release) are thought to control the blood vessel myogenic activity during this response by regulating vascular smooth muscle cells in the arterioles that feed into the capillaries [25]. However, endothelial function is reportedly not the sole contributor to changes in ischemic reactive hyperemia. In fact, only ¼ of the factors that govern

vasoactivity is attributed to the endothelium, even after ischemia [26]. In some cases, reactive hyperemia is independent of endothelial activity [27].

The dysregulated microvascular state is thought to arise, in part, from chronic leukocyte activity in the venules [24]. It has also been attributed to neutrophils and neutrophil-platelet aggregates that lead to dysregulation of the endothelium in the pre-capillary arterioles [28]. However, neutrophil/platelet binding to endothelium is debatable due to the high flows in the arterioles that reduce leukocyte residence times at the vessel wall and the high shears that avert firm binding. Thus, it has been suggested that an elevated state of inflammation in the blood translates, via soluble mediators, into arteriolar dysfunction [29].

Neutrophils in the face of hypercholesterolemia exhibit elevated surface expression of CD18 integrins, particularly Mac-1 (CD11b/CD18), as well as enhanced degranulation and ROS production [30-32]. As a result, enhanced neutrophil homotypic (i.e., binding to other neutrophils) and/or heterotypic (binding to other leukocytes or platelets) aggregation has been observed in hypercholesterolemia contributing to increased levels of rolling, adherent, and migrating neutrophils in the microvasculature [2, 33-36]. Notably, these phenotypic changes in microvasculature occur long before the appearance of fatty streak lesions in the large arteries of animals placed on high fat (HFD), i.e., a proatherogenic, diet [36, 37]. Therefore, neutrophils may contribute to the onset and/or progression of atherosclerosis by initiating or exacerbating microvascular dysfunctions. For instance, chronically activated neutrophils may disturb/reduce the blood flow in the microcirculation or vasa vasorum of the large vessels (e.g., aorta), leading to disrupted vascular (i.e., adventitial or medial) wall perfusion and resulting in

inflammation-related vessel tissue injury followed by atherogenesis (i.e., from within the vessel wall to the luminal surface) [38-40].

Despite the critical role of neutrophil activation in hypercholesterolemia-induced microvascular dysfunctions, the original triggers for cell activation are still controversial. In this regard, a defective mechanotransduction by neutrophils of fluid shear stress, which has been established as anti-inflammatory for leukocytes, may be a contributing factor for the elevated cell activity in the face of hypercholesterolemia.

2.3 Mechanobiological Regulation of Neutrophil Activity by Fluid Shear Stress

In general, the scientific literature describing vascular mechanotransduction and its role in vascular biology point to the following general paradigm, i.e., exposure of vascular cells to physiological levels of fluid flow-derived shear stress (the tangential force per unit area exerted on the cell surface) under normal non-inflamed (i.e., non-pathological, non-diseased, non-pathogenic) conditions correlates with quiescence (i.e., baseline activity) [5, 41]. For the blood borne neutrophils, fluid shear stress (1 – 10 dyn/cm^2) mechanobiologically restricts cell activation by minimizing pseudopod projection and cell surface expression of CD18 adhesion molecules. Other attributes of shear-induced neutrophil deactivation include depolymerization of the F-actin cytoskeleton, cell detachment, reduced phagocytic activity [42, 43] and a pro-apoptotic phenotype [44]. The role of fluid flow stimulation to control the activity of leukocytes is supported by the reported connection between impaired leukocyte shear responses and increased hemodynamic resistance in cardiovascular diseases [6, 7]. Taken together, the reported evidence points to a fundamental contribution of an impaired neutrophil shear

mechanotransduction in the dysregulation of microvascular blood flow under hypercholesterolemia.

2.3.1 Control of pseudopod activity in neutrophils by shear stress

Pseudopod retraction, the most overt (i.e., noticeable) morphological manifestation of the cell-inactivating effects of fluid shear stress, was first observed for non-cytokine-stimulated human neutrophils migrating on a glass surface and subjected to a non-uniform flow field imposed by a micropipette with a tip of diameter in the range of 4 – 8 μm [43]. This situation modeled brief and spontaneous periods of blood stasis followed by an acute onset of flow, a typical scenario in the microvessels particularly during post-ischemic reactive hyperemia. Within 2 min of the onset of micropipette flow, migrating neutrophils retracted their existing pseudopod and became rounded. Notably, using intravital microscopy, the same investigators demonstrated that neutrophils in the venules of the rat mesentery sediment, extend pseudopods, and migrate on the vascular endothelium during blood stasis. However, these same cells retracted pseudopods and detached into the flow field upon reintroduction of fluid flow [42, 43, 45]. The ability of shear stress to minimize pseudopod activity has also been further confirmed for non-adherent heterogeneous leukocyte populations [46] exposed to a constant shear field (5 dyn/cm^2) in a cone-plate viscometer.

Notably, the shear-induced pseudopod retraction response by neutrophils is depressed upon treatment of cells with threshold concentrations of biochemical agonists, such as fMLP (>10^{-8} M) and PAF (>10^{-7} M) [42]. This commits the neutrophils to an activated phenotype and leads to their entrapment in microvessels due to increases in

adhesivity, size, and stiffness [42, 43, 47]. Thus, during inflammation, the biochemical milieu of the neutrophil overrides mechanobiological deactivation. This relationship further substantiates an anti-inflammatory role for fluid shear stress in the physiological non-inflamed condition (i.e., in the absence of threshold levels of cell agonists).

Currently, the formyl peptide receptor (FPR), a cell membrane-bound GPCR implicated in the regulation of neutrophil chemotaxis, has been shown to be a critical mechanosensor for neutrophils to sense and respond to fluid shear stress. Overexpression of FPR in undifferentiated human promyelocytic leukemia (HL60) cells imparts on these cells the ability to form pseudopods as well as to retract them in response to fluid shear stress [48]. In contrast, differentiated neutrophilic HL60 (dHL60) cells, after being transfected with siRNA to block FPR expression, exhibit an attenuated pseudopod retraction response to shear exposure, despite the fact that these cells retain the ability to project pseudopods because of the presence of other cytokine-related GPCRs [48].

Since leukocyte pseudopod projection is controlled by the polymerization of F-actin, a mechanism involving GPCRs, receptors that control dynamics of the F-actin cytoskeleton, has been proposed for shear-induced pseudopod retraction response by neutrophils [5]. In fact, GPCRs, e.g., FPR, under shear flow may undergo a conformational shift from an active structure to an inactive configuration, leading to reductions in the activity of $G_{\alpha i}$ [48]. This reduces the cytosolic activity of the key small guanine triphosphate-binding phosphatases (e.g., Rac1, Rac2) involved in actin polymerization [45]. As such, fluid shear stress inhibits or interferes with the ability of neutrophils to form and sustain pseudopod projections. In support of this, a rapid

decrease in F-actin content in neutrophils has been observed in parallel with pseudopod retraction in response to fluid shear stress [49, 50].

2.3.2 Regulation of CD18 expression on the surface of neutrophils by shear stress

CD18 integrins make up a family of heterodimeric cell-cell adhesion molecules that share a common β_2-subunit; LFA-1 (i.e., CD11a/CD18) and Mac-1 (i.e., CD11b/CD18) are the primary subtypes that, during inflammation, mediate firm adhesion of leukocytes to other cells (e.g., ECs, platelets) [9, 51].

Fluid shear stress, in addition to modulating CD18 interactions with their ligands (e.g. ICAM-1) during inflammation [51, 52], regulates integrin dynamics on the neutrophil surface under conditions that mimic low activation states. Under non-inflamed conditions, migrating neutrophils exposed to shear flow redistribute their membrane-associated CD18 receptors from areas of high shear stress to regions where shear is minimal, i.e. at focal adhesions. Shear exposure also reduces the total amount of CD18 integrins on the surfaces of migrating and non-adherent neutrophils even in the presence of inflammatory mediators, e.g. fMLP [42, 53]. Considering the role of CD18 integrins, particularly LFA-1 and Mac-1, in strengthening neutrophil attachment to other cells or surfaces, shear-mediated reductions in CD18 appear to serve to either prevent cells from firm adhesion [54] or facilitate membrane detachment critical for an intact pseudopod retraction response by adherent neutrophils under shear [53]. Therefore, shear-induced down-regulation of CD18 is another anti-inflammatory mechanism for neutrophils in a non-inflamed environment to maintain neutrophils in a non-adhesive state.

The mechanism underlying shear-induced reductions in CD18 surface levels involves its proteolysis (i.e., cleavage) off the surfaces of migrating and suspended neutrophils. Reportedly, shear-induced cleavage of CD18 integrins by neutrophils requires lysosomal cysteine proteases (e.g., cathepsin B) that are released under shear [53, 54]. It also requires fluid flow-related conformational changes in the extracellular domains of CD18 integrins to expose the cleavage sites for cysteine proteases [53]. The exact role of CD18 integrin cleavage in the mechanobiology of the leukocytes as it relates to microvascular flow regulation, however, is still unclear.

2.3.3 Fluid shear stress mechanotransduction and implications in vascular pathophysiology

It is apparent that, by minimizing formation of pseudopods and surface expression of CD18 integrins, fluid shear stress mechanotransduction maintains neutrophils in a rounded, deformable, and non-adhesive state to ensure that these cells smoothly, optimally, and efficiently pass through the small vessels in the microcirculation with minimal disturbance. In doing so, shear stress mechanotransduction may serve to maintain circulation in a healthy state to avoid the detrimental effects of cell activation not only on the vascular tissues but also on microvascular blood flow (Fig. 2.1).

Along this line, attenuated neutrophil shear responses have been implicated in the microvascular pathobiology associated with hypertension [7]. Specifically, the blood from spontaneously hypertensive rats (SHRs) is characterized by elevated numbers of circulating neutrophils that project pseudopods but exhibit depressed expression of adhesion molecules (e.g., selectins, CD18) compared to cells from normotensive animals

[55-57]. Although the increased activity of SHR-derived neutrophils is not associated with increased adhesion to microvascular endothelium [5], their increased numbers raise peripheral vascular resistance [7]. One possible explanation is that circulating activated neutrophils in SHRs release vasoactive substances to promote vasoconstriction as documented for atherosclerosis [15, 58, 59]. Extensive evidence, however, points to a rheological effect of leukocyte activation on microvascular resistance above the effect that would occur due to the elevated leukocyte numbers, alone [8, 60, 61]. Specifically, pseudopod formation by the SHR-derived leukocytes likely further enhances resistance by increasing the tumbling of leukocytes and thus causing more disturbances in the flow profile of the surrounding RBCs. This effect on the apparent viscosity of blood would have an enhancing effect on hemodynamic resistance and upstream blood pressure (Fig. 2.1).

Notably, fluid flow mechanotransduction (i.e., pseudopod retraction in response to shear exposure) is lacking for neutrophils from SHRs. In some cases, cells extend cellular projections under flow stimulation, defined as a “reversed” pseudopod retraction response to fluid shear [7]. One possible mechanism underlying the abnormal shear mechanotransduction in SHRs relates to plasma glucocorticoids and their receptors on the neutrophil surface [62, 63]. Glucocorticoid-treated [6] rats, like SHRs, exhibit elevated peripheral resistance in the microcirculation with elevated numbers of neutrophils that lack a pseudopod retraction response to shear stress. Interestingly, it is possible that glucocorticoids may impact neutrophil responses to shear stress stimulation via their effects on receptor-mediated biological signaling. It, however, should be noted that glucocorticoids have pleiotropic effects including their ability to fluidize the cell

membrane. For example, Gerritsen et al showed that glucocorticoids influence eicosanoid generation in microvessel ECs via their effects on membrane composition and fluidity [64]. Moreover, glucocorticoids have been shown to reduce the membrane fluidity of leukemia cells [65].

As such, a defective shear mechanotransduction, e.g., due to a pathological blood environment, may not only lead to sustained neutrophil activation but also result in disturbed blood flow with reduced downstream tissue perfusion. Moreover, it is possible that the ability of fluid shear stress to alter neutrophil activation is related to not only the biological but also the physicochemical status of the leukocyte membrane.

2.4 Role of Cell Membrane in Regulating Neutrophil Shear Mechanosensitivity

The present study adopted an approach to define the shear mechanosensitivity of the neutrophil as a potential quantitative measure of the ability of fluid shear stress to deactivate neutrophils. The possibility that perturbations in leukocyte shear mechanosensitivity affect the regulation of leukocyte activity with impact on cell rheological behavior in the microvessels has implications in the reported chronic inflammatory phenotype of the microcirculation under disease conditions, such as hypercholesterolemia [2]. One way by which the extracellular factors influence neutrophil shear mechanosensitivity is through the cell membrane.

The cell membrane is strategically positioned at the interface between the intra- and extra- cellular milieu. Moreover, the cell membrane is enriched in putative fluid flow mechanosensors (e.g., various GPCRs [48, 66, 67], CD18 integrins [53, 54], ion channels [68], etc.) which undergo conformational changes under the actions of fluid shear stress.

As a supporting medium for these transmembrane proteins, cell membrane may play a critical role in regulating their sensitivity to fluid shear stimulation by influencing the ability of these molecules to undergo structural shifts that determine their functions.

In the case of hypercholesterolemia, the plasma membranes of vascular blood cells including the leukocytes and RBCs [69, 70] reportedly undergo changes in their lipid composition, i.e., increased membrane cholesterol. As a consequence, the biological and physical properties of cell membranes are also changed. For example, excess cholesterol in the membranes can disrupt the organization of lipid rafts [71], the cholesterol-rich microdomains that act as signaling platforms to orchestrate outside-in and inside-out signal transduction [4]. By dissociating the mechanosensory protein receptors from the lipid rafts, membrane cholesterol enrichment can interfere with their signaling cascades with effects on their response to fluid shear stress. In fact, lipid rafts (e.g., caveolae, a subtype of lipid rafts) have been implicated as mechanotransduction centers for the mechanotransduction of shear stress and pressure in ECs [72-74].

Alternatively, cholesterol enrichment can also influence the physical properties of membranes, among which the fluidity of lipid bilayer has received increasing attention. Specifically, increases in cholesterol reduce membrane fluidity [75, 76], thereby affecting the dynamics of membrane-associated protein sensors. This is because membrane fluidity can influence the mobility (e.g., lateral diffusion) [77] and structural flexibility [78] of membrane proteins. Membrane fluidity, in fact, has been reported to impact the conformation-dependent activation of GPCRs in ECs by affecting their structural changes [66]. Therefore, this physical property may influence neutrophil shear mechanosensitivity via its effects on shear-induced conformational activity.

In addition to the potential effects on membrane protein sensors, cell membranes may affect the mechanosensitive release or production of bioactive molecules that participate in or regulate the mechanotransduction processes in cells. For example, cholesterol-related membrane properties (e.g., permeability, fluidity) may affect the transmembrane release of cathepsin B, a key enzyme required for shear-induced CD18 cleavage [53, 54]. Moreover, it has been reported that O^{2-} blocks shear-induced pseudopod retraction response [46]. It is, therefore, possible that membrane fluidity influences neutrophil shear mechanotransduction by affecting the activity of membrane-associated NADPH oxidase [79], an main source of O^{2-}.

As such, the neutrophil mechanotransduction is tied to the state of the cell membrane. Along these lines, in the face of hypercholesterolemia, excess cholesterol in the cell membranes of neutrophils may contribute to abnormalities in their sensitivity to fluid shear stress to the point of impaired shear-induced cell deactivation and compromised microvascular flow. In this regard, the present study focused on the role of blood cholesterol milieu in the regulation of leukocyte mechanosensitivity of fluid shear stress. In doing so, the present study not only examined how cholesterol levels impact the mechanosensitive regulation of leukocyte activity, but also explored a potential mechanism related to the dysregulation of microcirculation in hypercholesterolemia.

CHAPTER 3 CHARACTERIZATION OF CELL-DEACTIVATING EFFECTS OF FLUID SHEAR STRESS

Pseudopod extensions and firm adhesion to other cells (e.g., ECs, platelets) are two major ways by which cellular activation impedes neutrophil transit through the microvessels of microcirculation (Fig. 2.1). In this regard, shear-induced pseudopod retraction and CD18 cleavage by neutrophils may serve to optimize their flow rheology in the microcirculation. To provide a basis for the assessment of this possibility, studies in the present section were designed to establish the basic characteristics (e.g., shear exposure time- and stress magnitude- dependence, dependence on cell and integrin subtypes) of the pseudopod retraction and/or CD18 cleavage responses to fluid shear stress. In order to demonstrate the anti-inflammatory role of CD18 cleavage by suspended cells, we further tested whether an impaired CD18 cleavage response influences fibrinogen-mediated neutrophil-platelet binding, which has been reported to exert dramatic effects on hemodynamic resistance of microvascular blood flow [19, 20].

3.1 Methods

3.1.1 Cells

3.1.1a Neutrophilic-like dHL60 cells

HL60 cells were purchased from the American Type Culture Collection (ATCC) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone) and 1% L-glutamine/penicillin/streptomycin solution (L-GPS; Sigma Aldrich) under standard incubator conditions (i.e., a humidified, 5%

CO_2/95% air environment maintained at 37 °C). Neutrophilic differentiation was induced by incubating HL60 suspensions (at $3x10^5$ cells/ml) with 1.25% DMSO (ATCC) for 6 days. Cells were then washed twice with isotonic phosphate-buffered saline (PBS; Mediatech) and cultured in fresh FBS/L-GPS-supplemented RPMI media for an overnight period of time prior to use. Neutrophilic differentiation was confirmed by the identification of multi-segmented nuclei stained with 4',6-diamidino-2-phenylindole (DAPI; MP Biochemicals).

3.1.1b Human peripheral blood neutrophils

All procedures used to collect and handle human blood samples in the present study were approved by the Institutional Review Board at the University of Kentucky (Lexington, KY).

Fresh human peripheral blood was drawn from asymptomatic volunteers into K_2-EDTA-coated vacutainers (Becton-Dickinson) using standard venipuncture. For experiments to examine shear-induced CD18 cleavage and its effects on leukocyte-platelet binding, blood was chilled at 4 °C for 30 min. The leukocyte-enriched plasma (containing neutrophils, monocytes, lymphocytes, platelets and sporadic RBCs) was then harvested after 1xg sedimentation at room temperature (RT) for 45 min.

To explore neutrophil-specific behavior, we also conducted experiments with purified neutrophils. In this case, human neutrophils were isolated by single-step gradient centrifugation in Polymorphprep™ solution (Axis-Shield) according to the

manufacturer's instructions. At the end of isolation procedures, cells were resuspended in PBS at $2x10^7$ cells/ml. Using this method, cell suspensions were >90% neutrophils.

3.1.2 Shear stress exposure

A custom cone-plate rheometer was used in the present study. This rheometer consisted of a stainless steel cone-plate setup (cone angle of 1°) that was fitted to a computer-controlled CNC mill (Sherline) as established previously [53, 54]. At RT, up to 3 ml of cell suspensions was placed on the plate and the cone in contact with cell suspensions was spun at a desired speed to expose cells to a constant shear stress field defined by Couette approximation. The shear stress created in this rheometer was calculated using the following equation:

$$\tau = \mu \times 2\pi\omega/(60 \sin\theta),$$

where τ is the shear stress (dyn/cm^2), μ is the viscosity (P), ω is the cone rotational rate (rpm), and θ is the cone angle in degrees [80]. For a cell suspension with an averaged viscosity of ~ 0.01 P, to achieve a shear stress of 2.5, 5, 7.5, or 10 dyn/cm^2, the cone rotational rate was set at 41, 82, 123, or 164 rpm, respectively. The shear time ranged from 1 to 10 min. Unsheared controls were cells maintained on the plate without spinning cone for a matched time period.

3.1.3 Analysis of shear-induced pseudopod retraction by dHL60 cells

3.1.3a Experimental details

Neutrophilic dHL60 cells were diluted 1:20 (v/v) with Hanks' buffered saline solution (HBSS; Invitrogen). To facilitate the analyses of cell-deactivating effects of shear stress, 10 nM fMLP (Sigma Aldrich) was used to mildly stimulate cells for 5 min; previous studies showed that fMLP at 10 nM results in elevated leukocyte activity without compromising the shear response [42]. Subsequently, cells were exposed to 5 dyn/cm^2 for 0 – 10 min, or to 0 – 10 dyn/cm^2 for 5 min. After experiments, cells were immediately fixed in 1% p-formaldehyde (Electron Microscopy Sciences) for >10 min.

3.1.3b Microscopic analysis of pseudopod activity

Fixed cells were permeabilized in 0.1% triton X-100 in PBS for 1 min, labeled with 2 µg/ml DAPI, and immediately observed with an IX-70 inverted fluorescence microscope (Olympus) at a 1000X magnification and ultraviolet illumination (excitation/emission wavelength: 358/461 nm). For each sample, at least 20 cells were visually assessed; the number of cells that expressed at least 1 cellular projection (Fig. 3.1) was counted and the percentage of cells with pseudopod(s) was used to quantify the cell pseudopod activity level.

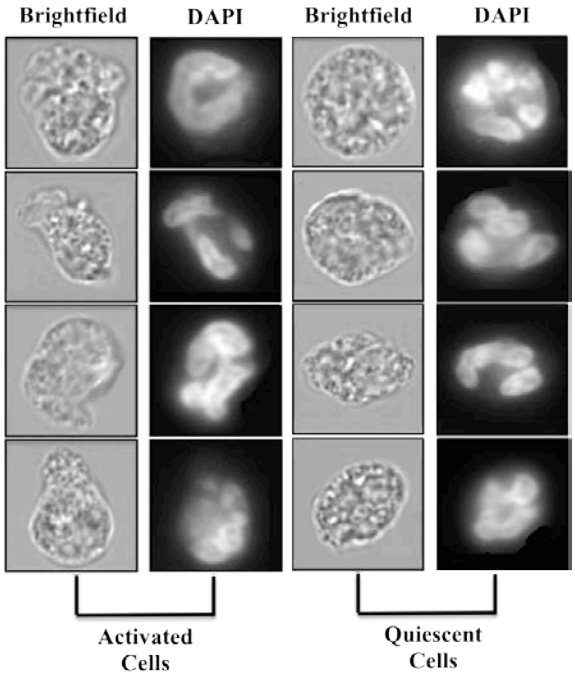

Figure 3.1 Representative images of quiescent and activated cells with pseudopod(s).

3.1.4 Analysis of shear-induced CD18 cleavage by suspended leukocytes

3.1.4a Experimental details

Within 3 h of blood collection, human leukocyte-enriched plasma was diluted 1:10 (v/v) in HBSS supplemented with 2.5 mM $CaCl_2$ (Sigma Aldrich) and subsequently exposed to 2.5, 5, or 10 dyn/cm^2 cone-plate shear stress at RT for up to 10 min. Controls were leukocyte populations maintained under no-flow, but otherwise similar experimental conditions.

To confirm the CD18 proteolysis, some experiments were conducted with cells pretreated with E64 (MP Biochemicals), a cysteine protease inhibitor known to block shear-induced reductions in CD18 surface expression [54]. For these studies, cells were

incubated in Ca^{2+}-supplemented HBSS containing 28 µM E64 for 10 min prior to shear exposure.

At the end of experiments, cells were fixed with 0.8% fresh p-formaldehyde for immunofluorescence analysis of CD18 expression or leukocyte-platelet binding (see section 3.1.4b).

To examine the effects of CD18 cleavage on fibrinogen binding, naïve or E64-treated purified neutrophils ($1x10^6$ cells/ml) in Ca^{2+}-supplemented HBSS were exposed to shear flow (10 dyn/cm^2; 10 min) followed by fibrinogen binding assay (section 3.1.4b).

3.1.4b Fluorescence-based flow cytometry

An LSR II flow cytometer (Becton-Dickinson) with Becton-Dickinson FacsDIVA software was used to analyze cell samples. For each sample, at least 10,000 leukocytes were acquired. Using WINMDI software (The Scripps Research Institute), populations of neutrophils, monocytes, and lymphocytes were gated based on their forward (FSC) and side scatters (SSC) (Fig. 3.2). On average, leukocyte populations tested in the present study contained approximately 61% neutrophils, 8% monocytes, and 31% lymphocytes typical of blood [81] under non-inflammatory/non-pathological conditions. We used this approach to examine CD18 cleavage, leukocyte-platelet binding, and neutrophil-fibrinogen binding, as follows.

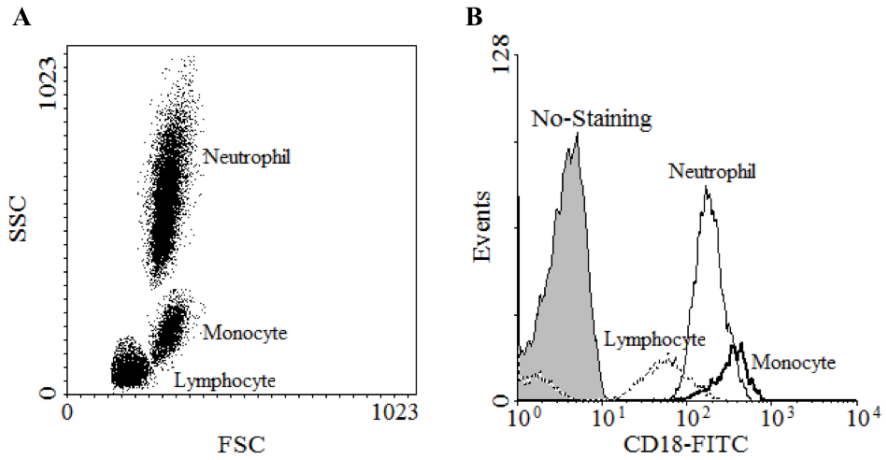

Figure 3.2 Representative analysis of antigen expression by flow cytometry.
A: Neutrophils, monocytes, and lymphocytes were gated based on FSC and SSC. B: Histograms of fluorescence intensity, i.e., CD18-FITC, were plotted for gated cell populations. The background fluorescence of unstained cells was also overlaid (No-Staining).

I. Quantification of CD18 cleavage

Fixed leukocytes were washed three times with PBS and blocked with 1% bovine serum albumin (BSA; Sigma Aldrich) at RT for 30 min.

To quantify CD18 surface expression, cells were stained with antibodies against CD11a (5 µg/ml; Alexa Fluor®647 conjugated clone 38; Serotec), CD11b (1:10 v/v; Alexa Fluor®488 conjugated clone ICRF44; Becton-Dickinson), or CD18 (1:10 v/v; FITC conjugated clone 6.7; Becton-Dickinson) in 1% BSA at 4 °C in the dark for 25 min [53, 54]. For the quantification of total amount of CD18 integrins (cleaved and uncleaved), a goat polyclonal antibody against the C-terminus of human CD18 (1 µg/ml; clone C-20; Santa Cruz) and Alexa Fluor®488 conjugated donkey anti-goat IgG (10 µg/ml; Invitrogen) secondary antibody were used to stain the cells using the same procedure but with 0.1% saponin (Sigma Aldrich) added to the blocking and wash buffers to permeabilize the cell membranes. After staining, cells were washed three times to

remove excess, unbound antibodies. The mean fluorescence intensity (MFI) of fluorescent label bound to each gated population was measured by flow cytometry to quantify the expression of CD18 integrins (Fig. 3.2).

II. Quantification of leukocyte-platelet binding

To quantify leukocyte-platelet adhesion, fixed cells were labeled with antibodies against platelet-specific CD41 (1:20 v/v; FITC conjugated clone VIPL3; Invitrogen) and/or leukocyte-specific CD45 (1:20 v/v; PE-Cy^{TM}7 conjugated clone HI30; Becton-Dickinson) in PBS at 4 °C in dark for 30 min. Non-specific binding was assessed using isotype-matched mouse IgG1 (1:20 v/v; Alexa Fluor®488 conjugates; Invitrogen). Using the LSR II flow cytometer and WINMDI software, the percentage of leukocytes that exhibited CD41-FITC labeling above a background level (i.e., unstained cells) was quantified and used as a measure of leukocyte-platelet binding [82, 83] (Fig. 3.3). Some experiments were conducted using two-color staining (i.e., CD41-FITC and CD45-PE-Cy^{TM}7) to verify that flow cytometry detected leukocyte-platelet interactions and not platelet-platelet aggregation.

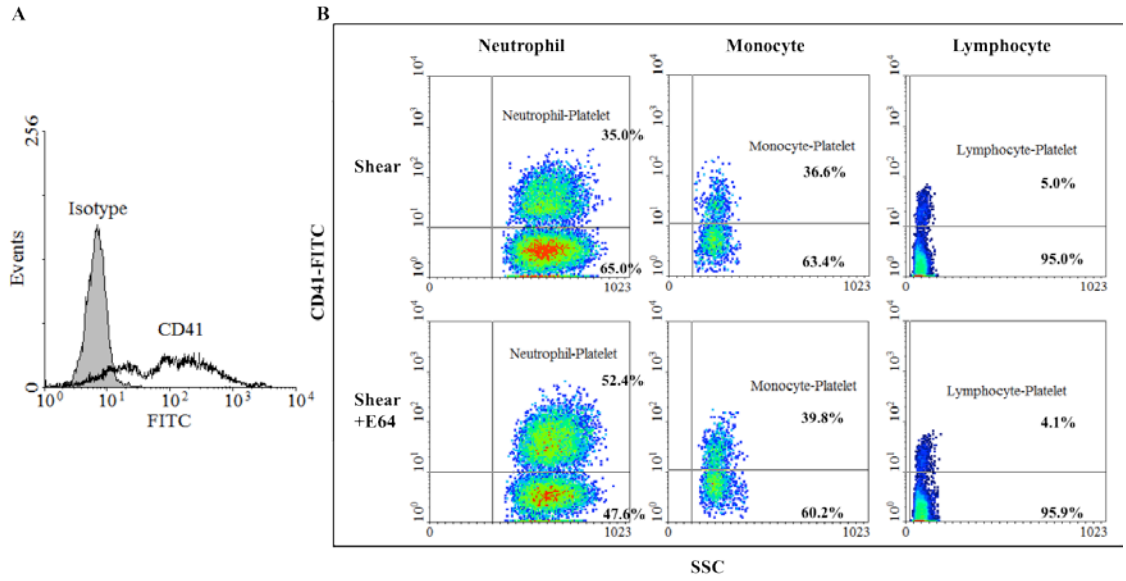

Figure 3.3 Representative analysis of leukocyte-platelet binding by flow cytometry. A: Leukocyte-platelet binding was analyzed using a monoclonal antibody against platelet-specific CD41. Non-specific binding was assessed with an isotype control. B: The distribution of CD41-FITC intensity was plotted against SSC for leukocytes under different experimental conditions. Particles exhibiting fluorescence above threshold levels were considered as leukocyte-platelet aggregates.

III. Quantification of neutrophil-fibrinogen binding

After shear experiments, live cells were chilled on ice for 2 min and subsequently incubated with 5 μg/ml Alexa Fluor®488 conjugated fibrinogen (Invitrogen) at 4 ℃ for 10 min with mild agitation followed by fixation with 0.8% p-formaldehyde on ice. Non-specific binding was assessed by performing the incubations of cells with Alexa Fluor®488 conjugated fibrinogen in the presence of a 10-fold excess of non-fluorescent fibrinogen (MP Biomedicals) [84]. The MFI of fibrinogen present on neutrophil populations was measured by flow cytometry as an indicator of neutrophil-fibrinogen binding.

3.2 Results

3.2.1 Pseudopod activity of non-adherent neutrophils is regulated by fluid shear stress

3.2.1a Shear-induced pseudopod retraction by neutrophils is an acute response

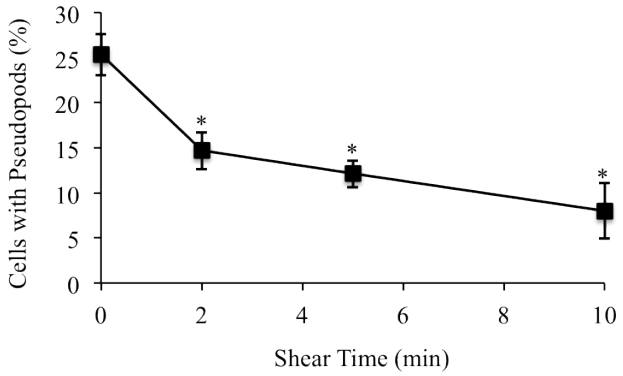

Figure 3.4 Shear-induced reductions in pseudopod activity depend on shear exposure time.
Percentage of dHL60 cells with pseudopods was visually counted after being sheared at 5.0 dyn/cm^2 for 0, 2, 5, and 10 min. Data are mean ± SEM from N = 3 experiments. *p < 0.05 compared to t = 0 using one way ANOVA with Bonferroni's method.

Under static no-flow (i.e., t = 0) conditions, the percentage of cells with pseudopods was approximately 25%. Despite this low level of pseudopod activity, a significant ($p < 0.05$) reduction in the numbers of cells expressing pseudopods in dHL60 cell populations was detected after only 2 min of exposure to 5.0 dyn/cm^2 cone-plate shear stress (Fig. 3.4). Shear exposure for 5 or 10 min also significantly ($p < 0.05$) reduced pseudopod formation by dHL60 cells, however, there was no difference in the percentage of cells exhibiting pseudopods among cell populations exposed to shear stress for 2, 5, or 10 min (Fig. 3.4).

3.2.1b Shear-induced pseudopod retraction by neutrophils occurs independently of shear magnitude within the range of 5 to 10 dyn/cm^2

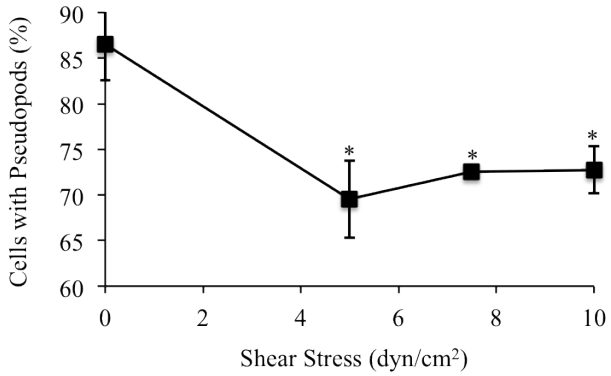

Figure 3.5 Shear-induced reductions in pseudopod activity occur independently of shear magnitudes within 5 – 10 dyn/cm^2.
Suspensions of dHL60 cells were subjected to 0 (control), 5, 7.5, and 10 dyn/cm^2 for 5 min following treatment with 10 nM fMLP for 5 min. Data are mean ± SEM from N = 3 experiments. *$p < 0.05$ compared to control using one way ANOVA with Bonferroni's method.

To facilitate detection of the magnitude dependent effects of fluid shear stress on pseudopod activity, 10 nM fMLP was used to elevate the pseudopod activity of cell populations above baseline levels. Stimulating cell populations with 10 nM fMLP for 5 min elevated the percentage of cells with pseudopods to approximately 86%. Pre-activation of cells in 10 nM fMLP buffer followed by exposure to 5.0 dyn/cm^2 in the same buffer for 5 min resulted in significantly ($p < 0.05$) reduced pseudopod activity (Fig. 3.5). Similar degrees of reductions in pseudopod activity of dHL60 cell populations were observed after 5-min exposure to 7.5 or 10 dyn/cm^2 (Fig. 3.5).

3.2.2 Shear-induced proteolysis regulates CD18 expression on the surfaces of leukocytes

3.2.2a Shear-induced down-regulation of CD18 surface expression is time-dependent

Under static conditions, the constitutive (i.e., baseline) surface expression of CD18 integrins by the three main leukocyte subpopulations remained constant (neutrophils: 216±54 MFI; monocytes: 386±110 MFI; lymphocytes: 80±24 MFI) within 3 h of blood collection (i.e., the time frame for all of our experiments) and was not affected by the stainless steel plates of our cone-plate rheometer.

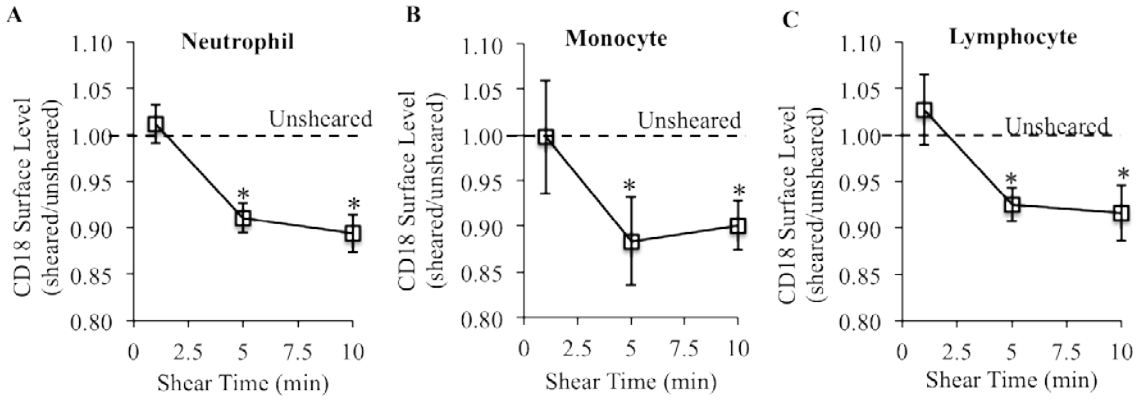

Figure 3.6 Fluid flow-mediated reductions in CD18 surface expression require threshold duration of shear exposure.
A – C: The surface levels of CD18 on neutrophils (A), monocytes (B), and lymphocytes (C) were measured after exposing diluted leukocyte-enriched plasma to 10 dyn/cm^2 shear stress for 1, 5, and 10 min, and normalized to levels of time-matched cell type-specific controls (Unsheared; dashed line). Data are mean ± SEM from N = 4 experiments. *p < 0.05 compared to controls using Mann-Whitney Rank Sum test.

Compared with cell populations maintained under static conditions (i.e., unsheared controls), heterogeneous leukocyte populations subjected to fluid shear stress (10 dyn/cm^2) for up to 10 min exhibited time-dependent reductions in CD18 surface levels (Fig. 3.6). Although there were no significant changes in CD18 expression on the surface of neutrophils, monocytes, or lymphocytes after being sheared for 1 min relative

to their respective time-matched controls, the CD18 surface levels for each leukocyte subtype were significantly ($p < 0.05$) reduced to similar levels (i.e., minimal levels) after 5 and 10 min of shear exposure.

3.2.2b Magnitude-dependence of shear-induced CD18 reductions is leukocyte subtype specific

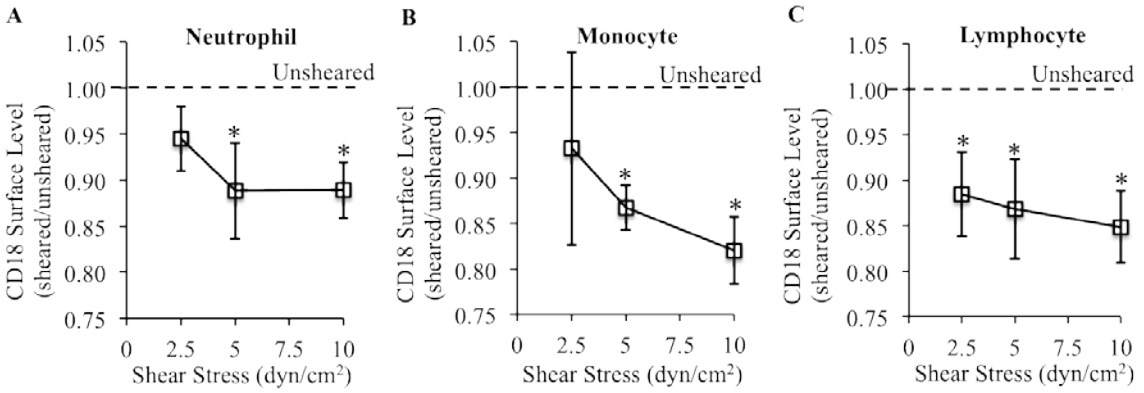

Figure 3.7 Fluid flow-mediated reductions in CD18 surface expression require exposure to a threshold magnitude of shear stress.
A – C: The surface levels of CD18 on neutrophils (A), monocytes (B), and lymphocytes (C) in the diluted leukocyte-enriched plasma were measured after 10-min exposure to 2.5, 5, and 10 dyn/cm^2 shear stress, and normalized to levels of time-matched cell type-specific controls (Unsheared; dashed line). Data are mean ± SEM from N = 4 experiments. *$p < 0.05$ compared to controls using Mann-Whitney Rank Sum test.

Fluid flow-mediated down-regulation of CD18 surface expression by leukocytes depended on the magnitude of the applied shear stress. Whereas exposure of neutrophils and monocytes to 2.5 dyn/cm^2 shear stress for 10 min did not lead to significant reductions in the surface levels of CD18, subjecting these cells to 5 or 10 dyn/cm^2 significantly ($p < 0.05$) reduced the total amount of surface CD18 after 10 min, relative to unsheared cell type-matched controls (Fig. 3.7). For lymphocytes exposed to the same

shear field, significant ($p < 0.05$) reductions in CD18 surface expression were detected under shear stresses as low as 2.5 dyn/cm^2 (Fig. 3.7).

3.2.2c Shear stress reduces CD18 expression on the leukocyte membranes via cleavage of the integrin ectodomain

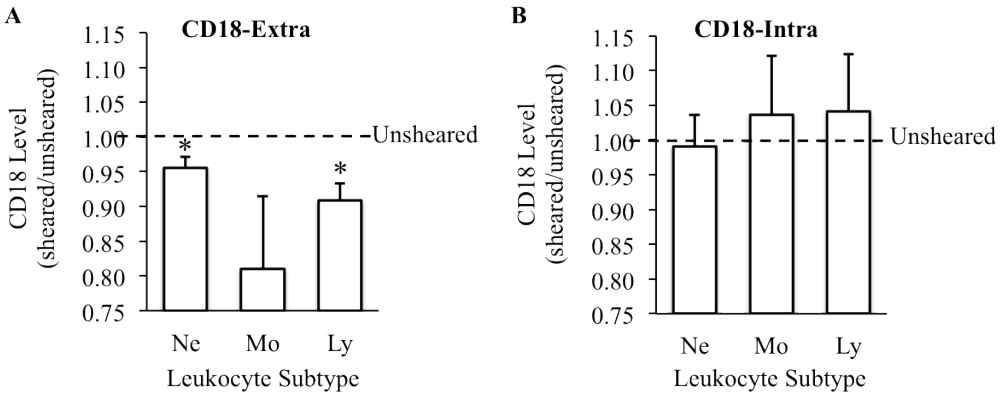

Figure 3.8 Shear stress-induced reductions in CD18 surface expression involve cleavage of the extracellular domain.
A and B: The expression levels of extra- (A) and intra- (B) cellular CD18 by neutrophils (Ne), monocytes (Mo), and lymphocytes (Ly) in the diluted leukocyte-enriched plasma were measured after 10-min exposure to 10 dyn/cm^2 shear stress, and normalized to levels of matched cell type-specific controls (Unsheared; dashed line). Data are mean ± SEM from N = 8 experiments. *$p < 0.05$ compared to controls using Mann-Whitney Rank Sum test.

For neutrophils and lymphocytes, exposure to 10 dyn/cm^2 shear stress for 10 min significantly ($p < 0.05$) reduced binding of antibodies targeting the extracellular domains of CD18 integrins, but had no detectable effects on the binding of antibodies recognizing cytosolic CD18 epitopes (Fig. 3.8). These results provide evidence that CD18 integrins were cleaved off neutrophils and lymphocytes in the extracellular domain due to shear exposure. A similar pattern of antibody binding was observed for monocytes although

differences in the amount of antibodies bound to the extracellular domain of CD18 between sheared and unsheared monocytes failed to reach significance (Fig. 3.8).

3.2.2d Shear-induced CD18 cleavage depends on the integrin subtype

Under static conditions, resting leukocytes constitutively expressed CD18 in an integrin subtype-dependent manner. The three leukocyte subtypes all expressed CD11a but at significantly ($p < 0.05$) different levels; monocytes exhibited the highest level of expression followed by lymphocytes and neutrophils (Fig. 3.9). In contrast, neutrophils and monocytes displayed similar surface levels of CD11b, while lymphocytes exhibited undetectable surface expression of this integrin subunit (Fig. 3.9).

In response to shear exposure (10 dyn/cm^2; 10 min), CD18 cleavage also occurred in an integrin subtype-specific fashion. For neutrophils, shear exposure significantly ($p < 0.05$) reduced the surface levels of CD11b and CD18, but not CD11a (Fig. 3.9). Although sheared monocytes on average exhibited lower levels of both CD11b and CD18 as compared to unsheared cells, these differences were not statistically significant (Fig. 3.9). In the case of lymphocytes, exposure to shear stress significantly ($p < 0.05$) reduced surface levels of CD11a and CD18 (Fig. 3.9). These results provide evidence that shear stress induced cleavage of Mac-1 on neutrophils and monocytes (less robust) but elicited proteolysis of LFA-1 on lymphocytes.

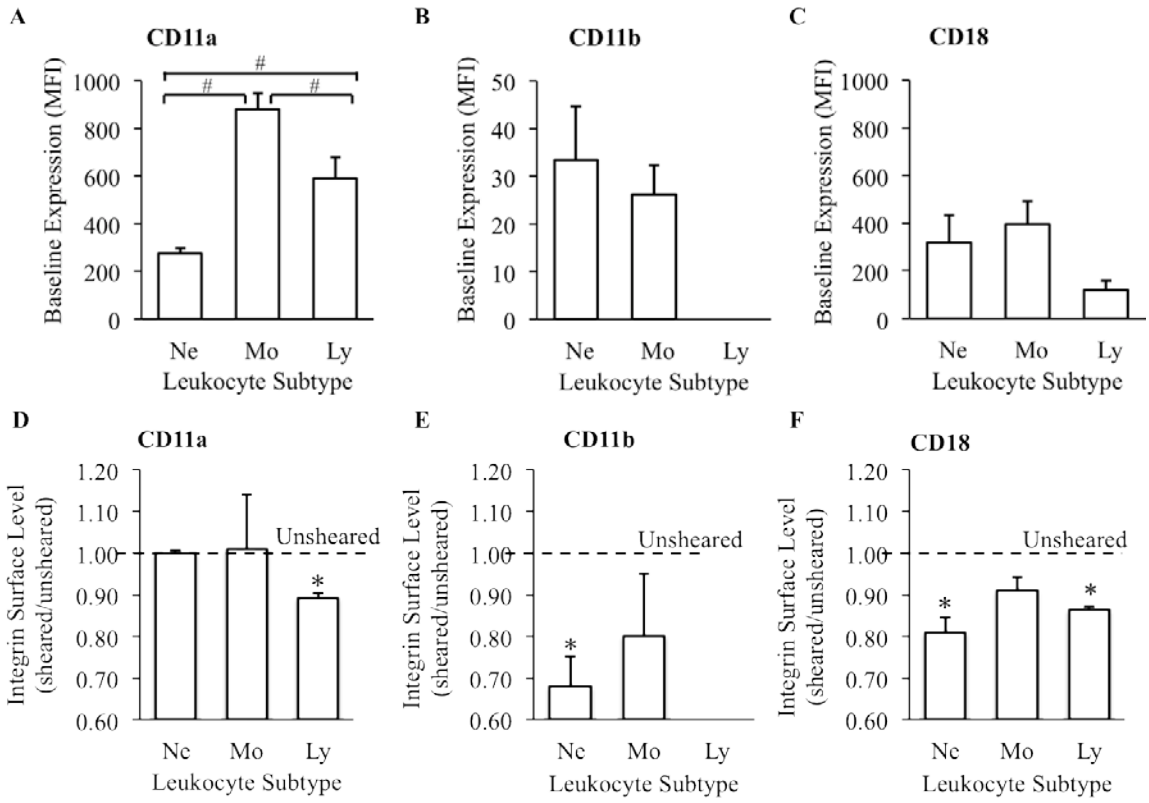

Figure 3.9 Shear-induced CD18 cleavage depends on the CD18 subtype.
A – C: The expression levels of CD11a (A), CD11b (B), and CD18 (C) on the surfaces of neutrophils (Ne), monocytes (Mo), and lymphocytes (Ly), under static condition, were measured as the mean fluorescence intensity (MFI) of related antibody. #$p < 0.05$ compared among the three leukocyte subsets using one way ANOVA with Bonferroni's correction. D – F: The surface levels of CD11a (D), CD11b (E), and CD18 (F) on different leukocyte subsets were measured after 10-min exposure to 10 dyn/cm^2 shear stress, and normalized to levels of matched cell type-specific controls (Unsheared; dashed line). Data are mean ± SEM from N = 3 experiments. *$p < 0.05$ compared to controls using Mann-Whitney Rank Sum test.

3.1.2e Protease inhibitor E64 differentially influences CD18 cleavage by leukocytes under shear

Treatment of leukocyte populations with 28 μM E64 had no effect on their baseline expression of CD18 integrins under static condition (data not shown). However, this protease inhibitor significantly ($p < 0.05$) enhanced surface expression of CD18 integrins by neutrophils subjected to shear stress (10 dyn/cm^2; 5 min) in a cone-plate

viscometer consistent with the blockade of shear-induced cleavage, as compared to untreated cells exposed to a similar shear field (Fig. 3.10). In contrast, E64 had no significant effect on the CD18 surface levels of sheared monocytes and lymphocytes (Fig. 3.10).

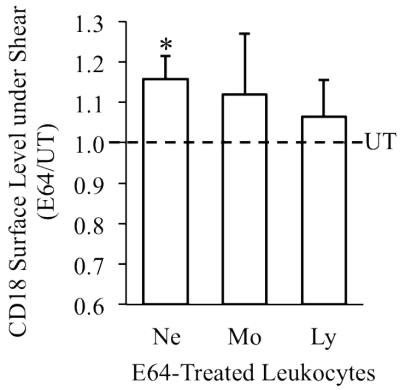

Figure 3.10 Protease inhibitor E64 differentially influences CD18 cleavage by leukocytes under shear flow.
The CD18 surface levels were quantified for E64-treated neutrophils (Ne), monocytes (Mo), and lymphocytes (Ly) after shear exposure (10 dyn/cm^2; 5 min) and normalized to those for cell type-matched controls (untreated cells or UT; dashed line) in the same shear field. Data are mean ± SEM from N = 5 experiments. *$p < 0.05$ compared to UT using Student's t-test.

3.1.2f Protease inhibitor E64 differentially influences leukocyte-platelet binding under shear

In the absence of exogenous chemical stimuli, heterogeneous leukocyte populations under shear flow (10 dyn/cm^2; 5 min) exhibited spontaneous platelet binding (Fig. 3.11A). In comparison, the percentage of cells with bound platelets for all the three subtypes of leukocytes increased significantly ($p < 0.05$) after shear exposure. Moreover, lymphocytes exhibited the lowest level of platelet binding under shear among the three subtypes.

Interestingly, whereas E64 did not affect the adhesive interactions between leukocytes and platelets under static condition (data not shown), treatment with this anti-protease promoted neutrophil-platelet binding under shear exposure. Notably, the percentage of E64-treated neutrophils binding at least one platelet were significantly ($p < 0.05$) higher than that observed for untreated cells when exposed to the same shear field (Fig. 3.11B). In the case of monocytes and lymphocytes, E64 had no detectable effects on their adhesion to platelets under shear flow (Fig. 3.11B)

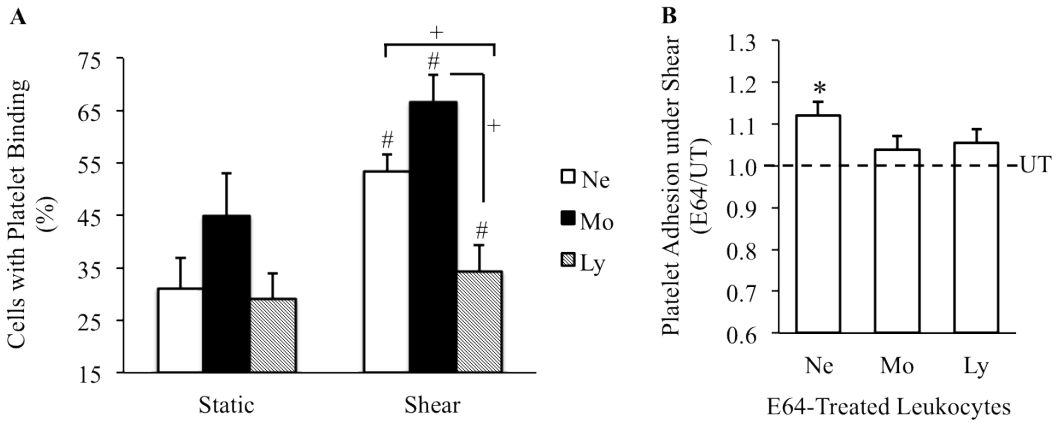

Figure 3.11 Protease inhibitor E64 differentially influences leukocyte-platelet binding under shear flow.
A: The percentage of naïve cells binding at least one platelet was examined for neutrophils (Ne), monocytes (Mo), and lymphocytes (Ly) under static and shear (10 dyn/cm^2; 5 min) conditions. #$p < 0.05$ compared to the same cell type under static condition using Student's t-test. +$p < 0.05$ compared among Ne, Mo, and Ly using one way ANOVA with Bonferroni's correction. B: Platelet binding to E64-treated cells was quantified after shear exposure and normalized to those of cell type-matched non-chemically treated controls (UT; dashed line) in the same shear field. Data are mean ± SEM from N = 5 experiments. *$p < 0.05$ compared to UT using Student's t-test.

3.1.2g Shear-induced CD18 cleavage reduces binding of neutrophils to fibrinogen

Fluid shear stress suppressed binding of neutrophils to fibrinogen, a Mac-1 ligand that mediates leukocyte-platelet interactions. The amount of fibrinogen bound to

neutrophils that had been sheared at 10 dyn/cm^2 for 10 min was significantly ($p < 0.05$) lower than that present on cells maintained under static no-shear conditions (Fig. 3.12). For cells pretreated with E64, there was no significant difference between sheared and unsheared samples (Fig. 3.12).

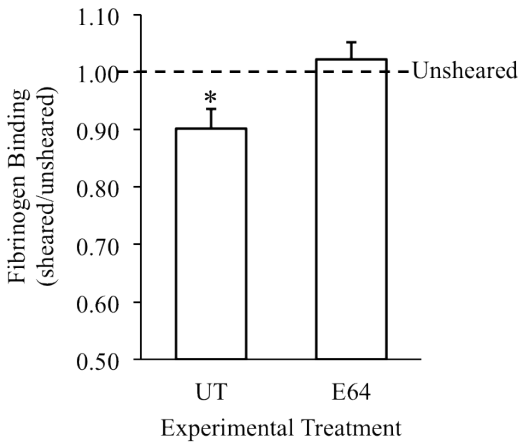

Figure 3.12 Shear stress-mediated CD18 cleavage influences neutrophil-fibrinogen binding.
The amount of fibrinogen bound to sheared neutrophils was measured and normalized to that of unsheared cells (dashed line). Data are mean ± SEM from N = 5 experiments. *$p < 0.05$ compared to unsheared controls using Mann-Whitney Rank Sum test.

CHAPTER 4 DEPENDENCE OF NEUTROPHIL SHEAR MECHANOSENSITIVITY ON MEMBRANE CHOLESTEROL

Cholesterol is an essential structural component of cell membranes that plays a critical role in regulating cell signaling and activity levels. Changes in the content and/or organization of cholesterol within the plasma membranes have been demonstrated to influence a number of cell functions for neutrophils or neutrophilic cells including cell polarization, shape change, actin polymerization, adhesion and migration [85-88]. The effects of membrane cholesterol modifications take place via impacts on lipid rafts and/or membrane fluidity.

Studies in this section were designed to explore the role of membrane cholesterol in the shear stress mechanoregulation of neutrophil pseudopod activity. For this purpose, cholesterol molecules conjugated with methylated β-cyclodextrin (MβCD) were used as membrane cholesterol-enhancing agents, particularly because MβCD in this conjugate has been shown to efficiently deliver cholesterol into the cells within a short period of incubation [89]. In order to confirm the involvement of membrane fluidity, benzyl alcohol (BnOH), which fluidizes plasma membranes by increasing membrane free volume [66], was also used.

Moreover, as an in vivo strategy of membrane cholesterol enrichment in leukocytes, a low-density lipoprotein receptor deficient ($LDLr^{-/-}$) murine model of hypercholesterolemia was used. This model is associated with diet-induced elevations in blood cholesterol levels that reportedly enhance the membrane cholesterol content of blood borne leukocytes [90].

4.1 Methods

4.1.1 Analysis of membrane cholesterol-dependent, shear-induced pseudopod retraction

4.1.1a Shear stress experiments with human whole blood

Whole blood was harvested from human subjects as described in section 3.1.1b. To increase the membrane cholesterol content of human leukocytes, aliquots of whole blood was treated with 0 – 10 μg/ml cholesterol:MβCD complexes (Sigma Aldrich) for 15 min, and subsequently incubated in HBSS (1:20 v/v) containing 10 nM fMLP and 0 – 7 mM BnOH (Acros Organics) for 10 min.

After membrane-modifying chemical treatments and cell pre-activation, blood cell suspensions were subjected to 5 dyn/cm^2 at RT for 10 min (see section 3.1.2). Controls were parallel cell suspensions maintained under no-flow, but otherwise similar experimental conditions. At the end of experiments, cells were fixed with 1% p-formaldehyde and subjected to analyses of pseudopod activity as described in section 3.1.3b. The shear response (%) was calculated using the following equation:

$$Shear\ Response\ (\%) = \frac{\%\ cells\ with\ pseudopods_{static} - \%\ cells\ with\ pseudopods_{shear}}{\%\ cells\ with\ pseudopods_{static}}\ \%$$

4.1.1b Cell viability

In separate experiments, the viability of cell populations was assessed immediately after the membrane-modifying chemical treatments. A Live/Dead Double Staining Kit (Calbiochem) that quantifies the uptake of calcein AM (live cell indicator)

and propidium iodide (dead cell indicator) was used following the manufacturer's instructions. Neutrophil viability remained unchanged at >90% after all membrane-modifying treatments tested in the present study.

4.1.1c Membrane cholesterol quantification

The free cholesterol in leukocyte membranes was quantified after membrane-modifying chemical treatments. Membrane cholesterol was extracted from leukocytes according to the method of Bligh and Dyer [91]. Briefly, cells ($1x10^6$ cells/ml) in 600 µl ice-cold HBSS were vortexed and incubated with 2.25 ml of a 1:2 v/v chloroform/methanol solution for 10 min followed by sequential addition of 750 µl of chloroform and 750 µl of diH_2O. After centrifugation at 1900xg for 10 min, the top alcoholic layer was aspirated and the organic layer was evaporated to dryness at 50 °C. The extracted cholesterol was reconstituted in 200 µl of 10% TritonX-100 in isopropyl alcohol. The reconstituted cholesterol was diluted (1:60 v/v) with the color reagent of the Free Cholesterol E kit (Wako) and incubated at 37 °C for 5 min; the absorbance was measured at 600 nm with a spectrophotometer (BioTek; µQuant). The concentration of free cholesterol was calculated from the absorbance readings according to a standard curve generated following the manufacturer's instruction.

4.1.1d Quantification of membrane fluidity

The effects of cholesterol:MβCD complexes on membrane fluidity were assessed using 1-pyrenedecanoic acid (PDA; Molecular Probes) as described previously [92].

Briefly, leukocyte-enriched plasma was incubated with 5 μM PDA in PBS containing 5 mM EDTA at 37 °C for 1 h, and then subjected to the experimental membrane-modifying treatments tested in the present study (see section 4.1.1a). Fluorescence emissions were immediately evaluated for each live cell preparation using a fluorescence spectrophotometer (Hitachi; F-2500) at wavelengths of 375 nm to quantify monomer (I_m) emission and 470 nm to measure excimer (I_e) emission under 344 nm excitation [92]. Membrane fluidity was expressed as a ratio of I_e/I_m. For this analysis, as I_e/I_m ratios increases, membrane fluidity increases.

4.1.2 Analysis of shear stress mechanosensitivity in hypercholesterolemia

All procedures used to collect and handle murine blood samples in the present study were approved by the Institutional Animal Care and Use Committee at the University of Kentucky (Lexington, KY).

4.1.2a Mice

Wild-type (WT) C57BL/6J (male; 7-wk age; Cat # 0664) and $LDLr^{-/-}$ mice (male; 7-wk age; B6.129S7-$Ldlr^{tm1Her}$; Cat # 2207) were purchased from the Jackson Laboratory. Animals were housed in a controlled environment, with up to 5 mice per cage, and provided water and food ad libitum. All mice were used after an acclimation period of 1 wk during which they were placed on a normal mouse diet (ND). WT mice were used primarily to generate baseline data for the planned experiments.

To induce hypercholesterolemia, acclimated LDLr$^{-/-}$ mice were fed a diet enriched in saturated fat (HFD) (21% wt/wt fat and 0.15% wt/wt cholesterol; Harlan Teklad; Cat # TD88137) ad libitum for up to 8 wk. Age-matched LDLr$^{-/-}$ mice fed a ND served as control animals. Animals underwent a weekly body weight check to ensure the uptake of food.

4.1.2b Murine blood harvest

After the prescribed diet durations, mice were anesthetized with a lethal dose of xylazine/ketamine mixture (90 and 10 mg/kg body weight; i.p.). When the mice lost consciousness (i.e., no toe-reflex), the chest cavity was surgically opened to expose the beating heart and blood (approximately 500 µl) was drawn from the left ventricle using a 23-gauge needle into a syringe containing EDTA (20 µl of 0.2 M). To ensure death, the right atrium was also cut to exsanguinate the animals until their hearts stopped beating. Blood was used for shear experiments within 3 h.

4.1.2c Analysis of murine neutrophil mechanosensitivity

Blood from WT or LDLr$^{-/-}$ mice that had been placed on either ND or HFD for 2, 4, and 8 wk was diluted 1:20 (v/v) in HBSS and subjected to 5 dyn/cm^2 cone-plate shear at RT for up to 10 min as described in section 3.1.2. The pseudopod activity of neutrophils was assessed with microscopy as described in section 3.1.3b.

To verify the influence of cell membrane fluidity on the pseudopod retraction responses of leukocytes, blood from LDLr$^{-/-}$ mice subjected to ND and HFD for 2 wk was

pretreated with 0 – 0.05 mM BnOH and 10 nM fMLP for 1 min prior to shear experiments (5 dyn/cm^2; 10 min).

4.1.2d Plasma cholesterol quantification

The plasma levels of total, free, and esterified cholesterol were quantified for all WT, ND, and HFD mice used in the present study. Briefly, plasma was harvested after centrifugation of whole blood under 1000xg at 4 °C for 5 min. Plasma samples were incubated (1:100 v/v) with Infinity Cholesterol Reagent (Sigma Aldrich) at 37 °C for 5 min; the absorbance was measured at 500 nm with a BioTek microplate reader and used to calculated the concentration of total cholesterol in plasma samples based on the standard curve generated following the manufacturer's instruction. The concentration of free cholesterol in plasma samples was quantified using the same Free Cholesterol E kit with the same spectrophotometer as described in section 4.1.1c. The mass concentration of esterified cholesterol was calculated as that of the sterol moiety plus fatty acid using the established relationship [93],

$$Esterified\ Cholesterol = (Total\ Cholesterol - Free\ Cholesterol) \times 1.67\ .$$

4.2 Results

4.2.1 The shear stress sensitivity of human neutrophils depends on the cholesterol-related fluidity of the cell membrane

4.2.1a Cholesterol governs shear stress-mediated reductions in neutrophil pseudopod activity by its influence on membrane fluidity

In line with neutrophil-like dHL60 cells (Figs. 3.4 and 3.5), populations of non-adherent human neutrophils pre-stimulated with 10 nM fMLP were deactivated by cone-plate shear stress (5.0 dyn/cm^2; 10 min) as reflected by significantly ($p < 0.05$) reduced percentage of cells with pseudopod formation (static: 62±9% versus shear: 28±5%; $p < 0.05$).

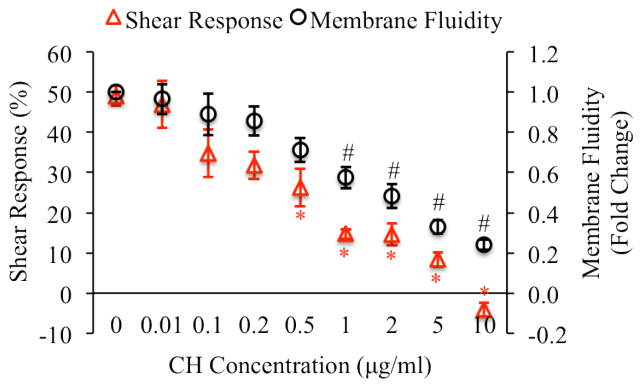

Figure 4.1 Membrane cholesterol-enhancing agents dose-dependently decrease membrane fluidity and impair shear-induced reductions in pseudopod activity. Shear responses (%; 5 dyn/cm^2; 10 min) were determined for human neutrophils pretreated with 0 – 10 μg/ml cholesterol:MβCD (CH) and 10 nM fMLP. Membrane fluidity for CH-treated cells was also measured and normalized to that of untreated cells. Data are mean ± SEM from $n \geq 3$ experiments. *, #$p < 0.001$ compared to samples without CH treatment using Student's t-test with Bonferroni's correction.

Treatment of neutrophils with cholesterol:MβCD conjugates, to enhance their membrane cholesterol content, had no detectable effects on their baseline pseudopod

activity under static conditions (percentages of cells with pseudopods in all samples fluctuated around 56% without significant changes). However, these treatments dose-dependently attenuated shear-induced pseudopod retraction (Fig. 4.1). Specifically, treating cells with increasing concentration of cholesterol complexes (between 0.5 - 10 μg/ml) resulted in gradual reductions in the ability of fluid shear stress to reduce the number of neutrophils with pseudopods (indicative of an impaired pseudopod retraction response). Notably, when treated with 10 μg/ml cholesterol conjugates, the responsiveness of neutrophils to shear was reversed; in other words, shear exposure (5 dyn/cm^2; 10 min) caused these cells to project pseudopods. The dose-dependent effect of cholesterol complexes on the shear responses of neutrophils was verified by linear regression analysis. A significant ($p = 0.0062$) negative correlation ($r = -0.8253$) was detected between concentrations of cholesterol:MβCD conjugates used to treat neutrophils and shear-induced reductions in the numbers of cells that projected pseudopods (Table 4.1).

Interestingly, the attenuating effect of cholesterol:MβCD conjugates on shear-induced pseudopod retraction was related to their influence on membrane fluidity. Incubation of neutrophils with cholesterol:MβCD decreased their membrane fluidity (Fig. 4.1). Specifically, the fluidity of neutrophil membranes negatively correlated ($r = -0.8494$; $p = 0.0038$) with the concentration of cholesterol conjugates used to treat neutrophils (Table 4.1). When cholesterol conjugates were greater than 1 μg/ml, the membrane fluidity of cholesterol-treated neutrophils was significantly ($p < 0.001$) lower than that of untreated naïve cells.

Table 4.1 Correlation analyses.

		CH (μg/ml)	Membrane Fluidity	Shear Response (%)
CH (μg/ml)	Pearson r p (two-tailed)	-	-	-
Membrane Fluidity	Pearson r p (two-tailed)	-0.8494 0.0038	-	-
Shear Response (%)	Pearson r p (two-tailed)	-0.8253 0.0062	0.9783 < 0.0001	-

Finally, a correlation analysis was conducted between the membrane cholesterol-dependent fluidity and the pseudopod retraction responses of cholesterol-treated neutrophils to shear. The responsiveness of neutrophils pretreated with 0 – 10 μg/ml cholesterol:MβCD to shear stress stimulation, in the form of reduced pseudopod activity, positively correlated ($r = 0.9783$; $p < 0.0001$) with their membrane fluidity (Table 4.1). These results therefore provide first evidence that neutrophil responsiveness to fluid shear stress depends on their membrane cholesterol-related fluidity.

4.2.1b Intact neutrophil responsiveness to shear stress requires an optimal level of membrane fluidity

To further verify the dependence of neutrophil mechanosensitivity to fluid flow on membrane cholesterol-related fluidity, the combined effects of cholesterol:MβCD conjugates and membrane fluidizer, BnOH, on the cell-deactivating effects of fluid shear stress were tested. It was confirmed that the fluidizing effect of BnOH was independent of any influence on membrane cholesterol levels (Fig. 4.2A). Specifically, treatment of

leukocytes with 2 μg/ml cholesterol:MβCD conjugates either alone or followed by 2 mM BnOH significantly ($p < 0.05$) increased cholesterol content in the cell membranes. In contrast, incubation of cells with 2 mM BnOH alone had no effect on membrane cholesterol levels.

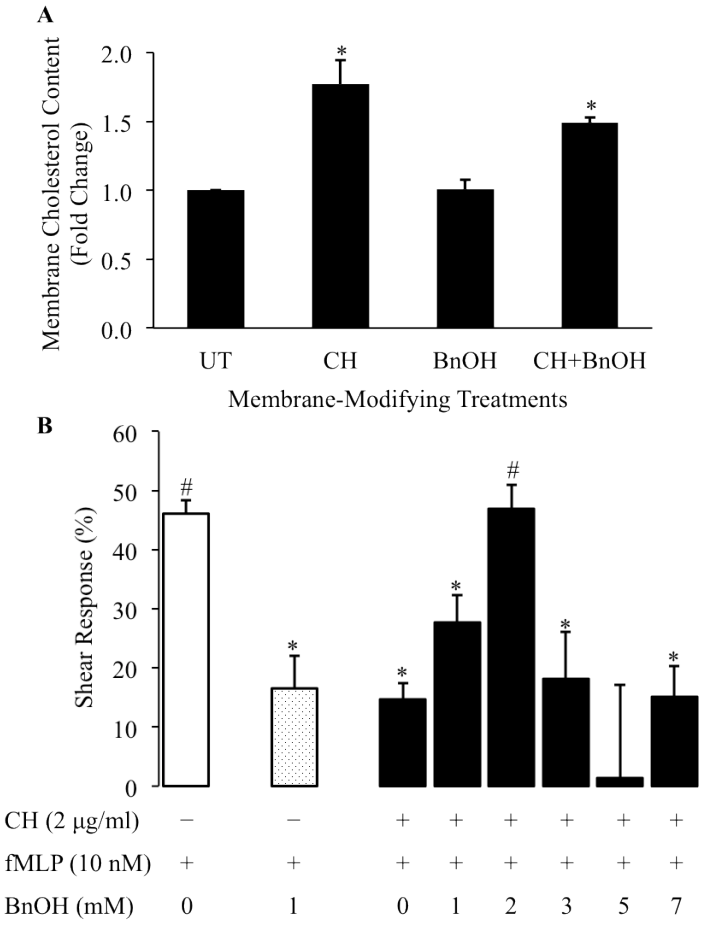

Figure 4.2 Membrane fluidizers selectively counteract the attenuating effects of membrane cholesterol enrichment on shear-induced reductions in pseudopod activity (I).
A: Normalized membrane cholesterol was measured for untreated cells (UT) and cells treated with 2 μg/ml cholesterol:MβCD (CH) and/or 2 mM BnOH. B: Shear responses (%; 5 dyn/cm^2; 10 min) were determined for neutrophils pretreated with different chemicals. Data are mean ± SEM from $n \geq 3$ experiments. Using one way ANOVA with Bonferroni's method, *$p < 0.05$ compared to samples without CH and BnOH treatment, #$p < 0.05$ compared to samples pretreated with CH alone.

Although treatment of neutrophils with cholesterol:MβCD conjugates and/or BnOH did not affect their pseudopod activity under static conditions (percentages of cells with pseudopods in all samples fluctuated around 48% without significant changes), these membrane modifications altered shear-induced deactivation (i.e., pseudopod retraction) by these cells (Fig. 4.2B). Notably, the neutrophil shear response was significantly ($p < 0.001$) attenuated by either 2 μg/ml cholesterol:MβCD or 1 mM BnOH alone. Addition of 2 mM BnOH to suspensions of cells pretreated with 2 μg/ml cholesterol conjugates restored their shear responses to levels exhibited by untreated cells. BnOH at concentrations either lower or higher than 2 mM (i.e., 1, 3, 5, and 7 mM), however, failed to counteract the attenuating effects of 2 μg/ml cholesterol complexes on neutrophil responses to shear.

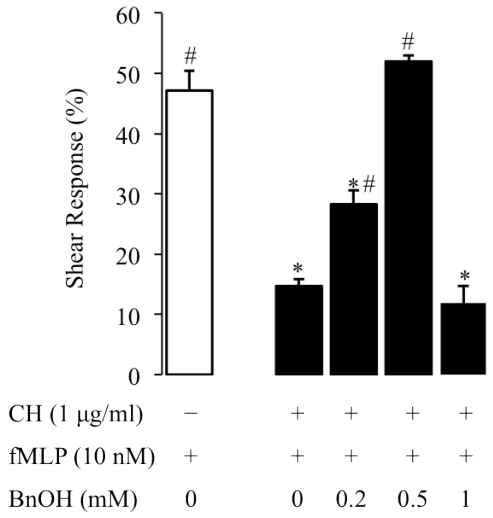

Figure 4.3 Membrane fluidizers selectively counteract the attenuating effects of membrane cholesterol enrichment on shear-induced reductions in pseudopod activity (II).
Shear responses (%; 5 dyn/cm^2; 10 min) were determined for neutrophils pretreated with different chemicals. Data are mean ± SEM from n = 3 experiments. Using one way ANOVA with Bonferroni's method, *$p < 0.05$ compared to samples without CH and BnOH treatment, #$p < 0.05$ compared to samples pretreated with CH alone.

When shear-induced pseudopod retraction responses by neutrophils were significantly ($p < 0.005$) blocked by 1 μg/ml cholesterol:MβCD, the concentration of BnOH needed to reinstate their responses was shifted to 0.5 mM (Fig. 4.3). In this case, BnOH at concentrations lower or greater than 0.5 mM (i.e., 0.2 and 1 mM) failed to completely recover the neutrophil shear responses depressed by pretreatment with 1 μg/ml cholesterol:MβCD. Thus, there existed an "optimal" level of membrane fluidity permissive for an intact pseudopod retraction response of neutrophils to shear stress stimulation.

4.2.2 Hypercholesterolemia impairs shear-induced neutrophil deactivation

4.2.2a $LDLr^{-/-}$ mouse is associated with diet duration-dependent elevations in blood cholesterol levels

$LDLr^{-/-}$ mice on ND exhibited significantly ($p < 0.05$) increased blood plasma levels of free, esterified, and total cholesterol compared with WT mice on the same diet (Table 4.2). However, whereas serum concentrations of esterified and total cholesterol in these $LDLr^{-/-}$-ND animals increased in a time-dependent fashion, the levels of free cholesterol remained constant throughout the 8-wk duration of the study (Table 4.2). When placed on HFD, the levels of cholesterol (both free and esterified) in $LDLr^{-/-}$ mice increased dramatically. Specifically, after 2 wk, the HFD group exhibited approximately 4-fold higher serum free and esterified cholesterol concentrations than those in the ND group (Table 4.2). As HFD progressed for up to 8 wk, plasma concentrations of total, esterified, and free cholesterol all rose in a diet duration-dependent fashion (Table 4.2).

Table 4.2 Cholesterol levels in the blood plasma of LDLr$^{-/-}$ mice.

Cholesterol Type	Diet	Cholesterol Concentration (mg/dl)		
		2-week	4-week	8-week
Total	ND	125 ± 11	207 ± 20 #	281 ± 43 #
	HFD	642 ± 33 *	852 ± 72 *#	1215 ± 228 *#
Free	ND	59 ± 3	62 ± 3	71 ± 4
	HFD	289 ± 21 *	374 ± 26 *#	449 ± 56 *#
Esterified	ND	110 ± 14	243 ± 31 #	350 ± 66 #
	HFD	590 ± 47 *	799 ± 94 *	1278 ± 309 *#

Note: [1]WT Mice on ND (reference): 53 ± 5 *(total); 27 ± 2 *(free); 44 ± 5 *(esterified).

[2]Data are mean ± SEM from N ≥ 4 animals. *p < 0.05 compared to ND; #p < 0.02 compared to 2-week; Student's t-test with Bonferroni's adjustment.

4.2.2b Hypercholesterolemia impairs the shear stress regulation of pseudopod activity in neutrophil populations

Consistent with the behavior of human neutrophils and dHL60 neutrophilic cells (Figs. 3.4 and 3.5), neutrophil populations from WT mice exhibited reduced pseudopod activity in response to fluid shear stress (5 dyn/cm^2). Compared to cell populations maintained under no-shear (t = 0) conditions, neutrophils in blood samples exposed to shear stress for 10, but not for either 2 or 5, min exhibited significantly ($p < 0.01$) reduced numbers of cells with pseudopod extension (Fig. 4.4). Similarly, neutrophils from LDLr$^{-/-}$ mice under ND conditions for all diet durations tested (i.e., 2, 4, and 8 wk) were deactivated by fluid shear stress with significantly ($p < 0.05$) reduced pseudopod extension detected after 10 min of shear exposure (Fig. 4.5A). The degree of shear-induced reductions in pseudopod activity remained constant over 8 wk of ND feeding and was comparable to that observed for neutrophils from WT mice.

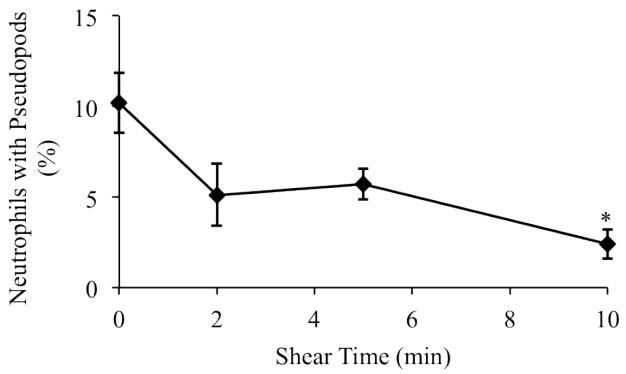

Figure 4.4 Shear induces time-dependent reductions in pseudopod activity of WT neutrophils.
Neutrophils with pseudopods (%) were counted after 0 (control), 2, 5, and 10 min of shear exposure (5 dyn/cm^2). Data are mean ± SEM from n = 4 experiments. *p < 0.05 compared to control using one way ANOVA with Bonferroni's method.

Under HFD conditions, both the baseline levels of pseudopod formation by neutrophils under static conditions and their shear-induced pseudopod retraction responses were altered. In contrast to mice subjected to ND for up to 8 wk (Fig. 4.5A), animals on HFD exhibited diet duration-dependent changes in their baseline levels of neutrophil activity (Fig. 4.5B). Compared to the blood from 2-wk HFD mice, the blood from 4-wk HFD mice exhibited significantly ($p < 0.05$; by over 2-fold) elevated numbers of activated neutrophils. Interestingly, the activated neutrophil counts in blood of 8-wk HFD mice were similar to that observed for 2-wk HFD animals.

In terms of mechanosensitivity to fluid flow, although the percentage of activated neutrophils from either 2-wk or 4-wk HFD mice was reduced by shear exposure, these reductions were not statistically significant (Fig. 4.5B). After another 4 wk of HFD (i.e., 8-wk HFD mice), neutrophil shear responses were completely reversed; shear exposure significantly ($p < 0.05$) increased the number of neutrophils with pseudopods (Fig. 4.5B).

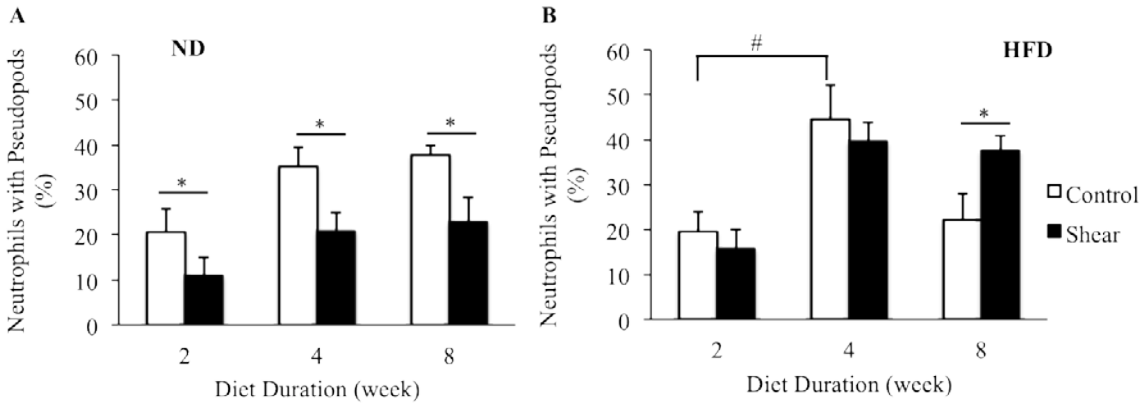

Figure 4.5 Neutrophils from LDLr$^{-/-}$ mice on HFD exhibit altered shear responses. A and B: Neutrophils with pseudopods (%) were counted for samples maintained under static or shear (5 dyn/cm^2; 10 min). Data are mean ± SEM from n ≥ 4 experiments. *p < 0.05 compared to control (static) using Student's t-test. #p < 0.05 using one way ANOVA with Bonferroni's method.

Linear regression analyses revealed significant ($p < 0.01$) correlations (total: $R^2 = 0.5$; free: $R^2 = 0.5$; esterified: $R^2 = 0.4$) between serum concentrations of cholesterol and neutrophil shear responses when combining data from both ND and HFD animals (independent of diet condition). Specifically, as serum cholesterol concentrations increased beyond some threshold level, shear-induced neutrophil deactivation decreased and eventually reversed; i.e., cells projected pseudopods (Fig. 4.6).

Linear regression analyses were also conducted for each diet group separately. In this case, whereas the neutrophil shear response for ND mice was independent of serum concentrations of any forms of cholesterol (free, total, or esterified), the shear responses of neutrophils from HFD animals exhibited significant ($R^2 = 0.5$; $p < 0.01$) correlations only with blood concentrations of free cholesterol (Fig. 4.6). These results provide evidence that free cholesterol is a dominant factor in the blood that governs the neutrophil mechanosensitivity to shear stress.

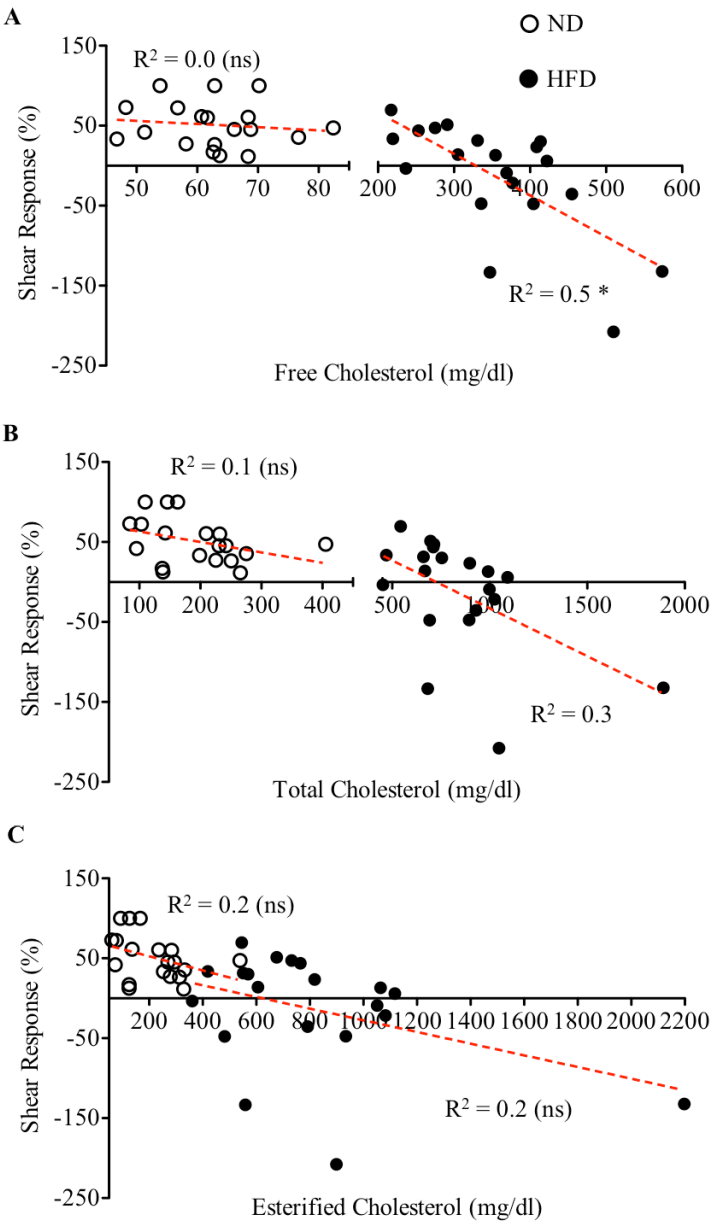

Figure 4.6 Neutrophil shear response correlates with the plasma level of free cholesterol.

A-C: Neutrophil shear response (%) was plotted versus the concentrations of free (A), total (B) or esterified (C) cholesterol in the plasma of LDLr$^{-/-}$ mice fed either a normal (ND), or a high fat diet (HFD) for up to 8 wk.

4.2.2c Benzyl alcohol influences shear-induced deactivation by neutrophils from LDLr$^{-/-}$ mice

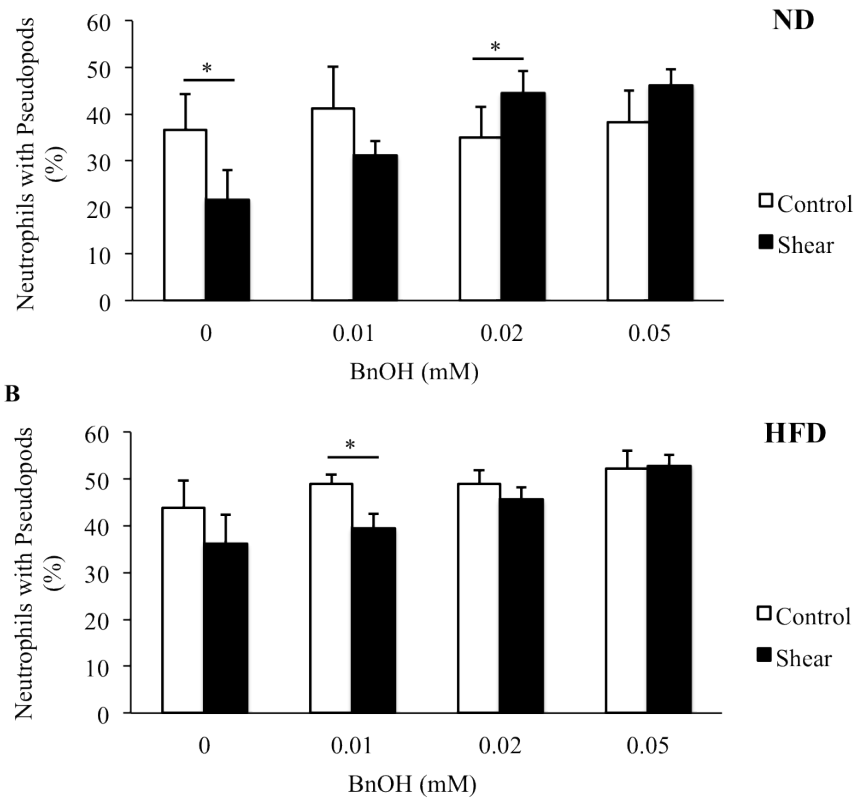

Figure 4.7 Membrane fluidizers influence the shear responses by neutrophils from LDLr$^{-/-}$ mice.
A and B: Neutrophils with pseudopods (%) were counted for samples maintained under static or shear (5 dyn/cm^2; 10 min). Data are mean ± SEM from n = 6 experiments. *p < 0.05 compared to control (static) using Student's t-test.

Whole blood from 2-wk ND mice stimulated with 10 nM fMLP exhibited significant ($p < 0.05$) reductions in pseudopod activity of neutrophils in response to shear exposure (5 dyn/cm^2; 10 min) (Fig. 4.7A). Exposure of similar blood samples to shear stress in the presence of 0.01 – 0.05 mM BnOH led to diminished and eventually reversed shear-induced pseudopod retraction response (Fig. 4.7A). Specifically, treatment of stimulated blood with 0.01 mM BnOH attenuated reductions in pseudopod formation

by neutrophils after shear exposure. Incubating similar blood samples with 0.02 mM BnOH, however, significantly ($p < 0.05$) increased pseudopod activity of neutrophils after shear exposure. When the same blood was sheared in the presence of 0.05 mM BnOH, a similar increase in the number of activated neutrophils was observed relative to unsheared samples, but this effect was not statistically significant.

Consistent with prior observation (Fig. 4.5B), exposure of fMLP-stimulated blood harvested from 2-wk HFD animals to shear failed to elicit significant reductions in the number of neutrophils with pseudopods. In contrast, the pseudopod activity of stimulated neutrophils was significantly ($p < 0.05$) reduced by shear exposure in the presence of 0.01 mM BnOH (Fig. 4.7B). BnOH at concentrations higher than 0.01 mM (i.e., 0.02 and 0.05 mM) completely abolished this shear response with similar levels of neutrophil pseudopod activity detected for both sheared and unsheared samples (Fig. 4.7B). These results suggest that hypercholesterolemia-induced blockade of shear-induced pseudopod retraction occurs, at least in part, via the effects of blood cholesterol elevations on neutrophil membrane fluidity.

CHAPTER 5 THE INFLUENCE OF MECHANOSENSITIVE CONTROL OF NEUTROPHIL ACTIVITY ON MICROVESSEL FLOW RHEOLOGY

Fluid shear stress mechanotransduction serves to deactivate neutrophils under physiological conditions and, therefore, may have significant effects on their rheological (e.g., tumbling) behavior in the microvascular blood flow. To link shear stress mechanotransduction (as reflected by pseudopod retraction, CD18 cleavage, or both) to leukocyte flow behavior, the apparent viscosity of cell suspensions, an important parameter describing flow rheology, was examined during shear exposure.

Additionally, a customized microfluidic network resistance chamber consisting of an array of microchannels that mimic the small vessels of microcirculation was fabricated and used to assess the effects of pseudopod formation by neutrophils on flow resistance of cell suspensions in vitro. These studies were conducted to highlight the critical role of shear-induced pseudopod retraction in promoting efficient microvascular flow.

Finally, the reactive hyperemia response in skeletal muscles (i.e., gastrocnemius) was assessed during the development of hypercholesterolemia in $LDLr^{-/-}$ mice. Reactive hyperemia is the transient increase in blood flow that occurs following brief ischemia. It reflects the regulation of microvascular blood flow (i.e., changes in the peripheral hemodynamic resistance). As such, results from these studies may provide in vivo insight into the contributions of an impaired neutrophil shear response to hypercholesterolemia-related microvascular flow dysregulation, a contributing factor for microvascular dysfunctions.

5.1 Methods

5.1.1 Kinetic analysis of the impact of shear stress mechanosensitivity on the leukocyte flow behavior

Human leukocyte-enriched plasma was harvested as described in section 3.1.1b. Leukocytes were incubated in HBSS (1:10 v/v) containing 10 nM fMLP for 10 min, and subsequently subjected to fluid shear stress (shear rate: 450 s^{-1}) for 10 min in the same buffer using a computer-interfaced DV-II+PRO digital cone-plate viscometer (Brookfield). This set up exposed cells to a constant shear stress field (~ 5 dyn/cm^2). The temperature of cell suspensions during viscometry was maintained at RT using a water jacket cooling system incorporated into the lower plate. The viscosity of cell suspensions during cone-plate flow exposure was recorded at 1-min intervals for the 10-min duration of experiments using Wingather 32 software (Brookfield).

In some experiments, leukocytes were treated with either membrane-modifying agents (cholesterol:MβCD conjugates or BnOH; see section 4.1.1a) or E64 (28 μM; see section 3.1.4a) prior to or during 10 nM fMLP stimulation. These studies assessed the involvement of membrane fluidity and CD18 cleavage-mediated platelet binding in kinetic viscosity measurements.

In separate experiments, pseudopod activity and platelet binding during shear exposure were examined for similar leukocyte suspensions with microscopy (see section 3.1.3b) and flow cytometer (see section 3.1.4b), respectively.

5.1.2 Analysis of the impact of neutrophil activation on microchannel flow resistance

5.1.2a Design of microfluidics flow chamber

A microfluidic network resistance chamber was developed based on Stokes approximation of creeping fluid flow where the Reynolds Numbers for planned flow rates are extremely small (< 1) ensuring laminar flow conditions [94]. The micronetwork chamber consisted of a single microchannel (w: 500 µm; h: 50 µm; square conduits) that eventually splits into a network of 20 microchannels (w: 20 µm; h: 50 µm; l: 1 cm) arranged in parallel. These parallel microchannels eventually reconnect into a single downstream channel (w: 500 µm; h: 50 µm) that drains to the atmosphere. A negative master of this design (Fig. 5.1A) was fabricated by Dr. Palaniappan Sethu (University of Louisville, KY) according to published procedures [95].

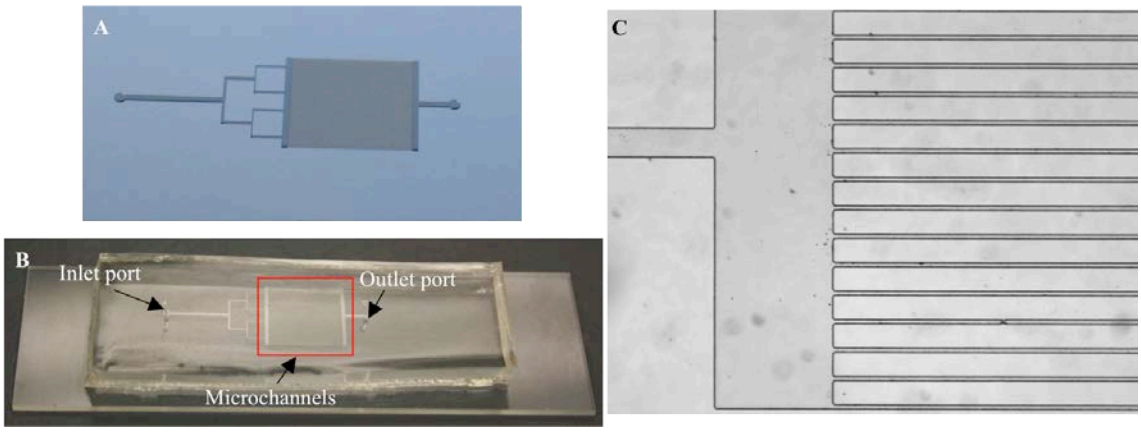

Figure 5.1 Design of the microfluidic network chamber used in the present study. A: A negative master of the desired microfluidic-based design for micronetwork chambers was fabricated using standard soft lithography. B: A PDMS replica was bonded to the glass slide. C: A region of the micronetwork chamber showing the parallel array of 20 x 50 µm microchannels.

5.1.2b Micronetwork chamber fabrication

Polydimethylsiloxane (PDMS) replicas of the flow chamber were generated by pouring a mixture of silicone elastomer and curing agent (10:1 v/v) onto the negative master and baking it at 65 °C for 1 h. Holes for inlet and outlet ports on the PDMS replicas were made using a blunt 20-gauge needle. After being cleaned with oxygen plasma (1% oxygen, 60 s) in a plasma cleaner (Harrick Plasma), PDMS replicas were bonded to glass slides to form the custom network flow chambers (Fig. 5.1B). This chamber design allowed for its placement on an inverted microscope to directly visualize the flow behavior of cells within the channels during flow experiments.

5.1.2c Cell preparations

Human neutrophil populations were purified from whole blood by two-step Histopaque-Percoll gradient centrifugation as previously established [96] and subsequently resuspended in PBS at $2x10^7$ cells/ml. The resultant cell suspensions contained >90% neutrophils.

Neutrophils ($2x10^6$ cells/ml) were stimulated with 10 nM fMLP for 10 min and fixed with 0.2% glutaraldehyde (Electron Microscopy Sciences). Cells without stimulation served as controls. In order to prevent pseudopod projections, neutrophils were incubated with 30 μM cytochalasin B (CTB) [97], an inhibitor of F-actin polymerization, for 30 min prior to fMLP stimulation. Fixed cells were diluted (1:10 v/v) in 10% FBS in PBS prior to use.

Neutrophil viability remained unchanged at 99% after CTB treatment as assessed by the Live/Dead Double Staining Kit (see section 4.1.1b).

5.1.2d Network chamber flow experiments

A Statham pressure transducer and a syringe were connected to the inlet of the micronetwork flow chamber via a 4-way stopcock and a piece of polyvinyl chloride (PVC) tubing (inner diameter: 0.010″). Downstream of the microchannel network, a piece of PVC tubing was connected to the outlet port to allow the perfusate to drain into an open waste beaker. The syringe was mounted on a syringe pump (PHD2000; Harvard Apparatus) to drive solutions into the flow chamber at controlled flow rates. The pressure transducer was interfaced to a carrier demodulator (Validyne) and a data acquisition device (NI USB-6008; National Instruments). The ability of the network flow chamber to detect changes in flow resistance as a function of perfusate viscosity was validated using 0 – 30% glycerol solutions (Sigma Aldrich).

Prior to each cell experiment, the microchannels within the network chamber were rinsed sequentially with 1 ml acetone, 2 ml diH_2O, 1 ml ethanol, and 2 ml diH_2O at a flow rate of 10 ml/h. Subsequently, the microchannel surfaces (to be exposed to flowing cell suspensions) within the network chamber were passivated with 10% FBS in PBS for 5 min to minimize non-specific cell binding.

For cell experiments, leukocyte suspensions were driven though the network chamber at three sequential flow rates: 1 ml/h for 5 min $\rightarrow$ 2 ml/h for 10 min $\rightarrow$ 1 ml/h for 10 min (Fig. 5.2). The infusing pressure at the inlet (relative to atmospheric pressure),

corresponding to the pressure drop across the microchannel bed, was monitored as voltage throughout the whole experiment by LabVIEW SignalExpress (National Instruments). The pressure monitoring setup was calibrated using water manometry prior to real-time data acquisition before experiments.

5.1.2e Data Analysis

Using Matlab (MathWorks), data (in voltage) was converted to pressure based on the calibration curve generated for each experiment. The raw pressure data over the last 3 min of each flow rate phase (i.e., P1, P2, and P3 in Fig. 5.2) were averaged as a steady pressure reading at each flow rate. To eliminate the variation associated with individual chamber, the steady pressure at each flow rate was normalized to the value obtained at the initial 1 ml/h phase (i.e., P1).

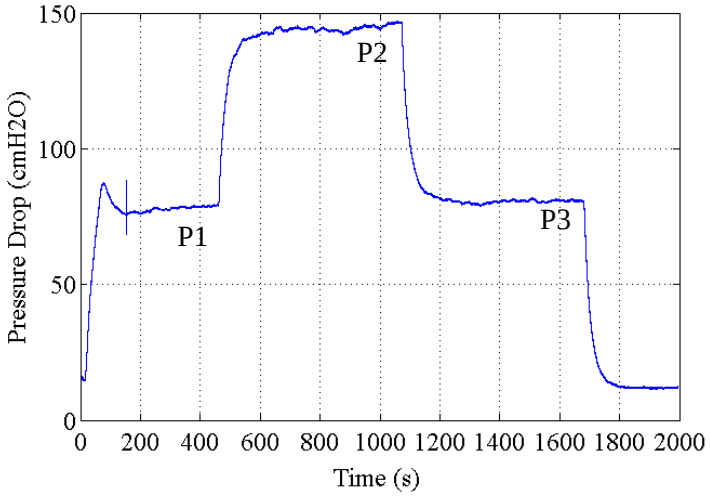

Figure 5.2 A representative flow curve of neutrophil suspensions through the micronetwork chamber.
Cells were perfused into the chamber at the following flow regimes: 1 ml/h for 5 min → 2 ml/h for 10 min → 1 ml/h for 10 min.

5.1.3 Analysis of reactive hyperemia in hypercholesterolemia

5.1.3a Mice

Hypercholesterolemia was induced in LDLr$^{-/-}$ mice for up to 8 wk as described in section 4.1.2a. Control animals were age-matched LDLr$^{-/-}$ mice fed a ND. At the end of dieting, mice were subjected to experimental ischemic reactive hyperemia and real-time tissue blood flow measurements for the gastrocnemius (thigh) muscle (see section 5.1.3b) followed by phlebotomy (see section 4.1.2b).

For some experiments, neutropenia was induced in LDLr$^{-/-}$ mice that had been placed on ND or HFD for 8 wk in order to confirm the role of neutrophils in the blood flow recovery response to transient ischemia. Briefly, 1.0 mg of anti-Ly6G (1A8; BioXCell), which depletes the mice of circulating neutrophils, was injected i.p. into animals 36 h prior to use [98]. The induction of neutropenia was confirmed using flow cytometry (section 3.1.4b); neutrophils in these animals accounted for < 1.0% of total leukocytes as compared to around 10% in regular mice.

5.1.3b Blood flow recovery response to transient ischemia: ischemic reactive hyperemia

The blood flow recovery response of the gastrocnemius to 5-min hindlimb cuff occlusion was examined for LDLr$^{-/-}$ mice using Near-Infrared (NIR) Diffuse Correlation Spectroscopy (DCS). This technology was developed by Dr. Guoqiang Yu (University of Kentucky, KY) and has been extensively validated to measure the relative change of blood flow (rBF) in various tissues against a number of current technologies (e.g., laser doppler, ultrasound doppler, fluorescence microsphere velocimetry, xenon-computed

tomography, arterial spin labeled-magnetic resonance imaging) for monitoring blood flow in deep tissues (up to centimeters below the skin) [99-101].

In the present study, mice were anesthetized by inhalation of 1% isoflurane (Butler Schein). Hair overlying the gastrocnemius muscle on the left hindlimb was shaved from the skin. A piece of PVC tubing (outer diameter: 0.05″) was loosely wrapped into a noose (i.e., cuff) around the proximal end of the limb without restricting blood flow. Distal to this, a DCS probe (i.e., a foam pad) housing a light source and a detector separated by a distance of 6 mm was super-glued to this bared skin site. The 6 mm separation distance ensured detection of rBF in tissue regions 3 mm under the skin surface deep within the muscle tissue [100, 101]. Fibers extending from the probe were then attached to the DCS flow unit and rBF was measured for 5 min to acquire a baseline. Cuff occlusion was then applied to the thigh by tightening the PVC tubing noose around the proximal hindlimb until rBF was reduced to less than 10% of baseline levels and maintained for 5 min, followed by cuff release (i.e., the PVC tubing was severed using a high temperature surgical cautery pen) (Fig. 5.3A). Real-time rBF was recorded during the whole process until it recovered to baseline (pre-occlusion) levels. Mice were maintained at 37 °C for the duration of blood flow measurements using a heating pad.

At the end of experiments, mice were sacrificed and their blood was harvested (section 4.1.2b) for analyses of neutrophil mechanosensitivity (section 4.1.2c) and quantification of plasma cholesterol levels (section 4.1.2d).

5.1.3c Data analysis of NIR-DCS-based measurements of reactive hyperemia

Using Matlab, the raw rBF data was plotted against time, and the highest value after cuff release was identified as peak flow. In order to minimize the variations (e.g., due to noise), all data fluctuating within 10% of the peak flow value for each curve were averaged and used as adjusted peak flow (APF) (Fig. 5.3B).

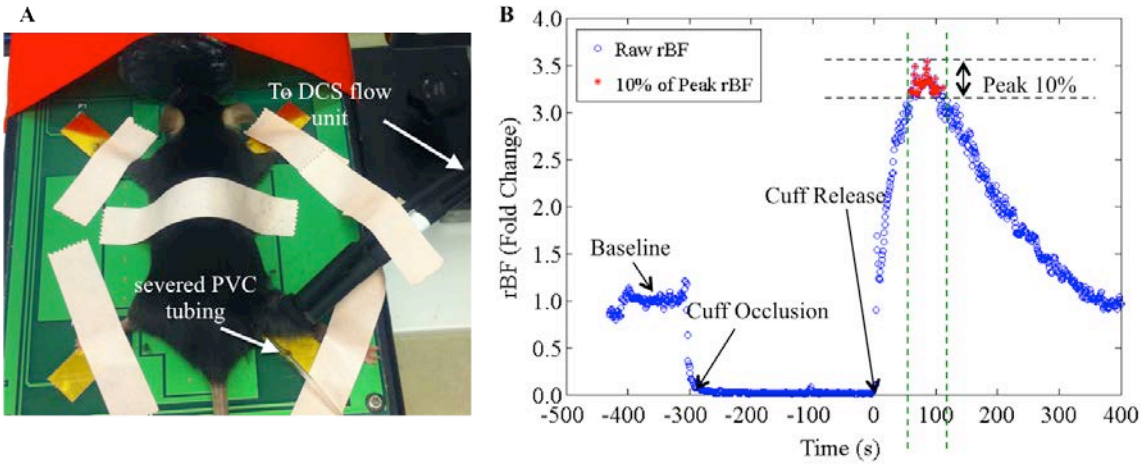

Figure 5.3 NIR-DCS-based relative blood flow (rBF) measurements during reactive hyperemia.
A: Demonstration of experimental setup. B: A representative rBF curve consists of pre-occlusion, occlusion, and post-occlusion phases.

5.2 Results

5.2.1 Shear stress mechanosensitivity influences leukocyte rheological behavior

5.2.1a Suspension viscosity of leukocytes relates to their pseudopod activity but not to their binding to platelets under shear exposure

At the initial time point (i.e., t = 30 s), suspensions of leukocytes in diluted plasma mildly stimulated with 10 nM fMLP exhibited significantly ($p < 0.05$) higher viscosity than cell-free plasma solution (1.19±0.05 cP for leukocyte suspensions versus

0.95±0.00 cP for cell-free plasma). In contrast to the constant viscosity exhibited by cell-free plasma solution, suspension viscosity of fMLP-stimulated leukocytes decreased in a time-dependent manner during 10-min exposure to a cone-plate shear flow (shear rate: 450 s^{-1}; shear stress ranged from approximately 4 to 6 dyn/cm^2) (Fig. 5.4). When shear exposure time was ≥ 3 min, the viscosities of cell suspensions were significantly ($p < 0.05$) reduced compared to that for the same cell populations at 30 s.

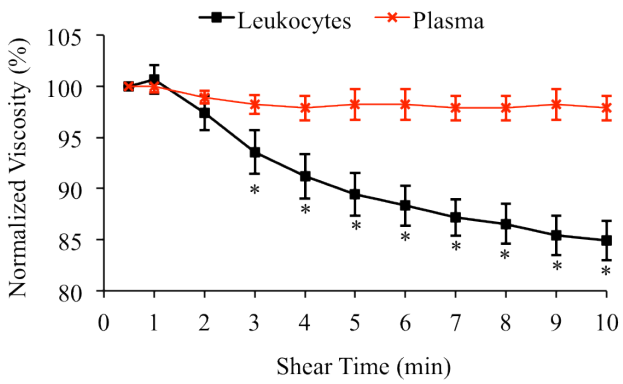

Figure 5.4 Shear induces time-dependent reductions in the apparent viscosity of leukocyte suspensions.
fMLP-stimulated cells were subjected to viscometry at 450 s^{-1}. Data are mean ± SEM from n = 4 experiments. *$p < 0.05$ compared to t = 30 s using one way RM ANOVA with Dunnett's method.

For similar cell suspensions exposed to a time course of shear stress, the percentage of neutrophils with pseudopods decreased in a time-dependent fashion. Significant ($p < 0.05$) reductions in pseudopod activity occurred after only 1 min and continued for the remaining shear exposure times (Fig. 5.5A).

In contrast, neutrophil-platelet adhesion in these suspensions increased during shear exposure to a plateau (approximately, after 5 min of flow exposure). Leukocyte suspensions exposed to shear stress for 5, 7.5, and 10 min exhibited significantly ($p <$

0.05) elevated percentages of neutrophils with bound platelets relative to similar suspensions maintained under static (t = 0) conditions (Fig. 5.5B). Further support for the lack of influence of platelet binding on leukocyte suspension viscosity is the observation that protease inhibitor E64, shown to enhance platelet binding under shear (Fig. 3.11B), had no effect on the apparent viscosity of fMLP-stimulated leukocyte suspension throughout the 10-min duration of shear exposure. Both naïve and E64-treated leukocytes exhibited significantly ($p < 0.05$) reduced suspension viscosity after 2 min of flow onset (Fig. 5.6).

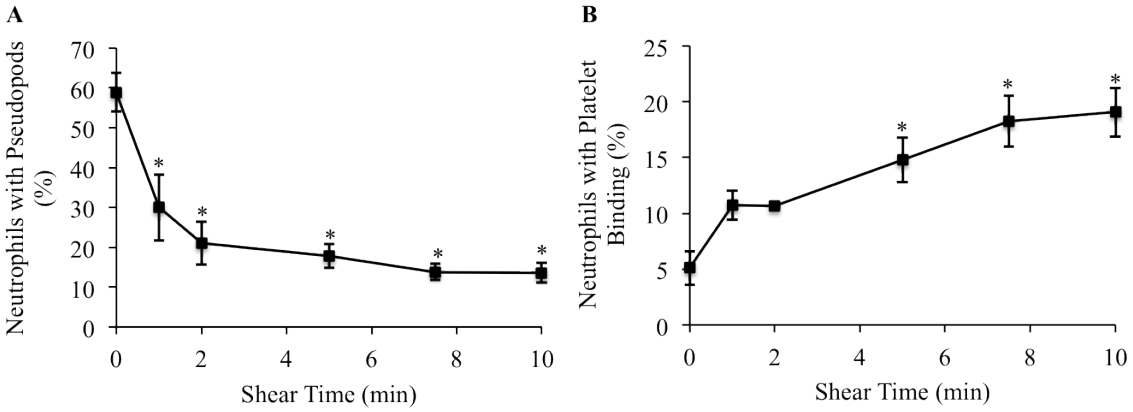

Figure 5.5 Shear induces time-dependent reductions in pseudopod activity and increases in neutrophil-platelet binding.
fMLP-stimulated cells were subjected to shear at 5 dyn/cm^2 for 0 (control), 1, 2, 5, 7.5, and 10 min. Data are mean ± SEM from n = 3 experiments. *$p < 0.05$ compared to t = 0 using one way ANOVA with Dunnett's method.

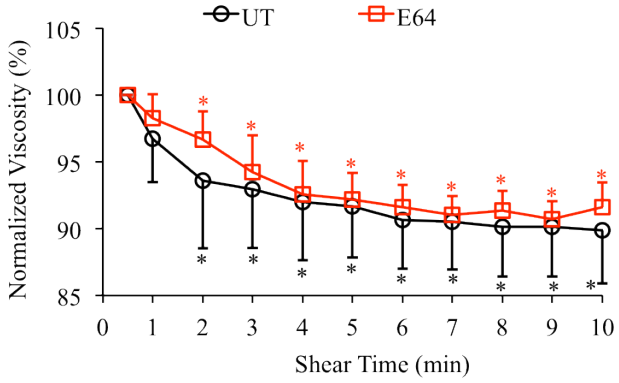

Figure 5.6 Protease inhibitor E64 had no effect on shear-induced reductions in leukocyte suspension viscosity.
fMLP-stimulated cells with/without treatment of E64 were subjected to viscometry at 450 s^{-1}. Data are mean ± SEM from n = 3 experiments. *,*p < 0.05 compared to t = 30 s using one way RM ANOVA with Dunnett's method.

5.2.1b Leukocyte rheological flow behavior is modulated by the physicochemical properties of the cell membrane

Leukocytes stimulated with fMLP in the absence (UT) or presence of 2 mM BnOH exhibited similar levels of suspension viscosity at 30 s of flow onset (1.16 ± 0.06 cP for UT cells versus 1.05 ± 0.03 cP for BnOH-treated cells). In contrast to the significant ($p < 0.05$ when $t \geq 4$ min) reductions in suspension viscosity exhibited by naïve leukocytes under shear, BnOH-treated cell suspensions did not exhibit significance reductions in viscosity over the 10-min duration of cone-plate exposure (Fig. 5.7).

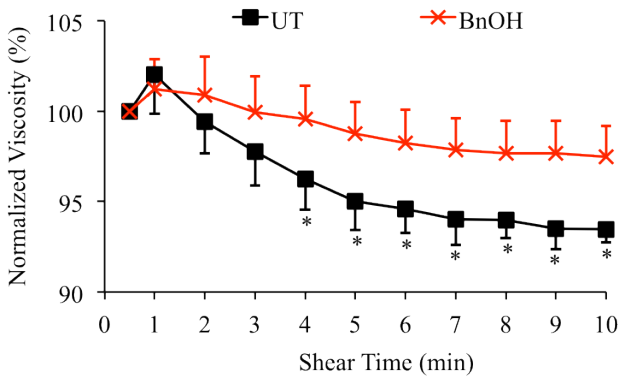

Figure 5.7 Membrane fluidizer attenuates shear-induced reductions in leukocyte suspension viscosity.
fMLP-stimulate cells with/without treatment of BnOH were subjected to viscometry at 450 s^{-1}. Data are mean ± SEM from n = 5 experiments. *p < 0.05 compared to t = 30 s using one way RM ANOVA with Dunnett's method.

Table 5.1 The apparent viscosity of leukocyte suspension at t = 30 s of flow onset.

Treatment		Viscosity (cP)	Note
CH (μg/ml)	0	1.15 ± 0.03	Data are mean ± SEM from N ≥ 5.
	2	1.09 ± 0.04	Non-significant among 0, 2, 5, and 10 ug/ml CH using one way ANOVA with Bonferroni;
	5	1.06 ± 0.02	
	10	1.10 ± 0.05	
No Cells	Plasma	0.95 ± 0.00	Non-significant between plasma and plasma+CH using Student's t-test.
	Plasma+CH	0.95 ± 0.01	

Moreover, although treatment of leukocytes with 0 – 10 μg/ml cholesterol:MβCD conjugates did not affect their suspension viscosity at t = 30 s of flow onset (Table 5.1), these membrane cholesterol-enhancing agents dose-dependently impaired shear-induced viscosity reductions (Fig. 5.8). Specifically, whereas suspensions of cells treated with 0, 2, and 5 μg/ml cholesterol complexes exhibited significantly ($p < 0.05$) reduced viscosity after 4 min of shear exposure, 10 μg/ml cholesterol-treated leukocyte suspensions

appeared to exhibit gradual increases in their viscosity after approximately 5 min of shear that were not statistically significant as compared to their initial viscosity at t = 30 s. Linear regression analyses revealed a significant correlation (R^2 = 0.95; $p < 0.05$) between the apparent viscosity of leukocyte suspensions after 10-min shear exposure and the concentration of cholesterol conjugates used to pretreat these cells.

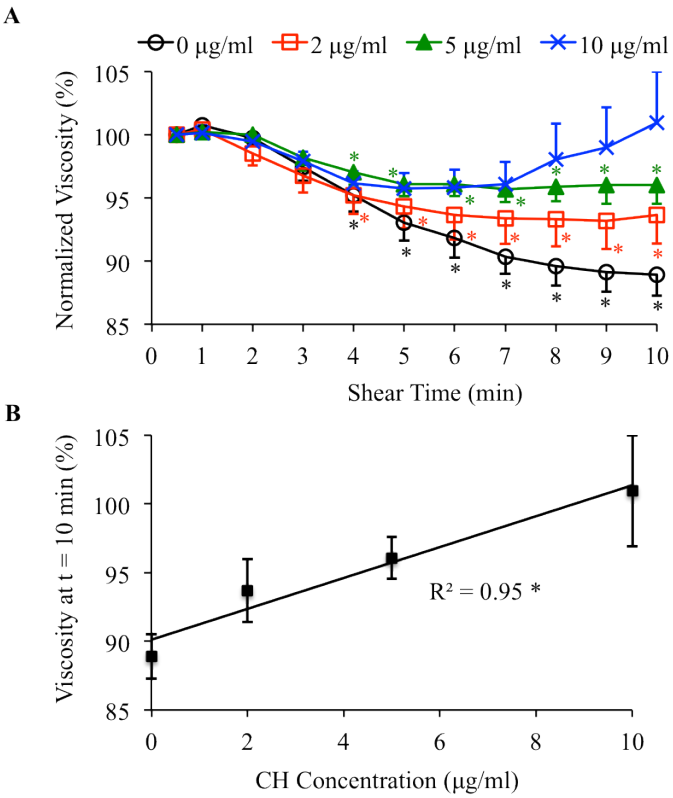

Figure 5.8 Membrane cholesterol-enhancing agents dose-dependently impair shear-induced reductions in leukocyte suspension viscosity.
0 – 10 µg/ml cholesterol:MβCD (CH)-pretreated cells were subjected to viscometry at 450 s^{-1} after fMLP stimulation. A: Time-course changes of viscosity. B: Correlation between the endpoint viscosity measurement and CH concentrations. Data are mean ± SEM from n ≥ 5 experiments. *p < 0.05 compared to t = 30 s using one way RM ANOVA with Dunnett's method.

5.2.2 Neutrophil pseudopod formation is linked to microchannel flow resistance

5.2.2a Perfusate viscosity influences microchannel resistance to flow

Perfusion of 0 – 30% glycerol solutions through the microfluidic flow chamber resulted in gradual increases in resistance (Fig. 5.9). Regression analyses pointed to a linear correlation (R^2 = 0.99; p = 0.0002) between flow resistance and viscosity within the range of 0.8 to 2.0 cP (Fig. 5.9). This range covers those observed for leukocyte suspensions during shear exposure (section 5.2.1). These results validated the use of flow resistance measurements from the microfluidic network chamber to detect changes in the apparent viscosity of the perfusate.

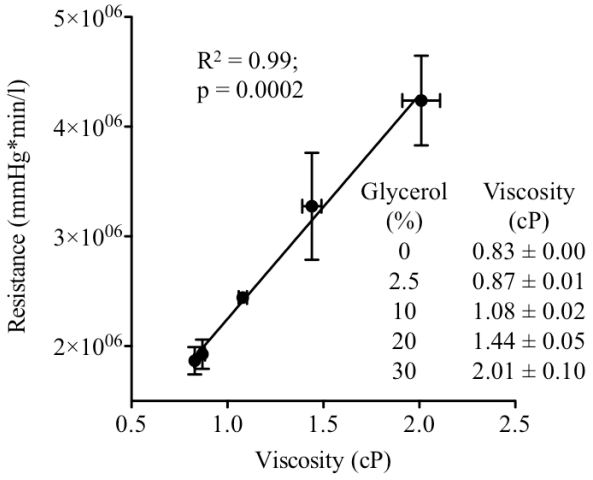

Figure 5.9 Microchannel resistance increases as the viscosity of perfusate rises.
0 – 30% glycerol solutions were perfused through micronetwork chamber at 0.5 ml/h for 5 min. Data are mean ± SEM from n =3 experiments.

5.2.2b Pseudopod formation alters flow resistance of neutrophil suspensions

After stimulation with 10 nM fMLP, the percentage of neutrophils with pseudopod projection increased significantly ($p < 0.05$) as compared to that of naïve

neutrophils without stimulation (Fig. 5.10). In contrast, CTB-treated neutrophils after fMLP stimulation exhibited pseudopod activity levels similar to those of unstimulated cells (Fig. 5.10).

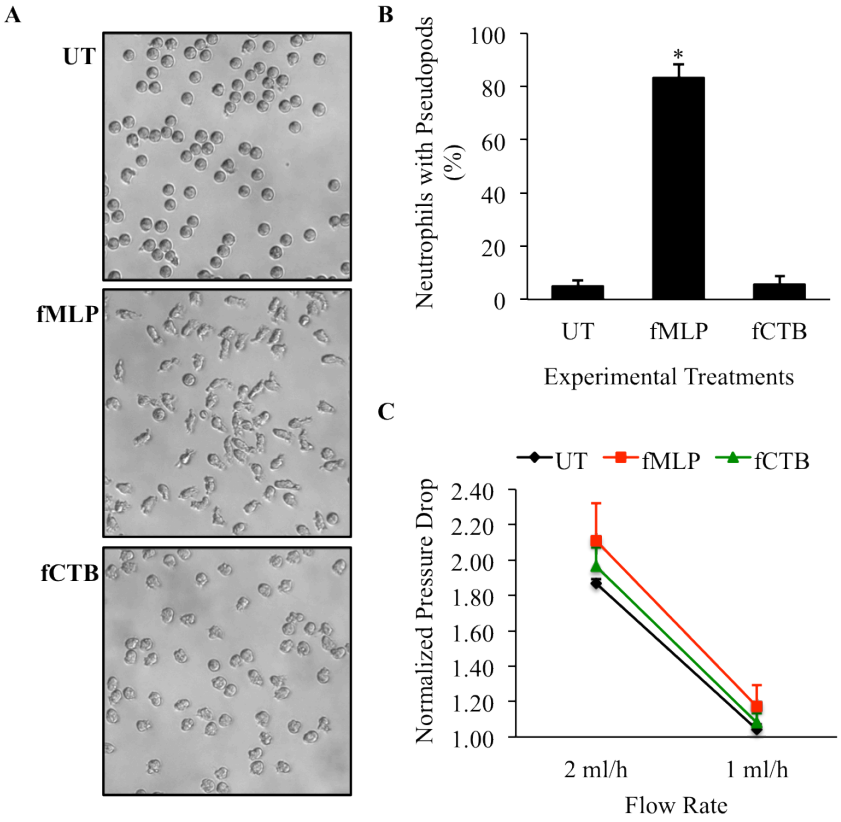

Figure 5.10 The effect of neutrophil pseudopods on microchannel resistance. Pseudopod formation (%) and pressure drop were assessed for untreated cells (UT), fMLP-stimulated cells, and cells treated with CTB prior to fMLP (fCTB). Data are mean ± SEM from n = 3 experiments.

During preliminary experiments, fMLP-stimulated neutrophils perfused through the microfluidic flow chamber at either 2 ml/h or 1 ml/h, on average, appeared to cause a small increase in pressure drop across the microchannel array; these differences, however, were not significant (Fig. 5.10). Interestingly, neutrophils that had been stimulated with fMLP following treatment with CTB (an inhibitor of F-actin

polymerization/pseudopod formation) and perfused through the microchannels induced pressure-drops that were, on average, similar to those observed for untreated suspensions (Fig. 5.10).

5.2.3 Neutrophil shear mechanotransduction is linked to microvascular flow regulation during reactive hyperemia in hypercholesterolemia

The present study explored the involvement of neutrophil shear mechanotransduction in the reactive hyperemia responses of hypercholesterolemic mice to transient ischemia (i.e., 5-min blood flow occlusion). Consistent with our prior studies using similar animals (Table 4.2), LDLr$^{-/-}$ mice fed a HFD exhibited time-dependent increases in plasma cholesterol levels (Table 5.2).

Table 5.2 Cholesterol levels in the blood plasma of LDLr$^{-/-}$ mice for blood flow measurements.

Cholesterol Type	Diet	Cholesterol Concentration (mg/dl)			
		2-week	4-week	8-week	8-week Neutropenia
Total	ND	251 ± 11	261 ± 20	271 ± 11	264 ± 12
	HFD	886 ± 49 *	1110 ± 55 *#	1297 ± 53 *#	1371 ± 69 *
Free	ND	70 ± 6	61 ± 4	71 ± 3	60 ± 3
	HFD	335 ± 18 *	396 ± 12 *#	439 ± 31 *#	472 ± 58 *
Esterified	ND	302 ± 8	334 ± 29	333 ± 18	341 ± 16
	HFD	921 ± 75 *	1192 ± 85 *	1432 ± 65 *#	1501 ± 161 *

Data are mean ± SEM from N ≥ 6 animals. *$p < 0.05$ compared to ND; #$p < 0.02$ compared to 2-week; Student's t-test with Bonferroni's adjustment.

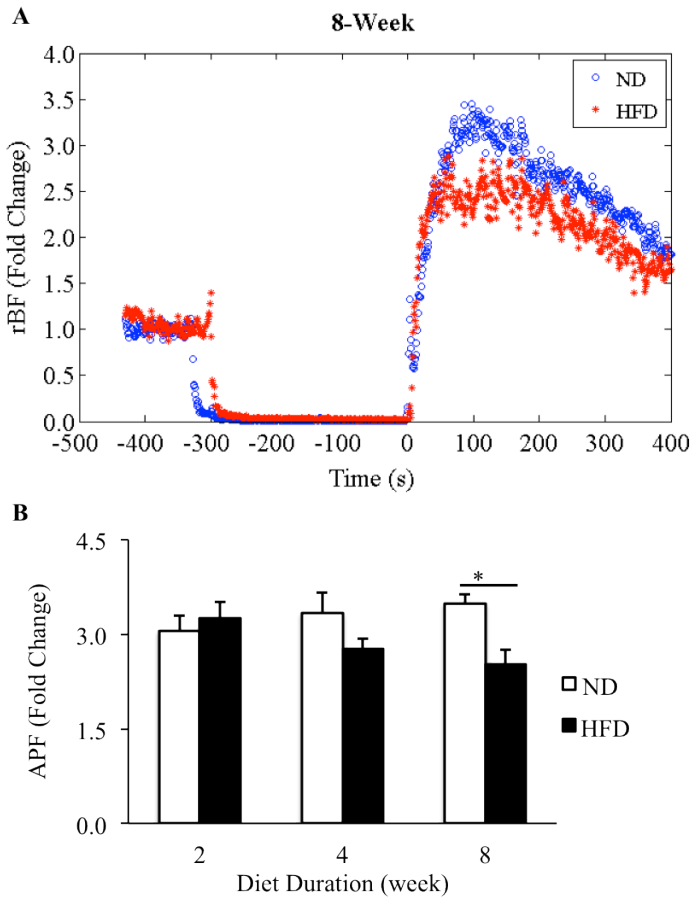

Figure 5.11 LDLr$^{-/-}$ mice on HFD exhibit a gradual attenuation in reactive hyperemia.
A: The rBF curves of 8-wk ND and HFD mice were overlaid. B: The adjusted peak flow (APF) was calculated for animals on ND or HFD for up to 8 wk. Data are mean ± SEM from n ≥ 6 experiments. *p < 0.05; 2-way ANOVA detected diet had effects, then student's t-test was performed between ND and HFD at each time point.

Measurements of rBF obtained from the gastrocnemius of mice fed either ND or HFD for up to 8 wk using NIR-DCS were consistent with reactive hyperemia and similar to that reported for humans [99, 100]. The rBF plot illustrated a stable baseline prior to cuff occlusion, near-zero flow during cuff occlusion, and a period of flow overshoot followed by recovery to baseline (pre-occlusion) flow levels after cuff release. Notably, mice fed a HFD for 8 wk exhibited a more blunted rBF curve as compared to their ND

counterparts (Fig. 5.11A). To quantify this difference, APF was calculated by averaging rBF data fluctuating within 10% of the peak overshoot flow; all of the rBF data points used to calculate APF occurred within approximately 1 – 2 min after cuff release (data not shown).

Based on APF values, animals subjected to HFD for 2, 4, and 8 wk exhibited a gradual impairment in their reactive hyperemia response to 5-min blood occlusion as compared to time-matched ND animals (Fig. 5.11B). Whereas there were no differences detected for the APF between ND and HFD group after 2 and 4 wk, the APF exhibited by 8-wk HFD animals was significantly ($p < 0.05$) lower than that observed for 8-wk ND mice.

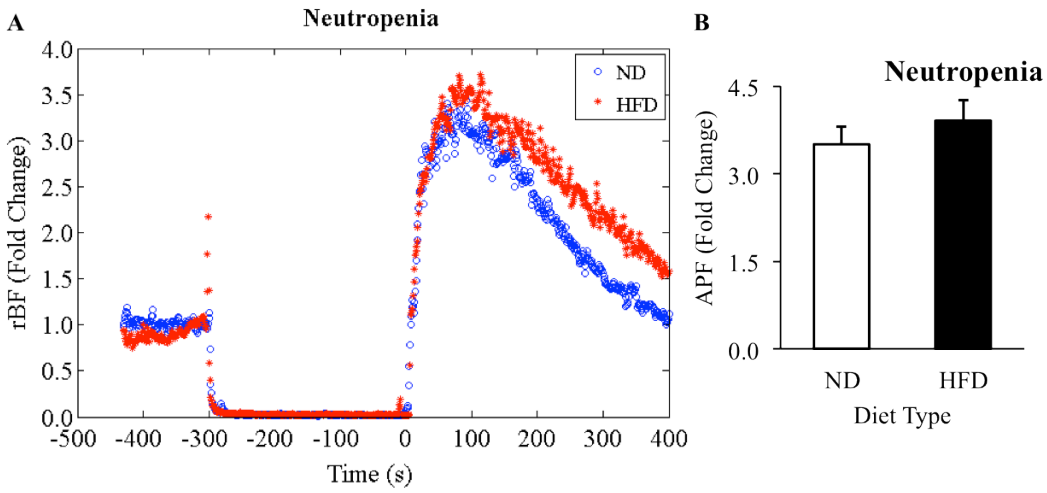

Figure 5.12 8-wk ND and HFD LDLr$^{-/-}$ mice subjected to neutropenia exhibit similar levels of adjusted peak flow.
A: The rBF curves of neutropenic ND and HFD mice were overlaid. B: The adjusted peak flow (APF) was calculated for neutropenic ND or HFD mice. Data are mean ± SEM from n = 6 experiments.

In order to assess the role of neutrophils in the reactive hyperemia responses of mice to transient blood flow occlusion, approximately 90% of neutrophils were removed

from 8-wk ND and HFD animals. Neutropenic HFD mice exhibited an rBF flow response curve similar to that of their ND counterparts (Fig. 5.12A). In fact, there was no difference in APF between ND and HFD mice subjected to neutropenia (Fig. 5.12B). These flow results, taken together, suggest a role for neutrophils in the hypercholesterolemia-induced impairment of reactive hyperemia response to transient ischemia.

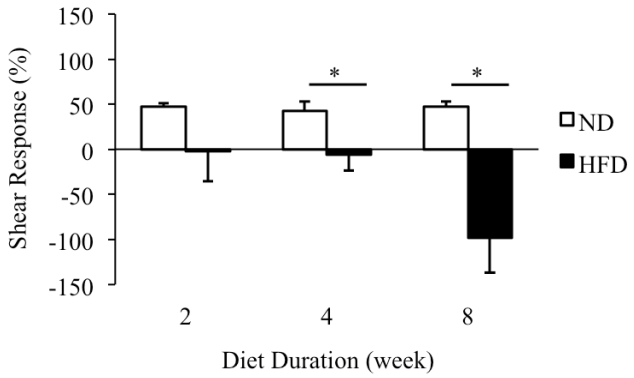

Figure 5.13 Neutrophils of HFD LDLr$^{-/-}$ mice exhibit attenuated shear-induced pseudopod retraction responses.
Shear responses (%) were assessed for animals on ND or HFD for up to 8 wk. Data are mean ± SEM from n ≥ 6 experiments. *p < 0.05; 2-way ANOVA detected diet had effects, then student's t-test was performed between ND and HFD at each time point.

Consistent with results from prior studies (Fig. 4.5A), neutrophils in blood from mice subjected to ND for 2, 4, and 8 wk exhibited similar reductions (around 50%) in pseudopod activity in response to 5 dyn/cm^2 shear stress for 10 min (Fig. 5.13). In contrast, neutrophils from HFD animals exhibited a gradual attenuation of this response (Fig. 5.13). Although neutrophil populations from 2-wk HFD mice appeared to exhibit a reduced shear response, this difference was not significant. Neutrophils in the blood of mice subjected to HFD for 4 and 8 wk exhibited significantly ($p < 0.05$) reduced shear

responses. In fact, at these time points, shear stress increased the number of neutrophils with pseudopods in the blood.

These data, combined, point to a role for the shear-sensitive control of neutrophil activity in microvascular flow regulation during reactive hyperemia in hypercholesterolemia.

CHAPTER 6 DISCUSSION

In the absence of chemical agonists, leukocytes exhibit spontaneous pseudopod activity as well as adhesion to other cells (i.e., leukocytes, platelets, ECs) under static (no-flow) conditions in the in vitro and in vivo settings [43, 83]. These cells, however, prevail in a quiescent state with few pseudopod projections, low surface expression of cell-cell adhesion molecules, and minimal binding to other leukocytes or platelets when flowing in the bloodstream of the physiological (non-inflamed, non-pathogenic) circulation [43]. This discrepancy points to the existence of in vivo mechanisms that are related to the fluid mechanics of blood flow and proactively prevent or minimize leukocyte activation under physiological conditions.

Recently, fluid shear stress mechanotransduction has been established to be anti-inflammatory in the physiologic circulation [5]. By inactivating leukocytes, particularly the neutrophils, fluid shear stress stimulation ensures these cells remain in a rounded, deformable, and non-adhesive state while flowing in the circulation. In doing so, shear stress deactivation of the neutrophils serves to optimize their transit through the small vessels of the microcirculation. Any factors that influence the sensitivity of leukocytes to fluid shear stimulation could potentially affect their activation status and rheological behavior to the point of compromising microvascular blood flow (e.g., enhanced flow resistance, impaired tissue perfusion). In this regard, the present study provides the first evidence that cholesterol enrichment impairs leukocyte shear mechanotransduction with detrimental effects on leukocyte flow rheology and microvascular resistance. Moreover, results of the present study point to the reduced mechanosensitivity of neutrophils to fluid shear stress as a potential contributing factor for the hypercholesterolemia-related

impairment of microvascular blood flow regulation during reactive hyperemia in response to transient ischemia.

6.1 Membrane Cholesterol Regulates Shear-Induced Neutrophil Pseudopod Retraction via Its Effects on Membrane Fluidity

Pseudopod retraction is the most obvious manifestation of the neutrophil-inactivating effects of fluid shear stress. It occurs for both adherent and suspended cells when subjected to a fluid shear field. In line with the observations of adherent neutrophils retracting existing pseudopods in response to micropipette flow [102], cell populations suspended in an uniform cone-plate shear field exhibited an acute response (within ~2 min of flow onset) with reduced pseudopod activity (Fig. 3.4). Moreover, once the shear stress magnitudes reached a threshold level (approximately 5 dyn/cm^2), the shear-induced reductions in pseudopod activity by neutrophil populations occurred independently of shear magnitudes up to 10 dyn/cm^2 (Fig. 3.5). This range of shear stress is similar to that typically found in the microcirculation [5].

Interestingly, pseudopod retraction by neutrophils in response to fluid shear stimulation depends on the cholesterol content and/or organization in their cell membranes. It has been shown that either increasing/reducing or sequestering cholesterol in plasma membranes of migrating neutrophils impairs their pseudopod retraction response to fluid flow stimulation [102]. In line with this, the present study demonstrated that non-adherent neutrophil populations also exhibit a membrane cholesterol-dependent pseudopod retraction response to shear stress stimulation (Fig. 4.1). Together, these data point to membrane cholesterol as an important modulator of cellular

mechanotransduction and suggest the existence of an optimal membrane cholesterol level permissive for an unimpaired fluid shear response for both non-adherent and adherent neutrophils.

As an indispensable component of cell membranes, cholesterol in the unesterified form (i.e., free cholesterol) modulates the properties of the lipid bilayer with profound effects on the functions of membrane signaling molecules as well as the cells. In general, these effects take place via two ways. For example, cholesterol can influence the formation and functionality of membrane lipid rafts, the signaling platforms that orchestrate outside-in and inside-out signal transduction [4]. Any changes in cholesterol content or organization can disrupt raft structure, leading to the dissociation of raft proteins [71]. The functional consequences of the lipid raft-related perturbations have been demonstrated extensively in neutrophils for chemokine-induced calcium signaling, extracellular regulated kinase activity, cell polarization, shape change, adhesion, migration, integrin expression and actin polymerization [85-87, 103, 104].

Alternatively, membrane cholesterol participates in cellular signaling through its impacts on membrane fluidity. Membrane fluidity (i.e., the inverse of microviscosity) refers to the ensemble of physical properties that govern the motion (e.g., rotational and lateral movements) of the phospholipid molecules in a membrane [77]. In this case, the cholesterol-dependent fluidity physically influences the dynamics (such as the lateral diffusion and structural changes) of membrane-associated proteins that drive downstream cell functions [77, 78]. For instance, membrane fluidity has been shown to affect the tertiary structure of GPCRs on ECs that undergoes conformational activation in response to fluid shear stress [66]. Moreover, it has been demonstrated that membrane fluidity

influences the chemotaxis of human neutrophils by regulating the conformation-dependent binding affinity of FPRs and thus their sensitivity to fMLP stimulation [105, 106].

In the present study, the involvement of membrane fluidity in membrane cholesterol-dependent shear mechanotransduction was initially highlighted by the parallel decreases in both membrane fluidity and shear-induced pseudopod retraction response of neutrophils treated with increasing concentrations of cholesterol conjugates (Fig. 4.1). In fact, the linear regression analyses revealed a strong positive correlation ($r = 0.9783$; $p < 0.0001$) between neutrophil shear responses and membrane fluidity during cholesterol enrichments (Table 4.1). This suggests that membrane cholesterol enrichment impairs neutrophil shear mechanotransduction, at least, in part, by reducing the cell membrane fluidity.

In order to further confirm the involvement of membrane fluidity in neutrophil shear mechanotransduction, the use of a membrane fluidizer, BnOH, was adopted in the present study. BnOH has been extensively used to fluidize the plasma membranes for a variety of cell types, including erythrocytes, lymphocytes, endothelial and epithelial cells [107-110]. Interestingly, BnOH, on its own, impaired shear-induced reductions in pseudopod activity by neutrophils (Fig. 4.2B). Thus, either rigidifying or fluidizing the cell membrane beyond its native state blocked neutrophil responsiveness to fluid shear stress (Fig. 4.2B). This, therefore, points to an optimal membrane fluidity level for neutrophil shear mechanotransduction that happens to fall within the physiologic range for the cells.

Furthermore, BnOH, in a concentration-selective manner, recovered the shear-induced pseudopod retraction response of neutrophils that had been blocked by membrane cholesterol enrichment (Figs. 4.2 and 4.3). In fact, only one concentration of BnOH completely recovered the shear responses of cholesterol-pretreated neutrophils. Also, the BnOH concentration required to reinstate the shear responsiveness of neutrophils that underwent membrane cholesterol enrichment depended on the concentration of cholesterol-enhancing agents used to treat the cells.

Finally, it should be noted that the influence of cholesterol-related fluidity of cell membranes on the neutrophil pseudopod retraction response to shear is independent of cell adhesion, because similar observations were also made for adherent neutrophils under micropipette flow [102]. Taken together, these data provide the first evidence that lipid bilayer fluidity is critical for neutrophil mechanotransduction and further confirm the existence of an optimal level of membrane fluidity permissive for an unimpaired shear response.

Moreover, these in vitro results strongly point to the cell membrane as a mechanotransducing compartment for neutrophils. This is reasonable considering its strategic location between the intra- and extra- cellular milieu as well as its enriched content of putative mechanosensors, e.g. FPR and CD18 integrins [48, 53]. There is, however, a possibility that the cell membrane itself acts as a mechanotransducer. In this respect, literature reports demonstrate increased membrane fluidity of either human or bovine ECs within 5 s of exposure to 10 – 26 dyn/cm^2 shear stress [66, 109, 111, 112]. However, the concept that the membrane, on its own, serves as a mechanotransducer lacks the specificity that would explain the diverse range of responses to shear stress

exhibited by different mammalian cell types. In line with the results of the present study, it is more likely that membrane fluidity serves to modulate the sensitivity of cell-specific mechanoreceptors to local shear stress distributions rather than to initiate cell mechanotransduction.

One pathological implication of the present study relates to hypercholesterolemia, a dominant risk factor for a variety of cardiovascular diseases including those associated with sustained leukocyte activation and a dysfunctional microcirculation [2, 36, 113]. In order to study the effects of hypercholesterolemia on the regulation of neutrophil activity by fluid shear stress, $LDLr^{-/-}$ mice were used in the present study. These animals, when placed on a diet supplemented with fat, developed gradually increasing plasma cholesterol levels over a period of 8 wk compared to those on regular chow (Table 4.2), as reported [114]. Presumably, this leads to the build up of a cholesterol concentration gradient across the outer leaflet of the cell membrane that gradually drives cholesterol into the neutrophil membranes predominantly via a receptor-independent pathway (e.g., endocytosis, direct surface exchange) that is incapable of saturation, as opposed to other receptor-mediated internalization processes [4, 115, 116]. In fact, it has been reported that neutrophils from humans or experimental animals exhibiting pathological elevations in blood cholesterol levels display enhanced membrane cholesterol abundance as well as reduced membrane fluidity levels [69, 117].

Consistent with the results from our in vitro membrane cholesterol enrichment studies (Fig. 4.1) and compared to cells from $LDLr^{-/-}$ mice on a ND, neutrophils from mice fed a HFD exhibited a reduced or even reversed shear response (Fig. 4.5). Notably, the blockade of the neutrophil pseudopod retraction response to shear occurred as early as

2 wk after placement on a HFD, which far precedes the appearance of pathological symptoms related to hypercholesterolemia such as atherosclerotic lesions [2]. This suggests that neutrophil shear mechanotransduction is highly sensitive to elevations in plasma cholesterol. It is also in line with the reported increases in leukocyte adhesion and emigration in the microcirculation of WT mice subjected to 2-wk of high cholesterol diet [36]. Moreover, the observed aberrant shear mechanotransduction by neutrophils was related to cholesterol-dependent membrane fluidity (Fig. 4.7).

It is important to point out that, in these animals, there may exist some other contributing factors apart from cholesterol for the impairment of shear mechanotransduction by neutrophils. For example, elevated circulating pro-inflammatory mediators (e.g., oxidized LDL [118]) associated with $LDLr^{-/-}$ mice on HFD may have raised neutrophil activity to levels that suppress shear-induced deactivation [42]. Contrary to this was the independence of shear responses on the baseline pseudopod formation by neutrophils from either ND or HFD animals (Fig. 4.5). It is also possible that an altered regulation of O^{2-} and NO related to hypercholesterolemia [119] impaired neutrophil responses to shear since both of these molecules are involved in the leukocyte shear mechanotransduction and exhibit membrane solubility [42, 46]. In addition, the reduced neutrophil shear responses may be attributable to an enhanced cleavage of membrane-bound receptors such as shear-sensitive FPRs [120]. However, the fact that half of the variances associated with neutrophil shear responses were solely attributed to changes in plasma concentration of free cholesterol (Fig. 4.6) with the remaining 50% representing the contributions from all the other possible factors further substantiates the important and dominant role of cholesterol.

In summary, these results provide evidence that the cell membrane plays a central role in neutrophil mechanotransduction of fluid shear stress. Specifically, membrane cholesterol influences shear-induced pseudopod retraction responses by neutrophils, at least in part, through its impact on membrane fluidity.

6.2 Shear Mechanotransduction Depresses Neutrophil-Platelet Adhesion by Inducing Protease-Mediated Cleavage of CD18 Integrins

Another way by which shear mechanotransduction deactivates the neutrophils is to down-regulate their surface expression of CD18 integrins. Under non-inflamed conditions, fluid shear stress induces proteolytic cleavage of the CD18 ectodomain on neutrophils, resulting in reduced levels of intact CD18 integrins [53, 54]. This process involves rapid (within 1 min of flow onset) structural shifts in the CD18 tertiary structure that likely expose cleavage sites for cysteine protease(s) released from intracellular lysosomes under the actions of fluid shear stress [53].

The present study provides further insight into this mechanotransduction process by demonstrating that fluid flow-induced down-regulation of CD18 depended on both the shear exposure time (Fig. 3.6) and stress magnitude (Fig. 3.7). This response occurred rapidly with CD18 surface expression reduced to a minimal level after 5 min of flow onset (Fig. 3.6). Its acuteness is consistent with the other cell-deactivating effects (e.g., pseudopod retraction) of fluid flow (Fig. 3.4) [5]. Notably, a detectable CD18 cleavage response required a threshold magnitude of shear stress within the range of 2.5 to 5 dyn/cm^2 (Fig. 3.7) which is similar to that reported for the microcirculation and upstream arteries/arterioles.

Further evidence that shear-induced CD18 down-regulation involved a cleavage process was the exclusive reductions in the extracellular, but not the cytosolic, domains of these integrins on sheared neutrophils (Fig. 3.8) confirmed a cleavage process occurring at the neutrophil surface. Under some conditions, cells reportedly down-regulate their surface receptor expression via internalization. However, the absence of any changes in neutrophil cytosolic CD18 staining in response to flow exposure, as reported by Fukuda and Schmid-Schönbein using confocal microscopy, precludes the possibility that shear stress lowered surface levels of CD18 integrins via receptor internalization [54]. In fact, these investigators reported similar reductions in CD18 surface expression by either human or rat neutrophils using different antibodies that target different epitopes on CD18 integrins (i.e., mAb MEM48 and Ab WT.3 against human and rat CD18, respectively) [54]. Thus, the observed shear-induced cleavage of CD18 was likely not due to flow-mediated changes in epitope accessibility.

Previous studies, using a panel of broad-spectrum and selective protease inhibitors, have identified cysteine proteases, particularly cathepsin B as key enzymes for the proteolysis of CD18 by neutrophils under shear [53, 54]. Notably, the degrees of shear-induced CD18 cleavage observed in the present study were comparable to those reported for either purified neutrophils or cells in undiluted whole blood exposed to 3 – 5 dyn/cm^2 shear stress [53, 54]. This further confirmed that CD18 cleavage resulted from shear-induced release of proteases from the cells and was not due to changes in the levels of endogenous inhibitors of leukocyte proteases present in the blood plasma under physiological (i.e., non-inflamed) conditions.

One novel finding related to the present study was that monocytes and lymphocytes, exposed to the same flow environments as neutrophils, also underwent CD18 surface cleavage in a similar shear time and magnitude dependent fashion (Figs. 3.6 – 3.8). However, lymphocytes appeared to be more sensitive to fluid flow stimulation requiring a lower shear magnitude to elicit significant ($p < 0.05$) reductions in CD18 surface expression, i.e., 2.5 dyn/cm^2 required by lymphocytes as compared to 5 dyn/cm^2 for neutrophils and monocytes (Fig. 3.7). Moreover, for monocytes, the reductions in CD18 surface expression sometimes failed to reach significance (Fig. 3.8), suggesting a less robust shear response by these cells. These results, therefore, point to these three leukocyte subtypes exhibiting different sensitivities to shear stress.

Several factors may contribute to the cell type-specific shear sensitivity. For example, neutrophils, monocytes, and lymphocytes exhibit quite different cell membrane topologies in the form of membrane folds and/or microvilli. Thus, the heterogeneous fluid stress distributions on the cell membrane imposed by the uniform macroscale shear field of our cone-plate viscometer may have differed among the three leukocyte subtypes with effects on the ability of flow to elicit either CD18 conformational activity or protease release. Differences in cell topology may also impact the residence time of proteases released by leukocytes in the vicinity of surface CD18 integrins. Since, at resting (i.e., non-inflamed) states, LFA-1 and Mac-1 are predominantly localized in the "valleys" between membrane folds/microvilli [121, 122], it would be expected that all of these CD18 molecules on a given leukocyte surface would be equally susceptible to cleavage independent of integrin subtype. In contrast, we observed an integrin subtype-dependent cleavage response; in other words, shear exposure elicited cleavage of LFA-1

(i.e., CD11a and CD18) on lymphocytes but proteolysis of Mac-1 (i.e., CD11b and CD18) on neutrophils and monocytes (less robust) (Fig. 3.9).

A more plausible explanation for the differential responsiveness of leukocytes to shear stress pertaining to CD18 cleavage may relate to the protease(s) required for proteolysis. Along this line, it is possible that only lymphocytes, which typically do not express detectable levels of Mac-1 [123], release protease(s) under shear to cleave LFA-1. Although neutrophils and monocytes are also capable of releasing proteases involved in LFA-1 shedding, this occurs only for activated cells during inflammation [124]. Our experiments, however, were conducted in the absence of inflammatory agonist stimulation.

Furthermore, although neutrophils and monocytes both exhibited Mac-1 cleavage under shear exposure (Fig. 3.9), they may do so by releasing different proteases. In fact, monocytes do not exhibit detectable extracellular enzymatic activity for cathepsin B, the specific proteases involved in shear-induced CD18 cleavage for neutrophils [53], until they differentiate into macrophages [125]. Therefore, monocytes under shear flow may mobilize other proteases, such as serine proteases (e.g., elastase, proteinase-3, cathepsin G) [126] and matrix metallo-proteinase-9 [127], all of which have been shown to be capable of truncating Mac-1.

In the present study, the cysteine protease inhibitor E64 significantly ($p < 0.05$) increased CD18 integrin levels on the surfaces of sheared neutrophils (Fig. 3.10) consistent with previous reports [54], but this anti-protease had no significant effect on CD18 surface cleavage for either monocytes or lymphocytes in the same suspension (Fig. 3.10). As such, the expression and enzyme kinetics of cell type-specific proteases may

contribute to the different shear sensitivities exhibited by the three types of leukocytes. Future work, however, is needed to confirm the involvement of proteases in shear-induced CD18 cleavage by the lymphocytes and monocytes.

It is also possible that the intrinsic structural properties of LFA-1 and Mac-1 influence their proteolysis following their conformational shifts under fluid flow. For example, differences in the secondary and tertiary structures of LFA-1 and Mac-1 [123] may impact cleavage sites and thus protease susceptibility rendering LFA-1 more prone to protease-specific cleavage than Mac-1. Alternatively, shifts in LFA-1 conformation may occur under lower shear stress magnitudes as compared to Mac-1. Interestingly, although LFA-1 and Mac-1 prevail in an inactive (i.e., closed-bent) tertiary configuration on resting leukocytes, the baseline level of LFA-1 in an open-extended (possibly protease-susceptible) conformation is reportedly almost 9-fold higher than that of spontaneously extended Mac-1 [51, 128]. Thus, the higher baseline of spontaneous conformational activity for LFA-1 on lymphocytes may reduce the need for shear-induced structural shifts that facilitate cleavage of CD18 by proteases.

Finally, the leukocyte-specific expression pattern of integrins may explain the dependence of shear-induced CD18 cleavage on cell type. Under static conditions, while resting neutrophils express LFA-1 and Mac-1 at equivalent levels [129, 130], monocytes express much more LFA-1 than Mac-1 at rest [123, 130]. As such, the substantial amount of LFA-1 expressed on monocytes that was not cleaved by shear stress (Fig. 3.6) accounted for a relatively higher proportion of the total CD18 integrins and thus may have increased the variability associated with measurement of shear-induced cleavage of CD18 (predominantly Mac-1) for these cells. Taken together, our results provide the first

evidence that the leukocyte sensitivity to shear stress depends on the subtype of CD18 integrins involved and the proteases mobilized by these cells.

Since CD18 integrins, particularly LFA-1 and Mac-1, play a critical role in the adhesive interactions between leukocytes and platelets [131-133], one physiological implication of shear-induced CD18 cleavage relates to its potential regulation of leukocyte-platelet binding under flow. Under non-inflamed conditions (i.e., in the absence of biochemical stimuli), leukocytes suspended in flow fields in vitro spontaneously interacted with platelets (Fig. 3.11). This observation agrees with previous reports demonstrating that shear flow not only promotes cell-cell collisions but also up-regulates P-selectin expression by platelets, both of which facilitate capture of leukocytes by platelets [134, 135]. While initiating these loose and transient selectin-mediated leukocyte-platelet interactions under non-inflammatory conditions, fluid flow may also serve to either restrict or prevent firm adhesion by cleaving CD18 integrins. A key piece of evidence supporting this possibility is the enhanced adhesion of neutrophils to platelets as well as to fibrinogen (a Mac-1 ligand immobilized on platelets [136]) by E64-related inhibition of shear-induced CD18 cleavage (Figs. 3.11 and 3.12).

Notably, the suppressing effect of shear-induced CD18 cleavage on neutrophil-platelet adhesion may have significant implications regarding microvascular blood flow where the vessel diameters are 100 μm or less. In fact, binding to platelets physically hinders neutrophil filtration through capillary-sized vessels [19, 20]. Moreover, neutrophil-platelet aggregates have been reported to impose the greatest effects on microvascular flow resistance in comparison to those by leukocyte deformability and RBC aggregation [20]. Thus, the observed 10 – 20% changes in CD18 surface levels and

platelet binding by neutrophils due to shear stress exposure may have a dramatic effect on microvascular blood flow.

In fact, it is not surprising that we observed such small changes in CD18 surface expression and platelet binding, as our studies were conducted with cells in the absence of inflammatory agonist stimulation that were inactivated or, at the most, mildly activated by our experimental manipulations. The fact that these changes were still associated with significance in such a reduced inflammatory background further suggests the importance of shear-induced CD18 cleavage as an anti-inflammatory mechanism in the physiological blood environment.

In the case of monocytes and lymphocytes that were suspended with neutrophils, the lack of an effect of cysteine protease inhibition (by E64) on platelet adhesion (Fig. 3.11) is consistent with the failure of E64 to influence CD18 surface expression under flow conditions (Fig. 3.10). This provides further support that: 1) cysteine proteases (e.g., cathepsin B) may not be the only proteolytic enzymes that exhibit shear-sensitive release and cleave CD18 integrins; 2) shear-induced CD18 cleavage by monocytes or lymphocytes involves non-cysteine proteases; and 3) flow-mediated leukocyte-platelet adhesion is closely related to shear-induced CD18 cleavage.

In summary, these results provide evidence that shear-induced proteolysis of membrane-associated CD18 serves to restrict spontaneous cell-cell binding under physiological conditions. This mechanotransduction process differs among the three major leukocyte subtypes contingent on their expression of CD18 subtypes and/or proteases.

6.3 Shear Mechanotransduction Influences Neutrophil Flow Rheology

The rheological properties (i.e., behavior in a flow field) of neutrophils, e.g., their cell size, potential irregularity of shape, stiffness, and adhesiveness, depend on their activation status [8, 10]. While up-regulation of CD18 integrins upon cell activation increases neutrophil adhesivity, projection of pseudopods by activated neutrophils renders these cells larger, irregular, and stiffer [8]. Shear-induced cell deactivation in the form of pseudopod retraction and/or CD18 cleavage, therefore, has important implications concerning the flow rheology of neutrophils particularly in the microcirculation where the vessel diameters are comparable to cell dimensions.

An important result of the present study was that transient reductions in the apparent viscosity observed for suspensions of mildly stimulated leukocytes under shear flow (shear rate: 450 s^{-1}; shear stress: 4 – 6 dyn/cm^2) (Fig. 5.4) tracked with the time-dependent reductions in pseudopod formation (Fig. 5.5A). One possible explanation for this relationship is that, when exposed to the linear flow velocity gradient created by cone-plate viscometry, the mildly activated neutrophils with pseudopods likely tumbled more and experienced more cell-cell collisions which disturbed the flow profile and raised the apparent viscosity. This possibility is in agreement with Helmke et al. who computationally showed the putative effects of leukocyte activation on the apparent viscosity of blood [18, 61]. The underlying mechanism for these effects was that activated leukocytes with increased tumbling in the flow field due to pseudopod projection would undergo hydrodynamic interaction with RBCs and disturb their flow profile [18, 61]. Considering that shear stress exposure elicits time-dependent reductions

in pseudopod activity, the transient reductions in the viscosity of leukocyte suspensions under shear likely reflect the neutrophil shear sensitivity.

In contrast, shear-induced transient reductions in leukocyte suspension viscosity did not track with neutrophil-platelet binding under shear stress exposure (Fig. 5.5B) and were not affected by blockade of shear-induced CD18 cleavage (Fig. 5.6). It is, therefore, possible that, although binding of platelets to neutrophils could also modify the cell geometry, these morphological changes are much smaller compared to those due to pseudopod projection. It should be noted that the number of platelets bound per cell is reportedly quite low for leukocytes mildly stimulated with 10 nM fMLP and it is less likely for leukocyte-platelet interactions to lead to the formation of larger multi-cellular aggregates [137]. Therefore, fluid shear stress appears to minimize the apparent viscosity of leukocyte suspensions predominantly by inducing pseudopod retraction.

Notably, the effects of leukocyte shear mechanotransduction on the apparent viscosity were influenced by modifications of membrane cholesterol/fluidity (Figs. 5.7 and 5.8) in a dose-dependent fashion similar to that observed for shear-induced pseudopod retraction (Figs. 4.1 – 4.3). As such, shear stress-related effects on leukocyte suspension viscosity also require the cells to exhibit an “optimal”, physiologic, level of membrane cholesterol and fluidity. Moreover, the observed changes in the kinetic viscosity measurements of neutrophils treated with membrane-modifying agents were not due to the physical actions of cholesterol:MβCD conjugates on viscosities of the suspending buffer (Table 5.1). In addition, the highest concentration of cholesterol:MβCD conjugates tested (i.e., 10 μg/ml) was far below the pathological levels (~ 240 mg/dl [1]) of cholesterol associated with cardiovascular diseases. Thus,

shear-induced reductions in the apparent (dynamic) viscosity of leukocyte suspensions, just like pseudopod retraction responses, are extremely sensitive to perturbations in the cholesterol environment.

The results of the present study, therefore, closely relate the suspension viscosity of leukocytes to their pseudopod activity. Through their impact on viscosity, the projections of pseudopods by leukocytes may also manifest as an effect on microvascular resistance. In support of this, a microfluidic network consisting of 20 microchannels with dimensions comparable to vessel diameters of microcirculation was used to show, on average, a slight increase in pressure drop across the microchannel network when neutrophils in the perfusate were stimulated to express pseudopods (Fig. 5.10). It is important to note that the recorded increases in pressure drop across the network due to cell activation were not statistically significant. However, the fact that CTB inhibition of F-actin polymerization (required for pseudopod projection) abrogated these small increases in pressure drop provides some indication that an effect was detected. In fact, it is not expected that viscosity changes due to cell activation would have a large impact on microchannel flow resistance considering for example that, based on Poiseuille flow, tube resistance is directly proportional to the viscosity of the perfusate. In comparison, cell adhesion that effectively reduces the cross-sectional area of microvessels to flow would have a more dramatic effect because of the dependence of flow resistance on the inverse 4^{th} power of the vessel radius. In addition, viscosity measures of leukocyte responses to shear (Figs. 5.4 – 5.8) were associated with a high degree of variation. Thus, it is likely that the inability to detect significant differences in recorded pressure drops due to cell activation may reflect the variations associated with cell suspensions and their influence

on viscosity. In the end, further refinement of the microchannel dimensions or optimizing the operating conditions (e.g., cell density) may lead to a more sensitive measure.

Despite a potentially small effect of leukocyte activation on the apparent viscosity of perfusate and microchannel resistance, it may have significance in the in vivo condition. Reportedly, elevations in microvascular resistance due to leukocytes can be significantly amplified by the interactions of leukocytes with RBCs [18, 61]. Specifically, the increased tumbling of activated leukocytes with pseudopods in the parabolic velocity gradient of microvessels could disturb the RBC motion as a result of increased intercellular tumbling collisions. This would then significantly elevate the apparent viscosity of the suspending buffer with an enhancing effect on the microvascular flow resistance. Along this line, pseudopod formation by leukocytes has been linked to elevated blood pressure in hypertension that occurs due to increased peripheral (micro)vascular resistance [138-140]. As such, shear mechanotransduction by neutrophils may serve to maintain optimal blood flow rheology and minimize resistance in the microcirculation by restricting pseudopod projection under physiological non-inflamed conditions.

In order to verify the rheological effects of neutrophil shear mechanotransduction in vivo, the reactive hyperemia response of gastrocnemius to acute ischemia (i.e., 5-min blood occlusion) was examined for hypercholesterolemic $LDLr^{-/-}$ mice. Reactive hyperemia is the transient increase in organ blood flow that occurs following temporary vessel occlusion [141]. It is typically assessed by quantifying the peak flow overshoot and the time required for blood flow to recover to pre-occlusion levels after reperfusion.

In the present study, diet duration-dependent increases in cholesterol concentrations (Table 5.2) in the blood of LDLr$^{-/-}$ mice tracked with a gradual attenuation of reactive hyperemia in response to HFD for up to 8 wk (Fig. 5.11). This attenuation was manifested as reduced peak flow (i.e., APF), which agrees with previous reports demonstrating that hypercholesterolemia impairs the peak flow of reactive hyperemia in microvessels [25, 142]. The impaired vasodilation of arterioles due to endothelial dysfunction has been implicated in this hypercholesterolemia-related microvascular dysfunction [25]. However, it is also possible that neutrophils within microvessels that are activated upon blood occlusion [43, 143] play a role in the impaired reactive hyperemia response by their effects on viscosity and flow resistance. In fact, neutrophils accumulate at sites of ischemia after reperfusion [144, 145], which is exacerbated in the face of hypercholesterolemia [113] and to the detriment of microvascular blood flow and tissue perfusion [12, 146]. A key piece of evidence supporting a neutrophilic involvement is our finding that depletion of neutrophils restored the peak flow in HFD mice to the level exhibited by their ND counterparts (Fig. 5.12). Thus, the presence of activated neutrophils influences the reactive hyperemia response, at least, in the early phase (i.e., ~ 1 – 2 min after cuff release).

Notably, the procedure used in the present study to induce reactive hyperemia, i.e. reperfusion response to transient ischemia, likely created a scenario in which neutrophils sediment, extend pseudopods, and migrate on the vascular endothelium during blood stasis and then retract pseudopods and detach into the flow field upon restoration of blood flow. Such a scenario has been mimicked using intravital microscopy examination of microvascular networks of rodents (e.g., mesentery, cremaster muscle) [42, 43, 45]. In

these experiments, activated neutrophils retracted their pseudopods immediately after release of upstream blood flow occlusion imposed by a blunt micropipette tip with dramatic changes in cell length/morphology observed within 1 – 2 min [43]. Interestingly, this time frame is well within the range where the peak flow appeared and where the reactive hyperemia response was affected by the presence of neutrophils (Figs. 5.11 and 5.12). Based on this, shear stress mechanotransduction may be linked to the peak blood flow of reactive hyperemia by its regulation on neutrophil rheology.

This connection was further supported by the observation that the time-course of reductions in peak flow tracked with the impairment of shear-induced pseudopod retraction responses by neutrophils in the blood of hypercholesterolemic mice (Figs. 5.11 and 5.13). Moreover, the fact that the defect in neutrophil shear-mechanotransduction was detectable at least 4 – 6 wk earlier than the time required for impairment of reactive hyperemia to develop during hypercholesterolemia (Figs. 5.11 and 5.13) [25] points to the shear mechanosensitivity of neutrophils as a potential predictor for hypercholesterolemia-related microvascular dysfunction.

In fact, the shear stress and viscometry experiments conducted for the present study may recreate the reactive hyperemia scenario for the neutrophils in the in vitro setting since neutrophils were exposed to shear immediately after activation under no-flow conditions. A potential difference is that our in vitro, flow-based analyses involved mildly stimulated neutrophils in suspension while reactive hyperemia may reflect the responses of migrating neutrophils. Both adherent and suspended neutrophils, however, respond to shear by reduced pseudopod activity within 10 min of the onset of flow exposure. Also, the effects of neutrophil shear responses on viscosity (Figs. 5.4 – 5.8)

occurred within the same time frame as reactive hyperemia. Thus, reactive hyperemia may probe the mechanosensitive impact of neutrophils on microvascular flow.

In summary, the results of the present study provide evidence that fluid shear stress mechanotransduction by neutrophils, particularly in the form of pseudopod retraction, modulates their rheological flow behavior. Furthermore, a reduced mechanosensitivity, e.g., due to membrane cholesterol enrichment, may be a contributing factor for the hypercholesterolemia-related impairment of microvascular blood flow regulation during reactive hyperemia.

CHAPTER 7 CONCLUSIONS

The present study provides further evidence that the mechanotransduction of fluid shear stress is an anti-inflammatory mechanism for leukocytes, particularly the neutrophils, in the physiological non-inflamed circulation. Specifically, fluid shear stress deactivates neutrophils not only by preventing their pseudopod formation, but also by inducing protease-mediated CD18 proteolysis to the point of minimal interactions with other cells. As such, this mechanobiological mechanism appears to optimize microvascular blood flow by regulating the rheological behavior of neutrophils. The results of the present study also provide the first evidence that membrane cholesterol plays a critical role in neutrophil shear mechanotransduction particularly as it relates to cellular mechanosensitivity. Along this line, membrane cholesterol-related perturbations, e.g., due to hypercholesterolemia, may affect leukocyte flow rheology through their effects on the cell sensitivity to fluid shear stress. The defective shear mechanotransduction by neutrophils, therefore, may expedite the initiation of microvascular dysfunction associated with hypercholesterolemia, contributing to obstruction of microvessels as well as reduced tissue perfusion.

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VITA

Personal Information:

Name: **Xiaoyan Zhang**

D.O.B.: Dec 24th, 1982 P.O.B.: Hubei, P.R.C

Education:

08/2008-Present	PhD candidate, Center for Biomedical Engineering, University of Kentucky, Lexington, Kentucky, U.S.A
09/2005-09/2006	MSc (Distinction), Advanced Biomedical Engineering, University of Warwick, Coventry, United Kingdom
09/2001-06/2005	BSc (Dean's List), Biological Engineering, Zhejiang University of Technology, Hangzhou, Zhejiang, P.R.C

Previous Positions:

02/2007-07/2008	Quality Inspector, Zaoyang Xiongji Water Supply Company, Hubei, P.R.C

Outreach Activities:

2011-2012	NSF-KY EPSCoR High School Teachers Fellowship Program
2009-2012	NSF-KY EPSCoR Research Experience for Undergraduates program
2009-2012	Lab Open House
2010	NSF-KY EPSCoR Science and Engineering Demonstration at Winburn Middle School

Scholastic and Professional Honors:

05/2012	The honor of Outstanding Graduate Student of Center for Biomedical Engineering, University of Kentucky
05/2005	The honor of Outstanding Graduate of Zhejiang University of Technology

Awards:

2010-2012	Conference Travel Support, University of Kentucky (three times)
08/2008-present	Research Assistantship, University of Kentucky
2002-2004	Undergraduate Student Scholarship, Zhejiang University of Technology (three times)
2002	The First Prize of the Third Physics Competition of Zhejiang University of Technology

Memberships in Professional Organizations:

2012-present	American Heart Association/American Stroke Association
2012-present	Biomedical Engineering Society

Publications

Peer-Review Journal:

1. Zhang, X; Zhan, D; Shin, HY. "Integrin Subtype-Dependent CD18 Cleavage Under Shear and Its Influence on Leukocyte-Platelet Binding." Journal of Leukocyte Biology, 2012 (In Printing).
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1. Zhang, X; Shin, HY. (2012). "Fluid Shear Mechanotransduction is Linked to Leukocyte Flow Behavior via Its Impact on Cell Activity." *BMES 2012 Annual Meeting*, Atlanta, GA.
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3. Zhang, X; Zhan, D; Shin, HY. (2012). "Regulation of Leukocyte-Platelet Binding by Shear Stress-Mediated CD18 Cleavage." *2012 NSF-KY EPSCoR HSTF Program*, Louisville, KY.
4. Zhang, X; Smith, J; Adams, S; Sethu, P; Shin, HY. (2012). "Linking Fluid Shear Mechanotransduction to Leukocyte Flow Rheology." *17th Annual KY EPSCoR Statewide Conference*, Lexington, KY.
5. Zhang, X; Zhan, D; Shin, HY. (2012). "Leukocyte Mechanosensitivity to Fluid Shear Stress Depends on the Subtypes of CD18 Integrins." *Experimental Biology 2012*, San Diego, CA.
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7. Zhang, X; Hurng, J; Rateri, DL; Daugherty, A; Shin, HY. (2011). "Leukocyte Sensitivity to Fluid Flow Stimulation Depends on Membrane Cholesterol-Dependent Fluidity." *BMES 2011 Annual Meeting*, Hartford, CT.
8. Zhang, X; Zhan, D; Shin, HY. (2011). "Identification of Quantitative Relationships that Serve as Measures of Neutrophil Mechanosensitivity." *2011 NSF-KY EPSCoR HSTF Program*, Louisville, KY.
9. Zhang, X; Zhan, D; Shin, HY. (2011). "Identification of Parameters to Quantitatively Assess Leukocyte Mechanosensitivity." *16th Annual KY EPSCoR Statewide Conference*, Louisville, KY.
10. Zhang, X; Hurng, J; Rateri, DL; Daugherty, A; Schmid- Schönbein, GW; Shin,

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