

Interplay between membrane cholesterol and ethanol differentially regulates neutrophil tether mechanics and rolling dynamics

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Received 3 January 2011

Accepted in revised form 25 March 2011

Abstract. Using microfluidic assays at a 100 s^{-1} wall shear rate, 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). Using methyl- β -cyclodextrin:cholesterol complexes, membrane cholesterol was increased over control by 4.6-fold (no ethanol), 3.6-fold (0.3% ethanol pre-loading), and 1.6-fold (0.3% ethanol post-loading). These treatments did not alter CD11b expression; however, PSGL-1 and L-selectin were lowered by cholesterol enrichment (\pm 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.

Keywords: Neutrophil membrane, membrane fluidity, cell adhesion, atherosclerosis, bead collision assay

1. Introduction

Neutrophils are important mediators of late-stage atherosclerotic plaque stability. Linear strings of firmly-arrested neutrophils have been found *in vivo* on mouse atherosclerotic plaques [14]. Neutrophil activation is a prerequisite for firm arrest and primes neutrophils to release enzymes like matrix metalloproteinases and elastase which can break down interstitial matrix and promote plaque instability [21]. Accordingly, neutrophils have been found in ruptured human plaques.

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The neutrophil adhesion cascade can be classified into three steps that precede diapedesis: capture via tethering, rolling and firm adhesion. Capture and rolling are mediated by selectins (L-selectin, constitutively expressed on neutrophil microvilli, and P-selectin, expressed on activated platelets and endothelial cells) and their counter-ligands (for the purposes of this study, PSGL-1, also constitutively expressed on neutrophil microvilli) [9,34]. Neutrophil homotypic tethering is controlled by PSGL-1 to L-selectin bonds, while neutrophil tethering and rolling on activated endothelium is mediated by PSGL-1 and P-selectin. Neutrophil rolling dynamics on activated endothelium are influenced largely by the number of force-shielding tethers pulled from the neutrophil membrane and the tether mechanics, and shear-induced cellular deformation, which increases contact area and accordingly, the number of adhesion molecules presented [17,25,29].

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 [1,8,11]. Firm adhesion of neutrophils to activated endothelium is controlled by the β_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–ICAM-3 and Mac-1–Mac-1 [19,32].

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 [26]. Similarly, neutrophils from hypercholesterolemic patients display increased adherence to human umbilical vein endothelial cells [30].

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 [27]. 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 [20]. In large doses, however, ethanol appears to increase the risk of cardiovascular disease [15].

Alteration of cell membrane cholesterol has been shown to have profound effects on cell membrane mechanics. Cholesterol depletion increases membrane stiffness in endothelial cells [2], and decreases lipid diffusivity [31]. Conversely, cholesterol enrichment increases lipid diffusivity in neutrophils, along with whole-cell deformability [24]. Cholesterol enrichment also decreases membrane surface viscosity in endothelial cells and decreases membrane–cytoskeleton adhesion energy [31]. Cholesterol depletion decreases neutrophil tethering to P-selectin-coated beads, along with tether length, lifetime, and growth velocity [24]. 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 [24]. Lipid diffusivity is significantly increased in neutrophils treated with ethanol [22]. Ethanol decreases neutrophil adhesion efficiency to P-selectin-coated beads, and decreases the likelihood of tethering [23]. 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 [16,20]. 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 [27]. Ethanol can form complexes with cholesterol [4] and may promote removal of cholesterol from cells [10]. 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.

2. Materials and 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 ($\text{M}\beta\text{CD}$; 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 h, in accordance with Institutional Review Board approval. Neutrophils were isolated by separation with Polymorphprep separation medium (Accurate Chemical) as previously described [14]. 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 $(1-2) \times 10^6$ cells/ml.

2.3. Membrane cholesterol enrichment and depletion

$\text{M}\beta\text{CD}$ was dissolved in RPMI1640 medium without phenol red or serum at a concentration of 5 mM. Neutrophils were depleted of cholesterol by 60 min incubation with $\text{M}\beta\text{CD}$ solution. Cholesterol enrichment was accomplished with $\text{M}\beta\text{CD}$ /cholesterol complexes at 8:1 and 4:1 molar ratios, as previously described [18]. Briefly, cholesterol stock solution was added to a glass scintillation vial, the solvent was evaporated, and an appropriate volume of $\text{M}\beta\text{CD}$ solution was added. The vial was sonicated for 20 min at 37°C and then placed in a shaking incubator overnight at 37°C.

2.4. Quantification of cholesterol levels

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 min, as controls for each time point, 0.3% ethanol by volume for 30 min, 5 mM M β CD solution for 60 min, 8:1 or 4:1 M β CD:cholesterol solutions for 60 min, 0.3% ethanol for 30 min before or after 5 mM M β CD solution for 60 min, and 0.3% ethanol for 30 min before or after 8:1 or 4:1 M β CD:cholesterol solutions for 60 min) 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 μ l aliquots into a black, polystyrene, 96-well tissue culture plate (Whatman) and 25 μ l of Amplex Red working solution (300 μ M 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 min 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. 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 non-specific 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 min, 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 [33].

2.6. Neutrophil-microbead collision 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- μ m-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 [5,28]. Rectangular glass capillaries (Vitrocom) with dimensions 0.2 \times 2.0 \times 70 mm and a wall thickness of 0.15 mm were used as flow chambers. P-selectin-coated beads (1.7×10^4 P-selectin/bead) were washed and incubated overnight for attachment to glass capillary flow chambers for a final concentration of 5400 P-selectin/ μ m². 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 [23].

2.7. P-selectin-coated surface perfusion assay

P-selectin-coated surfaces were prepared by incubating microcapillary flow chambers with recombinant human P-selectin (R&D Systems) in HBSS without Ca²⁺ or Mg²⁺ at a concentration of 1 μ g/ml for at least 3 h at room temperature. Excess unbound protein was removed by perfusing HBSS without Ca²⁺

or Mg^{2+} with 2% HSA through the chamber for 30 min. Under these conditions, the final P-selectin density was determined to be 10 sites/ μm^2 [29]. A wall shear rate of 100 s^{-1} was used for these experiments as well.

2.8. 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. 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 $\mu\text{g}/\text{ml}$ for at least 2 h 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 min. Neutrophils were perfused into the chambers using a syringe pump and allowed to adhere for 10 min under static conditions. Flow was then resumed at a wall shear rate of 100 s^{-1} , and primary and secondary neutrophil interactions were observed.

2.9. 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: $\gamma_w = 6Q/B^2W$. In this equation, Q represents the flow rate (cm^3/s), B is the total plate separation for the rectangular flow chamber and W represents the width of the chamber. For the microcapillary chamber, $B = 0.02 \text{ cm}$ and $W = 0.2 \text{ cm}$. For this aspect ratio of 10, the parallel plate approximation is suitable, yielding the previously-mentioned expression for wall shear rate. Flow studies were performed at a flow rate of 80 $\mu\text{l}/\text{min}$, corresponding to a wall shear rate of 100 s^{-1} , in the venous range.

2.10. Imaging and video analysis

Microcapillary flow chambers were imaged with an inverted microscope (Zeiss Axiovert 135). For P-selectin-coated bead and ICAM-1-coated surface secondary adhesion experiments, differential interference contrast (DIC) microscopy was used with a 63 \times objective (Plan Apochromat) while for P-selectin-coated surface experiments, phase contrast microscopy was used with a 20 \times objective (Plan Apochromat). Experiments were recorded using a JVC Professional Series Super VHS VCR and Sony Trinitron connected to a central processing unit/microscope/image processor system [13]. Video was captured at 28 frames per second (fps) and digitized using Windows Movie Maker (Microsoft). ImageJ software (National Institutes of Health) with the MTrackJ particle tracking plugin was used to analyze neutrophil tethering and rolling behavior.

The following definitions were used for analysis of P-selectin-coated bead experiments (adapted from [5]). 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 (ϵ), 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.

2.11. 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.

3. Results

3.1. Membrane cholesterol enrichment and depletion

An M β CD:cholesterol ratio of 4:1 resulted in a 4.6-fold ($p < 0.001$) increase in membrane cholesterol over control (Fig. 1A) whereas an 8:1 ratio elicited a 5.6-fold increase (data not shown). This increase is more than that observed in humans where cholesterol increases of 45% (postprandial) or 73% (hypercholesterolemic patients) have been measured [22]. 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 M β CD to cholesterol was chosen for further study because of the higher cholesterol enrichment seen in conjunction with ethanol treatment. M β CD 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).

3.2. Effect of ethanol and cholesterol on cell 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 1B 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

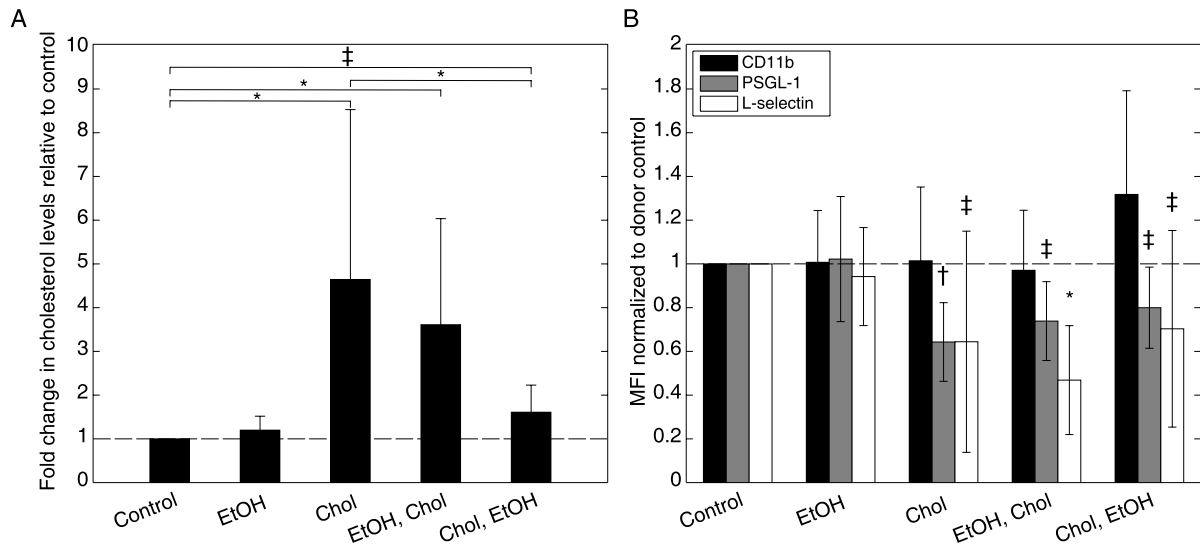


Fig. 1. Neutrophil cholesterol enrichment in conjunction with ethanol exposure (A) and mean fluorescence intensity (MFI) of FITC-conjugated anti-CD11b, PE-conjugated anti-PSGL-1, and Alexa Fluor 647-conjugated anti-L-selectin for neutrophils undergoing ethanol treatment, cholesterol loading and combined administration of ethanol and cholesterol (B). Cholesterol enrichment was accomplished by incubation with $M\beta CD$ /cholesterol complexes for 60 min. Cells were treated with ethanol by incubation in 0.3% solution for 30 min. Cholesterol and fluorescence levels normalized to control; data are presented \pm SD. $N \geq 4$ for (A), $N \geq 5$ for (B). ‡ $p < 0.05$, † $p < 0.01$, * $p < 0.001$ relative to control. Membrane cholesterol in control neutrophils was 3.3 ± 0.9 nmol/ 10^6 cells.

molecules. This is consistent with previous data from our lab [23]. 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 [1,8]. 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.

3.3. Effect of ethanol and cholesterol on neutrophil adhesion and tethering to P-selectin-coated microbeads

As seen previously [23], ethanol significantly ($p < 0.05$) decreased both neutrophil adhesion efficiency and tethering fraction relative to control (Fig. 2). In contrast, cholesterol enrichment increased ($p < 0.05$) adhesion efficiency and tethering fraction, also previously observed [24]. Ethanol significantly reduced cholesterol-enriched neutrophil adhesion efficiency relative to cholesterol enrichment alone by 47% ($p < 0.05$) when preceding cholesterol enrichment and 40% ($p < 0.05$) when following cholesterol enrichment. Ethanol did not have a significant effect on cholesterol-enriched neutrophil membrane tethering fraction relative to cholesterol treatment alone, however.

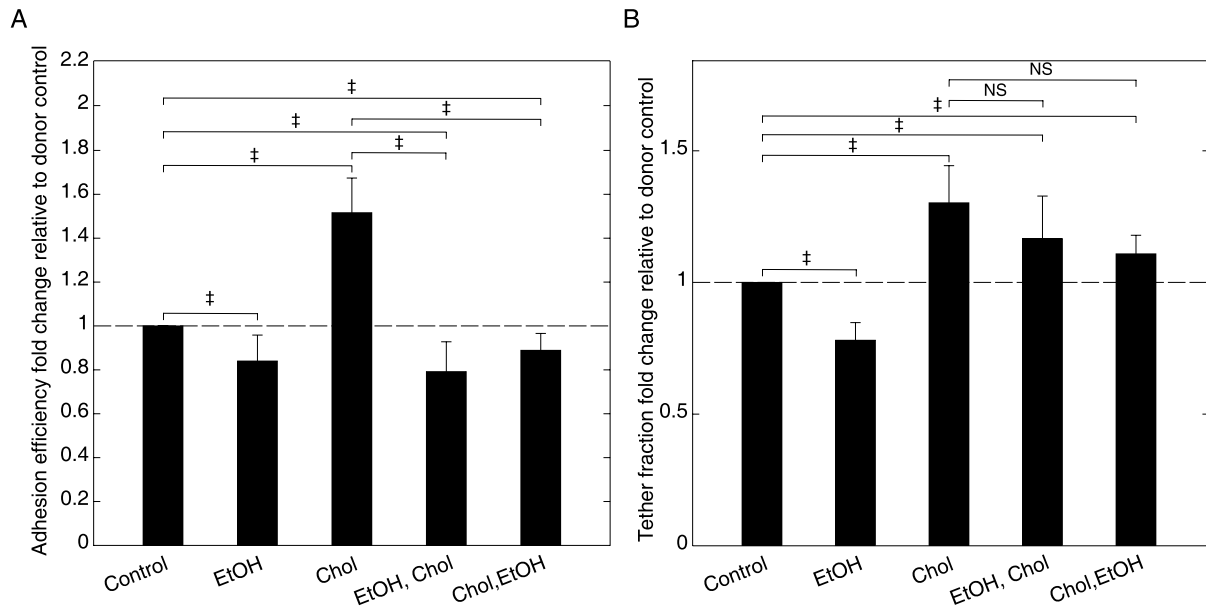


Fig. 2. Effect of cholesterol enrichment, ethanol, and combined administration on neutrophil adhesion efficiency (A) and tethering fraction (B) to P-selectin-coated microbeads. $\gamma = 100 \text{ s}^{-1}$. Values are mean \pm SD. ‡ $p < 0.05$, NS = not significant. $N = 5$ donors. Adhesion efficiency for control cells was 0.115 ± 0.014 . Tether fraction for control cells was 0.452 ± 0.025 .

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 (Fig. 3A), demonstrating that ethanol can enhance cholesterol's effect on neutrophil membrane fluidity. 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 (Fig. 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 (Fig. 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).

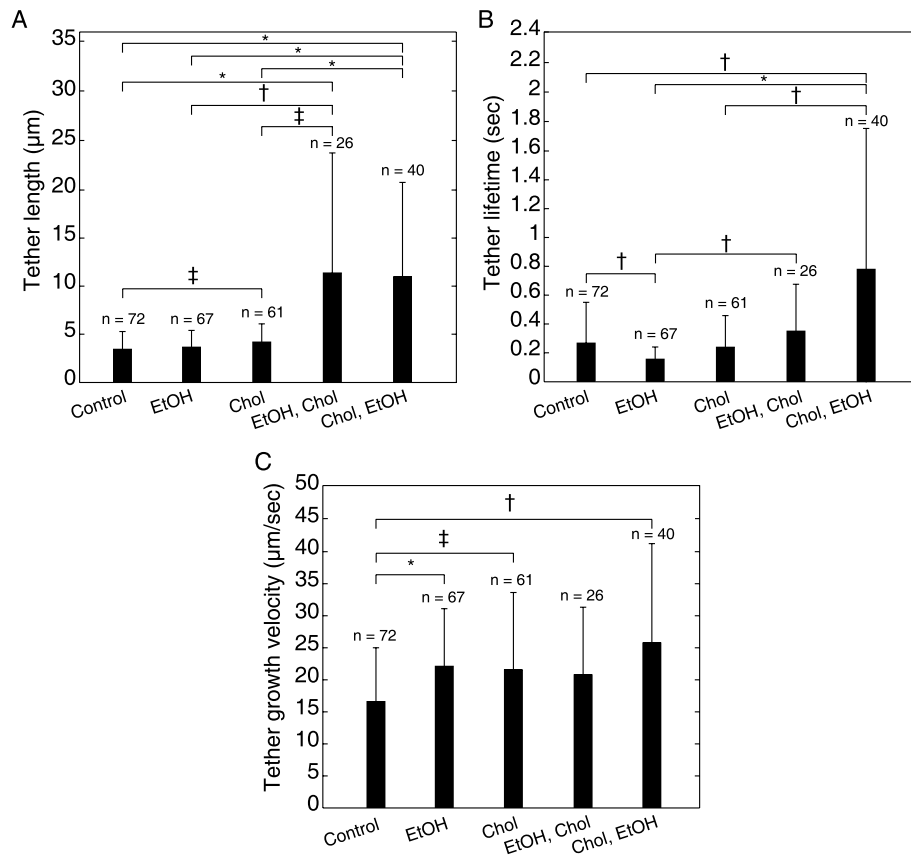


Fig. 3. Effect of cholesterol enrichment, ethanol and combined administration on neutrophil to P-selectin bead tether length (A), tether lifetime (B) and tether growth velocity (C). $\gamma = 100 \text{ s}^{-1}$. Values are mean \pm SD. ‡ $p < 0.05$, † $p < 0.01$, * $p < 0.001$. $N = 5$ donors.

3.4. 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 [23,24]. 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 (Fig. 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 devia-

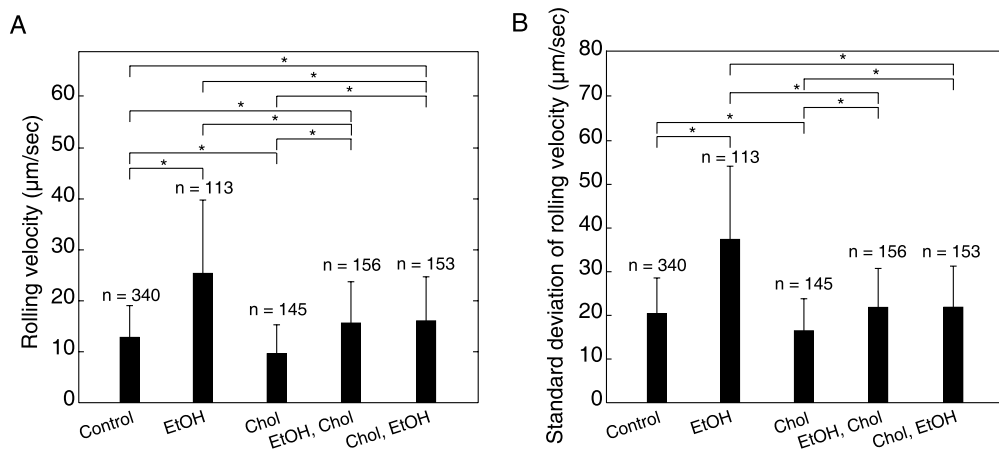


Fig. 4. Effect of cholesterol enrichment, ethanol, and combined administration on neutrophil rolling velocity (A) and standard deviation of rolling velocity (B) on a uniformly-coated P-selectin surface. $\gamma = 100 \text{ s}^{-1}$. Values are mean \pm SD. * $p < 0.001$. $N = 4$ donors.

tion relative to ethanol treatment, combined treatment significantly increased standard deviation over cholesterol enrichment by 30% ($p < 0.001$).

3.5. Effect of ethanol and cholesterol on neutrophil primary and secondary adhesion to pre-adherent neutrophils

Neutrophils exposed to ethanol experienced a significant decrease in homotypic adhesion efficiency relative to control (Fig. 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 (Fig. 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 (Fig. 5C) was not significantly altered by any treatments. Since none of the treatments significantly altered β_2 integrin expression (Fig. 1B) which mediates ICAM-1 adhesion, this was not surprising.

Cholesterol enrichment significantly increased homotypic tether length compared to control (Fig. 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 (Fig. 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

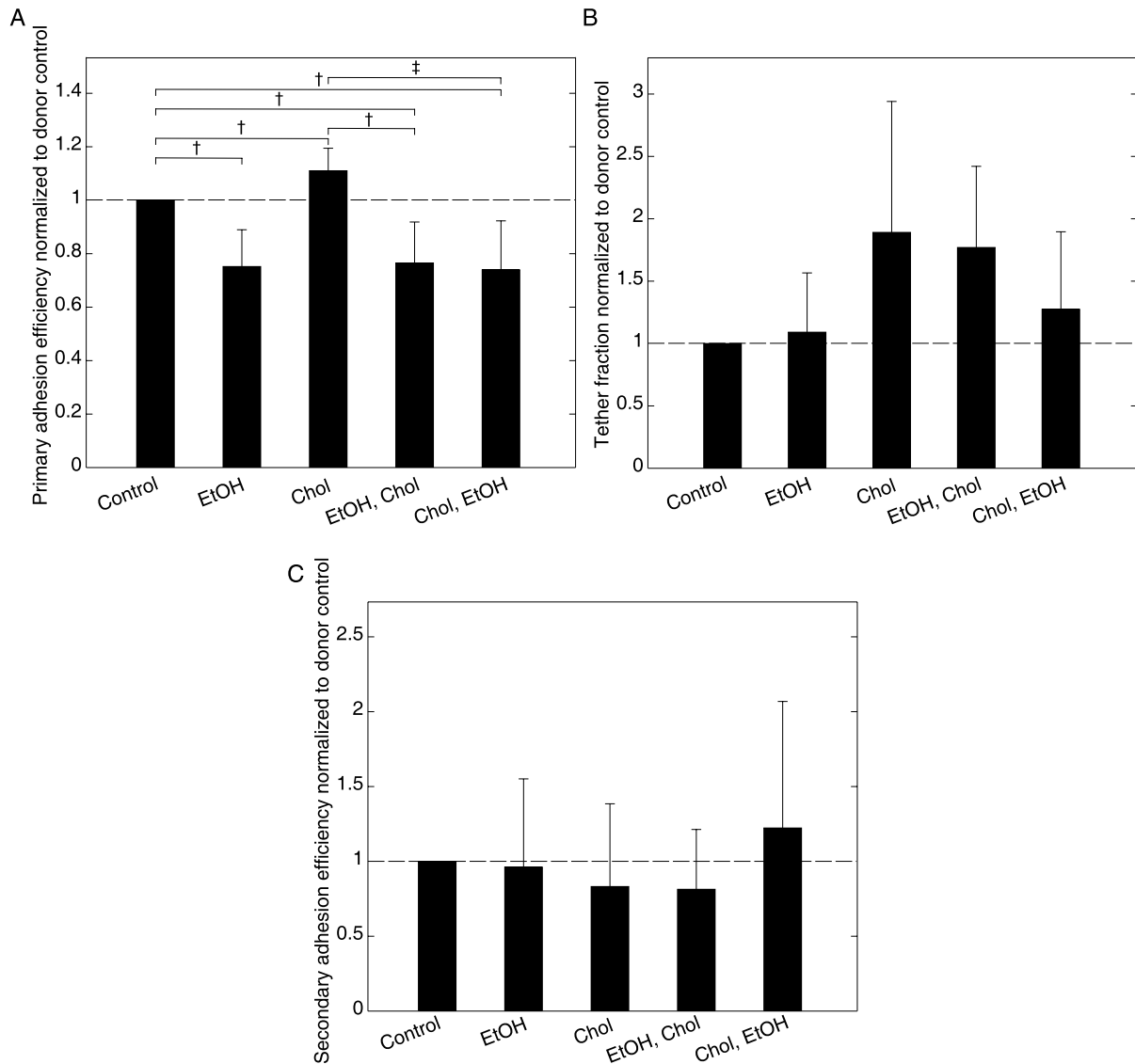


Fig. 5. Effect of cholesterol enrichment, ethanol, and combined administration on neutrophil homotypic adhesion efficiency (A) homotypic tethering fraction (B) and secondary adhesion efficiency to ICAM-1 (C). $\gamma = 100 \text{ s}^{-1}$. Values are mean \pm SD. ‡ $p < 0.05$, † $p < 0.01$. $N \geq 4$ 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 .

concert to affect neutrophil secondary adhesion lifetime (Fig. 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 (Fig. 6D) where there were no significant differences between any groups.

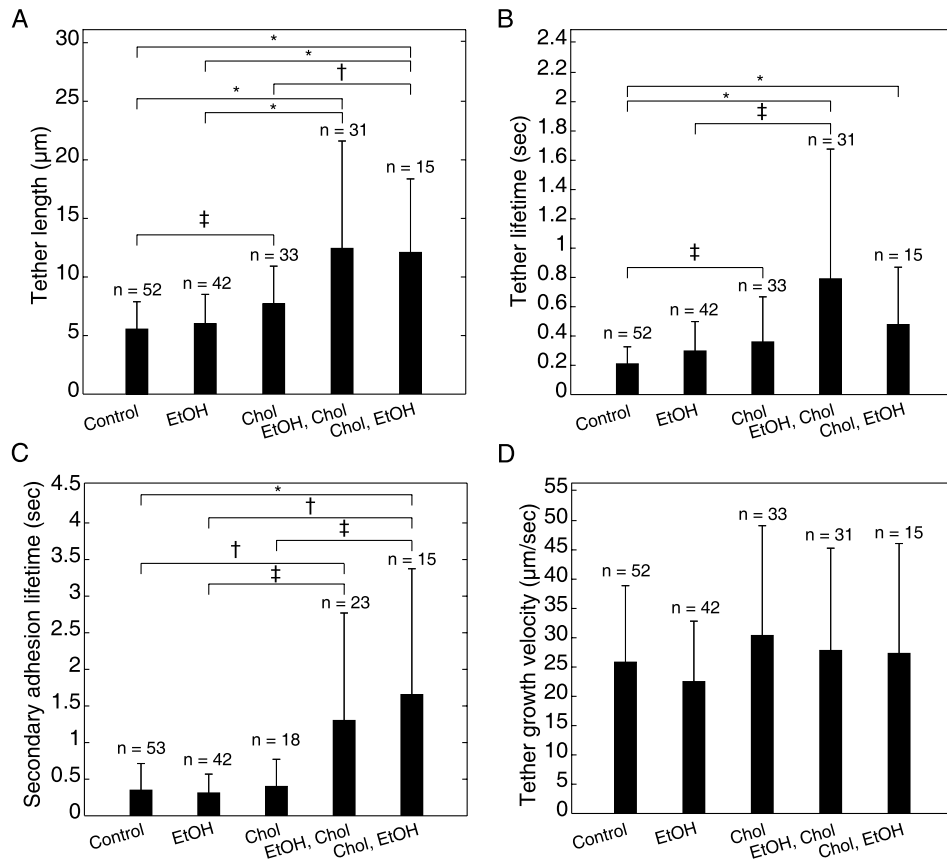


Fig. 6. Effect of cholesterol, ethanol, and combined administration on neutrophil homotypic tether length (A) tether lifetime (B) tether growth velocity (C) and secondary adhesion lifetime to ICAM-1 (D). $\gamma = 100 \text{ s}^{-1}$. Values are mean \pm SD. ‡ $p < 0.05$, † $p < 0.01$, * $p < 0.001$. $N \geq 4$ donors.

4. Discussion

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.

Neutrophil membrane cholesterol levels were successfully elevated by $M\beta CD$:cholesterol complexes. A statistically significant increase in cholesterol of 4.6-fold over control was seen with 4:1 $M\beta CD$: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 en-

richment increased membrane cholesterol only 1.6-fold. These results are consistent with previous data asserting that ethanol can promote cholesterol removal from cell membranes [10]. It has been shown that ethanol and cholesterol can bind in a 1:1 ratio [4] and it is conceivable that this binding is related to the cholesterol-removing effects of ethanol. In the ethanol, then cholesterol treatment regime, M β CD-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.

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 [23] 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 [9,34]. 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 β_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 [23]. On the other hand, cholesterol increases the likelihood for membrane-cytoskeleton dissociation by decreasing membrane-cytoskeleton adhesion energy [31]. 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 [3,6,24,31]. 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 [7], 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 neutrophil 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 [23], 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 [24], 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 [12]. 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 by 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.

In summary, 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 administered 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 primarily 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*.

Acknowledgement

This work was supported by National Institutes of Health Grant HL-103419.

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