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The Essentiality of PKCα and PKCβ$_I$ Translocation for CD14$^+$monocyte Differentiation Towards Macrophages and Dendritic Cells, Respectively

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Abstract Human peripheral CD14$^+$monocytes have been known to differentiate into monocyte-derived macrophages (MDMs) or dendritic cells (MoDCs) upon suitable stimulation. However, the key intracellular molecule(s) associated with their differentiation towards specific cell types was(were) not fully understood. This study was designated to determine the association of PKC isoenzymes with the differentiation of CD14$^+$monocytes into MDMs or MoDCs. Purified human peripheral CD14$^+$monocytes were cultured with GM-CSF, or GM-CSF plus IL-4 for 7 days to induce cell differentiation. The phenotypic changes were analyzed by Flow-Cytometry using various specific antibodies to cell type-specific surface markers. The immunological functions of these differentiated cells were determined by measuring the amounts of TNF-α secretion for MDMs, and the capacities of antigen-capturing and bacterial phagocytosis for MoDCs. The translocations of PKC isoenzymes in these cells from cytosol to plasma membrane were examined by Western Blot analysis and Confocal Microscopic observation. The treatment of CD14$^+$monocytes with either GM-CSF or PMA elicited PKCα translocation and consequently induced their differentiation into MDMs. The inclusion of PKCα/β$_I$ specific inhibitor, Go6976, greatly inhibited the GM-CSF-induced PKCα translocation and dose-dependently reduced the GM-CSF- induced MDM differentiation. On the other hand, the simultaneous pretreatment of CD14$^+$monocytes with Go6976 and PKCβ-specific inhibitor predominantly suppressed the GM-CSF/IL-4-induced generation of MoDCs. Further study demonstrated that GM-CSF/IL-4 selectively induced the translocation of PKCβ$_I$, not PKCα or PKCβ$_{II}$, in CD14$^+$monocytes. In conclusion, the cell fate commitment of CD14$^+$monocytes towards MDMs or MoDCs appears to be steered by the selective activation of PKCα or PKCβ$_I$, respectively. J. Cell. Biochem. 102: 429–441, 2007.

Key words: CD14$^+$monocytes; macrophages; dendritic cells; differentiation; protein kinase C

Granulocyte/macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) are known to stimulate the generation of monocyte-derived macrophages (MDMs) from peripheral CD14$^+$ monocytes [Akagawa, 1994; Akagawa, 2002]. However, GM-CSF- and M-CSF-induced MDMs are distinct from each other in their morphology, cell surface antigen expression, and sensitivity to human immunodeficiency virus, type I (HIV-I) infection [Akagawa, 1994; Akagawa, 2002]. GM-CSF-induced MDMs show a fried egg-like cell type, express CD14^{low} phenotype, and resistance to macrophage-tropic HIV-I infection [Andreesen et al., 1990; Kruger et al., 1996; Akagawa, 2002]. In contrast, M-CSF-induced MDMs are elongated and spindle-shaped cell type, express CD14^{high} phenotype, and are sensitive to macrophage-tropic HIV-I infection [Hashimoto et al., 1997; Akagawa, 2002]. On the other hand, phorbol-12-myristate-13-acetate (PMA), an activator for diacylglycerol (DAG)-sensitive protein kinase C (PKC) isoenzymes, induces the differentiation of CD14$^+$monocytes or THP-1 monocytic cells into macrophages [Vosper et al., 2003; Kohro et al., 2004]. Their phenotypic and morphological characteristics are similar to those found in the GM-CSF-induced MDMs [Kohro et al., 2004]. Based on these findings, we attempted to define which PKC isoenzyme(s) is(are) essential for the GM-CSF-induced

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differentiation of peripheral $CD14^+$monocytes into MDMs.

Recently, the combined treatment with cytokine GM-CSF and interleukin-4 (IL-4) was reported to induce the differentiation of peripheral $CD14^+$monocytes into immature dendritic cells, designated as type-1 dendritic cells (DC1s) [Liu, 2001] or monocyte-derived dendritic cells (MoDCs) [Mebouta-Nkamgueu et al., 2001]. Immature MoDCs exhibit a decreased expression of CD14 [Akagawa, 1994; Kiertscher and Roth, 1996] and reduced capacity of bacterial phagocytosis [Nagl et al., 2002], but show an increased expression of dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) [Colmenares et al., 2002; Geijtenbeek et al., 2002] and enhanced ability of pathogen-capturing [Mellman and Steinman, 2001]. Upon suitable stimulations, such as microbial pathogens, inflammatory agents etc., immature MoDCs undergo the progression of maturation and trigger T cell-dependent cellular immune responses. Previous studies demonstrated that the activation of PKCε is essential for the maturation of MoDCs [Aksoy et al., 2002]. Nevertheless, the critical PKC isoenzyme activation for MoDC differentiation is not yet reported.

PKC family members are serine-threonine protein kinases and divided into conventional (α, β_I, β_{II}, and γ), novel (ε, δ, μ, θ, and η) and atypical (ζ, ι, λ) isoenzymes depending on their responsiveness to Ca^{2+} and DAG stimulations [Mellor and Parker, 1998]. The conventional PKCs are activated by phosphatidylserine (PS) and DAG in a Ca^{2+}-dependent manner [Takai et al., 1979]. The novel PKCs are Ca^{2+}-insensitve, but activated by DAG in the presence of PS [Ono et al., 1988]. The atypical PKCs response to neither Ca^{2+} nor DAG [Ono et al., 1989]. The hallmark of PKC-activation in cells is its translocation from the cytosol to plasma membrane where it acquires co-activator, DAG [Oancea et al., 1998], and undergoes autophosphorylation [Feng and Hannun, 1998]. That the modulation of cellular individual PKC activity induces PKC-dependent cell differentiation has been reported in many cell types [Kang et al., 1996; Zauli et al., 1996; Park et al., 2002]. Therefore, this study was intended to delineate that the activation of which specific PKC isoenzyme(s) is essential for the differentiation of human peripheral $CD14^+$monocyte-derived macrophages or DCs.

MATERIALS AND METHODS

Reagents

Go6976 and PKCβ-specific inhibitor, 3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione, were purchased from Calbiochem (Merck Biosciences, Darmstadt, Germany). PMA and propidium iodide (PI) were from Sigma-Aldrich (St. Louis, MO). GM-CSF and IL-4 were from R&D System (MN). Except magnetic particle-conjugated CD14 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany), all other fluorochrome-conjugated antibodies of cell surface markers were from Becton Dickinson (BD, NJ). RPMI-1640 medium, fetal bovine serum (FBS) and other medium-related supplies were from Gibico, Invitrogen, CA. Ficoll-Pague solution was from Amersham Bioscience, Tokyo, Japan.

The Isolation of $CD14^+$monocytes

The buffy coat (provided by Chinese Blood Organization) was layered on the equal volume of Ficoll-Paque solution and centrifuged at 600*g* for 30 min at 18–20°C. The mononuclear blood cells were retrieved from the interphase. An aliquot (80 μl) of cells (1×10^7) was incubated with 20 μl of magnetic particle-conjugated anti-human CD14 antibodies for 20 min at 4°C. The mixture was set on a Magnetic Cell Sorter (BD) for 10 min at room temperature (RT) followed by several washes with RPMI 1640 media to remove non-$CD14^+$ cells. Then, the remaining bound $CD14^+$monocytes were collected and cultured in RPMI-1640 media containing 10% of FBS.

The Induction of MDMs and MoDCs

$CD14^+$monocytes (4×10^5/ml) were seeded in 3-cm or 10-cm culture plates and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 unit/ml of streptomycin/penicillin solution at 37°C. GM-CSF (10^3 IU/ml) combined with or without IL-4 (10^3 IU/ml), or PMA (10 nM) alone was added to culture media. Cells were then incubated for another 7 days to allow the differentiation of $CD14^+$monocytes into MDMs or MoDCs.

Flow Cytometric Analysis

Cells (2×10^5) were incubated separately with fluorochrome-conjugated antibodies against a panel of cell surface markers, includ-

ing CD14, CD11b (Mac-1), CD86, HLA-DR, and CD209 (DC-SIGN), for 45 min at 4°C. Cells were resuspended in Con's tube (BD) containing 200 μl of phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA) and then analyzed by Flow Cytometry using *Calibur* software (BD).

Liu's Stain

Cells (1×10^5) were spun onto slides by centrifugation at 1,000*g* for 2 min using a Cytospin apparatus. Cells were immediately stained with Liu A reagent for 30 s. Then, the Liu B reagent (2× volumes) was overlayed on the top of Liu A reagent and mixed well. After incubated for 90 s, the mixture of Liu reagents was discarded. The slides were further washed with running water to remove excessive reagent. Cell morphology was observed under microscope.

The Preparation of Membrane and Cytosol Protein Fractions

Membrane and cytosol protein fractions were purified using a commercial kit (PIERCE Biotech, Rockford) according to the manufacturer instructions. Briefly, Cells (1×10^7) were collected in a 1.5-ml eppendorf tube with 100 μl of buffer A (supplied in the kit). After incubated for 10 min at RT, an aliquot of 200 μl of buffer B/C (mixed 1 part of reagent B with 2 part of reagent C, supplied in the kit) was added, and followed by incubation for another 30 min on ice with vortexing every 5 min. After centrifuged at 10,000*g* for 2 min at 4°C, the supernatant was transferred into a new eppendorf tube and incubated at 37°C for 10 more min. Finally, the supernatant was further centrifuged at 10,000*g* for 2 more min at RT to separate the membrane fraction from cytosolic fraction in the hydrophobic and hydrophilic phases, respectively.

Western Blotting

Aliquots of membrane or cytosol proteins (100 μg) were boiled for 5 min in SDS sample buffer [62.5 mM Tris-base (pH 6.7), 1.25% SDS, 12.5% glycerol, and 2.5% β-mercaptoethanol]. Proteins were separated by SDS–PAGE on 10% gels and transferred to PVDF membrane. After blocked with 5% skim milk or 5% BSA (for avidin-biotin system) in Tris-buffered saline (TBS) plus 0.1% Tween-20 (TBST) for 1 h at RT, the PVDF membranes were incubated separately with designated antibodies specifically against each of PKC isoenzymes for 2 h at RT. The antibodies used including α, γ, δ, ε, η, θ, ι/λ (Transduction Laboratory, BD), β_I, β_{II}, and ζ (SantaCruz, CA), and GAPDH (Ambion, TX). The membranes were washed four times with TBST, and then incubated with respective HRP-conjugated secondary antibody for 1 h at RT. In some case, the primary antibody-treated transbloted membranes were further incubated with biotin-conjugated secondary antibody for 1 h at RT, followed by HRP-conjugated avidin complex (Vector Laboratories, CA) for another 30 min at RT. After washed four times with TBST, immunoreactive bands were visualized using ECL system (Amersham Bioscience). The densitometric analysis of immunoreactive band intensities was performed using Image^{plus} software (IBM, NY).

Confocal Microscopic Observation

Cells (1×10^5) were spun onto a round cover slide (22 mm in diameter and 0.17 mm in thickness, purchased from Sigma-Aldrich) at the speed of 1,000 rpm for 2 min at RT by using a Cytospin apparatus. Cells were then fixed with 4% formaldehyde for 15 min at RT. After washed twice with PBS, cells were treated with 95% EtOH/5% CH3COOH at −20°C for 15 min. Prior to blocking with 2% BSA/0.1% Triton X-100 for 2 h at RT, cells were washed twice with PBS. Subsequently, cells were incubated separately with PKCα or β_I antibody overnight at 4°C. After rinsed three times with PBS, cells were then incubated with biotin-conjugated secondary antibody (DAKO, Glostrup, Denmark) for 1 h at RT. After further washed three times with PBS, cells were incubated with Fluorescein-conjugated avidin complex (Vector Laboratories) for 30 min at RT. For nuclear staining, cells were incubated with PI for another 15 min at RT. After mounted with 50% glycerol/TBS (pH 7.0), cells were then observed with FluoView^{TM} Confocal Microscope Systems (Olympus, Tokyo, Japan).

TNF-α Determination

The concentration of tumor necrosis factor-α (TNF-α) in each collected medium was determined using a commercial Human TNF-α ELISA Kit (PIERCE Biotech) according to the manufacturer procedure.

Antigen Uptake Assay

Cells (4×10^5) were incubated with 10 μg/ml of fluorescein-labeled ovalbumin (Molecular Probe, Invitrogen, CA) for 1 h at 37°C. After incubation, cells were centrifuged at 1,000*g* at 4°C for 10 min and then resuspended in PBS/0.1% BSA. The uptake of fluorescein-labeled ovalbumin by cells was analyzed by Flow-Cytometry.

Phagocytosis Assay

Cells (4×10^5) were incubated with 50 μg of fluorescein-labeled *Escherichia coli* particles (Molecular Probe, Invitrogen) for 1 h at 37°C with gentle shaking. After incubation, cells were centrifuged at 1,000*g* at 4°C for 10 min and then resuspended in PBS/0.1% BSA. The phagocytosed fluorescein-labeled *E. coli* particles by cells were determined by Flow-Cytometry.

Statistical Analysis

Each datum point obtained from three independent experiments or an experiment of triplicate assay was presented as mean ± SEM. SEM. The statistical analysis was performed by One-way ANOVA and Duncan's Multiple Range Test.

RESULTS

The Morphological and Phenotypic Differences Among PMA-, GM-CSF-, and GM-CSF/IL-4-treated Human CD14$^+$monocytes

The induced differentiation-states of human CD14$^+$monocytes were examined morphologically and phenotypically after cells were cultured with PMA (10 nM), GM-CSF (10^3 U/ml), or GM-CSF/IL-4 (10^3 U/ml for each) for 7 days. Similar to GM-CSF, PMA induced the formation of adherent and fried egg-like cell types. On the other hand, the combination of GM-CSF and IL-4 caused CD14$^+$monocytes to differentiate into the semi-adherent cell type and formed cell clusters after cultured for 7 days (Fig. 1A). The results from Liu's stain revealed that the PMA- and GM-CSF-treated cells exhibited rough and irregular cell morphology, while both GM-CSF/IL-4-treated cells and untreated control cells showed smooth surface and round shape (Fig. 1A).

To characterize the differentiation-state of cytokine-treated CD14$^+$monocytes, various cell surface markers were used. CD14 is a marker for monocytes and abundantly presented on cell surface of monocytes. Mac-1, also called CD11b, is mainly presented on phagocytes. DC-SIGN, designated as CD209, is highly expressed by immature MoDCs [Colmenares et al., 2002] and also expressed by macrophages [Rappocciolo et al., 2006]. CD86 and HLA-DR are expressed by antigen-presenting cells. Our results demonstrated that the PMA- and GM-CSF-treated cells expressed a CD14^{low}, Mac-1$^+$, DC-SIGN$^+$, CD86$^+$ and HLA-DR$^+$ phenotype. On the other hand, GM-CSF/IL-4-treated cells showed a CD14$^-$, Mac-1$^+$, DC-SIGN^{high}, CD86$^{+/-}$ and HLA-DR^{high} phenotype (Fig. 1B).

The Activation of PKC Isoenzyme(s) by PMA- and GM-CSF-Triggered Signaling During the initial Stage of CD14$^+$monocyte Differentiation

By mimicking GM-CSF actions, PMA induced the differentiation of human CD14$^+$monocytes into macrophages. Experiments were performed to define the crucial PKC isoenzyme(s) activated during the early signaling of the GM-CSF-induced MDM differentiation. First of all, the constitutively expressed PKC isoenzymes in CD14$^+$monocytes were determined by Western blot analysis using respective specific antibodies for each PKC. As shown in Figure 2A, both conventional PKCs (α, β_I, β_{II}, and γ) and atypical PKCs (ι/λ and ζ) were detected in the lysate of CD14$^+$monocytes whereas novel δ, ε, η, and θ PKC isoenzymes were not detectable.

Because PMA is an analogue of diacylglycerol (DAG), it would selectively activate DAG-dependent PKCs. Consequently, which conventional PKC isoenzyme(s) is(are) activated in PMA- or GM-CSF-treated CD14$^+$monocytes was investigated. CD14$^+$monocytes were treated with PMA (10 nM) or GM-CSF (10^3 U/ml) for the designated time intervals. The isolated membrane and cytosolic proteins were analyzed by Western blotting using specific antibodies against PKCα, β_I, β_{II}, and γ. Figure 2B (left) shows that PMA induced the translocation of PKC α and γ (data not shown), but not β_I and β_{II} (data not shown) isoenzymes from the cytosol to cell membrane of CD14$^+$monocytes. However, the treatment of CD14$^+$monocytes with GM-CSF induced the translocation of only PKCα and reached a maximal level in the membrane fraction after 6 h of treatment (Fig. 2B, right). To further substantiate the translocation of PKCα from cytosol to cell membrane,

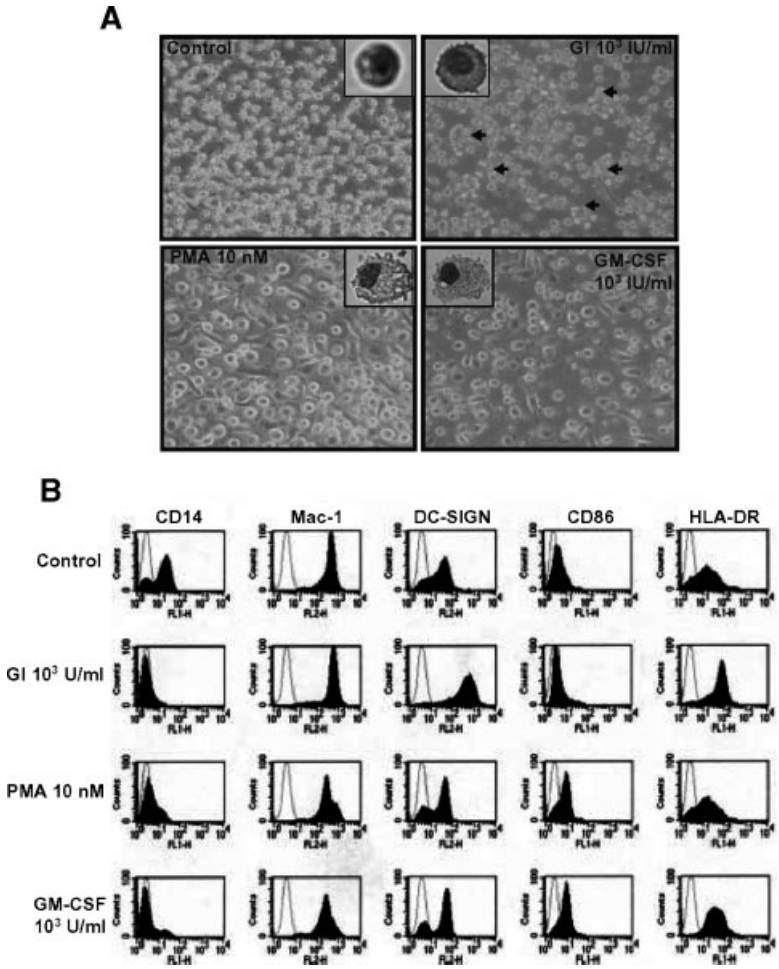

Fig. 1. The diverse morphology and phenotypes among the GM-CSF/IL-4-, PMA-, and GM-CSF-treated CD14$^+$monocytes. **A**: CD14$^+$monocytes were cultured with GM-CSF plus IL-4 (10^3 U/ml each) (GI), PMA (10 nM), or GM-CSF (10^3 U/ml) for 7 days. Cell morphologies were observed under an inverse microscope and appeared as shown at 400× magnification. The enlarged single cells in the inserts are cells processed with Liu's stain (fourfold enlargements of the original 400× magnified microscopic pictures). **B**: The phenotypic diversity was determined by Flow-cytometry using CD14, Mac-1, DC-SIGN, CD86, and HLA-DR specific monoclonal antibodies conjugated with fluorochrome. The X- and Y-axes represent the geometric fluorescent intensity and cell counts, respectively. In the histogram, the solid line and dark profile indicates the fluorescent intensity of the control isotype antibody and that of the designated antibody, respectively.

CD14$^+$monocytes were treated with GM-CSF for 6 h followed by fluorescence-immunocytochemical staining and Confocal Microscopic observation. As shown in Figure 2C, PKCα was clearly localized at the cell membrane after CD14$^+$monocytes were treated with GM-CSF for 6 h. In contrast, the fluorescence of immunostained PKCα proteins in the CD14$^+$monocytes without GM-CSF treatment was evenly distributed within the cytoplasm.

The Effects of PKC Inhibitors on the GM-CSF-Induced PKCα Translocation and MDM Formation

In order to delineate the essential role of PKCα in MDM differentiation, Go6976, a selective inhibitor for PKCα/β_I [Martiny-Baron et al., 1993], was used to block the GM-CSF-induced PKCα activation. Go6976 (0.1 or 1 μM) significantly ($P < 0.05$, n = 3) inhibited the GM-CSF-induced generation of CD14^{low}Mac-1$^+$MDM population as analyzed by Flow cytometry analysis (Fig. 3A,B). The effects of Go6976 on inhibiting the GM-CSF-induced generation of CD14^{low}Mac-1$^+$MDM population were dose-dependent (Fig. 3B). Go6976 exhibited a slight inhibition (17.5%) at 0.1 μM and 95% inhibition at 1 μM on the GM-CSF-induced generation of CD14^{low}Mac-1$^+$ MDM population. To further substantiate only the activation of PKCα is essential for the GM-CSF-induced MDM differentiation, a PKCβ-specific inhibitor,

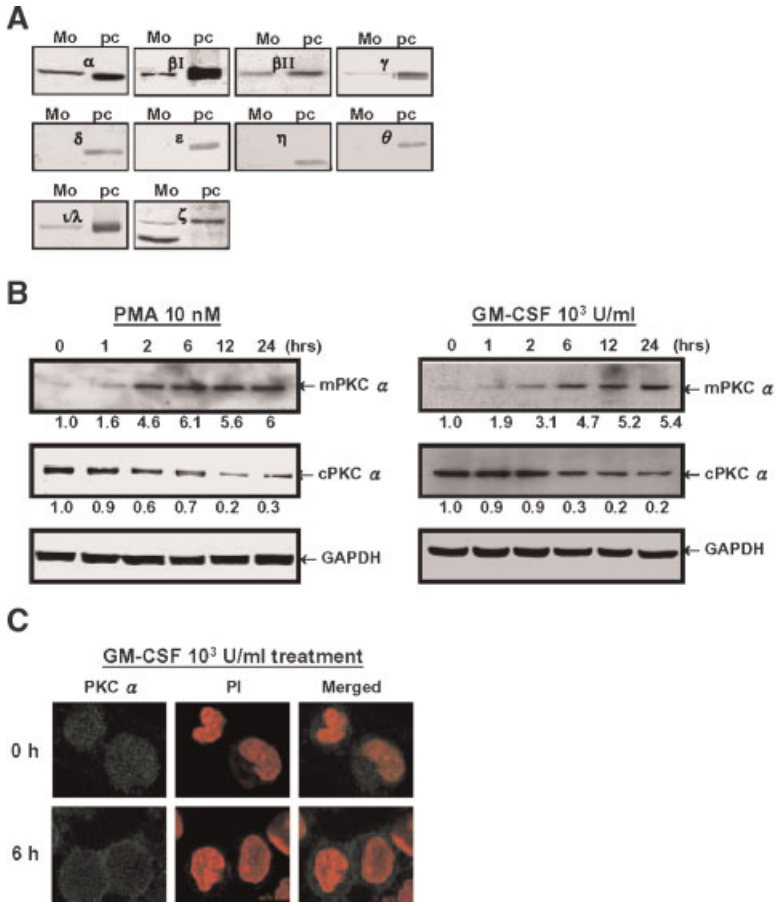

Fig. 2. The PMA- and GM-CSF-induced PKC translocation in $CD14^+$monocytes. **A**: Homogenated cell lysates (100 μg) were subjected to Western blot analysis using specific antibodies of respective PKC isoenzymes. ''Mo'' and ''pc'' represents the soluble proteins of $CD14^+$monocyte and PKC positive control cell lysates, respectively. The cell lysates of rat brain cells was used as positive controls for PKC α, β_I, β_{II}, γ, δ, ε, ι/λ, and ζ; that of WI38 cells was for PKC η; and that of Jurkat cells was for PKC θ. **B**: Fractionated membrane (100 μg) and cytosolic (100 μg) proteins from $CD14^+$monocytes treated with PMA or GM-CSF for designated intervals were analyzed by Western Blotting using PKCα monoclonal antibody. GAPDH was used as an internal control of cytosolic protein loading. Relative densities of protein bands were calculated and shown below the respective protein bands. **C**: After immunofluorescent staining with PKCα-specific antibody (green), the translocation of PKCα in $CD14^+$monocytes at 6 h post-treatment with GM-CSF was observed under Confocal Microscope (400× microscopic magnification for photoshot, followed by a fourfold enlargement for the print). Propidium Iodide (red) was used to stain the chromosomal DNA. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

3-(1-(3-Imidazol-1-yl-propyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione [Tanaka et al., 2004], was employed. The exposure of $CD14^+$monocytes to PKCβ-specific inhibitor did not affect the GM-CSF-induced generation of $CD14^{low}$Mac-1^+MDM population (Fig. 3A,B). On the other hand, the GM-CSF-induced generation of $CD14^{low}$Mac-1^+MDM population was not affected by the pretreatment of $CD14^+$monocytes with 0.1% (v/v%) dimethylsulfoxide (DMSO) (Fig. 3A,B), the solvent of Go6976 and PKCβ-specific inhibitor stock solutions.

GM-CSF was reported to stimulate the secretion of TNF-α from the $CD14^+$monocyte-derived cell types [Lu and Li, 2002]. Therefore, we examined the effects of Go6976 and PKCβ-specific inhibitor on the GM-CSF-induced TNF-α secretion in the media of 7-day cultured $CD14^+$monocyte-derived cells. PMA or GM-CSF significantly ($P < 0.05$, $n = 3$) induced a 10-fold elevation of TNF-α secretion into media (Fig. 4A). Go6976, not PKCβ-specific inhibitor and DMSO, significantly ($P < 0.05$, $n = 3$) reduced the GM-CSF-induced TNF-α secretion by $CD14^+$monocyte-derived cells in a dose-dependent manner (Fig. 4A). Go6976 at 0.1 μM reduced about 45% of the GM-CSF-induced TNF-α secretion while 1 μM Go6976 almost abolished the induced TNF-α secretion (reduced to 6.6%). Furthermore, Go6976 at

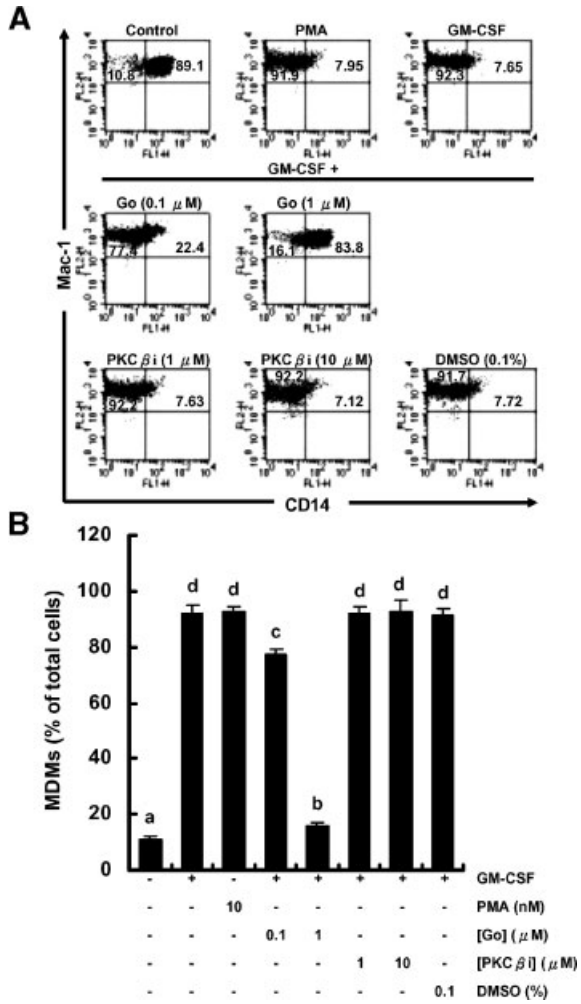

Fig. 3. The effects of Go6976 and PKCβ-specific inhibitor on GM-CSF-induced MDM formation. **A**: CD14$^+$monocytes were pre-incubated with or without Go6976 (Go), PKCβ-specific inhibitor (PKCβ$_I$) or DMSO at indicated doses for 40 min at 37°C prior to the GM-CSF treatment for 7 days. CD14$^+$monocytes were also treated with PMA (10 nM) alone for 7 days. On day 7, cells were collected and stained with FITC-conjugated CD14 and PE-conjugated Mac-1 antibodies. The stained cells were then analyzed by Flow-Cytometry. The length of FL1-H and the height of FL2-H represent the fluorescent intensities of FITC-CD14 and PE-Mac-1, respectively. **B**: The mean percentage of CD14^{low}-Mac-1$^+$macrophage population (upper left of dot plot figures) in the total cells derived from three independent experiments of each culture condition is shown as mean ± SEM. Different letters in serial sequences above the columns indicate significant differences between the means ($P < 0.05$). DMSO at 0.1% was used as solvent for both inhibitors.

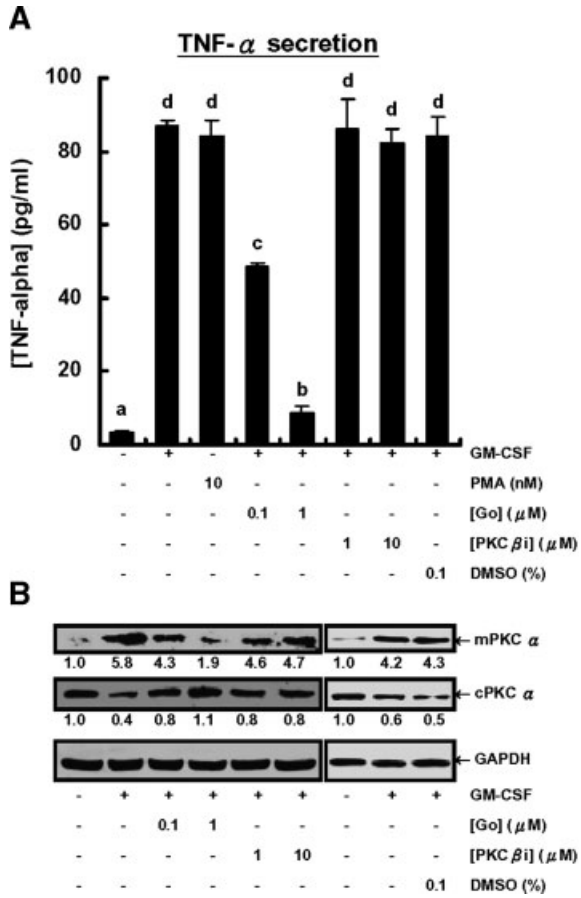

Fig. 4. The effects of Go6976 and PKCβ-specific inhibitor on the GM-CSF-induced secretion of TNF-α by CD14$^+$monocyte-derived macrophages after 7 days of culture. **A**: CD14$^+$monocytes were pretreated with or without Go6976 (Go), PKCβ-specific inhibitor (PKCβ$_I$), or DMSO at indicated doses for 40 min at 37°C prior to the GM-CSF treatment for 7 days. CD14$^+$monocytes were also treated with PMA (10 nM) alone for 7 days. On day 7, the culture media were colleted and subjected to the measurement for TNF-α concentration. Each column heights were derived from triplicate assays and presented as mean ± SEM. Different letters above the columns indicate significant differences between the means of consecutive sequences at $P < 0.05$. **B**: The fractionated membrane (100 μg) and cytosolic (100 μg) proteins of CD14$^+$monocytes with designated treatments for 6 h were analyzed by Western Blotting using specific PKCα antibody. GAPDH was used as an internal control of protein loading. The relative densities of protein bands were calculated and shown the below respective protein bands. DMSO at 0.1% was served as solvent for the inhibitors.

1 μM predominantly blocked the PKCα translocation (reduced to about one fifth of the GM-CSF-activated PKC translocation level) in GM-CSF-treated CD14$^+$monocytes after 6 h of exposure (Fig. 4B). However, the pretreatment with PKCβ-specific inhibitor or 0.1% DMSO did not change the GM-CSF-induced PKCα translocation. Consequently, the crucial role of PKCα in the GM-CSF-induced initial signaling during the differentiation of MDM was defined.

The Effects of Go6976 and PKCβ-Specific Inhibitor on GM-CSF/IL-4-Induced MoDC Differentiation

As to which PKC isoenzymes are associated with the GM-CSF/IL-4-induced MoDC differentiation was also explored. First, we examined the effects of Go6976 and PKCβ-specific inhibitor on the GM-CSF/IL-4-induced generation of $CD14^-$ DC-$SIGN^{high}$MoDC population by Flow-Cytometry analysis. Go6976 also significantly ($P < 0.05$, n = 3) reduced the GM-CSF/IL-4-induced generation of $CD14^-$DC-$SIGN^{high}$ MoDC population from $CD14^+$monocytes after 7 days of culture (Fig. 5A,B). At 1 μM, Go6976 inhibited approximately 60% of the GM-CSF/IL-4-generated MoDC population. Distinct from the effect of the GM-CSF-induced MDM differentiation, PKCβ-specific inhibitor exhibited a dose-dependent suppression ($P < 0.05$, n = 3), up to 85% repression observed at 10 μM concentration, in the GM-CSF/IL-4-induced generation of $CD14^-$DC-$SIGN^{hihg}$MoDC population (Fig. 5A,B). The pretreatment with DMSO (0.1%) did not affect the GM-CSF/IL-4-induced generation of $CD14^-$DC-$SIGN^{hihg}$MoDC population (Fig. 5A,B).

Immature MoDCs are thought to be the professional antigen processing machines in the acquired immunity [Mellman and Steinman, 2001]. However, the immature MoDCs exhibit a reduced capacity for bacterial phagocytosis as compared to those of $CD14^+$monomonocytes [Lu and Li, 2002]. Therefore, the effects of both PKC inhibitors on those immature MoDC immune functions were examined. Cells from designated experiments were incubated with FITC-labeled ovalbumin followed by Flow-Cytometry analysis to estimate their capacity for antigen uptake. The immature MoDCs exhibited about a fivefold increase in their capacity for antigen uptake than did the untreated $CD14^+$monocytes (Fig. 6A). The pretreatment of $CD14^+$monocytes with Go6976 or PKCβ-specific inhibitor, not DMSO (0.1%), dose-dependently ($P < 0.05$, n = 3) reduced the capacity of GM-CSF/IL-4-treated cell populations for the antigen uptake (Fig. 6A). Especially, the exposure of $CD14^+$monomonocytes to 10 μM of PKCβ-specific inhibitor completely abolished the GI-induced capacity of MoDCs for the antigen uptake, comparable to that of the untreated $CD14^+$monocytes.

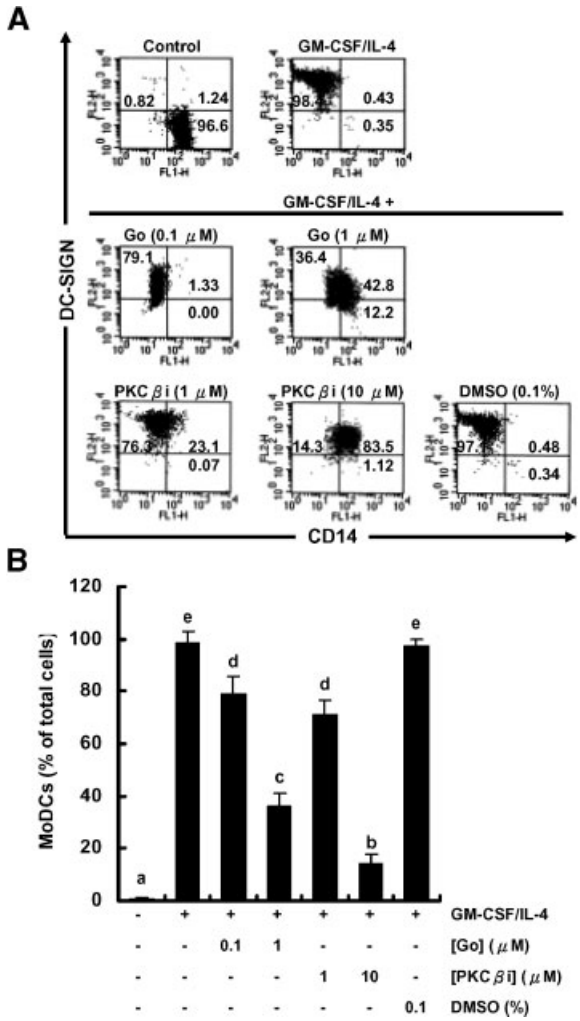

Fig. 5. The effects of Go6976 and PKCβ-specific inhibitor on $CD14^+$monocyte- derived MoDC differentiation. **A**: Prior to the administration of GM-CSF/IL-4 (10^3 U/ml each), $CD14^+$monomonocytes were pretreated with or without Go6976, PKCβ-specific inhibitor ($PKC\beta_I$), or DMSO for 40 min at 37°C. On day 7, cells were collected and stained with FITC-conjugated CD14 and PE-conjugated DC-SIGN antibodies. The stained cells were then analyzed by Flow-Cytometry. The fluorescent intensities of FITC and PE are indicated as FL1-H and FL2-H in the graphs, respectively. **B**: The percentages of $CD14^-$DC-$SIGN^{high}$ MoDC population (upper left of the dot plot figures) in total cells of three independent experiments are shown as means ± SEM. Different letters above the columns indicate significant differences between the means with consecutive consequences at $P < 0.05$. DMSO at 0.1% was used as solvent for both inhibitors.

To evaluate the cellular ability of bacterial phagocytosis, cells from the designated experiments were incubated with FITC-labeled *E. coli* particles. Their capacity for phagocytosing FITC-labeled *E. coli* particles was determined by Flow-Cytometry. The results revealed that the GM-CSF/IL-4-induced immature MoDCs

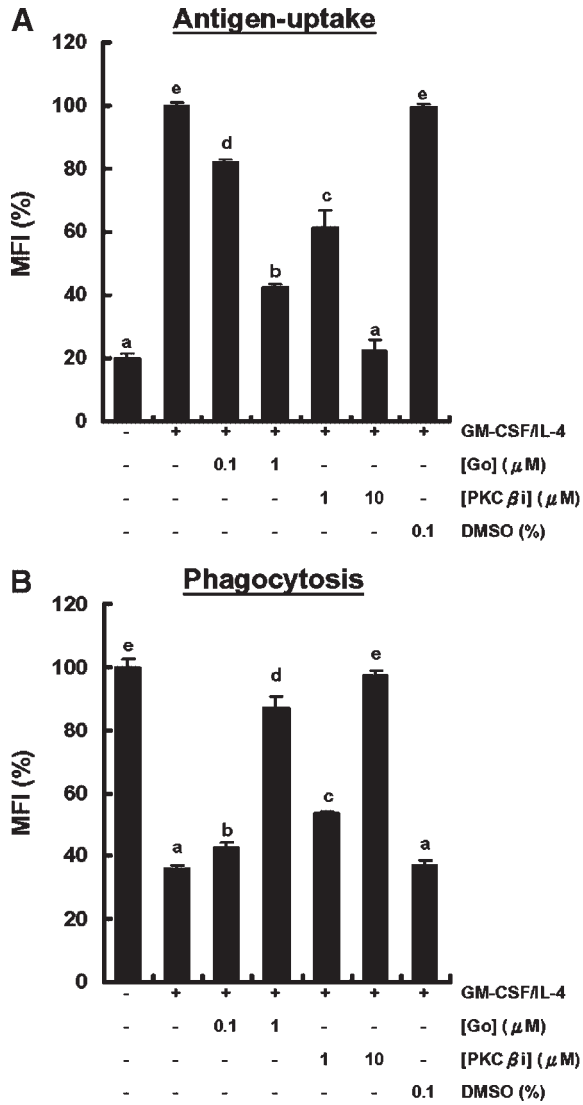

Fig. 6. The effects of Go6976 and PKCβ-specific inhibitor on immune functions of CD14$^+$monocyte-derived DCs. After cultured for 7 days as describe in Figure 5, cells from designated experiments were separately incubated with FITC-labeled ovalbumin (**A**), or *E. coli* particles (**B**) for 1 h at 37°C. The fluorescent intensities of endocytosed FITC-labeled ovalbumin or *E. coli* particles by various groups of cells were determined by Flow-Cytometry. The mean percentages derived from fluorescent intensities [MFI (%)] of three independent experiments are presented as means ± SEM. Different letters above the columns indicate significant differences between the means with consecutive consequences at $P < 0.05$.

lost about 60% of their capability for bacterial phagocytosis as compared with that of the untreated CD14$^+$monocytes (Fig. 6B). However, the pretreatment with Go6976 or PKCβ-specific inhibitor, not DMSO (0.1%), significantly ($P < 0.05$, n = 3) reverted the reduced immature MoDC capacity for bacterial phagocytosis (Fig. 6B). The reversion caused by the inhibitors was dose-dependent, indicating the GM-CSF/IL-4-induced generation of MoDCs was inhibited by the inhibitors.

The Crucial PKC Isoenzyme(s) Involved in the GM-CSF/IL-4-Induced Initial Signaling for the Generation of MoDCs

The above data indicate that either Go6976 or PKCβ-specific inhibitor suppressed the GM-CSF/IL-4-induced MoDC differentiation from CD14$^+$monocytes. To ascertain the activation of which PKC isoenzyme(s) (α, β_I or both) is(are) the crucial early events for MoDC differentiation, the isolated membrane and cytosol proteins of CD14$^+$monocytes treated with GM-CSF/IL-4 for designated intervals were subjected to Western blot analysis. Figure 7A shows that GM-CSF/IL-4 induced the translocation of PKCβ_I, but neither PKCα nor PKCβ_{II} (data not shown), from cytosol to plasma membrane of CD14$^+$monocytes. The translocation of PKCβ_I was observed at 1 h, reached the maximal level at 6 h, and sustained at least till 24 h of GM-CSF/IL-4 treatment (Fig. 7A). Subsequently, Confocal Microscopy of immunofluorescence-stained cells revealed that concentrated PKCβ_I distributed around cell membrane after CD14$^+$monocytes were treated with GM-CSF/IL-4 for 6 h (Fig. 7B). Furthermore, the pretreatment of CD14$^+$monocytes with either Go6976 or PKCβ-specific inhibitor, not DMSO (0.1%), dose-dependently inhibited the GM-CSF/IL-4-induced PKCβ_I translocation after 6 h of treatment (Fig. 7C).

DISCUSSION

The present study demonstrated that CD14$^+$monocytes could not differentiate into MDMs when the GM-CSF-induced translocation of PKCα from cytosol to plasma membrane was blocked by Go6976. On the other hand, phorbol-12-myristate-13-acetate (PMA) replicated the effect of GM-CSF on MDM differentiation and PKCα translocation. Go6976 also abolished the PMA-induced MDM differentiation as judged by alterations in cell surface markers and TNF-α secretion (unpublished results). These data reveal a crucial role of PKCα in MDM differentiation from CD14$^+$ monocytes. Numerous studies in the literature

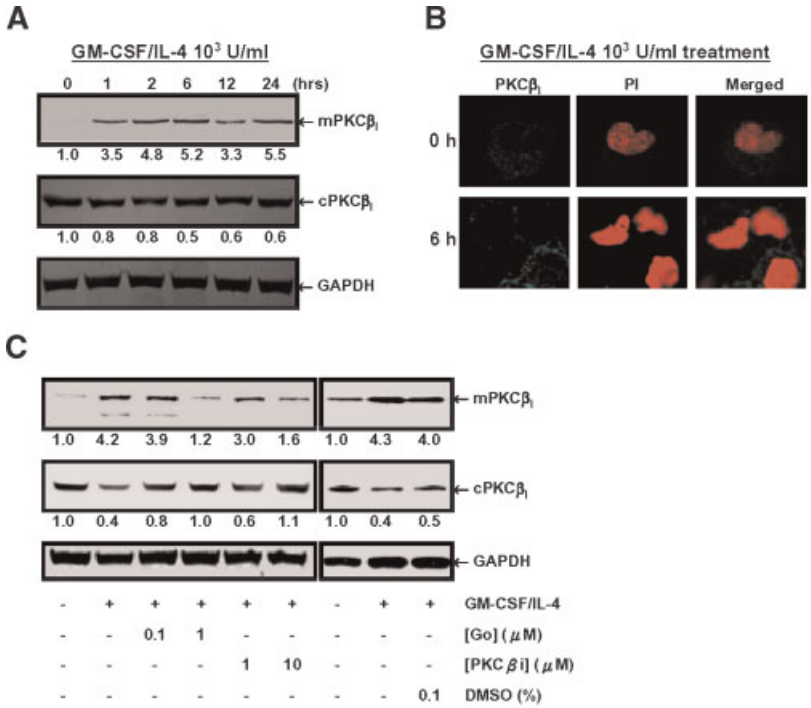

Fig. 7. The GM-CSF/IL-4-induced PKCβ translocation. **A**: $CD14^+$monocytes were treated with GM-CSF/IL-4 (10^3 U/ml each) for the designated time intervals. The fractionated membrane (100 μg) and cytosolic (100 μg) proteins were analyzed by Western Blotting using specific antibody of $PKC\beta_I$. GAPDH was used as internal indicator for the amount of protein loading. The relative densities of protein bands were calculated and shown below the respective protein bands. **B**: $CD14^+$ monocytes treated with or without GM-CSF/IL-4 for 6 h were stained with $PKC\beta_I$ specific antibody. The distributions of fluorescence-labeled $PKC\beta_I$ (green) were observed with the aid of a Confocal Microscope (at 400× microscopic magnification followed by a fourfold enlargement in the print). Propidium Iodide (red) was used to stain the chromosomal DNA. **C**: $CD14^+$monocytes were pretreated with or without Go6976 (Go), PKCβ-specific inhibitor ($PKC\beta_I$), or DMSO at the indicated doses for 40 min prior to the GM-CSF/IL-4 treatment for 6 h. The fractionated membrane (100 μg) and cytosolic (100 μg) proteins were analyzed by Western Blotting using specific antibody of $PKC\beta_I$. GAPDH was used as internal control of protein loading. The relative densities of protein bands were calculated and shown below the respective protein bands. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

also have reported the essentiality of PKCα activation for the differentiation of macrophage-like cells from various progenitor cell types [Schwende et al., 1996; Pierce et al., 1998; Dieter and Schwende, 2000]. In THP-1 monocytic cell line, PMA also induced the translocation of PKCα and elevated the secretion of TNF-α during the macrophage differentiation [Schwende et al., 1996]. The reduction in PKCα expression by its antisense cDNA inhibited the PMA-induced THP-1 cell differentiation toward macrophage-like cells and elevation of TNF-α secretion [Dieter and Schwende, 2000]. In addition, the differentiation of HL-60, human promyelocytic leukemia cells, into macrophage-like cells in response to PMA has been shown to be correlated with the up-regulation of PKCα [Edashige et al., 1992]. Other study also demonstrated that PMA promptly induced macrophage differentiation from PKCα-over-expressed mouse myeloid progenitor cell line which does not differentiate in response to PMA in the wild-type condition [Mischak et al., 1993]. Furthermore, the direct transfection of human hematopoietic progenitor cells with constitutively active form of PKCα also induced the macrophage differentiation [Pierce et al., 1998]. All these findings clearly demonstrate that the activation of PKCα is a crucial cell fate determining event for the generation of macrophages from their progenitor cells.

Our results reveal that $PKC\beta_I$ is a key molecule to initiate the GM-CSF/IL-4-induced differentiation of $CD14^+$monocytes towards MoDCs. The treatment of $CD14^+$monocytes with GM-CSF and IL-4 induced the translocation of $PKC\beta_I$, not PKCα, from cytosol to plasma membrane in the early event of MoDC differentiation. The blockage of $PKC\beta_I$ translocation by pretreatment with PKCβ specific inhibitor,

3-(1-(3-Imidazol-1-yl-propyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione, restricted the MoDC differentiation from CD14$^+$monocytes as verified by the changes in phenotypes and immune functions of GM-CSF/IL-4-differentiated MoDCs. Other researchers reported that PMA treatment induced the differentiation of CD34$^+$ hematopoietic progenitor cells to become bone marrow-derived DCs [Davis et al., 1998], another DC population [Liu, 2001]. The PKCβ_{II}-dependent signaling pathway was recently identified to mediate the differentiation of bone marrow-derived DCs [Cejas et al., 2005]. These findings demonstrate the critical role of PKCβ isoenzymes in the cytokine-induced DC differentiation from their progenitor or precursor cells.

According to Martiny-Baron et al. [1993] Go6976 inhibited the PKCα and β_I at nanomolar concentrations, whereas even micromolar concentrations of Go6976 had no effect on the activity of PKC isoenzymes δ, ε, and ζ. That Go6976 inhibited the GM-CSF-induced generation of CD14^{low}Mac-1$^+$ MDMs and the elevation of TNF-α secretion were resulted from the suppression of PKCα-dependent signaling pathway occurred early in the event of GM-CSF-induced MDM differentiation. To further verify that the specific induction of PKCα translocation cause macrophage differentiation, a PKCβ-specific inhibitor, 3-(1-(3-Imidazol-1-yl-propyl)- 1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione, was employed as negative control. The PKCβ-specific inhibitor did not affect the GM-CSF-induced PKCα translocation and MDM differentiation at all. These data further demonstrate the crucial role of PKCα-dependent signaling in the early event of MDM differentiation from CD14$^+$monocytes.

The pretreatment of CD14$^+$monocytes with Go6976 efficiently reduced the GM-CSF/IL-4-inducd MoDC differentiation. Although the PKCβ specific inhibitor did not affect the MDM differentiation, it predominantly inhibited the GM-CSF/IL-4-induced MoDC differentiation. Furthermore, GM-CSF/IL-4 obviously induced the translocation of PKCβ_I but not PKCα from cytosol to plasma membrane early in the MoDC differentiation by Western blot analysis. By Confocal Microscopic investigation, PKCβ_I was concentrated around plasma membrane, whereas PKCα (data not shown) was evenly distributed in the cytoplasm after CD14$^+$monocytes were treated with GM-CSF/IL-4. These results suggested that the suppression of PKCβ_I activity is correlated with the restriction of MoDC differentiation. In accord with the study by Terao et al., the PKCβ specific inhibitor efficiently reduced the activities of PKCβ_I and β_{II}, and exhibited a 15- and 60-fold, respectively, greater inhibitory effects on PKCβ_I and PKCβ_{II} than on PKC α, PKC γ, and PKC ε [Tanaka et al., 2004]. However, our results from Western blot analysis and Confocal Microscopic observation (data not shown) showed that the treatment with GM-CSF/IL-4 did not activate the PKCβ_{II} translocation in CD14$^+$monocytes. Therefore, the activation of PKCβ_I-dependent signaling pathway is crucial for the cell fate of CD14$^+$monocytes towards MoDCs.

The present study clearly demonstrates that the commitment of CD14$^+$monocytes to the differentiation path towards either MDMs or MoDCs is dictated by the selective translocation/activation of PKCα or PKCβ_I, respectively. These two intracellular PKC isoenzymes play pivotal roles in determining the cell fate of CD14$^+$monocytes towards either antigen-presenting cell types. This study provides a feasible strategy to manipulate the fate of CD14$^+$monomonocytes towards either MDMs or MoDCs through modulating the intracellular PKCα or PKCβ_I activity. Specific pharmacological activator for each individual PKC isoenzyme is currently not available. However, these results further offer an insight into the potential of manipulating CD14$^+$monocyte differentiation towards designated cell types (MDMs, MoDCs or other cells) for future medical/pharmaceutical applications.

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