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Abstract
Previous work from our lab has demonstrated that ethanol and cholesterol each have significant effects on neutrophil tethering, rolling, and adhesion. Since ethanol and cholesterol are often present within the body simultaneously, we endeavored to characterize their combined effects on neutrophil membrane mechanics and adhesion. The effect of ethanol on membrane cholesterol loading was ascertained by incubating neutrophils with 0.3% ethanol before or after cholesterol manipulation with methyl-beta-cyclodextrin (MβCD) or MβCD/cholesterol complexes. Microcapillary flow chamber assays at a wall shear rate of 100 s⁻¹ were used to examine the effects of ethanol on cholesterol-loaded neutrophils with respect to: (1) collision efficiency and membrane tethering to P-selectin-coated microbeads, (2) rolling on P-selectin-coated surfaces, and (3) primary and secondary interactions with neutrophils preadhered to intercellular adhesion molecule-1 (ICAM-1). We performed flow cytometry experiments to assess the effects of ethanol and cholesterol together on adhesion molecule expression. Microcapillary flow chambers presenting chamber-adherent, non-adhesive and P-selectin-coated 10 μm beads were used to assess neutrophil whole-cell deformation changes with separate ethanol and cholesterol treatment, and varied shear rate. The individual effects of ethanol and cholesterol on membrane fluidity, measured by membrane lipid diffusivity, were determined using fluorescence recovery after photobleaching (FRAP) experiments to obtain lipid diffusion coefficients. We found that membrane cholesterol was increased over control in the absence of ethanol; ethanol pre- or post- loading reduced this increase. Cholesterol enrichment did not alter CD11b expression; however, PSGL-1 and L-selectin expression were lowered by cholesterol enrichment plus or minus ethanol. Ethanol appeared to abrogate the pro-adhesive effects of cholesterol, but it did not impact cholesterol's ability to enhance tethering probability. Ethanol enhanced membrane fluidity of cholesterol-enriched neutrophils as indicated by tethering metrics. Ethanol and cholesterol enrichment both increased neutrophil deformation, while cholesterol depletion decreased neutrophil deformation. Neutrophil deformation increased in a dose-dependent manner with shear rate. Membrane lipid diffusivity was increased by ethanol administration and cholesterol enrichment, and decreased by cholesterol depletion. Our research shows that ethanol and cholesterol interact in a complex manner in the neutrophil membrane, sometimes in concert, and sometimes in conflict, to influence neutrophil adhesion via biomechanical and biochemical effects.

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INTERPLAY OF ETHANOL AND CHOLESTEROL IN THE NEUTROPHIL MEMBRANE: EFFECTS ON MEMBRANE AND CELL MECHANICS AND NEUTROPHIL ADHESION

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in
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INTERPLAY OF ETHANOL AND CHOLESTEROL IN THE NEUTROPHIL MEMBRANE: EFFECTS ON MEMBRANE AND CELL MECHANICS AND NEUTROPHIL ADHESION

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ABSTRACT

ETHANOL AND CHOLESTEROL INTERPLAY AND EFFECTS ON NEUTROPHIL MEMBRANE MECHANICS AND ADHESION

Matthew J. Furlow
Scott L. Diamond

Previous work from our lab has demonstrated that ethanol and cholesterol each have significant effects on neutrophil tethering, rolling, and adhesion. Since ethanol and cholesterol are often present within the body simultaneously, we endeavored to characterize their combined effects on neutrophil membrane mechanics and adhesion. The effect of ethanol on membrane cholesterol loading was ascertained by incubating neutrophils with 0.3% ethanol before or after cholesterol manipulation with methyl-beta-cyclodextrin (MβCD) or MβCD/cholesterol complexes. Microcapillary flow chamber assays at a wall shear rate of 100 s\(^{-1}\) were used to examine the effects of ethanol on cholesterol-loaded neutrophils with respect to: (1) collision efficiency and membrane tethering to P-selectin-coated microbeads, (2) rolling on P-selectin-coated surfaces, and (3) primary and secondary interactions with neutrophils pre-adhered to intercellular adhesion molecule-1 (ICAM-1). We performed flow cytometry experiments to assess the effects of ethanol and cholesterol together on adhesion molecule expression. Microcapillary flow chambers presenting chamber-adherent, non-adhesive and P-selectin-coated 10 µm beads were used to assess neutrophil whole-cell deformation changes with separate ethanol and cholesterol treatment, and varied shear rate. The individual effects of ethanol and...
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CHAPTER 1: Introduction

1.1 Endothelial cell activation and neutrophil inflammatory response

Vascular endothelium may become activated through exogenous or endogenous means, by chemical or mechanical stimuli. Depending on the duration and integration of these stimuli, activation may be acute or chronic in nature. Acute activation may contribute to the development of chronic inflammatory conditions like atherosclerosis. The nature of acute endothelial activation involves chemical changes (accumulation of cytokines and expression of adhesion molecules, for instance) and physical changes (vasodilation and increased endothelial permeability, for example) that can lead to accumulation of leukocytes and/or platelet aggregation and thrombosis. These cells are typically marginated to the periphery of the blood vessel by red blood cells, and they express adhesion molecules that bind to proteins expressed on the activated endothelium.

Neutrophils are the most abundant leukocyte in the circulatory system and play an active role in innate immunity. When activated, typically by chemical cues that have diffused into the bloodstream from surrounding tissue, neutrophils release proteolytic enzymes like neutrophil elastase and matrix metalloproteinases (MMP), as well as reactive oxygen species (ROS) that serve to destroy invading pathogens (Naruko, 2002).
1.2 Pathophysiological roles of neutrophils

1.2.1 Neutrophils in thrombosis

The abundance of adhesive ligands presented by neutrophils allows them to bind to a diverse array of circulating cells. While this has physiological utility, it can also have unintended, negative consequences, especially in the pathophysiology of thrombosis. Platelet-neutrophil complexes and aggregates have been found in hypercholesterolemic and hyperlipidemic patients (Sener, 2005; Tailor, 2004). These complexes promote vascular inflammation and restenosis after angioplasty (Simon, 2000). Neutrophils can also interact with red blood cells (Das, 2000) fibrin (Kujiper, 1997) and factor X through Mac-1 (Goel and Diamond, 2004) which provides them with further opportunities to contribute to thrombus growth, as well as aggregate formation that can promote regions of pro-inflammatory, recirculating vascular flow.

The proteolytic enzyme neutrophil elastase can block tissue-factor pathway inhibitor (TFPI) and de-encrypt plasma tissue factor, enabling it to interact with platelet surfaces in the process of thrombosis (Zillman, 2001). Neutrophils also release platelet-activating proteases (LaRosa, 1994) and can potentially serve as catalytic surfaces to large platelet aggregates because of their significantly larger surface area (Laurenzi and Diamond, 1999).
1.2.2 Neutrophils in atherosclerosis

Increased neutrophil levels in the circulation have been identified as an independent risk factor for coronary heart disease (CHD) (Madjid, 2004). Neutrophils have shown potential to contribute to the initiation and progression of atherosclerosis. Activated neutrophils release myeloperoxidases, which oxidize low-density lipoproteins (LDL) that are consumed by macrophages to form foam cells (Podrez, 1999). Dysfunctional release of superoxide by activated neutrophils can impair bioavailability of nitric oxide (NO), an athero-protective vasodilator that also contributes to platelet quiescence. Accordingly, neutrophil activation in humans has been correlated with reduced vasodilation and increased LDL oxidation (Sugano, 2005).

Neutrophils may also mediate late-stage atherosclerotic plaque stability. Linear strings of firmly-arrested neutrophils have been found in vivo on mouse atherosclerotic plaques (Kadash, 2004). Neutrophil proteolytic enzymes secreted upon activation can break down interstitial matrix and promote plaque instability (Naruko, 2002). Accordingly, neutrophils have been found in ruptured human plaques.

1.3 Leukocyte adhesion cascade

Marginated neutrophils can home to an activated endothelial surface by following chemoattractants released from or presented on the endothelium, like platelet-activating factor and leukotriene B4 (Witko-Sarsat, 2000). Once a neutrophil contacts an activated endothelial surface, the leukocyte adhesion
cascade (Figure 1.1) is initiated. This is a complex process featuring several key steps: capture via tethering, rolling, firm adhesion, and endothelial transmigration.

### 1.3.1 Neutrophil capture and rolling via selectins

Capture and rolling are mediated by selectins (L-selectin, constitutively expressed on neutrophil microvilli, P-selectin, expressed on activated platelets and endothelial cells, and E-selectin, also expressed on activated endothelial cells) and their respective carbohydrate ligands (Guyer, 1996; Witko-Sarsat, 2000). Initial neutrophil tethering and rolling on activated endothelium is mediated by P-selectin and its primary ligand PSGL-1. P-selectin is stored in platelet \( \alpha \)-granules and endothelial cell Weibel-Palade bodies, and is rapidly transported to the cell membrane in response to inflammatory stimuli (Witko-Sarsat, 2000). PSGL-1 is constitutively expressed on neutrophil microvilli, which are an interconnected series of ridges on the neutrophil surface 200-300 nm in height (Park, 2002). Subsequent, slow rolling is governed by E-selectin’s interaction with PSGL-1 and other glycoproteins (Yago, 2010). Neutrophil homotypic tethering is controlled by PSGL-1 to L-selectin bonds. The fast association and dissociation rates of selectin bonds contribute to their proficiency at mediating rolling under flow (Chang, 2000). These fast rates are a product of the specific domains that bind in the selectin-PSGL-1 interaction; PSGL-1 contains the tetrasaccharide sialyl Lewis\(^x\), which binds to the lectin domain of selectins. Force plays a significant role in these interactions, as there is a threshold shear stress
required for rolling (Lawrence, 1997; Alon, 1998) and leukocyte rolling velocities on endothelial cell monolayers and P-selectin-coated surfaces vary with shear stress (Lawrence, 1990; Lawrence, 1997). During the rolling period, chemical and mechanical signals are exchanged between the neutrophil and activated endothelium, which informs the neutrophil to transition to firm arrest.

1.3.2 Firm arrest and extravasation

The transition state between rolling and firm adhesion, initiated by activation of both neutrophil and endothelium, is marked by redistribution of PSGL-1 from the microvilli to the uropod, and cleavage of L-selectin from the microvilli (Bennett, 1996; Goel and Diamond, 2002; Itoh, 2007). Firm adhesion of neutrophils to activated endothelium is controlled by the $\beta_2$-integrin CD11a/CD18 (leukocyte function-associated antigen-1, LFA-1) on the neutrophil binding to intercellular adhesion molecule-1 (ICAM-1) on the activated endothelium. Neutrophil homotypic firm adhesion is mediated both by LFA-1 to ICAM-3 and Mac-1 to Mac-1 (Lynam, 1998; Taylor, 1996). Transmigration may occur via paracellular, or less-frequently, transcellular means. The process is complex and not well-characterized. There is evidence, however, for the role of platelet endothelial cell adhesion molecule-1 (PECAM-1) junctional adhesion molecules (JAMs) and endothelial cell-selective adhesion molecule (ESAM) in endothelial cell gap junctions supporting migration via integrin and/or homophilic interactions with the leukocyte (Ley, 2007).
Figure 1.1. Leukocyte adhesion cascade. Neutrophils, margined to the periphery of the blood vessel by red blood cells, can follow chemoattractants to the activated endothelial surface. Once at the endothelial surface, neutrophils can follow a multi-step process governed by adhesion molecules to eventually extravasate through the endothelium to the site of injury. Figure from Ley (2007) adapted by permission from author and Macmillan Publishers Ltd: [Nature Reviews Immunology], copyright 2007
1.4 Membrane mechanics and neutrophil adhesion

The adhesive steps of the leukocyte adhesion cascade, particularly initial capture and rolling, require a balance between hemodynamic forces exerted on the cell and cell-substrate binding forces. While changes in blood viscosity or shear rate alter the hemodynamic forces experienced by neutrophils and thus affect their adhesion and rolling processes, the rheological properties of neutrophils and their cell membranes also play an important role (Caputo and Hammer, 2005; Dong, 1999). Receptor-ligand bond forces may be influenced by force-shielding tethers pulled from the neutrophil membrane and their accompanying mechanics (Lei, 1999; Park, 2002, Schmidtke and Diamond, 2000). Additionally, shear stress can induce cellular deformation. Leukocytes rolling on endothelial cells \textit{in vivo} have demonstrated considerable deformation, reaching aspect ratios of up to 1.4 (Damiano, 1996). By reducing hydrodynamic drag, deformation can reduce bond force, which can in turn reduce the dissociation rate and prolong bond lifetime (Lei, 1999). Cellular deformation can also increase cell contact area and accordingly, the number of adhesion molecules presented to the substrate. This increases the probability of bond formation.

Membrane tethers are pulled from a cell when a force exerted on a point on the cell surface exceeds a certain threshold (greater than 45-61 pN for neutrophils) (Edmondson, 2005). This process, in which the cell membrane is pulled from the microvilli to form lipid tubes 100 to 200 nm in diameter, requires
dissociation of the membrane from the cytoskeleton. Since the lipid membrane can only expand in area up to 4%, the lipid for the tether comes from the cell body, rather than elastic stretching of microvillus-localized lipid (Needham and Nunn, 1990). Because drawing a membrane tether involves lipid flow from the cell body to the tether, membrane fluidity significantly impacts the tether extrusion process. This process has been visualized directly, during neutrophil rolling on a P-selectin-coated surface (Schmidtke and Diamond, 2000) and it has been found that changes in tether growth rate affect P-selectin/PSGL-1 bond lifetimes (Edmondson, 2005).

1.5 Membrane deformability- and fluidity-altering reagents

The role of the cytoskeleton on neutrophil adhesion has been probed with reagents that alter actin polymerization. Cytochalasin D and latrunculin, both actin depolymerizing agents, have been shown to reduce neutrophil cortical tension and membrane viscosity during tether formation in micropipette aspiration studies (Ting-Beall, 1995; Tsai, 1994; Marcus and Hochmuth, 2002). Jasplakinolide, which reduces membrane-cytoskeleton adhesion via plasmalemma blebbing, has been shown to facilitate membrane tether formation (Sheikh, 1997). Previous research from our lab has indicated that these reagents all increase membrane tether growth rate and P-selectin/PSGL-1 adhesion lifetime, while reagents that decrease cell compliance, like MβCD and formaldehyde, have opposite effects (Edmondson, 2005). Compliance-increasing reagents have been shown to alter neutrophil rolling both \textit{in vitro} and \textit{in silico};
neutrophils treated with cytochalasin were shown to deform more and roll slower on P-selectin (Finger, 1996; Sheikh and Nash, 1998) while computational studies have demonstrated that more compliant cells roll slower, with less variation (Pappu, 2008).

1.6 Effect of ethanol on neutrophils

Ethanol has complex, possibly dose dependent effects in vivo. Clinical studies have shown reduction in risk of cardiovascular disease with moderate (1-2 drinks per day a few days per week) alcohol consumption (Renaud and Delorgeril, 1992). Acute ethanol exposure, however, has been linked to a pro-inflammatory state and increased risk of cardiovascular disease (Kiechl, 1998; Radek, 2008). Some studies have found that acute ethanol exposure inhibits neutrophil chemotaxis (Spagnuolo, 1975; Astry, 1983; Zhang, 1997) and reduces surface adhesion molecule expression (Patarroyo, 1987; McKenzie, 2002; Nilsson, 1991) while other studies have shown no effect on these metrics under similar ethanol concentrations (Corberand, 1989; Borghese, 2006). Ethanol has also been shown to both block leukocyte recruitment and endothelial cell activation (Patel, 1996; Saeed, 2004) and enhance leukocyte-endothelial cell interactions (Kvietys, 1990). Non-physiological ethanol levels have been shown to reduce activation-induced integrin upregulation and morphological changes (Patel, 1996; Nilsson, 1991; Macgregor 1988).

Studies utilizing electronic paramagnetic resonance spectroscopy and fluorescence spectroscopy have demonstrated that ethanol has effects on
membrane fluidity (Colles, 1995). Ethanol has been shown to decrease compressibility and bending moduli, lysis tensions and lysis area strains in micropipette aspiration experiments with unilamellar vesicles (Tierney, 2005). These studies also found that ethanol increased area/molecule of membrane lipids and reduced membrane thickness.

Those results, along with research on membrane and cell-compliance altering reagents, motivated previous work from our lab studying ethanol’s effects on neutrophil tethering, rolling, and firm arrest. Generally speaking, ethanol was found to reduce both transient (selectin) and firm (integrin) neutrophil adhesion (Oh and Diamond, 2008). Ethanol also increased membrane fluidity, as measured by tether length, growth rate, and lifetime.

1.7 Effect of cholesterol on neutrophils

Many studies have linked cholesterol with changes in cell membrane mechanics. Decreased cholesterol has been shown to reduce protein mobility in skin fibroblast membranes (Kwik, 2003) and decrease lipid diffusion in endothelial cells (Sun, 2007). Endothelial cell membrane stiffness was increased by cholesterol depletion (Byfield, 2004).

Neutrophils from hypercholesterolemic (HC) subjects show an 89% increase in membrane cholesterol over healthy subjects (Seres, 2005). These increases may contribute to a pro-inflammatory state; HC neutrophils show increased superoxide secretion and enhanced adhesion to endothelial cells (Ludwig, 1982; Sugano, 2005). Acute dietary cholesterol can also increase
neutrophil adhesion, as evidenced by a two-week animal study (Petnehazy, 2006).

Our lab has also studied changes in neutrophil membrane tether dynamics and adhesion in response to membrane cholesterol manipulation. Cholesterol enrichment promotes P-selectin/PSGL-1 adhesion and neutrophil firm arrest on endothelial cells, while cholesterol depletion has opposite effects (Oh, 2009). Cholesterol enrichment also seems to reduce membrane-cytoskeleton adhesion, measured by tether formation probability. Like ethanol, cholesterol enrichment increases tether length, lifetime, and growth rate. Again, cholesterol depletion has opposite effects.

Little is known about the interplay between ethanol and cholesterol as it applies to neutrophils. Ethanol can form complexes with cholesterol (Daragan, 2000) and may promote removal of cholesterol from cells (Hannuksela, 2002). Ethanol is non-uniformly distributed within the lipid membrane, partitioning into the bilayer core (Colles, 1995). These factors alter membrane cholesterol homeostasis and accordingly, can affect membrane fluidity.
1.8 Thesis Aims

1.8.1 Aim 1: Characterize cholesterol loading of the neutrophil membrane in the presence of a physiological ethanol concentration

We will quantify membrane cholesterol levels for neutrophils treated with ethanol alone, enriched in cholesterol alone, and for neutrophils exposed to ethanol and enriched in cholesterol sequentially, in either order of administration.

1.8.2 Aim 2: Determine the effects of ethanol on cholesterol-enriched neutrophil tethering, rolling, and adhesion.

We will explore the effects of ethanol and cholesterol on individual bonding and tethering parameters with a P-selectin coated-microbead assay. Flow cytometry will be employed to assess changes in neutrophil surface adhesion molecule expression. We will also examine ethanol and cholesterol-exposed neutrophils rolling on uniformly-coated P-selectin surfaces. Finally, we will quantify how ethanol and cholesterol affect neutrophil homotypic adhesion and tethering, and secondary adhesion to uniformly-coated ICAM-1 surfaces.

1.8.3 Aim 3: Determine the effects of shear rate, ethanol and cholesterol on neutrophil deformability, and ethanol and cholesterol’s effects on neutrophil membrane fluidity.

We will assess separately the effects of ethanol and cholesterol enrichment and depletion on the membrane lipid diffusivity of neutrophils using fluorescence recovery after photobleaching (FRAP). We will also characterize
neutrophil deformation changes with altered shear rate, ethanol administration, and membrane cholesterol manipulation by analyzing neutrophil collisions with similarly-sized beads.
CHAPTER 2: Methods

2.1 Materials

Human serum albumin (HSA; Golden West Biologicals) Hank’s Balanced Salt Solution (HBSS; Invitrogen) both with and without Ca\(^{2+}\), Mg\(^{2+}\), and phenol red, and methyl-β-cyclodextrin (MβCD; Sigma-Aldrich) were stored according to manufacturer’s instructions. Cholesterol (Sigma-Aldrich) was dissolved at a concentration of 50 mg/mL in a 1:1 by volume chloroform:methanol solution for storage.

2.2 Neutrophil isolation

Human blood was obtained via venipuncture from healthy adult donors who had not taken any medications or consumed alcohol in the previous 72 hours, in accordance with Institutional Review Board approval. Neutrophils were isolated by separation with Polymorphprep separation medium (Accurate Chemical) as previously described (Kadash, 2004). Neutrophils were counted and diluted with a solution of 2% HSA in HBSS without Ca\(^{2+}\), Mg\(^{2+}\), or phenol red, to a final concentration of 1-2 × 10\(^6\) cells/mL.

2.3 Membrane cholesterol enrichment and depletion

MβCD was dissolved in RPMI1640 medium without phenol red or serum at a concentration of 5 mM. Neutrophils were depleted of cholesterol by 60 minute incubation with MβCD solution. Cholesterol enrichment was
accomplished with MβCD/cholesterol complexes at 8:1 and 4:1 molar ratios, as previously described (Levitan, 2000). Briefly, cholesterol stock solution was added to a glass scintillation vial, the solvent was evaporated, and an appropriate volume of MβCD solution was added. The vial was sonicated for 20 minutes at 37°C and then placed in a shaking incubator overnight at 37°C.

2.4 Quantification of membrane cholesterol

The Amplex Red assay from Molecular Probes was used to assess neutrophil membrane cholesterol content. After incubation regimens (RPMI1640 without serum for 30, 60, and 90 minutes, as controls for each time point, 0.3% ethanol by volume for 30 minutes, 5 mM MβCD solution for 60 minutes, 8:1 or 4:1 MβCD:cholesterol solutions for 60 minutes, 0.3% ethanol for 30 minutes before or after 5 mM MβCD solution for 60 minutes, and 0.3% ethanol for 30 minutes before or after 8:1 or 4:1 MβCD:cholesterol solutions for 60 minutes) cells were washed in PBS, resuspended in the Amplex Red reaction buffer (0.1 M potassium phosphate, 0.05 M NaCl, 5 mM cholic acid, 0.1% Triton X-100, pH 7.4) at a concentration of 10^6 cells/mL, and vortexed. Cell lysates were pipetted in 25 µL aliquots into a black, polystyrene, 96-well tissue culture plate (Whatman) and 25 µL of Amplex Red working solution (300 µM Amplex Red reagent, 2 U/mL horseradish peroxidase, 2 U/mL cholesterol oxidase, and 0.2 U/mL cholesterol esterase) was added to each well. After 30 minute incubation at 37°C, fluorescence was measured on a Fluoroskan Ascent FL fluorescent plate reader.
(Thermo) at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

2.5 Neutrophil-microbead perfusion assay

Neutrophil tethering behavior and bond mechanics were studied with the neutrophil-microbead collision assay, which probed interactions between flowing neutrophils and a small, point source of P-selectin. Protein A-coated, 1.05-µm-diameter polystyrene microspheres (Bangs Laboratories) were labeled with recombinant human CD62P/FC chimera P-selectin with IgG1 Fc region (R&D Systems) as previously described (Edmondson, 2005; Rodgers, 2000). Rectangular glass capillaries (Vitrocom) with dimensions 0.2 × 2.0 × 70 mm and a wall thickness of 0.15 mm were used as flow chambers. The flow chamber schematic can be found in Figure 2.1. P-selectin coated beads (1.7 × 10⁴ P-selectin/bead) were washed and incubated overnight for attachment to glass capillary flow chambers for a final concentration of 5400 P-selectin/µm². Before perfusion studies, bead-coated flow chambers were washed and blocked with HBSS with 2% HSA. Neutrophils were perfused through the chambers at a wall shear rate of 100 s⁻¹ using a syringe pump (Harvard Apparatus). Previous studies have shown that 0.3% ethanol has no effect on P-selectin coated beads themselves (Oh and Diamond, 2008).
2.6 P-selectin-coated surface perfusion assay

P-selectin-coated surfaces were prepared by incubating microcapillary flow chambers (Figure 2.1) with recombinant human P-selectin (R&D Systems) in HBSS without Ca\(^{2+}\) or Mg\(^{2+}\) at a concentration of 1 µg/mL for at least 3 hours at room temperature. Excess unbound protein was removed by perfusing HBSS without Ca\(^{2+}\) or Mg\(^{2+}\) with 2% HSA through the chamber for 30 minutes. Under these conditions, the final P-selectin density was determined to be 10 sites/µm\(^2\) (Schmidtke and Diamond, 2000). A wall shear rate of 100 s\(^{-1}\) was used for these experiments as well.
Figure 2.1. Microcapillary flow chamber schematic. Flow chambers coated with adhesion molecules or adhesive/non-adhesive beads are mounted on an inverted microscope with DIC and phase optics. Images are captured via CCD or high speed camera, where they are processed and either stored to CPU or video tape.
2.7 ICAM-1-coated surface secondary adhesion assay

Selectin-mediated, primary, neutrophil homotypic interactions and secondary, integrin-mediated neutrophil interactions with an adhesive surface were explored with ICAM-1 coated microcapillary chambers (Figure 2.1). ICAM-1-coated surfaces were prepared by incubating microcapillary flow chambers with recombinant human ICAM-1 (R&D Systems) in HBSS without Ca$^{2+}$ or Mg$^{2+}$ at a concentration of 1 µg/mL for at least 2 hours at room temperature. Flow chambers were washed and blocked by perfusing HBSS without Ca$^{2+}$ or Mg$^{2+}$ with 2% HSA through the chamber for 30 minutes. Neutrophils were perfused into the chambers using a syringe pump and allowed to adhere for 10 minutes under static conditions. Flow was then resumed at a wall shear rate of 100 s$^{-1}$, and primary and secondary neutrophil interactions were observed.

2.8 Whole-cell deformation assay

Neutrophil deformation was assessed via collision with 10 µm polystyrene “Polybeads” (Polisciences). Beads were either left non-adhesive, or coated with P-selectin as in the neutrophil-microbead perfusion assay. Beads were prepared for attachment to microcapillary flow chambers (Figure 2.1) via incubation with 200 µg/mL fibrinogen for two hours at 4°C, which supported bead adhesion to the flow chamber surface, but not to neutrophils (Edmondson, 2005). After incubation, beads were washed and diluted in HBSS with 2% HSA, then perfused into flow chambers to incubate overnight at room temperature. After this incubation, flow chambers were washed and blocked with HBSS with 2% HSA.
2.9 FRAP lipid diffusivity assay

A 1 mL sample of isolated neutrophils was labeled with 3 µL of the lipid analog dye DiO for 20 min at 37 °C, protected from light. Samples were loaded into Lab-Tek borosilicate coverglass chambers (Thermo Scientific-Nunc) for imaging. To obtain diffusion measurements in the neutrophil cell body, a circular area with a diameter of 4 µm was bleached by simultaneous full intensity illumination with the 488 nm and 458 nm lasers of the confocal microscope setup. Fluorescence recovery was imaged with attenuated excitation intensity (0.1-0.3%) of the 488 nm laser. Cells were imaged by fluorescence confocal microscopy (Olympus IX81), using a 60x, 1.2 NA water immersion objective (Olympus). Cells were imaged both near the cell equator, and near the top of the cell. Image analysis was performed using MATLAB (Mathworks) and ImageJ. Recovery data were analyzed according to Soumpasis (Soumpasis, 1983). Briefly, fluorescence recovery profiles were double-normalized (such that intensity ranged from 0 to 1) and fit to the fluorescence recovery equation derived by Soumpasis:

\[
f(t) = A \cdot \exp\left(-\frac{2\tau}{t}\right)\left[I_0\left(\frac{2\tau}{t}\right) + I_1\left(\frac{2\tau}{t}\right)\right]
\]  

(2.1)

where \(A\) is the mobile fraction, \(\tau\) is the characteristic diffusion time, and \(I_0\) and \(I_1\) are modified Bessel functions of the first kind of order zero and one, respectively. The MATLAB command nlinfit was used to perform the nonlinear regression and obtain the parameters \(A\) and \(\tau\). The diffusion coefficient can be calculated from the characteristic diffusion time with the relation below:
\[ D = \frac{w^2}{\tau} \]  

(2.2)

where \( w \) is the diameter of the FRAP bleach spot.

2.10 Flow Cytometry

After the incubation regimens described in the cholesterol enrichment and depletion section, neutrophils at \( 10^6 \) cells/mL were incubated with FITC-anti-CD11b (Ancell) PE-anti-CD162 (BD Pharmingen) and AlexaFluor647-anti-L-selectin. Appropriate mouse isotype controls were used to assess nonspecific binding to neutrophils (FITC-mouse IgG1, PE-mouse IgG1, AlexaFluor647-mouse IgG1, BD Pharmingen). Samples were incubated in the dark, at room temperature, for 30 minutes, then washed, centrifuged, and resuspended in 1% formaldehyde solution in PBS. Samples were then run on an Accuri C6 flow cytometer (Accuri Cytometers) and 10,000 neutrophils were collected for each sample. Neutrophils were identified by forward and side scatter properties as previously described (van Eeden, 1999).

2.11 Perfusion assay shear rate calculations

The wall shear rate was determined for the microcapillary flow chamber by the Navier-Stokes equation for laminar flow of a Newtonian fluid: \( \gamma_w = \frac{6Q}{B^2W} \). In this equation, \( Q \) represents the flow rate (cm\(^3\)/sec), \( B \) is the total plate separation for the rectangular flow chamber, and \( W \) represents the width of the chamber. For the microcapillary chamber, \( B = 0.02 \) cm and \( W = 0.2 \) cm. For this aspect
ratio of 10, the parallel plate approximation is suitable, yielding the previously-mentioned expression for wall shear rate. Most flow studies were performed at a flow rate of 80 µL/min, corresponding to a wall shear rate of 100 sec\(^{-1}\), in the venous range. Varying shear rate studies used flow rates from 20 to 160 µL/min, to obtain shear rates of 25 to 200 sec\(^{-1}\).

2.12 Flow chamber imaging and video analysis

Microcapillary flow chambers were imaged with an inverted microscope (Zeiss Axiovert 135). For P-selectin-coated bead, 10 µm bead, and ICAM-1-coated surface experiments, differential interference contrast (DIC) microscopy was used with a 63× objective (Plan Apochromat) while for P-selectin-coated surface experiments, phase contrast microscopy was used with a 20× objective (Plan Apochromat). Flow experiments were recorded using a JVC Professional Series Super VHS VCR and Sony Trinitron connected to a central processing unit/microscope/image processor system (Ji, 2008). Video was captured at 28 frames per second (fps) with a CCD camera for all experiments except neutrophil whole cell deformation experiments and digitized using Windows Movie Maker (Microsoft). For neutrophil whole cell deformation experiments, video was captured with a Motion-Corder Analyzer high-speed digital camera (Eastman Kodak) at a range of frame rates, from 120-400 fps, and played back onto a VCR at 30 fps. ImageJ software (National Institutes of Health) with the MTrackJ
particle tracking plugin was used to analyze neutrophil tethering, rolling, and deformation behavior.

The following definitions were used for analysis of P-selectin-coated bead experiments (adapted from Edmondson, 2005). Adhesive interactions refer to neutrophil-bead collisions that result in a visible pause (one frame or more). Tether-forming neutrophils are those neutrophils that, upon forming an adhesive interaction with a bead, translate downstream at a velocity below the hydrodynamic velocity and are quickly released. The following parameters were extracted from the bead experiment: adhesion efficiency ($\epsilon$), the number of adhesive interactions divided by the total number of neutrophil-bead collisions observed; tether fraction ($f$), the number of tether-forming adhesive interactions divided by the total number of adhesive interactions; lifetimes for tethering and all adhesive events; tether length, which is the distance from the center of the bead to the lagging edge of the adherent neutrophil; and tether growth velocity, which was calculated by dividing tether length by tether lifetime.

Two parameters were used to analyze neutrophil rolling on P-selectin coated surfaces. Average rolling velocity was calculated by dividing the distance traveled by a rolling cell by the adhesion lifetime. Standard deviation of rolling velocity was calculated by MTrackJ on a per-trajectory basis and described the fluctuations in cell velocity for a rolling neutrophil. Analysis of the ICAM-1-coated surface flow chamber experiments borrowed several metrics from the P-selectin-coated bead experiments, using pre-adhered neutrophils as reference points,
instead of P-selectin coated beads: adhesion efficiency, tethering fraction, and tether length, lifetime, and growth velocity. Additionally, lifetimes of secondary adhesive events were recorded.

For the whole-cell deformation assay, we analyzed the high-speed video data frame by frame. To quantify deformation, we tracked the centroid location (x,y) of the cells and measured the distance between the centroid of the cells and the centroid of the bead (d) while cells were in contact with the bead at angle θ. For two hard spheres during collision, this distance would remain identical throughout contact. This distance equals the sum of the radius of the cell and the radius of the bead: d0 = 9 µm. We calculated the difference between these two values as R = d - d0, which defines R = 0 for two non-deformable spheres, and tracked them during contact. A negative R value indicates deformation; for simplicity of comparison, we took the absolute value of R to represent deformation.

2.13 Statistical Analysis

In order to determine levels of statistical significance, data were first examined with the Kruskal-Wallis one-way analysis of variance test. This test was also used to identify and remove statistical outliers. Data from the Kruskal-Wallis test were passed to the MATLAB multiple comparison test to assess whether or not statistical differences existed among groups. Specific p values between groups were determined with the Wilcoxon rank-sum test. A p value of <0.05 was considered significant.
CHAPTER 3: Cholesterol loading of the neutrophil membrane in the presence of a physiological ethanol concentration

3.1 Abstract

We evaluated the role of a physiological concentration of ethanol on cholesterol loading of the neutrophil membrane. Cholesterol was removed from the neutrophil membrane via incubation with MβCD for 60 minutes, and loaded into the membrane via incubation with complexes of MβCD and cholesterol for 60 minutes. The effect of ethanol was ascertained by incubating neutrophils with 0.3% ethanol solution for 30 minutes before or after cholesterol loading. Membrane cholesterol levels were quantified using the Amplex Red assay. Membrane cholesterol was increased over control by 4.6-fold in the absence of ethanol, 3.6-fold with 0.3% ethanol pre-loading, and 1.6-fold with 0.3 % ethanol post-loading.
3.2 Introduction

Alteration of cholesterol levels in neutrophil and HL-60 (neutrophil-like cell line) cell membranes has been characterized previously, as shown in Figure 3.1 (Oh, 2009). Cholesterol depletion with MβCD, a cyclic oligosaccharide pictured in Figure 3.2 (Davis and Brewster, 2004) removed 35% of neutrophil membrane cholesterol, and 55% of HL-60 membrane cholesterol. Cholesterol enrichment increased in a dose-dependent manner with decreasing MβCD:cholesterol molar ratio, to a plateau around the 4:1 molar ratio.

Little is known about the interplay between ethanol and cholesterol as it applies to neutrophils, however. Ethanol can form complexes with cholesterol (Daragan, 2000) and may promote removal of cholesterol from cells (Hannuksela, 2002). This alters membrane cholesterol homeostasis and accordingly, can affect membrane fluidity.
Figure 3.1. Membrane cholesterol fold change relative to control for neutrophils and HL-60 cells exposed to a variety of cholesterol depletion and enrichment regimens. Figure from Oh, 2009, adapted by permission from authors and Lippincott Williams and Wilkins, Publisher: [Arteriosclerosis, Thrombosis, and Vascular Biology], copyright 2009
Figure 3.2. Chemical structure of MβCD, a cyclic oligosaccharide used in cholesterol depletion and enrichment protocols. The external surface of the molecule is polar and therefore soluble in cell culture medium and buffers. The internal cavity is hydrophobic, which allows cholesterol to reside and form a stable complex.
3.3 Results

A MβCD:cholesterol ratio of 4:1 resulted in a 4.6-fold ($p<0.001$) increase in membrane cholesterol over control (Figure 3.3) whereas an 8:1 ratio elicited a 5.6-fold increase (data not shown) (Furlow and Diamond, 2011). Pre-treating neutrophils with ethanol prior to cholesterol treatment lowered the cholesterol enrichment to 3.6-fold ($p<0.001$) above control for the 4:1 ratio (3.2-fold for the 8:1 ratio; not shown). Ethanol treatment after cholesterol loading resulted in cholesterol increase of 1.6-fold ($p<0.05$) over control (1.6-fold for the 8:1 ratio, not shown). This represented a significant decrease ($p<0.001$) in cholesterol enrichment compared to cholesterol loading alone. The 4:1 ratio of MβCD to cholesterol was chosen for further study because of the higher cholesterol enrichment seen in conjunction with ethanol treatment. MβCD treatment without cholesterol significantly ($p<0.05$) reduced membrane cholesterol compared to control by 75% and ethanol had no impact on this reduction (data not shown).
Figure 3.3. Neutrophil cholesterol enrichment in conjunction with ethanol exposure. Cholesterol enrichment was accomplished by incubation with MβCD/cholesterol complexes for 60 minutes. Cells were treated with ethanol by incubation in 0.3% solution for 30 minutes. Cholesterol levels normalized to control; data are presented ±SD. N ≥ 4. ‡ = p<0.05, * = p<0.001. Membrane cholesterol in control neutrophils was 3.3 ± 0.9 nmol/10^6 cells. Adapted from Furlow and Diamond, 2011.
3.4 Discussion

Neutrophil membrane cholesterol levels were successfully elevated by MβCD:cholesterol complexes. A statistically significant increase in cholesterol of 4.6-fold over control seen with 4:1 MβCD:cholesterol. Ethanol did not have a significant effect on cholesterol levels by itself. Ethanol did appear to affect cholesterol enrichment, however. Treatment of neutrophils with ethanol before enrichment resulted in an increase in membrane cholesterol of 3.6-fold, and ethanol treatment after enrichment increased membrane cholesterol only 1.6-fold. These results are consistent with previous data asserting that ethanol can promote cholesterol removal from cell membranes (Hannukse, 2002). It has been shown that ethanol and cholesterol can bind in a 1:1 ratio (Daragan, 2000) and it is conceivable that this binding is related to the cholesterol-removing effects of ethanol. In the ethanol, then cholesterol treatment regime, MβCD-cholesterol complexes encounter ethanol only in the neutrophil membrane. In this regime, ethanol would be the limiting reagent in the ethanol-cholesterol interaction. Eventually, the cholesterol binding and removal capacity of the ethanol in the neutrophil membrane could be exhausted, and cholesterol would then be loaded into the cell membrane without encountering a hindering concentration of ethanol. In the opposite incubation regime, however, ethanol would be in excess, and the cholesterol previously loaded into the neutrophil membrane would be the limiting reagent. Therefore, cholesterol could be bound and removed by ethanol to a greater extent. It is interesting that ethanol reduces
cholesterol levels in cholesterol-enriched neutrophils, yet has no effect on natural cholesterol levels in untreated neutrophils. This may be due to elevated extractability of added cholesterol due to membrane heterogeneity in enriched cells. It appears that ethanol removes excess cholesterol from neutrophil membranes, but not natural levels of cholesterol.
CHAPTER 4: Characterization of the effects of ethanol on cholesterol-enriched neutrophil tethering, rolling, and adhesion

4.1 Abstract

Using microfluidic assays at a wall shear rate of 100 s\(^{-1}\), we examined the effects of ethanol on cholesterol-loaded neutrophils with respect to: (1) collision efficiency and membrane tethering to P-selectin-coated microbeads, (2) rolling on P-selectin-coated surfaces, and (3) primary and secondary interactions with neutrophils pre-adhered to intercellular adhesion molecule-1 (ICAM-1). Cholesterol enrichment did not alter CD11b expression; however, PSGL-1 and L-selectin expression were lowered by cholesterol enrichment with or without the presence of ethanol. Cholesterol enrichment enhanced microbead collision efficiency, which was abrogated by ethanol. Ethanol had no effect on elevation of tethering fraction by cholesterol enrichment. Incubation of cholesterol-loaded neutrophils with ethanol resulted in significantly longer membrane tethers, due to tether lifetime enhancement. On P-selectin-coated surfaces, cholesterol-enriched neutrophils exposed to ethanol rolled faster and with more variability than cholesterol-enriched neutrophils. Ethanol reduced homotypic collision efficiency of cholesterol-loaded neutrophils without effect on tethering fraction or secondary collision efficiency. Tether length during cholesterol-loaded neutrophil homotypic collisions was enhanced by ethanol, in part due to increased L-selectin/PSGL-1 bond tether lifetime. Overall, ethanol attenuated cholesterol-induced adhesion increases while increasing membrane fluidity as indicated by tether length.
4.2 Introduction

In addition to contributing to fatty streak formation, elevated serum cholesterol may exert pro-atherosclerotic effects through altered neutrophil membrane biomechanics and adhesive behavior caused by increased membrane cholesterol content. Mice placed on a high cholesterol diet experience increased leukocyte adhesion and emigration (Petnehazy, 2006). Similarly, neutrophils from hypercholesterolemic patients display increased adherence to human umbilical vein endothelial cells (Sugano, 2005).

Ethanol, however, at least in moderate doses, appears to reduce the incidence of cardiovascular disease. Consumption of a “moderate dose,” 1-2 alcoholic beverages per day, has been correlated with decreased incidence of cardiovascular disease (Renaud and de Lorgeril, 1992). A separate study lasting 12 years, encompassing more than 38,000 healthy men, found that consumption of small amounts of alcohol 3-4 days a week significantly reduced the risk of myocardial infarction (Mukamal, 2003). In large doses, however, ethanol appears to increase the risk of cardiovascular disease (Kiechl, 1998).

Alteration of cell membrane cholesterol has been shown to have profound effects on cell membrane mechanics. Cholesterol depletion increases membrane stiffness in endothelial cells (Byfield, 2004), and decreases lipid diffusivity (Sun, 2007). Conversely, cholesterol enrichment increases lipid diffusivity in neutrophils, along with whole-cell deformability (Oh, 2009). Cholesterol enrichment also decreases membrane surface viscosity in endothelial cells and
decreases membrane-cytoskeleton adhesion energy (Sun, 2007). Cholesterol depletion decreases neutrophil tethering to P-selectin-coated beads, along with tether length, lifetime, and growth velocity (Oh, 2009). Cholesterol enrichment, on the other hand, increases neutrophil tether fraction, length, lifetime, and growth velocity. Cholesterol enrichment also increases adhesion efficiency to P-selectin coated beads. Neutrophils perfused over either a uniform P-selectin-coated surface, or over an IL-1-activated human aortic endothelial cell (HAEC) monolayer, experienced slower, smoother rolling after cholesterol enrichment compared to control, with a greater percentage of neutrophils converting to firm arrest; cholesterol depletion had opposing effects.

Whole-cell deformation of neutrophils is increased with ethanol treatment, but not to the same extent as cholesterol enrichment (Oh, 2009). Lipid diffusivity is significantly increased in neutrophils treated with ethanol (Oh, 2009). Ethanol decreases neutrophil adhesion efficiency to P-selectin-coated beads, and decreases the likelihood of tethering (Oh and Diamond, 2008). Tether length and tether growth velocity were increased by ethanol treatment in the same study. Ethanol slowed neutrophil rolling velocity on a uniform P-selectin-coated surface, but also decreased the rolling flux. On IL-1-activated HAEC, neutrophil rolling velocity was increased by ethanol treatment, though not to a significant extent. The percentage of neutrophils converted to firm arrest was significantly decreased by ethanol in that study.
Little is known about the interplay between ethanol and cholesterol as it applies to neutrophils. Clinically, light alcohol consumption can lower risk of MI by increasing HDL and decreasing LDL (Langer, 1992; Mukamal, 2003). This phenomenon seems to comprise part of the well-known French Paradox, wherein low mortality from cardiovascular disease despite high intake of saturated fat is attributed to daily, moderate alcohol consumption, particularly wine (Renaud and de Lorgeril, 1992). Ethanol can form complexes with cholesterol (Daragan, 2000) and may promote removal of cholesterol from cells (Hannuksela, 2002). This alters membrane cholesterol homeostasis and accordingly, can affect membrane fluidity.

Ethanol and cholesterol have significant effects on cardiovascular disease, appearing to work in opposite directions, for moderate doses of ethanol. Ethanol and cholesterol also both impact cell membrane mechanics and neutrophil function, sometimes in the same manner, and sometimes with opposing effects. From in vitro studies, it is known that ethanol and cholesterol interact within the cell membrane. There are little data on the interaction of ethanol and cholesterol applied to neutrophils, however. This interaction may be important, since ethanol and cholesterol are often present simultaneously in western diets. In light of this, we exposed cholesterol-enriched neutrophils to ethanol and examined neutrophil adhesion and tethering mechanics, as well as rolling and firm adhesion behavior.
4.3 Results

4.3.1 Effect of ethanol and cholesterol on surface adhesion molecule expression

To ascertain whether or not any of the ethanol and/or cholesterol incubation regimes activated neutrophils, we evaluated neutrophil surface adhesion molecule expression by flow cytometry. Figure 4.1 (Furlow and Diamond, 2011) shows the effect of ethanol, cholesterol-loading, and sequential administration on neutrophil CD11b, PSGL-1, and L-selectin expression. CD11b expression was not statistically different from control for any of the experimental groups, indicating that none of the treatments induced full neutrophil activation. Ethanol treatment alone did not significantly affect expression of any of the surface adhesion molecules. This is consistent with previous data from our lab (Oh and Diamond, 2008). Cholesterol enrichment, however, decreased PSGL-1 and L-selectin expression 35% compared to control. Both reductions were significant ($p<0.01$ and $p<0.05$, respectively) and did not appear to be significantly affected by ethanol. The observed decrease in L-selectin surface expression for cholesterol and combined ethanol and cholesterol treatments could be indicative of early activation, as evidence has been found for L-selectin shedding upon neutrophil activation (Bennett, 1996; Goel and Diamond, 2002). The decreases in PSGL-1 expression could be similar in nature, as PSGL-1 shedding has been observed upon stimulation of neutrophils with platelet activating factor and PMA. Despite these changes, neutrophils maintained a
rounded morphology. Since the antigen levels of CD11b, PSGL-1, and L-selectin were quite similar and not statistically different for the conditions of cholesterol loading, pre-ethanol + cholesterol, and cholesterol + post-ethanol, subsequent differences in adhesion between these three groups are not likely due to changes in antigen density.
Figure 4.1. Mean fluorescence intensity (MFI) of FITC-conjugated anti-CD11b, PE-conjugated anti-PSGL-1, and Alexa Fluor 647-conjugated anti-L-selectin for neutrophils undergoing ethanol treatment, cholesterol loading, and combined administration of ethanol and cholesterol. Fluorescence levels normalized to control; data are presented ±SD. N ≥ 5. ‡ = p<0.05, † = p<0.01, * = p<0.001 relative to control. Adapted from Furlow and Diamond, 2011.
4.3.2 Effect of ethanol and cholesterol on neutrophil adhesion and tethering to P-selectin-coated microbeads

As seen previously (Oh and Diamond, 2009), ethanol significantly ($p<0.05$) decreased both neutrophil adhesion efficiency and tethering fraction relative to control (Figure 4.2) (Furlow and Diamond, 2011). In contrast, cholesterol enrichment increased ($p<0.05$) adhesion efficiency and tethering fraction, also previously observed (Oh, 2009). Ethanol significantly reduced cholesterol-enriched neutrophil adhesion efficiency relative to cholesterol enrichment alone by 53% ($p<0.05$) when preceding cholesterol enrichment and 60% ($p<0.05$) when following cholesterol enrichment. Ethanol did not have a significant effect on cholesterol-enriched neutrophil membrane tethering fraction relative to cholesterol treatment alone, however.

Ethanol significantly increased the tether length of cholesterol-enriched neutrophils relative to control ($p<0.001$) ethanol incubation ($p<0.01$) and cholesterol enrichment ($p<0.05$) conditions (Figure 4.3A), demonstrating that ethanol can enhance cholesterol’s effect on neutrophil membrane fluidity (Furlow and Diamond, 2011). Specifically, ethanol incubation before cholesterol enrichment increased tether length relative to cholesterol enrichment alone 2.7-fold, and ethanol after cholesterol enrichment resulted in a 2.6-fold increase. This enhancement was also seen to an extent for tether lifetime (Figure 4.3B). Preceding cholesterol enrichment with ethanol treatment significantly ($p<0.01$) increased tether lifetime relative to ethanol treatment alone, by 2.2-fold, and
following cholesterol enrichment with ethanol treatment increased tether lifetime relative to control (p<0.01) ethanol (p<0.01) and cholesterol enrichment (by 3.3-fold, p<0.01). These effects were not seen for tether growth velocity (Figure 4.3C) however; ethanol, cholesterol enrichment, and cholesterol enrichment followed by ethanol treatment all significantly increased growth velocity over control, but there were no significant differences between those groups. While the average tether length would be expected to be equal to the average growth velocity multiplied by average lifetime, the large variability in events resulted in large standard deviations that were not normally distributed. Some neutrophils pulled long tethers with short lifetimes, other pulled long tethers with long lifetimes, some short tethers with short lifetimes, and some short tethers with long lifetimes.

To determine whether or not ethanol, as a small, readily-diffusible molecule, might leave the neutrophil membrane over the course of the perfusion experiments, matching experiments were performed with 0.3% ethanol in the perfusion medium for experimental groups that were incubated with ethanol. Results in these experiments were not statistically different from experiments with standard perfusion medium (data not shown).
Figure 4.2. Effect of cholesterol enrichment, ethanol, and combined administration on neutrophil adhesion efficiency (A) and tethering fraction (B) to P-selectin coated-microbeads. $\gamma = 100 \text{ s}^{-1}$. Values are mean ± SD. ‡ = $p<0.05$, NS = not significant. N = 5 donors. Adhesion efficiency for control cells was 0.115 ± 0.014. Tether fraction for control cells was 0.452 ± 0.025. Adapted from Furlow and Diamond, 2011.
Figure 4.3. Effect of cholesterol enrichment, ethanol, and combined administration on neutrophil to P-selectin bead tether length (A) tether lifetime (B) and tether growth velocity (C). $\gamma = 100 \text{ s}^{-1}$. Values are mean ± SD. ‡ = $p<0.05$, † = $p<0.01$, * = $p<0.001$. N = 5 donors. Adapted from Furlow and Diamond, 2011.
4.3.3 Effect of ethanol and cholesterol on neutrophil rolling on P-selectin-coated surfaces

Previous studies have shown that ethanol and cholesterol separately have significant effects on neutrophil rolling on P-selectin-coated surfaces (Oh and Diamond, 2008; Oh, 2009). These studies also indicated that ethanol and cholesterol alter neutrophil deformability, which in concert with selectin-governed tethering, governs neutrophil rolling. Informed by our findings on neutrophil membrane cholesterol content, surface adhesion molecule expression, and alterations in tethering behavior with ethanol and cholesterol incubation regimes, we examined neutrophil rolling on P-selectin-coated surfaces in response to ethanol and cholesterol treatment.

Ethanol doubled neutrophil rolling velocity and standard deviation of rolling velocity relative to control (Figure 4.4). Cholesterol enrichment, on the other hand, caused a significant reduction in neutrophil rolling velocity (30%) and standard deviation of rolling velocity (17%). In the combined treatment regime, ethanol significantly increased rolling velocity of cholesterol-enriched neutrophils relative to cholesterol enrichment alone (by 1.6-fold, \( p < 0.001 \)). This indicates that ethanol “rescues” cholesterol-enriched neutrophils from the slower, pro-adhesive rolling state brought about by cholesterol enrichment. Results were similar for standard deviation of rolling velocity, where despite lowering standard deviation relative to ethanol treatment, combined treatment significantly increased standard deviation over cholesterol enrichment by 30 % (\( p < 0.001 \)).
Figure 4.4. Effect of cholesterol enrichment, ethanol, and combined administration on neutrophil rolling velocity (A) and standard deviation of rolling velocity (B) on a uniformly-coated P-selectin surface. $\gamma = 100 \text{ s}^{-1}$. Values are mean ± SD.  

* = $p<0.001$. N = 4 donors. Adapted from Furlow and Diamond, 2011.
4.3.4 Effect of ethanol and cholesterol on neutrophil primary and secondary adhesion

Neutrophils exposed to ethanol experienced a significant decrease in homotypic adhesion efficiency relative to control (Figure 4.5A). Ethanol also significantly decreased homotypic adhesion efficiency for cholesterol-enriched neutrophils, relative to cholesterol enrichment, by about 30% ($p<0.01$). Ethanol treatment before cholesterol enrichment decreased adhesion efficiency relative to control, as well ($p<0.01$). These results parallel those from the P-selectin coated-microbead experiment; the anti-adhesive effects of ethanol also seem to dominate homotypic adhesion behavior in the combined treatment regime. Cholesterol enrichment and combined treatments increased homotypic tethering fraction (Figure 4.5B) over control and ethanol treatments, but not to a significant extent. These results were likely complicated by tether pulling between two deformable objects, which would alter the forces each tether experienced. Secondary adhesion efficiency (Figure 4.5C) was not significantly altered by any treatments. Since none of the treatments significantly altered $\beta_2$ integrin expression (Figure 4.1) which mediates ICAM-1 adhesion, this was not surprising.

Cholesterol enrichment significantly increased homotypic tether length compared to control (Figure 4.6A). Preceding cholesterol enrichment with ethanol treatment significantly ($p<0.001$) increased tether length relative to control and ethanol treatments, and following cholesterol enrichment with ethanol increased
tether length over control ($p<0.001$) ethanol ($p<0.001$) and cholesterol enrichment alone ($p<0.01$). Specifically, cholesterol enrichment, then ethanol treatment increased tether length 1.6 fold over cholesterol enrichment alone. As with tethering to microbeads, homotypic tethering after cholesterol treatment was enhanced by ethanol incubation. Homotypic tether lifetime (Figure 4.6B) was also significantly increased over control by cholesterol enrichment ($p<0.05$) and combined administration of ethanol and cholesterol ($p<0.001$). Combined administration increased tether lifetime over cholesterol enrichment, but not to a significant extent. Ethanol and cholesterol also appeared to work in concert to affect neutrophil secondary adhesion lifetime (Figure 4.6C). Neither ethanol nor cholesterol enrichment alone significantly affected secondary adhesion lifetime, but preceding cholesterol enrichment with ethanol treatment significantly increased lifetime over control ($p<0.01$) and ethanol ($p<0.05$) treatments, and following cholesterol enrichment with ethanol increased lifetime over all individual treatments ($p<0.001$, $p<0.01$, $p<0.05$ for control, ethanol, cholesterol enrichment, respectively). The complicating effects of double tether pulling were seen again for homotypic tether growth velocity (Figure 4.6D) where there were no significant differences between any groups.
Figure 4.5. Effect of cholesterol, ethanol, and combined administration on neutrophil homotypic adhesion efficiency (A) homotypic tethering fraction (B) and secondary adhesion efficiency to ICAM-1 (C).

γ = 100 s⁻¹. Values are mean ± SD. ‡ = p<0.05, † = p<0.01. N ≥ 4 donors. Primary adhesion efficiency for control cells was 0.367 ± 0.033. Secondary adhesion efficiency for control cells was 0.233 ± 0.090. Tether fraction for control cells was 0.211 ± 0.055. Adapted from Furlow and Diamond, 2011.

Figure 4.6. Effect of cholesterol, ethanol, and combined administration on neutrophil homotypic tether length (A) tether lifetime (B) tether growth velocity (C) and secondary adhesion lifetime to ICAM-1 (D).

γ = 100 s⁻¹. Values are mean ± SD. ‡ = p<0.05, † = p<0.01, * = p<0.001. N ≥ 4 donors. Adapted from Furlow and Diamond, 2011.
4.4 Discussion

Discrete bonding interactions between P-selectin and PSGL-1 were examined with the P-selectin coated-microbead assay. In the combined administration regime, the anti-adhesive effects of ethanol appeared to dominate, as cholesterol-enriched neutrophils exposed to ethanol had significantly lower adhesion efficiencies than neutrophils enriched in cholesterol alone. Ethanol did not significantly alter expression of any adhesion molecules by flow cytometry, and while combined treatments resulted in decreased PSGL-1 expression relative to control, cholesterol enrichment also decreased PSGL-1 expression relative to control, and actually increased adhesion efficiency relative to control. So, it appears that in these experiments, PSGL-1 levels themselves did not govern adhesive capability. It is more likely that PSGL-1 redistribution in response to ethanol (Oh and Diamond, 2008) was the reason for decreased adhesive capability with ethanol treatment.

The anti-adhesive effects of ethanol were demonstrated for neutrophil homotypic adhesion, also. Ethanol reduced cholesterol-enriched neutrophil homotypic adhesion efficiency relative to cholesterol enrichment alone; ethanol, then cholesterol treatment reduced adhesion efficiency relative to control, as well. Neutrophil capture by another neutrophil is governed by the interaction between L-selectin and PSGL-1 (Guyer, 1996; Witko-Sarsat, 2000). It is likely
that PSGL-1 redistribution is again responsible for the reduction in adhesion efficiency after ethanol treatment, as in the microbead experiments. Though decreases in PSGL-1 and L-selectin indicate early activation, it is unlikely that any treatments fully activated neutrophils, as CD11b expression was not significantly increased for any of the treatments. This was borne out by the secondary adhesion results, wherein none of the treatments produced a statistically significant change in secondary adhesion. Some treatments produced modest changes, likely due to alterations in cell deformability, but without changes in $\beta_2$ integrin expression required for adhesion to the secondary substrate, ICAM-1, significant changes in secondary adhesion were not seen.

Ethanol did not significantly impact tethering fraction of cholesterol-enriched neutrophils relative to cholesterol enrichment alone, and increased tethering fraction relative to control, indicating that cholesterol dictates the likelihood of neutrophil tethering in the combined administration regime. That ethanol slightly, but not significantly, decreased tethering fraction of cholesterol enriched neutrophils probably reflects a balance between two factors. Ethanol decreases PSGL-1-P-selectin bond forces to a transition region below that required for efficient tether-pulling (Oh and Diamond, 2008). On the other hand, cholesterol increases the likelihood for membrane-cytoskeleton dissociation by decreasing membrane cytoskeleton adhesion energy (Sun, 2007). This decreases the bond force required for tether formation.
Changes in homotypic tethering fraction, while not significant, followed the trends from the coated-bead assay results, indicating that alterations in membrane-cytoskeleton adhesion were likely at play there, as well. Tether pulling between two deformable objects and multiple tethers between the homotypic pair are possible confounding factors introducing extra variability into the data. Neutrophil tether length data from P-selectin coated-microbead experiments indicated strongly that ethanol enhances cholesterol’s effect on membrane fluidity, since combined treatments increased tether length significantly over control, ethanol, and cholesterol enrichment groups. This effect was seen to a lesser extent in tether lifetime data. These results make sense in terms of previous studies finding increased membrane diffusivity and fluidity with ethanol and cholesterol enrichment separately and decreased membrane viscosity with cholesterol enrichment (Cooper and Hausman, 2007; Garzetti, 1993; Oh, 2009; Sun, 2007). It is important to recognize, however, that tether length can be influenced by a number of interacting and interdependent factors such as membrane fluidity, tether lifetime, tether growth velocity, bonding lifetime, and tether force history.

Neutrophil homotypic tether length was increased significantly over control by cholesterol enrichment, and sequential ethanol and cholesterol treatment. Ethanol also significantly increased cholesterol-enriched neutrophil tether length relative to cholesterol enrichment, once again demonstrating ethanol and cholesterol working in concert to affect membrane fluidity. With two surfaces
capable of pulling tethers in the homotypic pair, the possibility for double tether pulling, which has been seen previously between neutrophils and endothelial cells (Girdhar and Shao, 2007), means that the fluidity- and viscosity-altering effects of ethanol and cholesterol may have even greater impact. Similar effects were seen for homotypic tether lifetime, though not as prominently as with tether length. Combined ethanol and cholesterol treatment increased tether lifetime significantly over control, and ethanol preceding cholesterol enrichment increased tether lifetime relative to ethanol, as well. Ethanol increased cholesterol-enriched neutrophil tether lifetime, but not to a significant extent. As with homotypic tethering fraction, tether growth velocity results were highly variable; neutrophils were occasionally observed to translate while tethered in a step-like manner, with an intermediate pause, indicative of double tether-pulling. Imaging magnification and resolution were not sufficient to directly image neutrophils tethers to confirm this, however. Cholesterol-enriched neutrophil secondary adhesion lifetime to ICAM-1 was significantly increased by ethanol, over cholesterol enrichment alone, ethanol, and control. This increase was reflective of an increased number of bonds to the ICAM-1 substrate, which was likely a result of increased contact area afforded by increased cell deformability in response to ethanol and cholesterol acting together.

In contrast to previous findings (Oh and Diamond, 2008), but in accordance with data from microbead experiments, ethanol was found to increase neutrophil rolling velocity on a uniformly-coated P-selectin surface. In
this case, it appears that the decreased PSGL-1-P-selectin adhesion as a result of ethanol-induced PSGL-1 redistribution was not countered by increases in force-shielding tethering behavior, as in the previous study. Ethanol decreased tethering fraction in this study, and did not significantly increase tether length or lifetime. These results also explain the decrease in rolling smoothness with ethanol treatment; lower tether lifetimes and fewer adhesions mean that a neutrophil will be less likely to find or maintain bonds close to existing bonds, which results in “jumpy” translation. Cholesterol significantly decreased rolling velocity and standard deviation of rolling velocity, as seen before (Oh, 2009), likely as a result of the increased deformation (and concomitantly, increased contact area with the adhesive surface) of the cholesterol-enriched cell membrane, and increased bond force-shielding resulting from increases in tethering metrics. This is supported by simulations demonstrating that more compliant cells roll more slowly and smoothly, and engage more bonds with an adhesive surface, due to increased contact area with substrate (Jadhav, 2005). Ethanol appeared to “rescue” cholesterol-enriched neutrophils from the more adhesion-prone, slower, smoother rolling state afforded by cholesterol enrichment. Sequential ethanol and cholesterol treatment significantly increased rolling velocity relative to cholesterol enrichment, and to control; standard deviation of rolling velocity was also increased relative to cholesterol treatment. This can be explained by returning to the data from the coated-bead assay. Ethanol effectively reduced the number of adhesive bonds between the
neutrophil and the substrate, inducing PSGL-1 redistribution. This decrease in adhesion countered the increase in tether fraction, length, and lifetime seen with cholesterol and combined treatments; though the probability of tether pulling may have been higher with combined treatment compared to control, and the resulting tethers may have been longer, with longer lifetimes, the absolute number of tethers was lower because of the decreased adhesive capability. Ethanol in the perfusion medium appeared to have little effect on neutrophil tethering and rolling behavior, establishing that ethanol does not leave the neutrophil membrane in appreciable quantities over the course of these experiments.
CHAPTER 5: Quantifying the effects of ethanol, cholesterol, and shear rate on neutrophil membrane fluidity and deformability

5.1 Abstract

To elucidate the roles of ethanol and cholesterol on altered neutrophil tethering, rolling, and adhesion behavior for combined administration regimes, we studied the effects of ethanol and cholesterol separately on neutrophil compliance, measured by whole cell deformability, and neutrophil membrane fluidity, measured by lipid diffusivity. Neutrophil deformation was measured by perfusing neutrophils through microcapillary flow chambers presenting adherent 10 µm beads absent of neutrophil-relevant adhesion molecules at a wall shear rate of 100 s\(^{-1}\) and observing non-adhesive collisions. Neutrophil-bead collisions were compared to theoretical rigid-sphere collisions to obtain deformation over the duration of neutrophil-bead contact. Deformation, and thus compliance, was increased over control for neutrophils exposed to 0.1% and 0.3% ethanol, and enriched in cholesterol with 8:1 and 4:1 M\(\beta\)CD:cholesterol molar ratio complexes. Cholesterol depletion with 5 mM M\(\beta\)CD decreased neutrophil deformation and compliance compared to untreated cells. The effects of variation in shear rate on neutrophil deformation were also investigated, with non-adhesive and P-selectin-coated beads, over a 25 s\(^{-1}\) to 200 s\(^{-1}\) wall shear rate range. Deformation increased in a dose-dependent manner with shear rate, and was similar in magnitude for collisions with non-adhesive and P-selectin-coated beads.
However, collisions with P-selectin-coated beads resulted in less variable deformations with dependence on neutrophil-bead collision angle. Neutrophil cell body membrane lipid diffusivity was assessed with FRAP experiments. We found that 0.3% ethanol and cholesterol enrichment with 4:1 MβCD:cholesterol complexes both increased membrane lipid diffusivity over untreated neutrophils. Membrane lipid diffusivity was decreased compared to control by cholesterol depletion with 5 mM MβCD. Together, these results characterizing ethanol and cholesterol's individual effects on neutrophil compliance and membrane fluidity can help explain how the two reagents interact to affect neutrophil tethering, rolling, and adhesion behavior when administered together.
5.2 Introduction

The complex results from the combined ethanol and cholesterol studies motivated us to examine the individual effects of ethanol and cholesterol on two factors influencing tethering and rolling behavior: membrane fluidity and neutrophil deformation. Lipid for membrane tethers necessarily comes from the neutrophil cell body, rather than elastic extension of microvillus lipid, since the lipid membrane can only stretch up to 4% (Needham and Nunn, 1990). This means that drawing a membrane tether involves lipid flow from the cell body to the tether; clearly, then, membrane fluidity can significantly impact the tether extrusion process. Additionally, leukocytes can be deformed by vascular shear stress. Deformation can reduce hydrodynamic drag on the leukocyte, which can in turn reduce bond force, bond dissociation rate, and prolong bond lifetime (Lei, 1999). Cellular deformation can also increase cell-substrate contact area, which can increase the number of adhesion molecules presented to the substrate and increase the probability of bond formation. Deformation and its effects have been theorized to be a contributor to the phenomenon of hydrodynamic thresholding (flow-enhanced adhesion) (Kadash 2004). With this in mind, we also studied how neutrophil deformation varied with shear rate.

5.2.1 Techniques to assess membrane fluidity

The two most common fluorescent microscopy techniques used to quantify membrane fluidity are fluorescence recovery after photobleaching (FRAP) and fluorescence polarization (FP). Both of these techniques yield
diffusion coefficients which can be used as a proxy for membrane fluidity; FRAP measures translational diffusivity of labeled membrane components, while FP characterizes molecular rotational diffusivity. FP has been used previously to determine membrane fluidity after cholesterol enrichment, with conflicting results; some studies have indicated that cholesterol reduces membrane fluidity (Seres, 2005; Goodwin, 2005) while other studies have indicated that cholesterol increases membrane fluidity (Cooper and Hausman, 2007; Sun, 2007; Garzetti, 1993). FP operates on a nanometer length scale, however, while FRAP operates on a micrometer length scale. Measuring diffusivity with FRAP allows sampling of larger areas of membrane that may incorporate heterogeneity (transmembrane proteins, lipid rafts, etc.). The FRAP length scale also captures the effects of membrane-cytoskeleton adhesion, which heavily influences the probability of tether formation (Sun, 2007). These factors make FRAP a more appropriate choice than FP for assessing membrane fluidity in the context of tether extrusion.

In FRAP experiments, the cell membrane component of interest is labeled with a fluorescent dye, and a focused laser beam is used to photobleach a small region of the cell. Diffusion of unbleached molecules into the bleached region is subsequently imaged with an attenuated excitation laser to obtain a fluorescence recovery curve. This curve can be fit to expected curves based on the diffusion equation to obtain the diffusion coefficient (D) of the component of interest.

FRAP has been used previously to characterize changes in neutrophil membrane lipid behavior, typically in the context of activation. Changes in
membrane mobility in fMLP-stimulated neutrophils in response to anesthetic
treatment have been studied by Hulse et al (1994). Distribution and diffusion of
quiescent vs. activated fMLP receptors has been characterized by Johansson et
in chemoattractant-exposed neutrophils. Preliminary studies from our lab (Oh,
2009) have demonstrated that ethanol and cholesterol may directly influence
diffusivity of membrane lipid in tethers from quiescent neutrophils.

5.2.2 Characterization of neutrophil deformation

Neutrophil deformation in flow has been characterized predominantly with
two different methods. Side-view flow chambers have been used in vivo and in
vitro to study deformation of leukocytes in rat venules and deformation of
neutrophil-like HL-60 cells in P-selectin-coated flow chambers, respectively
(Damiano, 1996; Lei, 1999). These studies indicate that under flow, neutrophils
deform to a tear-drop shape, and that cell-substrate contact length increases with
shear rate, to a plateau aspect ratio of 1.4.

Deformation of neutrophils has been studied previously in our lab using
microcapillary flow chambers coated with neutrophil-sized beads. The beads did
not present any neutrophil-relevant adhesion molecules, so collisions were non-
adhesive. Preliminary studies indicated that cholesterol enrichment, and to a
lesser extent, ethanol exposure, increased neutrophil deformation and collision
lifetime during collisions with beads (Oh, 2009).
5.3 Results

5.3.1 Whole-cell deformation variability with shear rate

5.3.1.1 Non-adhesive beads

To examine how neutrophil deformability varies over a range of venous shear rates, we perfused neutrophils into glass capillary flow chambers presenting adherent 10 µm beads absent of adhesion molecules for neutrophils. That the beads were sized similarly to neutrophils allowed us to examine whole-cell deformability, avoiding neutrophil phagocytic behavior with smaller beads, or flow disturbances from larger beads. As seen in Figure 5.1, there was a trend of increased deformation with increased wall shear rate, ranging from no deformation at 25 s\(^{-1}\) (or negative deformation, an artifact of variation in neutrophil and bead sizes) to roughly 2 µm of deformation at 200 s\(^{-1}\). Major differences were typically not evident over increments of 25 s\(^{-1}\), but were more apparent over increments of 50 s\(^{-1}\) or greater. Deformation appeared to be relatively uniform over all collision angles.

The maximum deformation at each wall shear rate can be found in Table 5.1. It is evident that the shear force imparted to the neutrophil at 25 s\(^{-1}\) is insufficient to induce any deformation. The maximum deformation at 50 s\(^{-1}\), 0.38 ± 0.45 µm, suggests microvillus compression rather than cell-body deformation, since the average lengths of microvilli on the neutrophil surface are roughly 0.2 to 0.3 µm (Park, 2002). Deformation at 50 s\(^{-1}\) was in this range over many collision
angles, though some collision angles exhibited negligible to slightly negative deformation. The maximum deformation at 75 s$^{-1}$ was more noticeable, at 0.95 ± 0.85 µm. This value suggests whole-cell deformation instead of microvillus compression, and was statistically different from deformation at 25 s$^{-1}$ ($p<0.05$).

Deformation increased in a dose-dependent manner with shear rate, to a maximum of 2.20 ± 0.54 µm at 200 s$^{-1}$. As in Figure 5.1, we found that significant changes in maximum deformation were seen over shear rate increments of 50 s$^{-1}$ or greater. This is consistent with previous data from our lab on neutrophil adhesion and tethering that also did not reflect significant changes over 25 s$^{-1}$ shear rate increments (Kadash, 2004; Oh and Diamond, 2008).

Table 5.1. Change in maximum neutrophil deformation with wall shear rate for collisions with non-adhesive beads. Values are mean ± SD. N = 5 donors; n = 54 neutrophils for 25 s$^{-1}$, 43 for 50 s$^{-1}$, 44 for 75 s$^{-1}$, 52 for 100 s$^{-1}$, 47 for 125 s$^{-1}$, and 87 for 200 s$^{-1}$. ‡ = $p<0.05$ relative to 25 s$^{-1}$, † = $p<0.001$ relative to 25 s$^{-1}$, * = $p<0.001$ relative to 50 s$^{-1}$, ‡‡ = $p<0.005$ relative to 50 s$^{-1}$, †† = $p<0.01$ relative to 75 s$^{-1}$, ** = $p<0.01$ relative to 100 s$^{-1}$.

<table>
<thead>
<tr>
<th>Wall Shear Rate</th>
<th>Maximum Deformation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 s$^{-1}$</td>
<td>-0.01 ± 0.57</td>
</tr>
<tr>
<td>50 s$^{-1}$</td>
<td>0.38 ± 0.45</td>
</tr>
<tr>
<td>75 s$^{-1}$</td>
<td>0.95 ± 0.85‡</td>
</tr>
<tr>
<td>100 s$^{-1}$</td>
<td>1.40 ± 0.56†‡</td>
</tr>
<tr>
<td>125 s$^{-1}$</td>
<td>1.78 ± 0.93†‡‡</td>
</tr>
<tr>
<td>200 s$^{-1}$</td>
<td>2.20 ± 0.54†‡‡‡</td>
</tr>
</tbody>
</table>
Figure 5.1. Changes in neutrophil whole-cell deformation with wall shear rate for collisions with non-adhesive beads, over a range of collision angles. Data for each shear rate were binned into 10-degree increments, with the mean angle for each increment represented on the plot. Horizontal error bars provide the standard deviation in degrees for each bin. Vertical error bars indicate the standard deviation in cell deformation for each binned angle. N = 5 donors; n = 54 neutrophils for 25 s$^{-1}$, 43 for 50 s$^{-1}$, 44 for 75 s$^{-1}$, 52 for 100 s$^{-1}$, 47 for 125 s$^{-1}$, and 87 for 200 s$^{-1}$. 
5.3.1.2 P-selectin-coated beads

Our next step was to determine whether or not P-selectin played a role in whole-cell deformability, by perfusing neutrophils into flow chambers presenting 10 µm P-selectin-coated beads. We then assessed neutrophil deformation over the same range of wall shear rates used in the non-adhesive bead experiments. These data can be found in Figure 5.2. Neutrophil deformation upon collision with P-selectin-coated beads followed the same trend as deformation upon collision with non-adhesive beads—increasing with increased shear rate. Deformation ranged from negligible to slightly negative at 25 s⁻¹ to 2.5 µm at 200 s⁻¹. In contrast to the non-adhesive bead experiments, deformation appeared to peak for most shear rates for collisions in the 75-85 degree range, and decrease from that range for larger or smaller collision angles.

Maximum deformation at each shear rate for neutrophils colliding with P-selectin-coated beads can be found in Table 5.2. Similar to experiments with non-adhesive beads, the maximum deformation for neutrophils colliding with P-selectin-coated beads at 25 s⁻¹ was negligible, and the maximum deformation at 50 s⁻¹, 0.20 ± 0.37 µm, appeared to entail microvillus compression rather than whole-cell deformation. The transition to whole-cell deformation appeared to occur around 75 s⁻¹ for collisions with P-selectin-coated beads as well, with a maximum deformation of 0.83 ± 0.43 µm. Maximum deformation increased concomitantly with shear rate to a maximum of 2.43 ± 0.48 µm at 200 s⁻¹. It is
interesting to note that collisions with P-selectin-coated beads produced less variable neutrophil deformations than collisions with non-adhesive beads.
Figure 5.2. Changes in neutrophil whole-cell deformation with wall shear rate for collisions with P-selectin-coated beads, over a range of collision angles. Data for each shear rate were binned into 10-degree increments, with the mean angle for each increment represented on the plot. Horizontal error bars provide the standard deviation in degrees for each bin. Vertical error bars indicate the standard deviation in cell deformation for each binned angle. N = 5 donors; n = 56 neutrophils for 25 s\(^{-1}\), 74 for 50 s\(^{-1}\), 66 for 75 s\(^{-1}\), 46 for 100 s\(^{-1}\), 51 for 125 s\(^{-1}\), and 41 for 200 s\(^{-1}\).
Despite differences in deformation dependence on collision angle and reduced variability in maximum deformations for neutrophil collisions with P-selectin-coated beads, collisions with non-adhesive beads and P-selectin-coated beads appeared to produce deformations of very similar magnitude. This similarity is evident in Figure 5.3, where maximum deformations for non-adhesive and P-selectin-coated bead collisions are compared. The values are very similar, with no statistically significant differences between bead conditions at any wall shear rate condition. A linear fit was applied to these data to obtain a predictive

**Table 5.2. Change in maximum neutrophil deformation with shear rate for collisions with P-selectin-coated beads.** Values are mean ± SD. N = 5 donors; n = 56 neutrophils for 25 s\(^{-1}\), 74 for 50 s\(^{-1}\), 66 for 75 s\(^{-1}\), 46 for 100 s\(^{-1}\), 51 for 125 s\(^{-1}\), and 41 for 200 s\(^{-1}\). ‡ = p<0.001 relative to 25 s\(^{-1}\), † = p<0.01 relative to 50 s\(^{-1}\), * = p<0.001 relative to 50 s\(^{-1}\), ‡‡ = p<0.05 relative to 75 s\(^{-1}\), †† = p<0.005 relative to 75 s\(^{-1}\), ** = p<0.001 relative to 75 s\(^{-1}\), ‡‡‡ = p<0.005 relative to 100 s\(^{-1}\), ††† = p<0.005 relative to 125 s\(^{-1}\).

<table>
<thead>
<tr>
<th>Wall Shear Rate</th>
<th>Maximum Deformation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 s(^{-1})</td>
<td>0.07 ± 0.54</td>
</tr>
<tr>
<td>50 s(^{-1})</td>
<td>0.20 ± 0.37</td>
</tr>
<tr>
<td>75 s(^{-1})</td>
<td>0.83 ± 0.43‡†</td>
</tr>
<tr>
<td>100 s(^{-1})</td>
<td>1.31 ± 0.53‡‡‡</td>
</tr>
<tr>
<td>125 s(^{-1})</td>
<td>1.55 ± 0.47‡‡‡</td>
</tr>
<tr>
<td>200 s(^{-1})</td>
<td>2.43 ± 0.48‡‡‡‡‡‡</td>
</tr>
</tbody>
</table>
relationship between wall shear rate and maximum deformation. For collisions with non-adhesive beads, this relationship was \( \dot{\text{def}} = 0.0120 \gamma \), with a \( R^2 \) value of 0.914. For collisions with P-selectin-coated beads, this relationship was 

\( \dot{\text{def}} = 0.0119 \gamma \), with \( R^2 \) value of 0.941. It appears, then, that deformation increases with shear rate in a linear fashion. Data from Oh et al 2009 shows that contact area increases with increased deformation, though the relationship is not linear. The relationship between deformation and contact area is also complicated by microvillus compression during deformation, and heterogeneous distribution of adhesion molecules between microvilli and the cell body (creating a concept of “functional contact area” containing relevant adhesion molecules).

From a coarse-grained perspective, however, increased shear rate increases neutrophil deformation to the same extent in the presence or absence of P-selectin, which results in increased contact area between neutrophil and substrate.
Figure 5.3. Maximum neutrophil deformation vs. wall shear rate for non-adhesive and P-selectin-coated beads. Data are ± SD. No significant differences in maximum deformation between non-adherent and P-selectin-coated beads for any shear rates. Differences among shear rates within a specific bead condition are given in Tables 5.1 and 5.2. N = 5 donors; for non-adhesive beads, n = 54 neutrophils for 25 s⁻¹, 43 for 50 s⁻¹, 44 for 75 s⁻¹, 52 for 100 s⁻¹, 47 for 125 s⁻¹ and 87 for 200 s⁻¹. For P-selectin-coated beads, n = 56 neutrophils for 25 s⁻¹, 74 for 50 s⁻¹, 66 for 75 s⁻¹, 46 for 100 s⁻¹, 51 for 125 s⁻¹, and 41 for 200 s⁻¹.
5.3.2 Whole-cell deformation variability with ethanol treatment and membrane cholesterol manipulation

In light of preliminary data from Oh 2009, and intrigued by results from rolling and adhesion experiments with combined ethanol and cholesterol treatment, we decided to assess neutrophil whole-cell deformation over a range of ethanol treatments and cholesterol manipulation regimes, using the 10 µm bead collision experiment, with non-adhesive beads. Figure 5.4 shows that cholesterol removal with MβCD reduced neutrophil deformation relative to control; as these experiments were performed at the same wall shear rate, 100 s⁻¹, we can infer that this reduction in deformation entails a reduction in neutrophil compliance. Deformation increased in a dose dependent manner for both ethanol administration and cholesterol enrichment. Deformation appeared to have a slight dependence on collision angle, with most conditions experiencing maximum deformation around 95 degrees.

Maximum deformation for each condition can be found in Table 5.3. Both cholesterol depletion and cholesterol enrichment had significant effects on maximum neutrophil deformation, decreasing (p<0.05) and increasing (p<0.05 for 8:1 and p<0.001 for 4:1) deformation, respectively. While deformation increased with cholesterol enrichment dose, the effect was not statistically significant. Ethanol also increased maximum neutrophil deformation significantly (p<0.05) over control, at each concentration. Deformation only marginally increased from 0.1% to 0.3% ethanol, however. Cholesterol induced greater maximum
deformation than ethanol, in accordance with preliminary data from Oh 2009, but this difference was not statistically significant, indicating that both reagents are important contributors to neutrophil compliance.

Table 5.3. Change in maximum neutrophil deformation with ethanol administration and membrane cholesterol manipulation for collisions with non-adhesive beads. Values are mean ± SD. N = 3 donors; n = 49 neutrophils for control, 43 for 0.1% EtOH, 32 for 0.3% EtOH, 40 for 4:1 MBCD:chol, 60 for 8:1 MBCD:chol, and 53 for 5 mM MBCD. ‡ = p<0.05 relative to control, † = p<0.001 relative to control.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximum Deformation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.38 ± 0.57</td>
</tr>
<tr>
<td>5 mM MβCD</td>
<td>0.98 ± 0.56‡</td>
</tr>
<tr>
<td>0.1% EtOH</td>
<td>1.87 ± 0.48‡</td>
</tr>
<tr>
<td>0.3% EtOH</td>
<td>1.92 ± 0.58‡</td>
</tr>
<tr>
<td>8:1 MβCD:chol</td>
<td>2.00 ± 0.78‡</td>
</tr>
<tr>
<td>4:1 MβCD:chol</td>
<td>2.27 ± 0.52†</td>
</tr>
</tbody>
</table>
Figure 5.4. Changes in neutrophil whole-cell deformation with ethanol treatment (0.1% and 0.3%) and cholesterol manipulation (removal with 5 mM MβCD and enrichment with 8:1 and 4:1 MβCD:cholesterol molar ratio complexes) for collisions with non-adhesive beads. Data for each shear rate were binned into 10-degree increments, with the mean angle for each increment on the plot. Horizontal error bars provide the SD in degrees for each bin. Vertical error bars indicate the SD in deformation for each binned angle. $\gamma = 100 \text{ s}^{-1}$. N = 3 donors; n = 49 neutrophils for control, 43 for 0.1% EtOH, 32 for 0.3% EtOH, 40 for 4:1 MBCD:chol, 60 for 8:1 MBCD:chol, and 53 for 5 mM MBCD.
5.3.3 Effect of ethanol treatment and manipulation of membrane cholesterol on lipid diffusivity

To help elucidate results from our tethering experiments, we performed FRAP experiments on neutrophils exposed to ethanol and membrane cholesterol manipulation regimes, separately. By labeling the neutrophils with a lipid analogue dye (DiO) we were able to assess lipid diffusivity (expressed by the lipid diffusion coefficient) with the FRAP experiments. In our experiments, we bleached a circular spot 4 µm in diameter on the neutrophil cell body. We investigated diffusivity within the neutrophil cell body because preliminary studies from our lab had addressed lipid diffusivity in neutrophil membrane tethers, and found that ethanol treatment and cholesterol manipulation had significant impact on diffusivity relative to control treatment (Oh, 2009). Since, as discussed previously, membrane tether lipid must necessarily derive from the neutrophil cell body (Needham and Nunn, 1990), we decided to explore cell body lipid diffusivity, as a way of sampling a much larger, heterogeneous region of neutrophil membrane lipid.

We found that membrane cholesterol manipulation affected neutrophil cell body lipid diffusivity in a dose-dependent manner (Table 5.4). Cholesterol depletion with MβCD decreased diffusivity by 42% relative to control ($p<0.05$), enrichment with 8:1 MβCD:cholesterol increased diffusivity by 37% over control (NS), and enrichment with 4:1 MβCD:cholesterol increased diffusivity by 74% ($p<0.05$). Our findings with cholesterol depletion differ from those in Oh et al.
2009, but these experiments probed the more heterogeneous cell body, rather than the membrane lipid tether, which is relatively homogenous in composition. Our findings concerning cholesterol enrichment concur, however. These results are consistent with our data showing increased membrane tether length and growth velocity in neutrophils enriched in cholesterol, and they support the idea that those tether metric changes were brought about by cholesterol increasing neutrophil membrane fluidity.

Dose-dependent effects on neutrophil cell body lipid diffusivity were also seen with ethanol treatment; 0.1% ethanol increased the lipid diffusion coefficient 21% over control (NS) while 0.3% ethanol increased diffusivity by 53% ($p<0.05$).

Table 5.4. Change in membrane lipid diffusivity with ethanol administration and membrane cholesterol manipulation. Values are mean ± SD. N = 3 donors; n = 8 neutrophils for control, 7 for 0.1% EtOH, 6 for 0.3% EtOH, 6 for 4:1 MβCD:chol, 6 for 8:1 MβCD:chol, and 9 for 5 mM MβCD. ‡ = $p<0.05$ relative to control.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diffusion coefficient (μm²/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.19 ± 0.08</td>
</tr>
<tr>
<td>5 mM MβCD</td>
<td>0.11 ± 0.05‡</td>
</tr>
<tr>
<td>0.1% EtOH</td>
<td>0.23 ± 0.08</td>
</tr>
<tr>
<td>0.3% EtOH</td>
<td>0.29 ± 0.05‡</td>
</tr>
<tr>
<td>8:1 MβCD:chol</td>
<td>0.26 ± 0.12</td>
</tr>
<tr>
<td>4:1 MβCD:chol</td>
<td>0.33 ± 0.09‡</td>
</tr>
</tbody>
</table>

The fact that ethanol increased neutrophil cell body lipid diffusivity sheds light on the nature of the additive effect of cholesterol and ethanol on membrane
tethering metrics. Additionally, our finding that cholesterol enrichment in isolation had more significant effects on tethering metrics than ethanol in isolation may be explained by the smaller increase in cell body diffusivity observed with 0.3% ethanol treatment vs. 4:1 MβCD:cholesterol enrichment.

The data from Table 5.4 are from FRAP experiments with the microscope objective focused on the cell equator. Since DiO is a membrane-localized dye, this imaging protocol resulted in low-intensity images. Since the double normalization process scales fluorescence recovery intensity from zero to one, however, it was hypothesized that this protocol did not impact the measurement of lipid diffusivity. To confirm this hypothesis, additional FRAP experiments were performed with the microscope objective focused near the top of the neutrophil. These data are in Table 5.5. Diffusion coefficients were nearly identical for each imaging condition, indicating that the imaging plane did not impact the measured lipid diffusivity.

Table 5.5. Membrane lipid diffusivity vs. measurement plane. Values are mean ± SD. n = 8 neutrophils for each condition. NS = not significant.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Diffusion coefficient (μm²/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, imaged at neutrophil equator</td>
<td>0.19 ± 0.08</td>
</tr>
<tr>
<td>Control, imaged near to of neutrophil</td>
<td>0.19 ± 0.07 (NS)</td>
</tr>
</tbody>
</table>

We also performed experiments varying bleach spot size to determine if incomplete fluorescence recovery was a result of an immobile fraction of...
membrane lipid (confined to lipid rafts, or connected to membrane cytoskeleton), or depletion of total membrane fluorophore. These data are in Table 5.6.

Table 5.6. Membrane lipid mobile fraction vs. bleach spot size. Values are mean ± SD. n = 4 neutrophils for 2 µm, 2 neutrophils for 3 µm and 2 neutrophils for 4 µm.

<table>
<thead>
<tr>
<th>Bleach spot size</th>
<th>Mobile fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µm</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td>3 µm</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>4 µm</td>
<td>0.53 ± 0.10</td>
</tr>
</tbody>
</table>

There was no discernible trend in mobile fraction with bleach spot size. If bleaching was depleting total fluorophore, then smaller bleach spots would have yielded larger mobile fractions, since there would have been more unbleached fluorophore available for diffusion. Since that trend was not seen, it appears that a real immobile fraction exists in neutrophils.

5.4 Discussion

To get a sense of the scale of neutrophil deformation under venous flow, we assessed neutrophil collisions with non-adhesive and P-selectin-coated beads adherent to microcapillary flow chambers. Collisions were observed over a venous wall shear rate range of 25 s⁻¹ to 200 s⁻¹, from pathologically low to intermediate shear (Goel and Diamond, 2002). Collisions at higher shear rates were outside of the imaging capability of our video system. For non-adhesive
bead collisions, neutrophil deformation increased gradually with shear rate, as would be expected, since greater shear force is applied to the neutrophils at increased shear rates. Significant differences between shear rates were only seen over increments of 50 s$^{-1}$ or greater. There was a clear demarcation, however, between shear rates considered to be pathologically low (25 and 50 s$^{-1}$), and shear rates considered to be “healthy” (≥100 s$^{-1}$).

The magnitude of neutrophil deformation for adhesive collisions was very similar to the magnitude of deformation for non-adhesive collisions. This makes sense, as P-selectin is involved in initial capture of neutrophils from flow and transient adhesion during rolling, rather than supporting spreading and migration, processes governed by integrins. Neutrophils colliding with P-selectin-coated beads did appear to be more sensitive to shear rate changes, however. It appears that the presence of P-selectin did not support additional deformation, but did reduce variability in deformation. As with non-adhesive bead collisions, there is a demarcation between low and normal shear rates, but with less variable deformations with adhesive bead collisions, finer distinctions can be made among the normal shear rates, with significant differences evident over smaller shear rate increments. Additionally, adhesive collision deformation exhibited angular dependence, which was not evident for non-adhesive collisions. It appears that though P-selectin may not alter the extent of deformation during collisions, it is necessary to provide specificity for neutrophils
to be able to respond sensitively to both shear rate magnitude and shear force direction changes.

We next exposed neutrophils to ethanol and manipulated neutrophil membrane cholesterol in separate bead collision experiments at a single wall shear rate of 100 s⁻¹, to assess ethanol and cholesterol’s effects on neutrophil whole cell deformation and compliance. Ethanol at 0.1% and 0.3% significantly enhanced neutrophil deformation, with little difference between the two concentrations. It is interesting that ethanol increased neutrophil deformation significantly, yet increased (destabilized) neutrophil rolling velocity and variability of velocity in P-selectin-coated surface perfusion assays (Furlow and Diamond, 2011) and decreased neutrophil rolling flux on P-selectin and firm arrest on activated endothelial cells (Oh and Diamond, 2008). This same study found that ethanol redistributed PSGL-1 from neutrophil microvilli to the cell body, however. So, despite increased neutrophil deformation and contact area with substrate with ethanol treatment, PSGL-1 on the neutrophil surface could be less accessible to P-selectin on the substrate (P-selectin-coated or endothelium). This would reduce the number of adhesions during rolling, increasing rolling velocity and flux, and reducing the likelihood of conversion to firm arrest (a process initiated by selectin-binding and/or proximity brought about by selectin-binding to soluble activating factors) (Ley, 2007).

The ethanol concentrations used in this study, while physiologically obtainable, bracket a range from too intoxicated to legally operate a vehicle to
potentially physically harmful intoxication (Oh and Diamond, 2008). That ethanol’s effect on neutrophil deformation and compliance appeared to stagnate at these high concentrations suggests that concentrations lower than 0.1% and more readily physiologically obtained could potentially increase neutrophil deformation to a significant extent. Ethanol at 0.02% (slightly less than one alcoholic beverage) has been shown to inhibit platelet adhesion to collagen-coated glass (Owens, 1990) so it is conceivable that similar concentrations could have other effects on vascular physiology.

Manipulation of membrane cholesterol also significantly impacted neutrophil deformation, from a 40% decrease from control with cholesterol depletion to a 64% increase with 4:1 MβCD:cholesterol enrichment. While this increase was larger than the increase afforded by ethanol, the difference was not statistically significant. These changes in deformation and compliance could partially explain previous results from rolling and adhesion studies with cholesterol-depleted and -enriched neutrophils. In P-selectin-coated surface perfusion studies, cholesterol depletion increased neutrophil rolling velocity and variance of rolling velocity, and decreased conversion to firm arrest on activated endothelium (Oh, 2009); cholesterol enrichment had opposite effects. Cholesterol manipulation did not significantly alter neutrophil integrin expression assessed via flow cytometry, however, and cholesterol enrichment actually decreased neutrophil PSGL-1 expression (which would theoretically result in decreased adhesion) (Furlow and Diamond, 2011). Thus, changes in adhesion molecule
expression cannot explain changes in neutrophil rolling and adhesion behavior brought about by membrane cholesterol manipulation. These results could be partially explained, however, in light of the effects of cholesterol manipulation on neutrophil compliance, however. Cholesterol-depleted neutrophils are less compliant than untreated neutrophils, which results in decreased contact area with substrate and accordingly, fewer bonding opportunities. Less compliant neutrophils also project higher into the vascular flow field, and thus experience greater hydrodynamic drag force and increased force loading of adhesive bonds with the substrate. Cholesterol-enriched neutrophils are more compliant than untreated neutrophils, and thus see increased contact area with substrate and reduced hydrodynamic drag. With increased bonding opportunities with the substrate and less force on each neutrophil-substrate bond, it is clear how cholesterol-enriched neutrophils experience slower and smoother rolling on P-selectin and increased conversion to firm arrest on activated endothelium.

The extrusion of neutrophil membrane tethers, like neutrophil compliance, can significantly impact neutrophil rolling behavior independent of altered adhesion molecule expression. Tether growth can alter bond force by changing the moment arm of the force on the bond, as evidenced by the following force and torque balances for neutrophils tethering to microbeads:

\[ F_s = F_B \cos(\theta) \quad 5.1 \]

\[ F_B \sin(\theta) \cdot l = F_s \cdot R + T_s \quad 5.2 \]
where $F_s$ is the shear force on the cell, $F_B$ is the bond force, and $\theta$ is the angle between the top of the microbead and point of tether extrusion from the neutrophil (Shao, 1998). Since shear force and shear torque remain constant for constant shear rate and neutrophil and microbead dimensions (Goldman, 1967; Oh and Diamond, 2008), it is clear that as the length of the bond force moment arm increases, the bond force decreases.

Since neutrophil membrane tether lipid is derived from the cell body (Needham and Nunn, 1990) we hypothesized that changes in cell body membrane fluidity (represented by changes in lipid diffusivity, measured by a lipid diffusion coefficient D) could be responsible for ethanol and cholesterol-derived changes in neutrophil tether length, lifetime, and growth velocity. Ethanol at 0.3% and cholesterol enrichment at a 4:1 MβCD:cholesterol molar ratio both significantly ($p<0.05$) increased lipid diffusivity over control by 53% and 74%, respectively. Lipid diffusivity did not appear to change with focal plane. These results provide a molecular-level mechanism for observed changes in neutrophil tethering metrics with ethanol treatment and cholesterol enrichment; as both agents increase membrane fluidity, their effects in isolation on heterotypic tethering metrics (Oh and Diamond, 2008; Oh, 2009) and additive effects on heterotypic and homotypic tether metrics (Furlow and Diamond, 2011) make sense. In contrast, cholesterol depletion with MβCD decreased lipid diffusivity 42% relative to control ($p<0.05$). This reduction in membrane fluidity explains reductions in tether length, lifetime, and growth velocity seen with cholesterol
depletion (Oh, 2009). Ranging from \(0.11 \pm 0.05 \mu m/sec^2\) to \(0.33 \pm 0.09 \mu m/sec^2\), diffusion coefficient values from this study fall within the range of diffusion coefficients for lipid vesicles and biological membranes defined in Soumpasis, 1983. We also found that neutrophil membrane lipid mobile fraction did not vary significantly with FRAP bleach spot size, indicating that incomplete fluorescence recovery was likely a result of a real immobile fraction, rather than depletion of total membrane fluorophore. Membrane lipid diffusion can be hindered by the presence of lipid rafts, and lipid-cytoskeleton adhesion (Sun, 2007).

Interestingly, previous FRAP studies have found that cholesterol depletion actually increased neutrophil membrane lipid diffusivity (Oh, 2009). Those studies were performed on neutrophil membrane tethers, however, which are relatively homogenous in composition (neutrophil membrane lipid) compared to the neutrophil cell body, which contains transmembrane proteins and lipid rafts. Since MβCD decreases neutrophil tethering fraction and cholesterol increases tether fraction (Oh, 2009) it is conceivable that tethers drawn for FRAP analysis from cholesterol-depleted cells may have originated from regions of locally high membrane cholesterol content, since those regions would be more likely to permit tether extrusion. The lipid diffusivity studies from this work sample a larger, more heterogeneous region of membrane lipid, and thus are more likely to provide an accurate picture of diffusivity in cholesterol-depleted neutrophils.
6.1 Conclusions

We investigated the effects of ethanol on cholesterol-enriched neutrophil adhesion, tethering, and rolling behavior, as compared to ethanol and cholesterol treatment in isolation, and control. Membrane cholesterol levels were assessed for neutrophils exposed to ethanol, enriched in cholesterol, or exposed to sequential ethanol and cholesterol treatment. Neutrophils exposed to these different incubation regimes were examined with several different microcapillary flow chamber assays. Chambers incubated with P-selectin-coated beads were used to probe selectin-based adhesion and tethering behavior. P-selectin-coated microcapillary chambers were used to assess neutrophil rolling velocity and standard deviation of velocity. We used ICAM-1-coated flow chambers to examine neutrophil primary, homotypic adhesion and tethering, as well as secondary, integrin-mediated adhesion.

Our results show that neutrophil behavior is affected in a complex manner when ethanol and cholesterol enrichment are combined. Ethanol reduced the efficacy of cholesterol enrichment when administrated before or after loading, possibly by binding with and removing cholesterol from the neutrophil membrane. Order of administration of ethanol and cholesterol appeared to affect cholesterol loading, but not tethering, rolling, or adhesion metrics. When administered in conjunction with cholesterol, ethanol appeared to control neutrophil adhesion, both to P-selectin-coated beads and pre-adhered neutrophils, while cholesterol
controlled tethering. Together, ethanol and cholesterol generally increased tethering metrics like length, lifetime, and growth velocity, associated with increased membrane fluidity and decreased membrane viscosity. Ethanol also appeared to “rescue” cholesterol-enriched neutrophils from slower, smoother rolling associated with a more adhesion-prone state, back to “healthy” control rolling behavior. The effects of ethanol and cholesterol together can be attributed to alterations in membrane fluidity and viscosity and redistribution of PSGL-1. These findings provide valuable insight into how ethanol and cholesterol might affect neutrophil adhesion, rolling, and tethering behavior when both are present in vivo.

To elucidate the complex results from our combined administration studies, we investigated the separate effects of ethanol and cholesterol in a dose-dependent manner on neutrophil whole cell deformation and membrane lipid diffusivity (as a measure of membrane fluidity), factors thought to influence neutrophil rolling and adhesion. Neutrophil whole cell deformation (and by extension, compliance) was assessed with perfusion experiments using microcapillary chambers presenting 10 µm beads adhered to the chamber surface, absent of neutrophil-relevant adhesion molecules. We also characterized deformation over a range of venous wall shear rates with the same assay using non-adhesive and P-selectin-coated 10 µm beads. Membrane lipid diffusivity was determined by performing FRAP experiments on neutrophil cell
bodies to obtain lipid diffusion coefficients via nonlinear regression of fluorescence recovery data.

We found that shear rate increased neutrophil whole cell deformation in a dose-dependent manner for collisions with both non-adhesive and P-selectin-coated beads, with very similar magnitudes of deformation for each bead condition. Collisions with P-selectin-coated beads produced less variable deformations with angular dependence, however, indicating that P-selectin potentially allows neutrophils to respond more sensitively to shear force magnitude and direction changes. In constant shear rate experiments, ethanol and cholesterol each increased neutrophil deformation in a dose-dependent manner, indicating increased compliance. That ethanol increased neutrophil compliance but had anti-adhesive and rolling de-stabilization effects on both untreated and cholesterol-enriched neutrophils indicates that the redistribution of PSGL-1 from microvilli to the cell body by ethanol has an even more significant impact on neutrophil rolling and adhesion behavior. Our findings that membrane cholesterol depletion decreases, and enrichment increases neutrophil compliance help explain how cholesterol manipulation influences neutrophil rolling and adhesion without concomitant changes in adhesion molecule expression.

From FRAP experiments, we found that ethanol administration and cholesterol enrichment both increased membrane lipid diffusivity. This explains previous observations showing increased membrane tether length, lifetime, and
growth velocity from application of these reagents, and sheds light on their 
additive effects on tethering metrics when administered in concert. We also found 
that cholesterol depletion decreased membrane lipid diffusivity, which helps 
explain observed reductions in tethering metrics for cholesterol-depleted 
neutrophils. This result conflicted with previous neutrophil FRAP studies, but that 
conflict was a result of studying different portions of the neutrophil (cell body vs. 
membrane tether) with different microscale compositions.

In summary, we have shown that ethanol and cholesterol interact in a 
complex manner to significantly alter neutrophil tethering, rolling, and adhesion 
behavior through a balance of biomechanical and biochemical factors. 
Cholesterol enrichment of neutrophils resulted in longer, faster growing 
membrane tethers (a result of increased lipid diffusivity) and increased whole-cell 
compliance. These mechanical factors stabilized neutrophil rolling on P-selectin 
and increased adhesion in the absence of associated changes in adhesion 
molecule expression. Ethanol, despite increasing membrane fluidity as measured 
by lipid diffusivity and tethering metrics, and increasing whole cell compliance, 
actually destabilized rolling in cholesterol-enriched neutrophils and decreased 
adhesion. These surprising findings can be explained by redistribution of PSGL-1 
from the neutrophil microvilli to the cell body after ethanol treatment. A decrease 
in PSGL-1 molecules on microvilli would decrease the probability of tethering 
events, resulting in faster, more jumpy translation during neutrophil rolling, and 
decreased conversion to firm arrest. These studies provide multiscale insight into
changes in neutrophil membrane mechanics and adhesive properties in the presence of ethanol and cholesterol, which are often present simultaneously in Western diets. These changes have relevance to the innate immune response and the pathophysiology of conditions like hypercholesterolemia and atherosclerosis.

6.2 Suggestions for future studies

6.2.1 Characterize the effects of moderate alcohol consumption on modulation of neutrophil tethering, rolling, and adhesion by acute dietary cholesterol.

Our results have demonstrated that ethanol and cholesterol have significant and complex effects on in vitro neutrophil function. A natural extension of this work would be to examine ethanol's effects on neutrophils enriched with dietary cholesterol in ex vivo studies. Post-prandial high cholesterol meal studies have been undertaken previously in our lab, examining tethering metrics and rolling and adhesive behavior. Tether length, rolling flux, and percent firm arrest were all significantly increased in high cholesterol post-prandial neutrophils (Oh, 2009). Epidemiological studies establishing beneficial cardiovascular effects of moderate ethanol intake, and our own in vitro results demonstrating that ethanol significantly impacts cholesterol's effects for acute administration of both reagents, indicate together that moderate ethanol intake during a high-cholesterol meal could noticeably alter post-prandial, cholesterol-enriched
neutrophil behavior. Since high cholesterol post-prandial neutrophils exhibited similar cholesterol enrichment and adhesive behavior to neutrophils from hypercholesterolemic patients (Oh, 2009) these studies could shed light on how hypercholesterolemic patients respond to alcohol intake. Since ethanol was shown \textit{in vitro} to mitigate some of cholesterol’s pro-adhesive effects on neutrophils, it would also be instructive to learn if those effects carried over to dietary ethanol and cholesterol interactions.

\textbf{6.2.2 Characterization of neutrophil-substrate contact area with Total Internal Reflectance Fluorescence Microscopy}

Since P-selectin’s bonding partner in capture, tethering, and rolling processes, PSGL-1, is localized to neutrophil microvilli, not all of the neutrophil membrane surface area is “functional” in terms of engaging with activated endothelium and other neutrophils. Unfortunately, the methods discussed in this work—whole cell deformation via bead collision, and side-view flow chambers, only provide indirect measures of functional contact area. It is logical that functional contact area scales monotonically with normal contact area, since microvilli are isotropically distributed on the neutrophil surface, but the ability to examine a neutrophil at its contact point with the surface would provide a significantly more accurate picture of functional contact area.

The most recent development in characterizing neutrophil-substrate contact area under flow involves the use of total internal reflectance fluorescence microscopy (TIRFM). In TIRFM, an excitation laser beam is reflected into the
junction between two media of different refractive indices (glass and cytosol, for instance) at an angle determined by the refractive indices of the two media, such that the beam undergoes total internal reflection within the medium of higher refractive index. This causes an evanescent excitation wave into the medium of lower refractive index that decays 100-200 nm into the medium. This allows visualization of features in contact or close association with the substrate, with little background interference from features out of plane. This technique was used by Sundd et al (2010) to visualize neutrophils tethering and rolling from the perspective of the substrate, with resolution sufficient to identify individual microvilli and tether anchorage points. This method could be employed to more accurately assess changes in neutrophil microvilli-substrate contact area with changes in shear rate, or contact area changes at constant shear rate for neutrophils exposed to ethanol or membrane cholesterol manipulation.

6.2.3 Computational simulation of neutrophil homotypic tethering

Leukocyte homotypic tethering, or “secondary capture,” mediated by L-selectin to PSGL-1 bonds, is a physiological strategy by which leukocytes not expressing ligands for E-selectin and P-selectin may reach sites of inflammation (Ley, 2007). However, secondary capture may lead to pathological aggregation of leukocytes, particularly neutrophils (Kadash, 2004). This process is influenced both by neutrophil compliance (homotypic tethering involves neutrophil collisions) and neutrophil membrane fluidity (tethers may be drawn from one or both of the neutrophils in the homotypic binding pair). We endeavored to characterize the
effects of ethanol administration and membrane cholesterol manipulation on the secondary capture process, but were stymied by the complex nature of tethering between two deformable, fluid objects, which raised issues unable to be resolved by our imaging system.

A computational approach to this process would facilitate the characterization of neutrophil homotypic tethering. Bagchi et al. developed a 3D model in 2008 that permits independent variation of tether viscosity and whole-cell deformability. This model treats the neutrophil as a liquid drop enclosed in an elastic membrane. The membrane follows the neo-Hookean law, and microvilli are modeled as viscoelastic strings. Receptor-ligand binding are simulated with the adhesive dynamics model (Hammer and Apte, 1992; Chang, 2000) and bond dynamics are handled by the Dembo model (Dembo, 1988). This method has been used to characterize the separate effects of ethanol and cholesterol on neutrophil tethering to P-selectin-coated microbeads (Oh, 2009). The model allowed for detailed studies of individual tethers from neutrophils with varied deformability and tether viscosity properties. With some adaptations, it could describe neutrophil homotypic tethering dynamics, and with extensions to the adhesive dynamics module, potentially describe secondary adhesion to ICAM-1-coated surfaces after homotypic tether rupture, and subsequent neutrophil string formation and aggregation (Kadash, 2004).
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