P-Selectin Glycoprotein Ligand-1 Is Expressed on Endothelial Cells and Mediates Monocyte Adhesion to Activated Endothelium

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Objective—The purpose of this study was to investigate the presence and functionality of P-selectin glycoprotein ligand-1 (PSGL-1) on activated endothelial cells (ECs).

Methods and Results—We show here that PSGL-1 is expressed at the mRNA and protein levels in umbilical vein and microvascular ECs. Furthermore, this endothelial PSGL-1 (ePSGL-1) is functional and mediates adhesion of monocytes or platelet-monocyte complexes (PMCs) to the activated endothelium in a flow model. ePSGL-1 expression was not affected by treating ECs with inflammatory stimuli (tumor necrosis factor α, interleukin-1β, thrombin, or histamine). However, the functional binding capacity of ePSGL-1 to monocytes or P-selectin/Fc chimera significantly increased by stimulation of the ECs with TNFα. By means of a siRNA approach to specifically knock-down the genes involved in the glycosylation of PSGL-1 we could show that tumor necrosis factor α-induced glycosylation of ePSGL-1 is critical for its binding capacity.

Conclusion—Our results show that ECs express functional PSGL-1 which mediates tethering and firm adhesion of monocytes and platelets to inflamed endothelium. (Arterioscler Thromb Vase Biol. 2007;27:1023-1029)

Key Words: P-selectin glycoprotein ligand-1  monocyte adhesion  platelet-monocyte complexes  endothelium  glycosylation

PSGL-1 is one of the best characterized selectin ligands known to date. PSGL-1 is expressed as a homodimer of two 120-kDa subunits that binds all three selectins, with the highest affinity for P-selectin,1 and is known to be constitutively expressed on the surface of platelets2 and most types of leukocytes.3 PSGL-1, besides playing a critical role in the inflammatory response by mediating leukocyte–leukocyte and leukocyte–endothelium interactions it also participates in the hemostatic process by mediating leukocyte–platelet interactions.4 In vivo studies showed that leukocyte PSGL-1 mediates rolling of leukocytes over E-selectin and P-selectin on activated ECs5 whereas PSGL-1 – L-selectin interactions mediate leukocyte secondary tethering at the activated endothelium.6

See page 990

PSGL-1–dependent interactions appear to enable the presence of inflammatory cells at the hemostatic thrombus by binding to P-selectin on activated platelets localized at the injured vessel wall.7 These platelet–leukocyte interactions, however, also occur when activated platelets are present in the circulation giving rise to circulating platelet–leukocyte complexes, mainly platelet–monocyte complexes (PMCs). PMCs are currently regarded not just as markers of vessel wall disease8,9 but also as thromboatherogenic particles with high adhesive capacity to activated endothelium.10,11

Only few studies have assessed the presence of PSGL-1 on the endothelium. Laszik et al described the presence of PSGL-1 in the small venules and capillaries of benign hyperplasia samples, although no vascular-associated staining could be detected in normal tissues or tissues undergoing acute inflammation. Also Sperandio et al failed to show PSGL-1 expression on resting or inflamed endothelium and platelets in mice. For many years the presence of PSGL-1 on ECs has not been considered to be important and therefore not further investigated. Recently, Ley et al12 demonstrated the presence of PSGL-1 in venules of the mesenteric lymph node and small intestine of mice. We show in this report that PSGL-1 is expressed at the mRNA and protein levels in human vein and foreskin microvascular ECs (HUVECs and FMVECs, respectively). Importantly, we also show that endothelial PSGL-1 plays an important role in mediating the rolling and adhesion of monocytes, platelets, and PMCs over activated endothelium. Further,
PSGL-1 expression was demonstrated on the endothelial lining of atherosclerotic coronary arteries, suggesting a role in the formation of the inflammatory infiltrate in this type of lesions. These findings reveal a new mechanism by which selectins and their ligands participate in the onset of inflammation and/or atherosclerosis.

Materials and Methods

Endothelial Cells
HUVECs were isolated from human umbilical cord veins as described. Immortalized HUVECs, EC-RF24 cells, were kindly provided by Prof. H. Pannekoek (Academic Medical Center, Amsterdam, The Netherlands). FMVECs were kindly provided by Prof. V.-W.M. van Hinsbergh (VU Medical Center, Amsterdam, The Netherlands). Cells were cultured in RPMI 1640 containing 20% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and streptomycin (Life Technologies) and grown to confluence in 5 to 7 days.

RNA Interference
The plasmids pSUPER/β4GALT-7, pSUPER/GST-1, pSUPER/GST-2, pSUPER/PSGL-1, and pSUPER/FX were generated and transfected into HUVECs as described in supplemental Methods (available online at http://atvb.ahajournals.org) to induce silencing of the genes β4GALT-7, GST-1, GST-2, PSGL-1, and FX.

Isolation of Blood Cells
Whole blood, anticoagulated with 0.4% trisodium citrate (pH 7.4), was obtained from healthy volunteers from the Sanquin Blood Bank (Amsterdam, The Netherlands). Monocytes were isolated by negative selection from human peripheral blood by means of a MACS monocyte isolation kit according to the manufacturer’s instructions (Miltenyi Biotech GMBH). This procedure resulted in more than 90% pure monocyte suspensions (measured as CD14-positive cells by flow cytometry).

Reverse Transcriptase Polymerase Chain Reaction
Total RNA was prepared from freshly isolated monocytes and untreated or IL-1β (4 hours) treated HUVECs or EC-RF24 cells with the Absolutely RNA kit (Stratagene). Total RNA (2 μg) was converted to cDNA using 0.5 μg of dT12–18 primer (Invitrogen), Superscript II (Invitrogen), and 20 U of RNAsin (Promega).

Western Blotting
Monocytes and HUVECs were lysed in 1.5% Triton X-100, 0.1% SDS, 0.1% NP-40, 100 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, and 1 mmol/L CaCl2 buffer. Proteins from the cell lysates (1×10⁶ monocytes and 2×10⁶ HUVECs) were separated on 7% SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and blotted with PL-1 antibody. The bound antibody was detected by using HRP-conjugated secondary antibody.

Flow Cytometry and Confocal Microscopy
PSGL-1 surface expression on ECs was investigated by flow cytometry (FACS; Vantage, Becton Dickinson) with cells from different passages, stimulated or not with TNF-α (10 and 30 minutes, 2, 6, 12, and 24 hours), IL-1β (6 hours), thrombin (5 and 10 minutes), or histamine (5 and 10 minutes). After stimulation, ECs were resuspended in washing buffer and incubated with a control antibody (fluorescein isothiocyanate [FITC]-labeled goat anti-mouse IgG), or an antibody against PSGL-1, P-selectin, E-selectin, vascular cell adhesion molecule (VCAM)-1, or PECAM-1 for 45 minutes at 4°C. The flow rate through the chamber was precisely controlled and the monocytes were perfused at 0.8 dyn/cm². The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/s.

Tissue Specimens: Immunohistochemistry and Immunofluorescence
Portions of coronary arteries were obtained from autopsy specimens at the Academic Medical Centre (Amsterdam, The Netherlands) according to institutional guidelines. Coronary arteries undergoing atherosclerosis were snap-frozen and sectioned using conventional techniques.

Statistical Analysis
Data are represented as the mean±SEM of at least 3 independent experiments and were compared with a two-tailed Student t test or a one-way ANOVA with Bonferroni correction. Probability values <0.05 were considered to be significant.

Results
Expression of PSGL-1 in Endothelial Cells
Because PSGL-1 is involved in leukocyte–endothelium interactions and there is controversy concerning the presence of PSGL-1 on inflamed endothelium, we investigated whether PSGL-1 is indeed expressed on ECs. Immunofluorescence analysis (flow cytometry and microscopy) showed that PSGL-1 is expressed on the surface of ECs (Figures 1A, 1B, and 2C). In contrast to E-selectin and VCAM-1, there was no increase in surface levels of PSGL-1 after EC activation using TNF-α (6 hours, Figures 1A, 1B, and 2C) or IL-1β (data not shown), at different incubation times (10 or 30 minutes, 2, 6, 12, or 24 hours; data not shown). PSGL-1 expression levels on FMVECs were similar to those obtained on HUVECs (data not shown). PSGL-1 was not upregulated by recruitment from intracellular stores as shown by stimulation with thrombin or histamine for 5 or 10 minutes (Figure 1C), in contrast to P-selectin (used as positive control).

PSGL-1 transcripts were shown by RT-PCR in untreated and TNF-α or IL-1β–treated primary HUVECs (Figure 2A), EC-RF24 cells (data not shown), and monocytes (positive control). No detectable differences between stimulated and unstimulated cells were observed. As a positive control for TNFα or IL-1β stimulation we analyzed intercellular adhesion molecule-1 (ICAM-1) mRNA which showed a dramatic increase in expression in response to these cytokines (data not shown). Quantitative real-time PCR did not show significant differences in the expression levels of PSGL-1 transcripts in both HUVECs and FMVECs (shown in supplemental Figure I). The expression of PSGL-1 was also confirmed by Western blot analysis (Figure 2B). Although the level of PSGL-1 protein in ECs was much lower than in monocytes, a protein of similar apparent molecular weight was observed in both cell types (~120 kDa).

Platelet Adhesion to Endothelial PSGL-1
To determine the functionality of endothelial PSGL-1, platelets were perfused over ECs and adhesion was quantified. Washed and labeled platelets were incubated with a control (W6/32) or a blocking P-selectin antibody (WASP 12.2) and perfused at high shear over untreated or TNF-α–treated (6 hours) ECs. Platelet adhesion was strongly increased after activation of ECs (Figure 3A). This effect was strongly inhibited when PSGL-1 on activated ECs or P-selectin on platelets was blocked with PL-1 or WASP12.2 antibodies, respectively.
Although similar amounts of PSGL-1 are present on the surface of unstimulated and stimulated ECs, only stimulated cells are able to support platelet adhesion. The P-Selectin/Fc chimera was used to test whether there is an increase in PSGL-1 affinity for its receptor on stimulation. Analysis by flow cytometry (Figure 3B) and immunofluorescence microscopy (Figure 3C) indeed showed that the P-selectin/Fc protein bound significantly more to stimulated than to unstimulated ECs. The binding of the P-selectin/Fc chimera to PSGL-1 was inhibited by a blocking antibody to PSGL-1, which underscored the specificity of the interaction between the P-selectin/Fc chimera and PSGL-1. These results indicate that, despite PSGL-1 being constitutively expressed on ECs, the affinity for its receptor is increased by cytokine stimulation of ECs.

PSGL-1 Expression in Atherosclerotic Coronary Arteries

Sections of coronary arteries undergoing acute inflammation such as atherosclerosis were examined for the expression of PSGL-1 (Figure 4). Expression of PECAM-1 was used as a marker for endothelial cells and a strong and regular staining was observed. Although not as regular, the sections also exhibited luminal staining with the anti–PSGL-1 antibody indicating clear PSGL-1 expression on the vascular endothelium of these arteries. In contrast, staining of the endothelium with an irrelevant mouse IgG1 MAb was not detected (Figure 4A). Simultaneous detection of PSGL-1 and PECAM-1, as an endothelial marker, show that these two molecules colocalize on the surface of activated endothelium (Figure 4B). Detection of an IgG-control antibody was at background levels.
Rolling/Adhesion of Monocytes and PMCs to ECs via Endothelial PSGL-1

To investigate whether endothelial PSGL-1 is also functional in mediating monocyte and platelet-monocyte complex (PMC) interactions with the endothelium under flow, monocytes were perfused over HUVECs (untreated or TNF-α-treated for 6 hours). Video recordings were analyzed for the number of adhered monocytes and for rolling velocity. Perfusions of monocytes or PMCs only resulted in rolling when the ECs had been treated with TNF-α. In the presence of PMCs, blocking PSGL-1 on ECs significantly inhibited monocyte adhesion by 30% (P < 0.05, Figure 5A, black bars) and strongly increased monocyte rolling velocity (Figure 5B, black bars). Simultaneous inhibition of PSGL-1 on ECs and on monocytes caused a synergistic reduction of monocyte adhesion (data not shown).

To test the role of endothelial PSGL-1 in the adhesion of monocytes in the absence of platelets, PMCs were removed from the cell suspension by immunodepletion. As previously reported, low levels of PMCs resulted in reduced monocyte adhesion to the endothelium. By blocking PSGL-1 on ECs, monocyte adhesion was further decreased 30% (Figure 5A, empty bars), whereas rolling velocity was significantly increased (Figure 5B, empty bars). As was shown before, blocking of P-selectin on the endothelium did not have any effect in cell adhesion.

To investigate whether endothelial PSGL-1 can interact with L-selectin on monocytes, an L-selectin–blocking antibody was used on a monocyte suspension containing 5% PMCs. To rule out a possible contribution of remaining platelets, the monocytes were, where indicated, incubated with an antibody to P-selectin to prevent PMC formation. When the cells were incubated with the DREG 56 antibody to L-selectin, adhesion to the endothelium was inhibited by 35% (P < 0.05, supplemental Figure IIIA). This effect was similar to that obtained by blocking PSGL-1 on ECs. Although not statistically significant, when both L-selectin on monocytes and PSGL-1 on ECs were blocked, monocyte adhesion was further inhibited. As a control we used a nonblocking antibody against PSGL-1 (PL-2) which did not affect monocyte adhesion to ECs (data not shown).

Previously it has been shown that the expression of L-selectin ligands in endothelial cells is modulated by sulfation, and that TNF-α upregulates the expression of two sulfotransferases implicated in the sulfation of L-selectin ligands. To investi-
gate whether the mechanism of increase in monocyte adhesion described here is dependent on the sulfation of PSGL-1 an RNA interference approach was designed. The genes targeted were GST-1 and -2, implicated in the sulfation of N- and O-linked glycans, ωGalT-7, involved in the initiation of the glycosaminoglycan chains, and FX, which controls the synthesis of GDP-Fucose (supplemental Figure I). Additionally, a knockdown for PSGL-1 and a sequence without homology in the human genome were used as a positive and negative control, respectively. The silencing of PSGL-1 results in a decrease in monocyte adhesion (supplemental Figure IIIB) and an increase in rolling velocity (data not shown), which are comparable to the effect of blocking with PL-1. Furthermore, the silencing of GST-1 was able to mimic the effects of silencing PSGL-1, whereas any of the other treatments were ineffective (supplemental Figure IIIB). In agreement, the binding of P-selectin/Fc to activated endothelial cells was also decreased when cells were transfected with pSUPER/PSGL-1, pSUPER/GST-1, and, to a lesser extent, with pSUPER/GST2 (shown in supplemental Figure II).

**Discussion**

The molecular mechanisms by which leukocyte recruitment to inflamed tissues occurs have been extensively studied over the past years. The initial tethering and rolling of monocytes along the vessel wall is generally accepted to be mediated by selectins and their ligands that are expressed on ECs, platelets, and leukocytes. PSGL-1, one of the primary selectin ligands, is known to be expressed on leukocytes and platelets. In this study we show that functional PSGL-1 is expressed on ECs on treatment with proinflammatory cytokines.

PSGL-1 expression was shown at the mRNA and protein level on primary microvascular and umbilical ECs and on an endothelial cell line. PSGL-1 is restricted to the surface of ECs and is not increased by stimulation with inflammatory cytokines such as TNF-α or IL-1β, in contrast to other cytokine-induced adhesion molecules such as ICAM-1, VCAM-1, and E-selectin. In addition, activators such as thrombin or histamine, which induce elevated surface expression of P-selectin on ECs, had no effect on the expression levels of endothelial PSGL-1. Thus, PSGL-1 is constitutively expressed in primary ECs and in immortalized endothelial cells, and is not stored in P-selectin–containing vesicles within ECs.

Endothelial PSGL-1, at higher shear stress, was able to interact with platelets and recruit them to the endothelium. This effect was inhibited by blocking P-selectin on platelets or PSGL-1 on ECs. The increase in affinity of endothelial PSGL-1 to P-selectin was further demonstrated by the strong binding of a P-selectin/Fc protein to stimulated HUVECs, which was abrogated by incubating the cells with a blocking antibody to PSGL-1.

Our flow system enabled us to show functionality of endothelial PSGL-1 as a ligand for selectins also on monocytes. When 10 to 20% PMCs were present in the monocyte suspension, we found a significant reduction (30%) in monocyte adhesive interactions with the endothelium, accompanied by an increase in cell rolling velocity when TNF-α–stimulated ECs were preincubated with a PSGL-1–blocking antibody.
antibody. Under low shear conditions, platelet interactions with the endothelium are mainly characterized by transient tethering and rolling, whereas firm adhesion rarely occurs.\textsuperscript{11,21} However, it is important to discern whether PSGL-1 on ECs interacts mainly with L-selectin on monocytes or might also interact with P-selectin on platelets. To investigate this, we used a PMC-free monocyte suspension. Blocking of endothelial PSGL-1 or L-selectin on monocytes increased monocyte rolling velocity and inhibited monocyte adhesion to ECs by 30\%, indicating that monocyte L-selectin is a primary receptor for endothelial PSGL-1. However, the contribution of molecular interactions, other than selectin-dependent, cannot be completely ruled out. P-selectin on platelets or platelet microparticles has been implicated in triggering monocyte arrest by deposition of chemokines, namely RANTES, on activated endothelium.\textsuperscript{22,23} Such mechanism could contribute to a more pronounced recruitment with PMC-rich monocyte suspensions. However, because preincubation of monocytes or platelets with a P-selectin-specific blocking antibody was able to block rolling to at least 50\%, it is possible to conclude that P-selectin–PSGL-1 interactions are involved in monocyte/platelet rolling over activated endothelial cells.

Interestingly, PSGL-1 was only functional after cytokine treatment of ECs. Although most lymphocytes express PSGL-1, only 10 to 20\% of cells are able to bind P-selectin,\textsuperscript{24} which shows that PSGL-1 expression does not necessarily imply functional relevance. The glycosylation of PSGL-1 is essential for functionality,\textsuperscript{25} and dramatic changes in endothelial cell glycosylation have been reported on TNF\textsubscript{α} treatment.\textsuperscript{19} Here we show that silencing of the sulfotransferase GST-1, and partially GST-2, mimics the effect of silencing PSGL-1 or using the blocking antibodies PL-1 or DREG 56. Altogether, these data indicate that TNF\textsubscript{α}-induced expression of functional PSGL-1 is dependent on the expression of GST-1, and partially GST-2, whereas fucosylation, or the expression of glycosaminoglycans do not contribute. These findings are in line with those of Li et al.,\textsuperscript{18} demonstrating the critical role of GST-1 and -2 in shear-resistant leukocyte rolling via L-selectin.

Additionally, we show the expression of PSGL-1 on the ECs of atherosclerotic lesions, suggesting a potential role in the recruitment of inflammatory cells to the lesion. Although expressed at low levels, PSGL-1 on activated ECs is able to functionally bind P- and L-selectin on platelets and monocytes.
Figure 5. PSGL-1 functionality on monocyte adhesion to TNF-α-activated endothelium. PMC-rich (10 to 20% PMC, filled bars) and -poor (<5% PMC, empty bars) monocyte suspensions were perfused over TNF-α-activated (6 hours) HUVECs for 5 minutes at 0.8 dyn/cm². Video images were evaluated for the number of adherent monocytes (A) and cell rolling velocity (B). Before perfusion, HUVECs were incubated either with W6/32 control antibody, with PL-1 (blocking antibody to PSGL-1), or WASP12.2 antibody (blocking antibody to P-selectin). Data represent the mean±SD (n=3, *P<0.01).

respectively, mediating monocyte initial tethering and platelet recruitment to the endothelium. Our results strongly suggest that PSGL-1 has a crucial role in monocyte/PMCs and platelet recruitment to the vascular endothelium and should be considered as an important participant in the onset of inflammation and/or atherosclerosis.

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Disclosures
None.

References
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Methods

*Reagents.* Human serum albumin (HSA) was purchased from Sanquin. Recombinant TNFα was from Boehringer Mannheim and Texas Red-phalloidin was from Molecular Probes. IL-1β and recombinant human P-selectin/Fc were purchased from R&D Systems. Thrombin and histamine were from Sigma. Washing buffer contained phosphate-buffered saline (PBS) supplemented with 0.5 % HSA and 13 mM trisodium citrate. Incubation buffer contained 20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM MgSO₄, 1.2 mM KH₂PO₄ supplemented with 5 mM glucose, 1.0 mM CaCl₂ and 0.5% (w/v) HSA. Tissue culture supplies were from Invitrogen.

*Monoclonal antibodies.* Monoclonal antibodies (MAbs) WASP 12.2 (CD62P, anti P-selectin), DREG 56 (CD62L, anti L-selectin) and W6/32 (anti HLA-A, B and C; control antibody) were isolated from the supernatant of hybridomas obtained from the American Type Culture Collection. Specific MAbs against human PSGL-1, PL-1 (blocking of PSGL-1 binding) and PL-2 (non-blocking) were kindly provided by Dr. Kevin L. Moore (University of Oklahoma, Oklahoma). MAb ENA2 (CD62E, blocking of E-selectin binding) was kindly provided by Dr. W. A. Buurman (University Hospital, Maastricht, The Netherlands). MAb against human VCAM-1 (4B2) was purchased from R&D Systems and MAb against PECAM-1 was from Dako. The following FITC-labeled MAbs were used: KPL-1 (Santa Cruz Biotechnology), (AK-6 anti-CD62P (Sanquin) and Cl26Cl0B7 anti CD62E (Bender MedSystems). The AlexaFluor-488-labeled goat-anti-mouse-Ig and AlexaFluor-546 goat-anti-mouse antibodies were purchased from
Molecular Probes and the FITC-conjugated goat-anti-human-IgG was from Jackson ImmunoResearch Laboratories.

**Endothelial cells.** HUVEC were isolated from human umbilical cord veins as described\(^{13}\). Immortalized HUVEC, EC-RF24\(^{14}\) cells, were kindly provided by Prof. H. Pannekoek (Academic Medical Center, Amsterdam, The Netherlands). FMVEC\(^{15,16}\) were kindly provided by Prof. V. W. M. van Hinsbergh (VU Medical Center, Amsterdam, The Netherlands). Cells were cultured in RPMI 1640 containing 20% (v/v) human serum, 200 μg/ml penicillin and streptomycin (Life Technologies) and grown to confluence in 5-7 days. Primary endothelial cells from the first, second or third passage were used in the experiments. TNFα (100 U/ml), interleukin-1β (10 μg/ml), thrombin (1 U/ml), or histamine (1 U/ml) was added directly to the medium at different time points prior to the experiments. For blocking experiments, EC were incubated with MAbs for 10 min at 37°C and washed with incubation buffer prior to perfusion.

**RNA interference.** The mammalian expression vector, pSUPER.retro.puro\(^{36,37}\) (a kind gift of Dr. R. Agami, Netherlands Cancer Institute, Amsterdam, The Netherlands) was used for expression of siRNA in HUVEC. The gene-specific insert identifies a 19-nucleotide sequence corresponding to nucleotides 758–777 (GAC ATT TCG CCA CCT GCA C) of β4GalT-7 (NM_007255), nucleotides 1037-1056 (ATA CGG CAC CGT GCG AAA C) of GST-1 (NM_003654), nucleotides 1535-1554 (CAG CCC TGA GGA GGT CAA A) of GST-2 (NM_004267), nucleotides 362-381 (GAC GAC CTA CCC GAT AGA T) of FX (NM_003313), nucleotides 510-529 (GCA CAG ACC ACT CAA CCC A) of PSGL-
1 (NM_003006), or a sequence with no significant homology to any human gene sequence, therefore used as a non-silencing control. The gene-specific insert was separated by a 9-nucleotide non-complementary spacer (TTC AAG AGA) from the reverse complement of the same 19-nucleotide sequence, and flanked by restriction sites for the enzymes Bgl II and Hind III, producing a final insert of 60 nucleotides. These sequences were inserted into the pSUPER.retro.puro backbone and transformed into XL Gold supercompetent cells (Invitrogen, USA), according to the manufacturer’s instructions. The different vectors were referred to as pSUPER/β4GalT-7, pSUPER/GST-1, pSUPER/GST-2, pSUPER/PSGL-1, pSUPER/FX, or pSUPER/Scrambled, respectively. Plasmids were transfected into HUVEC using the Basic Nucleofector Kit for Primary Mammalian Endothelial Cells (Amaxa, Germany) in an Amaxa Nucleofector (Amaxa, Germany), according to manufacturer’s instructions. Immediately after transfection, cells were seeded in glass coverslips coated with crosslinked gelatin (1%) and fibronectin (5 mg/mL). Transfection efficiency was higher than 90% as evaluated by flowcytometry analysis of HUVEC co-transfected pmax/GFP (Amaxa, Köln, Germany) and the different pSUPER constructs (data not shown). To test the efficiency of RNA interference, cells were lysed after 48 h, mRNA isolated (mRNA Capture Kit, Roche, Switzerland) and retrotranscribed into cDNA (Reverse Transcription System, Promega, USA), according to manufacturer’s instructions. Gene expression of β4GalT-7, GST-1, GST-2, FX, and PSGL-1 was assessed by means of quantitative real-time PCR in an ABI 7900HT platform (Applied Biosystems, USA) using the SYBR Green I chemistry (Applied Biosystems, USA), as previously described38. The primers used were: β4GalT-7 (Fwd: AGG TGG ACC ACT TCA GGT TCA, Rev: AGT CCG
TGC TGT TGC TGC T), GST-1 (Fwd: CCA CGT CCA GAA CAC GCT CAT C, Rev: CGG CGG CTT GAT GTA GTT CTC C), GST-2 (Fwd: GCT CTG GCT CGT CGT TCT TC, Rev: AGA GAG GTC GCA GCG GTA AAG), FX (Fwd: AGC CAT CCA GAA GGT GGT AGC, Rev: GAC GTG TGT GGG TTG GAC C), PSGL-1 (Fwd: TGA CAC CAC TCC TCT GAC TGG G, Rev: CTC CAT AGC TGC TGA ATC CGT G), and GAPDH (Fwd: AGG TCA TCC CTG AGC TGA ACG G, Rev: CGC CTG CTT CAC CAC CTT CTT G) as endogenous reference gene.

Isolation of blood cells. Whole blood, anticoagulated with 0.4% trisodium citrate (pH 7.4) was obtained from healthy volunteers from the Sanquin Blood Bank (Amsterdam, The Netherlands). Monocytes were isolated by negative selection from human peripheral blood by means of a MACS monocyte isolation kit according to the manufacturer’s instructions (Miltenyi Biotech GMBH). This procedure resulted in more than 90% pure monocyte suspensions (measured as CD14-positive cells by flowcytometry). To obtain PMC-poor monocyte suspensions, the monocytes were incubated with a mouse IgG against GPIIIa for 20 min at 4 °C. After one washing step, the cells were incubated with goat-anti-mouse-IgG microbeads (Dynabeads), at a ratio of two beads per platelet, for 20 min at 4 °C. After magnetic extraction of the beads, the presence of PMC was less than 5 % of the total amount of monocytes. After isolation, the cells were resuspended in incubation buffer. For blocking experiments, monocyte suspensions with or without PMC were incubated with MAbs for 10 min at 37 °C and washed in incubation buffer prior to the perfusion experiments. Platelet isolation has been previously described. Flowcytometry analysis with a P-selectin-FITC antibody confirmed the purity of the
platelet suspension (>90-95 % platelets) and the platelet activation status (>85 % of the platelets expressed P-selectin on their surface).

Reverse transcriptase PCR. Total RNA was prepared from freshly isolated monocytes and untreated or IL-1β (4 h) treated HUVEC or EC-RF24 cells with the Absolutely RNA kit (Stratagene). Total RNA (2 μg) was converted to cDNA using 0.5 μg of dT12-18 primer (Invitrogen), Superscript II (Invitrogen) and 20 units of RNAsin (Promega). For the PCR reaction, 5 % of the reaction volume served as template for the PSGL-1 primers (5’GGGATCTTCAGGGAAGGAAC3’ and 5’CTCCAGTGACCAGGAGAAGC3’, forward and reverse primers, respectively). The reaction mixture was denatured at 94 °C for 2 min and amplified in 35 cycles at 94 °C for 15 s, 60 °C for 20 s and 72 °C for 45 s. PCR products were resolved on a 1.5 % agarose gel.

Western blotting. Monocytes and HUVEC were lysed in 1.5 % Triton X-100, 0.1 % SDS, 0.1 % NP-40, 100 mM Tris-HCl pH 7.4, 150 mM NaCl, and 1 mM CaCl2 buffer. Proteins from the cell lysates (1x106 monocytes and 2x106 HUVEC) were separated on 7 % SDS-PAGE, transferred to a PVDF membrane and blotted with PL-1 antibody. The bound antibody was detected by using HRP-conjugated secondary antibody.

Flowcytometry and confocal microscopy. PSGL-1 surface expression on EC was investigated by flowcytometry (FACS Vantage, Becton Dickinson) with cells from different passages, stimulated or not with TNFα (10 and 30 min, 2, 6, 12 and 24 h), IL-1β
(6 h), thrombin (5 and 10 min) or histamine (5 and 10 min). After stimulation, EC were resuspended in washing buffer and incubated with a control antibody (FITC-labeled goat anti-mouse IgG), or an antibody against PSGL-1, P-selectin, E-selectin, VCAM-1 or GPIIbIIIa for 45 min at 4°C. Cells were washed with washing buffer before analysis. For confocal microscopy, EC seeded on fibronectin-coated glass coverslips were immediately stained and analyzed or fixed with 3.7% formaldehyde in PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} for 10 min at room temperature. After blocking with PBS containing 0.5% bovine serum albumin, 1 mM Ca^{2+} and 1 mM Mg^{2+}, PSGL-1 was detected with PL-1 antibody followed by an Alexa-488-labeled goat-anti-mouse-Ig antibody. In a similar way, specific antibodies were used to detect VCAM-1, E-selectin and PECAM-1 on EC.

The affinity of endothelial PSGL-1 for P-selectin was tested with a P-selectin/Fc chimera. EC, activated or not with TNFα, were incubated with P-selectin/Fc chimera for 20 min at 37°C. After washing, the cells were prepared for flowcytometry and confocal microscopy, as described above. P-selectin/Fc protein binding to EC was detected with a Alexa Fluor 488-conjugated goat-anti-human-IgG antibody. Fixed cells were counterstained for F-actin with Texas Red-phalloidin. Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope.

*Monocyte/platelet perfusion and evaluation of adhesion and rolling velocity.* Monocytes (2 x 10^6 cells/ml) were perfused over EC seeded on glass slides as previously described. The perfusion chamber was mounted on a microscope stage (Axiovert 25, Zeiss),
equipped with a B/W CCD video camera (Sanyo). The flow rate through the chamber was precisely controlled and the monocytes were perfused at 0.8 dyn/cm². The cut-off value to distinguish between rolling and static adherent cells was set at 1 μm/s. Platelets in suspension (10⁶ cells/ml in Krebs-Ringer buffer) were incubated with a control antibody (W6/32) or with a P-selectin blocking antibody (WASP 12.2). Residual P-selectin antibody was washed away prior to platelet staining with green calcein (Molecular Probes). Platelets were then perfused over EC in the same way as described for monocytes (see above) with minor modifications. In short, the flow rate through the chamber was maintained at 6 dyn/cm² and the flow chamber was mounted on a Zeiss LSM 510 confocal laser scanning microscope.

_Tissue specimens: immunohistochemistry and immunofluorescence._ Portions of coronary arteries were obtained from autopsy specimens at the Academic Medical Centre (Amsterdam, The Netherlands) according to institutional guidelines. Coronary arteries undergoing atherosclerosis were snap-frozen and sectioned using conventional techniques. The slides were incubated in 10 % horse serum to inhibit nonspecific antibody binding, followed by PL-1 or isotype MAb (2.5 μg/ml) diluted in PBS/1% BSA for 45 minutes, then incubated with biotin-conjugated goat anti-mouse secondary antibody for 30 minutes, followed by a chromogen/substrate reagent solution (diaminobenzidine/H₂O₂, Sigma) for 10 minutes. Hematoxylin was used for nuclear counterstaining. Between all incubation steps the slides were washed with PBS. For confocal microscopy, the slides were incubated with PBS containing 1 mM Ca²⁺ and 1 mM Mg²⁺ for 10 min at room temperature. After blocking with PBS containing 0.5%
bovine serum albumin, 1 mM Ca\(^{2+}\) and 1 mM Mg\(^{2+}\), PSGL-1 was detected with a FITC-labeled antibody. An unlabeled PECAM-1-specific antibody was detected by incubation with a goat-anti-mouse AlexaFluor-546 antibody.

**Statistical analysis.** Data are represented as the mean ± S.E.M. of at least 3 independent experiments and were compared with a two-tailed Student’s t-test or a one-way ANOVA with Bonferroni correction. P values < 0.05 were considered to be significant.
Figure 1. da Costa Martins et al.
Figure II. da Costa Martins et al.
Figure III. da Costa Martins et al.
**Figure I. mRNA silencing efficiency.** HUVEC were transfected with the plasmids pSUPER/PSGL-1 (black bars in A), pSUPER/GST-1 (black bars in B), pSUPER/β4Galt-7 (black bars in C), pSUPER/GST-2 (black bars in D), pSUPER/FX (black bars in E), or pSUPER/Scrambled (white bars in A, B, C, D, and E). After 24 h, cells were lysed, mRNA isolated, cDNA retrotranscribed and the transcript levels of b4GalT-7, GST-1, GST-2, PSGL-1, and FX were assayed by real-time PCR. The results show more than 50 % efficient silencing of the selected genes. Results are shown as average ± standard deviation of 3 separate experiments.

**Figure II. Influence of GST-knockdown on P-selectin affinity to endothelial cells.** HUVECs were transfected with the plasmids pSUPER/GST-1, pSUPER/GST-2, pSUPER/PSGL-1, or pSUPER/Scrambled (see Materials and Methods). 24 hrs after transfection, cells were treated with TNF-α for further 6 hrs and binding of P-Selectin/Fc was evaluated as described in Materials and Methods. Results are shown as the average ± standard deviation of the median fluorescence intensity in three independent experiments. The results (left) show how the binding of P-Selectin/Fc to activated endothelial cells decreases when the cells have been transfected with pSUPER/PSGL-1, pSUPER/GST-1, and, to a lesser extent with pSUPER/GST-2. The degree of inhibition correlates with the silencing effect of the transfection (Supplementary Material).

**Figure III. PSGL-1 functionality on monocyte adhesion to TNF-activated endothelium.** (A) PMC-poor (< 5 % PMC) monocyte suspensions were pretreated with WASP12.2 anti-P-selectin antibody prior to incubation with other antibodies. The
monocytes were perfused over TNF-activated (6 h) HUVEC for 5 min at 0.8 dyn/cm². Video images were analyzed for the number of adhered cells. Prior to perfusion, cells were incubated with W6/32 control antibody (monocytes and endothelial cells), anti L-selectin DREG 56 antibody (monocytes) or anti PSGL-1 PL-1 antibody (endothelial cells). Data represent the mean ± SD ($n = 3$, * $p < 0.05$). **(B)** PMC-poor (< 5 % PMC) monocyte suspensions were perfused over transfected-TNF-activated (6 h) HUVEC for 5 min at 0.8 dyn/cm². Video images were analyzed for the number of adhered cells. Prior to perfusion, cells were incubated with anti PSGL-1 PL-1 antibody (endothelial cells). Data represent the mean ± SD ($n = 3$, * $p < 0.05$).