P-Selectin Glycoprotein Ligand-1 Is Highly Expressed on Ly-6C^{hi} Monocytes and a Major Determinant for Ly-6C ^{hi} Monocyte Recruitment to Sites of Atherosclerosis in Mice

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P-Selectin Glycoprotein Ligand-1 Is Highly Expressed on Ly-6C^{hi} Monocytes and a Major Determinant for Ly-6C^{hi} Monocyte 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

Background—Ly-6C^{hi} monocytes are key contributors to atherosclerosis in mice. However, the manner in which Ly-6C^{hi} monocytes selectively accumulate in atherosclerotic lesions is largely unknown. Monocyte homing to sites of atherosclerosis is primarily initiated by rolling on P- and E-selectin expressed on endothelium. We hypothesize that P-selectin glycoprotein ligand-1 (PSGL-1), the common ligand of P- and E-selectin on leukocytes, contributes to the preferential homing of Ly-6C^{hi} monocytes to atherosclerotic lesions.

Methods and Results—To test this hypothesis, we examined the expression and function of PSGL-1 on Ly-6C^{hi} and Ly-6C^{lo} monocytes from wild-type mice, *ApoE*$^{-/-}$ mice, and mice lacking both *ApoE* and *PSGL-1* genes (*ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$). We found that Ly-6C^{hi} monocytes expressed a higher level of PSGL-1 and had enhanced binding to fluid-phase P- and E-selectin compared with Ly-6C^{lo} monocytes. Under in vitro flow conditions, more Ly-6C^{hi} monocytes rolled on P-, E-, and L-selectin at slower velocities than Ly-6C^{lo} cells. In an ex vivo perfused carotid artery model, Ly-6C^{hi} monocytes interacted preferentially with atherosclerotic endothelium compared with Ly-6C^{lo} monocytes in a PSGL-1–dependent manner. In vivo, *ApoE*$^{-/-}$ mice lacking PSGL-1 had impaired Ly-6C^{hi} monocyte recruitment to atherosclerotic lesions. Moreover, *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ mice exhibited significantly reduced monocyte infiltration in wire injury–induced neointima and in atherosclerotic lesions. *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ mice also developed smaller neointima and atherosclerotic plaques.

Conclusions—These data indicate that PSGL-1 is a new marker for Ly-6C^{hi} monocytes and a major determinant for Ly-6C^{hi} cell recruitment to sites of atherosclerosis in mice. (***Circulation*. 2008;117:3227-3237.)**

Key Words: atherosclerosis ■ cell adhesion molecules ■ endothelium ■ leukocytes

Atherosclerosis is characterized by inflammatory cell infiltration of the arterial wall.[1,2] Early atherosclerotic lesions are largely composed of lipid-laden macrophages known as foam cells, which are derived from circulating monocytes.[1,2] Thus, the recruitment of monocytes into the arterial wall plays decisive roles in the initiation and progression of atherosclerosis.

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In mice, monocytes are divided into Ly-6C^{hi} and Ly-6C^{lo} subsets. Ly-6C^{hi} monocytes are considered short-lived "inflammatory" cells that are actively recruited to inflamed tissues, whereas Ly-6C^{lo} monocytes have a longer half-life and home to noninflamed tissues to differentiate into resident macrophages.[3] Ly-6C^{hi} monocytes are preferentially recruited to atherosclerotic plagues and give rise to lipid-laden macrophages in apolipoprotein E–deficient (*ApoE*$^{-/-}$) mice.[4] Ly-6C^{hi} monocytes are recognized as the key monocyte subset in the development of atherosclerosis,[4,5] but the manner in which these monocytes selectively accumulate in atherosclerotic lesions is largely unknown.

During inflammation, monocyte recruitment follows the well-defined leukocyte trafficking cascade.[6–8] Circulating

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From the Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City (G.A., T.Y., J.M.M., S.M., L.X.); Department of Medicine, University of Minnesota Medical School, Minneapolis (H.W., R.T., Y.H.); and Department of Biochemistry and Molecular Biology and Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City (L.X.).

*Drs An and Wang contributed equally to this work.

The online-only Data Supplement, which contains Methods and figures, is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.771048/DC1.

Correspondence to Lijun Xia, MD, PhD, Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, 825 NE 13th St, Oklahoma City, OK 73104 (e-mail Lijun-Xia@omrf.org) or Yuqing Huo, MD, PhD, Department of Medicine, University of Minnesota Medical School, Minneapolis, MN 55455 (e-mail yuqing@umn.edu).

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leukocytes first tether to and roll on the activated endothelium, then firmly adhere and eventually transmigrate into the underlying tissues. These steps are regulated by adhesion molecules and chemokines. Murine Ly-6C^{hi} and Ly-6C^{lo} monocytes differ with regard to their expression of chemokine receptors and adhesion molecules. Ly-6C^{hi} monocytes are CCR2^{+}, CX3CR1^{lo}, L-selectin^{+}, CD44^{+}, LFA1^{+}, and VLA4^{+}, whereas Ly-6C^{lo} cells are CCR2^{-}, CX3CR1^{hi}, L-selectin^{-}, CD44^{+}, LFA1^{2+}, and VLA4^{+}.[3] Interestingly, only CCR2 and L-selectin are differentially expressed on Ly-6C^{hi} cells.[3,4] Although CCR2 is important for monocytes to enter atherosclerotic lesions,[5,9] it does not mediate the early adhesion steps such as tethering and initial rolling.[5,10] In blood flow conditions, especially arterial flow conditions, the initial steps serve as an anchoring system to capture flowing leukocytes and initiate rolling on the endothelium for subsequent firm adhesion and transmigration. The initial steps are primarily mediated by P-, E-, and L-selectin and their common ligand on leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1).[7] E-selectin and P-selectin are expressed on activated endothelial cells and/or platelets, whereas L-selectin is expressed on leukocytes. PSGL-1 binds to selectins with exceptionally high on- and off-rates, which favors interactions under flow. Moreover, shear stress actually strengthens the interactions of PSGL-1 with P- and L-selectin.[7,11] Ly-6C^{hi} cells exhibit an advantage over the Ly-6C^{lo} subset with regard to homing to atherosclerotic plagues.[4,5] This preferential homing could be a result of differential expression or function of PSGL-1. In addition, L-selectin–mediated rolling may also contribute to this difference because L-selectin is expressed on Ly-6C^{hi} but not on Ly-6C^{lo} monocytes.

To test this hypothesis, we examined the surface expression and function of PSGL-1 on Ly-6C^{hi} and Ly-6C^{lo} monocytes from wild-type (WT) and *ApoE*$^{-/-}$ mice. We also investigated PSGL-1–dependent interactions of Ly-6C^{hi} or Ly-6C^{lo} monocytes with P-, E-, and L-selectin under in vitro flow conditions and with early atherosclerotic endothelium using ex vivo perfusion of carotid arteries. To evaluate the role of PSGL-1 in the development of atherosclerosis, we studied atherosclerosis and wire injury–induced neointimal formation in the carotid artery in *ApoE*$^{-/-}$ mice and in mice lacking both *ApoE* and *PSGL-1* genes (*ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$). Our results demonstrate that PSGL-1 is highly expressed on Ly-6C^{hi} monocytes and is a major determinant for Ly-6C^{hi} monocyte recruitment to atherosclerotic lesions.

Methods

A full description of all methods can be found in the online-only Data Supplement.

Mice

ApoE$^{-/-}$/*PSGL-1*$^{-/-}$-deficient mice (C57BL/6J background) were generated after crossing *PSGL-1*$^{-/-}$ mice[12] with *ApoE*$^{-/-}$ mice. WT C57BL/6J and *ApoE*$^{-/-}$ mice were from the Jackson Laboratory (Bar Harbor, Me). Mice were kept in a specific pathogen-free facility. All mouse experiments were 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, Calif) unless specified otherwise. Mouse peripheral blood was used for monocyte analysis. Leukocytes that were positive for myeloid marker CD11b but negative for the rest of the lineage markers were defined as monocytes.[4] Anti-Ly-6C monoclonal antibody (mAb) (AL-21) 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}).[4] A rat anti-mouse PSGL-1 mAb (4RA10) was used to measure PSGL-1 expression.

The chemokine receptor CX3CR1 was originally used to classify monocytes.[3] In mice, the CX3CR1^{lo}CD11b^{+} monocyte subset is the equivalent of Ly-6C^{hi} monocytes.[3] To examine PSGL-1 expression and P- and E-selectin binding of CX3CR1^{lo}CD11b^{+} monocytes, C57BL/6 *CX3CR1*$^{GFP/+}$ mice were used. In *CX3CR1*$^{GFP/+}$ mice, 1 *CX3CR1* allele was replaced with the gene encoding green fluorescent protein (GFP).[13]

Human monocytes have CD14^{+}CD16^{-} and CD14^{-}CD16^{+} subsets, which resemble murine Ly-6C^{hi}CX3CR1^{lo} and Ly-6C^{lo}CX3CR1^{hi} subsets, respectively.[3] Human leukocytes that were HLA-DR and CD14 double-positive or HLA-DR and CD16 double-positive were analyzed for PSGL-1 expression with a mAb to human PSGL-1 (KPL1). The protocol was approved by the institutional review board committee of the University of Minnesota.

Flow cytometry was performed on a FACSCalibur (BD Biosciences). Data were analyzed with Summit Software v4.3 (Dako, Carpinteria, Calif).

In Vitro Flow Chamber Assay

To obtain sufficient cells, Ly-6C^{hi} and Ly-6C^{lo} monocytes from murine spleens were used. The spleen-derived monocytes are surrogates for circulating monocytes.[4] Ly-6C^{hi} and Ly-6C^{lo} monocytes were sorted from the monocyte population with the use of the inFlux V-GS Cytometer Work Bench (Cytopeia, Seattle, Wash).

Flow chamber experiments were performed as described.[11] Briefly, murine P-selectin-IgM, E-selectin-IgM, control CD45-IgM, biotinylated 2-glycosulfopeptide-6 (2-GSP-6; gift from Dr Richard Cummings, Emory University), or human L-selectin IgG chimera was captured on the dishes. 2-GSP-6 is modeled after the NH_2-terminal selectin-binding region of PSGL-1.[14] Human L-selectin is the equivalent of murine L-selectin because they share the same binding activity to murine PSGL-1. Sorted Ly-6C^{hi} or Ly-6C^{lo} monocytes (0.5×10^{6}/mL in Hanks' balanced salt solution with 0.5% human serum albumin) 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. Rolling cells were analyzed with the use of a Silicon Graphics workstation (Silicon Graphics, Sunnyvale, Calif).

Ex Vivo Perfusion of Murine Carotid Arteries

WT or *PSGL-1*$^{-/-}$ Ly-6C^{hi} or Ly-6C^{lo} monocytes were labeled with calcein AM (Molecular Probes, Eugene, Ore) and infused into the *ApoE*$^{-/-}$ carotid arteries at a rate of 10 mL/min (1×10^{6} cells/mL), resulting in a wall shear stress of 3.0 ± 0.1 dyne/cm^{2}.[10] 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.[5] Atherosclerotic lesions stained with Oil Red O were quantified by en face analysis of the whole aorta and by cross-sectional analysis of the proximal aorta.[15] For the carotid artery wire injury models, 8-week-old male *ApoE*$^{-/-}$ mice were fed the Western diet for 2 weeks, and carotid artery wire injury was performed.[16] For quantification of neointimas, ten 5-μm aortic sections that were stained with Movat pentachrome (Sigma, St Louis, Mo) were analyzed for each mouse. Images were analyzed with the use of NIH Image software.[10]

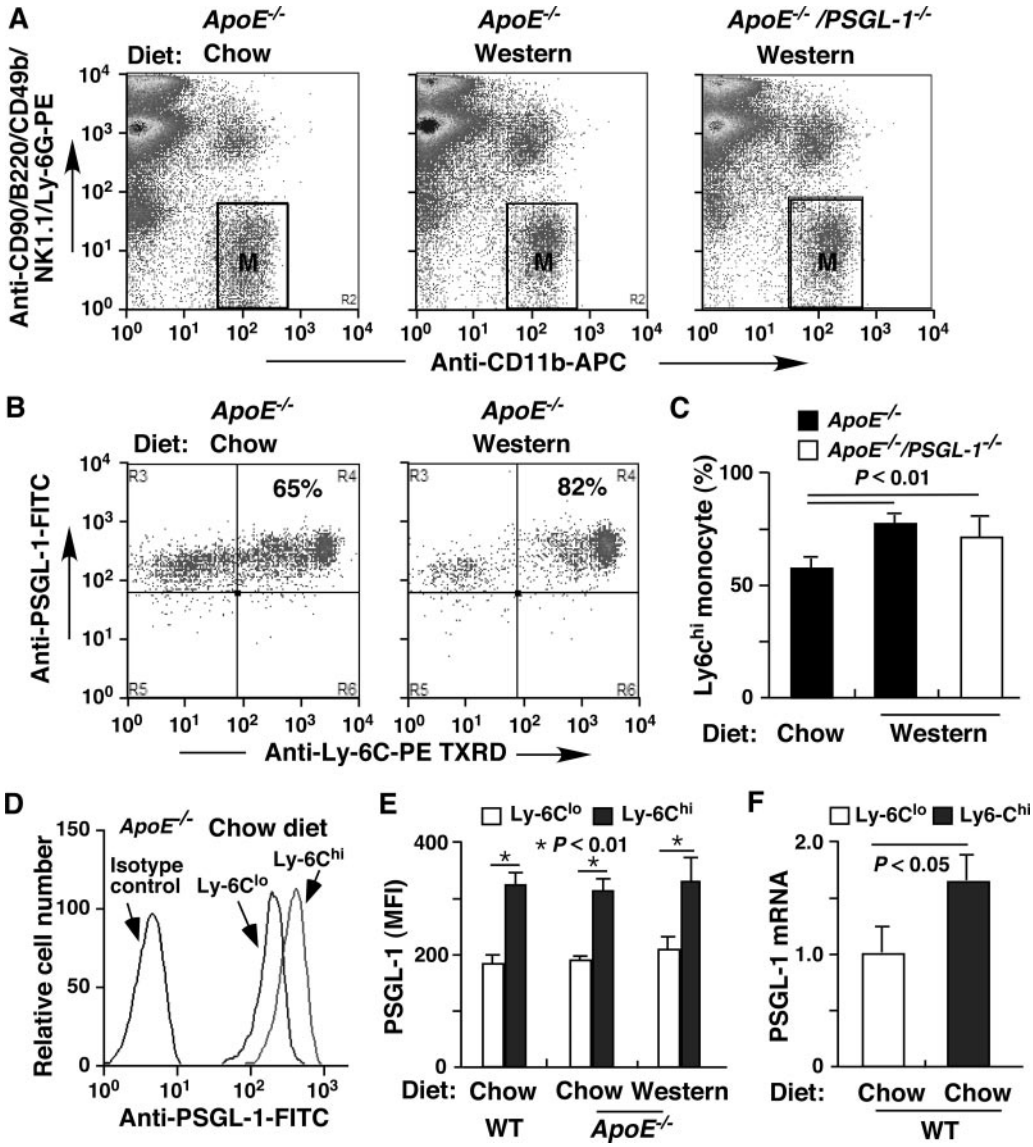

Figure 1. Ly-6C^{hi} monocytes have significantly increased PSGL-1 expression. A, Peripheral murine leukocytes were stained with mAbs to CD90, B220, CD49b, NK1.1, Ly-6G, and CD11b. Cells in gate M were defined as monocytes. B, Representative dot plots showing expression of Ly-6C and PSGL-1 in the total monocytes (gate M). Percentages in the upper right panels represent Ly-6C^{hi}PSGL-1^{hi} monocytes. C, Comparisons of the number of Ly-6C^{hi} monocytes in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ mice on the Western diet with *ApoE*$^{-/-}$ mice fed the chow diet. D, Representative PSGL-1 expression on Ly-6C^{hi} and Ly-6C^{lo} monocytes from an *ApoE*$^{-/-}$ mouse fed the chow diet. E, Quantification of PSGL-1 expression (mean fluorescence intensity [MFI]) on Ly-6C^{hi} and Ly-6C^{lo} monocytes from WT and *ApoE*$^{-/-}$ mice. F, Real-time polymerase chain reaction quantification (fold changes) of PSGL-1 transcripts of Ly-6C^{hi} and Ly-6C^{lo} monocyte subsets from WT mice. The fluorescent intensities of both axes (A and B) or *x* axis (D) are on a log scale. Data are mean±SEM (A to E, n=17 mice per group; F, n=9 mice per group).

Immunostaining

Cryosections of aortas from *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ mice were stained with anti-F4/80 mAb (MOMA-2) (Accurate Chemical, Westbury, NY).[17] Eight sections every 40 μm from each aorta root were examined. Digital images were used to quantify macrophages with NIH Image J software.

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

Statistical Analyses

Mean and SEM values are reported where appropriate. Data were analyzed by the Student *t* test. $P<0.05$ was considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Murine Ly-6C^{hi}CX3CR1^{lo} Monocytes and Human CD14^{+}CD16^{-} Monocytes Express High Levels of PSGL-1

We examined PSGL-1 expression on mouse Ly-6C^{hi} and Ly-6C^{lo} monocytes. We defined monocytes as CD11b^{+}

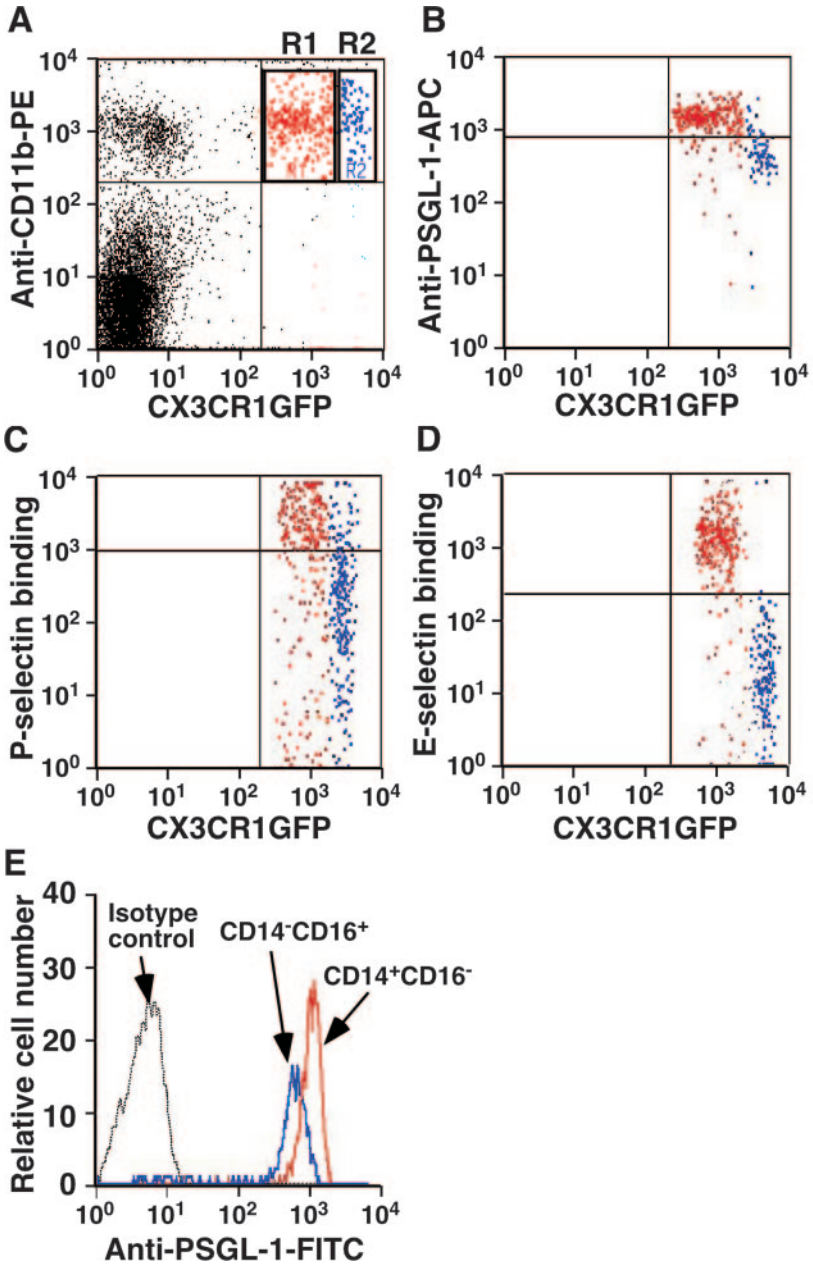

Figure 2. PSGL-1 is highly expressed on murine CX3CR1^{lo}CD11b^{+} monocytes as well as on human CD14^{+}CD16^{-} monocytes. A, Representative dot plot showing CX3CR1^{lo}CD11b^{+} (R1 region, red) and CX3CR1^{hi}CD11b^{+} (R2 region, blue) monocytes. Cells in the gated R1 and R2 regions were examined for expression of PSGL-1 (B) and for interactions with P-selectin (C) and E-selectin (D). Labels on both axes are on a log scale. The data represent at least 3 experiments. E, Representative histogram showing PSGL-1 expression on human CD14^{+}CD16^{-} monocytes. Labels on *x* axis are on a log scale. Results of 3 independent experiments are shown.

CD90^{lo}B220^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo} cells (Figure 1A).[4] Within this monocyte population, cells were classified into Ly-6C^{hi} and Ly-6C^{lo} subsets on the basis of their differential expression of Ly-6C (Figure 1B). The overall monocytes were not significantly different among *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ mice fed the Western diet for 12 weeks and *ApoE*$^{-/-}$ mice fed a standard chow (Figure 1A and data not shown). However, *ApoE*$^{-/-}$ mice fed the Western diet had an elevated number of Ly-6C^{hi} monocytes compared with the number of Ly-6C^{hi} monocytes from *ApoE*$^{-/-}$ mice fed chow (Figure 1B and 1C), which is consistent with published results.[4,5] The percentage of Ly-6C^{hi} monocytes was similar in *ApoE*$^{-/-}$ and in *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ mice fed the high-fat diet (Figure 1C). Remarkably, PSGL-1 was expressed at a much higher level on Ly-6C^{hi} than on Ly-6C^{lo} monocytes from *ApoE*$^{-/-}$ mice fed either chow or the Western diet (Figure 1D and 1E). The increase in PSGL-1 expression was not caused by the ApoE deficiency because Ly-6C^{hi} monocytes from WT mice also expressed more PSGL-1 (Figure 1E). Real-time polymerase chain reaction demonstrated that PSGL-1 mRNA transcripts in Ly-6C^{hi} monocytes were also expressed at a higher level than the transcripts in Ly-6C^{lo} monocytes (Figure 1F).

We confirmed that PSGL-1 was also highly expressed on CX3CR1^{lo}CD11b^{+} monocytes compared with its expression on CX3CR1^{hi}CD11b^{+} monocytes (Figure 2A and 2B). In addition, CX3CR1^{lo}CD11b^{+} monocytes exhibited greater binding to P- and E-selectin (Figure 2C and 2D) compared with CX3CR1^{hi}CD11b^{+} monocytes.

We also investigated PSGL-1 level on human monocytes. Interestingly, consistent with the expression patterns of PSGL-1 on murine monocytes, PSGL-1 level was also higher

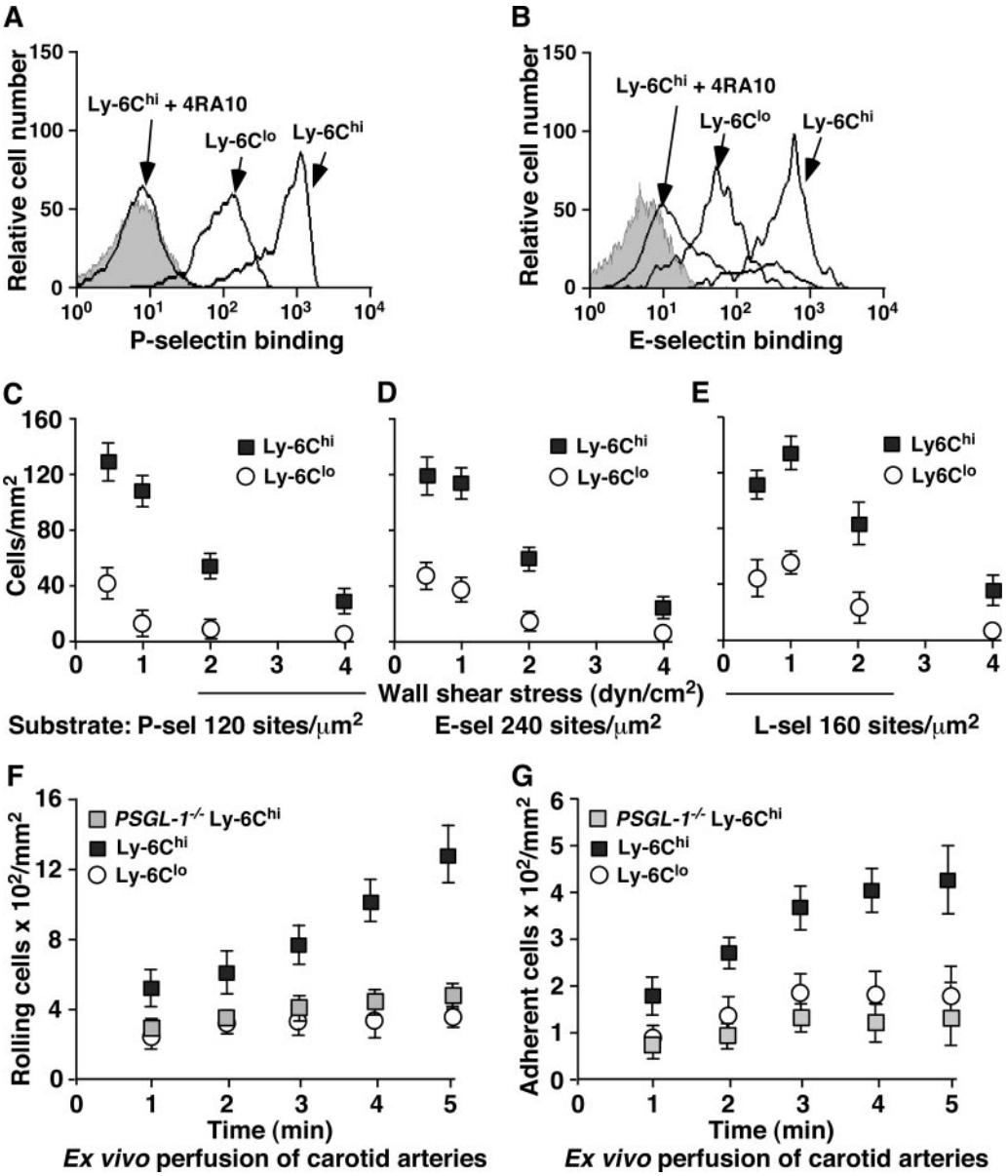

Figure 3. Ly-6C^{hi} monocytes exhibit enhanced rolling on P-, E- and L-selectin under flow and increased PSGL-1–dependent interactions with atherosclerotic endothelium. A and B, Representative histograms compare PSGL-1–dependent P-selectin and E-selectin binding activities of Ly-6C^{hi} and Ly-6C^{lo} monocytes from WT mice fed chow. 4RA10 is a blocking mAb to PSGL-1. Shaded histograms represent isotype controls. The fluorescent intensity of *x* axis is on a log scale. C, D, and E, Accumulation of rolling Ly-6C^{hi} and Ly-6C^{lo} monocytes over P-selectin (P-sel), E-selectin (E-sel), or L-selectin (L-sel). Shown are the mean±SEM values of 3 independent experiments. F and G, Rolling and adhesion of WT Ly-6C^{hi} and Ly-6C^{lo} as well as *PSGL-1*$^{-/-}$ Ly-6C^{hi} monocytes on atherosclerotic endothelium in an ex vivo carotid artery model. Mean±SEM values of 3 independent experiments are shown.

on human CD14$^+$CD16$^-$ monocytes than on CD14$^-$CD16$^+$ monocytes (Figure 2E; n=3; $P<0.01$).

Ly-6C^{hi} Monocytes Interact Preferentially With P-, E-, and L-Selectin as Well as 2-GSP-6 Under Flow

To evaluate the function of PSGL-1 on Ly-6C^{hi} monocytes, we first tested fluid-phase binding of P- and E-selectin to WT monocytes using flow cytometry. Compared with Ly-6C^{lo} monocytes, Ly-6C^{hi} monocytes had greater binding to P- and E-selectin (Figure 3A and 3B). The interaction between PSGL-1 and P-selectin was PSGL-1 dependent because 4RA10, a mAb that blocks PSGL-1 function, eliminated this interaction (Figure 3A). 4RA10 substantially reduced but did not abolish the PSGL-1 interaction with E-selectin (Figure 3B), which reflects E-selectin ligand activities other than PSGL-1 on Ly-6C^{hi} monocytes.[18] Ly-6C^{hi} monocytes from *ApoE*$^{-/-}$ mice fed the Western diet for 12 weeks had similar P- and E-selectin binding profiles (data not shown), suggesting that the 12-week high-fat diet did not significantly alter selectin ligand activities on monocytes. Similar levels of mRNAs of α-1,3-fucosyltransferase VII (FT-VII) and core 2 β1,6-glucosaminyltransferase-I (C2GlcNAcT-I), the protein products of which are essential for PSGL-1 function, were detected in Ly-6C^{hi} and Ly-6C^{lo} monocytes (Figure I in the online-only Data Supplement).

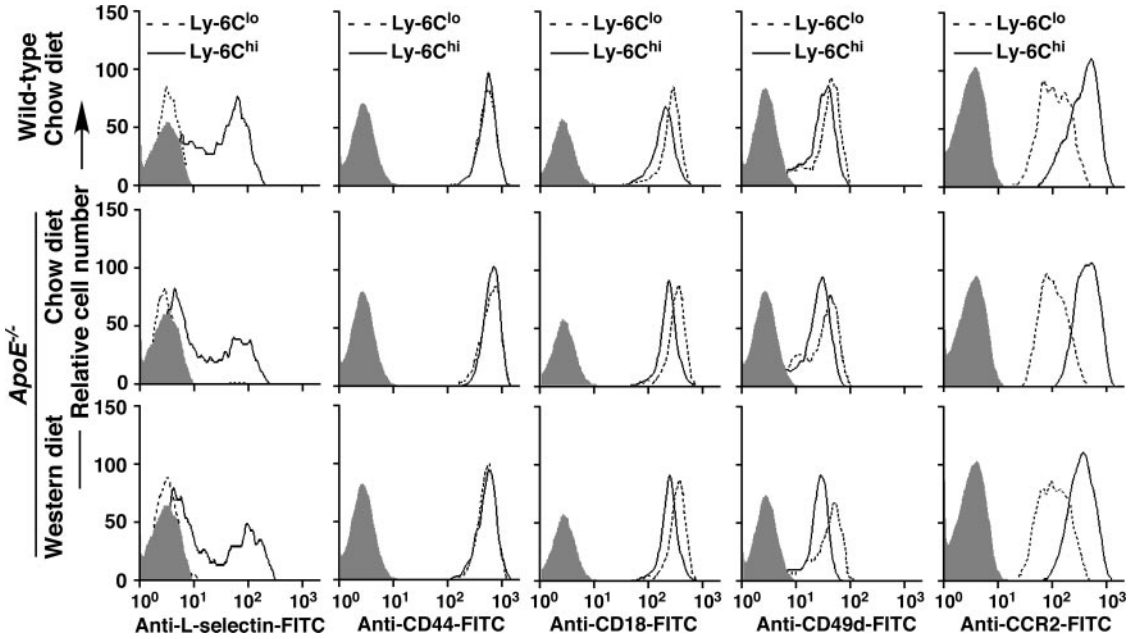

Figure 4. The Western diet does not change the expression profiles of other adhesion molecules or the chemokine receptor CCR2 on Ly-6C^{hi} and Ly-6C^{lo} monocytes. Representative histograms compare expression of L-selectin, CD44, CD18, CD49d, and CCR2 on both Ly-6C^{hi} and Ly-6C^{lo} monocytes from WT or *ApoE*$^{-/-}$ mice. Shaded histograms represent isotype controls. The fluorescent intensity of *x* axis of each histogram is on a log scale. Results of 3 independent experiments are shown.

We then compared the rolling of Ly-6C^{hi} and Ly-6C^{lo} monocytes, respectively, on immobilized P-, E-, or L-selectin under flow conditions. Comparatively more Ly-6C^{hi} than Ly-6C^{lo} monocytes rolled on P-, E-, and L-selectin at low shear stress (0.5 to 1 dyne/cm^2) (Figure 3C, 3D, 3E). Ly-6C^{hi} cells rolled more stably on P- and L-selectin, as manifested by their slow rolling velocities (μm/s; 1.37±0.48 for Ly-6C^{hi} versus 3.55±1.22 for Ly-6C^{lo} on P-selectin; 8.9±3.07 for Ly-6C^{hi} and 117.9±4.38 for Ly-6C^{lo} on L-selectin, at 0.5 dyne/cm^2; $P<0.01$; n=15). No significant difference was found in rolling velocities on E-selectin between Ly-6C^{hi} and Ly-6C^{lo} cells, which is consistent with previous studies showing that PSGL-1 is important for tethering to E-selectin and that other E-selectin ligand(s) such as CD44 other than PSGL-1 contributes to the slow rolling on E-selectin.[12,18] Rolling was PSGL-1 and selectin dependent because mAbs to PSGL-1 and P-, E-, or L-selectin reduced the number of rolling cells to the basal levels observed on the surfaces coated with control reagents (data not shown). Significantly, only Ly-6C^{hi} monocytes rolled on P-, E-, or L-selectin at relatively high shear stress (>2 dyne/cm^2) (Figure 3C, 3D, 3E).

Because L-selectin was preferentially expressed on Ly-6C^{hi} monocytes (Figure 4), we also compared rolling of Ly-6C^{hi} and Ly-6C^{lo} monocytes on immobilized 2-GSP-6, which is modeled after the NH_2-terminal L-selectin–binding region of PSGL-1.[13] At all shear stress levels tested, Ly-6C^{hi} cells rolled on 2-GSP-6 (32±4 cells/mm^2 at 1 dyne/cm^2) (Figure IIA in the online-only Data Supplement). Rolling was PSGL-1 dependent as it was blocked by mAb 4RA10 to PSGL-1 (Figure IIA in the online-only Data Supplement). In contrast, almost no Ly-6C^{lo} rolling on 2-GSP-6 was observed (Figure IIA and IIB in the online-only Data Supplement). This experiment suggests that L-selectin may contribute to the selective homing of Ly-6C^{hi} cells to atherosclerotic lesions.

Ly-6C^{hi} Monocytes Roll Preferentially on Early Atherosclerotic Endothelium in a PSGL-1–Dependent Manner

To test whether Ly-6C^{hi} monocytes have increased capacity to interact with early atherosclerotic endothelium, we used the ex vivo perfusion of *ApoE*$^{-/-}$ carotid artery model.[10] In this model, rolling of monocytes on early atherosclerotic endothelium of *ApoE*$^{-/-}$ mice is mediated primarily by P-selectin and vascular cell adhesion molecule-1.[10,19] Compared with Ly-6C^{lo} monocytes, more Ly-6C^{hi} monocytes rolled on atherosclerotic endothelium at 3 dyne/cm^2 (Figure 3F). Consequently, the number of adherent Ly-6C^{hi} monocytes was increased at all time points compared with Ly-6C^{lo} monocytes (Figure 3G). *PSGL-1*$^{-/-}$ Ly-6C^{hi} monocytes had dramatically reduced rolling and adhesion on atherosclerotic endothelium, indicating primarily PSGL-1–dependent interactions. The observed residual rolling of *PSGL-1*$^{-/-}$ Ly-6C^{hi} monocytes on endothelium may be from interactions between integrin VLA4 and vascular cell adhesion molecule-1.[20]

High-Fat Diet Does Not Change the Expression Profile of Other Adhesion Molecules or the Chemokine Receptor CCR2 on Ly-6C^{hi} Monocytes

To exclude the possibility that the increased recruitment of Ly-6C^{hi} monocytes into atherosclerotic lesions is a result of altered expression of other molecules resulting from consumption of the Western diet, we compared the expression of L-selectin, CD44, CD18 (β2 integrin), CD49d (VLA4), and CCR2 on both Ly-6C^{hi} and Ly-6C^{lo} mono-

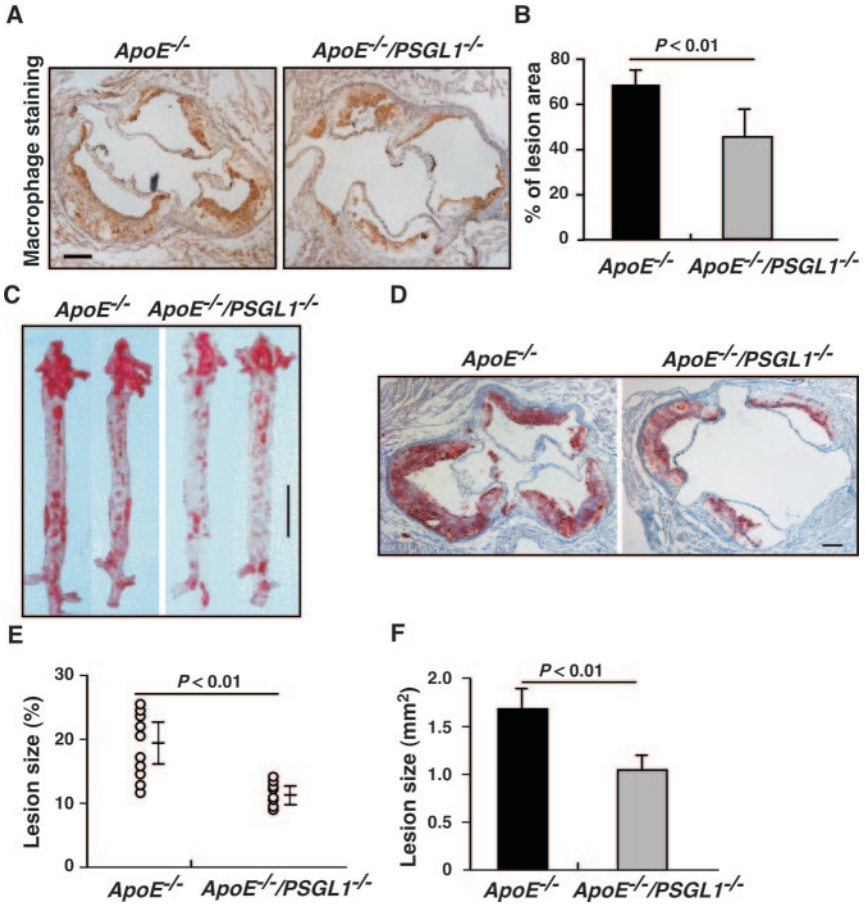

Figure 5. *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice have reduced macrophage accumulation in atherosclerotic lesions and develop smaller atherosclerotic plaques. A, Macrophage staining (light brown color) in aortic root cross sections of atherosclerotic lesions in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice that were fed the Western diet for 12 weeks. B, Quantitative analysis of the macrophages (n=8 mice per group). Data are mean±SEM. C and D, Oil-Red O–stained (red color) en face preparations of aortas (C) and aortic root cross sections (D) from atherosclerotic lesions in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice that were fed the Western diet for 12 weeks. E and F, Quantitative analysis of atherosclerotic lesion area in the entire aorta (E; n=10 mice per group) and aortic root cross sections (F; 8 sections per mouse; n=12 mice per group) with the use of NIH Image J software. Data are mean±SEM. Scale bars: A and D=100 mm; C=1 cm.

cytes. Consistent with previous reports,[3,5] expression of L-selectin and CCR2 was higher on Ly-6C^{hi} than on Ly-6C^{lo} monocytes, whereas CD18 and CD49d were lower on Ly-6C^{hi} than on Ly-6C^{lo} monocytes (Figure 4). Both Ly-6C^{hi} and Ly-6C^{lo} cells expressed a similar level of CD44 (Figure 4). Ly-6C^{hi} or Ly-6C^{lo} monocytes from WT or *ApoE*$^{-/-}$ mice fed either chow or the Western diet for 12 weeks express similar levels of these molecules (Figure 4), suggesting that diet does not affect the expression of these molecules.

ApoE$^{-/-}$*/PSGL-1*$^{-/-}$ Mice Have Reduced Monocyte/Macrophage Accumulation in Atherosclerotic Lesions and Develop Smaller Atherosclerotic Plaques

Ly-6C^{hi} monocytes are key contributors to atherosclerosis.[4] To examine whether PSGL-1 contributes to monocyte recruitment in atherosclerotic lesions and to the development of atherosclerosis, *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice were fed the Western diet for 12 weeks. Compared with *ApoE*$^{-/-}$ mice, *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice exhibited less monocyte/macrophage infiltration in the lesions (Figure 5A and 5B). In addition, the lesions in *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ aortas were significantly smaller than lesions in *ApoE*$^{-/-}$ aortas (Figure 5C, 5D, 5E, 5F). No significant difference was found between *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice in their plasma lipid levels (Figure III in the online-only Data Supplement). These data support the important role of PSGL-1 in mediating Ly-6C^{hi} monocyte recruitment into atherosclerotic lesions and in the development of early atherosclerotic plaques.

Lack of PSGL-1 Results in Impaired Ly-6C^{hi} Monocyte Recruitment to Injured Arterial Walls and Reduces Formation of Neointimal Lesions

Monocytes are critical in arterial neointimal formation.[21] We examined whether PSGL-1 deficiency reduces monocyte infiltration in neointima and the size of the lesions. Our experiments showed that *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice had less monocyte/macrophage infiltration in the neointima of carotid arteries after wire injury than *ApoE*$^{-/-}$ mice (Figure 6A and 6B). To examine Ly-6C^{hi} monocyte homing to injured arteries, cryosections of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ carotid arteries, which were harvested 7 days after wire-induced injury, were stained with an anti-murine Ly-6C mAb as described.[4] In this model, P-selectin expressed by regenerating endothelial cells and adherent platelets contributes to the monocyte recruitment into injured carotid arterial wall.[22] We found that *ApoE*$^{-/-}$ carotid arteries had substantial accumulation of Ly-6C^{hi} monocytes after wire-induced injury (Figure 6C). In contrast, Ly-6C^{hi} monocytes were rarely observed in wire-injured *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ carotid arterial sections (Figure 6D). These data suggest the critical contribution of PSGL-1 to the recruitment of Ly-6C^{hi} monocytes to neointimal lesions.

We also used this model to investigate the role of PSGL-1 in neointima formation. The average size of neointima in the carotid arteries of *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice after wire injury was dramatically reduced compared with that in *ApoE*$^{-/-}$ mice (Figure 6E and 6F), indicating an important role for PSGL-1 in this pathology.

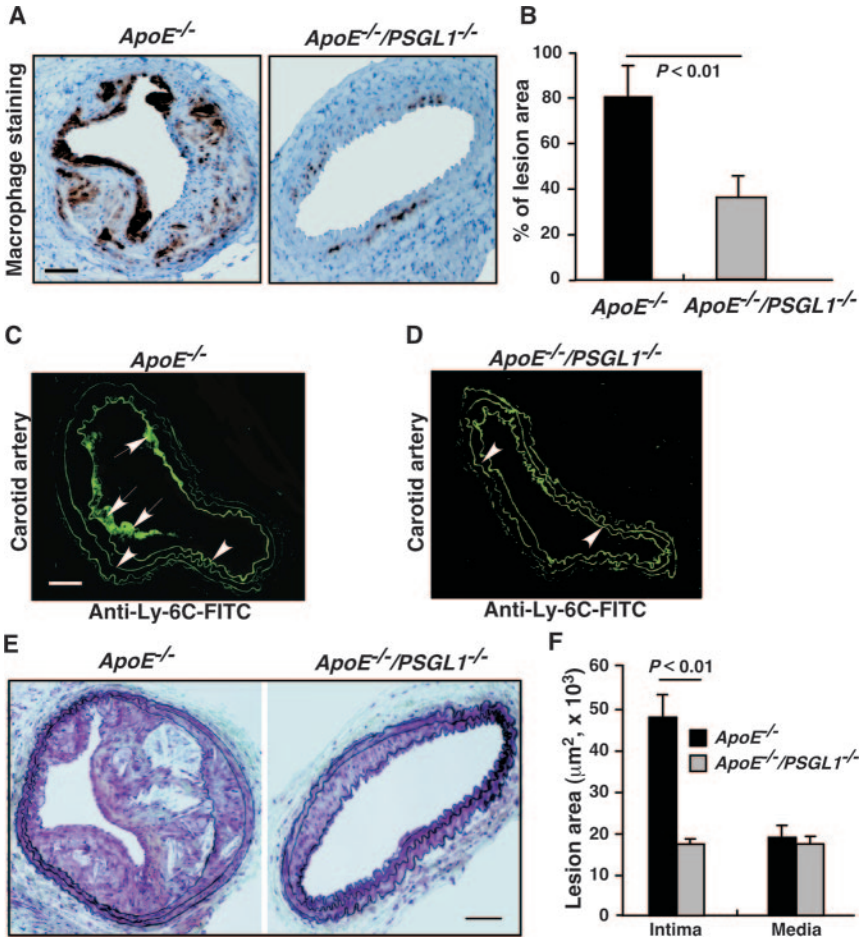

Figure 6. Lack of PSGL-1 results in reduced monocyte infiltration in neointima, impaired Ly-6C–positive monocyte recruitment to injured arterial walls, and reduced formation of neointimal lesions. A, Macrophage staining (dark brown color) in neointima of carotid arteries of *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ mice after wire-induced injury. B, Quantification of the macrophage staining of neointima. Data are mean±SEM (n=8 mice per group). C and D, Representative immunofluorescence staining of Ly-6C–positive monocytes (arrows) of *ApoE*$^{-/-}$ (C) or *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ (D) carotid arterial cryosections after wire-induced injury. Arrowheads indicate autofluorescence of vascular elastic membranes. E, Movat-stained neointimas in *ApoE*$^{-/-}$ and *ApoE*$^{-/-}$/*PSGL-1*$^{-/-}$ carotid arteries after wire injury. F, Quantification of the sizes of neointima and media (n=8). Scale bars: A, C, and E=100 mm.

Discussion

Our results demonstrate that PSGL-1 is highly expressed on both murine Ly-6C^{hi} and human CD14^{+}CD16^{-} monocytes and therefore is a new marker for this subset of inflammatory monocytes. PSGL-1 on Ly-6C^{hi} monocytes interacts with all 3 selectins and contributes significantly to the selective homing of Ly-6C^{hi} monocytes to atherosclerotic lesions.

Selective leukocyte homing to a particular tissue is primarily regulated by unique combinations of adhesion molecules and chemokines.[6,23] Regulated expression of functional PSGL-1 has been recognized as a primary mechanism for the tissue-specific homing of activated T cells.[23] All T cells express a similar level of PSGL-1, yet naive T cells do not interact with P-selectin because their PSGL-1 is not appropriately glycosylated and thus not functional. During activation, T cells acquire functional PSGL-1 on induced expression of glycosyltransferases FTVII and C2GlcNAcT-I, which add selectin-interacting carbohydrate groups to PSGL-1.[7,23–26] We sought to examine whether a similar mechanism regulates selective homing of Ly-6C^{hi} monocytes. Surprisingly, Ly-6C^{hi} monocytes expressed a higher level of PSGL-1, which interacted with all 3 selectins, compared with Ly-6C^{lo} monocytes. However, no difference was found in the level of FTVII and C2GlcNAcT-I transcripts in both Ly-6C^{hi} and Ly-6C^{lo} monocytes. Thus, unlike activated T cells that gain function of PSGL-1 as a result of upregulation of glycosyltransferases, Ly-6C^{hi} monocytes express a higher level of fully modified and functional PSGL-1 than Ly-6C^{lo} monocytes, which provides a molecular basis for their preferential homing to atherosclerotic plaques.

PSGL-1 is critical for leukocyte homing; it is a well-characterized ligand for all 3 selectins and enables circulating leukocytes to roll on activated endothelium under physiological flow conditions.[7] In humans, P- and E-selectin have been detected on the luminal surface of arteries with nascent or established atherosclerotic lesions.[27] In mouse models, P- and E-selectin are closely correlated with monocyte/macrophage infiltration and lesion formation.[27,28] Leukocytes can initiate rolling on endothelial cells in 3 ways.[7,8,23] First, leukocytes can directly contact the endothelium when entering venules from capillaries. Alternatively, leukocytes can be captured by the endothelium through PSGL-1–dependent tethering to P- and E-selectins, a process called primary tethering (Figure 7A, left panel).[6–8] Primary tethering is considered particularly important for leukocytes to initiate rolling in larger venules and arterial vessels because these vessels do not have upstream capillaries. The recruitment of Ly-6C^{hi} monocytes occurs in arteries during early atherosclerosis. Thus, PSGL-1–mediated primary tethering may be the key to Ly-6C^{hi} monocyte homing. PSGL-1 on Ly-6C^{hi} monocytes may interact with P-selectin on either activated endothelial cells or activated platelets adhered to injured arterial wall.[27] PSGL-1 may also mediate tethering to and rolling on E-selectin on

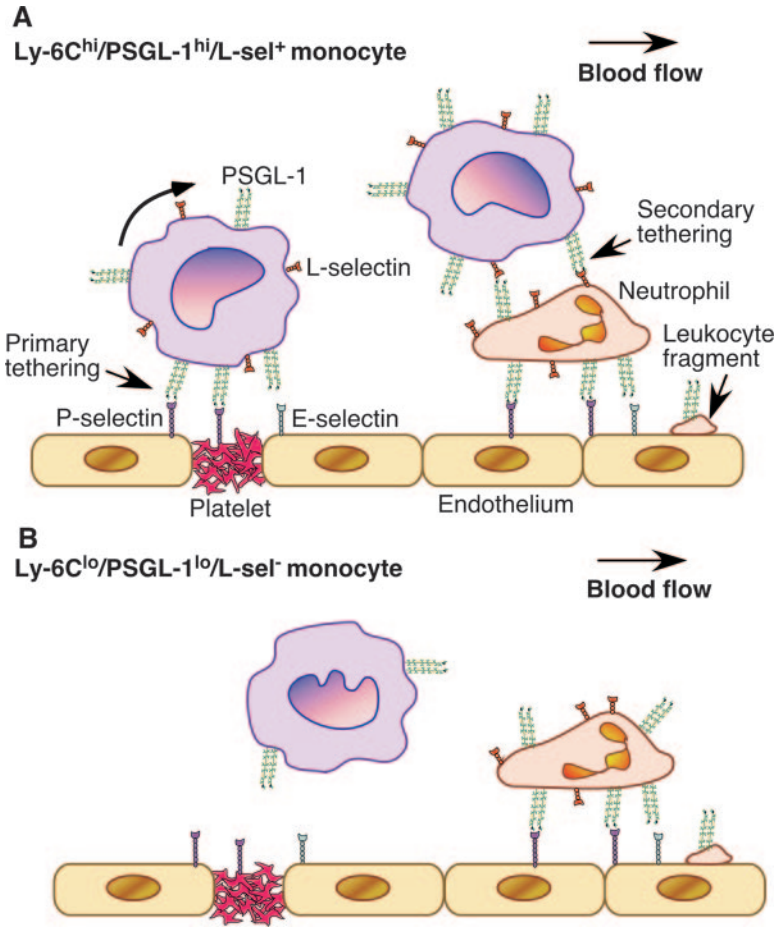

Figure 7. Model for PSGL-1–mediated selective homing of Ly-6C^{hi} monocytes. A, Ly-6C^{hi} monocytes that are also PSGL-1^{hi} and L-selectin (L-sel)$^{+}$ preferentially interact with P- and E-selectin on activated endothelium/adherent platelets (primary tethering) or with L-selectin on a rolling/adherent leukocyte or a leukocyte fragment (a neutrophil in this case, secondary tethering) under flow. B, Ly-6C^{lo} monocytes that are also PSGL-1^{lo} and L-selectin^{-} cannot efficiently interact with activated endothelium under flow because of low-level surface expression of PSGL-1 and, possibly, lack of L-selectin.

activated endothelium, either independently or in cooperation with other leukocyte E-selectin ligand(s).[7,12,18] Third, Ly-6C^{hi} monocytes may interact with the endothelium through secondary tethering in which a freely flowing leukocyte transiently interacts with a rolling or adherent leukocyte or adherent leukocyte fragment and subsequently rolls on the endothelium (Figure 7A, right panel).[29–31] Interestingly, Ly-6C^{hi} monocytes express L-selectin, whereas Ly-6C^{lo} monocytes do not. Ly-6C^{hi} monocyte L-selectin interacted well with 2-GSP-6, the functional domain of PSGL-1, under flow conditions. Reciprocal interactions between PSGL-1 and L-selectin on leukocytes are important for leukocyte adhesion to atherosclerotic lesions.[29] Limited by the availability of Ly-6C^{hi} and Ly-6C^{lo} monocytes, we were unable to analyze L-selectin–dependent secondary rolling. Nevertheless, secondary tethering may serve as another important mechanism for selective Ly-6C^{hi} monocyte homing. In vivo, the secondary tethering of a flowing Ly-6C^{hi} monocyte may occur at much higher frequencies on rolling or adherent neutrophils, which are known to interact with atherosclerotic lesions, than on rolling or adherent monocytes because of the dramatic difference in their number in circulation.[29] Our data demonstrate that Ly-6C^{hi} monocytes, which are PSGL-1^{hi} and L-selectin^{+}, use PSGL-1–dependent primary and possibly secondary capturing mechanisms to selectively home to atherosclerotic sites (Figure 7A), whereas Ly-6C^{lo} monocytes, which are PSGL-1^{lo} and L-selectin^{-}, are impaired in these homing mechanisms (Figure 7B).

Ly-6C^{hi} monocytes use the chemokine receptors CCR2 and CX3CR1 to enter atherosclerotic plaques.[5] However, CCR2 was found not to be involved in the early steps of monocyte adhesion,[10] and CX3CR1 is expressed at a lower level on Ly-6C^{hi} monocytes than on Ly-6C^{lo} monocytes.[3,5] Therefore, these chemokine receptors are less likely to contribute to the homing advantage of Ly-6C^{hi} monocytes. Other adhesion molecules such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 may not be key factors for this selective Ly-6C^{hi} cell homing process because their ligands are expressed at lower levels on Ly-6C^{hi} monocytes than on Ly-6C^{lo} monocytes.[3] Nevertheless, these chemokine receptors and adhesion molecules are important for later processes such as firm adhesion and transmigration once Ly-6C^{hi} monocytes initiate rolling on the endothelium.[5,8,20]

In our study, Ly-6C^{hi} monocytes exhibit PSGL-1–dependent rolling ability on selectins under relatively high shear stress (2 to 4 dyne/cm^2), whereas almost no Ly-6C^{lo} monocytes rolled under such conditions. Wall shear stress in vivo is higher in arteries than in veins. Atherosclerotic lesions are prone to develop at bifurcations, branchings, and curvatures where the shear stress ranges from 1 to 3 dyne/cm^2.[32,33] Neointimal lesions after wire injury develop at locations where shear stress can be much higher because of an angioplasty procedure.[32,33] Thus, the high level of functional PSGL-1 may give Ly-6C^{hi} monocytes a unique homing advantage under arterial flow conditions. Indeed, lack of

PSGL-1 protected *ApoE*$^{-/-}$ mice from developing severe wire injury–induced neointimal and atherosclerotic plaques, which may be attributable to diminished Ly-6C^{hi} monocyte recruitment. Significantly, lack of PSGL-1 provided *ApoE*$^{-/-}$ mice more protection from developing neointimas than atherosclerotic plaques, which supports the contention that PSGL-1 plays a more important role in the recruitment of monocytes in acute lesions than in chronic lesions. Our data indicate that PSGL-1 is also highly expressed on human CD14^{+}CD16^{-} monocytes. Thus, these results support the potential for PSGL-1 blockade as a therapeutic approach for the treatment of restenosis after angioplasty.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The recruitment of monocytes into the arterial wall plays decisive roles in the initiation and progression of atherosclerosis. In mice, monocytes are divided into Ly-6C^{hi} and Ly-6C^{lo} subsets. Ly-6C^{hi} monocytes are recognized as the key monocyte subset in the development of atherosclerosis, but the mechanisms by which these monocytes selectively accumulate in atherosclerotic lesions are largely unknown. In the present study, we identified that Ly-6C^{hi} monocytes expressed a higher level of P-selectin glycoprotein ligand-1 (PSGL-1) and had enhanced binding to P-, E-, and L-selectin compared with Ly-6C^{lo} monocytes under physiological conditions. *ApoE*$^{-/-}$ mice lacking PSGL-1 (*ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$) had impaired Ly-6C^{hi} monocyte recruitment to arterial injuries and exhibited significantly reduced monocyte infiltration in wire injury–induced neointima and in atherosclerotic lesions. Moreover, *ApoE*$^{-/-}$*/PSGL-1*$^{-/-}$ mice also developed smaller neointima and atherosclerotic plaques. These results indicate that PSGL-1 is a new marker for Ly-6C^{hi} monocytes and a major determinant for Ly-6C^{hi} cell recruitment to sites of atherosclerosis in mice.