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P-selectin Glycoprotein Ligand-1 Deficiency Leads to Cytokine Resistance and Protection against Atherosclerosis in Apolipoprotein E Deficient Mice

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Abstract

Adhesive interactions between endothelial cells and leukocytes contribute to atherosclerotic plaque growth. However, mechanism(s) responsible for endothelial priming and deactivation in inflammatory diseases such as atherosclerosis are not clear. Apolipoprotein E deficient mice were generated with deficiency of P-selectin glycoprotein ligand-1 (*Psgl-1*^{-/-}, *ApoE*^{-/-}). On both standard chow and Western diet, *Psgl-1*^{-/-}, *ApoE*^{-/-} mice were protected against atherosclerosis compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} controls. *Psgl-1*^{-/-}, *ApoE*^{-/-} mice also showed reduced leukocyte rolling and firm attachment on endothelial cells, however, adoptively transferred *Psgl-1*^{+/+}, *ApoE*^{-/-} leukocytes into *Psgl-1*^{-/-}, *ApoE*^{-/-} hosts displayed similar reduced rolling as *Psgl-1*^{-/-}, *ApoE*^{-/-} leukocytes. Hematopoietic deficiency of Psgl-1 conferred resistance to the effects of interleukin-1 β (IL-1 β) on leukocyte rolling along with reduced circulating levels of sP-sel and sE-sel. Antibody blockade of Psgl-1 also reduced endothelial activation in response to IL-1 β , eliminated leukocyte rolling, and was protective against atherosclerosis in *ApoE*^{-/-} mice. Monocyte depletion with clodronate restored the endothelial response to IL-1 β in *Psgl-1*^{-/-} mice. This study suggests that Psgl-1 deficiency leads to reduced atherosclerosis and adhesive interactions between endothelial cells and leukocytes by indirectly regulating endothelial responses to cytokine stimulation.

Keywords

atherosclerosis; apolipoprotein E; monocyte; interleukin-1; 4RA10

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Authorship Contributions

Contribution: D.T.E. designed research and wrote the paper; W.L. designed research, performed experiments, analyzed data, and wrote the paper; H.W., M.K.O., C.G., K.S. and J.W. performed experiments.

DISCLOSURES

None.

Introduction

Complications of atherosclerosis remain the leading cause of mortality in the US and many other countries¹. Since the majority of cardiac events are not prevented with conventional treatment², new therapeutic targets need to be identified and tested to reduce this residual vascular risk.

Leukocytes are major contributors to the initiation and growth of atherosclerotic plaques^{3,4}. Leukocyte infiltration into the vessel wall is preceded by leukocyte-endothelial (L-E) interactions such as leukocyte rolling and firm attachment^{4,5}. L-E interactions are increased in atherosclerosis, although specific factors responsible for mediating these increased interactions are unclear. P-selectin glycoprotein ligand-1 (Psgl-1) is an adhesive ligand expressed on leukocytes and endothelial cells, and Psgl-1 deficiency is associated with reduced leukocyte rolling and firm endothelial attachment^{6,7}. Since selectins may affect the phenotype of the leukocyte by initiating signaling cascades following binding to leukocyte Psgl-18, it is possible that the effects of Psgl-1 on adhesive characteristics of the endothelium may be via mechanisms other than direct binding of leukocyte Psgl-1 to endothelial selectins.

In the current study, we explored a novel mechanism by which Psgl-1 regulates L-E interactions in atherosclerosis. We also determined the effect of Psgl-1 deficiency on atherosclerosis and tested the effects of pharmacologic inhibition of Psgl-1 on the progression of atherosclerosis.

Material and Method

Mice

Apolipoprotein E deficient (*ApoE*^{-/-}), and *Psgl-1*^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME) on the C57BL6/J strain background. Mice were housed under specific pathogen-free conditions in static microisolator cages. *Psgl-1*^{-/-} mice were crossed to *ApoE*^{-/-} mice to generate *Psgl-1*^{+/-}, *ApoE*^{+/-} mice, which were then mated to generate *Psgl-1*^{-/-}, *ApoE*^{-/-} breeding pairs. From these breedings, *Psgl-1*^{-/-}, *ApoE*^{-/-} mice and *Psgl-1*^{+/-}, *ApoE*^{-/-} offspring were studied for analysis of atherosclerosis. One group of experimental animals was fed a standard laboratory rodent chow (#5001, TestDiet, Richmond, IN) ad libitum for 30 weeks of age and then sacrificed. Another group of mice was fed a Western diet (TD88137, Harlan, WI) from 4 to 13 weeks of age. For passive transfer experiments, leukocytes were prepared from 10 week old *Psgl-1*^{+/-}, *ApoE*^{-/-} mice and injected to either *Psgl-1*^{+/-}, *ApoE*^{-/-} or *Psgl-1*^{-/-}, *ApoE*^{-/-} recipients that were 24 weeks of age and had been fed a Western diet for 8 weeks. Anti- Psgl-1 antibody (4RA10)⁹ was administered to 15 week old *Psgl-1*^{+/-}, *ApoE*^{-/-} mice for analysis of cytokines and to 20 week old *Psgl-1*^{+/-}, *ApoE*^{-/-} mice with 2 weeks of Western diet for intravital microscopy studies. For monocyte depletion experiments, clodronate-containing liposomes were administered to 8 week old *Psgl-1*^{+/-} or *Psgl-1*^{-/-} mice. All procedures conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the University of Michigan Committee on Use and Care of Animals.

Bone marrow transplantation (BMT)

At 10 weeks of age, *ApoE*^{-/-} mice were irradiated and BMT was performed as previously described¹⁰. Mice were given a Western diet beginning 5 weeks following the BMT for an additional 7 weeks, after which mice were euthanized.

Atherosclerosis quantification

For quantification of atherosclerosis, animals were euthanized under intraperitoneal pentobarbital anesthesia (100mg/kg) and analyzed as previously reported¹¹. Briefly, animals were perfused with saline at physiological pressure and then fixed using formalin with a 25-gauge needle inserted into the left ventricle at a rate of 1 ml/min. The carcass was fixed in formalin >24 hours, and the arterial tree was then meticulously dissected and placed in 70% ethanol. Arterial trees were stained with oil-red-O, pinned on wax trays, and the surface area occupied by oil-red-O staining lesion was quantified throughout the arterial tree including the aortic arch, brachiocephalic trunk, right and left common carotid arteries, and right and left subclavian arteries with Image-Pro Plus software (Media Cybernetics, Bethesda, MD). The lesion area was expressed as a percentage of total surface area examined.

Plasma measurements

Plasma samples were collected by retro-orbital bleeding with heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA) or by ventricular puncture at time of euthanasia. Circulating levels of soluble E-selectin (sE-sel) and P-selectin (sP-sel) were assayed with respective murine ELISA kits (R&D systems, Minneapolis, MN) according to manufacturers' instructions.

Intravital microscopy

The intravital microscopy model consisted of a Nikon FN1 fixed stage microscopy system with X-cite for epi-fluorescence, Photometrics Coolsnap Cacade 512B color digital camera system, and MetaMorph premier software package and computer system¹². For analysis of cremaster vessels, male mice were anesthetized with pentobarbital (50 mg/kg) 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 (0.67mg/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 (35–50 μ m 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.

For adoptive transfer experiments donor leukocytes were obtained from *ApoE*^{-/-} mice after injection of pentobarbital (100mg/kg). Leukocytes were isolated from whole blood using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) according to manufacturer's instructions, then incubated with rhodamine 6G (0.067mg/ml) (Thermo Fisher Scientific, New Jersey).

Drug administration

Recombinant interleukin-1 β (IL-1 β) (Peprotech, Rocky Hill, NJ) was injected every other day for 2 weeks via tail vein (500ng in 200 μ l PBS) into mice beginning 8 weeks post-BMT

and 4 weeks after starting a Western diet. Serum was collected 6 hours after the first injection and 2 weeks later after injections were completed.

Rat anti-mouse Psgl-1 antibody (rat anti-mouse CD162 (4RA10)) or control antibody (rat anti-mouse IgG k, BD Pharmingen, Franklin Lakes, NJ)⁹ were injected via tail vein (50µg in 200ul PBS) into mice either 16 hours before or 2.5 hours following IL-1β injection.

Clodronate (dichloromethylene diphosphonate) encapsulated liposomes (Encapsula Nanosciences, Nashville, TN) have been previously shown to cause monocyte depletion after intravenous administration¹³. Mice were anesthetized with isoflurane and clodronate-containing liposomes (50µl) or PBS was injected via tail vein. Complete blood cells counting (CBC) were measured in 20µl of whole blood obtained by retro-orbital bleeding into Microtainer[®] blood collection tubes (BD Biosciences, San Jose, CA and enumerated using an automatic cell counter (HEMAVET; Drew Scientific).

Statistical analyses

Values are expressed as mean ± SEM. The statistical significance of differences between groups was determined by Student's *t* test. Values of *p* < 0.05 were considered significant.

Results

Effect of Psgl-1 on leukocyte-endothelial interactions

Psgl-1 may affect L-E interactions via direct interactions with selectins⁶ or by indirect regulatory effects on endothelial transcription¹². Furthermore, both endothelial and hematopoietic pools of Psgl-1 have been shown to promote leukocyte adhesion¹².

To determine whether deficiency of Psgl-1 limited to the hematopoietic-cellular pool was sufficient to reduce L-E interactions in *ApoE*^{-/-} mice following an 8 week challenge of a Western diet, BMT was performed from *Psgl-1*^{-/-}, *ApoE*^{-/-} and *Psgl-1*^{+/+}, *ApoE*^{-/-} donors to *Psgl-1*^{+/+}, *ApoE*^{-/-} recipients. 12 weeks following the BMT, leukocyte rolling and firm attachment were both markedly reduced in *Psgl-1*^{+/+}, *ApoE*^{-/-} mice that received *Psgl-1*^{-/-}, *ApoE*^{-/-} bone marrow compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} mice that received *Psgl-1*^{+/+}, *ApoE*^{-/-} marrow (Figure 1A). To test the hypothesis that the hematopoietic Psgl-1 deficiency state was capable of limiting the enhanced L-E interactions in response to inflammatory cytokine stimulation, we challenged *Psgl-1*^{+/+}, *ApoE*^{-/-} mice (standard chow) that had received *Psgl-1*^{-/-}, *ApoE*^{-/-} or control *Psgl-1*^{+/+}, *ApoE*^{-/-} bone marrow with intravenous recombinant IL-1β injection. Six hours following cytokine stimulation, L-E interactions (rolling and firm attachment) were markedly reduced in *Psgl-1*^{+/+}, *ApoE*^{-/-} mice that had received *Psgl-1*^{-/-}, *ApoE*^{-/-} bone marrow compared to those that received *Psgl-1*^{+/+}, *ApoE*^{-/-} marrow (Figure 1B). Thus, these studies demonstrate the importance of hematopoietic Psgl-1 in mediation L-E interactions induced by diet or cytokines.

To investigate whether the effects of hematopoietic Psgl-1 were due to acute interactions with endothelial selectins or to other indirect effects of hematopoietic Psgl-1 on endothelial adhesive characteristics, adoptive transfer experiments were performed with rhodamine-labeled leukocytes isolated from *Psgl-1*^{+/+}, *ApoE*^{-/-} donors infused into either *Psgl-1*^{+/+}, *ApoE*^{-/-} or *Psgl-1*^{-/-}, *ApoE*^{-/-} recipients. Despite the presence of Psgl-1, transferred labeled *Psgl-1*^{+/+}, *ApoE*^{-/-} leukocytes still displayed reduced rolling and firm attachment if the recipient was *Psgl-1*^{-/-}, *ApoE*^{-/-} compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} (Figure 1C).

To investigate potential therapeutic applications of Psgl-1 inhibition, a Psgl-1 antibody (4RA10) previously shown to block interactions between Psgl-1 and selectins was given 16 hours prior to IVM and found to reproduce the Psgl-1 deficiency state as *Psgl-1*^{+/+}, *ApoE*^{-/-}

mice treated with 4RA10 showed reduced L-E interactions compared to control antibody-treated mice (Figure 1D).

Effect of Psgl-1 Deficiency on circulating levels of sP-sel and sE-sel

Since the experiment described above using adoptive transfer indicated that the mechanism by which Psgl-1 deficiency affects L-E interactions may be indirect, the effects of Psgl-1 deficiency on circulating markers of endothelial adhesive properties were determined. Levels of sP-sel and sE-sel were measured from plasma of *Psgl-1*^{+/+}, *ApoE*^{-/-} and *Psgl-1*^{-/-}, *ApoE*^{-/-} mice. Circulating levels of sP-sel and sE-sel were markedly decreased in *Psgl-1*^{-/-}, *ApoE*^{-/-} mice compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} mice on both Western and standard diets (Figure 2A and B). This regulatory effect of Psgl-1 deficiency on generation of these circulating molecules was due to the hematopoietic pool as plasma from *Psgl-1*^{+/+}, *ApoE*^{-/-} mice that were transplanted with *Psgl-1*^{-/-}, *ApoE*^{-/-} marrow contained markedly reduced levels of sP-sel and sE-sel compared to the *Psgl-1*^{+/+}, *ApoE*^{-/-} mice that received the *Psgl-1*^{+/+}, *ApoE*^{-/-} bone marrow. This was evident in the basal state, and after challenge with both acute (6 hours post) and chronic (2 weeks of injections every 48 hours) administration of IL-1 β (Figure 2C and D).

Psgl-1 antibody blockade with 4RA10 mimicked the effect of the Psgl-1 deficiency state on generation of sP-sel and sE-sel. A single injection of 4RA10 reduced levels of sP-sel and sE-sel for at least 2 days (Figure 2E and F). *Psgl-1*^{+/+}, *ApoE*^{-/-} mice treated with 4RA10 also showed reduced sP-sel and sE-sel levels in response to IL-1 β compared to mice receiving control antibody (Figure 2E and F). To further determine whether this anti-adhesive effect of Psgl-1 blockade could deactivate endothelium already stimulated with IL-1 β , 4RA10 was administered 2.5 hours after IL-1 β stimulation at a time point when sP-sel levels were already rising in plasma. The antibody was capable of reversing the rising concentrations of sP-sel in plasma indicating deactivation of the endothelium occurs with Psgl-1 blockade (Figure 3).

Effect monocyte depletion with clodronate on endothelial response to IL-1 β in Psgl-1 deficient mice

To determine the potential role of monocyte Psgl-1 deficiency in mediating endothelial deactivation, *Psgl-1*^{+/+} and *Psgl-1*^{-/-} mice were given clodronate to reduce circulating monocytes and then challenged with IL-1 β . Monocytes were reduced from $0.7 \pm 0.03 \times 10^3/\mu\text{L}$ to $0.2 \pm 0.04 \times 10^3/\mu\text{L}$ following clodronate ($p < 0.003$). There were no significant changes in total WBC ($10.1 \pm 1.2 \times 10^3/\mu\text{L}$ to $9.02 \pm 0.33 \times 10^3/\mu\text{L}$, $p = \text{NS}$), neutrophils ($1.8 \pm 0.2 \times 10^3/\mu\text{L}$ to $2.1 \pm 0.2 \times 10^3/\mu\text{L}$, $p = \text{NS}$), or lymphocytes ($7.6 \pm 1.01 \times 10^3/\mu\text{L}$ to $6.7 \pm 0.22 \times 10^3/\mu\text{L}$, $p = \text{NS}$) following clodronate and no differences in response to control injection between *Psgl-1*^{+/+} and *Psgl-1*^{-/-} mice (data not shown). Control *Psgl-1*^{-/-} mice treated with PBS were completely resistant to the effects of IL-1 β on sP-sel levels, however *Psgl-1*^{-/-} mice became responsive to IL-1 β after monocytes were depleted with clodronate (Figure 4). *Psgl-1*^{+/+} mice were similarly responsive to IL-1 β challenge with and without clodronate (Figure 4). This indicates that Psgl-1 deficient monocytes are capable of blocking the endothelial effects of IL-1 β .

Effect of Psgl-1 deficiency on atherosclerosis

To determine the effect of Psgl-1 deficiency on progression of atherosclerosis in *ApoE*^{-/-} mice on both a standard chow and Western diet, quantitation of atherosclerosis by surface oil-red-O staining was performed and revealed reduced aortic surface area covered by atherosclerosis in *Psgl-1*^{-/-}, *ApoE*^{-/-} mice compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} mice on both standard chow and Western diet protocols (Figure 5A–E). There were no differences in body

weight or cholesterol E levels between *Psgl-1*^{-/-}, *ApoE*^{-/-} and *Psgl-1*^{+/+}, *ApoE*^{-/-} mice on either dietary protocol (data not shown).

Effect of Psgl-1 antibody blockade on atherosclerosis

Since Psgl-1 deficiency demonstrated a protective effect on atherosclerosis, a chronic pharmacologic intervention with the anti-Psgl-1 antibody, 4RA10, was tested. Weekly administration of 4RA10 to 14 week old *Psgl-1*^{+/+}, *ApoE*^{-/-} mice up to 20 weeks of age was associated with reduced atherosclerosis compared to mice receiving control antibody injections (Figure 6).

Discussion

Leukocyte recruitment to the arterial wall plays a prominent role in atherogenesis and complications of advanced atherosclerosis^{3,4}. Factors involved in promoting leukocyte recruitment to atherosclerotic plaques may therefore be useful therapeutic targets. However, the precise mechanisms by which leukocytes are recruited to growing atherosclerotic plaques remain unclear. For example, it is unknown how endothelial adhesive properties are switched on and off. While many cytokines and signaling pathway components have shown to be involved in endothelial activation, the mechanism by which the endothelium is turned off is not known. Elucidation of the molecular mechanism(s) by which the endothelium is deactivated could lead to development of agents designed to turn off inflammatory processes after they have been initiated.

Psgl-1 is a ligand for P, E, and L-selectin, and has been shown to directly mediate rolling of leukocytes on surfaces expressing P-selectin *in vitro*⁶. In addition, mice deficient in Psgl-1 have reduced rolling *in vivo* as assessed using intravital microscopy¹⁵. In *ApoE*^{-/-} mice on a Western diet, deficiency of Psgl-1 has been shown to be associated with reduced atherosclerosis and reduced neointima formation following carotid injury¹⁶. An et al showed that Psgl-1 was highly expressed on Ly6-C^{hi} monocytes and that these cells exhibited more interactions with immobilized selectins under flow conditions and more rolling on carotid arteries in an *ex vivo* flow model¹⁶. The increased rolling in the *ex vivo* model by Ly6-C^{hi} monocytes was attenuated if the monocytes were taken from a Psgl-1 deficient mouse¹⁶. Previous studies had shown that Western diet triggered an increase of circulating Ly6-C^{hi} monocytes and that these cells were the subtype found trafficking to atherosclerotic plaques¹⁷. Taken together, these previous studies suggest that the increased atherosclerosis triggered by Western diet in *ApoE*^{-/-} mice is due to increased L-E interactions mediated by Ly6-C^{hi} monocyte Psgl-1.

In the current study, the effect of Psgl-1 was examined on the development of atherosclerosis in *ApoE*^{-/-} mice on both standard and Western diets. Consistent with the previous study¹⁶, deficiency of Psgl-1 was associated with reduced atherosclerosis burden in mice challenged with Western diet. However, even on a standard chow, which doesn't induce the Ly6-C^{hi} monocytosis observed in Western diet-fed mice¹⁷, protection was noted in *Psgl-1*^{-/-}, *ApoE*^{-/-} compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} mice. This athero-protection was associated with reduced L-E interactions in *Psgl-1*^{-/-}, *ApoE*^{-/-} mice compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} mice. These results are consistent with Psgl-1-dependent leukocyte trafficking to sites of developing atheroma on both Western and standard diets.

To determine whether changes in the leukocyte versus the endothelium were directly responsible for driving the increased L-E interactions observed in Western diet-fed *ApoE*^{-/-} mice, adoptive transfer experiments were performed with rhodamine-labeled leukocytes obtained from *Psgl-1*^{+/+}, *ApoE*^{-/-} mice on standard chow diet and then analyzed using intravital microscopy in *Psgl-1*^{+/+}, *ApoE*^{-/-} or *Psgl-1*^{-/-}, *ApoE*^{-/-} mice on the Western

diet. When these cells were infused into control *Psgl-1*^{+/+}, *ApoE*^{-/-} mice, they were found to exhibit a similar increased rolling and firm attachment as observed in the endogenous Western diet-fed leukocyte population. When these Psgl-1-bearing labeled leukocytes were injected into recipient *Psgl-1*^{-/-}, *ApoE*^{-/-} mice, the leukocytes demonstrated reduced rolling similar to that observed in the endogenous Psgl-1 deficient leukocyte population. This finding suggested to us that in the setting of host Psgl-1 deficiency, adhesive properties of the endothelium are down-regulated and that changes in the endothelium are primarily responsible for the reduced L-E interactions observed in the Psgl-1 deficient state, in the setting of hyperlipidemia and atherosclerosis.

We have previously demonstrated in lean and obese mice that hematopoietic Psgl-1 deficiency leads to reduced generation of sP-sel, sE-sel, and Mcp-1^{12,14}. This unexpected regulatory role of hematopoietic Psgl-1 deficiency appears to involve transcriptional repression of endothelial adhesive and chemotactic factors¹². To determine the relevance of this mechanism in the setting of atherosclerosis, we measured levels of sP-sel, sE-sel in *ApoE*^{-/-} mice with variable Psgl-1 expression. Levels of sP-sel and sE-sel were markedly reduced in *Psgl-1*^{-/-}, *ApoE*^{-/-} mice compared to *Psgl-1*^{+/+}, *ApoE*^{-/-} mice on both standard and Western diets. Experiments involving the generation of chimeric mice using bone marrow transplantation into *ApoE*^{-/-} mice revealed that the effect of Psgl-1 on the generation of sP-sel and sE-sel was due to deficiency of the hematopoietic pool of Psgl-1. Thus, *Psgl-1*^{+/+}, *ApoE*^{-/-} recipient mice receiving *Psgl-1*^{-/-}, *ApoE*^{-/-} marrow had reduced levels of circulating selectins along with reduced L-E interactions.

Since IL-1 β has been used to induce L-E interactions and may contribute to increased L-E interactions in inflammatory states through effects on the endothelium^{18,19}, we hypothesized that the state of hematopoietic Psgl-1 deficiency might impair the ability of cytokines, such as IL-1 β , to stimulate the endothelium. Consistent with this hypothesis, mice with hematopoietic deficiency of Psgl-1 were resistant to the effects of systemic administration of IL-1 β on generation of sP-sel, sE-sel and on L-E interactions. Monocyte depletion with clodronate restored the endothelial response to IL-1 β in Psgl-1 deficient mice, indicating that the Psgl-1 deficient monocyte is responsible for attenuating the IL-1 β stimulatory effects on the endothelium.

The results with Psgl-1 on atherosclerosis indicate that therapeutic blockade of Psgl-1 may prevent endothelial activation and slow progression of atherosclerosis. To pursue the therapeutic potential of Psgl-1 blockade, an antibody was used that has previously been shown to be protective against neointima formation in a model of acute arterial injury⁹. Because of our observation that the Psgl-1 deficiency state is associated with reduced levels of sP-sel and sE-sel, we have an excellent means by which to determine efficacy of treatment with Psgl-1 antagonists. Using sP-sel as a biomarker of Psgl-1 expression, we found that a single dose of antibody reduced baseline levels of sP-sel within 24 hours of administration and levels remained significantly lower than control antibody-treated mice for 2 days after treatment. The antibody was effective in completely blocking the effects of IL-1 β on generation of sP-sel, sE-sel, and on L-E interactions. Even when given 2.5 hours following IL-1 β , after IL-1 β had initiated the increased generation of circulating sP-sel, the antibody was capable of reversing the rise in sP-sel levels. These findings indicate that induction of the Psgl-1 deficient state leads to rapid deactivation of the endothelium and support the hypothesis that the Psgl-1 inhibited leukocyte rapidly assumes potent anti-inflammatory properties. This effect could be mediated by secretion of an anti-inflammatory soluble factor or by leukocyte-endothelial cell-mediated contact inhibition of post IL-1 receptor signaling pathways. Adhesive interactions mediated via other molecules that were not measured in this study are likely also be affected by the Psgl-1 deficiency state. Although precise details of the downstream mechanism(s) responsible for this effect are

unknown at this time, these findings suggest a promising therapeutic potential for Psgl-1 inhibition in vascular diseases. To determine the potential for Psgl-1 inhibition in atherosclerosis, *ApoE*^{-/-} mice were treated with weekly injections of the anti-Psgl-1 antibody for 6 weeks. Compared to mice treated with the control antibody, anti-Psgl-1 treated mice demonstrated protection from atherosclerosis.

In conclusion, hematopoietic Psgl-1 deficiency prevents endothelial activation and reduces atherosclerosis in *ApoE*^{-/-} mice. These athero-protective effects can be achieved with antibody blockade of Psgl-1. Although additional mechanistic studies related to Psgl-1 deficiency may uncover other downstream targets, the current study supports consideration of anti-Psgl-1 therapy for patients at high risk of vascular complications.

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Figure 1. Effect of Psgl-1 status on leukocyte-endothelial (L-E) interactions

A) L-E interactions in *Psgl-1*^{+/+}, *ApoE*^{-/-} mice that received either *Psgl-1*^{+/+}, *ApoE*^{-/-} (white bar) or *Psgl-1*^{-/-}, *ApoE*^{-/-} bone marrow transplants (black bar) at baseline and B) 6 hours post IL-1 β challenge. C) Adoptively transferred leukocytes from *Psgl-1*^{+/+}, *ApoE*^{-/-} donors into *Psgl-1*^{+/+}, *ApoE*^{-/-} (white bar) or *Psgl-1*^{-/-}, *ApoE*^{-/-} recipients (black bar). D) *Psgl-1*^{+/+}, *ApoE*^{-/-} mice with control antibody (white bar) or 4RA10 (black bar). Representative intravital microscopy image of venule from *Psgl-1*^{+/+}, *ApoE*^{-/-} mouse treated with E) control antibody or with F) 4RA10. Arrows point to rhodamine labeled leukocytes. **P*<0.05. ***P*<0.001. ****P*<0.0001. N= 5 per group.

Figure 2. Effect of Psgl-1 status on circulating levels of sP-sel and sE-sel

A) sP-sel and B) sE-sel levels from 13 week old mice on Western diet for 9 weeks, and 30 week old mice maintained on standard diet; *Psgl-1*^{+/+}, *ApoE*^{-/-} (white bar) and *Psgl-1*^{-/-}, *ApoE*^{-/-} (black bar). C) sP-sel and D) sE-sel levels in *Psgl-1*^{+/+}, *ApoE*^{-/-} mice after either *Psgl-1*^{+/+}, *ApoE*^{-/-} (white bar) or *Psgl-1*^{-/-}, *ApoE*^{-/-} bone marrow transplantation (black bar). E) sP-sel and F) sE-sel levels in *Psgl-1*^{+/+}, *ApoE*^{-/-} mice treated with control antibody (white bar) or 4RA10 (black bar). *P<0.05. **P<0.001. ***P<0.0001. N=9 per genotype.

Figure 3. Effect of Psgl-1 antibody blockade given after IL-1 β on sP-sel levels
Circulating levels of sP-sel in *Psgl-1*^{+/+}, *ApoE*^{-/-} mice in which IV control antibody (dashed line) or 4RA10 (solid line) was administered (arrow) 2.5 hours after IL-1 β stimulation at a time point when sP-sel levels were rising in plasma. * $P < 0.02$. N=4 per genotype.

Figure 4. Effect of monocyte depletion on sP-sel levels in *Psgl-1*^{+/+}, *ApoE*^{-/-} and *Psgl-1*^{-/-}, *ApoE*^{-/-} mice

Circulating levels of sP-sel in *Psgl-1*^{+/+}, *ApoE*^{-/-} and *Psgl-1*^{-/-}, *ApoE*^{-/-} mice treated with PBS or clodronate liposome before (white bar) and after IL-1β (black bar). **P*<0.05.

***P*<0.001. N=3 per genotype.

Figure 5. Effect of Psgl-1 deficiency on atherosclerosis

Representative en face view of aortic trees stained with oil-red-O from A) *Psgl-1*^{+/+}, *ApoE*^{-/-} and B) *Psgl-1*^{-/-}, *ApoE*^{-/-} 13 week old mice on Western diet for 9 weeks; and C) *Psgl-1*^{+/+}, *ApoE*^{-/-} and D) *Psgl-1*^{-/-}, *ApoE*^{-/-} 30 week old mice on standard chow. E) Mean % surface area of aortic arch covered with lipid-rich atherosclerotic lesions. **P*<0.05. ***P*<0.001. N=11–15 per genotype.

Figure 6. Effect of Psgl-1 antibody blockade with 4RA10 on atherosclerosis

Representative en face view of aortic trees stained with oil-red-O from mice treated with 9 weekly injections of A) Control IgG k antibody or B) 4RA10. C) Mean % surface area of aortic arch covered with lipid-rich atherosclerotic lesions. * $P < 0.03$. N=8 per genotype.