PSGL-1 Regulates the Migration and Proliferation of CD8+ T Cells under Homeostatic Conditions

Krystle M. Veerman, Douglas A. Carlow, Iryna Shanina, John J. Priatel, Marc S. Horwitz and Hermann J. Ziltener

J Immunol published online 16 January 2012
http://www.jimmunol.org/content/early/2012/01/16/jimmunol.1103026

Why *The JI*?

- **Rapid Reviews! 30 days** from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

---

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2012 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
PSGL-1 Regulates the Migration and Proliferation of CD8+ T Cells under Homeostatic Conditions

Krystle M. Veerman,*,‡ Douglas A. Carlow,*,‡ Iryna Shanina,§§ John J. Priatel,†‡ Marc S. Horwitz,§§ and Hermann J. Ziltener*,‡

P-selectin glycoprotein ligand-1 (PSGL-1), a heavily glycosylated sialomucin expressed on most leukocytes, has dual function as a selectin ligand for leucocyte rolling on vascular selectins expressed in inflammation and as a facilitator of resting T cell homing into lymphoid organs. In this article, we document disturbances in T cell homeostasis present in PSGL-1null mice. Naive CD4+ and CD8+ T cell frequencies were profoundly reduced in blood, whereas T cell numbers in lymph nodes and spleen were at or near normal levels. Although PSGL-1null T cells were less efficient at entering lymph nodes, they also remained in lymph nodes longer than PSGL-1+/+ T cells, suggesting that PSGL-1 supports T cell egress. In addition, PSGL-1null CD8+ T cell proliferation was observed under steady-state conditions and PSGL-1null CD8+ T cells were found to be hyperresponsive to homeostatic cytokines IL-2, IL-4, and IL-15. Despite these disturbances in T cell homeostasis, PSGL-1null mice exhibited a normal acute response (day 8) to lymphocytic choriomeningitis virus infection but generated an increased frequency of memory T cells (day 40). Our observations demonstrate a novel pleiotropic influence of PSGL-1 deficiency on several aspects of T cell homeostasis that would not have been anticipated based on the mild phenotype of PSGL-1null mice. These potentially offsetting effects presumably account for the near-normal cellularity seen in lymph nodes of PSGL-1null mice. The Journal of Immunology, 2012, 188:000–000.

T cells develop in the thymus and exit upon maturation as naive T cells (1). Naive T cells travel through the bloodstream and enter secondary lymphoid organs (SLOs) including the spleen and lymph nodes. T cells enter lymph nodes through specialized blood vessels called high endothelial venules (HEVs) (2) and do so in a controlled process supported by the sequential engagement of adhesion molecules and chemotactic signals. Rolling and tethering of T cells on HEVs is initially facilitated by L-selectin on T cells interacting with peripheral node addressins on HEVs (3, 4). The traction from L-selectin/addressin interactions dramatically reduces T cell velocity, enabling chemokine CCL21 and CCL19 signaling through CCR7 on T cells. Chemokine signaling rapidly induces activation of LFA-1 on lymphocytes during the second step of T cell lymph node entry (5–7), allowing high-affinity LFA-1 binding to ICAM-1 on HEVs resulting in cell arrest. Once arrested, additional signals stimulate cell transmigration through the blood vessel wall and entry into SLOs (8). Entry of naive T cells into SLOs is mainly believed to occur via this route, whereas memory T cells enter lymph nodes via both the HEVs and the afferent lymphatics that drain directly from tissues (9).

Once in the lymph nodes, CCL21 and CCL19 gradients direct movement of T cells within the various lymph node compartments (10). Naive T cells encounter and survey APCs for presence of cognate Ag and also obtain survival signals from homeostatic cytokines, such as IL-7 produced by fibroblastic reticular cells (11), which promotes T cell survival via the Bcl-2 family apoptosis pathways (12). IL-15 is also found in lymph nodes and is necessary for survival of naive and memory T cells (13, 14). Homeostatic cytokines are constitutively produced within the lymph nodes and stimulate T cell survival (14); because of ongoing consumption by lymphocytes, these cytokines are normally present in low concentrations. Under lymphopenic conditions, less cytokine is used and homeostatic cytokine levels consequently increase in the lymph nodes to levels sufficient to stimulate both T cell survival and limited T cell proliferation. Increased exposure to IL-7, IL-15, IL-2, or IL-4 has been shown to induce such homeostatic expansion of T cells (15). In addition to cytokines, naive T cells require survival signals through interaction with self-peptide presented by MHC on APCs. T cells that fail to encounter their cognate Ag in a given lymphoid tissue are guided out of the lymph nodes into the efferent lymph by an S1P gradient (16) and re-enter the bloodstream only to repeat the process in another SLO.

Recently, we discovered that P-selectin glycoprotein ligand-1 (PSGL-1) on resting T cells is required for efficient entry into SLOs (17). PSGL-1 was found to facilitate lymph node entry through an interaction with CCL21 and CCL19, independent of its well-established role in selectin binding. To further elucidate the significance of this novel chemokine binding function of PSGL-1 on naive T cells, we examined how lack of PSGL-1 affected both T cell homeostasis in steady state and in mice responding to immune challenge with lymphocytic choriomeningitis virus (LCMV). We found that PSGL-1 influenced several aspects of general ho-
meostasis of CD8+ T cells through its effects on migration in and out of lymph nodes and on responsiveness to the homeostatic cytokines IL-2, IL-4, and IL-15. Furthermore, although absence of PSGL-1 did not affect the ability of T cells to mount an acute immune response against LCMV, it did influence the accumulation of memory T cells.

Materials and Methods

Mice

Mice were bred and housed in the specific pathogen-free animal unit at the Biomedical Research Centre. C57BL/6 and congenic C57BL/6 (Thy-1.1 or CD45.1) and PSGL-1−/− (B6.Cg-Selpkm1Fur/J stock number: 004201) backcrossed onto C57BL/6 background were purchased from The Jackson Laboratory. PSGL-1−/− mice were backcrossed in-house on the C57BL/6. Thy1.1 background. TCR transgenic mice expressing the male Ag-specific TCR (18) were maintained in the same facility. Mice were aged and sex-matched for experiments and ranged between 6 and 10 wk. LCMV-infected mice were housed at the Westbrook Animal Unit. Procedures used in this study were approved by the Animal Care Committee at the University of British Columbia.

Cell preparation

Mice were sacrificed using CO2. Tissues used included superficial cervical, brachial, inguinal, mesenteric, axillary lymph nodes, and spleen. Lymph nodes and spleens were minced and mechanically dissociated into single-cell suspensions as described using a stainless-steel sieve in complete RPMI 1640 (containing 10% [v/v] FBS, 2 mM L-glutamine, 50 μM 2-ME, penicillin, and streptomycin; Life Technologies) and kept at 4˚C. In cases where lymph node T cell counts were taken, lymph nodes were minced and digested with 0.5 mg/ml collagenase/dispace (Roche) for 2 h at 37˚C in HBSS on a turning rack with 10 μg/ml DNase (Roche). Peripheral blood was taken either by perfusion with PBS + 4% FBS + 2.5 μM EDTA or by cardiac puncture. RBC lysis was performed on all peripheral blood and spleen.

Flow cytometry

Cells were stained with combinations of CD4-PE/Cy7 (RM4-5; eBioscience), CD8-allophycocyanin-Cy7 (53-6.7; eBioscience), CD8β-biotin (H35-17.2; eBioscience), B220-FTTC (RA-6B2; hybridoma/ATCC), CD122/IL-2Rβ-biotin (SH4; eBioscience), CD132/γc-PE (TUGm2; BioLegend), CD251/IL-15Rα-allelophycocyanin (JM7A4; BioLegend), CD25/IL-2Rα-PE (PC61; Pharmingen), CD44-PE, -allelophycocyanin (IM7.8; hybridoma/ATCC), CD45.1-allelophycocyanin-Cy7, -PE (A20; eBioscience), hybridoma/ATCC, and Thy1.1-Pacific blue (H551; eBioscience) Abs in FACS buffer (PBS + 2% FBS + 2.5 μM EDTA + 1 × 10^6 beads) for 20 min at 4˚C (on ice) in the dark. They were washed twice in FACS buffer. Cells stained with biotinylated Abs were then stained with fluorochrome-conjugated streptavidin for 10 min at 4˚C (on ice) in the dark. Where indicated, propidium iodide (PI; 250 ng/ml) was included in the final sample volume to assess the viable cell population. Counting beads were added to all samples run on the FACS.

Data are represented as mean values and error bars are depicted using SD. Statistical significance was measured using an unpaired, two-tailed Student's t test. *p < 0.05, **p < 0.01, ***p < 0.001. Statistics were calculated using Microsoft Excel software.

Results

Naïve T cell frequency is substantially reduced in the blood of PSGL-1−/− mice

While examining the role of PSGL-1 in thymic progenitor homing, we discovered that PSGL-1−/− mice have a reduced thymic T cell output and decreased numbers of CD4+ and CD8+ T cells in the peripheral blood (22). These findings prompted us to examine how loss of PSGL-1 affects homeostasis within peripheral lymphocyte compartments. Lymphocytes from peripheral blood, peripheral lymph nodes, and spleen were enumerated in PSGL-1−/− and WT mice. As previously shown (22), the numbers of CD4+ and CD8+ T cells in the peripheral blood of PSGL-1−/− mice were found to be significantly reduced by ~45% relative to WT mice (Fig. 1A). Surprisingly, however, CD4+ and CD8+ T cell numbers within lymph nodes and spleen were comparable between PSGL-1−/− mice and WT mice. B220+ B cell numbers in WT versus PSGL-1−/− mice were also comparable in all the tissues analyzed, although a trend for increased B cell numbers in PSGL-1−/− mice was noted.

The observed T cell lymphopenia in the blood of PSGL-1−/− mice was explored further by analysis of the naive and memory T cell subsets to determine whether all subsets were similarly

In vivo proliferation assays

Donor lymph node cells were prepared as described earlier, then labeled with 2 μM CFSE in HBSS for 5 min at 25˚C. Cells were washed twice in complete RPMI 1640, then resuspended in HBSS at 25 × 10^6 cells/ml. Two hundred microliters of cells was injected into each mouse i.v. via the tail vein. In some experiments, lymphocytes were trapped in lymph nodes using 4’-deoxypyridoxine (DOP; Sigma), by including it at a concentration of 100 mg/L together with 10 g/L glucose to the drinking water at the time of injection (20). Water was refreshed weekly. In long-term proliferation assays, mice were sacrificed after 4–5 wk. Sublethally irradiated WT mice (350 rad) were sacrificed after 7 and 14 d. Lymphocytes were collected and analyzed as indicated earlier. In competition assays, donor cells were distinguished based on congenic marker expression using Abs against Thy1.1, Thy1.2, CD45.1, and/or CD45.2.

In vitro proliferation assay

Dissociated lymph node cells from WT Thy1.2 and PSGL-1−/−Thy1.1 mice were mixed at a 1:1 ratio, simultaneously labeled with 2 μM CFSE in HBSS, washed, and then added to prewarmed cytokine-enriched media in 96-well plates to a final density of 1 × 10^5 cells/ml. Cultures were maintained at 37˚C with 0.5% CO2. For in vitro experiments with purified naïve and memory T cells, cells were sorted on the basis of CD8β+ and CD44high and CD44low expression on a FACS Aria (BD Biosciences).

For sorting, cells were counted, combined at a 1:1 ratio, labeled with CFSE, and added to cytokine-enriched 96-well plate at a concentration of 5 × 10^3 cells/ml. After 3 d, cells were analyzed on the LSRII with counting beads and PI stain. All analyses were gated for viable PI- cells.

LCMV infections

Seven-week-old sex-matched mice were injected i.p. with 1 × 10^5 PFU LCMV-Armstrong. LCMV-specific MHC class I tetramers were constructed and purified as previously described (21). Spleens were harvested on either day 8 or 40 postinfection, and stained with PE-labeled H-2Db6-GP33 or PE-labeled H-2Dd-NP39 peptides in FACS buffer (PBS with 2% FBS [v/v] and 2.5 μM EDTA) for 40 min at 4˚C in the dark. They were then washed in FACS buffer and subsequently stained with CD8-allophycocyanin-Cy7 and CD4-PECy7 for 20 min at 4˚C in the dark. Samples stained by gp33 tetramer were immediately run on the LSRII. Samples stained by np39 peptide were fixed first with a 1% (w/v) paraformaldehyde/PBS solution for 15 min, then run on the LSRII. Counting beads were added to all samples run on the FACS.
affected. CD4+ and CD8+ naive T cells, defined as CD44low L-selectinhigh, in the blood of PSGL-1null mice were found to be reduced by 98 and 95%, respectively, relative to WT (Fig. 1B). By contrast, naive CD4+ and CD8+ T cell subsets in blood, lymph nodes, and spleen. Naïve T cells are defined as L-selectinhiCD44lo, central memory T cells as L-selectinhi CD44hi, effector T cells as L-selectinhiCD44lo, and effector memory T cells as L-selectinloCD44hi. Data are shown as absolute numbers in each tissue and represent a minimum of three independent experiments. Error bars represent SD. The unpaired Student t test was used to compare groups. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 1.** Naive CD4+ and CD8+ T cells are decreased in blood of PSGL-1null mice. Lymphocytes were isolated from tissues of WT and PSGL-1null mice, stained with mAbs, and analyzed by flow cytometry. A, Enumeration of CD4+ and CD8+ T cells, and B220+ B cells in blood, in pooled peripheral and mesenteric lymph nodes and in spleen. B, Enumeration of CD4+ and CD8+ T cell subsets in blood, lymph nodes, and spleen. NAIVE T CELLS ARE REINFORCED IN BLOOD BUT RETAINED IN Lymph nodes

The absence of naive T cells in blood of PSGL-1null mice was unexpected as we have previously shown that T cells deficient for PSGL-1 enter the lymph nodes at approximately half the rate of WT T cells (17). We therefore expected to find increased numbers of naive T cells in blood. The lack of naive T cells in blood of PSGL-1null mice suggested a critical role for PSGL-1 in the maintenance of naive T cells. To test this hypothesis, we co-injected lymph node-derived WT and PSGL-1null T cells, and measured their blood half-life in recipient mice (Fig. 2). T cells from PSGL-1null donors disappeared from the blood at a faster rate than T cells from WT donors. To address the possibility that the disappearance of PSGL-1null lymph node T cells might have occurred because of a pre-existing deficit in PSGL-1null resident lymph node T cell integrity, we used male Ag-specific HY TCR female donors for blood half-life experiments because they have an abundance of mature CD8+ thymocytes that possess a de facto naive phenotype. The disappearance of HY/PSGL-1null CD8+ thymocytes relative to HY/PSGL-1+/+ thymocytes was more dramatic than when non-TCR transgenic donor lymph node T cells were used (Fig. 2) and consistent with a preferential clearance of CD8+ PSGL-1null T cells with a naive status.

The comparable cellularity of lymph nodes in PSGL-1null mice relative to WT mice despite T cell lymphopenia in the blood, as well as the inability of PSGL-1null T cells to efficiently enter lymph nodes (17), led us to speculate that PSGL-1null T cells might have a decreased rate of lymph node egress. To test this hypothesis, we preloaded lymph nodes of recipient mice with a mixture of donor WT and PSGL-1null lymph node-derived T cells for 1 d and then used anti-integrin Abs to further block entry of T cells into lymph nodes. Integrin neutralization used in this way effectively blocks entry of PBLs into lymph nodes (23,
PSGL-1null CD8+ T cells disappear quicker from blood than WT CD8+ T cells. WT and PSGL-1null T cells or HY and HY/PSGL-1null CD8+ naïve T cells were injected into WT mice and ratios of injected cells in blood were compared over time. Data were measured by flow cytometry and cells were gated on CD8 and either Thy1 congenic markers (WT and PSGL-1null-T cells) or on CFSE labeling (HY and HY/PSGL-1null-T cells) to distinguish cell types. Data are a ratio of PSGL-1null T cells to WT T cells and are representative of at least three independent experiments. Error bars represent SD. *p < 0.05, **p < 0.001 (Student t test).

Adoptively transferred PSGL-1null CD8+ T cells proliferate spontaneously

Lymphopenia-associated homeostatic proliferation could be another compensatory mechanism to maintain T cell numbers in lymph nodes of PSGL-1null mice. To determine whether T cell lymphopenia in PSGL-1null mice induces homeostatic proliferation, we coinfected WT and PSGL-1null CFSE-labeled lymph node thymocytes (Fig. 3). By contrast, untreated mice showed no increase in ratio of PSGL-1null CD8+ T cells after 24 h (data not shown). Prolonged lymph node retention of PSGL-1null CD8+ T cells was also found when lymph node retention was compared between HY/PSGL-1null and HY/PSGL-1null CD8+ thymocytes (Fig. 3).

FIGURE 3. PSGL-1null CD8+ T cells persist in lymph nodes longer than WT T cells. WT and PSGL-1null T cells or HY and HY/PSGL-1null CD8+ naïve T cells were injected into WT recipient mice; 1 d later, lymph nodes were harvested from one set of mice to determine T cell ratio at time 0 h. The remainder of mice was treated with anti-integrin mAbs to block further T cell entry into lymph nodes, and ratios of CD8 T cells were determined 24 and 48 h after anti-integrin Ab treatment. Data were measured by flow cytometry and cells were gated based on CFSE labeling. Data are a ratio of PSGL-1null T cells to WT T cells and are representative of at least four independent experiments. Error bars represent SD. *p < 0.05, **p < 0.01 (Student t test).

To determine whether PSGL-1null CD8+ T cells responded better than WT CD8+ T cells in an “induced” homeostatic proliferation model, we coinfected CFSE-labeled T cells from WT and PSGL-1null mice into sublethally irradiated WT recipients. CFSE dilution was measured in T cells recovered from lymph nodes 7 and 14 d after adoptive transfer. CD8+ T cells from both WT and PSGL-1null donors divided at a similar rate at day 7, with PSGL-1null T cells showing slightly advanced proliferation (Fig. 4C). After 14 d, there was a larger proportion of PSGL-1null CD8+ T cells that had undergone three or more divisions than coinjected WT CD8+ T cells. WT CD8+ T cells appeared to slow their proliferation after 7 d, whereas PSGL-1null CD8+ T cells continued to divide. CD4+ T cells from both WT and PSGL-1null donors showed a maximum of one division at both time points and was equal between both genotypes (data not shown).

Because PSGL-1null T cells have an increased residence time in lymph nodes (Fig. 3), we considered whether enhanced exposure to cytokines such as IL-7, IL-15, and IL-2 that are known to support homeostatic T cell proliferation in lymph nodes (14) might contribute to the restoration of T cell cellularity in PSGL-1null lymph nodes. To address this question, we trapped CFSE-labeled WT and PSGL-1null T cells in lymph nodes for 5 wk using DOP, a drug that blocks T cell egress from lymph nodes by disrupting the sphingosine 1 gradient (20). CFSE dilution analysis of adoptively transferred cells showed that PSGL-1null CD8+ T cells maintained a proliferative advantage over WT CD8+ T cells even when spending equivalent lengths of time in the same lymph node microenvironment (Fig. 4D). Comparison of the percentage of divided cells also showed that PSGL-1null CD8+ T cells proliferated more than WT CD8+ T cells (Fig. 4E). Regardless of donor cell genotype, there was a greater percentage of dividing donor
cells in DOP-treated recipient mice relative to control untreated recipients, although this trend did not reach statistical significance. This observation was consistent with the possibility that lymph node residence time could be an additional factor contributing to increased expansion of PSGL-1null CD8+ T cells within the lymph nodes.

PSGL-null T cells are more sensitive to cytokine-induced proliferation

The cytokines IL-2, IL-7, and IL-15 have been shown to promote T cell survival when present at low concentrations and induce homeostatic proliferation of T cells at high concentrations (25). To determine whether the spontaneous in vivo PSGL-1null T cell proliferation, exemplified in Fig. 4, could be caused by altered responsiveness to homeostatic cytokines, we set up in vitro cocultures using CFSE-labeled lymph node cells from WT and PSGL-1null mice that were mixed at a 1:1 ratio and maintained in the presence of IL-2, IL-4, IL-7, and IL-15. After 4 d in culture, CD4+ T cells did not show CFSE dilution in response to any of the stimuli tested (data not shown). CD8+ T cells did not proliferate in response to IL-7 after 4 d (data not shown) but did proliferate in response to IL-2, IL-4, and IL-15 starting at day 3 and showed substantial amounts of dose-dependent proliferation by day 4. PSGL-1null T cells again exhibited a proliferative advantage in response to all three cytokines (Fig. 5A). IL-15 stimulated CD8+ T cells most efficiently, as there were ~4-fold more CD8+ T cells in cultures supplemented with IL-15 than in cultures with IL-2 or IL-4 (Fig. 5B).

Memory CD8+ T cells have been shown to express higher levels of IL-15Rα than naive CD8+ T cells and to proliferate more rapidly than naive T cells in response to IL-15 (26, 27). To eliminate the possibility that differences in memory and naive T cell ratios present in WT and PSGL-1null lymph node preparations might have caused the observed differences in cytokine responsiveness in vitro, we tested IL-15 responsiveness of purified CD8 memory (CD44hi) and CD8 naive (CD44low) T cells from WT and PSGL-1null donors. Although our data confirmed that memory T cells divide more rapidly than naive T cells, PSGL-1 deficiency was associated with increased proliferation in both naive and memory CD8+ T cells (Fig. 5C). Enhanced responsiveness of PSGL-1null CD8+ T cells was most clearly demonstrated for naive cells stimulated with 10 ng/ml IL-15 where blasting or cell division was observed in ~50% of input cells by day 3, whereas ~90% of naive WT CD8+ T cells remained in a nondividing, nonblasted state.

To examine whether increased proliferation of PSGL-1null T cells may be caused by differences in expression of γc family cytokine receptors, we used FACS to compare the cell surface expression of IL-15Rα, IL-2Rα, IL-2Rβ, and γc on CD8+ T cells. Our data show that there were no significant differences in the expression of these receptors between WT and PSGL-1null CD8+ T cells (Fig. 5D). Furthermore, expression of all receptors increased to a similar degree on both WT and PSGL-1null CD8+ T cells after stimulation with IL-15. After IL-15 stimulation, there was a small, statistically significant increase in the expression of γc receptors on PSGL-1null CD8+ T cells.
Nevertheless, the difference was small compared with the shift between T cells that have been stimulated with IL-15 compared with those that were not.

PSGL-1null mice mount a normal acute response to viral challenge but exhibit heightened numbers of antiviral memory T cells

To determine whether altered T cell homeostasis or lymphocyte trafficking associated with loss of PSGL-1 impacts CD8+ T cell immune responses in vivo, we challenged PSGL-1null and WT mice with LCMV-Armstrong. At the peak of the response 8 d postinfection, both WT and PSGL-1null CD8+ T cells were enumerated after 4 d cultured with IL-15, IL-2, or IL-4 at days 2, 3, and 4.

**FIGURE 5.** CD8+ PSGL-1null T cells are hyperresponsive to homeostatic cytokines in vitro. A, Percentages of divided CD8+ WT and PSGL-1null T cells are shown in cultures supplemented with IL-15, IL-2, or IL-4 at days 2, 3, and 4. B, WT and PSGL-1null CD8+ T cells were enumerated after 4 d cultured with homeostatic cytokines. C, Blasting and cell division of cocultured WT and PSGL-1null CD44hi memory CD8+ T cells and cocultured WT and PSGL-1null CD44low naive CD8+ T cells with IL-15. D, Cell surface expression of IL-2 and IL-15 receptor chains on WT and PSGL-1null CD8+ T cells ex vivo (upper panels) and after stimulation with IL-15 (lower panels). All experiments were done in triplicate and are representative of five (A–C) or three (D) independent experiments. Error bars represent SD. *p < 0.05, **p < 0.01, ***p < 0.001 (Student t test).
with WT mice. Next, we analyzed WT and PSGL-1null mice at day 40 postinfection with LCMV. In mice of either genotype, total CD8 T cell numbers were reduced to approximately one-fifth of those at day 8 postinfection (Fig. 6B). However, the numbers of GP33- and NP396-specific CD8 memory T cells in the spleens of PSGL-1null mice were found to be 2.4-fold higher than in WT mice.

**Discussion**

PSGL-1 has traditionally been recognized as a key regulator of leukocyte migration to areas of inflammation. Over the last few years, recognition of the functional importance of PSGL-1 has been widened significantly as PSGL-1 has been found to play a role in other contexts such as hematopoietic stem cell homing to the bone marrow (28), progenitor homing to thymus (29), and T cell homing to SLOs through interactions with chemokines (17). The latter reveals a novel PSGL-1 function associated with its nonselectin binding form.

Two PSGL-1 knockout mice have been generated and studied. Numbers of blood lymphocytes were reported to be normal in PSGL-1null mice generated by the laboratory of Bruce Furie (30), whereas PSGL-1null mice generated in Rodger McEver’s laboratory (31) were shown to have increased numbers of total lymphocytes in blood. In a follow-up study of these latter PSGL-1null mice, a decrease in CD4 and CD8 T cells but no differences in the number of total blood lymphocytes were reported (http://www.functionalglycomics.org/glycomics/publicdata/phenotyping.jsp). Our detailed analyses of T cell subsets in the Furie PSGL-1null mice confirmed that PSGL-1 deficiency is associated with a reduction in blood CD4 and CD8 T cells. This decrease in T cell numbers was only obvious when analysis of B cells and T cell subsets was performed as the reduction in T cell numbers was balanced by an increase in B cell numbers. We found that PSGL-1-associated T cell lymphopenia was compartment specific in that it is manifest in blood but not in spleen or lymph nodes. T cell lymphopenia in blood was due to a selective deficit of naive T cells (95–98% reduced), whereas PSGL-1null memory T cell numbers in blood were comparable with WT mice. The finding that PSGL-1-associated T cell lymphopenia was not generalized to the whole body demonstrates that PSGL-1 in its nonselectin binding state is required for the normal distribution of T cells in specific compartments and is crucial for the normal maintenance of circulating naive T cells.

We have previously found that PSGL-1null thymocytes exit the thymus at a rate 50% that of WT thymocytes (22). Although this may partially explain the decrease in naive T cells in the blood, the near complete lack of naive T cells in blood cannot be explained by this phenotype alone. Inefficient homing of PSGL-1null T cells to SLOs (17) constitutes another factor contributing to lymphopenia as it has been shown that inefficient SLO entry of naive T cells results in the decrease of peripheral T cells, likely because of their reduced exposure to homeostatic cytokines (11). Memory T cells would be less affected by inefficient SLO entry via HEV because they can enter lymph nodes through the afferent lymph and can therefore encounter homeostatic cytokines such as IL-15 in other organs such as the bone marrow (32).

Although naive T cell numbers were profoundly reduced in the blood of PSGL-1null mice, they were less strongly reduced in lymph nodes. New attributes peculiar to PSGL-1null T cells have emerged in our study that could help reconcile why naive T cell deficits were much less apparent in lymph nodes than in blood. First, we found that PSGL-1 deficiency on T cells was associated with reduced lymph node egress rates. Because directional cues within the lymph node are normally provided by CCL19 and CCL21 (10), and absence of PSGL-1 is known to compromise responsiveness to these chemokines (17), PSGL-1 deficiency could effectively result in lymphocyte trapping within the lymph node and thus permit extended exposure to homeostatic cytokines that are present. Parenthetically, naive T cell numbers were comparable between WT and PSGL-1null mice in the spleen, an organ without HEVs and where all T cells enter via the splenic artery. Indeed, T cell entry into spleen is only weakly reduced by loss of PSGL-1 (17), and homeostasis of PSGL-1null T cells may thus be less affected in the spleen. Because CCL21 and CCL19 are known to guide T cells within the spleen (33), it is, however, possible that T cell egress from the spleen may also be reduced for PSGL-1null T cells. Second, enhanced proliferative response of PSGL-1null CD8 T cells to homeostatic cytokines likely constitutes an additional mechanism that contributes to restoration of normal T cell numbers in lymphoid organs. We initially speculated that T cells adoptively transferred into PSGL-1null mice would homeostatically expand because of the observed T cell lymphopenia in the blood of these recipient mice. Instead, donor PSGL-1null CD8 T cells divided significantly more than WT CD8 T cells irrespective of whether they were injected into a WT or PSGL-1null recipient. Dividing cells were CD44high, a characteristic marker of cells undergoing homeostatic proliferation. In PSGL-1null mice, we noted a trend for an increase in CD44high T cells in the blood and lymph nodes, suggesting T cell proliferation may occur naturally within PSGL-1null mice. Enhanced proliferative responses of PSGL-1null CD8 T cells to the common
γ-chain receptor cytokines IL-2, IL-4, and IL-15, all known to induce homeostatic proliferation, was confirmed in vitro for both naïve and memory CD8 T cell populations. This observation was consistent with the observation that PSGL-1null CD8 T cells proliferated more extensively than WT T cells in vivo when injected into sublethally irradiated WT lymphopenic mice. Because PSGL-1null T cell hyperproliferation in response to mitogen was previously reported (34), the PSGL-1 effect on proliferation appears to be a general phenotype manifest in both homeostatic and inflammatory conditions.

How PSGL-1 affects proliferation of CD8 T cells is currently unknown. There are several possibilities. PSGL-1 could directly bind cytokines and induce an inhibitory effect on proliferation by sequestering the cytokines away from their receptors. PSGL-1, which is a large and heavily glycosylated protein, could in some way block or sequester the common receptor subunit that is shared by IL-2, IL-4, and IL-15, and thus inhibit response to these cytokines. PSGL-1 could also affect CD8 T cell proliferation indirectly in that it could negatively regulate cytokine receptor signaling via interference with elements of the downstream signaling pathways that are shared by these cytokines.

The increase in LCMV-specific memory T cell numbers we observed in PSGL-1null mice was also consistent with the enhanced responsiveness of PSGL-1null CD8 T cells to IL-2, known to be released during infection of LCMV, and to IL-15, required for memory T cell maintenance after viral clearance (35). Our findings that PSGL-1null mice are capable of mounting a normal CD8 T cell response are consistent with a study showing that 20% of T cells are sufficient to mount a full immune response to LCMV challenge (36). In this model, there was also an increase in LCMV-specific memory T cells after 6 mo. Similarly, mice deficient in fucosyltransferases IV and VII, which have impaired L-selectin–dependent T cell entry into lymph nodes and a decreased lymph node cellularity, also developed an increase in memory T cell frequency after LCMV infection (37). It was suggested that a combination of increased cytokine release during the viral infection and general lymphopenia favored the proliferation and increased frequency of memory T cells in both these mouse models.

PSGL-1null dendritic cells have been shown to express higher levels of MHC class II, CD40, and CD86 after stimulation with CpG than WT dendritic cells, and they induce a greater proliferation of naïve OT-II CD4 T cells when stimulated with OVA (38). Therefore, we cannot exclude the possibility that PSGL-1null dendritic cells in the LCMV model have an enhanced immunogenicity, which also might account for the increase in memory CD8 T cell numbers. However, because both our in vivo and in vitro proliferation studies were done in competitive experimental formats, our data highlight CD8 T cell intrinsic effects associated with PSGL-1 deficiency.

Because PSGL-1 was shown to interact with CCL21/CCL19 chemokines and enhance CCR7-dependent chemotaxis (17), we speculated that PSGL-1 may exert interference with migration and proliferative effects on resting T cells via direct or indirect interaction with CCR7. Although there are some similarities between phenotypes observed in CCR7null and PSGL-1null mice, there are also significant differences. Both knockout mice have reduced T cell homing to lymph nodes and Peyer’s patches, although CCR7null T cells are more affected than PSGL-1null T cells insofar as they exhibit reduced T cellularity in these organs, whereas PSGL-1null mice do not (39). CCR7null T cells are able to enter the spleen and CCR7null mice exhibit increased splenic T cell numbers, whereas PSGL-1null T cell entry into spleen is mildly reduced but cellularity is unaffected. Furthermore, PSGL-1null T cells are hyperproliferative to cytokine stimulation and mitogen stimulation (34), whereas CCR7null T cells were shown to proliferate less after mitogen stimulation (40). In addition, although PSGL-1null mice had normal levels of effector CD8 T cells and increased levels of LCMV-specific memory T cells after LCMV infection, CCR7null mice had approximately half the levels of LCMV-specific CD8 T cells both in the acute effector stage and in the memory stage (41). Therefore, although PSGL-1 and CCR7 are both required for migration of T cells, they appear to have opposing effects in proliferation and memory T cell production after LCMV infection.

In conclusion, PSGL-1 deficiency appears to influence CD8 T cell homeostasis in at least three different ways: 1) by interfering with lymph node entry of T cells, thereby limiting access to homeostatic cytokines and other prosurvival signals in the lymph node; 2) by prolonging lymph node residence time, thereby extending exposure of T cells to prosurvival signals therein; and 3) by increasing T cell sensitivity to cytokines. Although the first effect will negatively affect T cell homeostasis, the latter two effects will be positive. The mild net phenotype seen in PSGL-1null mice likely results from these potentially offsetting influences.

This study continues to explore the roles of PSGL-1 in its nonselective binding state. Our findings that PSGL-1 helps maintain the distribution of resting T cells to certain compartments, regulates proliferation of T cells in response to homeostatic cytokines, and enhances memory T cell formation/survival after LCMV challenge further extend our understanding of PSGL-1 functionality and introduce another facet of T cell homeostasis regulation.

Acknowledgments

We are grateful to Michael Williams for technical assistance and to Drs. Ninan Abraham and Pauline Johnson for helpful discussions throughout the project.

Disclosures

The authors have no financial conflicts of interest.

References
