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Expression of Functional P-Selectin Glycoprotein Ligand 1 on Hematopoietic Progenitors Is Developmentally Regulated

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T cell development requires periodic importation of hematopoietic progenitors into the thymus. The receptor-ligand pair P-selectin and P-selectin glycoprotein ligand 1 (PSGL-1) are critically involved in this process. In this study, we examined the expression of functional PSGL-1 on bone marrow hematopoietic progenitors. We demonstrate that functional PSGL-1 is expressed at low levels on hematopoietic stem cells, but upregulated on the cell surface of progenitors that bear other homing molecules known to be important for thymic settling. We found that progenitors able to home to the thymus expressed high levels of PSGL-1 transcripts compared with hematopoietic stem cells. We further demonstrate that hematopoietic progenitors lacking fucosyltransferase 4 and 7 do not express functional PSGL-1, and do not home efficiently to the thymus. These studies provide insight into the developmentally regulated expression of a critical determinant involved in progenitor homing to the thymus.

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tors in parallel to the established regulation of CCR7 and CCR9 is unknown.

In this study, we demonstrate that functional PSGL-1 is expressed on hematopoietic stem cells at very low levels, but up-regulated on downstream progenitors that are thought to home to and settle the thymus. We further show that glycosyltransferase enzymes, including Fuc-TIVI, specially regulate the formation of functional PSGL-1, thus contributing to T lymphopoiesis.

Materials and Methods

Mice

C57BL/6 (CD45.2) and B6.Ly5.5hi (CD45.1) mice were purchased from the National Cancer Institute animal facility. PSGL-1 knockout (KO) mice were purchased from The Jackson Laboratory. Rag1-Cre mice (23) were a gift of T. Rabbitts (University of Cambridge). Fuc-TVII/Fuc-TIV double-knockout (DKO) (24, 25) mice were obtained from J. Harris and P. Scott (University of Pennsylvania). Mice were used when 6–10 wk of age. Live animal experiments were performed according to approved protocols of the Office of Regulatory Affairs of the University of Pennsylvania in accordance with National Institutes of Health guidelines.

Cell preparations, flow cytometry, and cell sorting

BM isolated from femurs and tibias was treated with ACK lysis buffer (Lonzо) to remove RBC, and single-cell suspensions were made. Thymocytes were prepared as single-cell suspension. For thymic endothelial cell preparation, the thymus was treated with 40 mg/ml liberase (Roche) and 200 mg/ml DNase (Roche) in HBSS media at 37°C for 20 min. mAbs in the Lin mixture included anti-B220 (RA3-6B2), anti-CD19 (1D3), anti-CD11b (M1/70), anti-Gr-1 (KO2.5), anti-CD11c (HL3), anti-NK1.1 (PK136), anti-Ter-119 (Ter-119), anti-CD3 (2C11), anti-CD8a (53-6.7), anti-CD8 (53-5.8), anti-TcRβ (H57), and anti-TcRγδ (GL-3). Additional Abs used included anti-CD45.2 (104), anti-CD45.1 (A20), anti-Kit (2B8), anti-Scal (D7), anti-CD4 (GK1.5), anti-Flk-3 (A2F10), anti-IL-7Rα (A7R34), anti-CD25 (PC61.5), anti-Thy.1.2 (53-2.1), anti–PSGL-1 (2PH1), anti-Ly6D (A7R34), anti-CD4 (GK1.5), anti-Flk-3 (A2F10), anti-IL-7Rα (A7R34), anti-CD25 (PC61.5), anti-Thy.1.2 (53-2.1), anti–PSGL-1 (2PH1), anti-Ly6D (A7R34), and anti-CD4 (GK1.5). For P-selectin binding, a P-selectin-IgG Fc fusion protein (E Bioscience) was used, followed by allophycocyanin-conjugated anti-Fc (Jackson ImmunoResearch Laboratories). mAbs were directly conjugated to FITC, PE, PEcy5.5, PEcy7, allophycocyanin, allophycocyanin cyan5.5 (or Alexa 700), allophycocyanin Cy7 (or APCFluor780), Pacific Blue, or biotin, which were purchased from either eBioscience, BioLegend, or BD Pharmingen. Biotinylated Abs were revealed with streptavidin PE–Texas Red (BD Pharmingen).

Flow cytometric (FACS) analysis was performed on a CANTO or LSRII (BD Biosciences). Dead cells were excluded with DAPI. Doublets were excluded through forward scatter height by forward scatter width and side scatter height by side scatter width parameters. Data were analyzed using FlowJo version 4.6.2 (Tree Star). For cell sorting, BM was first enriched for progenitors, then stained and sorted on a FACS Aria II (BD Biosciences). Allquots of sorted cells were reanalyzed to ensure purity, which was usually >90%.

Competitive adoptive transfer

To generate mixed BM chimeras, host wild-type (WT) mice (expressing CD45.1) were lethally irradiated (9.5 Gy) and subsequently injected with T cell-depleted host-type BM mixed with either WT, PSGL-1 heterozygous, PSGL-1 KO, or Fuc-TVII/Fuc-TIV DKO BM (all expressing CD45.2) at a 1:1 ratio. T cells were depleted from BM by incubation with anti-CD4 and anti-CD8 Abs, followed by removal of Ab-bound cells with magnetic beads (Qiagen).

Intravenous transfers

For i.v. transfers into unirradiated mice, 1 × 10^5 sorted HSC, LMPP, Ly6Di CLP, and Ly6Di CLP were injected retro-orbitally. To prevent rejection, mice were given 0.1 mg anti-CD4 (GK1.5) the day before BM transfer and every 4 d thereafter (8, 26). Previous work by our laboratory has shown that this treatment does not affect BM or thymic engraftment by donor cells (8, 10).

Real-time PCR

RNA was prepared from indicated sorted populations using the RNeasy kit (Qiagen). cDNA was generated using the Superscript II kit (Invitrogen). Real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and primer/probe mixtures or Syber Green PCR Master Mix (Applied Biosystems) for genes and GAPDH (Applied Biosystems) and analyzed on an ABI Step One Plus Real-Time PCR System (Applied Biosystems). Relative expression levels were normalized using GAPDH transcript levels and calculated using the 2^ΔΔCT method.

TaqMan probes used were as follows: GAPDH (Mm99999915_g1); CCR7 (Mm00432608_m1); CCR9 (Mm02620030_s1); PSGL-1 (Mm01204601_m1); Fuc4 (Mm00487449_s1); B4galnt1 (Mm00480752_m1), and St6gal4 (Mm00510303_m1).

The primers for St6gal6 were forward, 5’-GCCCTTTCAAA-ACTGACAG-3’ and reverse, 5’-TCCCAACTTCCTCCTCATGG-3’, and for Fuc4 were forward, 5’-CAGTGTCTGATCGTTGCTCT-3’ and reverse, 5’-GCCACCGTATGGTACGAAAG-3’.

OP9 and OP9-DL4 cell culture

OP9-GFP (OP9) and OP9-DL4 cells were a gift of J. Carlos Zúñiga-Pflücker (University of Toronto) and were used as described (27, 28). Freshly sorted LMPP either from WT or Fuc-TVII/Fuc-TIV DKO were placed on 12- well plates containing OP9 and OP9-DL4 stromal layer in MEM (Invitrogen), supplemented with 20% FCS, IL-7 (1 ng/ml), and Flt3 ligand (5 ng/ml). Stromal cells were plated 2 d before initiation of culture at a concentration of 20,000 cells/ml in 24-well plates for bulk cultures.

Statistical analysis

Values are expressed as mean ± SEM. The p values were calculated using Microsoft Excel by Student t test.

Results

PSGL-1 in thymic homing

Previous work has shown the importance of PSGL-1 in T cell development (17). We have shown that the stage at which PSGL-1 is required in T cell development. We generated competitive BM chimeras by intravenously transferring both PSGL-1 KO BM (CD45.2) and WT BM (CD45.1) at 2:1 ratio into lethally irradiated WT hosts. As a control, we injected mixtures of PSGL-1 WT BM (CD45.2) and WT BM (CD45.1) to reconstitute irradiated mice. After hematopoietic reconstitution at 10 wk post-transplant, the BM and thymus of these mice were analyzed by flow cytometry to determine the donor-derived chimerism at successive stages of T cell development (Fig. 1A, 1B). Generation of ETP was almost comparable in control chimeras to the level expected based on the level of reconstitution by BM LSK progenitors (Fig. 1B, top panel). PSGL-1 KO HSC were able to engraft in the BM and differentiate into downstream MPP, LMPP, and CLP efficiently (Fig. 1B, bottom panel). However, the donor chimerism of early thymic progenitors derived from the PSGL-1 KO BM was significantly lower than the chimerism in the BM LSK (Lin− Kit+ Scal+) progenitors (Fig. 1B, bottom panel). These data confirm that the absence of PSGL-1 conferred a disadvantage in the generation of ETP in this competitive situation.

To determine whether PSGL-1 might be required for efficient responses to T-inductive Notch signaling rather than for thymic homing, sorted LMPP from PSGL-1 KO BM were cultured in vitro on OP9-DL4 stromal cells, which expresses the Notch1 ligand delta-like 4 (DL4) that supports T cell development (27). After 2 wk of culture, PSGL-1 KO LMPP and WT control LMPP generated similar numbers of progeny expressing T lineage markers CD25 and Thy1 (Fig. 1C, 1D). These data suggest that the lower number of ETPs in irradiation chimeras made with PSGL-1 KO BM cells was due to impaired thymic settling rather than defective intrathymic development, consistent with previous reports (17).

Expression of functional PSGL-1 is developmentally regulated in BM progenitors

PSGL-1 is expressed by all subsets of leukocytes (19). The binding of P-selectin chimaera Ig (IgG) Fc fusion protein was used to measure functional PSGL-1 levels on the cell surface. BM progenitor populations were gated, as indicated in Fig. 2A. Flow
cytometric analysis results revealed that expression of functional PSGL-1 was increased in CCR9+LP compared with HSC populations (Fig. 2B). We found that expression of functional PSGL-1 is low in HSC, gradually increases in MPP, and is highest in CCR9+LP and CLP. Functional PSGL-1 expression appeared bimodal in CLP, consistent with earlier reports of heterogeneity in this population (Fig. 2B) (11, 12). Progenitors previously suggested to settle the thymus, CCR9+LP and CLP, were those with the highest level of functional PSGL-1 on their surface. Interestingly, differences were also detected using anti–PSGL-1 Ab, indicating that there may be developmental regulation of PSGL-1 itself (Fig. 2C). Calculation of the mean fluorescence intensity (MFI) for each population showed that MFI of both functional PSGL-1 (Fig. 2D, left panel) and PSGL-1 (Fig. 2D, right panel) was significantly increased in CCR9+LP and CLP, putative thymic settling progenitors, as compared with HSC (Fig. 2D). These data indicate that populations thought to settle the thymus express increased levels of functional PSGL-1.

The expression of functional PSGL-1 separates CLP populations

Downstream of LMPP, CLP also settle the thymus and possess T cell potentials (8, 10, 29–31). Recently, it has been demonstrated that CLP in BM are a heterogeneous population, containing multiple subsets with different lineage potentials (11, 12). Previous work has assessed the in vivo T potential of CLP subsets in irradiated recipients (12). However, hematopoiesis following irradiation is far from physiologic, as irradiation has many secondary effects (8, 32, 33). To investigate the potential of CLP to undergo T cell development in physiological conditions, we assessed T cell development in unirradiated normal adult recipient mice. For these experiments, HSC, LMPP, Ly6D+CLP, and Ly6D+CLP cells were sorted from the BM of B6 donors, and 1 × 10^5 cells from each population were injected i.v. into each unirradiated B6.Ly5SJL congenic recipient. Two weeks after i.v. transfer of purified progenitors into mice, recipient thymi were analyzed for donor-derived DN3 and DP thymocytes (Fig. 3A). In all experiments, HSC failed to produce any DN3 or DP thymocytes (Fig. 3A). Both LMPP and Ly6D+CLP generated donor-derived thymocytes. The number of donor-derived DN3 and DP from Ly6D+CLP progenitor was significantly lower than that generated by LMPP and Ly6D+CLP (Fig. 3A). These results established that the Ly6D+CLP subset is an inefficient T cell progenitor in the unirradiated physiological thymus environment.
Because Rag-1 is highly expressed in the CLP population (34), we next examined Ly6d expression on CLP cells in Rag-1/YFP mice, which were created by crossing Rag1/Cre mice with mice in which YFP was knocked into the Rosa26 locus. This reporter allows for a more precise analysis of CLP subpopulations (35). Flow cytometric analysis of BM from Rag1-YFP mice showed that CLP expressing high levels of Ly6D also expressed high levels of Rag1-YFP, whereas many fewer Ly6D^2 CLP expressed YFP (Fig. 3B). About two-thirds of CLP in these experiments were Ly6D^+, and, of these, most expressed Rag1-YFP, as expected (11). We sought to analyze the expression of functional PSGL-1 in CLP subsets in the Rag1 reporter mouse. The expression of functional PSGL-1 was decreased by about half in Ly6D^+ Rag1-YFP marked cells, compared with Ly6D^- Rag1 low cells (Fig. 3C, 3D). The MFI seen with P-selectin fusion protein binding by flow cytometry was significantly decreased as cells progressed from Ly6D^-CLP to Ly6D^+CLP (Fig. 3E). These results indicate that functional PSGL-1 expression is downregulated within CLP coordinately with the loss of efficient T cell potential.

**Requirement of PSGL-1 in thymus settling**

Increases in expression of functional PSGL-1 from HSC to CCR9^+ lymphoid progenitor, and CLP. (B) BM from WT and PSGL-1 KO mice was analyzed by flow cytometry for P-selectin binding and (C) PSGL-1 on the indicated populations. Numbers represent the percentage of WT cells in the indicated gate. The gray histogram is from a PSGL-1 KO control sample. (D) The MFI of P-selectin fusion protein and PSGL-1 Ab on BM progenitor populations was determined by FACS, which was obtained from the gates applied in (B) and (C). Data are shown as mean ± SEM and are averages of three independent experiments performed on different days. The two-tailed Student t test indicated a significant increase of functional PSGL-1 and PSGL-1 protein on CCR9^+LP and CLP. *p < 0.05, **p < 0.01, ***p < 0.001.
acetylsyltransferase ($b_{1,4}$-GalT1) (37, 38), a2,3 sialyltransferase (ST3GalT4), and ST3GalT6 (39, 40). We examined the expression of glycosyltransferase enzymes in BM and early thymic progenitors. Quantitative RT-PCR confirmed that PSGL-1 and the glycosyltransferase enzyme Fuc-TII were expressed at higher levels in CCR9+LP cells and Ly6D$^2$CLP (Fig. 5), in agreement with

**FIGURE 3.** Expression of functional PSGL-1 in CLP. (A) Total number of donor-derived DN3 and DP thymocytes at 2 wk following i.v. injection of sorted HSC, LMP, Ly6D$^+$CLP, and Ly6D$^+$CLP into unirradiated congenic recipient mice. Shown are the means of three mice per group ± SEM. (B) BM from Rag1-Cre Rosa26-YFP reporter mice was analyzed by flow cytometry for Ly6D expression in CLP (Lin$^-$$Sca1^{low}$$Kitlow$$Flt3^{high}$$IL7Ra^+$). (C) BM from WT and (D) Rag1-Cre Rosa26-YFP reporter mice was analyzed for Ly6D and Rag expression in CLP subsets (top panel). P-selectin binding was used to determine functional PSGL-1 on the indicated populations (bottom panel). Numbers represent the percentage of WT cells in the indicated gate (bottom panel). The gray histogram in the bottom panel is from the PSGL-1 KO control sample. (E) The MFI of P-selectin fusion protein binding on CLP subpopulations was determined by FACS, which was obtained from the gates applied in (C), left panel. The two-tailed Student t test indicated a significant decrease of function PSGL-1 protein on Ly6D$^+$CLP. * p < 0.05, ** p < 0.01, *** p < 0.001.

**FIGURE 4.** Requirement for PSGL-1 in thymus settling. (A) Quantitative PCR analysis of total cellular RNA from indicated cell fractions purified from Gr-1, CD19, Mac-1–depleted BM of PSGL-1 heterozygous mice. We identified HSC as LSKFlt3$^+$ cells among LSK BM progenitors. MPP were LSKFlt3$^{low}$, LMP were LSKFlt3$^{low}$, and CLP were Lin$^-$$Sca1^{low}$$Kitlow$$Flt3^{high}$$IL7Ra^+$ cells in BM. Transcript levels of PSGL-1 were normalized to GAPDH mRNA levels and are indicated in arbitrary units. Data are mean ± SEM of three experiments. (B) BM from WT, PSGL-1 Het, and PSGL-1 KO mice was analyzed by flow cytometry for P-selectin binding on the indicated populations. The gray solid histogram is from a PSGL-1 Het control sample. The MFI of P-selectin fusion protein on BM population was determined by FACS, which was obtained from the open histogram in WT type and PSGL-1 Het mice. Data are shown as mean ± SEM and are representative of three experiments. * p < 0.05. (C) Mixed BM chimeras were generated using CD45.2 PSGL-1 WT BM and CD45.1 WT BM mixtures as controls (left panel), and CD45.2 PSGL-1 Het BM and CD45.1 WT BM mixtures (right panel). Chimeras were analyzed by flow cytometry after 10 wk using Abs to CD45.1 and CD45.2 to determine donor chimerism. Data are mean CD45.2 donor chimerism ± SEM for each indicated population in mixed BM chimeras. Numbers are from five mice per group.
their elevated surface expression of functional PSGL-1 (Fig. 2). In contrast, reduced expression of both of these genes in Ly6D+CLP cells (Fig. 5) suggests that both PSGL-1 and Fuc-TVII influence functional PSGL-1 levels in lymphoid progenitor cells, because functional PSGL-1 declines in the Ly6D+CLP population. The glycosyltransferase enzyme sialyltransferase ST3GalT6 is highly expressed in CCR9+LP cells; and sialyltransferase ST3GalT4, β1,4-galactosyltransferase β1,4-GalT1, fucosyltransferase 4 (Fuc-TIV), and core 2 β1,6-N-acetylglucosaminyltransferases C2GnT1 are expressed in CCR9+LP cells and also Ly6D-CLP lymphoid progenitor cells. We also examined expression of CCR7 and CCR9; consistent with previous reports, we found strong expression of CCR7 in CCR9+LP and CLP subsets (Fig. 5). Interestingly, CCR9 was highly expressed in CCR9+LP, as expected, but also in Ly6D+CLP (Fig. 5).

**FIGURE 5.** Glycosyltransferase enzyme gene expression profile in BM and early thymic progenitors. Quantitative PCR analysis of total cellular RNA from indicated cell fractions purified from Gr-1, CD19, Mac-1–depleted BM, and CD4, CD8–depleted thymus of C57BL/6 WT mice. We identified LT-HSC as CD150+CD34+ and ST-HSC as CD150−CD34− cells among LSK BM progenitors. MPP were LSKFlt3low, CCR9+LP were LSKFlt3highCCR9high, Ly6D+CLP were Lin−Sca1highKitlowFlt3highIL-7Rα+Ly6D+, and Ly6D−CLP were Lin−Sca1lowKitlowFlt3highIL-7Rα+Ly6D− cells in BM. To isolate thymic progenitors, we identified Flt3+ETP as Lin−Kit+CD25−Flt3+, Flt3−ETP as Lin−Kit−CD25−Flt3−, and DN3 as Lin−Kit−CD25− cells in thymus. Transcript levels of indicated genes were normalized to GAPDH mRNA levels and are indicated in arbitrary units. Means and SEs of the results obtained from three independent measurements are shown.

_**Fucosyltransferase enzyme 7 regulates thymus settling through formation of functional PSGL-1**_

PSGL-1 becomes functional (i.e., able to bind P-selectin) only after specific α1,3 fucosylation, which is accomplished by a Fuc-T, Fuc-TVII, with Fuc-TIV playing a supporting role (25, 41). We analyzed progenitor populations in the BM of Fuc-TVII/Fuc-TIV double-deficient mice and found that functional PSGL-1 expression was absent on LSK and LMPP progenitor populations (Fig. 6A, left panels), whereas PSGL-1 protein continued to be expressed on the surface of the indicated populations, as expected (Fig. 6B, left panels). The numbers of hematopoietic precursors were comparable in BM from WT and Fuc-TVII/Fuc-TIV DKO mice (Supplemental Fig. 1A). The absence of functional PSGL-1 on progenitor cells from Fuc-TVII/Fuc-TIV double-deficient mice indicates that expression of functional P-selectin on hematopoietic progenitor populations is dependent on functional PSGL-1.
etic progenitors is dependent on α1,3 fucosylation catalyzed by fucosyltransferase enzymes Fuc-TVII and/or Fuc-TIV, as has been previously described for neutrophils (41). It has been previously shown that inactivation of Fuc-TVII by homologous recombination results in an 80% decrease in lymphocyte homing to peripheral lymph nodes (25). We tested whether diminished expression of functional PSGL-1 on the surface of Fuc-TVI/(Fuc-TIV DKO double-deficient BM progenitors resulted in a thymic settling defect. We generated competitive BM chimeras by intravenously transferring both Fuc-TVI/(Fuc-TIV DKO BM (CD45.2) and WT BM (CD45.1) at a 2:1 ratio into lethally irradiated WT recipients. As a control, we injected mixtures of CD45.2 WT BM and CD45.1 WT BM mixtures (bottom panel). Chimeras were analyzed by flow cytometry after 10 wk using Abs to CD45.1 and CD45.2 to determine chimerism. The mean CD45.2 donor chimerism ± SEM for each indicated population is shown. (D) Sorted LMPP from WT or Fuc-TVII/Fuc-TIV DKO mice was cultured on OP9 (top panel) or OP9-DL4 (bottom panel) stromal layers in triplicate. After 10 d, cultured cells were analyzed by flow cytometry for expression of CD25 and Thy1.2. Representative FACS plots of cells gated on CD45+ are shown. (E) Total numbers of cells obtained from the cultures described in (D) for OP9-DL4. Data are mean ± SEM of three wells. **p < 0.01.

**FIGURE 6.** Fucosyltransferase enzyme plays role in thymus settling process. (A) BM from Fuc-TVII/Fuc-TIV DKO (left panels) and WT (right panels) mice was analyzed by flow cytometry for functional PSGL-1. (B) BM from Fuc-TVII/Fuc-TIV DKO (left panels) and WT (right panels) mice was analyzed by flow cytometry for surface PSGL-1 protein expression using mAb to PSGL-1. Numbers represent the percentage of WT cells in the indicated gate. (C) Mixed BM chimeras were generated using CD45.2 WT BM and CD45.1 WT BM mixtures as controls (top panel), and CD45.2 Fuc-TVII/Fuc-TIV DKO BM and CD45.1 WT BM mixtures (bottom panel). Chimeras were analyzed by flow cytometry after 10 wk using Abs to CD45.1 and CD45.2 to determine chimerism. (D) Sorted LMPP from WT or Fuc-TVII/Fuc-TIV DKO mice was cultured on OP9 (top panel) or OP9-DL4 (bottom panel) stromal layers in triplicate. After 10 d, cultured cells were analyzed by flow cytometry for expression of CD25 and Thy1.2. Representative FACS plots of cells gated on CD45+ are shown. (E) Total numbers of cells obtained from the cultures described in (D) for OP9-DL4. Data are mean ± SEM of three wells. **p < 0.01.

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It has been previously shown that inactivation of Fuc-TVII by homologous recombination results in >80% decrease in lymphocyte homing to peripheral lymph nodes (25). We tested whether diminished expression of functional PSGL-1 on the cell surface of Fuc-TVII/Fuc-TIV double-deficient BM progenitors resulted in a thymic settling defect. We generated competitive BM chimeras by intravenously transferring both Fuc-TVII/Fuc-TIV DKO BM (CD45.2) and WT BM (CD45.1) at a 2:1 ratio into lethally irradiated WT recipients. As a control, we injected mixtures of CD45.2 WT BM and CD45.1 WT BM. After 10 wk to allow for hematopoietic reconstitution, BM and thymi were analyzed by flow cytometry to determine the donor chimerism within defined progenitor populations (Fig. 6C, upper panel). Fuc-TVII/ Fuc-TIV double-deficient HSC were able to engraft in the BM and differentiate into MPP, LMPP, and CLP normally; however, the donor chimerism was significantly decreased in early intrathymic T cell progenitors (Fig. 6C, bottom panel). Indeed, progenitors lacking Fuc-TVII/Fuc-TIV showed a severe defect in thymic settling that was comparable in magnitude to the defect seen with PSGL-1−/− mixed BM chimeras (Fig. 1B). Defects in thymic immigration and immunodeficiency have been linked to increases in thymic receptivity mediated by increased frequencies of P-selectin+ thymic endothelial cells (18). However, frequencies of P-selectin+ thymic endothelial cells appeared unaffected in Fuc-TVII/Fuc-TIV DKO mice (Supplemental Fig. 1B, 1C). When cultured on OP9-DL4 stromal cells, Fuc-TVII/Fuc-TIV DKO LMPP generated similar number of CD25 and Thy1.2 DP T cells on per cell basis compared with WT controls, indicating equivalent responses to T-inductive Notch signals (Fig. 6D, 6E). These findings establish an important role for fucosylation-mediated functional PSGL-1 in thymic settling in a competitive situation.

**Discussion**

We have established that expression of functional PSGL-1 is regulated on BM-derived hematopoietic progenitor cells, and investigated the molecular basis of this regulation. We found that functional PSGL-1 is expressed at low levels on hematopoietic stem cells, but that high levels are found on downstream progenitors that are thought to home to and settle the thymus. Finally, we found that
Fuc-TVII/Fuc-TIV–deficient BM progenitors demonstrate thymic settling defects, due to the abrogation of fucosyltransferase-mediated modifications necessary to form functional PSGL-1.

The ability to settle the thymus is selectively achieved by progenitors downstream of HSC, and includes LMPP and CLP (7, 8, 29, 42). One molecular basis of this selectivity was previously shown to be the expression of the chemokine receptors CCR7 and CCR9 on CLP and subsets of LMPP, and progenitors lacking both of these molecules are almost completely unable to contribute to T lymphopoiesis in a competitive situation (8, 10, 15, 16). Earlier work had established that the selectin ligand PSGL-1 also plays a role in facilitating thymic settling and efficient homing of T cells to secondary lymphoid organs (17, 43). We have demonstrated that progenitors lacking PSGL-1 contribute inefficiently to the migration of progenitors to the thymus, we performed transplantation assays using BM from Fuc-TVII/Fuc-TIV double-deficient mice. In competition with wt cells, Fuc-TVII/Fuc-TIV double-deficient cells were clearly disadvantaged in thymic settling. Thus, our data suggest that PSGL-1 is the main substrate of Fuc-TVII/Fuc-TIV involved in thymic homing. However, some progenitor cells can get into thymus without Fuc-TVII, and it remains possible that other Fuc genes, including Fuc-TVI (46) or residual PSGL-1 interactions with Fuc-TVII–independent ligands such as E-selectin, may play a role (47). For this reason, we did not see a clear increase in frequencies of P-selectin* thymic endothelial cells, which has been reported to underlie the increased thymic reactivity seen in PSGL-1−/− mice and IL-7R−/− mice (18). However, the phenotype of Fuc-TVII/Fuc-TIV double-deficient cells otherwise largely mirrors the phenotype of PSGL-1–deficient progenitors. The occurrence of some T cell development from PSGL-1–deficient progenitors and also Fuc-TVII/Fuc-TIV double-deficient progenitors suggests that receptor ligand pairs distinct from PSGL-1 and its ligands may also exist that support thymic settling; these remain to be discovered.

In conclusion, the current study established that expression of functional PSGL-1 on BM progenitor is dynamically regulated. Together with the known selective expression of CCR7 and CCR9 chemokine receptors, selective expression of functional PSGL-1 therefore contributes to the regulated basis of selective thymic settling by T cell progenitors.

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Disclosures

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