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Broxmeyer

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P-Selectin Enhances Generation of CD14⁺CD16⁺ Dendritic-Like Cells and Inhibits Macrophage Maturation from Human Peripheral Blood Monocytes¹

Geling Li, Young-June Kim, Charlie Mantel, and Hal E. Broxmeyer²

Endothelial cells play a critical role in monocyte differentiation. Platelets also affect terminal maturation of monocytes *in vitro*. P-selectin is an important adhesion molecule expressed on both endothelial cells and activated platelets. We investigated its effects on human peripheral blood monocyte differentiation under the influence of different cytokines. Generation of dendritic-like cells (DLCs) from peripheral blood monocytes was promoted by immobilized P-selectin in the presence of M-CSF and IL-4 as judged by dendritic cell (DC) morphology; increased expression of CD1a, a DC marker; low phagocytic activity; and high alloreactivity to naive T cells. In contrast to typical DCs, DLCs expressed CD14 and Fc γ RIII (CD16). These features link the possible identity of DLCs to that of an uncommon CD14⁺CD16⁺CD64⁻ monocyte subset found to be expanded in a variety of pathological conditions. Functionally, DLCs generated by P-selectin in combination with M-CSF plus IL-4 primed naive allogeneic CD4⁺ T cells to produce significantly less IFN- γ than cells generated by BSA in the presence of M-CSF and IL-4. P-selectin effects on enhancing CD14⁺CD16⁺ DLC generation were completely abrogated by pretreatment of cells with the protein kinase C δ inhibitor rottlerin, but not by classical protein kinase C inhibitor Gö6976. Immobilized P-selectin also inhibited macrophage differentiation in response to M-CSF alone as demonstrated by morphology, phenotype, and phagocytosis analysis. The effects of P-selectin on macrophage differentiation were neutralized by pretreatment of monocytes with Ab against P-selectin glycoprotein ligand 1. These results suggest a novel role for P-selectin in regulating monocyte fate determination. *The Journal of Immunology*, 2003, 171: 669–677.

Terminal differentiation of monocytes *in vitro* and *in vivo* is regulated by their interaction with various cell types (1). In a model of transendothelial trafficking, human peripheral blood monocytes, after initial transmigration on endothelial monolayer, migrate in basal-to-apical directions to become dendritic cells (DCs),³ whereas those that remain in the subendothelium mature into macrophages (2, 3). *In vivo*, monocytes are the primary leukocytes to interact with activated platelets by forming aggregates with them (4, 5). Intact platelets strongly induce monocyte differentiation under serum-free conditions (1). Interaction of the adhesion molecule P-selectin and its primary ligand P-selectin glycoprotein ligand-1 (PSGL-1), a disulfide-bonded homodimeric mucine-like glycoprotein expressed on leukocytes (6), is involved in direct contact of circulating leukocytes with endothelium and activated platelets (7, 8). Whether P-selectin plays a role in monocyte differentiation beyond mediating passive adhesion is unclear. P-selectin belongs to the selectin family of cell adhesion molecules that includes two other members, E-selectin and L-selectin. They

are structurally related integral membrane glycoproteins that participate in mediating adhesion of leukocytes on endothelium (9). L-selectin is constitutively expressed on leukocytes (10). P-selectin is constitutively expressed at variable levels on resting endothelial cells and is rapidly up regulated on endothelium and platelets on inflammatory stimuli (7, 8). E-selectin expression is induced on endothelium by inflammatory activation (11). Even though the E-selectin gene is silent in *in vitro*-cultured endothelial cells, low levels of E-selectin are expressed in most tissues *in vivo* and regulate leukocyte homeostasis together with constitutively expressed P-selectin (8, 12). Mice lacking both P-selectin and E-selectin display severe leukocytosis and develop spontaneous skin infections (12). P-selectin negatively modulates myeloid cell differentiation (granulocyte-macrophage CFU) from hemopoietic stem cells (13). These observations suggest that selectins might also regulate cell growth beyond mediating rolling and adhesion of leukocytes.

Human peripheral monocytes constitute a heterogeneous population that can be divided into at least four subsets with overlapping, but distinct functions based on their size, density, and surface Ag characteristics (14). CD14⁺ monocytes, representing a large pool of circulating precursors, have the capacity to differentiate into macrophages (the scavengers of the host immune systems) (15, 16), DCs (the most potent APCs) (17–19) and osteoclast-like cells (20), depending on the cytokine environment. *In vitro*, CD14⁺ monocytes give rise to macrophages, DCs, and osteoclast-like cells in response to M-CSF (15), GM-CSF and IL-4 (18, 19), and M-CSF and IL-4 (20) respectively.

Another subset of monocytes with the CD14⁺CD16⁺CD64⁻ phenotype, representing <10% of the total monocyte population in normal peripheral blood, is increased up to 40% of the monocyte population in a variety of pathogenic conditions, including HIV infection, autoimmune disease, sepsis, and systemic vasculitis (14,

Department of Microbiology and Immunology, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202; and Walther Cancer Institute, Indianapolis, IN 46208

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^{2,2} Address correspondence and reprint requests to Dr. Hal E. Broxmeyer, Walther Oncology Center, Indiana University School of Medicine, Building R4, Room 302, 1044 West Walnut Street, Indianapolis, IN 46202-5254. E-mail address: hbroxmey@iupui.edu

³ Abbreviations used in this paper: DC, dendritic cell; DLC, dendritic-like cell; PKC, protein kinase C; DAG, diacylglycerol; PI, phagocytic index; PP, phagocytic percentage; PSGL-1, P-selectin glycoprotein ligand 1; rh, recombinant human.

21–24). Compared with common monocytes, this subset displays lower phagocytic activity, but higher alloreactivity, and has enhanced capacity to secrete TNF- α on stimulation (21, 25). This monocyte subset is predisposed to become migratory DCs in a model tissue setting (26). During HIV infection, increased circulating CD14⁺CD16⁺ monocytes are associated with high risk to develop HIV encephalopathy. This cell population might preferentially enter the brain and expose neural cells to toxic factors (27, 28). The exact functions and mechanisms of their expansion in pathogenic conditions are still unclear. Recently, it was demonstrated that incubation of CD16⁻ monocytes with TGF β 1 could generate phenotypically similar cells (26).

PKC consists of a group of cellular serine/threonine kinases that have been implicated in regulation of cell proliferation and differentiation. There are 11 identified closely related protein kinase C (PKC) isoforms that are encoded by different genes except for PKC β I and β II, which are the products of alternative splicing (29). These 11 PKC isoforms have been classified into 3 groups based on their activation requirement by Ca²⁺ and diacylglycerol (DAG) (29). The classical PKC- α , - β I, - β II, and - γ isoforms are activated by Ca²⁺ and DAG. The novel PKC- θ , - η , and - δ are DAG dependent but Ca²⁺ independent; the atypical PKC- ζ and - ι are both Ca²⁺ and DAG independent (30). PKC isoforms are distributed differently in tissues and organs, implying diverse functions of different isoforms. Among them, PKC- δ has been shown to be involved in myeloid differentiation (31, 32) and activated on M-CSF stimulation (33, 34). PKC- δ is also involved in β ₂ integrin-mediated cell adhesion (35). In addition, P-selectin-mediated adhesion of monocytes may change PKC- δ mRNA levels (36).

We investigated the role of P-selectin in regulating monocyte differentiation induced by different cytokines in an *in vitro* culture system. The studies reveal a novel dual role for P-selectin in enhancement of the generation of CD14⁺CD16⁺ dendritic-like cells (DLCs), but inhibition of macrophage differentiation from human peripheral blood monocytes, and imply possible involvement of PKC- δ in expansion of the CD14⁺CD16⁺ monocyte subset induced by P-selectin.

Materials and Methods

Monocyte isolation

PBMCs were isolated from fresh buffy coats (<6 h old) obtained from the Indiana Blood Center (Indianapolis, IN) by density gradient centrifugation on Ficollpaque^{Plus} (1.077g/ml; Pharmacia, Piscataway, NJ). Monocytes were purified from PBMCs using CD14⁺ magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. The purity of the bead-labeled CD14⁺ cells was >98% as analyzed by flow cytometry.

Cord blood T cell isolation

Heparinized umbilical cord blood was collected according to institutional guidelines. CD34⁺ cells were selected out from PBMC with the MACS CD34⁺ isolation kit (Miltenyi Biotec). T cells were then isolated with Lympho-Kwik Lymphocyte isolation kits (One Lambda, Canoga Park, CA) from CD34⁻ PBMC for MLR. CD4⁺ T cell were isolated by negative selection using the MACS CD4⁺ T cell isolation kit (Miltenyi Biotec).

P-selectin coating

Recombinant soluble P-selectin and E-selectin were purchased from R&D Systems (Minneapolis, MN). Flat-bottom culture plates (96 wells; Costar, Corning, NY) were coated with 10 μ g/ml P-selectin diluted in PBS⁺ (containing 1 mM Ca²⁺ and 1 mM Mg²⁺) for 6–8 h at 4°C or 1 h at room temperature. Control wells were coated with equal amounts of E-selectin or BSA (low endotoxin, IgG free, cell culture tested; Sigma-Aldrich, Saint Louis, MO) in PBS⁺. Wells were washed twice with PBS⁺ and blocked with 1% BSA in RPMI 1640 (BioWhittaker, Walkersville, MD) for 1 h at room temperature. Wells were washed three times with culture medium consisting of RPMI 1640, 10% FBS (HyClone, Logan, UT), 100 U/ml penicillin, and 100 μ g/ml streptomycin before deposit of purified monocytes into the wells.

Cell culture

Purified CD14⁺ monocytes were resuspended to a concentration of 5 \times 10⁵ cells/ml with appropriate cytokine combinations at the following concentrations: 1000 U/ml recombinant human (rh) M-CSF (Cetus/Chiron, Emeryville, CA) or 100 ng/ml rhM-CSF (Peprotech, Rock Hill, NJ), 200 U/ml rhGM-CSF (Immunex, Seattle, WA), 10 ng/ml rhIL-4 and 10 ng/ml rhTGF β 1 (Peprotech). Aliquots (100 μ l) of the cell suspensions were loaded into the coated wells, and plates were then centrifuged at 1200 rpm for 3 min to sediment cells before culture at 37°C. Cells were harvested by incubation with 250 μ M EDTA in PBS for 15 min on ice to detach adherent cells.

For blocking study, cells were preincubated with 100 μ g/ml anti-PSGL-1 Ab (clone CHO131; R&D Systems) for 30 min at 4°C and washed with culture medium before being deposited into the coated wells. For inhibition studies, cells were resuspended in culture medium with appropriate cytokines and 3 μ M rottlerin (Calbiochem, La Jolla, CA) or 6.2 nM Gö6976 (Calbiochem) before being deposited into each well.

Flow cytometry

Harvested cells were washed twice with PBS supplemented with 1% BSA. FcRs on the cells were preblocked with excess human IgG (Sigma-Aldrich) at room temperature for 15 min. Subsequently, cells were stained for 30 min at 4°C with the following FITC-conjugated Abs: anti-CD14, anti-CD80, anti-CD86, and anti-HLA-DR from Caltag (Burlingame, CA); anti-CD1a, anti-CD64, and anti-CD11b from eBioscience (San Diego, CA); anti-CD11b and anti-CD16 from BD PharMingen (San Diego, CA); and PE-conjugated Abs: anti-CD14 from BD PharMingen. Murine IgG1-FITC, IgG2a-FITC, IgG2a-PE, and rat IgG2b-FITC from Caltag or murine IgG1-FITC from BD PharMingen were used as isotype controls. After two washings, cells were analyzed for surface expression of Ags with a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA). Dead cells were gated out according to light scatter patterns.

MLR

Stimulator cells were irradiated at 1000 rad and cultured with 1 \times 10⁵ cord blood T cells/well in 96-well U-bottom cell culture plates (Costar) at E:T ratios ranging between 1:10 and 1:2500. Cells were pulsed with [*methyl*-³H]thymidine (1 μ Ci/well; Amersham, Piscataway, NJ) for 16 h before the end of the culture on day 6 and harvested onto a glass fiber filter. Incorporated thymidine was measured using a Microbeta scintillation counter (PerkinElmer Life Sciences, Turku, Finland).

Phagocytosis assay

SRBC (10⁹/ml; Biowhittaker) were opsonized with 92 μ g/ml rabbit anti-SRBC IgG (Accurate Chemical & Scientific, Westbury, NY) for 1 h at 37°C. After two washings with PBS, opsonized SRBC were incubated with cells differentiated from CD14⁺ monocytes at a 200:1 ratio at 37°C for 30 min. Nonphagocytosed SRBC were then lysed by short pulse with 0.25% PBS for 20 s. After two washings with PBS, cells were collected by cytospin and stained with Wright-Giemsa, modified (Sigma-Aldrich). Phagocytosis was measured under microscopy as phagocytic index (PI), the number of SRBC ingested per 100 cells; and phagocytic percentage (PP), the number of cells with internalized SRBC per 100 cells. At least 300 cells were counted in each experiment.

T cell differentiation assay

Allogeneic CD4⁺ T cells (1 \times 10⁵/well) were cocultured with cells (1 \times 10⁴/well) derived from monocytes in the presence of M-CSF and IL-4 on BSA-coated plates and P-selectin-coated plates as described above in 96-well U-bottom cell culture plates (Costar) for 6 days in culture medium. T cells were washed and stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 1 μ g/ml ionomycin (Sigma-Aldrich) in the presence of 10 μ g/ml brefeldin A (Sigma-Aldrich) for 5 h. Cells then were fixed and permeabilized with a Cytotfix/Cytoperm Kit (BD PharMingen) and incubated with FITC-labeled anti-IFN- γ (clone 25723.11) and PE-labeled anti-IL-4 (clone 3010.211) Abs (BD Immunocytometry Systems, San Jose, CA). Samples were then analyzed with a Calibur flow cytometer and CellQuest software.

BrdU cell proliferation assay

Cell proliferation was measured by detecting incorporation of BrdU with a BrdU flow kit (BD PharMingen) according to manufacturer's instructions. In brief, at the end of the 3-day cell culture of monocytes, a final concentration of 10 μ M BrdU solution diluted in culture medium was added into the cell culture medium. Treated cells were then incubated at 37°C for 16 h,

cells were harvested, and incorporated BrdU was detected with a FITC-conjugated anti-BrdU Ab. Total cellular DNA was stained with 7-amino actinomycin D. Cell proliferation was analyzed by flow cytometry.

Statistical analysis

The paired Student *t* test (two-tailed) was used to determine statistical significance. Values of $p < 0.05$ were considered significant.

Results

Enhancement of $CD14^+CD16^+CD1a^+$ dendritic-like cells generation by P-selectin

M-CSF expands the $CD14^+CD16^+$ monocyte subset in vivo (37), and IL-4 down-regulates monocyte phagocytic activity and increases MHC-II expression (38, 39). The effects of the combination of M-CSF and IL-4 on monocyte differentiation in short term cultures (less than one week) have not been reported although it has been shown that osteoclast-like multinucleated giant cells can be generated from monocytes in response to M-CSF and IL-4 at 2 wk of culture (20). We cultured freshly isolated monocytes with M-CSF and IL-4 in the presence of BSA for 3 and 6 days and generated a mixed cell population composed of two types of cells with distinct morphology. The majority of cells had a macrophage-like morphology (large cells, strongly adherent with irregular or spindle-like shape); the rest had a DLC morphology (less adherent and veiled with small dendrites) (Fig. 1A, left panel). On LPS stimulation, M-CSF- and IL-4- cultured monocytes acquired a typical DC phenotype ($CD83^+$; high expression level of HLA-DR and costimulatory molecules and high alloreactivity; G. L. Li and H. E. Broxmeyer, manuscript in preparation).

We then examined the effects of immobilized P-selectin on this in vitro differentiation process. To exclude a possible indirect effect of P-selectin on monocyte differentiation solely due to the enhanced adhesion of cells to the plates, E-selectin known to induce adhesion of monocytes (40, 41) was used as a control, in addition to BSA. Interestingly, cells with dendritic-like morphology were increased to $>90\%$ when monocytes were cultured on P-selectin- but not on BSA- or E-selectin-coated plates in the presence of M-CSF plus IL-4 (Fig. 1). Correspondingly, cells with macrophage-like morphology greatly diminished on P-selectin-

coated plates. The appearance of dendritic-like morphology began after 3 days of cell culture and persisted to 6 days of cell culture. To compare P-selectin-induced DLCs with DCs and macrophages that were developed from monocytes in vitro in a standard culture period, we cultured DLCs for 6 days. To clarify whether P-selectin drove cell morphology changes by inducing proliferation of one subset of monocytes, we did BrdU incorporation assay at day 3 of cell culture. No BrdU incorporation was detected regardless of whether cells were cultured with M-CSF plus IL-4 in the presence of BSA, P-selectin, or E-selectin.

The significant morphological difference led us to evaluate phenotypic differences among cells cultured on BSA, P- or E-selectin in the presence of M-CSF and IL-4. We focused on a series of surface molecules involved in DC and macrophage development and function including lineage markers, CD14, CD16, CD1a, and CD64, as well as on markers involved in Ag presentation, CD80, CD86, HLA-DR, and also on the adhesion molecule CD11b. In preliminary studies, we found that IL-4 at 10 ng/ml was optimal for $CD14^+CD16^+$ DLC generation. Increasing IL-4 concentration was accompanied by decreased generation of DLCs with a CD14 and CD16 double-positive phenotype. This observation was consistent with previous reports that IL-4 decreased the expression level of CD14 and CD16 on monocytes (42). The majority of DLCs generated by P-selectin with M-CSF and IL-4 (10 ng/ml) were CD14 and CD16 double-positive cells (Fig. 2A). In comparison, significantly fewer $CD14^+CD16^+$ cells were observed on E-selectin-coated plates (Fig. 2A). DLCs driven by P-selectin in the presence of M-CSF and IL-4 (Fig. 2B) had significantly higher levels of CD1a and CD80 but significantly decreased levels of CD86 and CD11b than cells cultured on BSA and E-selectin. All the cells were $CD64^{dim}$ or $CD64^-$. To our knowledge, this is the first report that demonstrates that CD1a expression can be induced in M-CSF-containing cell cultures and that costimulatory molecules CD80 and CD86 are so significantly and differently regulated in a cell type. To exclude the possibility that endotoxin contamination caused morphological and phenotypic changes induced by P-selectin, we tested effects of LPS from *Salmonella minnesota* (0.001–100 ng/ml, serial 1/10 dilutions; Sigma-Aldrich) on

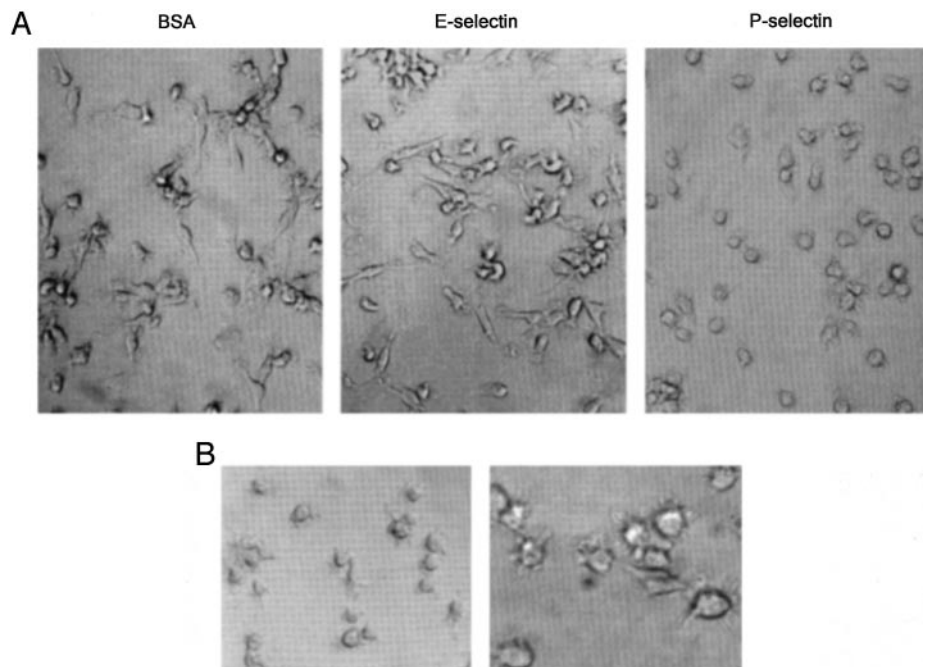


FIGURE 1. P-selectin induced dendritic-like morphological changes of monocytes cultured with M-CSF and IL-4. **A**, Phase contrast microscopy of cells derived from monocytes cultured with M-CSF and IL-4 for 6 days on BSA-, E-selectin-, or P-selectin-coated plates at $\times 400$ magnification. **B**, Numerous dendrites of DLCs derived from monocytes with P-selectin in the presence of M-CSF and IL-4 for 6 days at $\times 400$ (left) and $\times 800$ (right) magnifications. These results are representative of at least eight independent experiments.

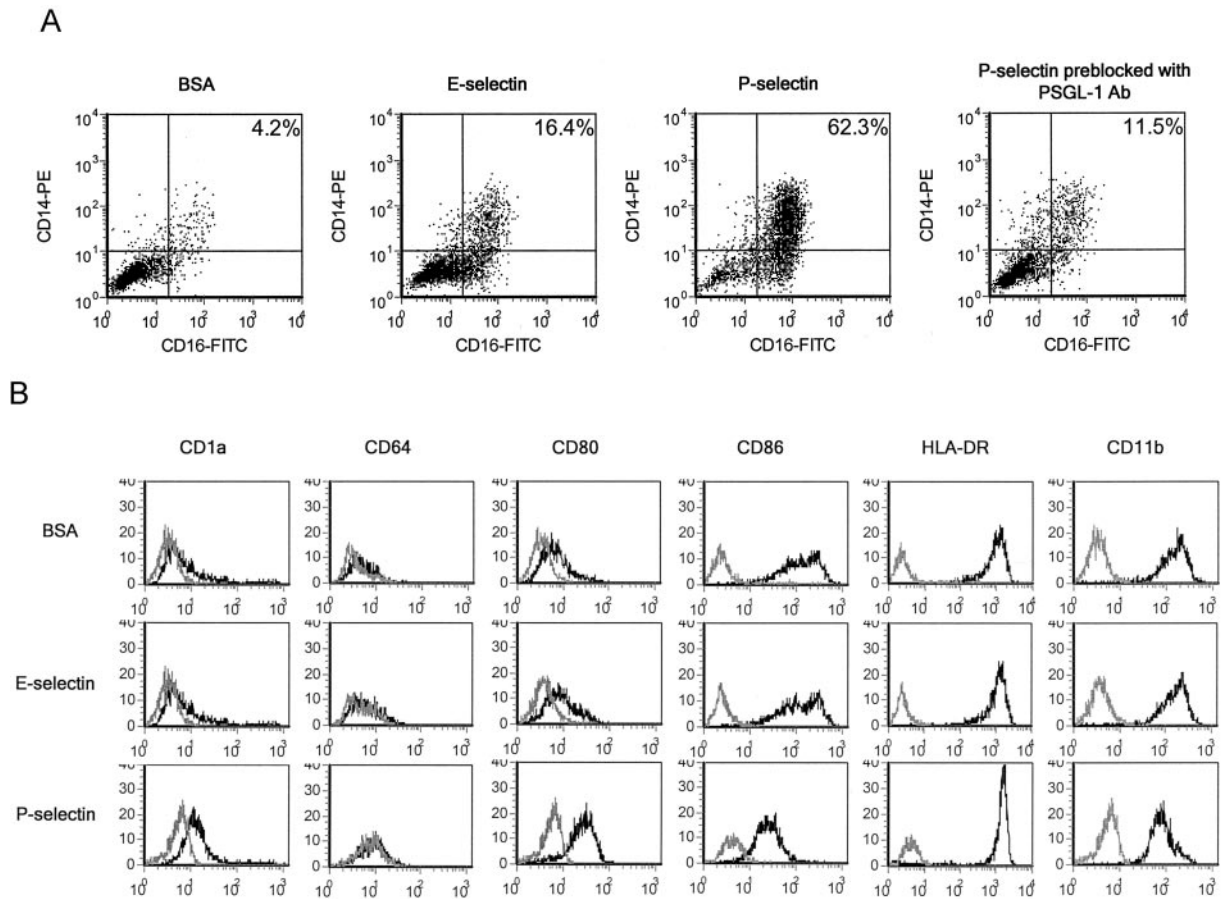


FIGURE 2. Effects of P-selectin on cell surface phenotypic changes of monocytes cultured with M-CSF and IL-4. **A**, P-selectin-induced DLCs derived from monocytes in the presence of M-CSF and IL-4 for 6 days had significantly increased population of CD14⁺CD16⁺ cells ($n = 8$ independent experiments); this was neutralized by Abs against PSGL-1 ($n = 3$ independent experiments). Monocytes harvested from BSA, E-selectin-, or P-selectin-coated plates were double labeled with anti-CD14-PE and anti-CD16-FITC. For anti-PSGL-1 Ab blocking study, monocytes were preincubated with anti-PSGL-1 at 4°C for 30 min and then washed before being deposited on P-selectin-coated plates. **B**, Phenotypic comparison of cells generated on BSA, P-selectin, and E-selectin by single staining with mAbs (black lines) indicated in the figure. Surface staining with isotype-matched control Abs are shown by gray lines. Data are representative of four independent experiments.

CD14⁺CD16⁺ DLC generation in our culture environment ($n = 2$). In contrast to P-selectin, LPS promoted macrophage-like cell generation and failed to increase either CD14 or CD16 expression level. The specificity of the effects of P-selectin was further demonstrated by an Ab blocking study. Pretreatment of monocytes with Abs against the primary ligand for P-selectin, PSGL-1, significantly blocked the effects of P-selectin on CD14⁺CD16⁺ cell generation by >80% (Fig. 2A). In contrast, pretreatment of monocytes with isotype control Ab failed to block the effects of P-selectin (data not shown).

The CD14⁺ and CD16⁺ phenotype and high HLA-DR expression link the possible identity of such DLCs to a monocyte subset with a CD14⁺CD16⁺ phenotype, which is expanded markedly in various pathological conditions including HIV infection, autoimmune disease, sepsis, and systemic vasculitis (14, 21–24). Compared with other monocyte subsets, CD14⁺CD16⁺ monocytes are characterized by their higher alloreactivity (high MHC-II expression) and lower phagocytic capability (14). The dendritic morphology of these DLCs might facilitate them to contact T cells just like DCs, the numerous dendrites of which may be of great advantage in interactions with T cells (43). Therefore, we compared DLCs for their capability to stimulate alloreactive cord blood naive T cells to macrophages and DCs derived in vitro from monocytes (Fig. 3). The DLCs were much more efficient ($p < 0.001$) than macro-

phages in stimulating cord blood naive T cells in MLR. In addition, they induced significantly higher MLR ($p = 0.014$) at the highest E:T tested (1:10) and comparable MLR at lower E:T ratios (1:50–1:1250) compared with in vitro generated DCs.

We also evaluated the phagocytic activity of these DLCs. CD14⁺ monocytes cultured with M-CSF and IL-4 on P-selectin-coated plates and macrophages generated from monocytes with M-CSF only were incubated with opsonized SRBC for 30 min, and phagocytic ability was determined. The DLCs derived from monocytes in the presence of P-selectin had significantly lower ability to engulf opsonized SRBCs than macrophages ($p < 0.05$; Fig. 4). Therefore, P-selectin-induced DLCs in the presence of M-CSF and IL-4 are similar to the CD14⁺CD16⁺ monocyte subset in both phenotype and function. Because P-selectin in the presence of M-CSF and IL-4 enhanced DLC generation, we were interested in whether P-selectin could activate and induce maturation of DCs. Therefore, we generated DCs from monocytes cultured with GM-CSF and IL-4 and exposed the immature DCs to P-selectin- or BSA-coated plates. Exposure to P-selectin failed to drive maturation of immature DCs based on morphology and phenotypic staining of CD83 and HLA-DR (data not shown).

Because M-CSF- and IL-4-cultured monocytes acquired a DC phenotype upon LPS stimulation and P-selectin significantly changed the phenotype of the M-CSF- and IL-4- generated potent

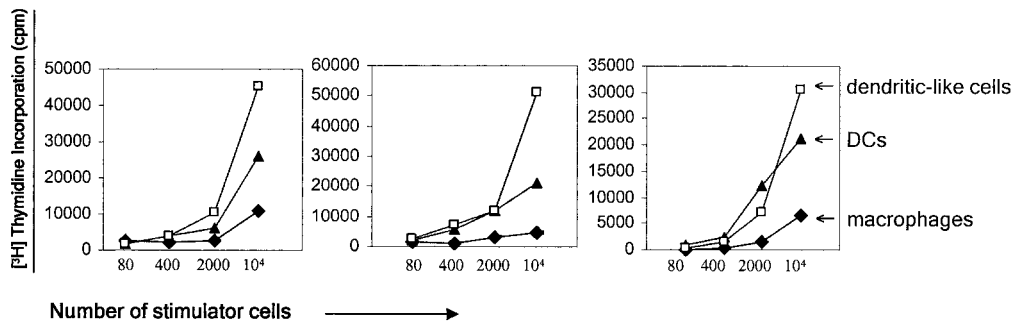


FIGURE 3. Comparison of MLR of DLCs, macrophages, and DCs. Monocytes cultured for 6 days with P-selectin, M-CSF, and IL-4 (DLCs; □), M-CSF (macrophages; ◆), or GM-CSF and IL-4 (DCs; ▲) were used as stimulators for allogeneic cord blood T cells. Increasing amounts of irradiated stimulator cells were cocultured with cord blood T cells (1×10^5) for 5 days, and uptake of [3 H]thymidine for the last 16 h was assessed. Values are the mean \pm SD obtained from triplicate wells. The DLCs show significantly higher MLR than in vitro-generated DCs when 10^4 stimulator cells (E:T 1:10) were used ($p = 0.014$). The DLCs are much more potent than macrophages in MLR ($p < 0.001$) at all the E:T ratios tested. Data are three representatives of five independent experiments using monocytes from five donors.

APCs, it was of interest to learn whether the APCs generated with M-CSF and IL-4 in the presence of P-selectin or control BSA differed in their capacity to support Th cell differentiation. Naive CD4⁺ T cells from cord blood were cocultured with cells derived from monocytes cultured with M-CSF and IL-4 on BSA- and P-selectin-coated plates for 6 days, and the T cells were subsequently activated with PMA and ionomycin to analyze IFN- γ and IL-4 production. As shown in Fig. 5, IL-4 was barely detected in T cells cocultured with both cell types. Interesting, T cells cultured in the presence of DLCs produced significantly less IFN- γ than control cells harvested from BSA-coated plates (Fig. 5).

Because M-CSF expands the CD14⁺CD16⁺ monocyte subset in vivo (37) and PKC- δ is an important signaling molecule of M-CSF (33, 34), we hypothesized that PKC- δ might be an important intracellular molecule for expansion of CD14⁺CD16⁺ cells. We took advantage of the PKC- δ inhibitor rottlerin to test whether P-selectin up-regulated CD14⁺CD16⁺ expression through activa-

tion of PKC- δ . We pretreated monocytes with the PKC- δ inhibitor, rottlerin, at 3 μ M (IC_{50} 3–6 μ M) or a classical PKC inhibitor, Gö6976, at 6.2 nM (IC_{50} 2.3 nM for PKC- α ; 6.2 nM for PKC- β) before culturing them with M-CSF and IL-4 on P-selectin-coated plates. As a control, another group of cells were pretreated with equal amount of DMSO before being deposited on BSA- and P-selectin-coated plates. Treatment of cells with rottlerin, Gö6976, and DMSO did not affect cell viability. In the presence of 3 μ M rottlerin, but not in the presence of 6.2 nM Gö6976, the effects of P-selectin on enhancing CD14⁺CD16⁺ cell generation were markedly diminished to background levels comparable with those of cells on BSA-coated plates (Fig. 6A).

TGF β 1 has been previously shown to promote generation of CD16⁺ monocytes (26, 44) and PKC- δ has been involved in TGF β 1 signaling in primary astrocytes (45) and fibroblasts (46). Therefore, we further examined whether the PKC- δ inhibitor rottlerin also inhibited the generation of CD16⁺ monocytes induced by TGF β 1 alone. As expected, rottlerin at 3 μ M also significantly blocked the generation of CD16⁺ monocytes (Fig. 6B). In contrast, 6.2 nM Gö6976 did not block TGF β 1-induced CD16⁺ expression (data not shown). These results suggest that PKC- δ might be one intracellular mediator of the expansion of CD14⁺CD16⁺ monocytes.

Inhibition of macrophage differentiation by P-selectin

Because P-selectin specifically enhanced DLC generation in the presence of M-CSF and IL-4, we also evaluated whether P-selectin played

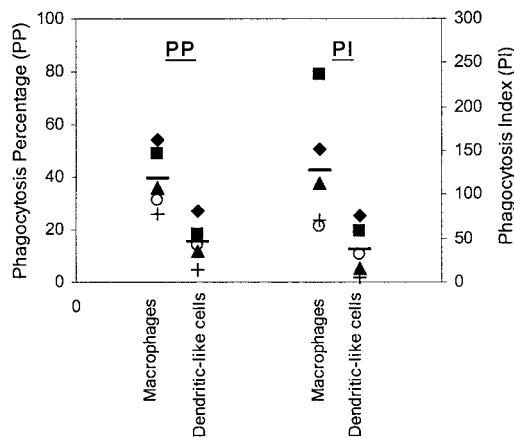


FIGURE 4. Comparison of phagocytic activity of DLCs and macrophages. DLCs derived from monocytes by P-selectin in M-CSF and IL-4 had significantly lower phagocytic activity than macrophages derived from monocytes cultured with M-CSF and BSA for 7 days ($p < 0.05$). DLCs and macrophages were incubated with SRBC for 30 min at 37°C. PP and PI values were examined under the microscope. ■, ◆, ▲, +, and ○ represent the results from five different individuals. Statistical analysis by paired t test (two-tailed) indicates that DLCs harvested from P-selectin-coated plates have lower PI and PP values ($p < 0.05$ vs macrophages). The PP and PI scales are respectively depicted on the left and right ordinates. The average values of five independent experiments are expressed as a horizontal line.

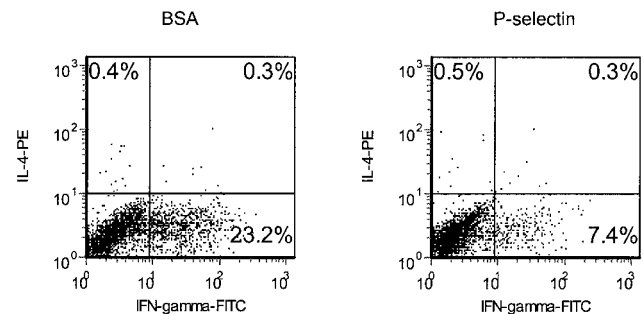


FIGURE 5. Two-color immunofluorescence intracellular analysis of IL-4 and IFN- γ production by naive cord blood CD4⁺ T lymphocytes primed with allogeneic cells derived from monocytes by P-selectin and BSA in the presence of M-CSF and IL-4. CD4⁺ T cells were cocultured with allogeneic cells at a 10:1 ratio for 6 days followed by a 5-h restimulation with PMA and ionomycin. Results are one representative of three experiments.

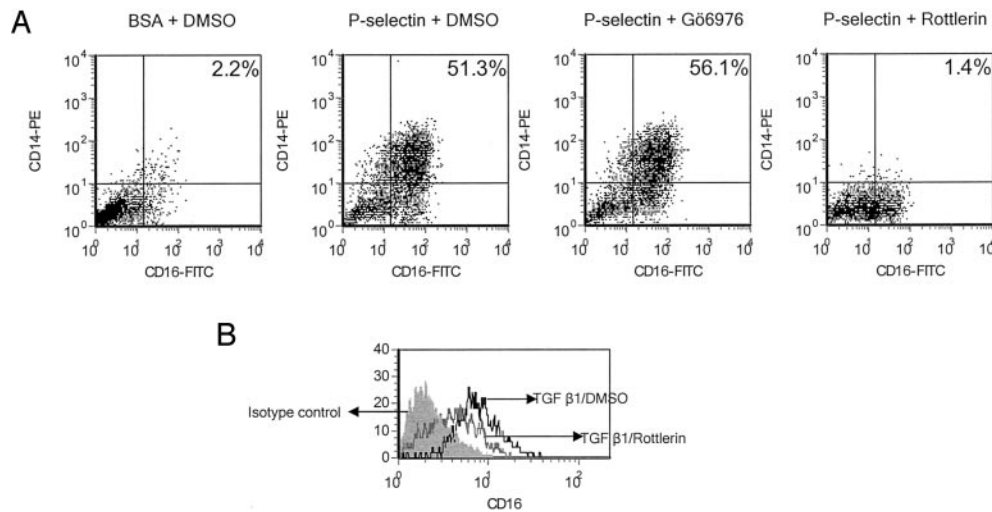


FIGURE 6. Effects of PKC- δ inhibitor rottlerin on CD14⁺CD16⁺ cell generation. *A*, 3 μ M rottlerin (IC₅₀ 3–6 μ M for PKC- δ) completely inhibited CD14⁺CD16⁺ DLC generation induced by P-selectin in combination of M-CSF and IL-4. In contrast, 6.2 nM Gö6976 (IC₅₀ 2.3 nM and 6.2 nM for PKC- α and TGF β 1, respectively) did not. Freshly isolated monocytes were preincubated with 3 μ M rottlerin, 6.2 nM Gö6976, or DMSO as control for 15 min on ice before deposit into each well indicated in the figure. Cells were double stained for CD14 and CD16 at day 4 of cell culture. Data are representative of three independent experiments. *B*, Rottlerin at 3 μ M partially but significantly blocked TGF β 1-induced CD16⁺ monocyte generation. Monocytes cultured with 10 ng/ml TGF β 1 for 48 h in the presence of rottlerin (dark line) or equal amount of DMSO (gray line) were stained with CD16 Abs. Data are representative of three independent experiments.

a regulatory role in monocyte-to-macrophage differentiation induced only by M-CSF. After 6-day culture with M-CSF in the presence or absence of BSA, monocytes acquired the typical morphology of previously described M-CSF-induced macrophages (15). Most of the cells became elongated and strongly adhered to plastic with a spindle-like morphology (Fig. 7*A*, left panel). Monocytes cultured on P-selectin-coated plates became less adherent than cells grown on BSA after 3 days of cell culture. Most of cells remained round, and spindle-like macrophages were sporadically detected (Fig. 7*A*, middle panel). Immobilized P-selectin alone (10 μ g/ml) had no effect on monocyte survival and differentiation (data not shown).

Because strong phagocytic capability is a special feature of mature macrophages, we compared the phagocytic ability of M-CSF-driven macrophages developed on BSA or P-selectin-coated plates. Consistent with changes in morphology, macrophages recovered from P-selectin-coated plates had significantly less phagocytic activity (lower PI and PP values; $p < 0.0001$) than those recovered from BSA-coated plates (Fig. 7*B*).

We further compared the expression levels of the macrophage maturation marker, CD11b, on M-CSF-induced macrophages recovered from BSA- and P-selectin-coated plates. Cells recovered from P-selectin-coated plates had higher autofluorescence and isotype staining than those harvested from BSA-coated plates (Fig. 7, *C* and *D*). P-selectin-treated macrophages had significantly lower levels of CD11b than BSA-treated macrophages (Fig. 7, *C* and *D*). Combined with the morphological and phagocytosis assays, this indicates that P-selectin down-regulated M-CSF-induced monocyte differentiation into macrophages.

To further confirm that the inhibition of macrophage maturation is through immobilized P-selectin, fresh monocytes were preblocked with Abs against PSGL-1 (100 μ g/ml) for 30 min on ice and then washed before being loaded on P-selectin-coated wells. Pretreatment with anti-PSGL-1 Abs neutralized the effects of P-selectin on inhibition of macrophage maturation (Fig. 7*A*, right panel). Our prior experiments had shown that preblockage with isotype control Ab failed to blocked effects of P-selectin. Monocytes preblocked with anti-PSGL-1 manifested typical spindle-like morphology (Fig. 7*A*, right panel) and high expression of maturation marker CD11b (Fig. 7*D*)

at a level comparable with that of BSA, even when cultured on P-selectin-coated plates.

Discussion

Monocytes represent a heterogeneous population and have the potential to further differentiate. Based on evidence for roles of endothelial cells and platelets in the monocyte differentiation process (1–3), the present study was undertaken to investigate effects of P-selectin, an important adhesion molecule expressed on endothelium and activated platelets, on differentiation of monocytes into CD14⁺CD16⁺ DLCs and macrophages. Our studies noted the following effects of P-selectin in combination with M-CSF and IL-4 on differentiating monocytes into DLCs with a CD14⁺CD16⁺CD64⁻CD1a⁺ phenotype: typical dendrites; expression of HLA-DR, CD80, and CD86; low phagocytic activity; and high alloreactivity to cord blood naive T cells. However, DLCs (CD14⁺CD16⁺CD64⁻) are distinct from typical DCs derived from monocytes cultured with GM-CSF plus IL-4, because they express two macrophage markers, CD14 and CD16. Therefore, these CD14⁺CD16⁺ DLCs might be a cell population at an intermediate stage of differentiation between DCs and macrophages. In support of this possibility of CD14⁺CD16⁺ representing an intermediate stage, it was recently reported that the combination of GM-CSF, IL-4, and IL-10 led to the generation of a CD14⁺CD16⁺⁺ monocyte subset with DC features (CD1a⁺, high MLR) (47). Our induction conditions were quite different compared with theirs with the exception that both included IL-4. Because in vivo administration of M-CSF in primates and humans leads to the expansion of CD16⁺ monocytes (37), our data suggest that P-selectin expressed as a membrane-bound form in synergy with cytokines M-CSF and IL-4, which might mimic an in vivo scenario, could possibly play an important role in expansion of CD14⁺CD16⁺ monocytes. It was noted that E-selectin also had small effects on CD14⁺CD16⁺ cell generation. However, the differences noted between P- and E-selectin were substantial at a morphological level and with all the cell surface markers analyzed. These differences may result from P- and E-selectin binding to different sites and to the structure of PSGL-1 that undergoes

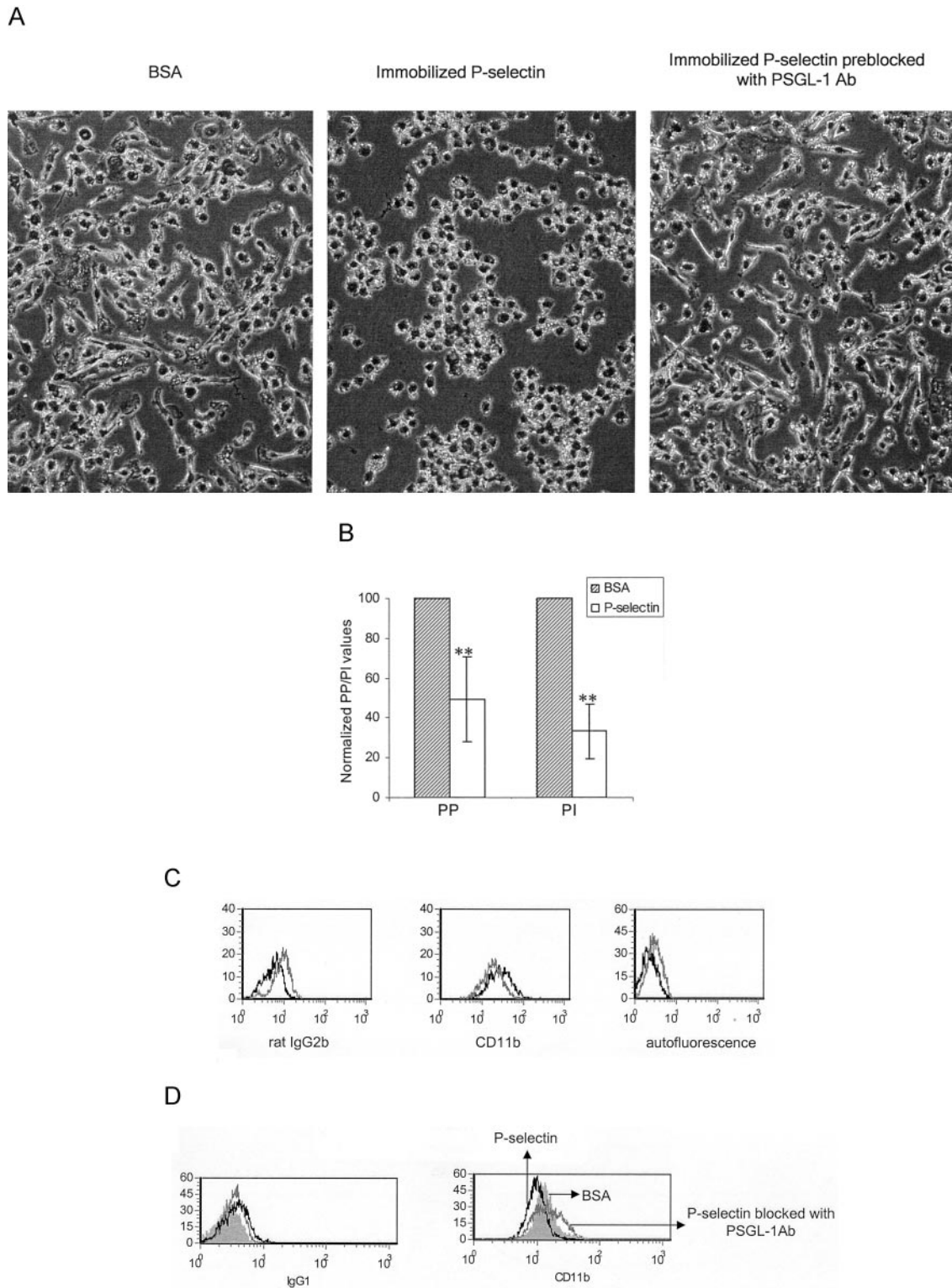


FIGURE 7. P-selectin inhibited macrophage maturation induced by M-CSF only. *A*, P-selectin inhibited typical macrophage-like morphological changes of monocytes cultured with M-CSF. *Left panel*, Phase contrast microscopy of typical macrophages derived from monocytes cultured with M-CSF for 7 days on BSA-coated plates. *Middle panel*, Typical macrophage-like cells are greatly decreased on P-selectin-coated plates ($n = 8$). *Right panel*, Anti-PSGL-1 Abs blocked the inhibitory effects of immobilized P-selectin on macrophage-like morphological changes ($n = 3$). Original magnification, $\times 400$. *B*, Comparison of phagocytic activity of monocytes cultured with M-CSF on BSA and P-selectin-coated plates. PP and PI values of P-selectin treated cells are normalized to BSA values set at 100. **, Statistically significant ($p < 0.0001$) compared with BSA-treated cells. Data are representative of five independent experiments. *C*, P-selectin decreased macrophage maturation marker CD11b (clone M1/70, rat IgG2b) expression on M-CSF-induced macrophages. Monocytes cultured with M-CSF were harvested from BSA and P-selectin-coated plates on day 7 and stained with rat IgG2b and CD11b or stained with no Abs to detect autofluorescence. Data are representative of three independent experiments. The black and gray lines represent cells treated with BSA and P-selectin, respectively. *D*, P-selectin decreased CD11b (clone ICRF44, mouse IgG1) expression, which was neutralized by PSGL-1 Abs. Equal numbers of monocytes were preblocked with anti-PSGL-1 Ab and washed with culture medium before being deposited on P-selectin-coated plates. The silver-filled histogram represents cells cultured on BSA, the black lines represent cells cultured on P-selectin, and the gray line represents cells cultured on P-selectin preblocked with anti-PSGL-1 Ab. Data are representative of two independent experiments.

various posttranslational modifications and their different affinity and kinetics of interaction with monocytes (7, 9, 48–50). Moreover, E-selectin may bind to other ligands expressed on monocytes in addition to PSGL-1 (48). Therefore, P-selectin appears to be a dominant selectin for CD14⁺CD16⁺ cell generation. Besides the combination of IL-10, GM-CSF, and IL-4, TGFβ1 alone has been reported to promote CD16⁺ monocyte generation (26, 44). However, we found that TGFβ1-generated CD16⁺ monocytes expressed little CCR5 (data not shown), whereas high CCR5 expression is one of the characteristics of circulating CD14⁺CD16⁺ monocytes (51). Thus, even though TGFβ1 can expand monocytes with a CD16⁺ phenotype, TGFβ1 alone might not be sufficient to fully recapitulate the onset of the CD16⁺ expansion in vivo. Our findings that P-selectin in combination with M-CSF and IL-4 enhances generation of CD16⁺ monocytes with high CCR5 expression (our unpublished data) may have relevance to immunological disorders found in various pathological conditions based on previous reports of substantially increased levels of phenotypically similar cells in HIV infection, autoimmune disease, sepsis, and systemic vasculitis (14, 21–24).

Considering that the frequency of CD14⁺CD16⁺ monocytes in the blood varies substantially in association with various disease states, it is important to identify intracellular mechanisms that may modulate their expansion, given that this may allow the identification of new methods to manipulate CD14⁺CD16⁺ monocyte generation. PKC-δ is involved in signaling of M-CSF (33, 34), which is an important growth factor for CD14⁺CD16⁺ monocyte expansion in vivo (37). Therefore, we hypothesized that PKC-δ might be essential for M-CSF-induced CD14⁺CD16⁺ monocyte generation. Our hypothesis was tested with the PKC-δ inhibitor, rottlerin. Here, we showed that the PKC-δ inhibitor, rottlerin, but not the classical PKC inhibitor, Gö6976, specifically and completely abrogated CD14⁺CD16⁺ monocyte generation induced by P-selectin. Furthermore, TGFβ1-induced CD16⁺ generation was also partially blocked by 3 μM rottlerin with an IC₅₀ of 3–6 μM. Therefore, PKC-δ might be one of the intracellular molecules involved in regulation of CD16⁺ monocyte generation, and its inhibitor rottlerin or other inhibitors of PKC-δ could potentially have therapeutic value for treatment of various diseases associated with CD16⁺ monocyte expansion. This possibility remains to be evaluated.

A biological consequence of our finding was further demonstrated by the reduced capability of DLCs generated by P-selectin in combination with M-CSF and IL-4 to drive Th cells to secrete Th1-type cytokine IFN-γ compared with cells generated by BSA. Whether this effect is due to a different expression of CD80 and CD86 costimulatory molecules or to a different capability of these two cell types to secrete cytokines requires further investigation. Published data on roles of CD80 and CD86 on Th cell differentiation are conflicting (52–54). However, DLCs can be a suitable and physiological generated cell type to further study whether CD80 and CD86 play a distinct or overlapping role on T cell activation and differentiation. Our unpublished data also suggest that M-CSF- and IL-4-generated cells are potent APCs. Therefore, P-selectin, instead of merely existing as an adhesion molecule, might actively regulate host immunological responses by manipulating functions of APCs generated by M-CSF and IL-4.

In contrast to effects of P-selectin on the enhancement of generation of DLCs, P-selectin strongly blocked M-CSF-driven differentiation of CD14⁺ monocytes into macrophages with typical macrophage-like morphology, phenotype and high phagocytic capability. This inhibitory effect of immobilized P-selectin was blocked by pretreatment of fresh isolated monocytes with Abs against P-selectin ligand PSGL-1, which is highly expressed on monocytes, demonstrating the activity of P-selectin.

The dual effects of P-selectin described by us might have relevance to HIV pathology. AIDS dementia and opportunistic infections are major complications in HIV infection. Due to greatly decreased CD4⁺ T cell numbers during the progression of AIDS (28), macrophages are major cellular sources for anti-infection in the host immune system. Considerably increased circulating CD14⁺CD16⁺ monocytes in HIV infection, especially in AIDS-related dementia, may play a role in transmitting HIV and in the neurodegenerative pathology associated with HIV by crossing the blood-brain barrier, thus releasing neurotoxic factors (27, 28). If the DLCs in culture that we have noted are identical with the circulating monocyte subsets found in patients with HIV, then P-selectin might play a dual role in promoting HIV pathology by enhancing generation of DLCs, thus inducing AIDS dementia, and by inhibiting macrophage maturation, which may endanger their capability to kill various opportunistic pathogens. Further studies are required to determine whether the CD14⁺CD16⁺ monocyte subset increased in HIV-infected peripheral blood and the in vitro-generated DLCs are identical cell populations. It would be of interest to examine whether P-selectin levels are increased in HIV infection and whether they are related to the severity of HIV infection.

It is not entirely clear how much time is required for P-selectin stimulation to alter the monocyte differentiation pathway. P-selectin-induced morphological changes have been detected at day 3 of cell culture (data not shown). Monocyte interactions with endothelium may persist for only a few minutes in physiological conditions in vivo. However, in atherosclerosis, monocytes may encounter P-selectin for a relatively long period of time, especially after extravasation, because P-selectin is also likely to be expressed on subluminal surface of inflammatory endothelium (55, 56). In the circulating blood of patients with unstable atherosclerosis (57–60), stable coronary disease (4) and hypercholesterolemia (61, 62), where activated platelets were found to be present, interaction of P-selectin with monocytes may last long enough to alter monocyte differentiation fate.

In summary, we have demonstrated a novel role for P-selectin in the generation of macrophages and CD14⁺CD16⁺ monocytes and have suggested the possible involvement of PKC-δ in the expansion of CD14⁺CD16⁺ monocytes, which could possibly have clinical implications for diseases with increased numbers of CD14⁺CD16⁺ monocytes.

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