Supporting Information

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SI Materials and Methods

Cell Lines. All cell lines were cultured at 37 °C, containing 5% CO_2 under humidifying conditions. The THP-1 monocytic cell line was purchased from the American Type Culture Collection (LGC Standards) and passaged in RPMI medium containing 10% FCS, 1% antibiotics (penicillin/streptomycin), and 50 μM β-mercaptoethanol. HUVECs were purchased from Lonza and maintained in endothelial cell growth media (EGM-2) supplemented with growth factors and antibiotics provided within their "bullet kits." Cells were initially expanded for six or fewer passages and were harvested and stored in liquid nitrogen for final use in flow assays or Western blotting. Confluent HUVECs were split with trypsin/EDTA solution and seeded onto 10 μg/mL bovine-derived fibronectin. HEK 293T cells were a kind gift from Yolanda Calle, University of Roehampton, London, United Kingdom. Cells were maintained in RPMI-1640 medium supplemented with 10% FCS and 1% antibiotics (penicillin/streptomycin). Cells were routinely passaged at a 1:3 ratio on the third day. Cos-7 cells were maintained in DMEM supplemented with 10% FCS and 1% antibiotics (penicillin/streptomycin). One day before transfection, Cos-7 cells were seeded to reach 70–80% confluence. Cells were then collected from triple-vent 14-cm dishes (Greiner Bio-One) using trypsin/EDTA (Invitrogen) and washed with 5 mL of cold electroporation buffer [120 mm KCl, 10 mm K_2PO_4·KH_2PO_4 (pH 7.6), 25 mm Hepes, 2 mm $MgCl_2$, and 0.5% Ficoll]. Following centrifugation, the trypsinizing solution was aspirated; cells were washed twice in ice-cold PBS and then finally resuspended in 250 μL of cold electroporation buffer and electroporated at 250 V and 960 μF (Bio-Rad electroporator) with 2 μg of DNA for each construct. Ezrin and moesin band 4.1-ezrin-radixin-moesin (FERM) domains and L-selectin constructs were both cloned into pcDNA3.1 vectors (Invitrogen) and have been described in a previous report (1).

Primary Monocyte Isolation. Human peripheral blood mononuclear cells (PBMCs) were isolated from 100 mL of fresh venous blood using Ficoll-Paque (GE Healthcare). Monocytes were enriched from the PBMCs using immunomagnetic beads (Pan Monocyte Isolation Kit; Miltenyi Biotec). Briefly, PBMCs were depleted of nonmonocytic cells, including T cells, natural killer cells, B cells, and basophils, allowing for the selection of an "untouched" whole-monocyte population. Monocyte purity and viability were assessed before further experiments by staining for CD14 and 7-aminoactinomycin D, respectively. Cells were analyzed using a MACSQuant flow cytometer (Miltenyi Biotec). Purity was assessed to be at or above 90%.

Lentiviral Vector Construction. Human L-selectin cDNA was amplified by PCR assay from a pCMV6-AC-GFP vector (OriGene). PCR primers were designed with BamHI and XhoI restriction sites engineered at the 5′ and 3′ ends, respectively. PCR products amplified by Pfu DNA polymerase (Stratagene) were excised from 0.8% agarose gels and purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The pHR′SIN-SEW lentiviral backbone vectors were provided by Adrian Thrasher, Institute of Child Health, University College London, London, United Kingdom, and were carrying either enhanced GFP (hereafter referred to as GFP) or monomeric RFP (hereafter referred to as RFP) C-terminal tags. The lentiviral vectors were linearized by double digestion with BamHI and XhoI restriction enzymes (New England Biolabs) according to the manufacturer's protocol. Digests were resolved on a 0.8% agarose gel, and the vector was purified with a QIAquick Gel Extraction Kit according to the manufacturer's protocol. Purified vectors were dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs) for 1 h at 37 °C and cleaned from the reaction mixture using a QIAquick Gel Extraction Kit according to the manufacturer's protocol. The vectors were then ligated with the insert, with a molar vector-to-insert ratio of 1:3. Ligation was performed using T4 DNA ligase (Promega) in a 10-μL reaction at 4 °C overnight before transformation. Single colonies were picked and cultured for plasmid purification using a QIAprep Miniprep DNA Isolation Kit (Qiagen).

The template vector was further mutated to produce mutant forms of L-selectin via PCR-based mutagenesis kits (QuikChange; Agilent Technologies).

The primers (all written in 5′–3′ format) used in this study include the following:

WT L-selectin (for PCR cloning of human L-selectin)

Forward (Fwd): GAGAGAGGATCCGGTACCGAGGAGA

Reverse (Rev): GAGAGACTCGAGATATGGGTCATTCATACTTCTC

PCR primers for cloning and mutagenesis of L-selectin

Two-step cloning of ΔM-N mutant

ΔMIKE (first step)

Forward: CAAAAGTTTCTCACCCCTCTTCATTC

Rev: GAATGAAGAGGGGTGAGAAACTTTTG

ΔMIKEGDYN (second step)

Fwd: GACAAAAGTTTCTCAGGTGATTATAACCCC

Rev: GGGGTTATAATCACCTGAGAAACTTTTGTC

S364A

Fwd: GATTAAAAAAAGGCAAGAAAGCCAAGAGAAGTATGAATGACC

Rev: GGTCATTCATACTTCTCTTGGCTTTCTTGCCTTTTTTTAATC

S364D

Fwd: GGCAAGGAGATTAAAAAAAGGCAAGAAAGACAAGAGAAGTATGAATGACCCATATCAC

Rev: GTGATATGGGTCATTCATACTTCTCTTGTCTTTCTTGCCTTTTTTTAATCTCCTTGCC

S367A

Fwd: GGCAAGAAATCCAAGAGAGCTATGAATGACCCATATCAC

Rev: GTGATATGGGTCATTCATAGCTCTCTTGGATTTCTTGCC

S367D

Fwd: GGCAAGAAATCCAAGAGAGATATGAATGACCCATATCAC

Rev: GTGATATGGGTCATTCATATCTCTCTTGGATTTCTTGCC

PCR primers designed to clone CaM-RFP

Fwd: GAGAGAGGATCCATGGCTGACCAACTG ACTGAA

Rev: GAGAGACTCGAGCTTTGCTGTCAT CATTTG

Generation of Lentiviral Particles. Lentiviruses were generated in HEK 293T cells through transfection of the psPAX2 (envelope) and pMD.G (packaging) vectors, together with the lentiviral vector. Approximately 10–15 × 10^6 HEK cells were plated into dishes with a diameter of 14 cm. HEK cells were transfected in 4 mL of OPTIMEM (Life Technologies) containing 30, 10, and 40 μg of psPAX2, pMD, and pHR′SIN vector DNA, respectively. Another tube containing 4 mL of OPTIMEM with 1 μL of 10 mM polyethylene imine was then added to the first tube, mixed, and incubated at room temperature for 15 min. Following aspiration of HEK cell media (RPMI-1460 containing 10% FCS and penicillin/streptomycin), the 8-mL transfection mix was added in a drop-wise manner to the HEK cells. After 4 h of incubation, the transfection media were aspirated and replaced with fresh HEK cell media. Cell supernatants containing lentivirus particles were collected 48 h and 72 h after transfection. Both harvests were pooled, filter-sterilized using a 0.45-μm filter (MILLEX GP), concentrated by ultracentrifugation (50,000 × *g*), aliquoted, and stored at −80 °C. Viral titers were established by infecting 80% confluent HEK cells with a series dilution of concentrated virus. FACS analysis of trypsinized HEK cells was performed on day 3 postinfection and used to quantify the percentage of GFP/RFP-positive cells.

Transduction of THP-1 Cells. THP-1 cells were cultured at a density of 0.4 × 10^6 cells per milliliter 1 d before transduction. On the following day, 1 × 10^6 cells were harvested and resuspended in 1 mL of fresh media into which lentivirus particles were added. The volume of concentrated virus that was added per transduction was calculated on the basis of the titer and desired multiplicity of infection (MOI) according to the following equation: (Number of cells transduced) × (MOI)/(Titer) = (Milliliters of virus). The MOIs used were consistent between all cell lines and never exceeded a value of 20 per lentiviral construct. Following infection, cell lines were then placed in a cell culture incubator and fed fresh media 2 d after infection. Four days after transduction, the media were replaced entirely and cells were cultured as normal, maintaining cell densities from 0.5–0.9 × 10^6 cells per milliliter.

FACS Analysis and Cell Sorting. All cells were sorted using either a FACSAria II or MoFlo cell sorter housed within King's College London's Biomedical Research Centre. All cell lines were sorted to match expression levels between WT and mutant L-selectin–expressing cell lines (an example is provided in Fig. S3).

SEM. SEM was performed using an in-house service, and followed similar procedures that have been published by our group (2). In brief, THP-1 cells were allowed to adhere to poly-L-lysine–coated coverslips, fixed in a Karnovsky mixture (3% glutaraldehyde and 1% PFA in 0.08 M sodium cacodylate buffered to pH 7.4 with 0.1 M HCL), rinsed in 0.1 M sodium cacodylate buffer, and osmicated for 1 h in 1% aqueous osmium tetroxide before dehydration in ascending steps of (50–100%) alcohols. Cells were subjected to two 5-min changes of hexamethyldisilazane and were then resuspended in a 500-μL volume. Dried cells were sputter-coated with gold-palladium before imaging. Note that samples were carbon-coated in experiments where immunogold labeling of DREG56 (an anti-human L-selectin monoclonal antibody) was used. Where immunogold labeling of THP-1 cells was used, THP-1 cells stably expressing WT L-selectin–GFP were labeled with primary and secondary antibodies on ice for 30 min before adhesion on to poly-L-lysine–coated coverslips and fixation.

Immunoprecipitation. Cell lysates were generated from 2 × 10^7 THP-1 cells. After pelleting cells by centrifugation at 400 × *g*, cells were lysed in 1 mL of ice-cold lysis buffer [25 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40 (Fluka), 1% ovalbumin, protease inhibitor mixture, and Ser/Thr and Tyr kinase/phosphatase inhibitor mixture] and left to incubate for 15 min on ice. Lysates were centrifuged at 14,000 × *g*, and the supernatant was retained and incubated with 25 μL of pre-equilibrated GFPTrap beads (Chromotek). The lysate/bead mixture was left to rotate at 4 °C overnight. Beads were washed four times in ice-cold lysis buffer containing 0.1% Nonidet P-40, and subsequently resolved on polyacrylamide gels for Western blotting.

Western Blotting. Gels were transferred to a PVDF Immobilon-P (Millipore) transfer membrane, which had been presoaked in methanol and then equilibrated in NuPAGE transfer buffer (Invitrogen), set at a constant rate of 25 V for 2 h. Membranes were blocked in 5% milk buffer in Tris-buffered saline (TBS) containing 0.1% Nonidet P-40 for at least 1 h at room temperature. The primary antibody of interest was added at a dilution of 1:1,000 in blocking solution and incubated at 4 °C with continuous agitation overnight at 4 °C. Membranes were washed in TBS for 5 min and then in 0.1% Nonidet P-40/TBS for 5 min before being washed again in TBS. The membrane was blocked again for at least 60 min at room temperature, after which HRP-conjugated secondary antibody was added at a dilution of 1:5,000 for a further 60 min. For detection of protein in immunoprecipitated samples Trueblot (Rockland) antibodies, which specifically recognize nondenatured antibody, were used to reduce signal generated from immunoprecipitating antibody. For detection of CaM, the PVDF membranes were incubated for 15 min at room temperature in 20 mL of 1% glutaraldehyde in PBS before the first blocking step. This technique prevented CaM protein from being washed from the membrane during the labeling procedure. After glutaraldehyde fixation, the membrane was thoroughly washed, as described above, before starting the blocking and labeling process. Signal detection was performed using a chemiluminescent reagent (PerkinElmer) as directed by the manufacturer, followed by exposure of membranes to X-ray films (Fuji). Films were developed using a Compact 4× automatic X-ray film developer (Xograph). Developed films were scanned with a commercially available Canon scanner for image processing.

Western Blot Assay of L-Selectin–GFP Shedding in Static Transmigration Assays. HUVEC monolayers were grown to confluence in six-well plastic dishes and stimulated overnight with 10 ng/mL recombinant human TNF-α (R&D Systems). THP-1 cell suspensions were added to each well at a density of 2 × 10^5 cells per milliliter and incubated for 0, 5, 10, 20, 30, or 60 min. The supernatant (unbound THP-1 fraction) and HUVEC fraction (bound THP-1 fraction) were then harvested, washed once with PBS, and lysed in an appropriate volume of Cytobuster Protein Extraction Reagent (Merck Millipore) supplemented with protease inhibitor mixture. Samples were boiled to 95 °C for 5 min and resolved on polyacrylamide gels. L-selectin–GFP was detected by Western blotting using anti-GFP antibody (Chromotek).

Confocal Microscopy. All specimens were fixed in 4% (vol/vol) PFA and imaged using an inverted SP5 confocal microscope (Leica Microsystems). Unless otherwise stated, all images were acquired using a 63× objective (N.A. = 1.4 oil immersion). GFP was excited at λ = 488 nm with an argon laser, whereas TRITC-phalloidin and Alexa Fluor 633 were excited with helium-neon lasers at λ = 543/568 nm and 633 nm, respectively. Images were acquired as single *z* planes (0.75 μm) or as series of Z-stacks.

Acquired Z-stacks were saved as a series of tagged image file format files and imported into Imaris imaging software (Bitplane AG). Three-dimensional reconstruction was performed manually using the "volume rendering" task for both green and red channels, which corresponded to L-selectin–GFP–stained and TRITC-phalloidin–stained actin, respectively.

Wide-Field Fluorescence Microscopy. All leukocyte/endothelial interactions under flow were performed at 37 °C and acquired using an Olympus IX81 time-lapse inverted fluorescence microscope connected to a Hamamatsu C10600 ORCA-R2 digital video camera. All images were acquired using a Plan Fluor 10× objective lens (N.A. = 0.30), with acquisition rates set to four frames per minute. At least three different fields of view were acquired for each flow assay, representing regions at the beginning, middle, and end of the flow chamber.

Spinning Disk Confocal Microscopy. An inverted Nikon TI-E microscope was used, equipped with a Yokogawa CSU-X1 Spinning Disk Confocal unit, as well as an Andor NEO EM-CCD camera and a Sutter filter wheel. For laser excitation, the 488-nm line of an Agilent MLC laser was used and the emission filter was set at 525/30 (Chroma). Z-series were acquired using a Mad City Labs nanodrive-controlled Piezo stage using a Nikon apo 40× 1.25-water immersion N.A. objective. The movie is a maximum intensity projection of a Z-series, using Z-steps of 0.75 μm, an original Z-range of 26 μm in depth, and a selected Z-range of ∼15 μm.

Disuccinimidyl Suberate Cross-Linking of CaM/L-Selectin Peptides. DSS (disuccinimidyl suberate; Pierce) is a homobifunctional chemical cross-linker. Each end of the chemical is capped with an amine-reactive NHS ester, separated by an 11.4-Å spaced arm. The two NHS groups within a single DSS molecule can react with amines with the distance provided by the spacer. Cross-linking experiments were performed using fixed concentrations of DSS (0.1 mM) and CaM (4.6 μM). L-selectin peptide concentrations are indicated in the legend of Fig. 4. The DSS cross-linker was dissolved in DMSO and was added to the reaction tube at a maximum volume of 0.625 μL. This volume equates to a 2.5% (vol/vol) final DMSO content in each reaction, an amount that did not affect the stability/electrophoretic mobility of CaM or the L-selectin tail, as determined with control experiments conducted with an equivalent volume of DMSO only.

Micropipette Assay. Assays were followed according to Volpe et al. (3). Fibronectin solution was added to glass-bottomed dishes 5 cm in diameter (glass thickness = 0.17 mm/no. 1.5) in 5 μg/mL fibronectin for 1 h at 37 °C. Excess fibronectin was removed through two rounds of PBS washes. Before the addition of cells, dishes were further incubated in imaging media (PBS containing calcium and magnesium supplemented with 1% FBS, penicillin/streptomycin, 40 μM sodium pyruvate, 1 mg/mL fatty acid-free BSA, and 1 mg/mL glucose) to block nonspecific binding of cells to glass bottoms. Cells were seeded at a density of 1×10^6 cells per milliliter in warm imaging media left to adhere for 30 min at 37 °C. Excess unbound cells were removed before imaging. All experiments were conducted at 37 °C and performed within 1 h. Images were acquired at the rate of one frame every 10 s using an inverted 40× objective lens attached to a Nikon Eclipse TE2000-U epifluorescence microscope.

Micropipettes were manufactured in-house. Borosilicate glass capillaries with an i.d. of 0.5 mm were pulled using a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company). Pipettes were pulled using the following parameters: heat = 500, pull = 500, velocity = 30, and time = 250. The micropipette was attached to a pressure system [Narishige IM300 microinjector (0.7 psi)]. Avoiding air bubbles, the pipette tip was filled with 200 nM MCP-1 diluted in imaging media supplemented with a 10-kDa TRITC-dextran tracer to observe gradient formation and that the needle did not become blocked during the assay. Optimal polarization responses were obtained by culturing THP-1 cells in RPMI 1640 GlutaMAX (Gibco, Life Technologies), supplemented with 10% (vol/vol) HyClone FBS (GE Healthcare) and 1% (vol/vol) penicillin/streptomycin.

1. Killock DJ, et al. (2009) In Vitro and in Vivo Characterization of Molecular Interactions between Calmodulin, Ezrin/Radixin/Moesin, and L-selectin. *J Biol Chem* 284(13): 8833–8845.
2. Burns SO, et al. (2010) A congenital activating mutant of WASp causes altered plasma membrane topography and adhesion under flow in lymphocytes. *Blood* 115(26): 5355–5365.
3. Volpe S, Thelen S, Pertel T, Lohse MJ, Thelen M (2010) Polarization of migrating monocytic cells is independent of PI 3-kinase activity. *PLoS ONE* 5(4):e10159.

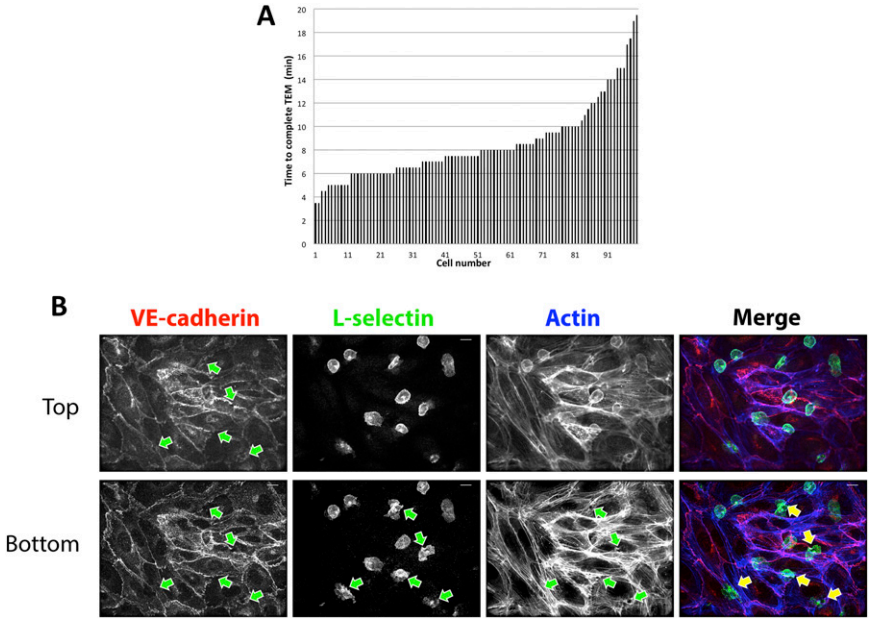


Fig. S1. TEM of primary monocytes under static and flow conditions. (*A*) TEM of primary monocytes across TNF-activated HUVECs performed under static conditions. Analysis was performed exactly as outlined for Fig. 1*B*. The average time taken for monocytes to transmigrate is 8.5 min. The additional 2.5 min is taken up by monocyte crawling before TEM. This observation suggests that recruitment from flow may act to localize monocytes better to sites of emigration than under static conditions. (*B*) Extended view of monocytes transmigrating from Fig. 1*F*. Green arrows are pointing to five monocytes captured in the process of TEM. Moreover, their protruding pseudopods are expressing L-selectin. Arrows indicate a breach in continuity of the VE-cadherin signal and suggest TEM (paracellular) is actively taking place. "Holes" can be seen in the phalloidin (actin) stain, identifying the route taken by the transmigrating leukocyte. Yellow arrows are used in the merged channels image to avoid confusion with green L-selectin signal.

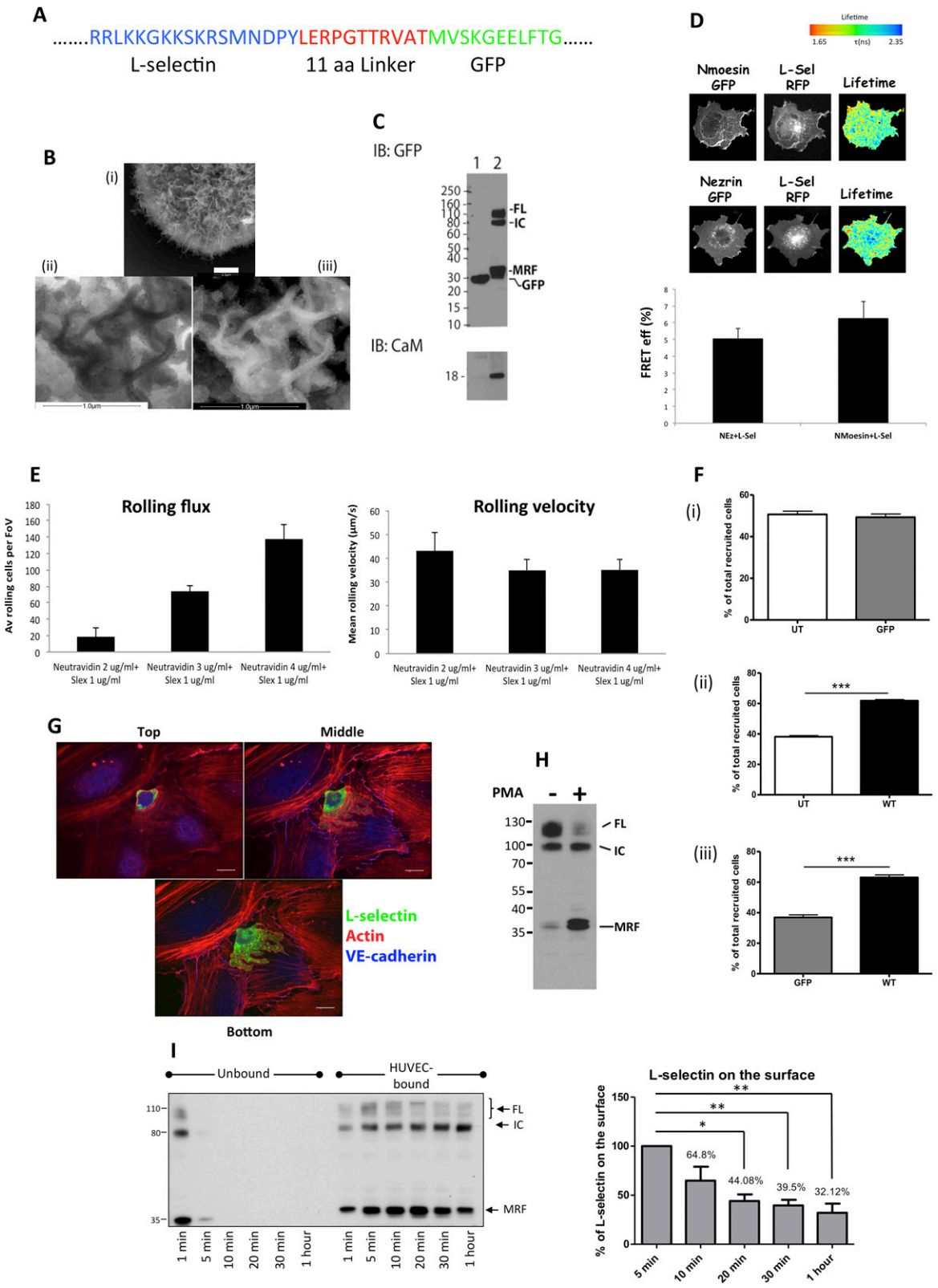


Fig. S2. Characterization of a cellular model to study L-selectin shedding during TEM. (*A*) Amino acid sequence corresponding to the junction of the 17-aa L-selectin cytoplasmic tail (blue) and GFP (green). The 11-aa linker is indicated in red. (*B*, *i*) Typical SEM image of a THP-1 cell possessing abundant microvilli when seeded and fixed onto poly-L-lysine–coated glass coverslips. Nontransduced THP-1 cells were found to possess similar microvillar structures. (Scale bar: 2 μm.) (*B*, *ii* and *iii*) Close-up views of THP-1 microvilli. L-selectin was labeled with DREG56 monoclonal antibody, followed by 10-nm immunogold labeling with secondary antibody. Positive (*ii*) and negative (*iii*) images allow for easier detection of immunogold particles on microvilli. (Scale bar: 1 μm.) (*C*) Two anti-GFP immunoprecipitation experiments are resolved in lanes 1 and 2 using lysates derived from THP-1 cells expressing GFP alone (lane 1) or full-length WT L-selectin–GFP

Legend continued on following page

(lane 2). (*Upper*) Anti-GFP immunoblot reveals the three major molecular mass species of WT L-selectin–GFP in lane 2. IB, immunoblot; FL, full length; IC, intracellular form of L-selectin–GFP; MRF, membrane-retained fragment (after shedding). (*Lower*) Anti-CaM immunoblot shows endogenous CaM coprecipitating with WT L-selectin–GFP, but not GFP alone. (*D*) N-terminal domain of moesin-GFP or ezrin-GFP was coexpressed with WT L-selectin (L-Sel)–RFP in Cos-7 cells, and FRET efficiency between the two fluorochromes was measured by FLIM (*Materials and Methods*). The lifetime of fluorescence is expressed in nanoseconds as a pseudocolor scale ranging from red (low lifetime with a very high probability of interaction) to blue (high lifetime with a very low probability of interaction). Note that donor (GFP) lifetime is 2.3 ns, and any value lower than this value is calculated as FRET efficiency. (*E*) THP-1 cells expressing WT L-selectin–GFP were perfused over increasing concentrations of immobilized biotinylated sLe^x (*Materials and Methods*) at 1.25 dyn/cm^2. Av, average; FoV, field of view. (*F*) Coperfusion experiments were performed using untransduced THP-1 cells (UT) and THP-1 cells expressing GFP alone (GFP) or WT L-selectin–GFP (WT). (*i* and *ii*) Control experiments for (*iii*). Results are expressed as the percentage of cells recruited after 15 min of flow. Three independent coperfusions were performed for each pair of cell lines. Error bars represent SEM. A two-tailed, unpaired Student's *t* test was used for statistical analysis. ***$P < 0.001$. (*G*) Optical sections (0.75 μm) of a THP-1 cell undergoing TEM. The continuous VE-cadherin stain in blue (Alexa Fluor 633 secondary antibody) is breached specifically where the THP-1 cell is undergoing TEM. HUVEC-derived actin stress fibers are clearly seen to overlay monocyte pseudopods. Note that monocytes do not produce actin stress fibers. (Scale bar: 5 μm.) (*H*) Anti-GFP immunoblot of extracts derived from THP-1 cells expressing WT L-selectin–GFP, stimulated with or without 10 nM phorbol myristate acetate (PMA) for 10 min. An increase in the cleaved product and a concomitant loss of the full-length (FL) form are observed in the right-hand lane, indicating robust shedding in response to PMA stimulation. MRF, membrane-retained fragment. (*I*, *Left*) Similar Western blots performed on THP-1 cells bound to TNF-activated HUVECs over a time course (static assay; *SI Materials and Methods*). Lysates were generated from bound and unbound fractions of THP-1 cells, and reveal that the majority of cells bind to activated HUVECs between 1 and 5 min under static conditions. Equal volumes of extract were generated so that lanes could be compared and quantified by densitometric analysis over time. Quantitation of the surface or FL form was performed only on the bound fraction of cells. Note that shedding peaks by ~20 min. (*I*, *Right*) Loss of FL signal in the Western blot is expressed as the percentage of L-selectin remaining on the surface in the bar graph. All values were normalized against an actin loading control. One-way ANOVA, followed by Tukey's posttest, was used for statistical analysis, and is the sum of at least three independent experiments. *$P < 0.05$; **$P < 0.01$.

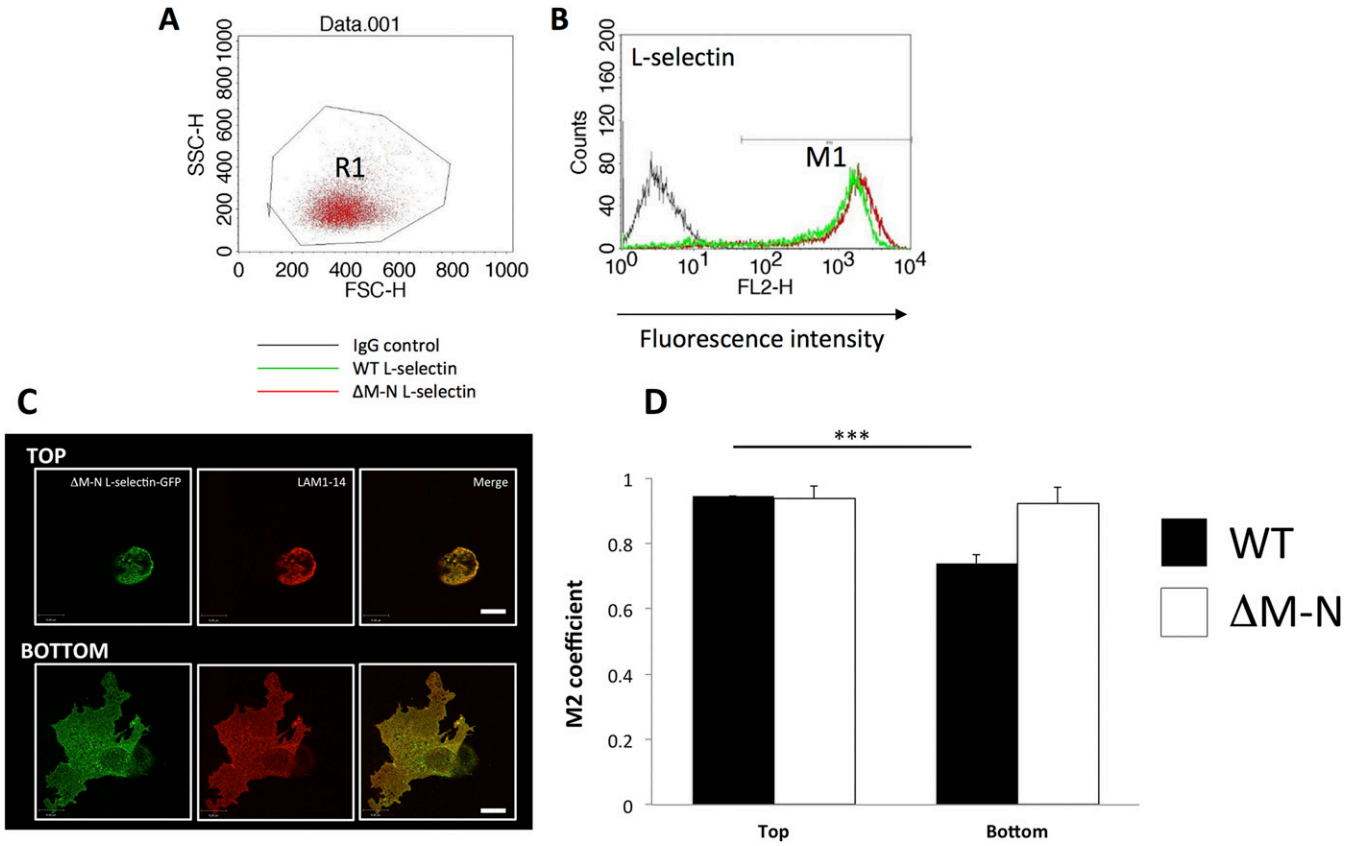


Fig. S3. Characterization of THP-1 cell lines expressing WT and ΔM-N L-selectin. Following lentiviral transduction, all THP-1 monocytes were sorted by flow cytometry to express matched levels of L-selectin–GFP using an in-house cell sorting service. To verify that surface expression levels were matched between cell lines, DREG56 monoclonal antibody, followed by phycoerythrin (PE)-conjugated secondary monoclonal antibody, was used to analyze mean fluorescence intensities. (*A*) Forward and side scatter profiles of THP-1 cells. The R1 gate depicts the gate within which cells were analyzed. (*B*) PE fluorescence intensity histogram with overlaid profiles of WT L-selectin–GFP, ΔM-N L-selectin–GFP, and THP-1 cells labeled with an isotype-matched control. All L-selectin Ser to Ala/Asp mutants were similarly sorted against THP-1 cells expressing WT L-selectin–GFP. FL2H, phycoerythrin fluorescence channel. (*C* and *D*) Manders' colocalization coefficiency (M2) between LAM1-14 and L-selectin–GFP remains the same above and below the endothelial monolayer in ΔM-N cells (white bars in *D*), which is in stark contrast to cells expressing WT L-selectin (black bars in *D*). A two-tailed unpaired Student's *t* test was used to calculate differences between TOP and BOTTOM optical sections, and error bars represent SEM. ***$P < 0.001$. (Scale bar: 9 μm.)

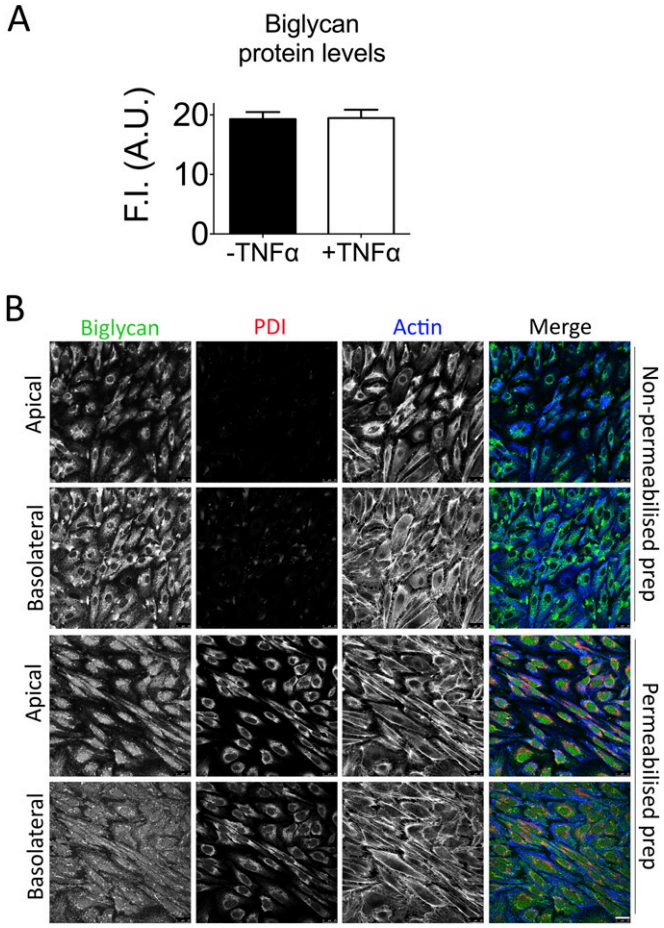


Fig. S4. (*A*) Whole-cell lysates were prepared from HUVEC monolayers treated with or without TNF-α for 16 h and biglycan levels were detected from each group by Western blotting. Densitometric analysis was performed on the biglycan band as indicated in Fig. 2*H*, and values were normalized against actin loading controls for each group. No change in biglycan expression levels was observed before and after TNF stimulation. Data are expressed from five independent experiments. A.U., arbitrary units; F.I., fluorescence intensity obtained from quantification of IRDye secondary antibodies (LI-COR Biosciences). (*B*) Immunofluorescence staining of biglycan in TNF-activated HUVEC monolayers reveals a higher expression in the basolateral compartment. HUVECs were seeded onto fibronectin-coated glass coverslips 13 mm in diameter (10 $\mu g \cdot mL^{-1}$) and stimulated for 16 h with 10 $ng \cdot mL^{-1}$ TNF. Cells were fixed in 4% (vol/vol) PFA solution, and one set of samples was subjected to permeabilization with ice-cold PBS containing 0.1% Nonidet P-40 for 3 min, whereas the other group was left untreated. Immunostaining of biglycan revealed a stronger signal in the basolateral compartment, most notably in the nonpermeabilized preparation (prep), which should specifically detect the extracellular fraction of biglycan. PFA fixation was sufficient to permit phalloidin staining, in the absence of permeabilization, but not antibody staining. This phenomenon was likely due to a size exclusion phenomenon, because phalloidin is much smaller than whole antibody. Protein disulfide isomerase (PDI) is an intracellular endoplasmic reticulum marker that was used to control for immunofluorescence staining between permeabilized and nonpermeabilized cells. Anti-PDI antibody was obtained from Abcam [mouse monoclonal anti-PDI antibody (RL90)]. Note that instrument parameter settings were kept constant for all acquisitions. Immunofluorescence staining of actin (phalloidin-Alexa Fluor 633), biglycan (Alexa Fluor 488), and PDI (Alexa Fluor 568) was used to stain all preparations. Images were acquired with a 63× objective lens and are representative of three independent experiments. (Scale bar: 25 μm.)

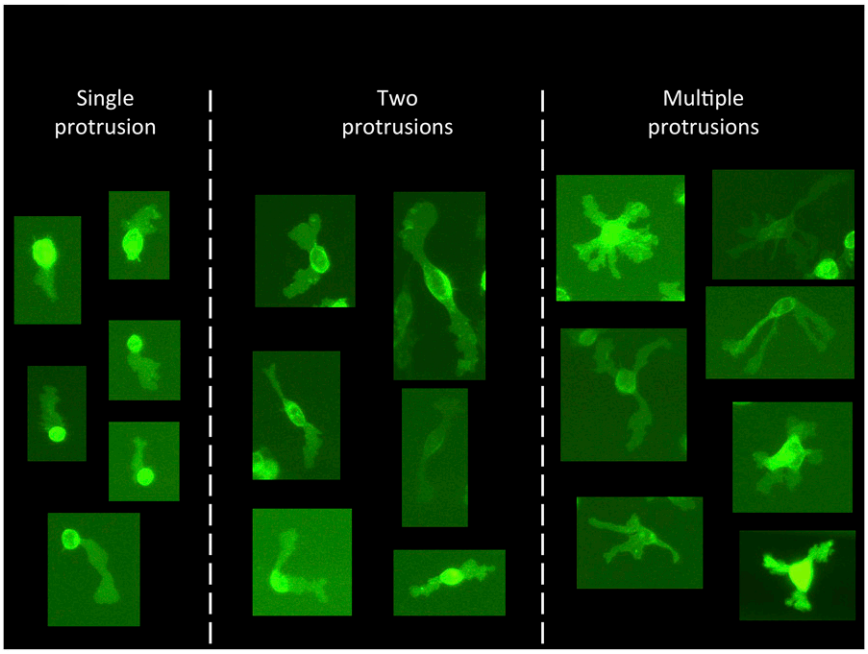


Fig. S5. Typical examples of morphological changes that THP-1 cells undergo as they protrude beneath the endothelial monolayer. Still images were grabbed from time-lapse recordings of flow assays (using Volocity software). GFP signal refers to the GFP tag on L-selectin.

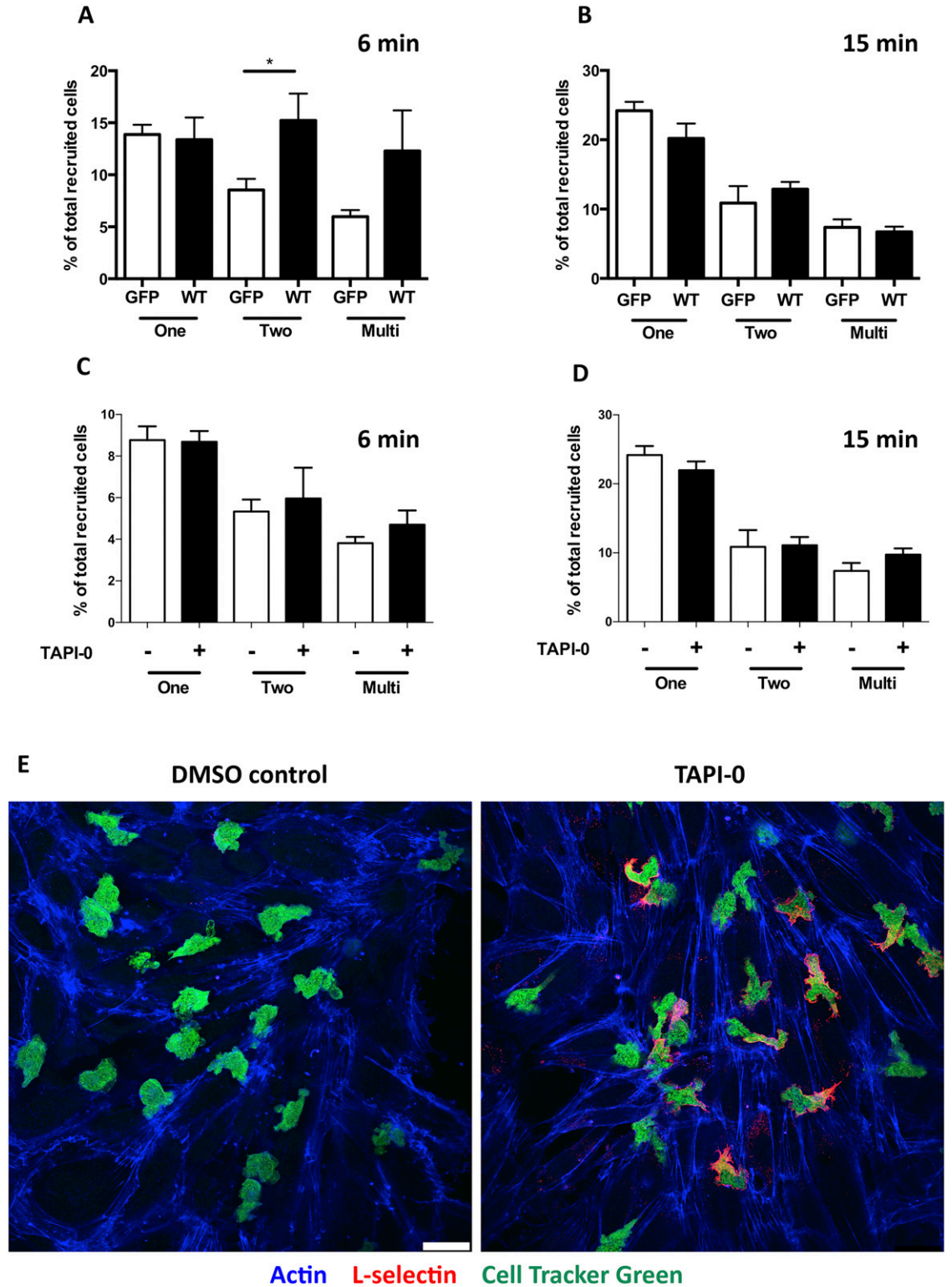


Fig. S6. (*A* and *B*) Expression of L-selectin in THP-1 cells potentiates proinvasive behavior, which is significant only at early time points. It is thought that shedding of L-selectin may "rein-in" excessive protrusive behavior at later time points. Cells expressing GFP alone (GFP) or WT L-selectin–GFP (WT) were perfused over TNF-activated HUVECs for 6 or 15 min. Cells were scored for having one, two, or multiple (Multi) protrusions (as in Fig. S4). All other cells were deemed to be adherent, but not protruding. Values are given as the percentage of the total number of cells adhered at the 15-min time point. (*C* and *D*) TAPI-0 (10 μM) does not alter protrusive behavior in THP-1 cells that do not express L-selectin. Cells expressing GFP alone were perfused over TNF-activated HUVECs in the same manner as in *A* and *B*. (*E*) Primary monocytes were labeled with Cell Tracker Green (Molecular Probes, Life Technologies) and perfused over TNF-activated HUVECs for 10 min, followed by a further 15 min with perfusion media. Samples were fixed and stained for L-selectin (using LAM1-14 and Alexa Fluor 555) and actin (phalloidin-Alexa Fluor 633). Note that the cell outlines appear more irregular in the TAPI-0–treated monocytes and that transmigrated cells have retained L-selectin expression (red signal). These still images reflect the time-lapse recordings provided in Movies S1 and S7. (Scale bar: 25 μm.)

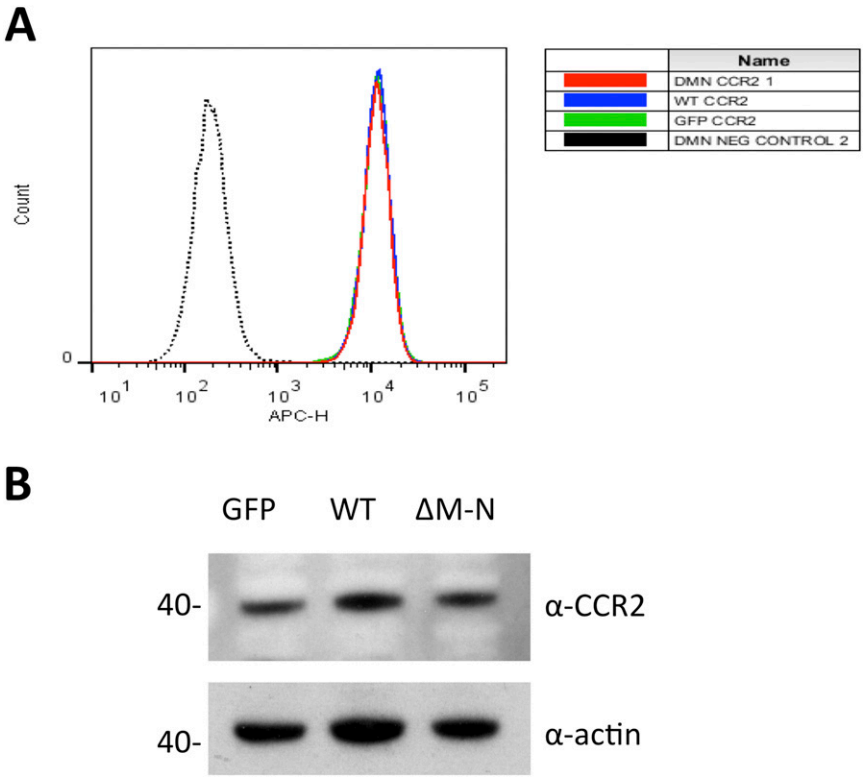
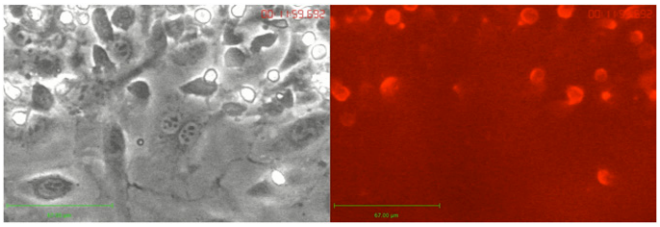
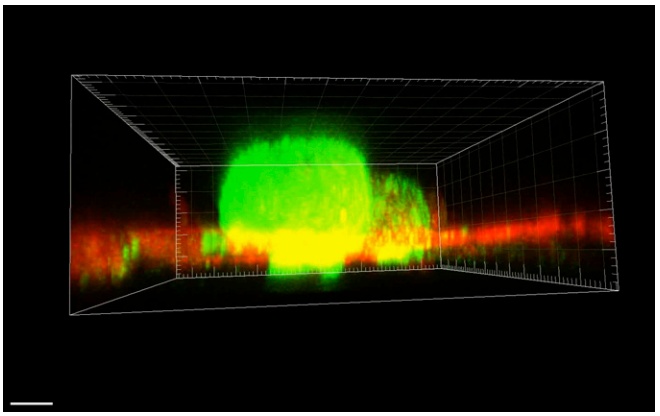


Fig. S7. Expression of CCR2 remains unchanged between different THP-1 cell lines. (*A*) Fluorescence intensity profiles of THP-1 cells expressing WT or ΔM-N L-selectin–FP or GFP alone. In all cases, cells showed matched expression levels of CCR2. The ΔM-N (DMN) cells were stained with an isotype-matched control antibody to identify the negative (NEG) profile. APC-H, allophycocyanin fluorescence channel. (*B*) Western blotting of whole-cell lysates also confirmed matched expression levels. Taken together, these results indicate matched surface level expression of CCR2 and at the whole-cell lysate level.



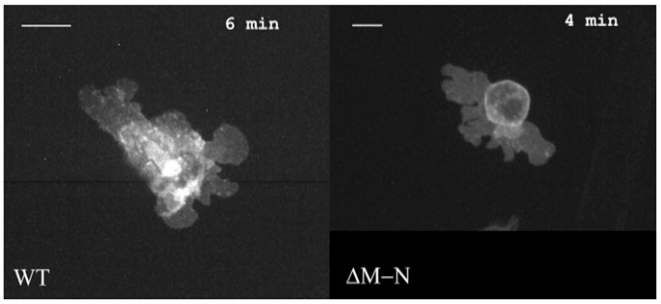
Movie S1. Monocytes isolated from healthy donors, labeled with LAM1-14 directly conjugated to Alexa Fluor 555, were perfused over TNF-activated HUVECs. Phase and wide-field fluorescence images were acquired using an Olympus IX-81 fluorescence time-lapse microscope. Images were acquired at a rate of four frames per minute over a 25-min period using an inverted 10× objective lens. (Scale bar: 45 μm.)

Movie S1



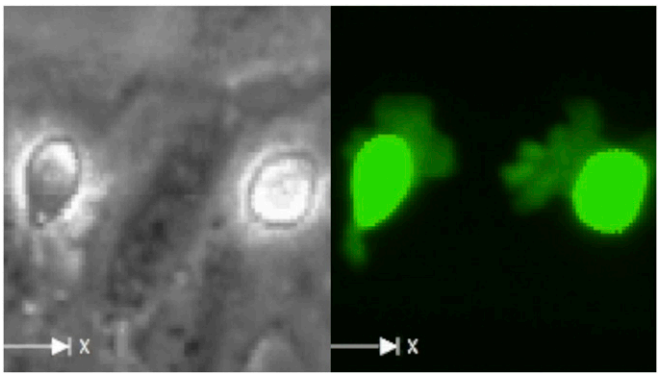
Movie S2. Primary human monocytes were perfused over TNF-activated HUVECs for 6 min, fixed, and stained for L-selectin (LAM1-14 + Alexa Fluor 488 secondary) and TRITC-phalloidin. Imaris software used to render 3D, multiple 0.75-μm confocal sections revealed that the pseudopod contains L-selectin (green) and is penetrating beneath the endothelial monolayer (red). (Scale bar: 3 μm.)

Movie S2



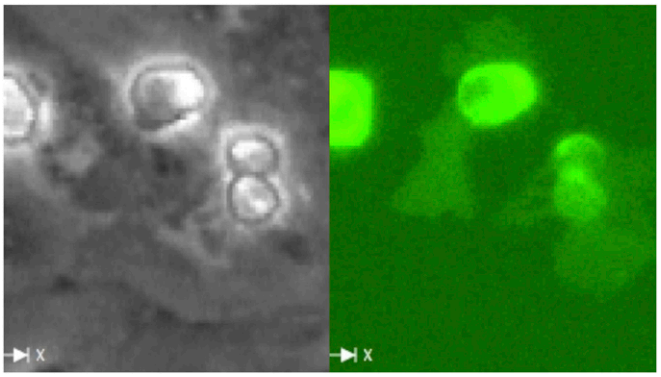
Movie S3. THP-1 cells expressing matched levels of either WT or ΔM-N L-selectin–GFP were perfused over TNF-activated HUVECs for 20 min, and events were recorded using a Nikon spinning disk confocal microscope. Images were acquired as a series of Z-stacks and reconstituted into a 4D movie. Note that GFP-positive spots appear in pseudopods specifically within the WT cell line at later time points, which was not observed in the ΔM-N cell line. (Scale bar: 10 μm.)

Movie S3



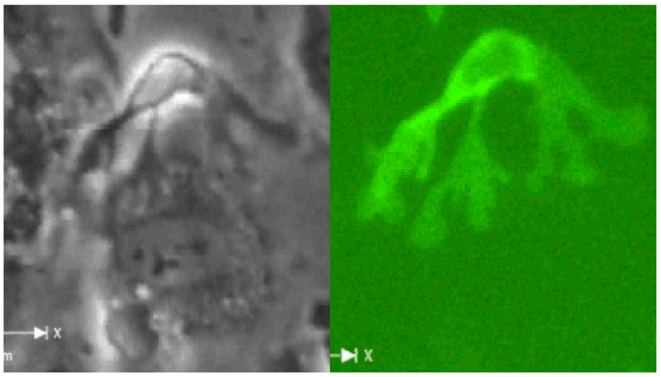
Movie S4. THP-1 cells stably expressing GFP alone were perfused over TNF-activated HUVECs for 15 min. Side-by-side phase and GFP channels are shown. The frame rate is four frames per minute.

Movie S4



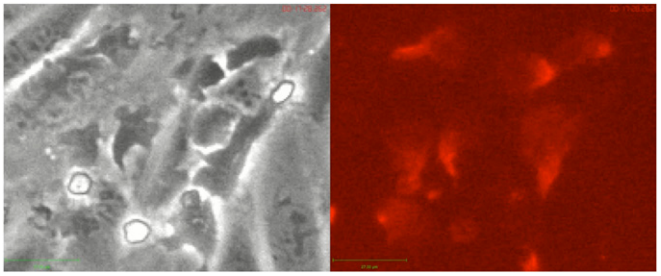
Movie S5. THP-1 cells stably expressing WT L-selectin–GFP were perfused over TNF-activated HUVECs for 15 min. Side-by-side phase and GFP channels are shown. The frame rate captured at a rate of four frames per minute. Note that cells produce multiple protrusions that are then consolidated into one or two as time progresses.

Movie S5



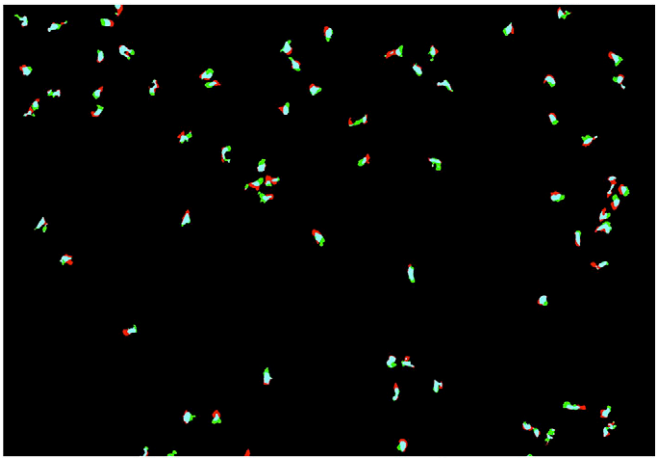
Movie S6. THP-1 cells stably expressing ΔM-N L-selectin–GFP were perfused over TNF-activated HUVECs for 15 min. Side-by-side phase and GFP channels are shown. Time lapse recordings were taken at four frames per minute for 15 min. Note that ΔM-N cells produce multiple pseudopods and fail to reduce the extent to which they protrude pseudopods.

Movie S6



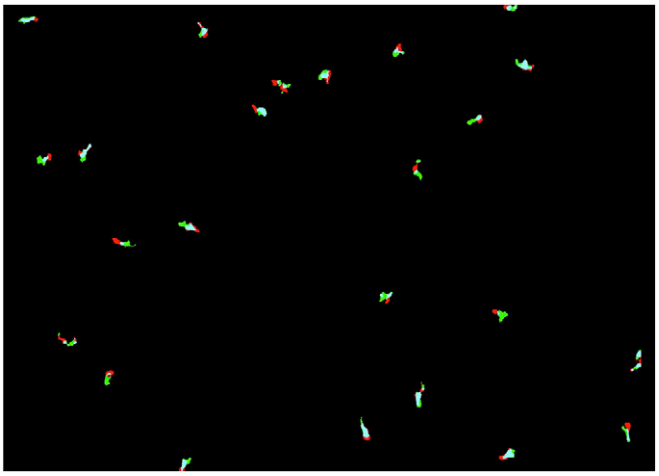
Movie S7. Primary monocytes were labeled with LAM1-14 monoclonal antibody, as in Movie S1, and subsequently treated with 10 μM TAPI-0 for 10 min and perfused over TNF-activated HUVECs for 25 min. Phase and wide-field fluorescence images were acquired using a 10× objective at four frames per minute. Note the retention of the L-selectin signal in transmigrated monocytes and their altered morphology and directionality following TEM. Similar shapes are seen in Fig. S6*E*, where monocytes have not been prelabeled with LAM1-14. (Scale bar: 45 μm.)

Movie S7



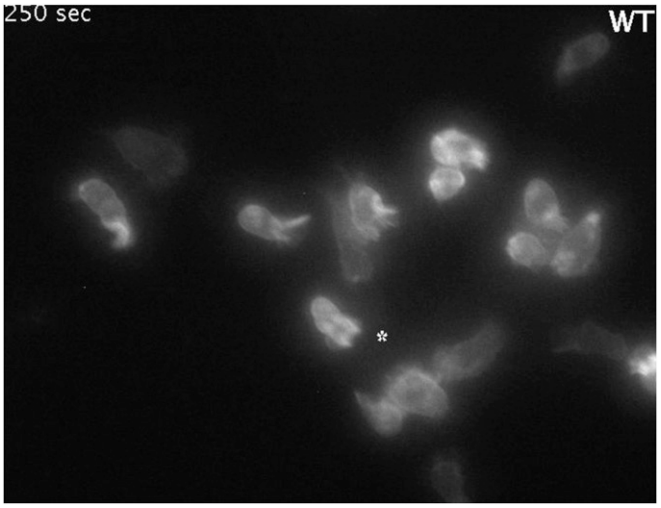
Movie S8. Frame-by-frame recording of DMSO-treated monocytes perfused over TNF-activated HUVECs. Cells were perfused for the first 10 min, followed by 15 min of perfusion with media containing DMSO. Protrusion/retraction maps were generated for every nontouching monocyte. Each frame was used to provide a mean cell volume, mean protrusion area (green), and mean retraction area (red).

Movie S8



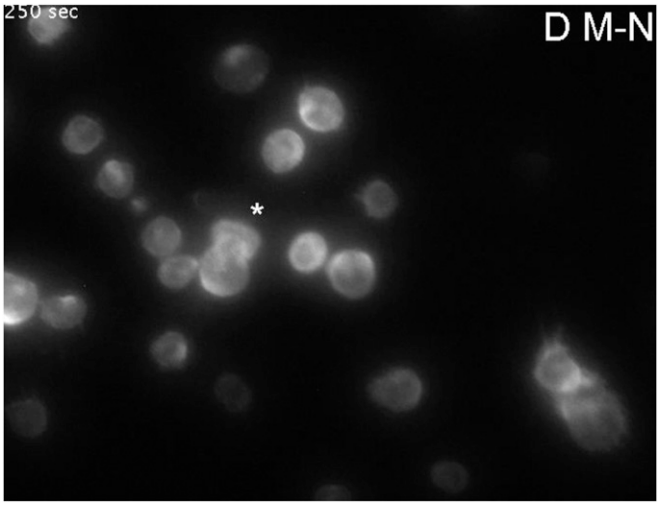
Movie S9. Frame-by-frame recording of TAPI-0–treated monocytes perfused over TNF-activated HUVECs. Cells were perfused for the first 10 min, followed by 15 min of perfusion with media containing 10 μM TAPI-0. Protrusion/retraction maps were generated for every nontouching monocyte. Each frame was used to provide a mean cell volume, mean protrusion area (green), and mean retraction area (red).

Movie S9



Movie S10. Micropipette assay. THP-1 cells expressing WT L-selectin–GFP were challenged with 200 nM CCL2 (MCP-1) for ~10 min. The asterisk denotes the position of the micropipette tip.

Movie S10



Movie S11. Micropipette assay. THP-1 cells expressing ΔM-N L-selectin–GFP were challenged with 200 nM CCL2 (MCP-1) for ~10 min. The asterisk denotes the position of the micropipette tip.

Movie S11