PSGL-1 is highly expressed on Ly-6C^{hi} monocytes and a major determinant for Ly-6C^{hi} monocytes recruitment to sites of atherosclerosis in mice

Guangyu An, MD, PhD; Huan Wang, MD, PhD; Rong Tang, MD, PhD; Tadayuki Yago, MD, PhD; J. Michael McDaniel, MS; Samuel McGee, BS; Yuqing Huo, MD, PhD; Lijun Xia, MD, PhD

Online Data Supplements

METHODS

Mice

PSGL-1$^{-/-}$ mice were generated as described.[1] *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ double-deficient mice were generated after crossing *PSGL-1*$^{-/-}$ mice with *ApoE*$^{-/-}$ mice (The Jackson Laboratory, Bar Harbor, ME). WT C57BL/6J mice were purchased from the Jackson Laboratory. Mice were kept in a specific pathogen-free facility, and all mouse experiments were performed in compliance with protocols approved by the Institutional Animal Care and Use Committees of the Oklahoma Medical Research Foundation and the University of Minnesota.

Flow cytometry

All antibodies were obtained from BD Biosciences (San Diego, CA) unless otherwise specified. Peripheral blood collected through the retro-orbital bleeding of mice was used for monocyte analysis. After lysis of red blood cells (RBC) with RBC lysis solution containing 150 mmol/L, NH_4Cl, 10 mmol/L $NaHCO_3$, 1 mmol/LEDTA, the leukocytes were incubated with a cocktail of monoclonal antibodies (mAbs) to T cells (CD90-PE, 53-2.1), B cells (B220-PE, RA3-6B2), natural killer (NK) cells (CD49b-PE, DX5 and NK1.1-PE, PK136), granulocytes (Ly-6G–PE, 1A8), and myeloid cells (CD11b-APC, M1/70).[2] Cells that were positive for CD11b but negative for the rest

of the lineage markers were defined as monocytes. Biotinylated anti-Ly-6C mAb (AL-21), which was detected with streptavidin-PE Texas Red, was used to classify monocytes into subsets with either high expression of Ly-6C (Ly-6C^{hi}) or low expression of Ly-6C (Ly-6C^{lo}). A rat anti-mouse PSGL-1 mAb (4RA10) and FITC-labeled anti-rat IgG were used to measure PSGL-1 expression. A rat anti-CCR2 (gift from Dr. Matthias Mack, University of Munich, Germany) followed by FITC-labeled a goat anti-rat antibody was used to measure CCR2 expression. FITC-labeled mAbs to L-selectin, CD44, CD18, or CD49d were used to analyze the expression of these molecules. In all cases appropriate isotype controls were used. CD16/CD32 (2.4G2) was used as an Fc blocker where appropriate.

For the P- and E-selectin binding assay, leukocytes were incubated with murine soluble P- or E-selectin/human IgM chimera.[3] The bound P-selectin and E-selectin were then detected with FITC-labeled goat anti-human IgM (Chemicon International, Temecula, CA). CD45/human IgM chimera (CD45/IgM) was used as the negative control. PSGL-1-dependent interactions were examined in the presence of mAb 4RA10 that blocks PSGL-1 function.

To examine PSGL-1 expression and P- and E-selectin binding of CX3CR1^{lo}CD11b^{+} monocytes, peripheral blood from C57BL/6 *CX3CR1*$^{GFP/+}$ mice was used.[4] CD11b^{+} and GFP-expressing monocytes were analyzed for PSGL-1 expression and P- and E-selectin binding as described above.

Human peripheral blood samples were stained with FITC-conjugated anti-CD16, PerCp-conjugated anti-HLA-DR, APC-conjugated anti-CD14 and PE-conjugated anti-PSGL-1 mAbs. Cells that were HLA-DR and CD14 double-positive, or HLA-DR and CD16 double-positive, were gated as human inflammatory and resident monocytes, respectively. PSGL-1 expression was analyzed based on these double-positive cells. The protocol was approved by the IRB committee of the University of Minnesota.

Incubations were performed on ice for 20 minutes and were followed by three washes with Hanks' balanced salt solution (HBSS, Mediatech, Herndon, VA). Flow

cytometry was perfomed on a FACSCalibur (BD Biosciences). Data were analyzed using Summit Software v4.3 (Dako, Carpinteria, CA).

Real-time PCR

Total RNA from Ly6C^{hi} and Ly6C^{lo} monocytes was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using the SuperScript First-Strand Synthesis System kit (Invitrogen). The following primers were used: PSGL-1 (forward, 5'-TGAGGCAGAGTCGTTTGCTTC-3; reverse, 5'-CCAAGATGGTCAGCAGCACA-3'), FTVII (forward, 5'-CTACAGTTCAAGGGTACCAC-3'; reverse, 5'-TAAGGATGGTGAGTGTGGAC-3'), C2GlcNAcT-I (forward, 5'-TCTAAACGTGATATCTTGTCCC-3'; reverse, 5'-GAACATTCAGAGGCTTCCTG-3'), and β-actin (forward, 5'-TGAGAGGGAAATCGTGCGTGAC-3', reverse, 5'-GAGGAAGAGGATGCGGCAGTG-3'). PCR was performed on an ABI Prism 7000 spectrofluorometric thermal cycler (Applied Biosystems, Foster City, CA) using SYBR-green as a double-strand DNA–specific binding dye. The relative amount of each gene in each sample was estimated by the $\Delta\Delta C_T$ method according to manufacturer's protocol. Each sample was assayed in triplicate.

In vitro flow chamber assay

To obtain a sufficient number of cells for this assay, Ly-6C^{hi} and Ly-6C^{lo} monocytes from murine spleens were used. The spleen-derived monocytes are surrogates for circulating monocytes.[2] Spleens from euthanized mice were gently crushed using a 3-ml syringe plunger in HBSS at 4°C. The cells were filtered through a 70-μm nylon mesh (BD Biosciences). As described above, cells were incubated with the cocktail of mAbs against lineage markers and mAbs to CD11b and Ly-6C for 20 minutes on ice. Ly-6C^{hi} and Ly-6C^{lo} monocyte subsets were sorted from the CD11b^{hi}CD90^{lo}B220^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo} population using the inFlux V-GS Cytometer Work Bench (Cytopeia, Seattle, WA).

Flow chamber experiments were carried out as described.[3, 5] Briefly, goat antibody to the Fc portion of human IgM (10 μg/mL, CALTAG, Burlington, CA), streptavidin (100 μg/mL, Pierce, Rockford, IL), or goat anti-human IgG Fc (50

μg/mL, Chemicon, Pittsburgh, PA) was adsorbed on 35-mm polystyrene dishes for 2 hours at 4°C. The dishes were blocked for 2 hours with 1% human serum albumin (HSA) in HBSS, and then murine P-selectin-IgM, E-selectin-IgM, control CD45-IgM, biotinylated 2-glycosulfopeptide-6 (2-GSP-6), or human L-selectin IgG chimera was captured on the dishes. 2-GSP-6 is modeled after the NH_2-terminal P-selectin–binding region of PSGL-1.[6] Human L-selectin is the equivalent of murine L-selectin because they share the same binding activity to murine PSGL-1. Site densities were measured by binding of ^{125}I-labeled anti-P-selectin mAb RB40.34 (Dietmar Vestweber, Max-Planck-Institute of Molecular Biomedicine, Munster, Germany), anti-E-selectin mAb 9A9 (Barry Wolitzky, MitoKor, San Diego, CA), or anti-human L-selectin mAb DREG56 (ATCC, Manassas, VA). Sorted Ly-6C^{hi} or Ly-6C^{lo} monocytes (0.5×10^6/mL in HBSS with 0.5% HSA) were perfused over P-selectin, E-selectin, control CD45-IgM, 2-GSP-6, or L-selectin in dishes mounted in a parallel-plate flow chamber at different wall shear stresses. For some experiments, dishes were preincubated with mAbs that block functions of P-selectin (RB40.34), E-selectin (9A9), PSGL-1 (4RA10), or L-selectin (DREG56). Rolling cells were analyzed using a Silicon Graphics workstation (Silicon Graphics, Sunnyvale, CA).

Ex vivo perfusion of murine carotid arteries

As described, *ApoE*$^{-/-}$ mice were fed the Western diet (21.2% fat/weight, 0.2% cholesterol, TD.88137, Harlan Teklad) for 6 weeks. The carotid arteries were isolated and perfused with heparinized MOPS-buffered physiological salt solution supplemented with 1% HSA. Ly-6C^{hi} or Ly-6C^{lo} monocytes were isolated from WT mice or from *PSGL-1*$^{-/-}$ mice as described above. These cells were labeled with calcein AM (Molecular Probes, Eugene, Oregon), and were infused into the carotid arteries at a rate of 10 ml/minute (1×10^6 cells/mL), resulting in a wall shear stress of 3.0 ± 0.1 dyne/cm^2. Cell rolling and adhesion were recorded on videotape by stroboscopic epifluorescence illumination with an intravital microscope (Axioskop, 10× water immersion objective, NA 0.5, Carl Zeiss, Thornwood, NY).

Atherosclerosis and carotid artery wire injury models

To induce atherosclerosis, *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ or *ApoE*$^{-/-}$ mice at 5 weeks of age were fed the Western diet for 12 weeks. The age-matched control mice consumed a regular chow diet during this time.

For the carotid artery wire injury models, 8-week-old male *ApoE*$^{-/-}$ mice were fed the Western diet for 2 weeks and then anesthetized, and carotid artery wire injury was performed on mice as described.[7] Briefly, a 0.014-inch flexible angioplasty guide wire was used. Complete and uniform endothelial denudation was achieved in the left carotid artery by five passes with a rotating motion. After surgery mice were given the Western diet for 4 more weeks, and then euthanized for collection of injured arteries.

Quantification of atherosclerotic lesions

Atherosclerotic lesions were quantified by en face analysis of the whole aorta and by cross-sectional analysis of the proximal aorta as described.[8] Briefly, for en face preparations of the aorta, anesthetized mice were perfused with 4% paraformaldehyde and aortas were collected between the subclavian and iliac branches. After fixation with 4% paraformaldehyde, the aortas were rinsed in 60% isopropanol and stained for 10 minutes in a filtered Oil red O solution (Newcomer supply, Middleton, WI). Stained aortas were digitally photographed. The percentage of lesion areas was determined by Oil red O–covered areas divided by total aorta area. For cross-sectional analysis of the aortas, hearts with ascending aortas were isolated from mice and embedded in OCT compound. Beginning at the aortic valve, 8-μm serial sections of the proximal aorta were made. Sections were stained with Oil red O and counterstained with hematoxylin. For quantification, total area of 10× magnification from eight sections every 40 μm of the aortic sinus from each mouse was analyzed using the NIH Image J software.

For quantitative comparisons of acute neointimal formation, ten 5-μm aortic sections within a standardized distance (1200 μm) from the bifurcation to the common carotid artery that were stained with Movat pentachrome (Sigma, St. Louis, MO) were analyzed for each mouse. The areas of the lumen, internal elastic lamina, and external elastic lamina were determined by using planimetry with the NIH Image software.

Quantitative immunostaining of monocytes/macrophages

Cryosections of aortas from *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice were incubated first with anti-F4/80 mAb (MOMA-2) (Accurate Chemical, Westbury, NY), and subsequently with biotin-conjugated anti-rat IgG, and finally with horseradish peroxidase (HRP)-streptavidin (Vector Laboratories, Burlingame, CA).[9] The sections were developed with a diaminobenzidine substrate and counterstained with hematoxylin. Eight sections every 40 μm from each aorta root were examined microscopically (ECLIPSE E600, 10× objective, NA 0.45, Nikon, Melville, NY). Digital images were used to quantify macrophages with NIH Image J software.

Ly-6C^{hi} monocytes in cryosections (5 μm) of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ carotid arteries were examined using an FITC-conjugated anti-mouse Ly-6C mAb (AL-21, 1:100 dilution, BD Biosciences) .[2] Peripheral Ly-6C^{hi} or Ly-6C^{lo} monocytes, which were cytocentrifuged on glass slides, were stained with the same FITC-conjugated mAb and used as controls for the identification of Ly-6C^{hi} cells. Fluorescence was detected using a fluorescence microscope (4× objective, NA 0.3, Olympus America, Center Valley, PA).

Statistical analyses

Mean and SEM are reported where appropriate. Data were analyzed by the Student's t test. $P < 0.05$ was considered significant.

References

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LEGENDS TO SUPPLEMENTAL FIGURES

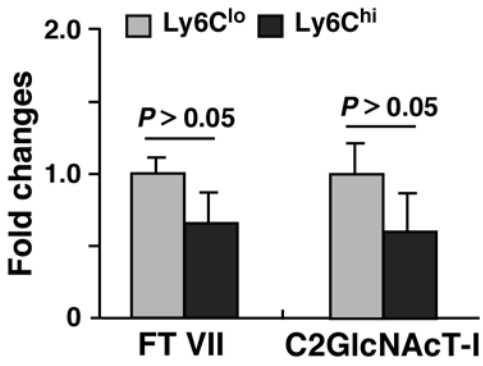
Supplemental figure legends

Figure S1. There is no significant difference in FVTII and C2GlcNAcT-1 expression between Ly-6C^{hi} and Ly-6C^{lo} monocytes. Real-time PCR quantification of FVTII and C2GlcNAcT-I transcripts in Ly-6C^{hi} and Ly-6C^{lo} monocytes (n = 9 mice). Data are means ± SEM.

Figure S2. Ly-6C^{hi} but not Ly-6C^{lo} monocytes roll on 2-GSP-6 under flow. A. Comparison of Ly-6C^{hi} and Ly-6C^{lo} monocytes rolling on 2-GSP-6 at 1.0 dyn/cm^2 with and without the presence of 4RA10, a mAb that blocks PSGL-1 function. B. Ly-6C^{hi} and Ly-6C^{lo} monocytes rolling under different shear stress. Shown are the mean ± SEM of three independent experiments.

Figure S3. *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice have similar plasma lipid levels. Plasma cholesterol and triglyceride concentrations of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice that were fasted overnight after fed the high fat diet for 12 weeks (mean ± SEM, $P > 0.05$, n = 14 mice). Choelsterol was measured using a kit from Wako Diagnostics, Richmond, VA, while triglyceride was measured using reagents from Sigma, according to the manufacturers' instructions.

Fig. S1


Ly6C^lo
Ly6C^hi
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1.0
0
Fold changes
P > 0.05
P > 0.05
FT VII
C2GlcNAcT-I

Fig. S2

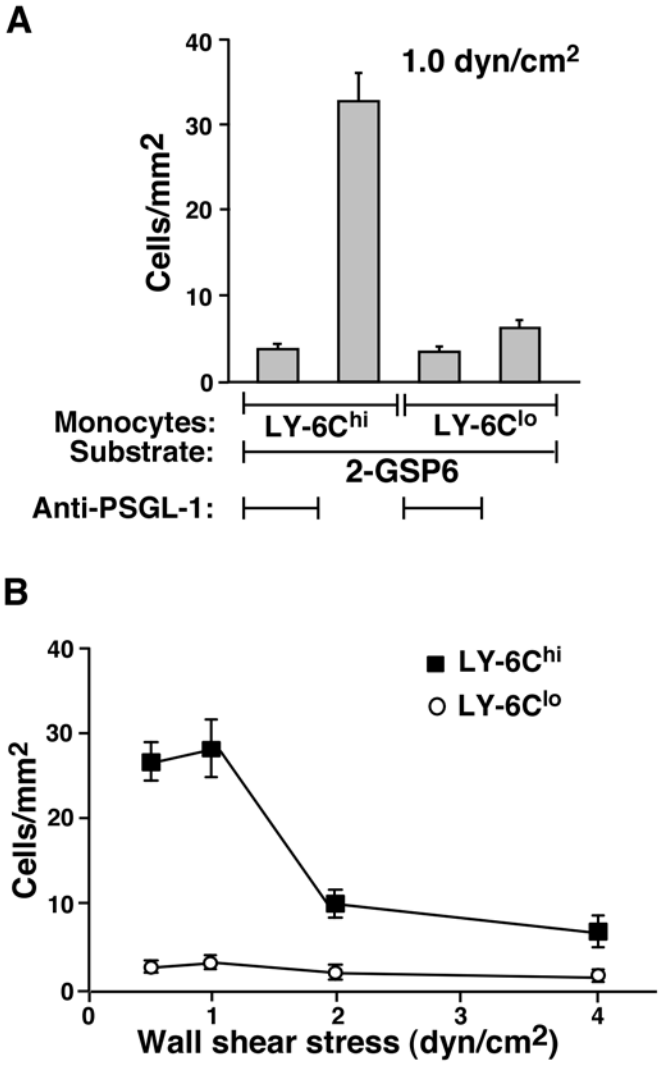
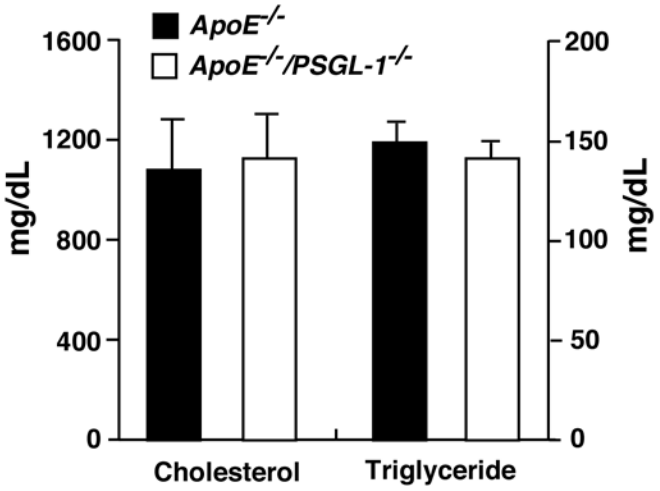

A
1.0 dyn/cm2
Cells/mm2
40
30
20
10
0
Monocytes:
LY-6Chi
LY-6Clo
Substrate:
2-GSP6
Anti-PSGL-1:
B
LY-6Chi
LY-6Clo
Cells/mm2
40
30
20
10
0
0
1
2
3
4
Wall shear stress (dyn/cm2)

Fig. S3


ApoE-/-
ApoE-/-/PSGL-1-/-
1600
1200
800
400
0
mg/dL
200
150
100
50
0
mg/dL
Cholesterol
Triglyceride