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# Functional Role of P-Selectin Glycoprotein Ligand 1/P-Selectin Interaction in the Generation of Tolerogenic Dendritic Cells[1]

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**Dendritic cells (DCs) have a key role in both the generation of the immune response and the induction of tolerance to self-Ags. In this work, the possible role of P-selectin glycoprotein ligand 1 (PSGL-1) on the tolerogenic activity of human DCs was explored. We found that the engagement of PSGL-1 by P-selectin on DCs induced the expression of c-Fos, IDO, IL-10, and TGF-$\beta$ genes. Remarkably, stimulation of DCs through PSGL-1 with P-selectin enhanced their capability to generate $CD4^+CD25^+Foxp3^+$ regulatory T cells, which expressed high levels of TGF-$\beta$1 mRNA, synthesized IL-10, and suppressed the proliferation of autologous $CD4^+CD25^-$ T cells. Accordingly, we found that DCs from PSGL-$1^{-/-}$ mice expressed higher levels of MHC class II molecules, and exhibited an enhanced immunogenicity compared with wild-type mice. In addition, the percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the thymus of PSGL-1-deficient animals was significantly reduced. Our data reveal an unexpected role of PSGL-1 on the tolerogenic function of DCs, and the regulation of the immune response.** *The Journal of Immunology,* **2007, 179: 7457–7465.**

Bone marrow-derived dendritic cells (DCs)[3] are potent APCs that have a key role in the induction and the regulation of the immune response (1–3). The turnover of these cells has been studied in detail. Immature blood DCs migrate toward tissues, mainly skin and other epithelia, to accomplish tissue replenishment (4, 5). Under environmental danger signals (infection, inflammation, tissue damage), these cells capture Ags and migrate toward the regional lymph nodes. This migration is accompanied by a maturation process, increasing the expression of MHC class II molecules and costimulatory receptors and becoming highly immunogenic (1, 2, 4, 5). In contrast, in steady-state conditions, tissue DCs remain immature, exhibiting a high internalization capability, a reduced expression of molecules involved in Ag presentation, and a very low immunogenic capability (1, 2, 4, 5). When these immature DCs interact with T lymphocytes, they induce anergy or deletion, acting as negative regulators of the immune response (6). In addition, immature or tolerogenic DCs are able to induce the generation of regulatory T (Treg) cells (7). Therefore, DCs exert a key regulatory activity on the immune system, initiating the immune response under inflammation or infection, and favoring immunological tolerance in the steady state. Because the same cell type performs these opposite activities, the factors that determine the generation of immunogenic and tolerogenic DCs have key roles in the immune system.

Migration of DCs from the bloodstream to different tissues follows the same sequential process described for other leukocytes. Thus, it has been shown that DCs, attracted by different chemokines, tether and roll on endothelium and then transmigrate through the endothelial cell lining (8, 9). As in the case of other leukocytes, selectins and their receptors play an important role in the early steps of DC-endothelial cell interactions. In this regard, it has been shown that E- and P-selectin mediate tethering and rolling of DCs on endothelium in vitro and in vivo (8). In addition, it has been reported that P-selectin glycoprotein ligand 1 (PSGL-1, CD162) is expressed by DCs (10). Although it has been reported that PSGL-1 is not required for the extravasation of DCs to inflamed tissues (11), other works suggest that, under steady-state conditions, this receptor is involved in the transendothelial migration of these cells (8, 12). At least two glycoforms of this selectin receptor, recognized by the HECA-452 and M-DC-$8^+$ mAbs, are expressed by DCs (13, 14). The PSGL-1 form recognized by HECA-452 (denominated as cutaneous lymphocyte Ag) interacts mainly with E-selectin, whereas the form lacking the cutaneous lymphocyte Ag epitope largely interacts with P-selectin (15, 16). Cutaneous lymphocyte Ag is a skin homing receptor and has been detected on Langerhans cells and on a high proportion of freshly isolated blood DCs (8, 17, 18). In contrast, the M-DC8+ form of PSGL-1 lacks the cutaneous lymphocyte Ag epitope and is expressed by a subset of blood DCs that shows a potent capacity to prime T cells in vitro (14). Recently, it has been shown that differentiated peripheral DCs can return to blood and travel to different organs (spleen, liver, lungs, and bone marrow) (19). Furthermore, circulating DCs show bone marrow tropism that is dependent, in part, on microvascular P- and E-selectins (19).

Different effects of PSGL-1 engagement and intracellular signaling events have been described, mainly in myeloid cells and

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[3] Abbreviations used in this paper: DC, dendritic cell; Treg, regulatory T; PSGL-1, P-selectin glycoprotein ligand 1; LC, Langerhans-like DC; mDC, monocyte-derived DC; pDC, plasmacytoid DC; cDC, conventional DC; Flt3L, Flt3 ligand.

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neutrophils (20–22). However, there is scarce information on the functional consequences of PSGL-1 engagement in DCs. Therefore, in this work we have explored the functional consequences of PSGL-1 engagement on both human monocyte-derived DC (mDCs) and Langerhans-like DCs (LCs) generated in vitro. In addition, we have studied the status of DCs in PSGL-1-deficient mice. Our results strongly suggest that PSGL-1 can behave as a tolerogenic receptor in human and murine DCs.

## Materials and Methods

### *Abs and reagents*

FITC-, allophycocyanin-, and PE-conjugated anti-human CD1a, CD3, CD4, CD14, CD25, DC-SIGN (CD209), HLA-DR, CD80, CD83, CD86, and PSGL-1 as well as anti-E-cadherin mAbs and conjugated anti-mouse mAbs were obtained from BD Biosciences. Human recombinant E- and P-selectins, IL-4, TNF-$\alpha$, and TGF-$\beta$ were obtained from R&D Systems. The anti-PSGL-1 (CD162) PL1 mAb was purchased from Upstate Biotechnology, and the anti-CD28 mAb from BD Pharmingen. GM-CSF and Flt3 ligand (Flt3L) from PeproTech, and PMA, ionomycin, brefeldin A, LPS, fibronectin-80 and the CpG oligonucleotide were obtained from Sigma-Aldrich. BSA was obtained from PAA Laboratories. FITC-labeled anti-human and anti-mouse Foxp3 staining kits were purchased from eBioscience.

### *Mice*

PSGL-1-deficient mice (males and females 6- to 8-wk-old) backcrossed on the C57BL/6 background (23) and C57BL/6 control mice from different suppliers were obtained. Transgenic OT-II mice expressing a TCR specific for $\text{OVA}_{323–339}$ peptide (OVA peptide) on a C57BL/6 background were purchased from Charles River Breeding Laboratories.

### *Cells*

PBMC were obtained from buffy coats of healthy donors by Ficoll-Hypaque (Sigma-Aldrich) density gradient centrifugation. Then, monocytes were isolated by plastic adherence or by positive selection with anti-CD14-coated microbeads (Miltenyi Biotec), following the manufacturer's instructions. To generate mDCs and LCs, monocytes were incubated at 37°C for 5–7 days in RPMI 1640 (Invitrogen Life Technologies) culture medium, containing 10% FCS and supplemented with 50 ng/ml GM-CSF and 20 ng/ml IL-4. In the case of LCs, the culture medium was additionally supplemented with 10 ng/ml TGF-$\beta$. The phenotype of mDCs (HLA-$\text{DR}^+$, $\text{CD1a}^+$, DC-$\text{SIGN}^+$, and $\text{CD14}^-$), and LCs (HLA-$\text{DR}^+$, $\text{CD1a}^+$, DC-$\text{SIGN}^+$, $\text{CD14}^-$, and E-$\text{cadherin}^+$) was confirmed by flow cytometry analysis. To obtain mature mDCs and LCs, immature cells were washed and incubated for 24 h with 20 ng/ml LPS.

Human naive $\text{CD4}^+$ T cells and Treg cells were isolated from PBMC by negative selection using AutoMacs magnetic cell sorter kits (Miltenyi Biotec), following the manufacturer's instructions.

Murine DCs from thymus and lymph nodes as well as Flt3L-driven DCs generated from bone marrow precursors were obtained as described (24). Maturation of Flt3L-derived plasmacytoid (pDCs) and conventional DCs (cDCs) was induced on day 7 of differentiation by culture for additional 24 h in the presence of 6 $\mu$g/ml CpG ODN 1826. $\text{CD4}^+$ T lymphocytes were obtained from the spleens and lymph nodes of OT-II transgenic mice by magnetic cell sorting-mediated negative selection using a $\text{CD4}^+$ T cell isolation kit (Miltenyi Biotec).

### *Immunofluorescence microscopy analysis*

Monocyte-derived DCs or LCs were allowed to migrate onto coverslips precoated with 20 $\mu$g/ml fibronectin-80 for 20 min at 37°C. Cells were then fixed with 2% paraformaldehyde, washed, and stained for 1 h with the PL-1 anti-PSGL-1 mAb (20 $\mu$g/ml) for 30 min, followed by a rhodamine X-conjugated goat anti-mouse IgG Ab. For double staining, cells were fixed again, permeabilized for 2 min with 0.5% Triton X-100, and incubated with the anti-moesin polyclonal Ab 90:2/3, followed by a goat anti-rabbit Alexa Fluor 488-conjugated Ab. Before immunostaining, Fc receptors were blocked with human gammaglobulin (100 $\mu$g/ml for 20 min). Finally, cells were analyzed using a Leica DMR photomicroscope with a ×63 oil immersion objective and an inverted epifluorescence Leica TCS-SP confocal microscope equipped with argon and helium/neon laser beams.

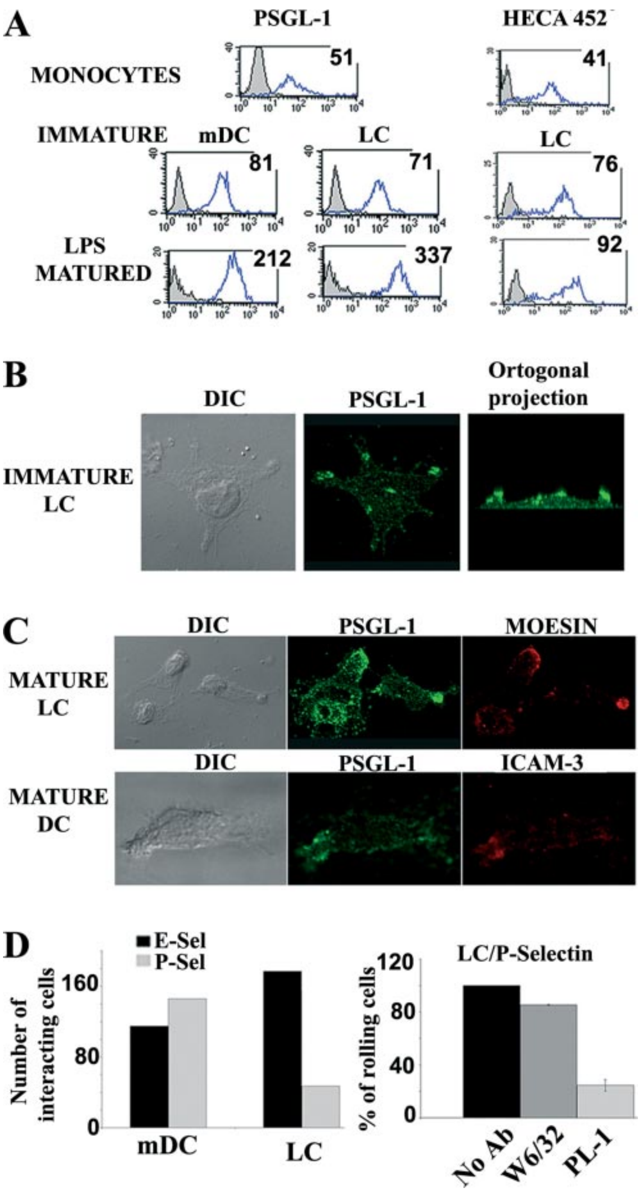

**FIGURE 1.** Expression and subcellular localization of PSGL-1 in DCs. *A*, The mDCs and LCs were generated in vitro as described in *Materials and Methods*. Then, the expression of PSGL-1, and its carbohydrate epitope HECA-452 was determined by flow cytometry in immature cells and in those induced to mature with LPS. Cells incubated with an isotype-matched irrelevant mAb (gray-filled histogram). Values shown indicate the geometric mean fluorescence staining of positive cells. *B* and *C*, The mDCs and LCs were allowed to migrate onto fibronectin-80-coated coverslips. Then, cells were fixed, immunostained for PSGL-1 (*B*) or PSGL-1 (green) and ICAM-3 or moesin (red) (*C*), and analyzed by immunofluorescence microscopy, as described in *Materials and Methods*. Original magnification at ×630. Data are from one representative experiment of five performed. *D*, In vitro rolling of DCs on selectins. Glass coverslips precoated with 10 $\mu$g/ml P- or E-selectin were placed in a parallel-plate flow chamber mounted on an inverted microscope. The mDCs or LCs were perfused to the chamber at 1.5 dynes/$\text{cm}^2$ flow rate using a syringe pump, and the cells were recorded using a video camera. The number of rolling cells was calculated in 10 different fields per experiment. When indicated, assays were performed with cells preincubated with the control (W6/32, an anti-HLA class I mAb) or the PL-1 (anti-PSGL-1) mAbs.

### *DC rolling assay*

This assay has been described in detail elsewhere (8). Briefly, glass coverslips precoated with 10 $\mu$g/ml P- or E-selectin were placed in a parallel-plate flow chamber mounted on an inverted Nikon Diaphot microscope.

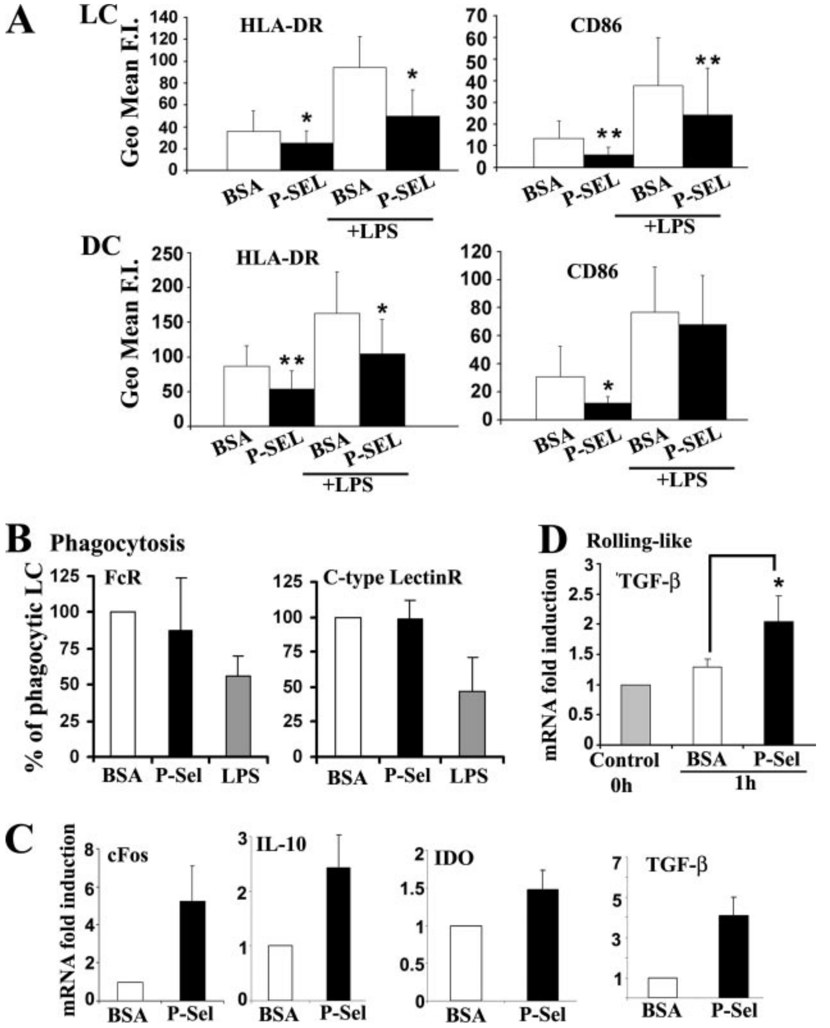

**FIGURE 2.** Effect of PSGL-1 engagement on HLA-DR, CD80 and CD86 expression, phagocytosis, and transcription factor and cytokine gene induction by DCs. *A*, The mDCs and LCs were cultured for 24 h on plates precoated with P-selectin or BSA, in the absence or presence of 20 ng/ml LPS, and then analyzed by flow cytometry for the expression of the indicated molecules. The arithmetic mean of the geometric mean fluorescence intensity and SD obtained in eight independent experiments is shown for each molecule. The significant differences in the expression level between BSA and P-selectin-treated DCs were obtained by applying the Student's *t* test. $*$, $p < 0.05$; $**$, $p < 0.01$. *B*, After incubation with BSA, P-selectin, or LPS plus BSA, LCs were assayed for Fc receptor- or C-type-lectin phagocytosis, as described in *Materials and Methods*. The arithmetic mean and SD of four independent experiments is shown. *C*, LCs were incubated for 24 h in BSA- or P-selectin-coated plates. Then, cells were collected and analyzed by semiquantitative real-time RT-PCR for c-Fos, IL-10, TGF-β, and IDO mRNAs expression. The arithmetic mean and SD of four independent experiments is shown. *D*, LCs were incubated in rolling-like conditions for 1 h on BSA or P-selectin-coated plates and then were harvested and analyzed for mRNA expression by semiquantitative real-time RT-PCR. The arithmetic mean and SD of three independent experiments is shown.

Then, mDCs or LCs were perfused into the chamber and allowed to interact under static conditions for 3 min. Flow was initiated at defined rates using a syringe pump (model 44; Harvard Apparatus), and cells were recorded for 6 min using a video camera. Those cells that traveled slowly were considered to be rolling. The number of rolling cells was calculated in four different fields at each time point of every independent experiment. When indicated, assays were performed with cells preincubated for 20 min with the PL-1 anti-PSGL-1 (10 μg/ml) mAb.

### *Stimulation of mDCs and LCs through PSGL-1*

The 24-well tissue culture plates were coated with 10 μg/ml E- or P-Selectin (R&D Systems) or with 0.5% BSA by overnight incubation at 4°C. Then plates were washed, and immature mDCs or LCs were cultured in them without or with LPS for 24 h at 37°C. For rolling-like experiments, plates were shaking at 60 rpm during the time of incubation. Afterward, cells were collected and analyzed for the expression of HLA-DR, CD80, and CD86 by flow cytometry. In addition, c-Fos, IDO, IL-10, and TGF-β1 gene expression as well as the secretion of IL-12 and IL-10 were determined in these cell cultures, as stated below.

### *Cytokine assays*

Synthesis of IL-12p70, IL-10, IL-4, and IFN-γ in cell cultures was determined by ELISA. Briefly, cell culture supernatants were collected, and cytokine concentration was analyzed by specific solid phase sandwich enzyme immunoassay (Elipairs; Diaclone Research), following the manufacturer's instructions. For the intracellular staining of IL-10 after interaction with P-selectin, cells were stimulated for 6 h with PMA (50 ng/ml) and ionomycin (1 μg/ml), and in the last 4 h in the presence of 50 μg/ml brefeldin A before labeling for flow cytometry analysis.

### *Phagocytosis assays*

Particles used were either SRBC (Biomerieux) or latex beads (3 μm; Sigma-Aldrich). For FcγR-mediated phagocytosis, SRBC ($10^7$ cells/ml) were opsonized as previously described (25) and loaded with 5 μM CFSE (Molecular Probes). For C-type lectin R phagocytosis, latex beads were opsonized by overnight incubation with 5 mg/ml OVA (Sigma-Aldrich). Then, $1 \times 10^6$ LCs were incubated with CFSE-labeled SRBC or OVA beads in a 1:20 ratio at a final volume of 200 μl for 30 min at 37°C. To distinguish internalized from bound particles, noningested SRBC or OVA beads were lysed or removed by treatment with $NH_4Cl$ or glycine buffer (150 mM glycine (pH 2.3)), respectively, during 1 min. Cells were then washed once with PBS, fixed with 1% paraformaldehyde, and stained for HLA-DR or OVA beads with specific mAb labeled with allophycocyanin or Alexa Fluor 488 (Molecular Probes). The uptake of stained particles was determined by flow cytometry.

### *Flow cytometry analysis*

Fc receptors of DCs or PBMCs were saturated with human gammaglobulin (100 μg/ml) or anti-CD16/CD32 mAb (BD Pharmingen) at 4°C for 15 min, and then cells were stained with the indicated mAbs (20 μg/ml) for 15 min at 4°C. The expression of Foxp3 was analyzed by intracellular staining with FITC-conjugated anti-mouse or anti-human Foxp3 mAb (clones FJK-16s and PCH101 from eBioscience) after fixation and permeabilization of the cells with the reagents provided by the manufacturer. Finally, cells were washed, fixed, and analyzed in a FACSCalibur flow cytometer (BD Biosciences), using the CellQuest software.

### *Allogenic mixed cell proliferation assays and cytokine synthesis*

To analyze the stimulatory potential of DCs on allogenic T cells, $2 \times 10^5$ naive $CD4^+$ T cells were cocultured for 5 days with $2 \times 10^4$ mDCs or LCs that had been pretreated or not for 24 h with P-selectin. Then, cells were collected, lysed, and analyzed for gene expression. Supernatants of these cell cultures were analyzed by ELISA to determine the concentration of IL-10 and IFN-γ. T cell proliferation was assessed after 5 days of coculture by [*methyl*-$^3$H]thymidine ([$^3$H]TdR, 5.0 μCi/ml) incorporation in a 16-h pulse. For this purpose, cells were harvested with a semiautomated device,

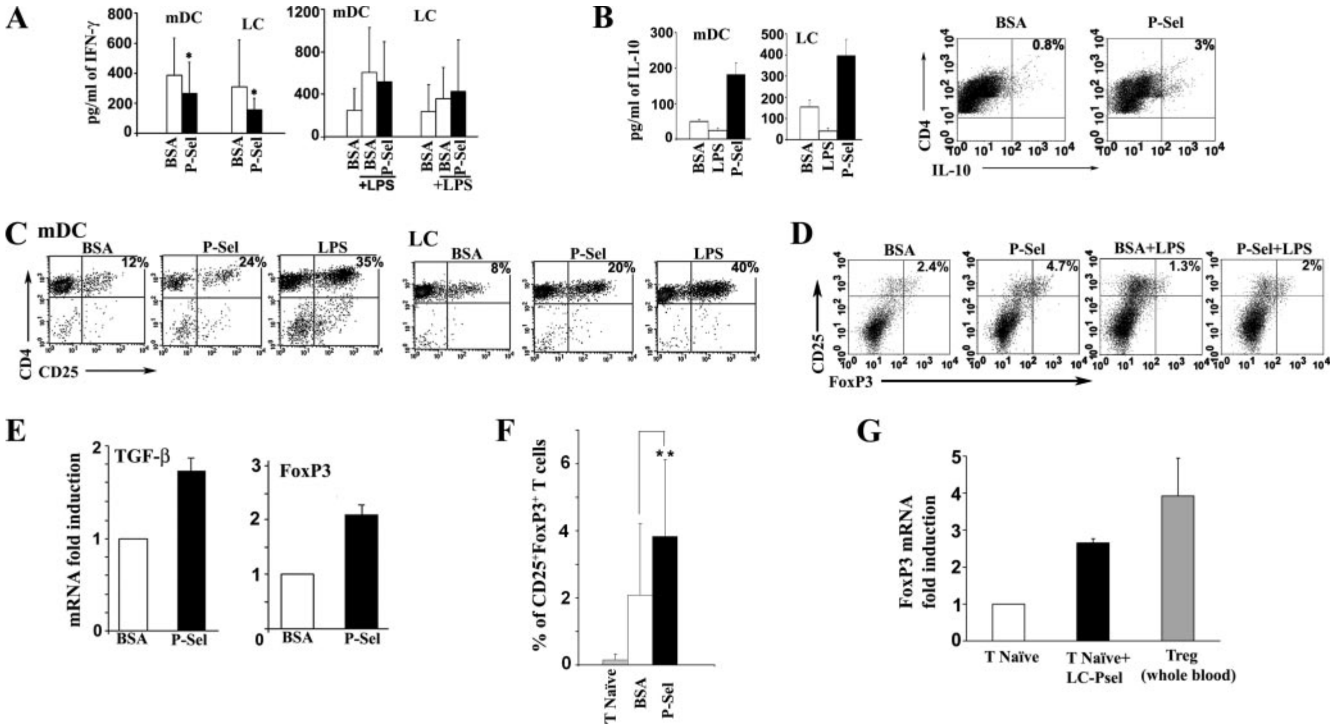

**FIGURE 3.** Effect of PSGL-1 engagement on the immunogenic activity of DCs. *A–F*, The mDCs or LCs were incubated in plates precoated with P-selectin or BSA for 24 h, in the absence or presence of LPS, and then cocultured with allogenic naive $CD4^+$ T lymphocytes for 5 days. *A*, Culture supernatants were analyzed by ELISA for IFN-γ concentration. The arithmetic mean and SD of five independent experiments is shown. The significant differences in the IFN-γ production induced by BSA and P-selectin-treated DCs were obtained by applying the Student's *t* test. $*$, $p < 0.05$. *B*, IL-10 concentration in cell supernatants was determined by ELISA and the intracellular content of this cytokine in $CD4^+$ T cells by flow cytometry. A representative experiment of five performed is shown. *C* and *D*, Flow cytometry analysis of $CD4^+CD25^+$ and $CD25^+FoxP3^+$ cells generated after coculture of naive $CD4^+$ lymphocytes with BSA or P-selectin-treated mDCs and LCs (*C*) or only LCs (*D*). The percentage of positive cells is indicated, and results correspond to a representative experiment of four independent performed. *E*, After coculture with LCs, the TGF-β and FoxP3 mRNA expression was analyzed by semiquantitative real-time RT-PCR. Data are from a representative experiment of three performed. *F*, Percentage of $CD25^+Foxp3^+$ T cells generated in cocultures of naive $CD4^+$ lymhocytes with control or P-selectin-treated LCs. The arithmetic mean and SD of four independent experiments is shown. The significant differences in the percentage of $CD25^+Foxp3^+$ T cells generated by BSA- and P-selecetin-treated DCs were obtained by applying the Student's *t* test. $**$, $p < 0.01$. *G*, Level of mRNA Foxp3 in cocultures of naive $CD4^+$ cells with P-selectin-treated LCs. Results of Foxp3 mRNA in isolated naive $CD4^+$ lymphocytes, and peripheral blood $CD4^+$ $CD25^+$ Treg cells are also shown. Data correspond to the arithmetic mean and SD of two independent experiments.

and the incorporation of [$^3$H]TdR was determined in a liquid scintillation counter. All these experiments were conducted by triplicate, and results were expressed as cpm incorporated. For CFSE dilution assays, $CD4^+$ lymphocytes were labeled with CFSE (5 μM) before their coculture with DC, and with anti-CD25-PE after 5 days of culture with DCs. In these assays, results were expressed as CFSE histograms and the percentage of $CD25^+$-divided cells.

### *Suppression assay*

To analyze the suppressive function of the Treg lymphocytes induced in vitro by DCs, autologous mixed cell cultures were performed. Briefly, $CD4^+$ T lymphocytes recovered after 5 days of coculture with DCs (T1 lymphocytes), were maintained in culture for two additional days in the presence of IL-2 (5.0 U/ml), and then mixed with autologous naive $CD4^+$ T lymphocytes ($1 \times 10^5$) and stimulated with anti-CD3 (5 μg/ml) plus anti-CD28 (1 μg/ml) mAb. After 2 days of cell culture, 5.0 μCi/ml [$^3$H]TdR was added, and cells were harvested 18 h later. Results were expressed as cpm incorporated.

### *Murine $CD4^+$ T cell proliferation assays*

Transgenic OT-II $CD4^+$ T cells were cocultured for 72 h in 96-well plates with either immature or mature pDCs or cDCs at a 10:1 T cell to DC ratio in the presence of OVA peptide. T cell proliferation was then analyzed by [$^3$H]TdR incorporation as stated.

### *Real-time PCR assays*

Cells were lysed and total RNA extracted using the Ultraspec RNA isolation system (Biotecx Laboratories). cDNA was obtained with the ImProm-II Reverse Transcriptase kit (Promega), following the manufacturer's recommendations. These samples were then analyzed for the expression of c-Fos, Foxp3, TGF-β, IL-10, IDO, and GAPDH genes by SYBR Green real-time PCR (Roche Diagnostics), using a DNA LightCycler rapid thermal cycler system (Roche). The sequences of the specific primer pairs used in each case were as follows: GAPDH (forward) 5′-GAA GGT GAA GGT CGG AGT C-3′, (reverse) 5′-GAA GAT GGT GAT GGG ATT TC-3′; c-Fos (forward) 5′-AGG AGA ATC CGA AGG GAA AGG-3′, (reverse) 5′-TCC GCT TGG AGT GTA TCA GTC A-3′; Foxp3 (forward) 5′-GAG AAG CTG AGT GCC ATG CA-3′, (reverse) 5′-GGA GCC CTT GTC GGA TGA T-3′; TGF-β1 (forward) 5′-GGA CAC CAA CTA TTG CTT CAG-3′, (reverse) 5′-TCC AGG CTC CAA ATG TAG G-3′; IL-10 (forward) 5′-CCT TCC AGT GTC TCG G-3′, (reverse) 5′-AGA CGG GGT TTC ACC A-3′; and IDO (forward) 5′-AGA GTC AAA TCC CTC AGT CC-3′, (reverse) 5′-AAA TCA GTG CCT CCA GTT CC-3′. Results were normalized for GAPDH expression, measured in parallel in each sample.

### *Statistical analysis*

Student's *t* test, the nonparametrical Mann-Whitney *U* test, and ANOVA analysis were used to determine significant differences. A value for $p <$ 0.05 was considered as significant.

## Results

### *Expression and function of PSGL-1 in mDCs and LCs*

To explore the possible role of PSGL-1 in the regulation of DC function, we first analyzed its expression during the differentiation of monocytes to mDCs and LCs. As shown in Fig. 1*A*, the expression of PSGL-1 was higher in mDCs compared with monocytes,

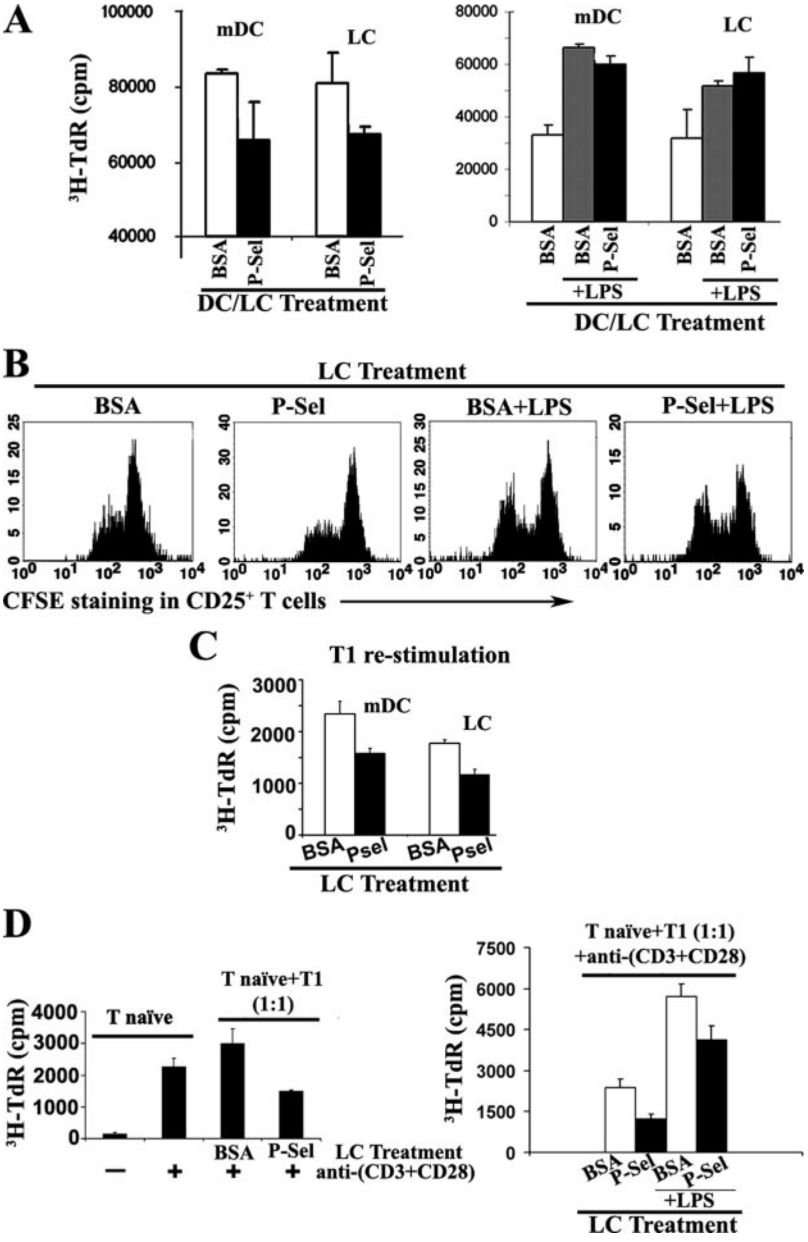

**FIGURE 4.** P-selectin-treated DCs are able to induce functional Treg cells. The mDCs and LCs, after interaction for 24 h with P-selectin or BSA in the absence or presence of LPS, were cocultured with allogenic nonfractionated CD4$^+$ T cells (*A* and *C*) or naive CD4$^+$ lymphocytes (*B* and *D*) for 5 days. *A*, Results of cell proliferation ([$^3$H]Tdr incorporation assays) are shown. Data are from a representative experiment of five performed. *B*, Naive CD4$^+$ lymphocytes isolated from blood were loaded with CFSE and then cocultured with control or treated LCs; after 5 days of culture, cells were stained for CD25 and analyzed by flow cytometry. CFSE data of CD25$^+$ cells are from a representative experiment of four performed. The percentage of divided cells are 57% for T lymphocytes activated by BSA-treated DCs and 39% for T lymphocytes activated by P-selectin-treated DCs. *C*, After 5 days of coculture with DCs, T cells (T1) were additionally cultured for 2 days with 5 U/ml IL-2, and then restimulated with anti-CD3/CD28 mAbs and analyzed for [$^3$H]Tdr incorporation. Data are from a representative experiment of three performed. *D*, Suppressive activity of T1 cells. T1 lymphocytes were cocultured with autologous naive CD4$^+$ T cells in the presence of anti-CD3/CD28 mAbs, and cell proliferation was assessed by [$^3$H]Tdr incorporation. Data are from a representative experiment of three performed. In *A*, *C*, and *D* data correspond to the arithmetic mean and SD of triplicates.

indicating PSGL-1 up-regulation during cell differentiation. A further enhancement in PSGL-1 expression was found upon DC maturation with bacterial LPS or with TNF-α (Fig. 1*A* and data not shown). Accordingly, the expression of the HECA-452 epitope of PSGL-1 also increased during the differentiation of LCs and its maturation induced with LPS (Fig. 1*A*).

We then studied the subcellular localization of PSGL-1 in immature and mature mDCs and LCs by immunofluorescence microscopy during their migration on fibronectin-80. PSGL-1 was localized at the rear pole of immature mDCs, mainly at the membrane microspikes and microvilli (data not shown), whereas in immature LCs this receptor was clustered in different regions of the cell membrane (Fig. 1*B*). In contrast, in mature LCs and mDCs, PSGL-1 was localized at both poles of the cell, although in a higher extent at the rear pole, where it colocalized with ICAM-3 (Fig. 1*C*). Because in T lymphocytes PSGL-1 interacts with ezrin-radixin-moesin proteins (21), we decided to analyze their subcellular localization in mature mDCs. As shown in Fig. 1*C*, PSGL-1 (green fluorescence) and moesin (red fluorescence) were localized at the same motility-associated protrusive structures of both mDCs and LCs during their migration on fibronectin-80.

Flow chamber cell rolling experiments performed on coverslips coated with selectins showed that, as described (8), mDCs efficiently rolled on selectins P and E, whereas LCs were able to roll with high efficiency mainly on E-selectin (Fig. 1*D*). As expected, most DCs lost their capability to roll on P-selectin when were pretreated with a blocking anti-PSGL-1 mAb (Fig. 1*D*).

### *Effect of PSGL-1 engagement on gene expression by immature DCs*

The possible role of PSGL-1 on the expression of molecules involved in the immunogenic capability of DC was subsequently explored. We found that the interaction of mDCs or LCs with P-selectin significantly diminished the up-regulation of HLA-DR and costimulatory molecules, even when this interaction was performed in the presence of LPS (Fig. 2*A*). In contrast, PSGL-1 engagement did not change the phagocytic activity of these cells

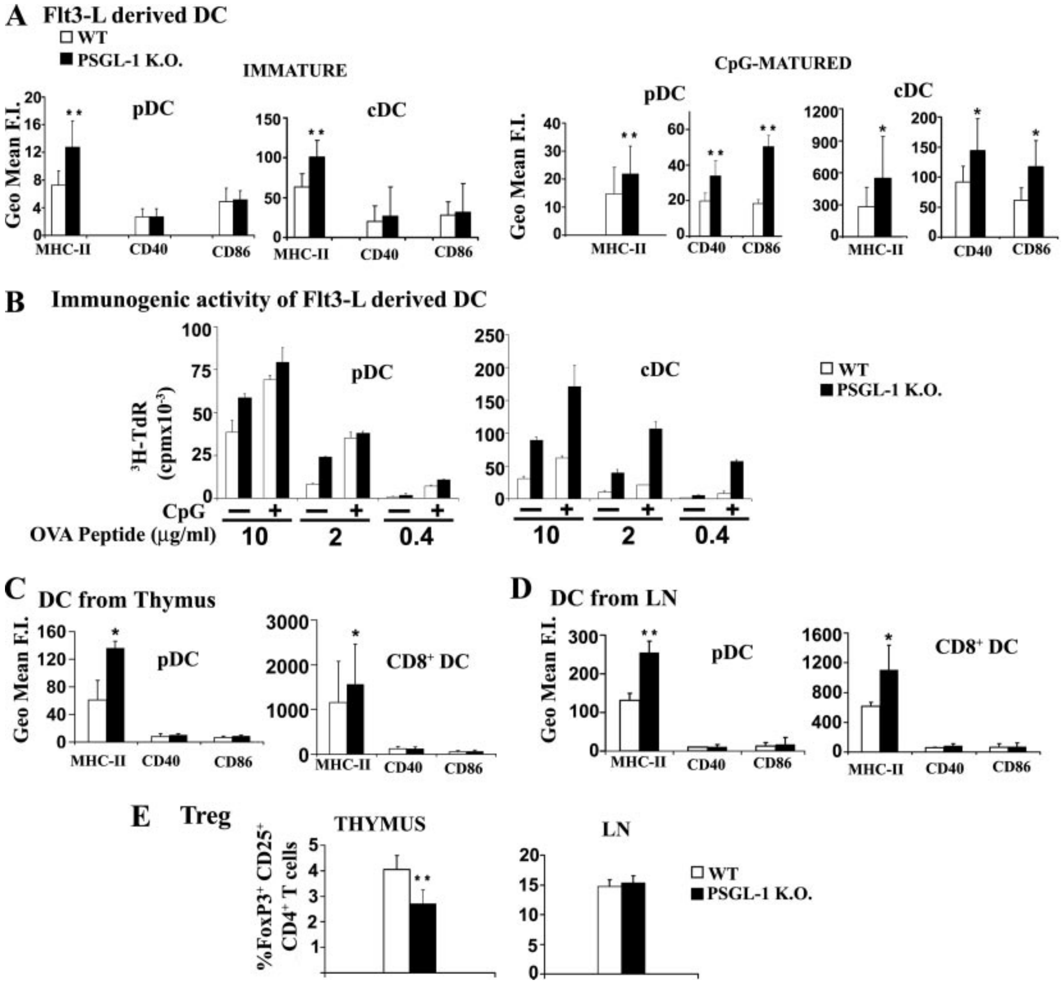

FIGURE 5. Analysis of DCs and Treg cells in PSGL-1-deficient mice. *A*, The pDCs and cDCs, derived from Flt3L-driven bone marrow cultures (FL-DCs) from wild-type or PSGL-1-deficient mice before and after CpG-induced maturation, were analyzed for MHC class II, CD40, and CD86 expression by flow cytometry. Data correspond to the arithmetic mean and SD of three independent experiments. Each experiment was conducted with the bone marrow of four wild-type and four PSGL-1-deficient mice. *B*, Immunogenic capability of DCs from wild-type and PSGL-1-deficient mice. Immature or CpG-matured pDCs or cDCs were pulsed with the OVA peptide, cocultured with transgenic OT-II CD4$^+$ cells, and 3 days later, the cell proliferation was assessed by [$^3$H]TdR incorporation. Data are from a representative experiment of four performed. Each experiment was conducted with the bone marrow of four wild-type and four PSGL-1-deficient mice. *C* and *D*, Expression of MHC class II and costimulatory molecules (CD40 and CD86) was determined by flow cytometry in pDCs and cDCs freshly isolated from the thymus and peripheral lymph nodes from wild-type and PSGL-1-deficient mice. Data are from a representative experiment of three performed. Each experiment was conducted with four wild-type and four PSGL-1-deficient mice. *E*, Cell suspensions obtained from thymus and lymph nodes from PSGL-1-deficient and wild-type mice were stained for CD4, CD25, and Foxp3 and analyzed by flow cytometry. Data correspond to the arithmetic mean and SD of 16 PSGL-1-deficient and 21 wild-type mice. *, $p < 0.05$; **, $p < 0.01$ between PSGL-1-deficient and wild-type control mice obtained by applying the Student's *t* test.

(Fig. 2*B*), suggesting that they remained with an immature functional phenotype.

We have previously found that the engagement of PSGL-1 on myeloid cell lines induces the activation of Syk and the transcription of c-Fos gene (21). In addition, it has been described that the synthesis of IL-12 and IL-10 by DCs is under the control of ERK and Syk (26–30). We therefore investigated the effect of PSGL-1/selectin interaction on the activation of c-Fos gene in mDCs and LCs. Upon interaction of DCs with selectins, there was an intracellular accumulation of c-Fos mRNA (Fig. 2*C*), and kinetics analysis showed that cFos mRNA induction was already observed 1 h after interaction with selectins and persisted for at least 24 h (data not shown).

To further assess the effect of PSGL-1 engagement in DCs, we studied, by real-time semiquantitative RT-PCR, the expression of different genes implicated in the function of these cells. We found that P-selectin-treated LCs showed a significant induction of different genes involved in immune tolerance, as TGF-β, IL-10, and IDO (Fig. 2*C*). Similar results were obtained when TGF-β mRNA was analyzed in LCs cultured on P-selectin, under rolling-like conditions (Fig. 2*D*). In contrast, no significant changes in the expression of IL-4 or IL-12p40 genes were detected upon PSGL-1 engagement (data not shown).

### *Effect of PSGL-1 engagement on the immunogenic capability of DCs*

We next investigated whether PSGL-1 engagement could regulate the immunogenic capability of DCs. For this purpose, the potential to stimulate allogenic CD4$^+$ T cells by P-selectin-treated mDCs and LCs was determined. As shown in Fig. 3*A*, engagement of PSGL-1 by P-selectin significantly diminished the capability of mDCs and LCs to induce the synthesis of IFN-γ by allogenic CD4$^+$ T cells. In contrast, under such experimental conditions, IL-10 production was clearly enhanced (Fig. 3*B*), whereas the production of IL-4 was not significantly affected (data not shown). Additional experiments showed that PSGL-1 engagement did not modify the capability of DCs treated with LPS to induce the synthesis of IFN-$\gamma$ by T cells (Fig. 3*A*).

Interestingly, when coculture experiments were conducted with naive CD4$^+$ T lymphocytes, the engagement of PSGL-1 on DCs enhanced the percentage of CD4$^+$CD25$^+$ T lymphocytes (Fig. 3*C*). However, a higher increase in CD4$^+$CD25$^+$ was induced by DCs in the presence of LPS, suggesting that most of these cells corresponded to activated T lymphocytes. To further explore this point, we detected the presence of CD4$^+$Foxp3$^+$ in these cell cocultures. As shown in Fig. 3, *D* and *F*, P-selectin-treated DCs induced higher levels of CD4$^+$Foxp3$^+$ lymphocytes than untreated cells, and in this case, the presence of LPS reduced the generation of CD4$^+$Foxp3$^+$ cells. Accordingly, P-selectin-treated DCs induced a significant increase in the expression of Foxp3 gene (Fig. 3, *E* and *G*) as well as of TGF-$\beta$ (Fig. 3*E*).

### *P-selectin-treated DCs promote the generation of T cells with regulatory activity*

We next assessed the capacity of DCs to induce the proliferation of allogenic T cells. As shown in Fig. 4*A* (*left*), P-selectin-treated DCs exhibited a diminished capability to induce T cell proliferation compared with untreated cells. However, when DCs were exposed to LPS, PSGL-1 engagement did not affect their ability to induce T cell proliferation (Fig. 4*A*, *right*). Additional experiments showed that in cocultures of naive CD4$^+$ T cells (labeled with CFSE) and P-selectin-treated DCs, the percentage of divided cells expressing CD25 was lower compared with those cultures with untreated DCs (Fig. 4*B*). However, this difference was not observed when cell cultures were performed in the presence of LPS (Fig. 4*B*). Additional experiments showed that the T lymphocytes cultured with P-selectin-treated DCs (T1 cells) exhibited a diminished cell proliferation when restimulated through CD3 and CD28 (Fig. 4*C*).

Because these results strongly suggested that P-selectin-treated DCs were able to induce the generation of Treg lymphocytes, T1 cells were tested in a conventional suppression assay. As shown in Fig. 4*D*, T1 cells were able to suppress the proliferation of autologous naive CD4$^+$ cells, indicating the presence of T cells with regulatory activity. However, as in other experiments, the tolerogenic activity of P-selectin-treated DCs was less evident when these cells were stimulated with LPS. All these data indicated that, under steady-state conditions and upon PSGL-1 engagement, immature DCs enhance their tolerogenic properties and are able to promote the generation of T lymphocytes with regulatory phenotype and activity.

### *Analysis of DCs and Treg cells in PSGL-1$^{-/-}$ mice*

Our findings on the tolerogenic behavior of human selectin-treated DCs, prompted us to analyze DCs and Treg cells in PSGL-1-deficient mice. For this purpose, the expression of MHC class II and costimulatory molecules by in vitro-differentiated and freshly isolated DCs from PSGL-1 knockout mice were determined. As shown in Fig. 5*A*, immature DCs obtained from bone marrow cultures of PSGL-1-deficient mice, exhibited higher levels of MHC class II than cells from wild-type mice. Furthermore, after stimulation with the TLR9 ligand CpG, both mature pDCs and cDCs from PSGL-1 knockout mice, expressed higher levels of CD40, CD86, and MHC II molecules than those derived from control mice (Fig. 5A). We next investigated the capacity of mature pDCs and cDCs to induce the proliferation of naive transgenic OT-II CD4$^+$ T cells in vitro. We found that, in the presence of OVA peptide, DCs from PSGL-1-deficient mice showed a higher immunogenic activity compared with DCs from wild-type mice (Fig. 5*B*). Accordingly, thymic or lymph node pDCs and cDCs from PSGL-1$^{-/-}$ mice expressed higher levels of MHC class II molecules than cells from control mice (Fig. 5, *C* and *D*).

Finally, we found that the percentage of CD4$^+$CD25$^+$Foxp3$^+$ cells in the thymus of PSGL-1$^{-/-}$ mice was ~33% lower ($p <$ 0.0001) than in control mice (Fig. 5*E*). In contrast, under these steady-state experimental conditions, no significant differences were observed in the proportion of Treg cells in peripheral and mesenteric lymph nodes or spleen from PSGL-1-deficient and control mice (Fig. 5*E* and data not shown).

## Discussion

Bone marrow-derived DCs are potent APCs that play a dual role in the immune response, participating in its induction and in the development and maintenance of immune tolerance (9, 31). Although the regulatory role of DCs has been widely recognized, the mechanisms involved in this activity have not been fully characterized (9, 31, 32). Experimental evidence indicates that DCs exert their regulatory effect through two main mechanisms, the induction of anergic T lymphocytes, and the generation of Treg cells. In addition, it has been found that this tolerogenic effect can be performed by immature and mature DCs (9, 31, 32). In any case, these regulatory phenomena involve Ag presentation by DCs to naive T cells and, therefore, the interaction of DCs with these cells.

PSGL-1, by interacting with selectins, has an important role in adhesion phenomena among leukocytes, endothelial cells, and platelets (33). In this regard, our data, in agreement with a previous report, demonstrate the role of PSGL-1 in the tethering and rolling of DC (8). As other adhesion receptors, PSGL-1, upon interaction with its counterreceptors, generates different intracellular signals and induces key phenomena, including integrin activation and programmed cell death (34, 35). Accordingly, it has been reported that PSGL-1 interacts with Syk and cortical cytoskeleton through the actin linking proteins moesin and ezrin (21, 36, 37), and very recently it has been described the role of PSGL-1/Syk signaling in the selectin-dependent rolling and in the integrin activation induced by E-selectin (38, 39).

In this study, we have found that PSGL-1 exerts, in steady-state conditions, a novel and very interesting effect on the immune system. Our data show that PSGL-1 engagement induces the generation of tolerogenic DCs that are able to induce the differentiation of naive CD4$^+$ lymphocytes into CD4$^+$Foxp3$^+$ Treg. These tolerogenic DCs have an immature-like phenotype, showing phagocytic activity, low expression of MHC class II and costimulatory molecules, and enhanced synthesis of IL-10, IDO, and TGF-$\beta$ mRNAs. Because PSGL-1 has been widely considered as an adhesion receptor that generates activating signals in leukocytes, our findings are unexpected. However, there are previous reports on a regulatory role of PSGL-1 on bone marrow-derived cells, mainly on the proliferation of CD34$^+$ hemopoietic progenitor cells and on the cell survival of activated T lymphocytes (34, 40, 41). The possible role of PSGL-1 on DCs in central lymphoid tissues is of interest and it is very feasible that during their differentiation in the bone marrow, immature DCs are exposed to the engagement of their PSGL-1 molecules by their ligands, mainly E- and P-selectins. Our data indicate that the signals generated through PSGL-1 in these immature cells, in the absence of proinflammatory stimuli, may contribute to their tolerogenic behavior. The phenotypic characteristics of DCs generated from bone marrow precursors of PSGL-1-deficient mice further support this possibility. In addition, it has been reported that thymic endothelial cells constitutively express P-selectin, and that its interaction with

PSGL-1 is important for the recruitment of thymic progenitors (42). Moreover, it has been very recently described that the entry of peripheral DCs into the thymus, controlled by P-selectin, has a role in central tolerance by inducing Ag-specific clonal deletion (12). It is therefore tempting to speculate that the interaction of DCs with endothelium in the thymus, through PSGL-1/P-selectin binding, may also contribute to the local development of tolerogenic DCs and the generation of natural $CD4^+Foxp3^+$ Treg cells. This possibility is supported by our findings showing that in the thymus of PSGL-1-deficient mice, DCs express higher levels of MHC class II molecules and, accordingly, the percentage of $CD4^+CD25^+Foxp3^+$ T cells is significantly diminished. In this sense, it has already been suggested in the literature that thymic DCs might be the APC responsible for the induction of central tolerance (4, 43, 44).

The ability of selectin-treated DCs to induce Treg cells is also of interest regarding peripheral tolerance. Although it was originally proposed that $CD4^+CD25^{bright}$ Treg cells expressing Foxp3 are generated in the thymus ("natural regulatory cells"), different evidences indicate that cells with similar phenotypic and functional features may arise in the periphery from $CD4^+CD25^-$ naive T lymphocytes (45). Our data suggest that, upon PSGL-1 engagement, DCs may contribute to the generation of Treg in the periphery through their interaction with $CD4^+$ naive T cells. Although this effect was not evident in vivo, in the mesenteric and peripheral lymph nodes of PSGL-1-deficient mice under steady-state conditions, it is very feasible that it could be observed under infection or autoimmunity. However, it is evident that the lack of PSGL-1 exerts an important effect on both the phenotype of thymic and lymph node DCs, and the number of Treg cells in the thymus, suggesting a functional role of PSGL-1 in DCs on the generation of natural Treg. In this regard, it has been widely described that after their migration from bone marrow to blood, immature DCs extravasate to replenish different tissues and it is feasible that during their transmigration through endothelium PSGL-1 is engaged, mediating the tethering and rolling of these cells. Our data suggest that under such conditions, PSGL-1 would contribute to the immune homeostasis by maintaining the tolerogenic activity of the immature DCs. It is also conceivable that the engagement of PSGL-1 during the recirculation and extravasation of mature DCs may contribute to regulate their immunogenic activity, either in steady-state conditions or under inflammatory conditions, including allograft rejection (46). Importantly, our data provide a possible mechanism to explain the observations that P-selectin plays in vivo a protective role in the development of different experimental inflammatory conditions such as glomerulonephritis, intestinal inflammation, collagen-induced arthritis or chronic ulcerative dermatitis (47–51).

In summary, our data strongly suggest that, in addition to its key role in endothelial-leukocyte interactions, PSGL-1 may exert an interesting effect in the immune system, through the induction of tolerogenic DCs, which in turn trigger the differentiation of Treg cells that resemble natural Treg lymphocytes. Because it has been described that DCs expressing the PSGL-1 glycoform recognized by the M-DC8 mAb are highly immunogenic, our data also suggest that different forms of this receptor may exert distinct functional roles. We consider that our findings will contribute to further understand the physiological role of PSGL-1 in the immune system, and the complexity of the different stimuli for the induction of immune tolerance. In addition, our findings may have practical relevance because it has been proposed that PSGL-1 is a potential target for the therapy of autoimmune and inflammatory diseases (41, 52, 53).

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## Disclosures

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