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Brief Definitive Report

P-Selectin Glycoprotein Ligand 1 (PSGL-1) Is a Physiological Ligand for E-Selectin in Mediating T Helper 1 Lymphocyte Migration

By Takako Hirata, Glenn Merrill-Skoloff, Melissa Aab, Jing Yang, Barbara C. Furie, and Bruce Furie

From the Center for Hemostasis and Thrombosis Research, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Abstract

P-selectin glycoprotein ligand 1 (PSGL-1) is a sialomucin expressed on leukocytes that mediates neutrophil rolling on the vascular endothelium. Here, the role of PSGL-1 in mediating lymphocyte migration was studied using mice lacking PSGL-1. In a contact hypersensitivity model, the infiltration of $CD4^+$ T lymphocytes into the inflamed skin was reduced in PSGL-1–deficient mice. In vitro–generated T helper (Th)1 cells from PSGL-1–deficient mice did not bind to P-selectin and migrated less efficiently into the inflamed skin than wild-type Th1 cells. To assess the role of PSGL-1 in P- or E-selectin–mediated migration of Th1 cells, the cells were injected into E- or P-selectin–deficient mice. PSGL-1–deficient Th1 cells did not migrate into the inflamed skin of E-selectin–deficient mice, indicating that PSGL-1 on Th1 cells is the sole ligand for P-selectin in vivo. In contrast, PSGL-1–deficient Th1 cells migrated into the inflamed skin of P-selectin–deficient mice, although less efficiently than wild-type Th1 cells. This E-selectin–mediated migration of PSGL-1–deficient or wild-type Th1 cells was not altered by injecting a blocking antibody to L-selectin. These data provide evidence that PSGL-1 on Th1 cells functions as one of the E-selectin ligands in vivo.

Key words: cellular immunity • contact hypersensitivity • P-selectin • E-selectin • knockout mice

Introduction

The regulated migration of leukocytes from the bloodstream into tissues in response to inflammatory stimuli is a critical component of both innate and adaptive immune responses. This process involves a variety of adhesion and signaling molecules whose coordinate action defines the selective migratory behavior of particular classes of leukocytes. The initial adhesive events in this process, characterized by the capture of leukocytes followed by their rolling on the endothelial surface under vascular shear flow, are mediated by selectins: L-selectin expressed on most leukocytes and E- and P-selectin expressed on activated endothelium. Previous studies using blocking antibodies or gene-targeted mice have demonstrated that all three selectins coordinately regulate leukocyte trafficking in vivo. Cooperative functioning of P-selectin with either E- or L-selectin is essential for efficient neutrophil rolling and migration into sites of inflammation (1–4). In a model of contact hypersensitivity, a form of delayed-type hypersensitivity induced by cutaneous application of a reactive hapten (5), P- and E-selectin may cooperatively mediate T cell migration into the skin during the efferent phase (6–8).

All selectins recognize a sialylated and fucosylated glycan on their counterreceptors. The presentation of the carbohydrate on the protein backbone contributes to the specificity and affinity of selectin binding. A major question concerning selectin ligands is whether they mediate physiologically relevant cell–cell interaction (9). Multiple studies have established the physiologic role of P-selectin glycoprotein ligand 1 (PSGL-1) as a predominant ligand for P-selectin on neutrophils, as is most clearly demonstrated by the deficient P-selectin–mediated neutrophil rolling in PSGL-1–deficient mice (10). PSGL-1 on other classes of leukocytes such as monocytes, T lymphocytes, and mast cells as well as on hematopoietic stem cells is likely to be a predominant ligand for P-selectin (11–14), although in vivo evidence for this remains to be established. Although a number of reports have shown that PSGL-1 is capable of binding to E-selectin (15–22) and L-selectin (23–26), no

Address correspondence to Bruce Furie, Research East #319/Beth Israel Deaconess Medical Center, Boston, MA 02215. Phone: 617-667-0620; Fax: 617-975-5505; E-mail: bfurie@caregroup.harvard.edu

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direct evidence for the physiologic roles of these interactions has yet emerged. The availability of PSGL-1–deficient mice should help to define the physiological role of PSGL-1, specifically the role as a ligand for each of the selectins in various cell types.

In this study, we analyzed the role of PSGL-1 in lymphocyte trafficking using PSGL-1–deficient mice. We demonstrate that PSGL-1 plays an important role in Th1 lymphocyte migration into the inflamed skin in an oxazolone-induced contact hypersensitivity model. We confirm that PSGL-1 on Th1 cells functions as the sole ligand for P-selectin in vivo. Our data also indicate that PSGL-1 on Th1 cells is an E-selectin ligand in vivo. This is the first example of a physiologic role for the interaction of PSGL-1 with E-selectin.

Materials and Methods

Mice. Mice homozygous for the PSGL-1 targeted mutation (PSGL-$1^{-/-}$ mice) have been described (10). 8–12-wk-old PSGL-$1^{-/-}$ mice and control PSGL-$1^{+/+}$ mice on the same mixed genetic background were used. C57BL/6J (B6) mice, P-selectin–deficient mice on the B6 background, B6 × 129S3/SvImJ (129S) F2 mice, E-selectin–deficient mice on the B6/129S background, and P- and E-selectin double-deficient mice on the B6/129S background were purchased from The Jackson Laboratory and used at similar ages. All procedures were approved by the Animal Care and Use Committee of Beth Israel Deaconess Medical Center.

Selectin–IgG Chimeras. Mouse P- and E-selectin–IgG chimeras were constructed from mouse P- and E-selectin cDNAs provided by Dr. D. Vestweber (University of Münster, Münster, Germany). Mouse L-selectin–IgG chimera was constructed from mouse spleen cDNA. The fragments corresponding to their lectin, epidermal growth factor, and two complement repeat domains were amplified by PCR and ligated to CD5 leader–IgG1 vector provided by Dr. B. Seed (Massachusetts General Hospital, Boston, MA) at NheI and BamHI sites. These selectin–IgG plasmids were transfected into CHO cells with pSV2neo (CLONTECH Laboratories, Inc.), and stable transfectants were selected in the presence of G418 (GIBCO BRL). Selectin–IgG chimeric proteins were purified from culture supernatants using protein A–Sepharose (Sigma-Aldrich).

Generation of Th1 Cells. Splenic $CD4^+$ T cells were isolated using Mouse T cell CD4 Subset Column Kit (R&D Systems). The enriched population was >95% positive for CD4 staining as determined by flow cytometry. Purified $CD4^+$ T cells were cultured on 24-well tissue culture plates (Costar) coated with 20 μg/ml anti-CD3ε (145-2C11; R&D Systems) and 10 μg/ml anti-CD28 (37.51; BD PharMingen) for 2 d in the presence of 4 ng/ml IL-2 (R&D Systems), 2 ng/ml IL-12 (R&D Systems), and 4 μg/ml anti–IL-4 (11B11; BD PharMingen). The cells were then transferred to uncoated plates and cultured for an additional 4 d.

Cell Adhesion Assays. 96-well plates (Flow Laboratories) were coated with 10 μg/ml selectin–IgG chimeras for 2 h at 37°C and blocked with 1% BSA in PBS overnight at 4°C. The cells were labeled with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein (BCECF; Molecular Probes), added to each well (5×10^5 per well), and incubated for 20 min at 4°C under rotation (60 rpm). The plates were washed three times with HBSS with $CaCl_2$ and $MgCl_2$, and bound cells were quantitated using a Cytofluor 2300 (Millipore). In some experiments, the cells were incubated with 10 μg/ml rabbit anti–PSGL-1 (10), control rabbit IgG (Sigma-Aldrich), 50 μg/ml anti–L-selectin mAb MEL-14 (BD PharMingen), or control rat IgG (IgG2a; BD PharMingen) for 30 min on ice and washed.

Induction of Contact Hypersensitivity. Mice were sensitized by the application of 100 μl of 2% (wt/vol) oxazolone (Sigma-Aldrich) in 4:1 acetone/olive oil (Sigma-Aldrich) on the abdominal skin on day 0. Some mice were painted with acetone/olive oil alone. Mice were challenged on day 6 by applying 20 μl of 0.5% (wt/vol) oxazolone on the left ear (10 μl per side). The right ear was painted with the vehicle only. Ear swelling responses were measured using a dial thickness gage (Mitutoyo).

Histologic Analyses and Immunohistochemistry. Ear specimens were taken 24 h after challenge. Histological analyses were performed on formalin-fixed, paraffin-embedded sections stained with hematoxylin and eosin. For immunohistochemistry, cryostat sections were fixed in acetone and stained using rat anti-CD4 (Caltag), biotinylated rabbit anti-rat IgG (Vector Laboratories), and ABC-alkaline phosphatase reagent (Vector Laboratories). Sections were developed using Vector Red alkaline phosphatase substrate and counterstained with methylene green.

In Vivo Migration Assays. Th1 cells were harvested after 6 d of culture, and dead cells were removed by centrifugation on Histopaque 1083 (Sigma-Aldrich). The cells (10^7/ml) were radiolabeled with 100 μCi/ml sodium [^{51}Cr]chromate (NEN Life Science Products) for 1 h at 37°C, washed twice, and resuspended in PBS. Cells ($2–4 \times 10^6$) were injected into the tail veins of mice that had been sensitized 7 d before and challenged on the left ear 24 h before. In some experiments, MEL-14, anti-E-selectin mAb 9A9 provided by Dr. B. Wolitzky (Coelacanth Corp., East Windsor, NJ) or its isotype control (200 μg/mouse) was injected together with the cells. Mice were killed 3 h after injection, and the radioactivity in the ear was measured using a Packard gamma scintillation counter.

Results and Discussion

PSGL-1–deficient Mice Exhibit Reduced $CD4^+$ T Cell Infiltrate in the Inflamed Skin in a Contact Hypersensitivity Model. PSGL-1 is highly expressed on T cells and to a much lower degree on B cells. Flow cytometric analyses of thymocytes, splenocytes, LN cells, and blood lymphocytes revealed no alterations in the expression of CD3, CD4, CD8, TCR-α/β, or B220 in PSGL-$1^{-/-}$ mice (data not shown). The normal lymphoid architecture in PSGL-$1^{-/-}$ mice is consistent with the fact that L-selectin is the major homing receptor for naive lymphocytes (27) and that PSGL-1 is expressed in a nonfunctional form on naive lymphocytes and gains selectin-binding activity during cell activation (28). Therefore, we studied the role of PSGL-1 in immune responses after antigen stimulation using an oxazolone-induced contact hypersensitivity model. PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ mice were sensitized with oxazolone on the abdominal skin, and 6 d later they were challenged on the left ear. The kinetics of ear swelling measured over 72 h after challenge was not significantly different between PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ mice (data not shown). Histological analysis of the skin sections from the ears 24 h after challenge showed characteristic changes of inflammation including interstitial edema, cellular infiltrate in the dermis

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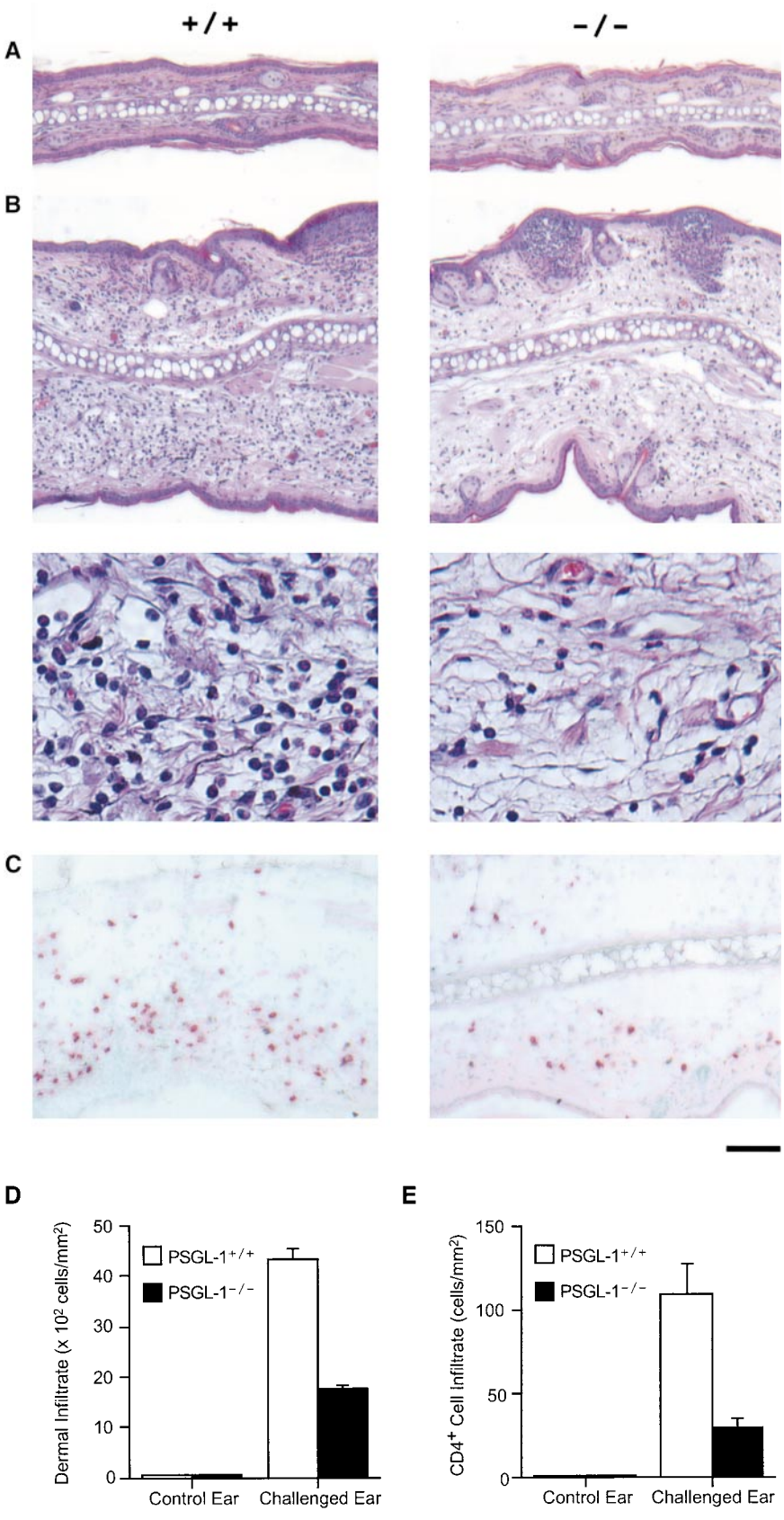

Figure 1. Histological analysis of contact hypersensitivity response. (A–C) Hematoxylin and eosin–stained sections from control (A) or challenged ears (B) and anti-CD4–stained frozen ear sections from the challenged ears (C) from sensitized PSGL-$1^{+/+}$ (left panel) or PSGL-$1^{-/-}$ mice (right panel). Ear specimens were taken 24 h after challenge. The scale bar indicates 100 μm for A, B top panel, and C; 25 μm for B bottom panel. (D) The number of infiltrating cells in the dermis of the control and challenged ears was quantitated. Data are means ± SEM from six mice. (E) The number of infiltrating $CD4^+$ T cells was quantitated on frozen sections stained with anti-CD4. Data are means ± SEM from four mice.

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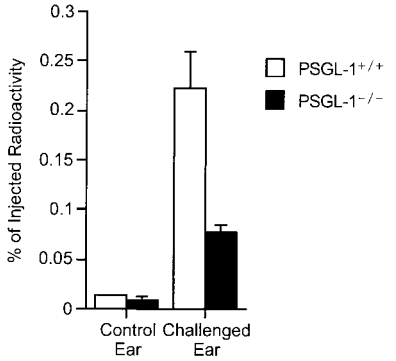

Figure 2. Migration of PSGL-1–deficient Th1 cells into the inflamed skin is impaired. ^{51}Cr-labeled PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ Th1 cells were injected into the tail veins of PSGL-$1^{+/+}$ mice. The mice had been sensitized 6 d before with oxazolone and challenged 24 h before on the left ear. The mice were killed 3 h after injection, and the radioactivity in the control and challenged ears was counted. Values are means ± SEM from four mice.

comprised predominantly of mononuclear cells and neutrophils, and microabcesses in the epidermis (Fig. 1 B). These histologic changes were not observed in unchallenged ear sections (Fig. 1 A) or challenged ear sections from unsensitized mice. Although the edema in PSGL-$1^{-/-}$ mice was similar to that in PSGL-$1^{+/+}$ mice, the cellular infiltrate in the dermis was reduced by 60% in PSGL-$1^{-/-}$ mice (Fig. 1, B and D). In addition, immunohistochemical analysis showed that $CD4^+$ T lymphocyte infiltration was reduced by 72% in PSGL-$1^{-/-}$ mice (Fig. 1, C and E). These results suggest that PSGL-1 may play a role in the migration of the inflammatory cells including $CD4^+$ T lymphocytes into the inflamed skin in a contact hypersensitivity model.

Migration of PSGL-1–deficient Th1 Cells into the Inflamed Skin Is Impaired. There are two functionally distinct $CD4^+$ T lymphocyte subsets, Th1 and Th2, characterized by distinct cytokine production profiles. Previous studies have shown that both P- and E-selectin mediate the migration of Th1 cells but not Th2 cells to sites of delayed-type hypersensitivity (8) and that antibodies against PSGL-1 partially inhibit the migration of Th1 cells into these sites (12). Based on these observations, the migratory behavior of PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ Th1 cells was studied. PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ Th1 cells generated in vitro exhibited indistinguishable profiles of cytokine production and expression of L-selectin, integrin α4, and integrin β2 (data not shown). These cells were labeled with ^{51}Cr and injected into mice previously sensitized with oxazolone and challenged on the left ear. The right ear served as control skin. 3 h after injection, the radioactive cells that accumulated in the ears were measured. Similar to results using anti–PSGL-1 antibodies (12), the migration of PSGL-$1^{-/-}$ Th1 cells into the inflamed ear of PSGL-$1^{+/+}$ mice was reduced by 66% compared with PSGL-$1^{+/+}$ Th1 cells, while the migration into the control ear was comparable between PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ cells (Fig. 2). No migration was observed when the cells were injected into mice lacking both P- and E-selectin.

PSGL-1–deficient Th1 Cell Binding to P-Selectin but Not to E-Selectin Is Abolished. PSGL-1 on Th1 cells binds to P-selectin in vitro (12). The observation that the migration of PSGL-$1^{-/-}$ Th1 cells into the inflamed skin is impaired raises a question whether PSGL-1 on Th1 cells involves only a P-selectin–mediated process, an E-selectin–mediated process, or both. To explore this, we tested Th1 cells for their ability to bind selectins using selectin–IgG chimeras. No detectable binding was observed for unstimulated $CD4^+$ cells from PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ mice to either P-selectin–IgG or E-selectin–IgG (Fig. 3 A). PSGL-$1^{+/+}$ Th1 cells bound P-selectin–IgG and E-selectin–IgG (Fig. 3 A). No binding to L-selectin–IgG by Th1 cells was detectable. In contrast to the complete absence of binding of PSGL-$1^{-/-}$ Th1 cells to P-selectin, the binding of PSGL-$1^{-/-}$ Th1 cells to E-selectin was measurable, albeit reduced

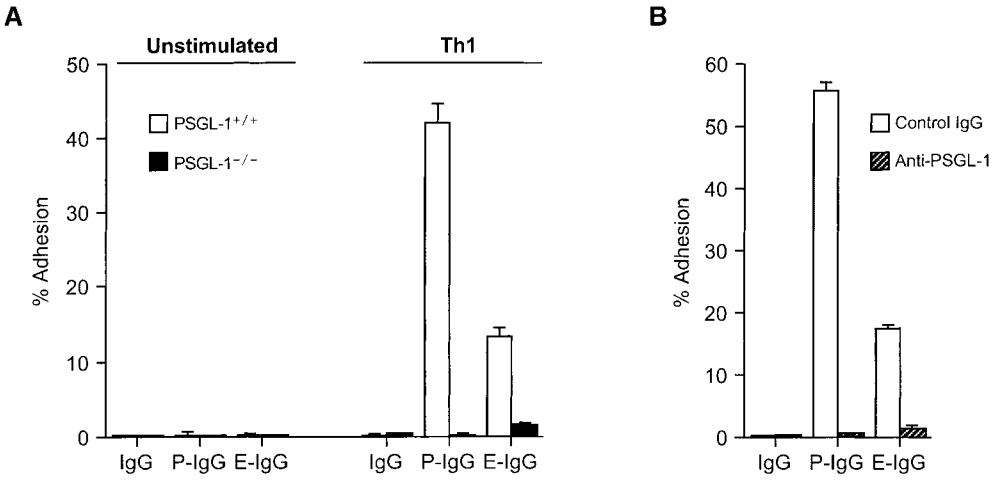

Figure 3. Selectin-binding activities of PSGL-1–deficient Th1 cells. In vitro–generated Th1 cells from PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ mice were labeled with BCECF and added to 96-well plates coated with human IgG, P-selectin–IgG chimera (P-IgG), or E-selectin–IgG chimera (E-IgG). The plates were incubated for 20 min at 4°C under rotating conditions, unbound cells were removed, and the fluorescence per well was determined. Percent adhesion equals 100 × bound cells/total cells added. (A) Adhesion of PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ unstimulated $CD4^+$ cells and Th1 cells to selectin–IgG chimeras. (B) Adhesion of PSGL-$1^{+/+}$ Th1 cells treated with anti–PSGL-1 antibodies to selectin–IgG chimeras. PSGL-$1^{+/+}$ Th1 cells were incubated with either rabbit anti–PSGL-1 or nonimmune IgG for 30 min on ice and washed before addition to the wells. Values are means ± SEM from triplicate wells.

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by 89% compared with PSGL-$1^{+/+}$ cell binding to E-selectin (Fig. 3 A). Similarly, the binding of PSGL-$1^{+/+}$ Th1 cells to E-selectin–IgG was inhibited by 91% by pretreatment of the cells with anti–PSGL-1 antibodies directed against the NH_2-terminal region of PSGL-1 (Fig. 3 B). Therefore, PSGL-1 plays a role in Th1 cell binding to E-selectin in vitro. However, PSGL-1 does not appear to be the sole ligand for E-selectin, as shown by the residual binding of PSGL-$1^{-/-}$ Th1 cells to E-selectin–IgG. These in vitro assays demonstrate that PSGL-1 on Th1 cells is the sole ligand for P-selectin and has a potential role in Th1 cell binding to E-selectin in vivo. The finding that anti–PSGL-1 antibodies inhibit adhesion of Th1 cells to E-selectin also suggests that the NH_2-terminal region of PSGL-1 may be involved in Th1 cell binding to E-selectin.

Migration of PSGL-1–deficient Th1 Cells into the Inflamed Skin of E-Selectin–deficient Mice Is Abolished. To test whether the in vitro observation that PSGL-1 on Th1 cells is the predominant ligand for P-selectin is relevant during the in vivo migration of cells into the inflamed skin, PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ Th1 cells were injected into the sensitized and challenged E-selectin$^{-/-}$ mice. This experiment allows analyses of P-selectin–dependent cell migration in the absence of E-selectin. The migration of PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ Th1 cells into the skin of wild-type B6/129S control mice was similar to that shown for PSGL-$1^{+/+}$ mice (Fig. 4 A). Ear swelling of E-selectin$^{-/-}$ mice 24 h after challenge was comparable to that of B6/129S control mice. The migration of PSGL-$1^{+/+}$ Th1 cells into the inflamed skin of E-selectin$^{-/-}$ mice was reduced by 37% compared with that in B6/129S control mice (Fig. 4 A). The migration of PSGL-$1^{-/-}$ cells into the inflamed skin of E-selectin$^{-/-}$ mice was almost abolished. This confirms that PSGL-1 is the sole ligand for P-selectin on Th1 cells in vivo and that no other ligands serve as a P-selectin ligand in the absence of PSGL-1. Previously, we demonstrated that PSGL-1 is the sole P-selectin ligand during neutrophil rolling (10). Thus, for neutrophil–endothelial cell interaction and for lymphocyte migration, PSGL-1 is the sole P-selectin ligand. Whether cell adhesion during other P-selectin–mediated processes is PSGL-1 dependent remains to be determined.

Migration of PSGL-1–deficient Th1 Cells into the Inflamed Skin of P-Selectin–deficient Mice Is Not Abolished. If PSGL-1 is the sole ligand for P-selectin on Th1 cells in vivo, then the migration of PSGL-$1^{-/-}$ cells observed when they were injected into PSGL-$1^{+/+}$ mice must be mediated by

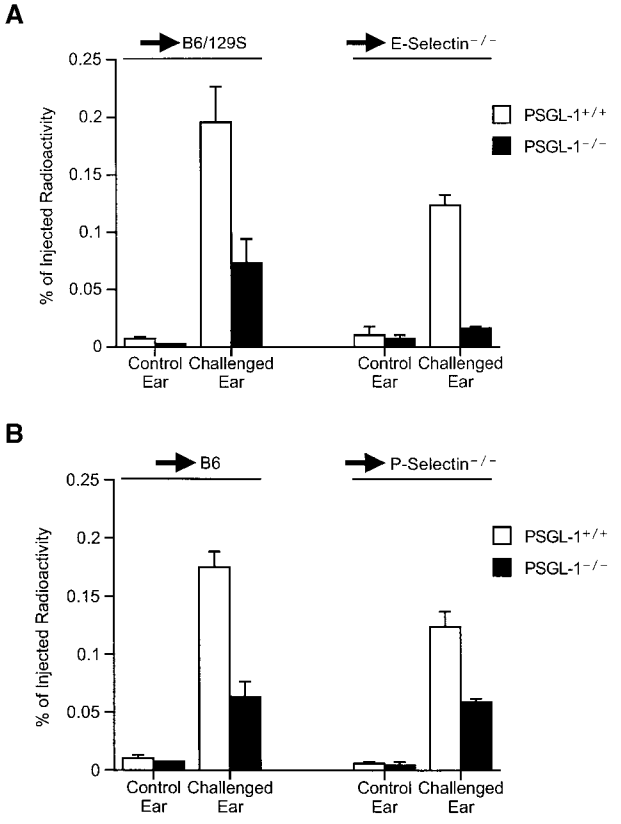

Figure 4. Migration of PSGL-1–deficient Th1 cells into the inflamed skin of E-selectin– or P-selectin–deficient mice. ^{51}Cr-labeled PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ Th1 cells were injected into the tail veins of E-selectin$^{-/-}$ mice and wild-type B6/129S F2 mice (A) or P-selectin$^{-/-}$ mice and wild-type B6 mice (B). The mice were killed 3 h after injection, and the radioactivity in the control and challenged ears was assayed. Values are means ± SEM from four mice.

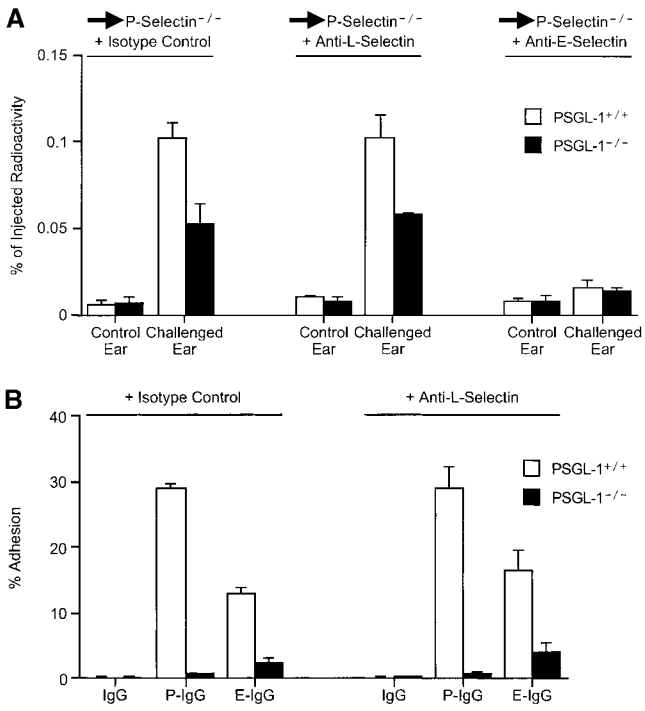

Figure 5. Effect of an anti–L-selectin antibody on Th1 migration in vivo and adhesion in vitro. (A) ^{51}Cr-labeled PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ Th1 cells were injected together with anti–L-selectin mAb MEL-14, anti–E-selectin mAb 9A9, or its isotype control. The mice had been sensitized 6 d before with oxazolone and challenged 24 h before on the left ear. Values are means ± SEM from three mice. (B) Adhesion of PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ Th1 cells treated with MEL-14 to selectin–IgG chimeras. PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ Th1 cells were incubated with either MEL-14 or its isotype control for 30 min on ice and washed before addition to wells. Values are means ± SEM from triplicate wells.

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E-selectin, as P- and E-selectin together mediate Th1 cell migration into the inflamed skin (8). This predicts that PSGL-1 is not required for E-selectin–mediated migration of Th1 cells. The adhesion assays also suggested the existence of E-selectin ligands other than PSGL-1 (Fig. 3 A). To prove this hypothesis, we tested whether PSGL-1 plays a role in an E-selectin–mediated process by injecting PSGL-$1^{-/-}$ Th1 cells into P-selectin$^{-/-}$ mice. This allows E-selectin–dependent migration to be studied in the absence of P-selectin. The migration of PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ Th1 cells in wild-type B6 controls was similar to that in PSGL-$1^{+/+}$ mice (Fig. 4 B). The ear swelling response in P-selectin$^{-/-}$ mice measured 24 h after challenge was comparable to that in B6 controls. In contrast to the abrogation of migration when injected into E-selectin$^{-/-}$ mice (Fig. 4 A), the migration of PSGL-$1^{-/-}$ cells into the inflamed skin of P-selectin$^{-/-}$ mice was not abolished (Fig. 4 B). These results confirm that PSGL-1 on Th1 cells is not the sole ligand for E-selectin in vivo. However, comparison of the migration of PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ cells revealed that PSGL-$1^{-/-}$ cell migration into the inflamed skin of P-selectin$^{-/-}$ mice was reduced by 52% compared with PSGL-$1^{+/+}$ cells (Fig. 4 B). Thus, PSGL-1 plays a role other than as a P-selectin ligand.

PSGL-1 Is an E-Selectin Ligand in Th1 Cell Migration into the Inflamed Skin. In view of in vitro results showing that PSGL-1 binds E-selectin (15–22) and L-selectin (23–26), we hypothesized that PSGL-1 is serving as an E-selectin ligand to cooperate with other E-selectin ligands to promote cell migration, as an L-selectin ligand to mediate leukocyte–leukocyte interaction, or both. Leukocyte–leukocyte interaction mediated by L-selectin–PSGL-1 interaction has been suggested as a mechanism of amplifying accumulation of leukocytes including lymphocytes on E-selectin under flow (13, 29, 30). To analyze these possibilities, we compared the migration of PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ Th1 cells into the inflamed ears of P-selectin$^{-/-}$ mice in the presence of anti–L-selectin mAb MEL-14. MEL-14 did not affect the migration of either PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ cells into the inflamed skin of P-selectin$^{-/-}$ mice; anti–E-selectin mAb 9A9 inhibited the migration of both PSGL-$1^{+/+}$ and PSGL-$1^{-/-}$ cells (Fig. 5 A). These results suggest that leukocyte–leukocyte interaction mediated by L-selectin has little effect on the migration of cells and that the less efficient migration of PSGL-$1^{-/-}$ cells in an E-selectin–mediated process is due to the lack of PSGL-1–E-selectin interaction. Indeed, in vitro adhesion assays showed that MEL-14 did not affect the binding of PSGL-$1^{+/+}$ or PSGL-$1^{-/-}$ cells to either P-selectin or E-selectin (Fig. 5 B). Thus, the cell accumulation measured in the adhesion assays reflects the direct binding of cells to the selectins and leukocyte–leukocyte interaction does not contribute to the overall cell accumulation. Together, these results indicate that PSGL-1 on Th1 cells interacts directly with E-selectin both in vitro and in vivo. This is the first demonstration of the in vivo role of PSGL-1 as an E-selectin ligand. The molecular nature of E-selectin ligands other than PSGL-1 on T cells needs to be determined.

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