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Phosphatidic Acid Induces the Differentiation of Human Acute Promyelocytic Leukemic Cells Into Dendritic Cell-Like

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Abstract We investigated whether phosphatidic acid (PA) can differentiate the promyelocytic leukemia (PML)-retinoic acid receptor α (RARα)-expressing acute promyelocytic leukemic cell line, NB4, to dendritic cell (DC)-like cells. Dioctanoyl-PA alone upregulated the expression of DC markers. The expression of DC markers on NB4 cells was potentiated by the overexpression of phospholipase D and upregulation was blocked by the addition of *n*-butanol, an inhibitor of PA production. The expression of CD11c, CD83, and CCR7 in PA-treated NB4 cells was further increased by tumor necrosis factor (TNF)-α treatment. Increased functional capacities were also found in PA-differentiated and TNF-α-activated NB4 cells with respect to changes in T-cell proliferation, cytokine production, endocytic activity, and cytolytic capacity against undifferentiated NB4 cells. PA alone increased the phosphorylation of extracellular signal-regulated kinase (ERK)-1/2. The expression of DC markers was downregulated by PD98059, a specific inhibitor of ERK kinase or transient transfection of mutant-ERK. The level of PML-RARα fusion protein was decreased by PA treatment and PD98059 blocked the decrease of PML-RARα. These results suggest that PA induces differentiation of NB4 cells into DC-like cells and that the upregulation of antigen presenting cell markers is mediated by the activation of ERK and the downregulation of PML-RARα levels. J. Cell. Biochem. 100: 191–203, 2007.

Key words: NB4; promyelocytic leukemia; phosphatidic acid; dendritic cell; phospholipase D

Human acute promyelocytic leukemia (APL) accounts for 10% of all acute myeloid leukemia. The retinoic acid receptor α (RARα) gene fuses to the promyelocytic leukemia (PML) gene to yield the fusion protein PML-RARα [de The et al., 1990]. A human APL cell line, NB4 cells, express the characteristic chromosomal translocation (15;17) [Lanotte et al., 1991]. NB4 cells undergo differentiation via the granulocytic pathway when exposed to all-*trans* retinoic acid (ATRA) [Lanotte et al., 1991].

Dendritic cells (DCs) are potent antigen-presenting cells and play major roles in the regulation of immune responses to various antigens [Banchereau et al., 2000]. DCs can develop from myeloid progenitors in the bone marrow or from circulating blood monocytes [Ardavin et al., 2001]. Functional DCs can also be produced from blasts in patients affected by acute myeloid leukemia, and it has been shown that at least 10% of leukemia cells from APL patients were differentiated to DCs in the presence of granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-4, and tumor necrosis factor (TNF)-α [Choudhury et al., 1999; Cignetti et al., 1999; Rigolin et al., 2001]. Furthermore, the T-cell

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stimulatory properties of leukemic cells were also found to be upregulated by the CD40 ligand [Schultze et al., 1995].

Phosphatidic acid (PA) is a lipid second messenger that participates in various intracellular signaling events and phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine into PA and choline [Jones et al., 1999; Liscovitch et al., 2000]. PA can be further metabolized to diacylglycerol by PA phosphohydrolase or lyso-PA by phospholipase A_2, and PA can also be generated through non-PLD pathways, for example, diacylglycerol phosphorylation by diacylglycerol kinase or by the acylation of glycerol 3-phosphate. Moreover, PLD-generated PA can activate a number of physiological events, such as secretion, neutrophil superoxide generation, and cytoskeletal reorganization [Jones et al., 1999; Liscovitch et al., 2000]. Exogenous PA added to cell culture media incorporates rapidly into cellular membranes and subsequently participates in cellular functions [Fukami and Takenawa, 1992; Reeves et al., 2000]. It has been reported that PLD activity increases with the maturation and differentiation of myeloid cells into granulocytic or monocytic lineages [El Marjou et al., 2000].

The mitogen-activated protein kinase (MAPK) family of enzymes is comprised of an extracellular signal-regulated kinase (ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and a p38-kinase [Chang and Karin, 2001]. It has been shown that PA production plays a central role in the activation of the ERK pathway and the generation of DCs from monocytes depends on the activation of Raf/MEK/ERK and phosphoinositide-3 kinase/AKT signaling pathways [Andresen et al., 2002; Xie et al., 2005]. In this study, we have found that PA alone will induce the expression of surface markers of DC and that this effect is associated with the ERK pathway and degradation of PML-RARα.

MATERIALS AND METHODS

Reagents and Antibodies

Fluoresceine isothiocyanate (FITC)-conjugated anti-CD83 (IgG1), anti-CD80 (IgG1), anti-CD86 (IgG1), anti-CD11c (IgG1), anti-CCR-7 (IgG1), anti-CD14 (IgG1), anti-CD66b (IgG1), anti-MHC-I (IgG1), and anti-MHC-II antibodies (IgG1), PE-conjugated anti-CD11b (IgG1) and anti-CD54 antibodies (IgG1), and isotype control antibodies (IgG1 and IgG2a) were purchased from BD Pharmingen (Franklin Lakes, NJ). PAs, including dioctanoyl-PA and lyso-PA (LPA), and FITC-dextran were obtained from Sigma (St. Louis, MO). PD98059, SB203580, and SP600125 were purchased from Calbiochem (San Diego, CA). Antibodies against ERK-1/2, phospho-ERK-1/2, and phospho-p38-kinase were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against phospho-JNK, phospho-c-jun, and RARα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant TNF-α and IL-4 were obtained from R&D Systems, Inc. (Minneapolis, MN).

Cell Culture

NB4 cells were kindly provided by Dr. Lanotte (Saint Louis Hospital, Paris). The NB4 cells were cultured in RPMI-1640, 10% FBS, 2 mM glutamine, 100 units of penicillin, and 100 μg/ml of streptomycin at 37°C in 5% CO_2. Cells were treated alone or in combination with various concentrations of PA. The cell viability was determined by trypan blue exclusion. The level of apoptosis was measured using an annexin V-FITC apoptosis detection kit (Oncogene Research Products, Boston, MA) and analyzed using a FACSCalibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ).

Morphologic Assessment of NB4 Cells

Cells incubated with or without PA were centrifuged on a glass slide in a cytospin (Shandon, Pittsburgh, PA), fixed with methanol and stained with Giemsa staining solution.

Flow Cytometry

Cells (1×10^6) were first harvested, washed with phosphate-buffered saline (PBS) buffer containing 0.5% BSA, and labeled with either FITC- or PE-conjugated antibodies by incubation on ice for 30 min followed by washing with PBS buffer. The cells (1×10^4) were subsequently analyzed using a Flow cytometer.

Transient Transfection of Plasmids

The cells were washed and resuspended in 500 μl of electroporation buffer with 20 μg of plasmid DNA (pCDNA3.1 and pCDNA3.1-h

PLD2) [Ahn et al., 2003]. The green fluorescent protein (GFP-PLD) constructs were kindly provided by Dr. Sung-Ho Ryu (Pohang University, Korea). Following electroporation (two pulses at 1 KV, 200 ms), the cells were placed at 4°C for 10 min, resuspended in RPMI-1640 and then incubated for 3 days. NB4 cells that overexpressed GFP-tagged hPLD2 were analyzed for their expression of GFP and CD86 by confocal microscopy (Carl Zeiss LSM 510).

Western Blotting

Cultured cells (2×10^6) were suspended in a lysis buffer containing 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% Triton X-100, 50 mM NaF, 2.5 mM Na_3VO_4, and protease inhibitors. Lysates (25 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes which were then blocked for 1 h at 25°C with blocking buffer (10 mM Tris-HCl, 0.15 M NaCl, 0.1% sodium azide, and 5% skim milk) and incubated with a primary antibody (1:1,000 dilution) in blocking buffer overnight at 4°C. Secondary antibodies directed against IgG were diluted 1:5,000 in blocking buffer and incubated for 1 h. Signals were detected by ECL chemiluminescence.

Mixed Leukocyte Reaction

T-cells were purified from the peripheral blood of healthy volunteers and purity was determined by staining with FITC-conjugated anti-CD3 antibody. Informed consent was obtained from all volunteers and the Institutional Review Board at Dong-A University Hospital approved this study. Cultured NB4 cells were treated with 50 μg/ml of mitomycin-c for 1 h and then added to 1×10^5 T-cells in 96-well microtiter culture plates. Cell mixtures were cultured for 5 days at 37°C in 5% CO_2 and then pulsed for 18 h with 1 μCi of [^{3}H]thymidine (NEN-DuPont, Boston, MA). The cells were then harvested and the incorporated radioactivity was determined.

Phagocytosis Assay

To analyze endocytosis, 1×10^5 cells were incubated at 37°C for 1 h with 1 mg/ml of FITC-dextran (42,000 Da). The cells were washed twice with cold HBSS after this incubation and analyzed by flow cytometry. Parallel experiments were also performed at 4°C.

Cytokine Assay

IL-10, IL-12(p70), interferon (INF)-γ, and TNF-α levels were assayed in supernatants derived from NB4 cells, T-cells, and mixtures of NB4 and T-cells by ELISA sets (BD Pharmingen). Standard cytokine preparations were used as internal controls in all tests.

Cytotoxic Assay

The ^{51}Cr release assay was used to examine the cytotoxic potential of the stimulated T-cells, used as effect cells. NB4 target cells were labeled with 200 μCi $Na_2{}^{51}CrO_4$ (NEN Life Science Products, Inc.) for 1 h at 37°C. After washing, the labeled target cells were mixed with effector T-cells at a 25:1 effector: target ratio for 6 h. Prelabeled target cells which had been cultured without effector cells were used to determine spontaneous ^{51}Cr release. The cytotoxicity activities were calculated as the percentage of specific ^{51}Cr release using the following equation: $\%\ \text{specific lysis} = (\text{cpm}_{\text{experimental}} - \text{cpm}_{\text{spontaneous}})/(\text{cpm}_{\text{maximal}} - \text{cpm}_{\text{spontaneous}}) \times 100$.

Statistical Analyses

The results are presented as a mean ± standstandard deviation (SD). The Wilcoxon's rank sum test was used to analyze data for significant differences. P-values of <0.05 were regarded as significant.

RESULTS

Upregulation of DC-Markers in PA-Treated NB4 Cells

Differences of cell morphology, judged by phase contrast and Giemsa staining between control cultures or cultures with addition of PA, could be observed as early as Day 3 (Fig. 1A). The dioctanoyl-PA-treated NB4 cells showed morphologic evidence of differentiation, for example, nuclear indentation and more abundant cytoplasm. As shown in Figure 1B, the growth rate of the dioctanoyl-PA-treated NB4 cells decreased. We analyzed the effects of PA on the surface expression of differentiation-related molecules on NB4 cells using flow cytometer. The NB4 cells expressed little or no DC markers, including MHC-I, MHC-II, costimulatory molecules, and CD83 on the cell surface. Treating the NB4 cells with dioctanyol-PA for 3 days markedly increased the surface expression of the DC lineage marker, CD11c, and the costimulatory

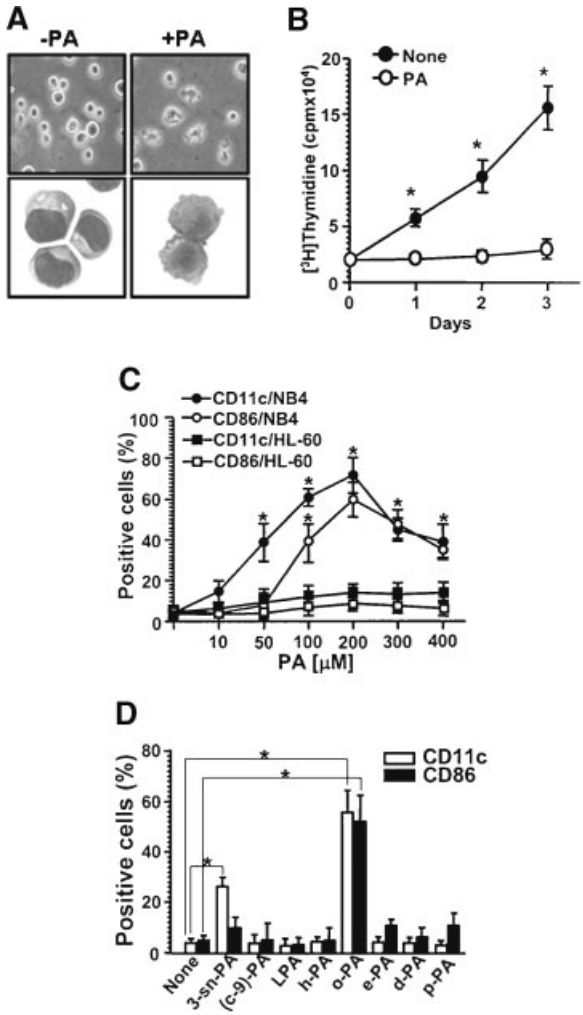

Fig. 1. PA induces differentiation of NB4 cells. **A**: In the upper panel, NB4 cells (1×10^6) were cultured with (+PA) or without (−PA) 200 μM dioctanoyl-PA for 3 days (400×). In the **lower panel**, the cells were harvested and stained with a Giemsa solution (1,000×). Photomicrographs are representative of four independent experiments. **B**: Cell proliferation was measured by the incorporation of [^{3}H]thymidine over the last 18 h of culture of PA-treated (PA) or -untreated (None) cells. **C**: The cells were incubated with increasing concentrations of dioctanoyl-PA for 3 days and the expression levels of CD11c and CD86 were measured. **D**: The expression of CD11c and CD86 were analyzed after NB4 cells were treated for 3 days with 200 μM of 3-sn-PA, (*cis*-9-octadecenoyl)-PA ((c-9)-PA), lyso-PA (LPA), 1,2-diheptanoyl-PA (h-PA), 1,2-dioctanoyl-PA (o-PA), 1,2-dioleyl-PA (e-PA), 1,2-didecanoyl-PA (d-PA), and 1,2-dipalmitoyl-PA (p-PA). Results are expressed as the mean ± SD of three or four independent experiments. *$P < 0.05$ versus without PA.

molecule, CD86, in a dose-dependent manner. However, there was no increase in CD11c and CD86 expression in the HL-60 cells treated with the same concentrations of PA (Fig. 1C). Exogenous PA (100–500 μM) has been shown to have various biological functions [Murakami-Murofushi et al., 2002; Park et al., 2002; Sakai et al., 2002]. PA alone did not affect the viability of the cells nor did it induce apoptosis of NB4, as measured on Day 3 (data not shown).

When PA containing different fatty acids was tested to determine the effect of acyl composition on the expression of DC markers, dioctanoyl-PA but no other commercially available isotypes, had little effect on the expression of CD11c and CD86 (Fig. 1D). Therefore, we used dioctanoyl-PA as the source of PA for this experiment. We considered the possibility that exogenously added PA might be metabolized into LPA by phospholipase A_2. The treatment of NB4 cells with LPA for 3 days, however, had no effect on the expression of CD11c and CD86. When we also co-treated the cells with propranolol, which prevents PA dephosphorylation by PA phosphohydrolase and thus causes the accumulation of PA, this PA upregulatory effect was not changed (data not shown). Taken together, these results suggest that PA itself rather than PA-derived metabolites affects the expression of CD11c and CD86.

NB4 cells were harvested after 1, 3, or 5 days of treatment and the expressions of the DC markers were determined. Expression levels of these surface markers on untreated NB4 cells (Day 0) were below 5% (Fig. 2A). The expression of MHC-I, MHC-II, CD11c, CD54, CD80, CD86, and CD83 was detected from Day 1 after treatment and the PA-mediated effects were highest the third day of culture (Fig. 2A). NB4 cells showed low CD11b expression, a cell surface marker of both granulocytic and monocytic differentiation but expression of CD11b was enhanced by dioctanoyl-PA. Upregulation of CD66b or CD14, however, did not occur for the culture conditions including dioctanoyl-PA. Moreover, dioctanoyl-PA treatment did not increase the nitro blue tetrazolium-reducing activity which is a phenotypic marker of granulocytic maturation, when compared to ATRA treatment (data not shown). In contrast to NB4 cells, UF-1 cells (a stable RA-resistant cell line from Dr. Kizaki, Keio University, Japan) and other leukemic cells including KG-1, K562, U937, and THP-1 failed to differentiate into myeloid cells when cultured under identical conditions (Fig. 2B). These results suggest that PA by itself is capable of inducing the differentiation of the NB4 cells into DC-like cells.

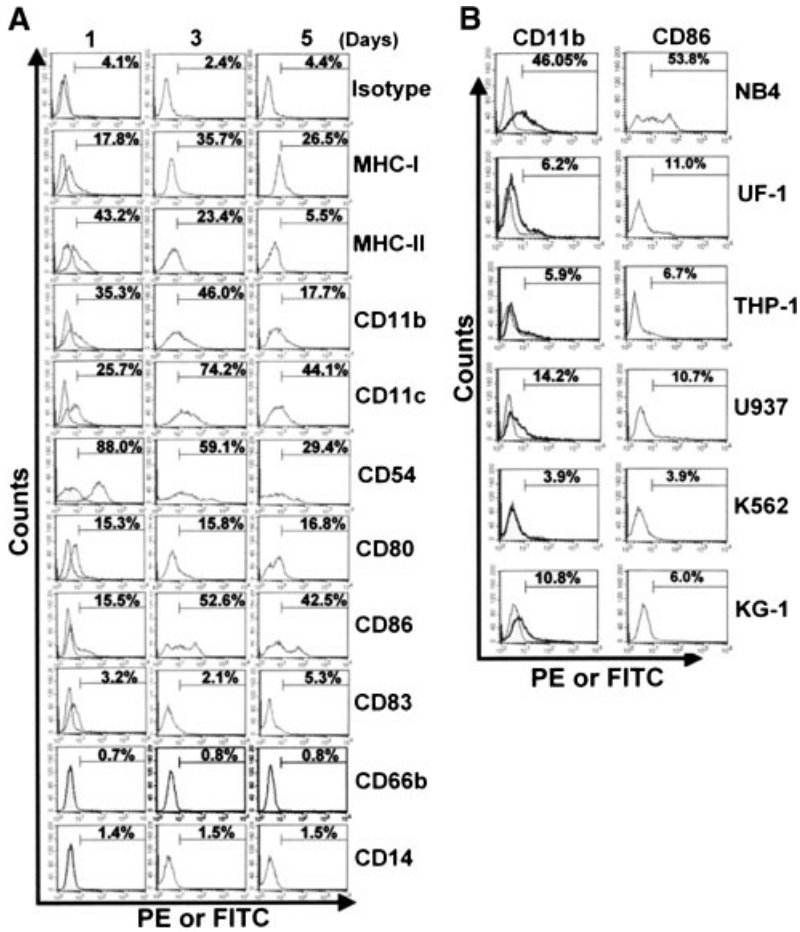

Fig. 2. PA upregulates DC cell surface markers on NB4 cells. **A**: NB4 cells were cultured for the indicated times with 200 μM dioctanoyl-PA. Cells were then stained with PE- or FITC-conjugated antibodies and expression levels were analyzed by flow cytometry. Shadow areas indicate untreated cells (0 day). Results are representative of three or four independent experiments. **B**: Various myeloid leukemic cells were tested for differentiation in the presence of 200 μM dioctanoyl-PA for 3 days. Shadow area indicates isotype control. The data shown are representative of three independent experiments.

Increased Expression of DC Markers in PLD-Transfected NB4 Cells

Since intracellular PA is produced mainly by PLD, we measured the effect of PLD on DC marker expression in NB4 cells. PLD2-transfections induced a substantial increase in basal PLD activity [Kim et al., 1999]. As shown in Figure 3A, transient transfection of PLD2 plasmid enhanced the expression of CD11b, CD11c, CD80, and CD86. CD83 expression was not increased by PA, an observation consistent with the transfection of PLD plasmids. Fluorescent microscopy analyses after a 72 h transfection interval of GFP-PLD plasmids indicated that CD86 expression was enhanced with the expressions of GFP-PLD2 in the cells (Fig. 3B). As shown in Figure 3C, the addition of *n*-butanol, a selective inhibitor of the production of PA by PLD, markedly blocked the upregulation of CD11c and CD86 in PLD-transfected NB4 cells, whereas *t*-butanol exhibited little or no effects. This observation suggests that the PLD-derived PA induces differentiation of NB4 cells into DC-like cells.

Enhancement of DC Marker Expression by TNF-α in PA-Differentiated NB4 Cells

As shown in Figure 2A, the expression level of DC markers was decreased after 5 days of cell culture. When IL-4 was added, the decreased expression of CD11b and CD11c was not restored after 5 days in the presence of PA (Fig. 4A). The expression of surface antigens is known to depend on the maturation stage of the

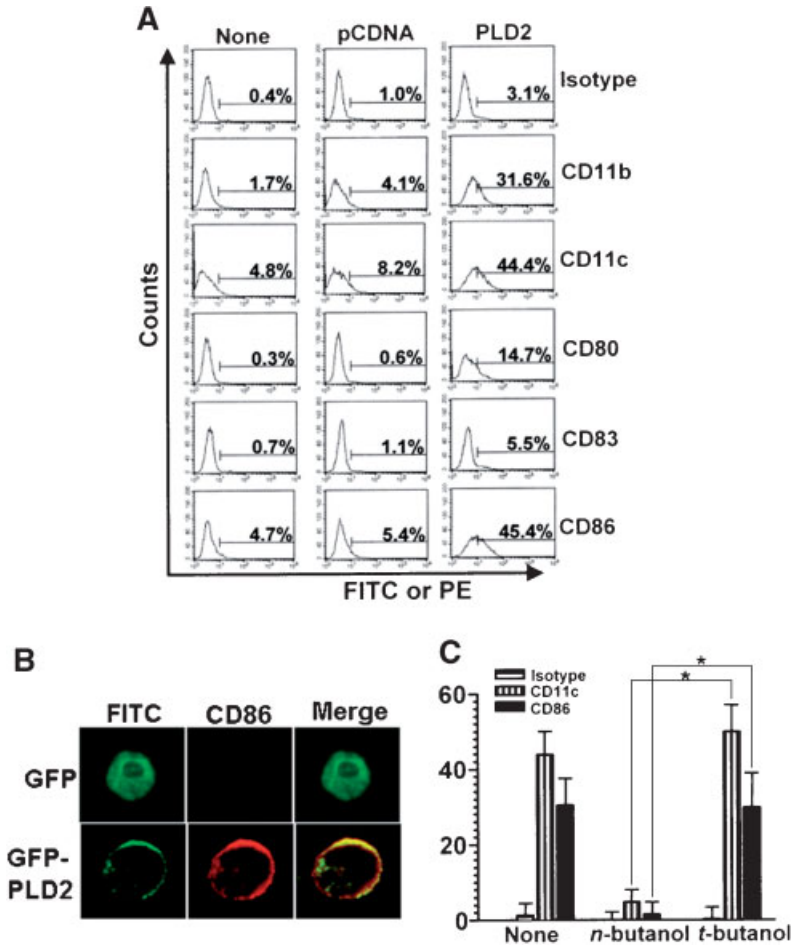

Fig. 3. Increased expression of DC markers by the overexpression of PLD in NB4 cells. **A**: The cells were transiently transfected with plasmid DNA (pCDNA and pCDNA-hPLD2). Following electroporation, the cells were incubated for 3 days. The expression levels were measured by flow cytometry. **B**: GFP-tagged hPLD2 was transfected into NB4 cells as in Panel A and the overexpressed proteins were analyzed for the expression of GFP and CD86 by confocal microscopy (1,000×). **C**: Following electroporation of PLD2 plasmid, the cells were resuspended in RPMI-1640 and incubated with 0.5% *n*-butanol or *t*-butanol for 3 days. The expression levels of CD11c and CD86 in the transfected cells were measured by flow cytometry. Results are expressed as the mean ± SD of three independent experiments. $^*P < 0.001$ versus *t*-butanol.

DCs. TNF-α has been shown to cause DCs to mature [Whiteside and Odoux, 2004]. NB4 cells did not express DC markers on their surfaces by the addition of TNF-α in the absence of PA (data not shown). When PA-differentiated NB4 cells were stimulated with TNF-α for 48 h, these cells enhanced the expression of CD83, a known marker of mature DC (Fig. 4B). We also observed increases in the expression of CCR7 which are also known to accompany DC maturation. The expression of MHC-I, -II, and CD11c was also enhanced by TNF-α. These results suggest that the phenotype of a PA-differentiated and TNF-α-activated NB4 cell resembles that of mature DCs.

Effect of PA on Cytokine Production of NB4 Cells

We sought to determine whether differentiated NB4 cells could achieve the functional capabilities of normal mature DCs. The concentrations of immunosuppressive IL-10 were increased in PA-treated cells compared to untreated control cells (Fig. 5A). The production of IL-10, however, was reduced by treatment of PA-differentiated NB4 cells with TNF-α. When we analyzed the bioactive IL-12(p70) production by PA-differentiated NB4 cells, very low levels of the cytokine were detected but the production was significantly increased by

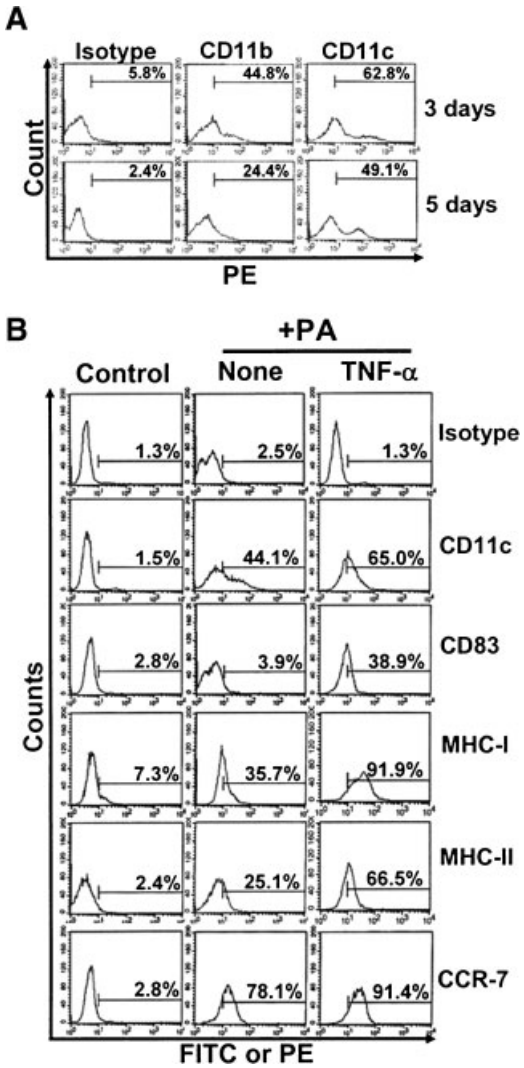

Fig. 4. Enhanced expression of mature DC markers by TNF-α in PA-treated NB4 cells. **A**: NB4 cells (1×10^6) were cultured with dioctanoyl-PA in the presence of 20 ng/ml IL-4 for the indicated times. The expression of CD11b and CD11c were determined by flow cytometry. Results are representative of three or four independent experiments. **B**: NB4 cells were cultured for 3 days with PA, washed with culture media, and further cultured with (+TNF-α) or without (None) TNF-α (10 ng/ml) for 2 days. NB4 cells were then stained with FITC- and PE-conjugated antibodies and expression levels were analyzed by flow cytometry.

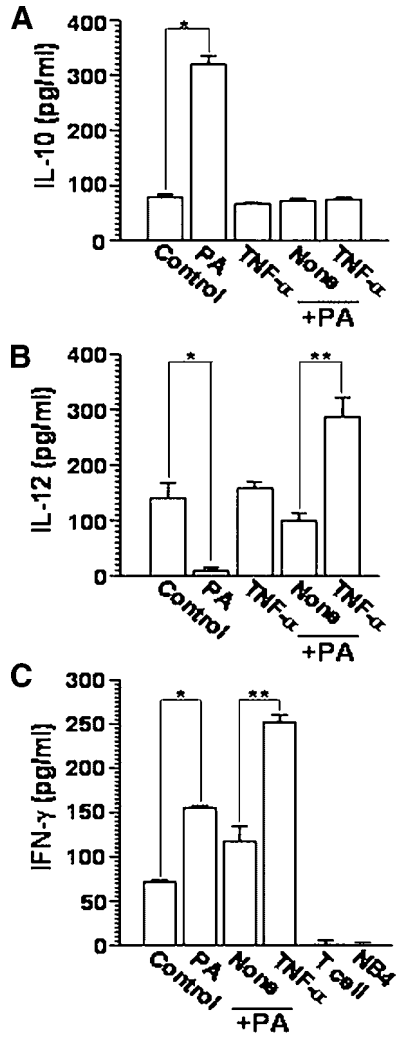

Fig. 5. Changes of cytokine production of NB4 cells by PA. Culture fluid was withdrawn after treatment of NB4 cells with PA and TNF-α as in Figure 4B and the concentrations of IL-10 (**A**) and IL-12(p70) (**B**) were measured by ELISA. **C**: PA and TNF-α-treated or -untreated NB4 cells as in Figure 4B were mixed with T-cells (1×10^5) in a 1:25 ratio. T-cells alone (T-cell), NB4 cells alone (NB4), and the cell mixtures were cultured for 5 days and the concentrations of INF-γ were measured. Results are presented as the mean ± SD of triplicate assays from two separate cultures. $^*P < 0.05$ versus control. $^{**}P < 0.05$ versus none in the presence of PA.

TNF-α stimulation (Fig. 5B). We found that the production of IL-12(p70) was conversely related to the production of IL-10 in PA-treated cells. As shown in Figure 5C, INF-γ production by T-cells was increased when the T-cells were co-cultured for 5 days with PA and TNF-α-treated NB4 cells. Untreated control NB4 cells did not produce these cytokines by the addition of TNF-α alone.

T-Cell Proliferative Activity of PA-Differentiated NB4 Cells

When FITC-dextran was used to examine the cellular antigen uptake capacity, PA-treated cells (3 days) showed higher phagocytic ability in comparison to control NB4 cells (Fig. 6A). Moreover, after induction of cell maturation by TNF-α, the endocytic activity of PA-treated NB4

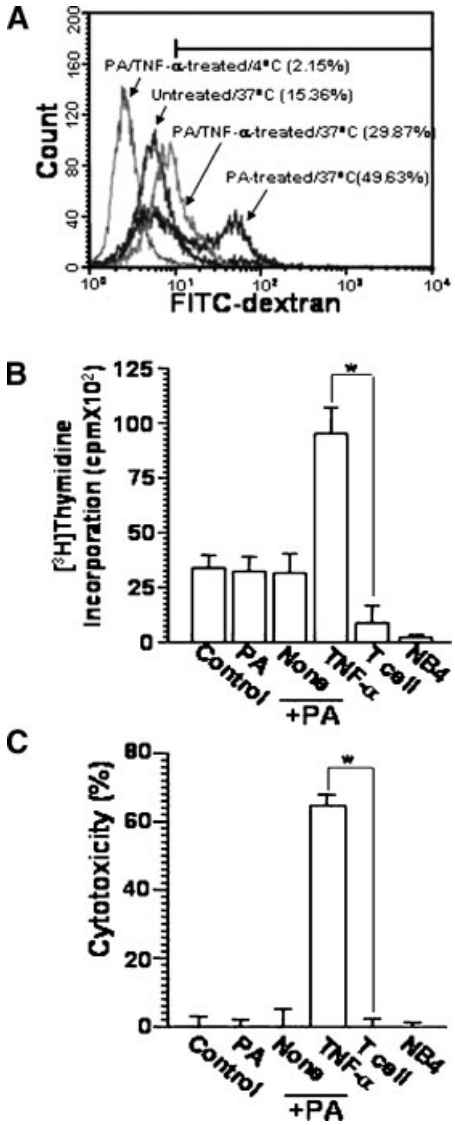

Fig. 6. Activation of T-cells by PA-treated NB4 cells. **A**: PA-differentiated cells were treated or untreated for 2 days with TNF-α, harvested, and then incubated with 1 mg/ml of FITC-conjugated dextran for 30 min at 37°C. After this incubation, the cells were washed twice with cold HBSS and the cellular uptake of dextran was analyzed by flow cytometry. The bold lines represent the cells incubated with FITC-dextran at 4°C. The data shown are representative of three independent experiments. **B**: NB4 cells were treated as in Figure 4B, harvested, and washed twice with media. These NB4 cells were pretreated with mitomycin c and mixed with T-cells (1×10^5) in a 1:25 ratio. T-cells alone (T-cell), NB4 cells alone (NB4), and the cell mixtures were cultured for 5 days. The cells were further cultured for 18 h after adding 1 μCi [^{3}H]thymidine. The incorporation of [^{3}H]thymidine was determined using a scintillation counter. **C**: T-cells were cultured for 5 days with or without the differentiated and activated NB4 cells and isolated to be used as effector cells. Untreated NB4 cells were labeled with ^{51}Cr and used as target cells. ^{51}Cr release was assayed as described in Materials and Methods. Results are presented as the mean ± SD of triplicate assays. *$P < 0.05$ compared with T-cell alone.

cells was significantly reduced. Next, NB4 cells were tested for their ability to support T-cell activation. PA-differentiated and TNF-α-activated NB4 cells, but not untreated- or PA-differentiated NB4 cells without TNF-α activation, stimulated T-cell proliferation when co-cultured for 5 days at a 1:25 ratio (Fig. 6B). As shown in Figure 6C, the T-cell-mediated cytotoxicity towards undifferentiated NB4 cells was only observed with PA-differentiated and TNF-α-activated NB4 cells.

Activation of ERK-1/2 by PA

MAPK phosphorylation was assessed by immunoblotting with the respective anti-phosphorylated MAPK antibodies. As shown in Figure 7A, the treatment of NB4 cells with PA alone for 10 min dose-dependently caused phosphorylation of ERK-1/2. The incubation of NB4 cells with PA for 30 min increased the phosphorylations of JNK, but PA alone failed to phosphorylate p38-kinase. These results suggest that PA differentially modulates the activation of MAPKs and it is not likely that the concentrations of PA used in our system acted non-specifically. PD98059 is known to prevent the activation of MAPK kinase, an upstream activator of ERK-1/2. PA-induced ERK-1/2 phosphorylation was suppressed by the treatment of NB4 cells with PD98059 but not with the inhibitor of the p38-kinase pathway, SB203580 (Fig. 7B). In contrast, PD98059 blocked the phosphorylation of JNK and c-jun. The inhibitor of JNK phosphorylation, SP600125 abolished the phosphorylation of JNK and c-jun but had no effect on the phosphorylation of ERK-1/2 and p38-kinase, suggesting that the JNK pathway may be a downstream effector of ERK-1/2 activation by PA in NB4 cells. As shown in Figure 7C, PA-induced activation of ERK-1/2 was not blocked by the inhibitors of Ras and Raf1 kinase, upstream activators of ERK-1/2.

Decrease of PML-RARα Fusion Protein Levels in PA-Treated NB4 Cells

ATRA is known to induce degradation of PML-RARα and RARα [Yoshida et al., 1996]. By using an anti-human RARα antibody that recognizes both the RARα and PML-RARα fusion proteins, treatment with ATRA was shown to result in a significant diminution of the level of both RARα and PML-RARα. Treatment of NB4 cells for 3 days with PA caused the

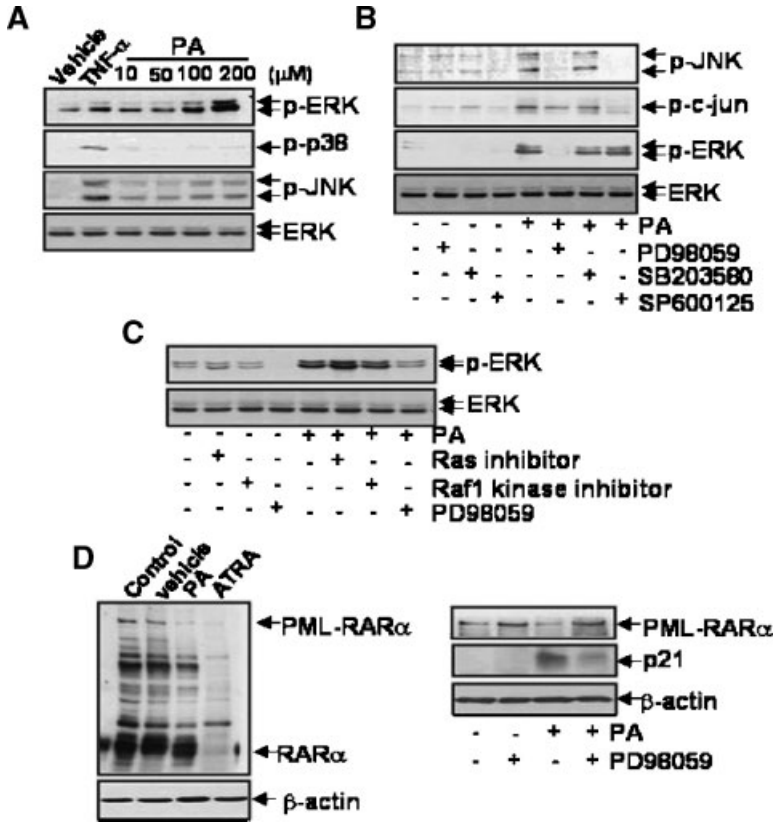

Fig. 7. Effect of PA on the activation of MAPK and protein levels of PML-RARα in NB4 cells. **A**: NB4 cells (2×10^6) were treated for 10 min (p-ERK-1/2 and p-p38) or 30 min (p-JNK) with or without increasing concentrations of dioctanoyl-PA or 10 ng/ml TNF-α. Cell lysates (25 μg) were immunoblotted with antibodies against the phosphorylated forms of ERK-1/2 (p-ERK), p38-kinase (p-p38), and JNK (p-JNK). Anti-ERK-1/2 antibody was used as a control. Results are representative of three independent experiments. **B**: NB4 cells were preincubated with 10 μM PD98059, 1 μM SB203580, or 10 μM SP600125 for 10 min and then incubated for another 10 min or 30 min with 200 μM PA as shown in panel A. **C**: NB4 cells were preincubated with 10 μg/ml Ras inhibitor peptide, 10 nM Raf1 kinase inhibitor, or 10 μM PD98059 for 10 min and treated with or without PA. **D**: In the **left panel**, NB4 cells were cultured for 3 days in the presence (PA) or absence (Vehicle) of PA or cultured for 4 days in the presence of 1 μM ATRA (ATRA). Sub-cultured NB4 cells were used as a control. In the **right panel**, NB4 cells were pretreated for 1 h with 10 μM PD98059 and cultured with PA for 12 h or 72 h for detecting P21 or PML-RARα, respectively. Cell lysates (25 μg) were immunoblotted with antibodies against RARα or P21.

disappearance of the bands corresponding to PML-RARα but the levels of those corresponding to RARα remained as the control (Fig. 7D). The PA-induced downregulation of PML-RARα was restored to the control levels by addition of PD98059. The level of $p21^{WAF1/Cip1}$ cyclin-dependent kinase inhibitor (P21) increased in NB4 cells treated with PA for 12 h and the increase was abrogated by preincubation with PD98059.

ERK-1/2 Pathway in PA-Induced Differentiation of NB4 Cells

Figure 8A shows that the treatment of NB4 cells for 3 days with PD98059, but not with SB203580 and SP600125, abrogated the effect of PA on morphological changes. Moreover, PA-induced upregulation of the DC surface markers (e.g., CD11c, CD80, and CD86) was blocked by PD98059 but not by SP600125 (Fig. 8B). As seen in the pharmacological ERK inhibition experiments, the dominant negative mutant of ERK-1 also reduced the expression of CD11c that was induced by PA (Fig. 8C), whereas the transfection of wild type ERK-1 did not affect CD11c expression. Wild type or the dominant negative mutant form of JNK, however, did not affect the expression level of CD11c. The PA-induced enhancement of the expression of CD11c and CD86 on NB4 cells was not affected by the inhibitors of Ras and Raf1 kinase (data not shown). We also examined the ability of inhibitor-treated cells to produce cytokines and as indicated in Figure 8D, PD98059 abrogated the effect of PA on the production of IL-10, TNF-α, and IL-12(p70). In contrast, the inhibitors of p38-kinase and JNK

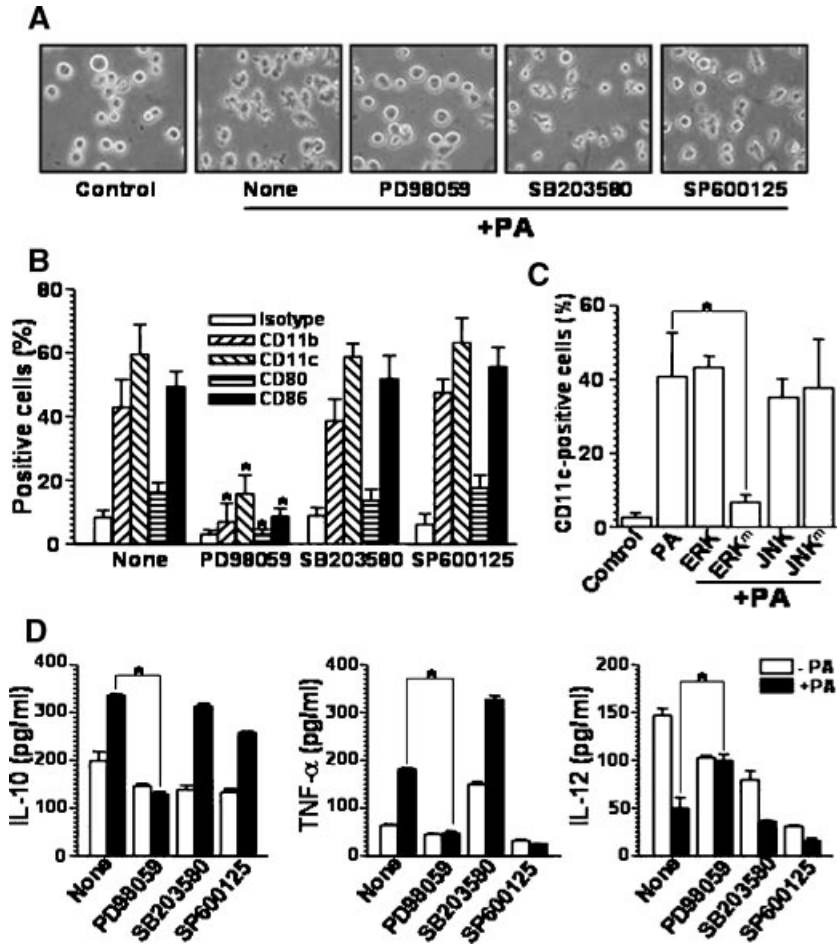

Fig. 8. PA-induced differentiation of NB4 cells was blocked by ERK inhibition. **A**: The NB4 cells (1×10^6) were treated for 3 days with 200 μM dioctanoyl-PA in the presence or absence of MAPK inhibitors. Photomicrographs are representative of four independent experiments (400×). **B**: NB4 cells were treated as in panel A and the expression levels of DC markers on the NB4 cell surface were measured. The data are presented as percentages of the marker-positive cells. **C**: NB4 cells were transfected with plasmid of the wild type ERK-1, dominant negative mutant form of ERK-1 (ERK^m), the wild type of JNK, or the dominant negative type of JNK (JNK^m) for 2 days and treated for another 3 days with 200 μM PA. **D**: NB4 cells were treated as in panel A. Culture fluid was withdrawn and concentrations of IL-10, TNF-α, and IL-12(p70) were measured by ELISA. Results are reported as the mean ± SD of three experiments. $^*P < 0.001$ versus None.

increased the production of IL-10 and TNF-α in the PA-treated cells. These results suggest that ERK-1/2 but not JNK is involved in the differentiation of NB4 cells by PA, although these two MAPKs are also activated by PA.

DISCUSSION

Malignant myeloid precursor cells are capable of developing into cells with DC characteristics when they are cultured with combinations of cytokines. Although little is known about the signal transduction pathways involved in the differentiation and the maturation of DCs, the signaling pathways preferentially regulate the generation of surface markers in leukemia cell differentiation. In this study, we determined that PA alone was able to induce APL cells into a DC immunophenotype. NB4 cells, but not other leukemic cells, upregulated DC markers in response to PA. The selective upregulation of the DC markers with the PA treatment of NB4 cells expressing PML-RARα and the degradation of PML-RARα by the PA treatment suggest that the downregulation of PML-RARα or a related signaling pathway(s), including ERK, may mediate the differentiation signals in the NB4 cells exposed to PA. While ERK activation induces DC-like differentiation, other signaling pathways are necessary for induction because the activation of ERK is involved in the process of myeloid differentiation

that is triggered by ATRA in the HL-60 cells [Yen et al., 1998], as well as in the process of DC-like differentiation by the overexpression of the myeloid leukemia-associated protein SET in U937 cells [Kandilci and Grosveld, 2005]. In our experiments, PA also induces the activation of ERK-1/2 in HL-60 cells but failed to induce the cells into the DC-like cells (data not shown).

In matured DCs, the ability to incorporate and process antigen is lost but the ability to present antigen is increased. The ability to take up dextran beads increased in the PA-differentiated cells and lessened after activation of the differentiated cells by TNF-α. The production of IL-12, T-cell stimulation, and T-cell-mediated cytotoxicity were also increased in the PA-differentiated and TNF-α-activated NB4 cells, indicating that PA-differentiated NB4 cells are capable of a characteristic phenotypic and a functional DC maturation response to TNF-α. On the other hand, the level of the DC markers decreased at Day 5, and this decrease was not blocked by IL-4. IL-4 appears to have played a key role as a regulator of monocyte differentiation into DCs, by blocking macrophage development. It should be noted that not all NB4 cells simultaneously differentiated into DC-like cells. Therefore, undifferentiated cells might exist at the late culture stages or the cells might acquire different phenotypic properties. Montesoro et al. [2006] reported that the capability of the monocyte precursor cells from a hematopoietic progenitor culture to differentiate into DCs in the presence of IL-4 and GM-CSF was completely lost at the end of the culture when the majority of cells were macrophage-like.

In this experiment, PA containing other fatty acid chains had less effect on the upregulation of DC markers than dioctanoyl-PA, suggesting that the acyl chain of PA may be important in the induction of differentiation of NB4 cells. A mixture of dioleoyl-PA and dipalmitoyl-PC has been shown to bind Raf-1 kinase [Ghosh et al., 1996], but the dioleyl-PA failed to upregulate DC markers in this study. Alternatively, overexpression of PLD enhanced the level of DC markers on NB4 cells, an effect that was abrogated by *n*-butanol, thereby suggesting that endogenously produced PA by PLD is involved in the differentiation of NB4 cells into DC-like cells. This study showed a similar effect with the exogenous addition of PA. The molecular mechanism and appropriate cellular locations of the exogenously added PA is unclear. However, it has been shown that added PA is incorporated into the membranes of various cell types [Wang et al., 2006]. It is possible that the incorporation of PA in the cellular membranes may be due to its characteristics as a lipid anchor and fusogenic lipid or an alteration in the composition of the membrane microdomains leading to changes in the physical properties of the cellular membranes. In comparison, it was suggested that injected PA has no functional effect due to the formation of micelles, which are not effectively incorporated into the membranes [Fukami and Takenawa, 1992]. Direct interaction between PA and cell proteins has previously been demonstrated [Andresen et al., 2002] and other groups have shown that PA facilitates the recruitment of Raf to the plasma membrane to participate in activation of the MAPK pathway [Rizzo et al., 1999]. PA alone, however, cannot activate Raf-1 although Raf-1 binds PA [Rizzo et al., 1999]. PA-induced ERK-1/2 phosphorylation and upregulation of DC markers were not abrogated by the inhibitors of Ras and Raf kinase, which suggests that the downstream signaling pathway of Raf kinase may be involved in the PA-induced differentiation of NB4 cells.

PA-dependent signaling involves several protein kinases, including ERK-1/2. The functional maturation of monocyte-derived DCs could be inhibited by SB203580 but enhanced by PD98059, suggesting that the ERK and p38-kinase signaling pathways differentially regulate the maturation of monocyte-derived DCs [Puig-Kroger et al., 2001]. Kim et al. [2004] have shown that PA alone fails to induce MAPK phosphorylation and expression of CD83 in KG1 cells but PA increases the phosphorylation of ERK-1/2 and induces the expression of CD83 in the presence of TNF-α. We found that PD98059 and the dominant negative mutant of ERK-1 significantly inhibited the PA-induced upregulation of DC markers, cell morphology, and cytokine release. Lim et al. [2003] reported that PA activates AKT and rapamycin-sensitive mTOR pathway, but AKT was not phosphorylated by PA in NB4 cells and PA-induced upregulation of CD11c was not abrogated by LY294002, an inhibitor of phosphoinositide 3-kinase and rapamycin (data not shown). In contrast, combined treatment of NB4 cells with LY294002 and PA induced apoptosis of NB4 cells. JNK may be the downstream signaling

effector of ERK-1/2 because the inhibition of ERK-1/2 blocked the phosphorylation of JNK and c-jun by PA. The JNK inhibition by SP600125 and transfection of dominant negative mutant JNK, however, failed to affect the PA-induced expression of DC markers. More research is required to identify the downstream target of ERK-1/2 that is involved in the PA-induced differentiation of NB4 cells into the DC-like cells.

This study suggests that there is a differentiation pathway of promyelocytes into DCs. The treatment of cells by PA reduced the level of PML-RARα and its reduction was blocked by the MEK inhibitor. PML-RARα protein acts as a dominant negative inhibitor of myeloid differentiation [Grignani et al., 1993]. It is possible that the ERK pathway may be one of the signaling pathways that directly contribute to transcriptional activation for differentiation to the DC lineage or via a PML-RARα. HL-60 cells have a similar nature to NB4 cells but do not have a PML-RARα fusion protein. However, this study showed that HL-60 and UF-1 cells failed to differentiate to DC-like cells in the presence of PA. The expression levels of CD66b and CD14 were not enhanced by PA before DC marker expression, suggesting that PA induces the differentiation of NB4 cells into DC-like in the absence of granulocytic or monocytic differentiation. PA-induced differentiation may be independent from the action of ATRA. It has been shown that ATRA binding directs both RARα and PML-RARα along the proteasome degradation pathways [Yoshida et al., 1996]. In this study, PA treatment reduced the level of PML-RARα protein but did not affect RARα and the reduction of PML-RARα level was not blocked by the proteasome inhibitor MG132 (data not shown). Among the genes encoding RARs, RARα has been identified with myeloid development and the overexpression of RARα was shown to block myeloid differentiation [de The et al., 1989; Grignani et al., 1996]. We cannot eliminate the possibility that residual RARα after degradation of PML-RARα may be involved in inducing a gene critical for differentiation into DC. The degradation of PML-RARα has also been suggested to be related to a caspase system leading the cells to apoptosis [Wang et al., 1998a]. However, the pancaspase inhibitor, zVAD-fmk, failed to block both the degradation of fusion proteins and the upregulation of DC markers by PA (data not shown). It has been shown that cell proliferation can be decreased by increasing the expression of p21, which is related to degradation of PML proteins [Wang et al., 1998b]. PA-triggered growth arrest may result from the induction of p21 because PA increases the protein level of p21 as measured by Western blot.

In summary, the strategy to convert leukemic cells into DCs can be used whereby the leukemia antigen is made available for the treatment of acute or chronic leukemia. The development of a therapy targeting novel differentiation pathways using PA in APL cells and the possibility of developing an anti-leukemia immunotherapy using leukemia-derived DCs may offer a significantly improved treatment for this disease.

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