Shear-Induced Resistance to Neutrophil Activation via the Formyl Peptide Receptor

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SUPPORTING MATERIAL

Reagents

FITC-conjugated mouse anti-human CD62L specific for human L-selectin, FITC-conjugated mouse IgG1 isotype control antibody, APC-conjugated mouse IgG2b isotype control antibody, APC-conjugated mouse anti-human CD181 specific for CXCR1, APC-conjugated mouse IgG1 isotype control antibody, and APC-conjugated mouse anti-human CD182 specific for CXCR2 were purchased from BD Biosciences (San Jose, CA). PE-conjugated anti-human CBRM1/5 and PE-conjugated mouse IgG1 isotype control antibody were purchased from Biolegend (San Diego, CA). Primary goat anti-human FPR, which binds to the extracellular epitope of FPR, and FITC secondary donkey anti-goat IgG antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Formyl-methionyl-leucyl-phenylalanine (fMLP) and interleukin-8 (IL-8) were purchased from R&D Systems (Minneapolis, MN). Ca^{2+} and Mg^{2+} free DPBS (Invitrogen, Carlsbad, CA), Ca^{2+} and Mg^{2+} free HBSS (Invitrogen), endotoxin-free human serum albumin (Sigma Aldrich, St. Louis, MO), calcium carbonate (Sigma Aldrich), endotoxin-free water (MO BIO Laboratories, Carlsbad, CA), and low endotoxin (1 ng/mg), essentially γ-globulin-free BSA (Sigma-Aldrich) were purchased to make buffer solutions for neutrophil isolation and viscometer assays. Tumor necrosis factor α (TNF-α) protease inhibitor-0 (TAPI-0; Peptides International, Louisville, KY), 1,10-phenanthroline (1,10-Ph; Sigma Aldrich), and GM6001 (Chemicon, Temecula, CA) were purchased for protease inhibition studies. Quantum simply cellular (QSC) anti-mouse IgG beads were purchased from Bangs Laboratories, Inc. (Fisher, IN).

Neutrophil Isolation

Human peripheral blood was obtained via venipuncture from healthy consenting blood donors and collected into sterile sodium heparin-containing tubes (BD Biosciences) after informed consent. Neutrophils were then isolated by centrifugation at 480 X *g* for 50 min at 23°C in a Marathon 8K centrifuge (Fisher Scientific, Pittsburgh, PA) using 1-Step™ Polymorphs (Accurate Chemical & Scientific Corporation, Westbury, NY). This centrifugation method creates a density gradient to separate blood into visible layers of plasma, mononuclear cells, neutrophils, and erythrocytes and platelets. The neutrophils were extracted and washed in Mg^{2+} and Ca^{2+} free HBSS to remove any remaining polymorph. Any remaining red blood cells in the suspension were lysed hypotonically. Neutrophils were resuspended at a concentration of 0.5 x 10^6 cells/mL in HBSS containing 0.5% HSA, 2 mM Ca^{2+}, 1 mM Mg^{2+}, and 10 mM HEPES (Invitrogen), buffered to pH 7.4. For protease inhibition studies, neutrophils were incubated with 25 μM GM6001, 5 mM 1,10-Ph, or 35 μM TAPI-0 for 30 min prior to the onset of shear.

Cone-and-Plate Viscometer Assay

To study the shear stress response of neutrophils in a controlled, uniform environment, experiments were performed using a cone-and-plate device consisting of a stationary plate beneath a rotating cone (0.8° angle) maintained at 23°C or 37°C by a circulating water bath

(Brookfield, Middleboro, MA). The cone-and-plate viscometer design allows for a uniform shear rate to be applied to the entire sample. The shear rate, G, does not depend on the distance from the cone center, and is given by:

$$G = \frac{\omega}{\tan\theta}$$

where ω is the angular velocity of the cone (rad/s) and θ is the angle of the cone (rad). A laminar flow field is expected for all experimental conditions. Under these conditions for a Newtonian fluid, the shear stress, τ, is proportional to the shear rate being applied:

$$\tau = \mu G$$

where μ is the viscosity of the medium. Prior to the experiments, the stationary plate and rotating cone were incubated with 5% BSA at room temperature for 1 hour to block non-specific adhesion of neutrophils to the cone-and-plate surfaces. Neutrophil suspensions of 500 μL were placed on the plate at a concentration of 0.5 x 10^6 cells/mL, and were allowed to equilibrate for 1 min before the onset of shear. Shear stress was varied from 0.1-4.0 dyn/cm^2 for 1-120 min in duration. To maintain a constant shear rate while increasing the shear stress, the medium viscosity was increased by adding varying amounts of ultra-high molecular weight dextran polymer to the medium (2 x 10^6 MW; Sigma Aldrich) of neutrophils under both shear and static conditions. Increasing the shear rate alone caused neutrophils to collide more frequently and activate (Fig. S1), most likely due to L-selectin binding to P-selectin glycoprotein ligand-1 (PSGL-1) (1). After shearing, aliquots of neutrophils were immediately exposed to 0.5 nM fMLP, 5 nM IL-8, or no chemoattractant for a period of 10 min. The 10 min incubation period was chosen to observe a measurable amount of L-selectin shedding and $\alpha_M\beta_2$ integrin activation, given the low concentrations of chemoattractants that were utilized.

Flow Cytometry

After chemoattractant exposure, both sheared and non-sheared neutrophils were immediately labeled with FITC-conjugated anti-human CD62L monoclonal antibody and PE-conjugated anti-human CBRM1/5 to quantify L-selectin expression and activated CD11b subunits of β_2 integrins. Sheared and non-sheared control samples of neutrophils were labeled with FITC- and PE-conjugated mouse IgG1 isotype antibodies to distinguish non-specific from specific antibody staining. All labeled neutrophil samples were incubated for 30 min at 4°C and then washed twice with cold Ca^{2+} and Mg^{2+} free DPBS. Samples were then analyzed using an Accuri C6 flow cytometer (Accuri Cytometers Incorporated) and flow cytometry plots were created with Accuri CFlow Plus and FCS Express V3 (De Novo Software, Thornhill, Canada) software.

Brightfield Microscopy and Image Analysis

Unlabeled neutrophils were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in Ca^{2+} and Mg^{2+} free DPBS at 4°C for 30 min and then washed twice with cold

Ca^{2+} and Mg^{2+} free DPBS. Cells were then placed on coverglass and imaged by bright field and phase contrast microscopy using an Olympus IX81 inverted microscope (Olympus America Inc., Center Valley, PA). Outlines of neutrophils were created from thresholded images using edge-detection functions in Metamorph (Universal Imaging Corp., West Chester, PA). Changes in neutrophil shape were determined using the 'shape factor' program in Metamorph, where the shape factor is given by:

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

where P is the perimeter and A is the area of the object (neutrophil). Shape factor values close to 1 represent a perfect circle, while values closer to zero represent elongated or ruffled shapes. All shape factor data were imported into Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA).

Surface Receptor Quantification

The average number of FPR, CXCR1, and CXCR2 receptors on the surface of neutrophils was determined using flow cytometry with QSC beads. Beads were incubated for 45 min with a FITC- or APC- conjugated antibody specific to the antigen on the beads. A mixture of beads with varying numbers of antigen binding capacities (ABCs) was run through a flow cytometer. Populations of beads corresponding to increasing numbers of ABCs yield increasingly fluorescent peaks in the FITC or APC fluorescence channel. The median value of each fluorescence peak was obtained using Accuri CFlow Plus software, and was used along with the ABC values reported by the manufacturer to generate a calibration curve using QuickCal v2.3 (Bangs Labs, Fisher, IN).

Immediately following the calibration step, both sheared and non-sheared neutrophils were incubated with primary anti-human FPR for 45 min at 4°C. To examine the number of IL-8 surface receptors, separate sheared and non-sheared neutrophils were labeled with anti-CD181 and anti-CD182 for 30 min at 4°C. All neutrophil samples were washed twice with cold Ca^{2+} and Mg^{2+} free DPBS, and neutrophils labeled with anti-FPR were incubated with an additional secondary IgG-FITC antibody for 30 min at 4°C. All samples were washed and then analyzed using a flow cytometer. The peak fluorescence channel was recorded from each sample, and the peak was converted into the number of receptors using the calibration curve in QuickCal.

Confocal Microscopy

Neutrophils exposed to either shear or static conditions were fixed with 4% paraformaldehyde in Ca^{2+} and Mg^{2+} free DPBS at 4°C for 30 min, and then washed twice with cold Ca^{2+} and Mg^{2+} free DPBS. Neutrophil samples were then distributed onto slides using a Shandon CytoSpin III centrifuge (Shandon, Pittsburgh, PA) at 750 RPM for 5 min. Slides were allowed to dry for 5 min, and were then rehydrated in DPBS. Samples were permeabilized in 0.2% Triton X-100 for

5 min, and then incubated in 1% BSA for 1h. Slides were incubated in primary anti-human FPR for 12 h in a humidified chamber, and then washed twice with 0.2% Tween in DPBS for 5 min each. All samples were incubated with a secondary IgG-FITC antibody for 30 min 4°C, washed twice with 0.2% Tween in DPBS, and mounted onto coverslips using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Samples were examined with a Zeiss 710 Spectral Confocal Microscope System (Carl Zeiss MicroImaging GmbH, Jena, Germany) at 65X magnification with a FITC filter. Both individual and Z-stack images were taken and processed using Zeiss ZEN software (Carl Zeiss MicroImaging GmbH). Metamorph software was used to examine FPR internalization and fluorescence intensities within neutrophils. To measure fluorescence intensity within the cell, the cell membrane was thresholded using edge detection functions to exclude the fluorescent membrane from calculations (Fig. 8*C*). Fluorescent measurements within the cell were then recorded and averaged for 100 neutrophils from each of n=3 donors.

Statistical Analysis

Flow cytometry and shape factor data were plotted and analyzed using Prism 5.0b for Microsoft (GraphPad software, San Diego, CA). A two-tailed paired t-test was used for comparisons between two groups with $p < 0.05$ being considered significant.

Figure S1

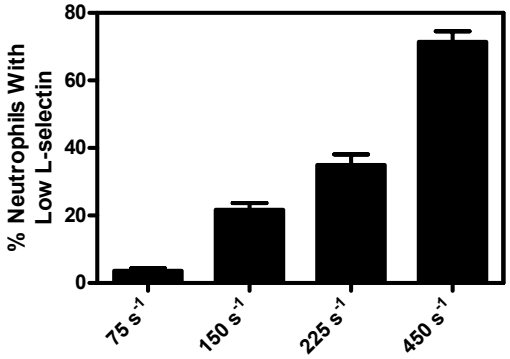

Figure S1: Loss of neutrophil L-selectin under various shear rates. Neutrophils were exposed to shear rates of 75 s^{-1}, 150 s^{-1}, 225 s^{-1}, or 450 s^{-1} in a cone and plate viscometer 2 h at 23°C and were analyzed for L-selectin shedding due to shear alone. n = 3 donors for each shear rate value. Error bars represent 95% confidence intervals.

Figure S2

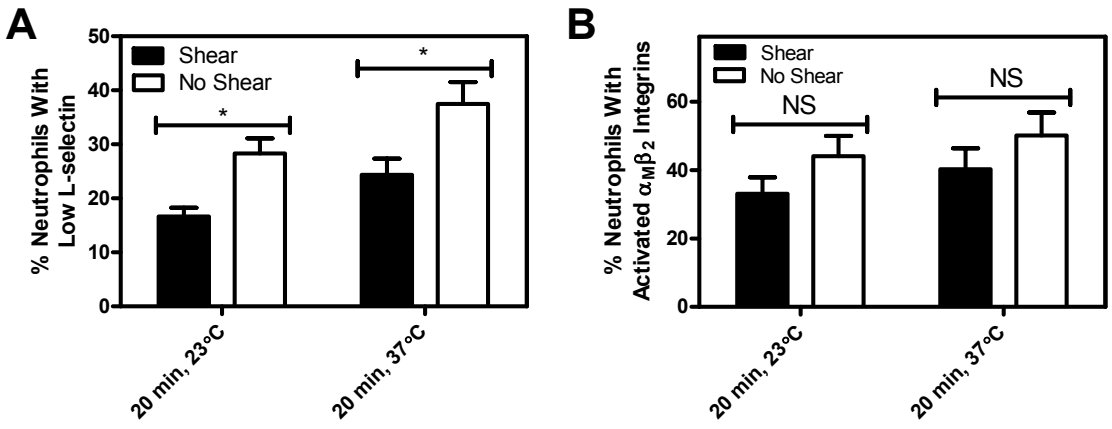

Figure S2: Neutrophil shear-induced resistance to activation at 23°C and 37°C. Neutrophils were exposed to either shear (4.0 dyn/cm^2) or static conditions for 20 min at 23°C or 37°C, and then analyzed for L-selectin shedding (*A*) and $\alpha_M\beta_2$ integrin activation (*B*). A 20 min duration was used to minimize sample evaporation. n = 5 donors for each shear stress value. Error bars represent 95% confidence intervals. *P < 0.05 for all measurements. NS: not significant.

Figure S3

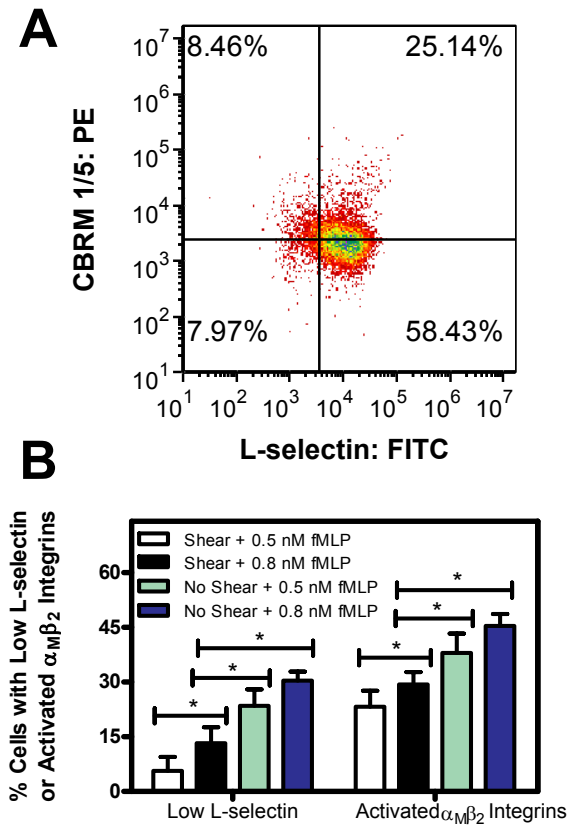

Figure S3: Changes in FPR surface expression contribute to the neutrophil resistance to activation. Sheared neutrophils at 4.0 dyn/cm^2 for 2 h were stimulated with 0.8 nM fMLP (*A*), to approach the C_{eq} of nonsheared neutrophils stimulated with 0.5 nM, based on an assumption of simple monovalent receptor-ligand binding. Nonsheared neutrophils stimulated with 0.5 nM and 0.8 nM fMLP and sheared neutrophils exposed to both 0.5 nM and 0.8 nM fMLP (*B*) were compared for L-selectin shedding and $\alpha_M\beta_2$ integrin activation with n=3 donors. Error bars represent 95% confidence intervals. *P < 0.05 for all measurements.

Supporting References

1. Guyer, D., K. Moore, …, and L. A. Sklar. 1996. P-selectin glycoprotein ligand-1 (PSGL-1) is a ligand for L-selectin in neutrophil aggregation. *Blood*. 88: 2415-2421.