Supplemental Information

Supplemental Methods

Cells

Blood was collected from healthy volunteers with a protocol approved by the Institutional Review Board of the Oklahoma Medical Research Foundation. Human neutrophils were isolated as described[1]. Umbilical cords were provided by the Pathology Department of Mercy Lab Oklahoma with a protocol approved by the Institutional Review Board of Mercy Lab Oklahoma. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described[1]. HUVEC were passaged 2 times or less for all experiments.

Mouse bone marrow leukocytes were isolated as described[2]. Briefly, cells were isolated by gently flushing femurs and tibias with 10 ml Hanks' balanced salt solution (HBSS) without Ca^{2+} or Mg^{2+}. After lysing red blood cells in 150 mM NH_4Cl, 10 mM $NaHCO_3$, and 1 mM EDTA, the cells were washed with HBSS and resuspended at 2 x 10^6/ml in HBSS containing 1.26 mM Ca^{2+}, 0.81 mM Mg^{2+}, and 0.5% human serum albumin. Neutrophils were isolated from bone marrow leukocytes by a density gradient method[3].

Isolation of mouse lung endothelial cells (MLEC) was performed as described previously[4,5], with minor modifications. Briefly, lungs from two or three mice were washed, minced into 1- to 2-mm^2 pieces in 10% FBS-DMEM with antibiotics, and digested with collagenase type I (2 mg/ml) at 37˚C for 1 h with occasionally vortex. The digested tissues were filtered through a 70-μm cell strainer and centrifuged. The cells were cultured on gelatin-coated dishes with DMEM containing 20% FBS and 100 μg/ml endothelial cell growth factors (Sigma-Aldrich). After 48 h, the cells were harvested with trypsin and EDTA, and incubated with biotin-

conjugated rat anti-mouse CD102 mAb (BD Biosciences) followed by streptavidin-conjugated microbeads (Miltenyi Biotec). The CD102-positive cells were collected with a magnetic separator (STEMCELL Technologies), and cultured on gelatin-coated plates with DMEM containing 20% FBS and 100 μg/ml endothelial cell growth factors. As ascertained by flow cytometry, ~90% were endothelial cells that stained with FITC-conjugated rat anti-mouse CD31 mAb.

Antibodies and reagents

Mouse anti-human P-selectin mAbs S12 and G1[6], anti-human gp130 mAb 4B11[7], and anti-human PSGL-1 mAbs PL1 and PL2[8] were described. Goat anti-P-selectin IgG, which binds to both human and mouse P-selectin, was described[9]. Mouse mAb to human α-adaptin, PE-conjugated mouse mAbs to human CD3, CD14, and CD16b, and FITC- or PE-conjugated rat mAbs to mouse β2 integrin (GAME-46), mouse L-selectin (MEL-14), mouse PSGL-1 (2PH1), mouse CD44 (IM7), mouse Ly6G, macrophage colony-stimulating factor (M-CSFR; CD115), and CD3 were from BD Biosciences. Human OSM, non-immune goat IgG, goat anti-gp130 IgG, goat anti-OSM IgG, rat anti-mouse gp130 mAb, rat anti-mouse ICAM-1 mAb, and mouse anti-human OSM mAb were from R&D Systems. Alexa Fluor 488- or 555-conjugated donkey anti-mouse IgG, and Alexa Fluor 546-conjugated donkey anti-goat IgG were from Molecular Probes. Rat anti-mouse P-selectin mAbs RB40.34 and 5H1 and rat anti-mouse PSGL-1 mAb 4RA10 were described[10-12].

Mice

All mice were backcrossed with C57BL/6J mice at least 10 times. C57BL/6J mice were used as wild-type (WT) controls. *Osm*$^{-/-}$ mice lacking OSM[13] were provided by Dr. Atsushi Miyajima (University of Tokyo). *Selplg*$^{-/-}$ mice lacking PSGL-1 were generated as described[14].

gp130$^{flox/flox}$ mice[15] were crossed with transgenic mice expressing Cre recombinase ER^{T2} under control of the promoter for the *cdh5* gene that encodes VE-cadherin (VECad-Cre-ER^{T2})[16]. WT mice injected with tamoxifen were used as controls. Cre-mediated recombination was induced by intraperitoneal injection of tamoxifen (2 mg/mouse) for 5 consecutive days. Ten days after the last injection, the deletion of gp130 on endothelial cells was evaluated by injecting Fluoresbrite Red microspheres (0.5 mm diameter, Polysciences, Inc.) coated with control rat IgG or rat anti-gp130 mAb as described[5]. Briefly, 10^{10} mAb-coated microspheres were injected through the retro-orbital venous plexus. After 20 min, the cremaster muscle or the inferior vena cava (IVC) was exteriorized, and adhesion of microspheres to endothelial cells was visualized with spinning-disk microscopy[17]. For postcapillary venules of the cremaster muscle, mice were pre-injected 1 h before tissue exteriorization with 50 μg/ml anti-PSGL-1 mAb 4RA10 to block leukocyte rolling on P-selectin. After 20 min, adhesion of microspheres in postcapillary venules (cremaster muscle) or the inferior vena cava (IVC) was observed with spinning-disk microscopy[17]. The digital images were captured by the NIH acquisition software Micromanager. The covered area of the bound microspheres in the images was analyzed using Element digital image-analysis software (Nikon).

All mouse protocols were approved by the Institutional Animal Care and Use Committee of the Oklahoma Medical Research Foundation.

Flow cytometry

Human heparinized blood from healthy volunteers was collected by venipuncture, and red blood cells were removed by dextran sedimentation. Mouse heparinized blood was obtained from the facial vein, and red blood cells were lysed. Human or mouse peripheral blood leukocytes were washed in HBSS with 0.5% human serum albumin. The cells were pre-

incubated with 5 μg/ml Fc blocking antibody (BD Biosciences) for 20 min at 4˚C, and washed in HBSS with 0.5% human serum albumin. Both human and mouse cells were fixed and permeabilized with a Cytofix/Cytoperm fixation and permeabilization kit (BD Biosciences). The cells were then washed with BD Perm/Wash buffer (BD Biosciences). Mouse anti-human OSM IgG2a or non-immune control mouse IgG2a was labeled using an Alexa Fluor 488 mouse antibody labeling kit (ThermoFisher). Goat anti-mouse OSM IgG or non-immune control goat IgG was labeled using an Alexa Fluor 488 goat IgG labeling kit (ThermoFisher). For human cells, the fixed and permeabilized cells were stained with PE-conjugated isotype control mouse IgG2a, anti-CD3, anti-CD14, or anti-CD16b, plus Alexa Fluor 488-conjugated mouse anti-mouse OSM IgG or control mouse IgG. For mouse cells, the fixed and permeabilized cells were stained with 5 μg/ml PE-conjugated isotype control rat IgG, anti-Ly6G, anti-M-CSFR, or anti-CD3, plus Alexa Fluor 488-conjugated goat anti-mouse OSM IgG or control goat IgG. The neutrophil, monocyte, or T cell population was identified by its scatter properties and by CD16b, CD14, or CD3 staining for human cells, or Ly6G, M-CSFR, or CD3 staining for mouse cells, respectively. In some experiments, mouse peripheral blood leukocytes were pre-treated with Fc blocking antibody and then incubated with FITC-conjugated anti-Ly6G, anti-M-CSFR, or anti-CD3, plus control rat IgG or rat anti-mouse gp130 mAb followed by PE-conjugated goat anti-rat IgG, or plus PE-conjugated anti-PSGL-1, anti-β2 integrin, anti-CD44, or anti-L-selectin.

In other experiments, thrombi collected from the IVC 24 h after stenosis were minced into small pieces and digested in HBSS with Ca^{2+} and Mg^{2+} containing 1 mg/ml collagenase type IV (Worthington) and 100 μg/ml DNAse I (Roche) for 15 min at 37˚C, with occasional vortex. After digestion, the single-cell suspension was passed through a 100-μm cell strainer and washed in HBSS without Ca^{2+} and Mg^{2+}, containing 5 mM EDTA and 1% human serum albumin. The

cells were pre-incubated with 5 μg/ml Fc blocking antibody for 20 min at 4˚C, and then incubated with 5 μg/ml PE-conjugated anti-Ly6G or anti-M-CSFR for 20 min at 4˚C. Six-μm polystyrene beads (PolySciences) (1 x10^5) were added to 500 μl of cell suspension. The neutrophil or monocyte population was identified by its scatter properties and by Ly6G or M-CSFR staining. The number of neutrophils or monocytes per thrombus was quantified.

OSM release from human or mouse neutrophils

Human neutrophils (2 x 10^7 in 1 ml HBSS/0.5% human serum albumin with Ca^{2+} and Mg^{2+}) or neutrophils isolated from bone marrow of WT or *Selplg*$^{-/-}$ mice (10^7 in 1 ml HBSS/0.5% human serum albumin with Ca^{2+} and Mg^{2+}) were incubated, respectively, on immobilized human platelet-derived P-selectin[18] or on mouse P-selectin-IgM or control CD45-IgM coated plates with or without movement on a rotary shaker at 70 rpm for 30 min at room temperature. In some experiments, human neutrophils were preincubated with 10 μg/ml of anti-PSGL-1 mAb PL1 or PL2. After incubation, the buffer was collected and the adherent cells were lysed with 1 ml of 1% Triton X-100, 125 mM NaCl, 50 mM Tris pH 8.0, 10 mM EDTA, 0.1% SDS, and a protease inhibitor cocktail (1:50; Thermo Fisher Scientific). OSM in buffer or lysate was measured with a human OSM ELISA kit or a mouse OSM ELISA kit (R&D Systems).

Intravital microscopy

Intravital video microscopy of the cremaster muscle of anesthetized mice was performed as described[14,17,19]. Exteriorization of the cremaster muscle was completed within 10 min. In some experiments, 10 μg goat anti-ICAM-1 IgG, goat anti-gp130 IgG, goat anti-OSM IgG, non-immune goat IgG, or rat anti-P-selectin mAb was injected intravenously 10 min before cremaster exteriorization. In some experiments, neutrophils isolated from WT or *Osm*$^{-/-}$ bone marrow leukocytes were labeled with red fluorescence dye (PKH26) or far red fluorescence dye (CellVue

Claret). Labeled WT cells, *Osm*$^{-/-}$ cells, or a 1:1 mixture of WT and *Osm*$^{-/-}$ cells (5 x 10^6 labeled cells of each genotype in 200 μl saline) were injected into *Osm*$^{-/-}$ mice intravenously just before exteriorization of the cremaster muscle. The vessels were stained by intravenous injection of 5 μg Alexa Fluor 488-labeled anti-CD31 mAb 1 h before exteriorization of the cremaster muscle. In all experiments, leukocyte rolling and adhesion were recorded in 3 to 5 venules from each mouse. Microvessel diameters, length, and centerline velocity were comparable in mice from all genotypes. Mean leukocyte rolling velocities and rolling fluxes were analyzed offline.

Bone marrow transplantation

Bone marrow transplantation was performed as described[5]. Briefly, bone marrow cells from WT or *Osm*$^{-/-}$ mice were isolated, and 2 x 10^6 cells were injected intravenously into lethally irradiated *Osm*$^{-/-}$ or WT mice, respectively. After 8 weeks, reconstitution of hematopoietic cells was evaluated by intracellular staining of OSM in Ly6G-positive neutrophils from peripheral blood collected from facial vein.

Flow chamber assay

Rolling of human neutrophils on confluent monolayers of HUVEC or on immobilized human P-selectin was performed as described[1,20]. Neutrophils (10^6/mL in HBSS containing 0.5% human serum albumin) were perfused in flow chambers at a wall shear stress of 1 dyne/cm^2 in the presence of 10 μg/ml mAb to human gp130 or OSM. Histamine (0.1 mM) or thrombin (1 U/ml) was introduced 4 min after perfusion was initiated. In some experiments, HUVEC were pre-treated with 10 μg/ml anti-human gp130 mAb. In other experiments, 2 x 10^6 neutrophils, pre-fixed with 1% paraformaldehyde for 10 min, were suspended in hypertonic medium (HBSS plus 0.5% human serum albumin containing 0.45 M sucrose) or control isotonic medium (without 0.45 M sucrose), with or without 10 ng/ml OSM. They were then perfused as described

above. Rolling of mouse bone marrow neutrophils on confluent monolayers of MLEC was performed similarly. To mobilize P-selectin to the cell surface, MLEC were preincubated with 10 μM calcium ionophore A23187 for 30 min and then washed with HBSS containing 0.5% human serum albumin. Bone marrow neutrophils (2 x 10^6/mL in HBSS containing 0.5% human serum albumin) were perfused in flow chambers at a wall shear stress of 1 dyne/cm^2 in the presence of 10 μg/ml mAb to mouse gp130, OSM, or P-selectin. Rolling, arrest, and spreading of mouse bone marrow neutrophils on immobilized P- or E-selectin with or without co-immobilized ICAM-1 and CXCL1 was performed as described[3].

Site density measurement

HUVEC were incubated for 10 min in the presence or absence of 0.1 mM histamine or 10 ng/ml exogenous human OSM. The surface density of P-selectin on HUVEC was measured using ^{125}I-labeled mAb S12 as described[20].

Internalization assay

HUVEC were incubated for 10 min in the presence or absence of 0.1 mM histamine or 10 ng/ml exogenous human OSM. The rate of internalization of P-selectin was measured by the ability of an acidic buffer to remove ^{125}I-labeled mAb G1, prebound to the cell surface at 4˚C, after warming to 37˚C for various intervals[20]. The cell-bound radioactivity remaining at each time point represented the amount of internalized P-selectin and was presented as a percentage of the initial cell-bound radioactivity.

Immunofluorescence confocal microscopy

HUVEC were incubated for 10 min in the presence or absence of 0.1 mM histamine or 10 ng/ml exogenous human OSM. The cells were then fixed and permeabilized, and confocal

microscopy was used to measure colocalization of cell surface P-selectin with α-adaptin as described[1]. In some experiments, nuclei were stained with DAPI.

Deep vein thrombosis

Deep vein thrombosis induced by flow restriction of the IVC was performed as described[21,22]. Mice were placed under anesthesia by continuous inhalation of 1-2% isoflurane in 100% oxygen. Intestines were exteriorized and covered with saline-moistened gauze to prevent drying after laparotomy. The IVC was gently separated and permanently ligated over a 30-gauge needle with a 7.0 nylon, non-absorbable suture (Braintree Scientific, Inc.). The needle spacer was then removed to prevent complete vein occlusion. Side branches were not ligated. Control mice underwent sham surgery without occlusion of the IVC. The peritoneum and skin were then closed.

Ultrasonography was performed with a Vevo 2100 system with 40 MHz mouse scan head (VisualSonics) to confirm flow restriction and to monitor thrombus progression, including the frequency and size of the thrombus. Color Doppler mode or pulse-wave Doppler mode was used for measuring the flow direction or the flow velocity, respectively.

In some mice, spinning-disk intravital microscopy of the IVC was performed as described[21,23]. The IVC was superfused with thermocontrolled (35˚C) bicarbonate-buffered saline 3 h after occlusion. One hour before microscopy, 4 μg PE-conjugated anti-Ly6G mAb or 4 μg PE-conjugated anti-mouse M-CSFR mAb in 200 μl saline was injected through the retro-orbital venous plexus. The PE-labeled neutrophils or monocytes in the IVC were observed 1 mm below the ligation in the experimental group or 1-2 mm below the left renal vein in sham-surgery control mice. Rolling or adherent cells were defined as described[21].

Thrombi, collected from mice sacrificed 24 h after ligation, were used for measurement of weight, for flow cytometry, and for Western blots.

Statistical analysis

Statistical differences between groups were analyzed using the unpaired and two-sided tail Student's *t*-test or with 1-way analysis of variance with the post hoc multiple-comparison test. Thrombus frequencies were analyzed using X^2 tests of contingency tables. Values were considered significant at $P < 0.05$.

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Table S1

Genotype	Total leukocytes	Neutrophils	Lymphocytes	Monocytes	Red blood cells	Hemoglobin	Platelets
	$x10^3/\mu l$	$x10^3/\mu l$	$x10^3/\mu l$	$x10^3/\mu l$	$x10^6/\mu l$	g/dl	$x10^6/\mu l$
WT	4.5 ± 0.5	1.1 ± 0.1	3.4 ± 0.5	0.1 ± 0.0	9.5 ± 1.0	12 ± 2.0	1.0 ± 0.1
Osm$^{-/-}$	6.5 ± 0.4*	1.4 ± 0.2	5.1 ± 0.7*	0.1 ± 0.0	7.5 ± 1.0*	8.5 ± 2.0	0.7 ± 0.1*
WT *to Osm*$^{-/-}$	4.3 ± 0.3	0.9 ± 0.2	3.2 ± 0.5	0.1 ± 0.1	9.0 ± 1.5	11 ± 1.5	0.9 ± 0.1
Osm$^{-/-}$ to WT	6.3 ± 0.5*	1.3 ± 0.3	5.0 ± 0.3*	0.1 ± 0.1	7.1 ± 1.2*	8.3 ± 1.0	0.7 ± 0.1*
WT/tamoxifen	4.0 ± 0.5	0.8 ± 0.1	3.1 ± 0.5	0.1 ± 0.0	9.2 ± 1.1	11 ± 2.5	0.9 ± 0.1
gp130$^{flox/flox}$VECad-Cre-ER^{T2} /tamoxifen	4.1 ± 0.7	1.0 ± 0.1	3.0 ± 0.4	0.1 ± 0.0	9.0 ± 1.0	10 ± 1.5	1.1 ± 0.1

WT *to Osm*$^{-/-}$: Bone marrow cells from WT mice were transplanted into irradiated *Osm*$^{-/-}$ mice.

Osm$^{-/-}$ to WT: Bone marrow cells from *Osm*$^{-/-}$ mice were transplanted into irradiated WT mice.

WT/tamoxifen and *gp130*$^{flox/flox}$VECad-Cre-ER^{T2}/tamoxifen: Blood counts were measured 10 days after completion of tamoxifen treatment.

The data represent the mean ± SEM from 15-40 mice in each genotype. * $P < 0.05$ vs. WT.

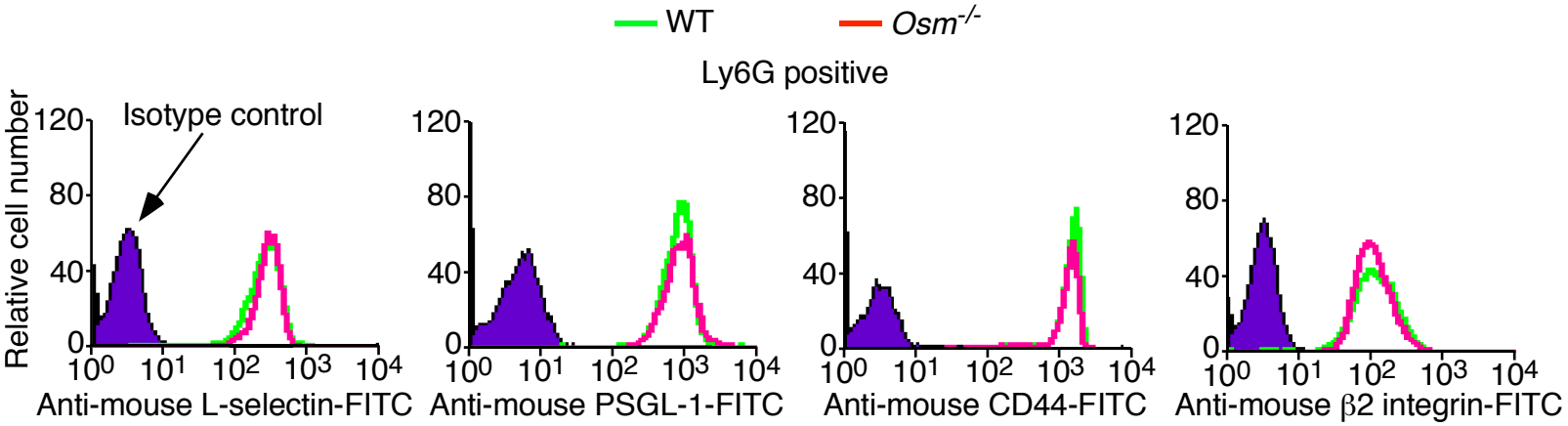
Supplemental figure legends

Fig. S1. **Neutrophils from *Osm*$^{-/-}$ mice express normal levels of PSGL-1, β2 integrins, CD44, and L-selectin.** Ly6G-positive peripheral blood neutrophils from WT or *Osm*$^{-/-}$ mice were stained with the indicated antibody and analyzed by flow cytometry. The data are representative of 3 experiments.

Fig. S2. **Neutrophils from *Osm*$^{-/-}$ mice have normal selectin- and chemokine-induced integrin activation under flow.** (**A and C**) Rolling velocities of neutrophils of the indicated genotype on P- or E-selectin with or without co-immobilized ICAM-1 in the presence or absence of anti-ICAM-1 mAb. (**B and D**) Percentages of neutrophils of the indicated genotype rolling, arrested and round, or arrested and spread on co-immobilized P- or E-selectin, ICAM-1, and CXCL1. The data represent the mean ± SEM from 3 experiments, with 3 mice in each experimental group.

Fig. S3. **Tamoxifen treatment does not alter gp130 expression in hematopoietic cells of *gp130*$^{flox/flox}$VECad-Cre-ER^{T2} mice.** Cells from the indicated genotype were stained with isotope control IgG or with anti-gp130 mAb. Each cell population was defined by the indicated surface marker and by light scatter during flow cytometry. (**A**) Ly6G-positive neutrophils. (**B**) M-CSFR-positive monocytes. (**C**) CD3-positive lymphocytes. The data are representative of 3 experiments.

Figure S1


WT
Osm-/-
Ly6G positive
Relative cell number
Isotype control
120
80
40
0
10^0 10^1 10^2 10^3 10^4
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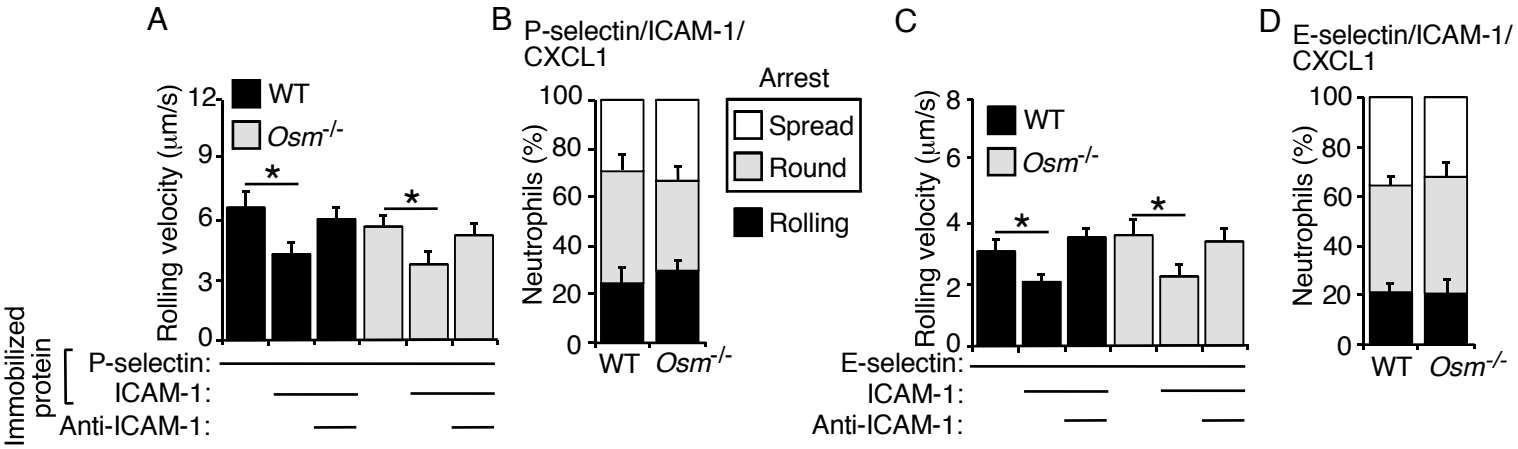
Anti-mouse PSGL-1-FITC
Anti-mouse CD44-FITC
Anti-mouse β2 integrin-FITC

Figure S2


A
B
P-selectin/ICAM-1/
CXCL1
C
D
E-selectin/ICAM-1/
CXCL1
WT
Osm-/-
Rolling velocity (μm/s)
Neutrophils (%)
Arrest
Spread
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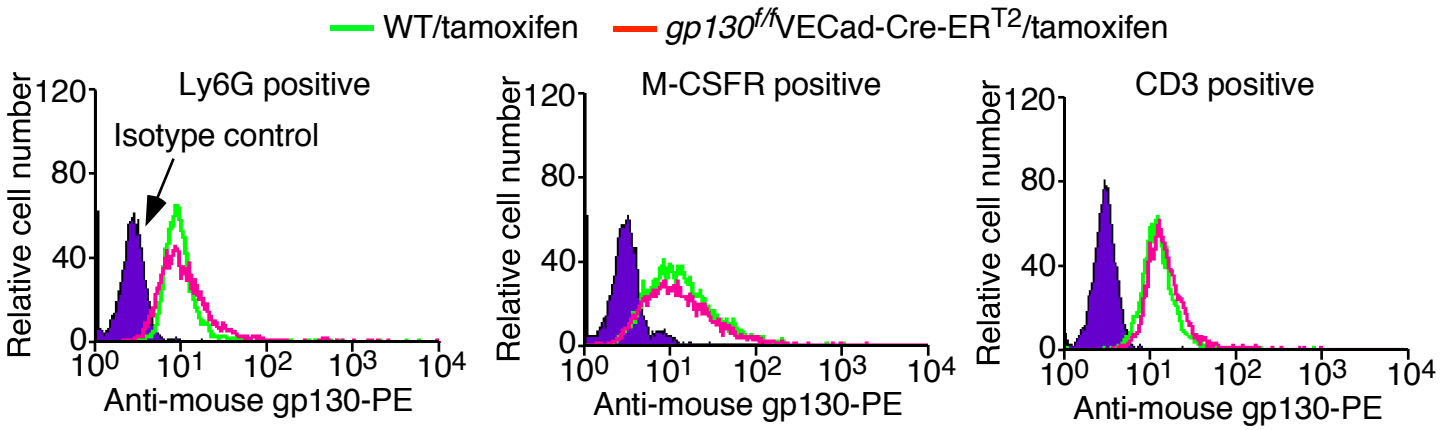
Rolling
Immobilized protein
P-selectin:
ICAM-1:
Anti-ICAM-1:
E-selectin:
*

Figure S3


WT/tamoxifen
gp130f/fVECad-Cre-ERT2/tamoxifen
Ly6G positive
Isotype control
M-CSFR positive
CD3 positive
Relative cell number
120
80
40
0
100
101
102
103
104
Anti-mouse gp130-PE