P-Selectin Glycoprotein Ligand-1 Regulates Adhesive Properties of the Endothelium and Leukocyte Trafficking Into Adipose Tissue

Hana M. Russo,* Kevin J. Wickenheiser,* Wei Luo, Miina K. Öhman, Luigi Franchi, Andrew P. Wright, Peter F. Bodary, Gabriel Núñez, Daniel T. Eitzman

Rationale: Adhesive interactions between endothelial cells and leukocytes affect leukocyte trafficking in adipose tissue. The role of P-selectin glycoprotein ligand-1 (Psgl-1) in this process is unclear.

Objective: The goal of this study was to determine the effect of Psgl-1 deficiency on adhesive properties of the endothelium and on leukocyte recruitment into obese adipose depots.

Methods and Results: A genetic model of obesity was generated to study the effects of Psgl-1 deficiency on leukocyte trafficking. Leukocyte-endothelial interactions were increased in obese leptin receptor mutant mice (Leprdb/db,Psgl-1/+), but not obese Psgl-1–deficient mice (Leprdb/db,Psgl-1−/−), when compared with lean mice (Lepr+/+,Psgl-1+/+). This effect of Psgl-1 deficiency was due to indirect effects of Psgl-1, because Psgl-1+/+ adoptively transferred leukocytes did not exhibit enhanced rolling in Lepr db/db,Psgl-1−/− mice. Additionally, circulating levels of P-selectin, E-selectin, monocyte chemoattractant protein-1, and macrophage content of visceral adipose tissue were reduced in Lepr db/db,Psgl-1−/− compared with Lepr db/db,Psgl-1+/+. Reduced leukocyte-endothelial interactions and macrophage content of visceral adipose tissue due to Psgl-1 deficiency was also observed in a diet-induced obese mouse model. Psgl-1−/− mice were resistant to the endothelial effects of exogenous IL-1β, suggesting that defective cytokine signaling contributes to the effect of Psgl-1 deficiency on leukocyte-endothelial interactions. Mice deficient in the IL-1 receptor also had reduced levels of circulating P-selectin, similar to those observed in Psgl-1−/− mice.

Conclusions: Deficiency of Psgl-1 is associated with reduced IL-1 receptor-mediated adhesive properties of the endothelium and is protective against visceral fat inflammation in obese mice. (Circ Res. 2010;107:388-397.)

Key Words: inflammation ■ obesity ■ endothelium ■ leukocytes ■ cell adhesion molecules

Obesity has been characterized as a chronic, low-grade inflammatory disease that is marked by an increase in macrophage content and macrophage activity in adipose tissue.1–4 Adhesive interactions between leukocytes and endothelial cells play a critical role in leukocyte trafficking during inflammatory diseases.5 Animal models of obesity have demonstrated an increase in leukocyte-endothelial interactions in visceral adipose tissue that is mediated by increased endothelial expression of P-selectin (P-sel) and E-selectin (E-sel).6 The mechanism by which the endothelium is activated in obese adipose tissue is unclear.

P-selectin glycoprotein ligand-1 (Psgl-1) is the primary leukocyte ligand for P-sel2 and an important ligand for E-sel.8 Leukocytes from Psgl-1−/− mice show reduced selectin-dependent rolling.9,10 Thus, interactions between Psgl-1 and endothelial selectins may contribute to the influx of macrophages into adipose tissue, which may in turn trigger the chronic low-grade inflammatory state associated with obesity.2,4

To determine the contribution and mechanism(s) of Psgl-1 toward leukocyte trafficking into adipose tissue, we analyzed leukocyte-endothelial interactions and fat inflammation in genetically and diet-induced obese mice with and without Psgl-1. Our findings indicate that Psgl-1 affects leukocyte trafficking, but that this occurs indirectly via Psgl-1–mediated regulation of endothelial adhesive properties.

Methods

Animals and Breeding

Psgl-1−/− mice were from Jackson Laboratory (Bar Harbor, Maine) and backcrossed to the C57BL/6J strain for a total of 10 generations before use in cross-breeding. Leprdb/db mice on the C57BL/6J strain background were purchased from Jackson Laboratory. To generate Lepr db/db,Psgl-1−/− mice, Lepr db/db males were...
crossed to Psgl-1\(^{-/-}\) females. Lepr\(^{db/db}\), Psgl-1\(^{-/-}\) were then crossed to Psgl-1\(^{-/-}\) mice to produce Lepr\(^{db/db}\), Psgl-1\(^{-/-}\) offspring. These offspring were then intercrossed to generate Lepr\(^{db/db}\), Psgl-1\(^{-/-}\) mice. Lepr\(^{db/db}\), Psgl-1\(^{-/-}\) mice were generated from an intercross between Lepr\(^{db/db}\), Psgl-1\(^{+/+}\) parents. IL-1R\(^{-/-}\) and C57BL/6J control mice were from Jackson Laboratory.

Mice were fed either a standard laboratory rodent diet (No. 5001, TestDiet, Richmond, Ind) or a high-fat, high-sucrose rodent diet (D12451, 45 kcal% fat, Research Diets Inc, New Brunswick, NJ) in specific pathogen-free facilities. For the diet-induced obesity study, Psgl-1\(^{-/-}\) and Psgl-1\(^{+/+}\) mice were started on a high-fat, high-sucrose diet at 5 weeks of age and remained on it for 10 weeks. All procedures complied with the Principles of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.

### Analysis of Serum Factors

Serum samples were collected by retroorbital bleeding with nonheparinized capillary tubes (Fisher Scientific, Pittsburgh, Pa). Circulating levels of soluble P-selectin (sP-select), soluble E-selectin (sE-select), monocyte chemotactic protein (MCP-1), and soluble Intercellular adhesion molecule-1 (sICAM-1) were assayed with murine ELISA kits (R&D systems, Minneapolis, Minn) according to manufacturers’ instructions. An Ultra Sensitive Rat Insulin Elisa kit (Crystal Chem Inc, Downers Grove, Ill) was used to measure overnight fasted insulin levels, and overnight fasted glucose levels were determined using an Ascensia Contour Blood Glucose Meter and Ascensia Contour test strips (Bayer HealthCare LLC, Tarrytown, NY).

### Weight and DEXA Measurement

Body weight and percent body fat were measured using a Dual-Energy X-ray Absorptiometry (DEXA) scanner Lunar PIXimus2 (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) in diet-induced and genetically obese mice at 15 weeks of age.

### Immunohistochemistry

For details, please see the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org. Macrophages in adipose cross-sections were identified with a rat antimonocyte Mac-3 monoclonal antibody (1:100 dilution; BD Biosciences, San Jose, Calif).

### Real-Time Polymerase Chain Reaction

For details, please see Supplemental Materials and Methods. P-select, E-select, MCP-1, and tumor necrosis factor α (TNFα) Real-time polymerase chain reaction (RTPCR) primers were used. 7000 System SDS Software and the 2\(^{-\Delta\Delta CT}\) method\(^{11}\) were used to analyze the results.

### Intravitral Microscopy

For details, please see Supplemental Materials and Methods. For adoptive transfer experiments, leukocytes were isolated from whole blood of Psgl-1\(^{-/-}\) mice with Histopaque-1077 (Sigma-Aldrich, St Louis, Mo) according to the manufacturer’s instructions. The isolated leukocytes were resuspended in 1 mL of PBS and then incubated with 100 μL of 10X stock rhodamine 6G for 30 minutes. Leukocytes were centrifuged at 250g for 10 minutes. Cells were washed twice in PBS and ultimately resuspended in 1 mL PBS. 1x10\(^6\) leukocytes were then injected into Lepr\(^{db/db}\), Psgl-1\(^{-/-}\) and Lepr\(^{db/db}\), Psgl-1\(^{+/+}\) mice.

To determine the type of leukocytes that were adoptively transferred into mice using this protocol, analysis of leukocytes was performed using a Bayer Advia 120 Hematology System.

### Bone Marrow Transplantation

For details, please see Supplemental Materials and Methods. Bone marrow transplantation (BMT) was performed as previously described.\(^{14}\) 8- to 10-week-old Lepr\(^{db/db}\), Psgl-1\(^{+/+}\) mice were used as recipients for BMT from Psgl-1\(^{-/-}\), Psgl-1\(^{-/-}\), or Psgl-1\(^{-/-}\) donors. Psgl-1\(^{-/-}\) mice received marrow from Psgl-1\(^{-/-}\) donors, Psgl-1\(^{+/+}\) mice received marrow from Psgl-1\(^{-/-}\) donors. IL-1R\(^{-/-}\) mice received marrow from IL-1R\(^{-/-}\) donors, and IL-1R\(^{-/-}\) mice received marrow from IL-1R\(^{-/-}\) donors.

### Recombinant IL-1β

For details, please see Supplemental Materials and Methods. Recombinant IL-1β (Peprotech, Rocky Hill, NJ) was injected to mice via tail vein. Mice were bled for serum 5 hours after IL-1β injection.

### Immunoblotting

For details, please see Supplemental Materials and Methods. The antibodies for mouse IκBα, phospho-IκBα, and p38 were from Cell Signaling Technology (Danvers, Mass).

### Statistical Analyses

Values are expressed as means±SEM. The statistical significance of differences between groups was determined by Student t test. Probability values <0.05 were considered significant.

### Results

#### Metabolic Profile of Diet-Induced and Genetically Obese Psgl-1–Deficient Mice

Mice deficient in leptin receptor signaling (Lepr\(^{db/db}\), Psgl-1\(^{-/-}\)) develop severe obesity.\(^{13}\) No differences between Lepr\(^{db/db}\), Psgl-1\(^{-/-}\) mice and leptin receptor deficient mice without Psgl-1 (Lepr\(^{db/db}\), Psgl-1\(^{+/+}\) in body weight (49.6±0.9 g, n=9 versus 47.7±1 g, n=12, respectively, P=NS), or percent fat mass (56.9±1.1% versus 56.5±0.5%, respectively, P=NS) were observed at 15 weeks of age. Similarly, in diet-induced obesity, no differences between Psgl-1\(^{-/-}\) mice and Psgl-1\(^{-/-}\) mice in body weight (31.4±1 g, n=5 versus 27.7±2.9 g, n=5, respectively, P=NS) or percent fat mass (30.3±3% versus 34.2±4.6%, respectively, P=NS) were observed at 15 weeks of age after 10 weeks on high-fat, high-sucrose diet.

Fasting glucose levels were significantly reduced in Lepr\(^{db/db}\), Psgl-1\(^{-/-}\) mice (n=19) compared with Lepr\(^{db/db}\), Psgl-1\(^{+/+}\) mice (n=11) at 10 weeks of age (280.2±19.1 versus 430±27.4 mg/dL, respectively, P<0.001), whereas fasting insulin levels were not different (5.8±0.9 (n=15) versus 5.1±1 ng/mL (n=19), respectively, P=NS). In the diet-induced obesity model, glucose and insulin levels were not significantly different between Psgl-1\(^{-/-}\) (n=5) and Psgl-1\(^{-/-}\) (n=4) mice at 10 weeks of age (glucose: 266±30.7 versus 227.5±25.1 mg/dL, P=NS; insulin: 0.34±0.1 versus 0.43±0.2 mg/mL, P=NS).

### Non-standard Abbreviations and Acronyms

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<thead>
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<th>Acronym</th>
<th>Description</th>
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<tr>
<td>BMT</td>
<td>bone marrow transplant</td>
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<tr>
<td>DEXA</td>
<td>dual energy x-ray absorptiometry</td>
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<tr>
<td>IL-1β</td>
<td>interleukin-1 beta</td>
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<tr>
<td>IL-1R</td>
<td>interleukin-1 receptor</td>
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<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
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<td>P-selectin glycoprotein ligand-1</td>
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<td>TNFα</td>
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Effect of Psgl-1 Deficiency on Obesity-Induced Leukocyte-Endothelial Interactions in Cremaster Venules

Intravital microscopy was used to analyze leukocyte-endothelial interactions in cremaster venules of 15-week-old Lepr$^{db/db}$Psgl-1$^{+/+}$ mice. Leukocyte rolling was markedly increased in Lepr$^{db/db}$Psgl-1$^{+/+}$ mice (n=4) compared with lean wild-type mice Psgl-1$^{+/+}$ mice (n=4) (108.7±14.4 versus 40.8±11.8 cells/mm, respectively, P<0.01). Lepr$^{db/db}$Psgl-1$^{-/-}$ mice (n=4) exhibited significantly reduced rolling compared with Lepr$^{db/db}$Psgl-1$^{+/+}$ mice (9.1±2.2 versus 108.7±14.4 cells/mm, respectively, P<0.001). No significant difference in firm attachment was observed in Lepr$^{db/db}$Psgl-1$^{-/-}$ mice compared with Lepr$^{db/db}$Psgl-1$^{+/+}$ mice (17.2±2.9 versus 30.6±6.9 cells/mm, respectively, P=0.1).

In diet-induced obesity, Psgl-1$^{-/-}$ mice exhibited significantly reduced rolling and firm attachment compared with Psgl-1$^{+/+}$ mice (rolling: 5.3±1.2 versus 35.5±8 cells/mm, respectively, P<0.01; firm attachment: 16.4±4.5 versus 54.7±8.9 cells/mm, respectively, P<0.01).

Circulating Levels of P-Selectin, E-Selectin, and MCP-1 in Obese Psgl-1–Deficient Mice

To determine whether reduced leukocyte-endothelial interactions in genetically and diet-induced Psgl-1 deficient mice were associated with reduced circulating levels of soluble adhesion molecules and MCP-1, sP-sel, sE-sel, and MCP-1 were measured from Lepr$^{db/db}$Psgl-1$^{-/-}$ and Lepr$^{db/db}$Psgl-1$^{+/+}$ mice (n=7 to 13). Figure 1A through 1C. A, sP-sel levels. B, sE-sel levels. C, MCP-1 levels. Psgl-1$^{-/-}$ (black bars) (n=5) and Psgl-1$^{+/+}$ (white bars) (n=3 to 5) on high-fat, high-sucrose diet. D, sP-sel levels. E, sE-sel levels. F, MCP-1 levels. *P<0.05, **P<0.01, ***P<0.001.
Effect of Psgl-1 Deficiency on Leukocyte Recruitment and Inflammatory Cytokine Expression In Adipose Tissue in Obese Psgl-1–Deficient Mice

To determine if the decreased leukocyte-endothelial interactions observed in Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice compared with Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice were associated with reduced adipose tissue macrophage content, macrophage immunostaining was performed on visceral (perigonadal and pericardial) and subcutaneous adipose depots retrieved from 15-week-old obese Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> and Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice. The macrophage content of perigonadal and pericardial fat was significantly reduced in Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice compared with Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice (Figure 2A, Online Figure 1 A-F). No significant difference in macrophage content was observed in subcutaneous fat between Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> and Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice (Figure 2A, Online Figure 1 A-F). Similarly, Psgl-1<sup>−/−</sup> mice on a high-fat, high-sucrose diet for 10 weeks had a significant reduction in macrophage content of perigonadal fat compared with Psgl-1<sup>+/+</sup> control mice (Figure 2B through 2F). No significant difference in macrophage content was observed in subcutaneous fat between these mice (Figure 2B through 2F).

To determine the effect that Psgl-1 deficiency in obesity has on macrophage infiltration in different adipose depots compared with lean mice, macrophage content of subcutaneous and perigonadal fat in Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> and Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice was compared with lean Psgl-1<sup>−/−</sup> and Psgl-1<sup>+/+</sup> mice. There was no difference in macrophage content of perigonadal fat in Psgl-1<sup>−/−</sup> (n=5) versus Psgl-1<sup>−/−</sup> (n=3) lean mice (14.9±1.0% versus 16.4±0.8% mac3+ cells, respectively, P=NS). There was also no difference in macrophage content of subcutaneous fat in Psgl-1<sup>−/−</sup> (n=4) versus Psgl-1<sup>−/−</sup> (n=3) lean mice (9.7±0.6 versus 8.9±0.1% mac3+ cells, respectively, P=NS). Both lean Psgl-1<sup>−/−</sup> and Psgl-1<sup>+/+</sup> had a significantly higher macrophage content in perigonadal fat compared with subcutaneous fat (Psgl-1<sup>−/−</sup>: 14.9±1.0% versus 9.7±0.6% mac3+ cells, respectively, P<0.01; Psgl-1<sup>+/+</sup>: 16.4±0.8% versus 8.9±0.1% mac3+ cells, respectively, P<0.001). There was no difference in macrophage content of subcutaneous fat in Psgl-1<sup>−/−</sup> compared with Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice (9.7±0.6% versus 14.8±3.2% mac3+ cells, respectively, P=NS) or Psgl-1<sup>−/−</sup> compared with Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice (8.9±0.1 versus 15.8±3.9% mac3+ cells, respectively, P=NS). There was a significantly higher macrophage content in perigonadal fat in Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> compared with Psgl-1<sup>−/−</sup> mice (32.8±4.7% versus 14.9±1.0% mac3+ cells, respectively, P<0.05) and in Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> compared with Psgl-1<sup>+/+</sup> mice (66.1±2.7% versus 16.4±0.8% mac3+ cells, respectively, P<0.001); however, the rise in macrophage infiltration into perigonadal fat with obesity was significantly higher between Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> and Psgl-1<sup>−/−</sup> mice compared with Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> and Psgl-1<sup>+/+</sup> mice (49.8±2.7% versus 17.8±4.7% increase in mac3+ cells, respectively, P<0.001).

The expression of MCP-1 and TNFα was measured in perigonadal adipose tissue retrieved from 15-week-old obese Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> (n=14) and Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> (n=13) mice. Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice had reduced expression of MCP-1 and TNFα in perigonadal adipose tissue compared...
Figure 3. Circulating sP-sel, sE-sel, MCP-1, and sICAM-1 and expression of P-sel, E-sel, and MCP-1 in lung and perigonadal fat 5 hours after IL-1β injection. Psgl-1+/+ (n=4), Psgl-1−/− (n=3), and IL-1R−/− mice (n=3). A, sP-sel levels, B, sE-sel levels, C, MCP-1 levels, and D, sICAM-1 levels in Psgl-1+/+, Psgl-1−/−, and IL-1R−/− mice before IL-1β challenge (black bar) and after IL-1β challenge (white bar). Expression of P-sel, E-sel, and MCP-1 in Psgl-1+/+ Psgl-1−/− and IL-1R−/− in E, lung and in F, perigonadal fat after PBS injection (black bar) and after IL-1β challenge (white bar). * P<0.05, ** P<0.01, *** P<0.001.
with Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> controls (MCP-1: 2.33±0.3 versus 4.05±0.7 relative expression units, respectively, P<0.05; TNFa: 3.4±0.4 versus 5.84±0.6 relative expression units, respectively, P<0.01).

**Effect of Adoptive Transfer of Wild-Type Leukocytes Into Obese Psgl-1-Deficient Mice**

To determine whether the effect of Psgl-1 on leukocyte rolling was due to lack of direct, acute Psgl-1 interactions with endothelial selectins or due to an indirect, more chronic effect of Psgl-1 on adhesive properties of the endothelium, adoptive transfer of rhodamine-labeled leukocytes from Psgl<sup>-/-</sup> donors into Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> (n=3) and Lepr<sup>db/db</sup>, Psgl<sup>+/+</sup> (n=5) recipients was performed. Transferred leukocytes consisted of 91.5±3.3% lymphocytes, 0.65±0.5% monocytes, and 5.5±3.2% neutrophils (n=3).

Control adoptive transfer experiments showed that rhodamine-labeled Psgl<sup>+/+</sup> donor leukocytes infused into Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice exhibited similar leukocyte rolling characteristics as endogenous rhodamine-labeled Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> leukocytes (96.8±12.9 versus 108.7±14.4 cells/mm, respectively, P=NS). When rhodamine-labeled Psgl<sup>-/-</sup> donor leukocytes were infused into Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice, the donor leukocyte rolling was markedly less than that observed when Psgl<sup>-/-</sup> donor leukocytes were infused into Lepr<sup>db/db</sup>, Psgl<sup>+/+</sup> mice (2.1±0.7 versus 96.8±12.9 cells/mm, respectively, P<0.001). The donor leukocyte firm attachment was also less in Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice compared with Lepr<sup>db/db</sup>, Psgl<sup>+/+</sup> mice (8.8±2.7 versus 18±2.9 cells/mm, respectively, P<0.05).

**Effect of Bone Marrow-Derived Psgl-1 on sP-sel Levels and Leukocyte-Endothelial Interactions in Obese Mice**

Because the adoptive transfer experiments suggested that Psgl-1 deficiency affects the activation state of the endothelium, BMT experiments were performed to identify the potential source of Psgl-1 responsible for regulating the endothelium. The BMT involved transplanting Psgl<sup>-/-</sup> or Psgl<sup>-/-</sup> bone marrow into Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> recipients. To identify the contribution of the hematopoietic Psgl-1 pool on the generation of sP-sel in obesity, sP-sel was measured 4 weeks after BMT. sP-sel was markedly reduced in Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice receiving Psgl<sup>-/-</sup> marrow (n=6) compared with Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice receiving Psgl<sup>-/-</sup> marrow (n=3) (45.7±3.8 versus 166.5±5.7 ng/mL, respectively, P<0.001). Ten weeks post-BMT, the extent of leukocyte-endothelial interactions was measured in these mice using intravital microscopy. Leukocyte rolling and firm attachment was markedly reduced in Lepr<sup>db/db</sup>, Psgl<sup>+/+</sup> mice receiving Psgl<sup>-/-</sup> marrow (n=5) compared with Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice receiving Psgl<sup>-/-</sup> marrow (n=4) (rolling: 3.6±1.1 versus 87.0±7.7 cells/mm, respectively, P<0.001; firm attachment: 9.0±2.2 versus 36.0±6.3 cells/mm, respectively, P<0.001). These results implicate the hematopoietic Psgl-1 in regulating the activation state of the endothelium.

Because both platelets and endothelial cells are rich sources of P-sel, bone marrow transplants were performed from wild-type (Psel<sup>+/+</sup>) or P-sel deficient (Psel<sup>-/-</sup>) donors into Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> recipients to determine the predominant cellular source of sP-sel. Circulating sP-sel levels in obese mice receiving Psel<sup>-/-</sup> bone marrow (n=6) were not different from mice receiving Psel<sup>-/-</sup> marrow (153.2±3.7 versus 166.5±5.7 ng/mL, respectively, P=NS), implicating the endothelium as the major source of sP-sel in obese mice.

**Psgl-1 Regulates Response to Recombinant IL-1β**

Because IL-1β has previously been shown to induce selectin-dependent leukocyte rolling,

\[
\text{IL-1β} \rightarrow \text{selectins} \rightarrow \text{MCP-1} \rightarrow \text{leukocyte rolling}.
\]

In this study, the possibility that leukocyte Psgl-1 might affect endothelial expression of selectins and MCP-1 in response to IL-1β stimulation was explored. Therefore, either Psgl<sup>-/-</sup> or Psgl<sup>-/-</sup> mice were stimulated with recombinant IL-1β. As a control, mice deficient in the IL-1 receptor (IL-1R<sup>-/-</sup>) were also analyzed for potential nonselective effects of the recombinant IL-1β preparation.

Five hours after an intravenous injection of recombinant IL-1β (500 ng), sP-sel, MCP-1, sE-sel, and sICAM-1 levels were measured from serum. IL-1β-induced serum levels of sP-sel, sE-sel, MCP-1, and sICAM-1 were completely blocked in IL-1R<sup>-/-</sup> mice, as expected (Figure 3A through 3D). Compared with serum from Psgl<sup>-/-</sup> mice, IL-1β-induced changes in Psgl<sup>-/-</sup> mice were either completely blocked (sP-sel, MCP-1) or reduced (sE-sel and sICAM-1) (Figure 3A through 3D). Surprisingly, basal levels of sP-sel were reduced in IL-1R<sup>-/-</sup> mice similar to that observed in the Psgl<sup>-/-</sup> mice, indicating that even basal sP-sel levels are regulated via the IL-1 receptor (IL-1R) (Figure 4).

To confirm that Psgl-1 deficiency in the setting of obesity was also associated with resistance to IL-1β, Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice (n=4) and Lepr<sup>db/db</sup>, Psgl<sup>+/+</sup> mice (n=3) were given 500 ng of recombinant IL-1β. Similar to lean mice, there was no increase in sP-sel in Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice after IL-1β injection (83.3±14.1 pre-IL-1β versus 83.9±3.5 ng/mL after IL-1β, P=NS), whereas there was a marked increase in sP-sel in Lepr<sup>db/db</sup>, Psgl<sup>-/-</sup> mice (183.2±3.5 pre-IL-1β versus 533.1±38.3 ng/mL after IL-1β, P<0.001).

To determine the effect of Psgl-1 deficiency on transcriptional regulation of endothelial selectins (P-sel and E-sel) and MCP-1 after stimulation with recombinant IL-1β, lung tissue (as a rich source of endothelial cells) and perigonadal fat were removed for RNA isolation 5 hours after intravenous IL-1β challenge.

**Figure 4. Circulating levels of sP-sel in mice at baseline.** Wild-type (WT) (black bar) (n=11), Psgl<sup>-/-</sup> (white bar) (n=10), and IL-1R<sup>-/-</sup> mice (diagonal striped bar) (n=15). ***P<0.001.
**Relevant IL-1R Pools for Regulation of Endothelial Selectins**

To further characterize the cellular pool of IL-1R relevant for the response to IL-1β, chimeric IL-1R−/− mice were generated by BMT. After IL-1β challenge, sP-selectin levels increased in IL-1R+/+ mice with IL-1R−/− marrow and did not change in Psgl-1+/+ mice with Psgl-1−/− marrow, indicating that hematopoietic Psgl-1 is regulating the endothelial response to IL-1β via endothelial IL-1R signaling (Figure 5A).

Psgl-1−/+ mice injected with IL-1β had a higher expression of P-selectin, E-selectin, and MCP-1 in the lung compared with Psgl-1−/+ mice injected with PBS (Figure 3E). There was no difference in P-selectin, E-selectin, and MCP-1 expression in the lung between Psgl-1−/− and IL-1R−/− mice injected with IL-1β compared with Psgl-1−/+ and IL-1R−/− mice injected with PBS (Figure 3E). Psgl-1−/+ mice injected with IL-1β also had a higher expression of P-selectin and MCP-1 and a trend toward higher expression of E-selectin in perigonadal fat compared with Psgl-1−/+ mice injected with PBS (Figure 3F). There was no difference in P-selectin, E-selectin, and MCP-1 expression in perigonadal fat among Psgl-1−/− and IL-1R−/− mice injected with IL-1β compared with Psgl-1−/+ and IL-1R−/− mice injected with PBS (Figure 3F).

Psgl-1−/+ mice were also resistant to the effects of recombinant IL-1β on leukocyte rolling compared with Psgl-1−/+ mice (2.8±1 versus 88.5±10.5 cells/mm, respectively, P<0.001).

**Discussion**

Adipose tissue inflammation may contribute to comorbidities associated with obesity. Leukocyte-endothelial interactions are increased in visceral adipose tissue depots in obese mice. These increased leukocyte-endothelial interactions are associated with increased expression of P-selectin and E-selectin in endothelial cells from visceral adipose depots. Furthermore, antibody blockade of P-selectin and E-selectin reduced leukocyte rolling and firm attachment in obese visceral adipose tissue. Psgl-1 is the primary ligand for P-selectin and a major ligand for E-selectin. Leukocytes from Psgl-1−/− mice show reduced selectin-dependent rolling.

To further explore the implications of selectin-ligand interactions in adipose tissue inflammation, we studied Psgl-1 deficiency in genetic and diet-induced mouse models of obesity. Lepr<sup>ob/db</sup> mice lack leptin receptor signaling and become obese...
and hyperglycemic at an early age. Obese mice lacking leptin or a functional leptin receptor have been shown to develop increased macrophage infiltration in visceral fat tissue. Thus, this model is suitable for studying factors involved in obesity-related adipose tissue macrophage trafficking. In the current study, Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice and Psgl-1<sup>−/−</sup> mice on a high-fat, high-sucrose diet developed normally up to 15 weeks of age, with a similar gain in body weight and fat mass, compared with Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice. Psgl-1<sup>−/−</sup> mice on a high-fat, high-sucrose diet, respectively. To determine if deficiency of Psgl-1 affected leukocyte-adipose tissue interactions in obesity, we first needed to establish a model where interactions between leukocytes and endothelial cells were increased in the setting of obesity. Nishimura et al previously demonstrated that leukocyte-endothelial interactions were increased locally within visceral adipose depots in obese mice. In the current study, we found that obesity also induces marked increases in leukocyte rolling in cremaster vessels of obese Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice at 15 weeks of age compared with lean Lepr<sup>−/−</sup>,Psgl-1<sup>−/−</sup> mice. Thus, the effect of obesity on leukocyte-endothelial interactions may be more systemic than previously suggested, although we cannot rule out a local effect of the adipose tissue surrounding the cremaster vessels. Irrespective of the mechanism, the cremaster vessels appear to be suitable to study leukocyte-endothelial interactions triggered by obesity in mice and are more suitable for analysis using standard intravital microscopy techniques. We found that the effect of genetic and diet-induced obesity on leukocyte-endothelial rolling was neutral in the setting of Psgl-1 deficiency suggesting that interactions between Psgl-1 and endothelial selectins are important for obesity-induced leukocyte rolling.

To determine if the decreased leukocyte-endothelial interactions observed in genetically or diet-induced obese Psgl-1<sup>−/−</sup> mice were associated with reduced adipose tissue macrophage content, macrophage immunostaining was performed on visceral and subcutaneous adipose tissue from those mice. Psgl-1 deficiency in both models of obesity resulted in reduced macrophage content in visceral adipose tissue, while no differences were observed in subcutaneous adipose tissue. Macrophage content was also analyzed in visceral and subcutaneous adipose tissue from lean Psgl-1<sup>−/−</sup> and Psgl-1<sup>+/+</sup> mice. Psgl-1 deficiency was found to decrease the obesity-induced rise in macrophage infiltration found in visceral fat. Obesity did not result in an increase in macrophage infiltration into subcutaneous fat, and Psgl-1 deficiency had no effect on macrophage content in subcutaneous fat in lean or obese mice, indicating differential regulation of adipose tissue macrophage infiltration between different adipose depots.

MCP-1 and TNFα expression has been shown to increase in visceral fat from obese mice. Psgl-1 deficiency in obese mice was associated with reduced MCP-1 and TNFα expression in visceral fat, perhaps due to decreased macrophage content in visceral fat.

Because sP-sel levels are positively regulated by leukocyte Psgl-1 in the basal state, we determined if sP- and sE-sel levels in both genetic and diet-induced obesity were also reduced in the setting of Psgl-1 deficiency. In this study, sP-sel and sE-sel were lower in Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice and Psgl-1<sup>−/−</sup> mice on a high-fat, high-sucrose diet compared with Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice and Psgl-1<sup>−/−</sup> mice on a high-fat, high-sucrose diet, respectively. MCP-1 was also lower in Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> mice compared with Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice. Collectively, these data indicate a regulatory role of Psgl-1 toward the generation of these soluble molecules in obesity.

To determine whether the effects of Psgl-1 on leukocyte-endothelial interactions in obesity were due to direct, acute interactions with endothelial selectins or to other, more chronic, effects of Psgl-1 on endothelial adhesive characteristics, adoptive transfer of rhodamine-labeled leukocytes was performed from Psgl-1<sup>−/−</sup> mice into obese Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> and Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> mice. Unexpectedly, donor Psgl-1<sup>−/−</sup> leukocyte rolling was markedly reduced in the Lepr<sup>db/db</sup>,Psgl-1<sup>−/−</sup> recipients compared with the Lepr<sup>db/db</sup>,Psgl-1<sup>+/+</sup> recipients. One explanation for these findings is that leukocytePsgl-1 chronically upregulates endothelial adhesive molecules by an unclear mechanism.

BMT was performed to determine the cellular source of Psgl-1 in obese mice responsible for altering the adhesive properties of the endothelium. Genetically obese mice receiving bone marrow from Psgl-1<sup>−/−</sup> mice displayed reduced sP-sel levels in addition to reduced rolling and firm attachment compared with genetically obese mice receiving wild-type bone marrow, indicating that hematopoietic Psgl-1 is responsible for chronically altering the adhesive characteristics of the endothelium. BMT with P-sel deficient donors indicated the predominant tissue source of sP-sel in the obese state was nonhematopoietic.

The findings above suggested that hematopoietic Psgl-1 plays a major role in regulating adhesive characteristics of the endothelium in obesity. Because cytokines such as IL-1β have been shown to induce selectin dependent rolling, we tested the hypothesis that Psgl-1 would positively regulate the endothelial response to IL-1β, while mice deficient in Psgl-1 would be resistant to IL-1β stimulation. Consistent with this hypothesis, sP-sel and MCP-1 levels in wild-type mice markedly increased after administration of recombinant IL-1β, whereas sP-sel and MCP-1 levels in Psgl-1<sup>−/−</sup> mice were unchanged after IL-1β stimulation. The reduced circulating levels of these proteins were associated with corresponding reduced gene transcription in lung and perigonadal adipose tissue. The cell source of the transcriptional differences were attributed to the endothelial cells within lung and perigonadal adipose tissue because the factors measured were either exclusively or primarily expressed by the endothelium. Lung tissue was used for transcriptional studies because it is a rich source of endothelial cells, and inflammation in the lung is regulated by IL-1β. Overall, the response of Psgl-1<sup>−/−</sup> mice to IL-1β was similar to that of mice with complete deficiency of the IL-1R. To further confirm the critical role of IL-1R signaling for regulation of sP-sel under basal, unstimulated conditions, sP-sel levels were markedly reduced in IL-1R<sup>−/−</sup> mice.

Although several cell types have been shown to express Psgl-1 and the IL-1R, the BMT experiments between Psgl-1<sup>−/−</sup> mice and Psgl-1<sup>+/+</sup> mice and between IL-1R<sup>−/−</sup>.
Figure 7. Hematopoietic Psgl-1 regulates endothelial response to IL-1β.

and IL-1R\(^{+/−}\) mice indicate that hematopoietic Psgl-1 (ie, leukocyte) is regulating the nonhematopoietic IL-1R signaling (ie, endothelium). The precise cellular source and further downstream mechanism(s) by which Psgl-1 regulates the adhesive properties of the endothelium will require additional study. However, it appears that the presence of Psgl-1 facilitates or the deficiency of Psgl-1 induces a block in pathways leading to NFκB activation, which probably are responsible for the subsequent changes in adhesion molecule expression (Figure 7).

The effect of Psgl-1 on glucose regulation in genetically obese mice may be secondary to the reduced macrophage content of visceral adipose depots as preclinical studies have demonstrated that inflammatory fat leads to insulin resistance.\(^{25}\) This effect on glucose was not observed in the diet-induced obese mice suggesting a more severe state of diabetes may be required to reveal this protective effect of Psgl-1 deficiency on hyperglycemia. Other potential mechanisms for improved glucose regulation may involve IL-1R signaling effects on pancreatic beta cell insulin secretion.\(^{26}\) Of clinical interest, trials with IL-1R inhibitors are associated with improved glucose regulation in humans.\(^{27}\)

Taken together, these results indicate an important role of hematopoietic Psgl-1 deficiency in the regulation of the adhesive properties of the endothelium via attenuation of endothelial signaling pathways. As a result, Psgl-1 deficiency is protective against visceral adipose tissue inflammation in obesity. Further elucidation of mechanisms by which leukocytes regulate the endothelial response to cytokines may lead to new treatments aimed at reducing the chronic inflammatory state associated with obesity.

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Disclosures

None.

References


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**Novelty and Significance**

**What Is Known?**

- P-selectin glycoprotein ligand-1 (Psgl-1) is a ligand on white blood cells that mediates binding to adhesion molecules on endothelial cells and other cell types.
- Deficiency of Psgl-1 reduces inflammation in some disease states.
- Obesity is associated with inflammation in visceral fat depots.

**What New Information Does This Article Contribute?**

- Deficiency of Psgl-1 leads to reduced visceral fat inflammation in obesity.
- Psgl-1 plays an important role in controlling adhesive interactions between blood cells and endothelial cells through an indirect mechanism.
- Psgl-1 deficiency leads to reduced endothelial activation in response to cytokine stimulation.
- Both Psgl-1−/− deficient mice and mice deficient in the IL-1 receptor have similarly low levels of circulating P-selectin in the basal state.

Inflammation in obese visceral fat may contribute to the adverse consequences of obesity. Identification of the factors responsible for fat inflammation may lead to new treatments aimed at reducing the risks associated with obesity. This study indicates that a leukocyte ligand, Psgl-1, plays an important role in visceral fat inflammation. Furthermore, the absence of Psgl-1 on blood cells leads to reduced activation of the endothelial cells after cytokine stimulation. This is associated with reduced transcription of some adhesion molecules and reduced leukocyte rolling on endothelial cells. Therapeutic antagonism of Psgl-1 may be beneficial in reducing the chronic inflammatory state associated with obesity. In addition, elucidation of the precise mechanism by which Psgl-1 affects adhesive properties of endothelial cells may lead to new targets for the treatment of inflammatory disease states.
P-Selectin Glycoprotein Ligand-1 Regulates Adhesive Properties of the Endothelium and Leukocyte Trafficking Into Adipose Tissue

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In the article that appears on page 388 of the August 6, 2010, issue, the name of one of the authors, Gabriel Núñez, was listed incorrectly. The full correct author list is reprinted below:

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The publisher regrets this error and it has been noted in the online version of the article, which is available at http://circres.ahajournals.org/cgi/content/full/107/3/388

Reference


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SUPPLEMENTAL MATERIALS AND METHODS

Immunohistochemistry
For histological analyses, fat pads were removed and fixed in zinc formalin for \(\geq 24\) hours. Macrophages in adipose cross-sections were identified with a rat anti-mouse Mac-3 monoclonal antibody (1:100 dilution; BD Biosciences, San Jose, CA), followed by detection with biotin-anti-rat IgG (Accurate Chemical & Scientific Corp., Westbury, NY). Stained cells were counted manually from four randomly chosen fields using Image-Pro Plus software (Media Cybernetics) and expressed as a percentage of total cells per field.

Real-Time Polymerase Chain Reaction (RTPCR)
Mice were sacrificed via cardiac puncture, and then perfused with PBS. RNA was isolated from 100 mg of perigonadal fat using 1 mL of QIAzol Lysis Reagent (QIAGEN Inc., Valencia, CA) and following the RNeasy Lipid Tissue Mini Handbook protocol (QIAGEN Inc., Valencia, CA). RNA was isolated from 30 mg of lung tissue using a QIAGEN RNeasy Mini Kit. RTPCR was performed using an ABI Prism 7000 Sequence Detection System from Applied Biosystems, Foster City, CA. 100 ng of RNA and 1µl of primer were used per reaction. P-sel, E-sel, MCP-1, and Tumor necrosis factor-alpha (TNFα) RTPCR primers were used (Applied Biosystems, Foster City, CA). 7000 System SDS Software and the \(2^{-\Delta\Delta C_T}\) method \(^1\) were used to analyze the results.

Bone marrow transplantation
Bone marrow transplantation (BMT) was performed as previously described \(^2\). 8-10 week-old Lepr\(^{db/db}\), Psgl-1\(^{+/+}\) mice were used as recipients for BMT from Psgl-1\(^{+/+}\), Psgl-1\(^{-/-}\) or Psel-1\(^{-/-}\) donors. Psgl-1\(^{-/-}\) mice received marrow from Psgl-1\(^{+/+}\) donors, and Psgl-1\(^{+/+}\) mice received marrow from Psgl-1\(^{-/-}\) donors. IL-1R \(^+/+\) mice received marrow from IL-1R \(^{-/-}\) donors, and IL-1R \(^{-/-}\) mice received marrow from IL-1R \(^{+/+}\) donors. Bone marrow was harvested from the donor mice by flushing their femurs and tibias with RPMI medium (Gibco/Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA). Cells were then centrifuged at 300 x \(g\) and resuspended in PBS before injection. Each recipient mouse was irradiated (2 x 650 rad [0.02 x 6.5 Gy]) and injected with 4 x 10\(^6\) bone marrow cells via the tail vein. Four weeks after transplantation, blood was drawn from the retro-orbital sinus.

Intravital microscopy
The intravital microscopy model consisted of a Nikon FN1 fixed stage microscopy system with X-cite for epi-fluorescence, Photometrics Coolsnap Cascade 512B color digital camera system, and MetaMorph premier software package and computer system. For analysis of cremaster vessels, male mice were anesthetized with pentobarbital (67mg/g) and positioned supine securely with tape. An incision was made in the scrotal skin to expose the left cremaster muscle, which was then removed from the surrounding fascia. A lengthwise incision was made on the ventral surface of the cremaster muscle, and the testicle and epididymis were separated from the underlying muscle and reintroduced into the abdominal cavity. The muscle was then spread over an optically clear viewing pedestal and secured along the edges with 3-0 suture. The exposed tissue was superfused with warm bicarbonate-buffered saline (pH 7.4). The cremaster microcirculation was observed through the intravital microscope with a 10x eyepiece and 40x objective lens. To visualize white blood cells, rhodamine 6G (0.3 mg/kg) (Sigma Chemical, St Louis, MO) was injected into the tail vein immediately prior to visualization. At this dose, rhodamine 6G labels leukocytes and allows detection of all rolling leukocytes. Rhodamine
6G-associated fluorescence was visualized by epi-illumination with a 510-560 nm emission filter. Single unbranched venules (20-40 µm in diameter) were selected for study and images of the microcirculation were digitally recorded.

Rolling leukocytes were defined as leukocytes that rolled at a velocity slower than red blood cells. Firm leukocyte adhesion was detected if leukocytes remained stationary for 30 seconds or longer. The number of rolling and firmly adherent leukocytes during each 35 second video was counted and expressed as cells/mm length of vessel. Three venules were analyzed from each mouse. Intravital microscopy was performed on diet-induced and genetically obese mice.

For adoptive transfer experiments, leukocytes were isolated from whole blood of Psgl-1+/+ mice with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO). Whole blood was layered onto histopaque brought to room temperature in a 1:1 ratio. Tubes were centrifuged at 400 x g for 30 minutes at room temperature. The upper layer was discarded and the opaque interface was transferred to another tube. 10mL of PBS was added to the tube and mixed by gentle aspiration. The solution was centrifuged at 250 x g for 10 minutes. The supernate was aspirated and the pellet was resuspended in 5mL of PBS. The solution was centrifuged at 250 x g for 10 minutes. The supernate was discarded and the pellet was resuspended in 1mL of PBS and then incubated with 100ul of 10X stock rhodamine 6G for 30 minutes. White blood cells were centrifuged at 250 x g for 10 minutes. Cells were washed twice in PBS and ultimately resuspended in 1mL PBS. 1x10⁸ leukocytes were then injected into Leprdb/db,Psgl-1+/+ and Leprdb/db,Psgl-1+/+ mice.

**Recombinant IL-1β**

Recombinant IL-1β (Peprotech, Rocky Hill, NJ) was injected into IL-1R−/−, Psgl-1−/−, Psgl-1+/+, Leprdb/db,Psgl-1+/+, and Leprdb/db,Psgl-1−/− mice (500ng in 200µl 1X PBS) via tail vein. Recombinant IL-1β was also injected into the following mice four weeks after BMT: Psgl-1−/− mice receiving marrow from Psgl-1+/+ mice; Psgl-1+/+ mice receiving marrow from Psgl-1−/− mice; IL-1R−/− mice receiving marrow from IL-1R−/− mice; and IL-1R−/− mice receiving marrow from IL-1R+/+ mice. Mice were bled for serum 5 hours post IL-1β injection.

**Immunoblotting**

Lung was homogenized and lysed in a buffer containing 150mM NaCl, 5mM EDTA, 50mM Tris-HCl pH 7.4, 1mM DTT, 1% NP-40 (Ipagel), 1% Triton X-100, supplemented with protease inhibitor cocktail (Roche), PMSF, and phosphatase inhibitor cocktail 1 and 2 (SIGMA, St. Louis, MO). Lysates were resolved by SDS-PAGE and transferred to PVDF membranes by electro-blotting. The antibodies for mouse IκBα, phospho-IκBα, and p38 were from Cell Signaling Technology, Danvers, MA.

**SUPPLEMENTAL ONLINE FIGURE**

Legend for Online Figure I.

**Online Figure I.** Macrophage content of perigonadal, pericardial, and subcutaneous adipose tissue. Representative cross sections of perigonadal adipose tissue from A) Leprdb/db,Psgl-1−/− and B) Leprdb/db,Psgl-1+/+ mice, pericardial adipose tissue from C) Leprdb/db,Psgl-1−/− and D) Leprdb/db,Psgl-1+/+ mice, and subcutaneous adipose tissue from E) Leprdb/db,Psgl-1−/− and F) Leprdb/db,Psgl-1+/+ mice. Staining with Mac3 antibody, magnification 40X, scale bar 200 µm, arrows showing stained cells. *p<0.05, **p<0.01, ***p<0.001.
SUPPLEMENTAL REFERENCES
