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Original Article

CD44 clustering is involved in monocyte differentiation

Guoliang Zhang[1†], Huizhen Zhang[2†], Yiwen Liu[1], Yiqing He[1], Wenjuan Wang[1], Yan Du[1], Cuixia Yang[1*], and Feng Gao[3*]

[1]Department of Molecular Biology, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China
[2]Department of Pathology, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China
[3]Department of Molecular Biology and Clinical Laboratory, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China
[†]These authors contributed equally to this work.
*Correspondence address. Tel: +86-21-64369181 Ext 58752; Fax: +86-21-63701361; E-mail: dr.steven@163.com (C.Y.)/Tel: +86-21-64369181 Ext 58702; Fax: +86-21-63701361; E-mail: gao3507@126.com (F.G.)

Differentiation of monocytes into macrophages is an important process under physiological and pathological conditions, but the underlying mechanism of monocyte differentiation is not completely clear. Some adhesion molecules have been reported to play an important role in cell differentiation. CD44 is an important adhesion molecule that mediates cell–cell and cell–matrix interaction, and participates in a wide variety of cellular functions. As CD44 has been reported to show different activated states between monocytes and macrophages, we propose that CD44 may be involved in monocyte differentiation. In this study, we explored the role of CD44 in monocyte differentiation and further studied the mechanisms that were involved in. THP-1 cells (human monocytic leukemia cell line) were induced with phorbol 12-myristate 13-acetate (PMA) to establish the model of monocyte differentiation *in vitro*. It was found that CD44 expression and binding capacity to hyaluronic acid were increased significantly, and the distribution of CD44 was converted into clusters during differentiation. The PMA-induced CD44 clustering and CD44 high expression were suppressed by blocking CD44, which resulted in the inhibition of CD14 expression. PMA-induced phosphorylation of ERK1/2 signal was also suppressed by blocking CD44. Our results suggested that CD44 was involved in monocyte differentiation. The mechanisms of monocyte differentiation following CD44 activation may include CD44 high expression and clustering which in turn lead to phosphorylation of ERK1/2.

Keywords monocyte differentiation; CD44; activated state; clustering; p-ERK1/2

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Introduction

Monocyte is a type of white blood cell in peripheral blood, and a part of the innate immune system. When monocytes migrate into the tissues, they mature into different types of macrophages at different anatomical locations. Macrophages play important roles in the inflammatory and repair processes associated with tissue injury through the ability to exercise diverse functions, including phagocytosis and release of proteases, cytokines, mesenchymal growth factors, and other mediators [1]. However, the underlying mechanism of monocyte differentiation is not completely clear. Considering the difference between monocytes and macrophages and the important role of macrophages in immune response, it is of importance to study the molecules involved in monocyte differentiation. During the maturation of monocytes into macrophages, there are characteristic changes in the expression of adhesion molecules, such as integrins, immunoglobulin (Ig)-related selectins, and other adhesion molecules [2]. Some adhesion molecules have been reported to participate in cell differentiation, such as integrins, E-cadherin, immunoglobulin superfamily recognition molecule L1, and CD44 [3–6].

CD44 is an important adhesion molecule that mediates cell–cell and cell–matrix interaction, and participates in a wide variety of cellular functions such as lymphocyte recirculation and homing, T-cell activation, hematopoiesis, and tumor metastasis [7–10]. CD44 is a major cell surface receptor for hyaluronic acid (HA), which has an N-terminal link module homology domain that is responsible for binding to HA [11]. Some CD44-positive cells fail to exhibit HA-binding capacity, but can be induced to interact with HA when activated by inflammatory stimuli. Through interaction with HA, CD44 is involved in a variety of biological functions of macrophages, such as adhesion to extracellular matrix, phagocytosis, migration to inflammatory sites, and secretion of cytokines [12–15]. However, CD44 in freshly isolated peripheral blood monocytes is in a quiescent state and does not have the capability to bind HA until they differentiate into mature macrophages [16,17]. This phenomenon indicated that CD44 in monocytes may be activated during differentiation, but the underlying mechanism is unknown. Recent studies have shown that

CD44 also plays an important role in cell differentiation [6,18]. These findings suggest that CD44 may involve in monocyte differentiation.

THP-1, a human monocytic leukemia cell, can be converted into mature cells with functions of macrophages after treatment with phorbol 12-myristate 13-acetate (PMA) [19]. This cell line is usually used as a model for the study of maturation or differentiation from monocytes to macrophages. In this study, we stimulated THP-1 cells with PMA to establish the model of monocyte differentiation *in vitro* [19,20]. Then, CD44 activation and alterations were investigated in this monocyte differentiation model. It was found that CD44 became activated by binding with its ligand, HA, and the distribution of CD44 was converted into clusters. To determine its role in monocyte differentiation, CD44 was blocked during differentiation. Our data suggested that CD44 may play a vital role in monocyte differentiation. To further study the mechanism underneath CD44-induced monocyte differentiation, we also analyzed the phosphorylation of ERK1/2 which may signal the inner activation of cellular differentiation.

Materials and Methods

Reagents

RPMI1640 medium was purchased from Gibco (Carlsbad, USA). PMA was obtained from Merck (San Diego, USA). Anti-CD44 mAb and anti-CD14 mAb for flow cytometry analysis were purchased from eBioscience (San Diego, USA). Purified NA/LE mouse anti-CD44 (clone IM7) was obtained from eBioscience. Fluorescence-labeled HA (FL-HA) was obtained from Calbiochem (San Diego, USA). Anti-phospho-ERK1/2 mAb and anti-CD44 mAb were purchased from Cell Signaling Technology (Beverly, USA). Anti-GAPDH antibody, horseradish peroxidase (HRP)-conjugated polyclonal secondary antibodies were obtained from Jackson ImmunoResearch Company (West Grove, USA). Enhanced plus chemiluminescence assay kit and BS^3 Crosslinker were purchased from Thermo Scientific (Waltham, USA). FITC-labeled phalloidin was from Sigma (St Louis, USA). All other chemicals were of reagent grade or higher.

Cell culture and differentiation

THP-1 cell line (human monocytic leukemia cell line) was purchased from American Type Culture Collection (ATCC, Manassas, USA). Cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in the presence of 5% CO_2. THP-1 cells (5×10^5/ml) were differentiated using 320 nM PMA (Merck) for 72 h.

Immunocytochemistry analysis of F-actin distribution

THP-1 cells were seeded in 24-well plates, treated with or without PMA for 72 h. Then, the cells were fixed with 4% paraformaldehyde for 10 min and washed two times by phosphate-buffered saline (PBS). The cells were incubated with FITC-labeled phalloidin overnight at 4°C. After incubation, the cells were washed three times with PBS, and the F-actin was visualized by the inverted fluorescence microscope.

Flow cytometry analysis

Cultured cells were harvested and washed with washing buffer (PBS supplemented with 2% bovine serum albumin, pH 7.4). For detection of cell surface markers, monoclonal mouse anti-human antibodies CD14-APC and CD44-PE or the relevant isotypes were incubated with samples containing 1×10^6 cells for 30 min at room temperature. After incubation, the samples were washed three times and resuspended in PBS. Ten thousands cells were analyzed per sample in a flow cytometer (Beckman-Coulter, Brea, USA).

Binding of exogenous HA

Cells were incubated with 40 μg/ml FL-HA (Calbiochem) for 1 h at 37°C, and then the cells were washed three times with PBS. After fixation with 1% paraformaldehyde, plasma membrane-bound FL-HA was observed under an inverted fluorescence microscope (Olympus IX70, Tokyo, Japan) and analyzed using a flow cytometer (Beckman-Coulter).

Chemical cross-linking for CD44 association at the cell surface

Cells were treated with or without PMA for 72 h, and then the cells were washed three times with ice-cold PBS. CD44 cross-linking was performed by incubation with 2 mM BS^3 for 1 h at 4°C and quenched by incubation with 20 mM Tris, pH 7.5, for 15 min at room temperature [21]. Cells were washed twice with PBS and lysed with cell lysis buffer.

Western blot analysis

Cells were harvested and homogenized in ice-cold RIPA lysis buffer. Total cell lysates were collected, and equal quantities of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride (PVDF) membrane. The PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk powder for 1 h at room temperature, and then incubated with CD44 mAb (1 : 1000), phospho-ERK1/2 mAb (1 : 1000), or GAPDH mAb (1 : 1000) overnight at 4°C. Membranes were washed with TBS/T for three times (5 min each time) and incubated with HRP-conjugated polyclonal secondary antibody (1 : 5000) for 1 h at room temperature. The membranes were developed with the enhanced plus chemiluminescence assay kit according to the manufacturer's instructions. The intensity of the individual band was quantified by densitometry (Image-Pro Plus 6.0) and normalized to the corresponding input control (GAPDH) band.

Statistical analysis

The data presented are the representative of three independent biological replicates. Data are presented as mean ± standard deviation. All statistical analysis was performed using SPSS 16.0. Statistical significance was examined by unpaired Student's *t*-test and one-way analysis of variance (ANOVA) with *post hoc* test. $P < 0.05$ was considered significant.

Results

Establishment of monocyte differentiation model *in vitro*

The differentiation of THP-1 cells was assessed by observing the change of morphology and measuring the increase of CD14 antigen expression which is specific to the mature monocyte and macrophages. THP-1 cells showed a large, round, and single-cell morphology, grown in suspension

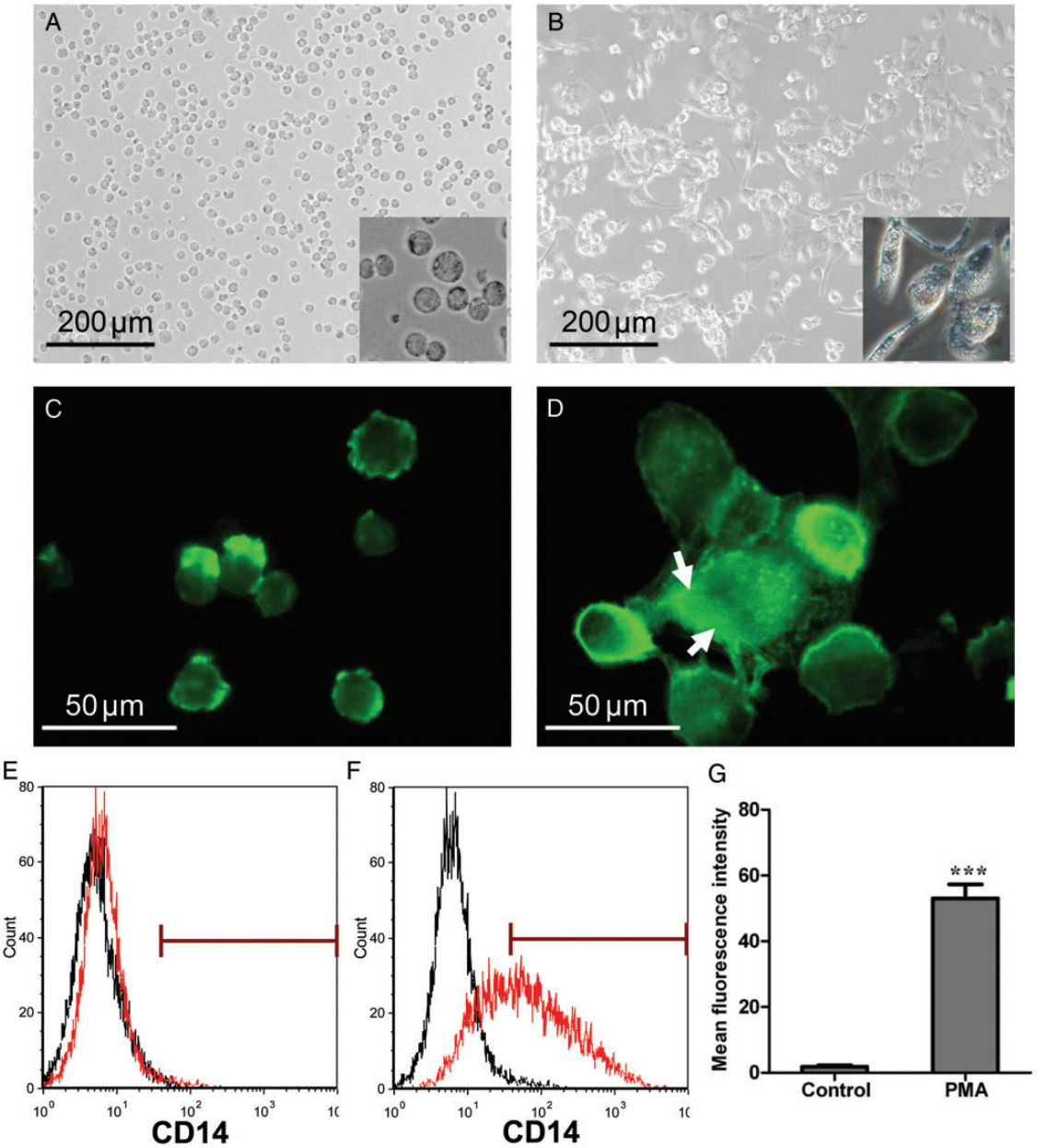

Figure 1. PMA induced THP-1 cells differentiation into macrophages (A,B) THP-1 cells develop characteristic macrophage morphology after exposure to PMA. THP-1 suspension cells were treated with RPMI 1640 (A) or PMA at 320 nM (B) for 72 h. THP-1 cells became adherent and showed macrophage-like morphology. The representative microscopy images are shown. (C,D) F-actin redistribution in THP-1 cells. THP-1 cells were treated with RPMI 1640 (C) or PMA at 320 nM (D) for 48 h. After treatment, cells were fixed, and then stained using FITC-labeled phalloidin. Fluorescence microscopy was used to visualize the rearrangement of F-actin and the changes in cell morphology. Cells treated with PMA had more intense F-actin stress fibers (arrows) and extended pseudopodia. (E–G) Expression of CD14 on THP-1 cells. THP-1 cells were stimulated with or without PMA (320 nM) for 72 h. Cell surface expression of CD14 in THP-1 cells was assessed by flow cytometry. Histograms indicate log fluorescence intensity (*x*-axis) vs. relative cell number (*y*-axis). Cells were stained with mAb against CD14 (red line) or with isotype control Ig (black line). (E) Control cells. (F) PMA-treated cells. (G) MFI of cells stained with anti-CD14 mAb (isotype controls subtracted). ***$P < 0.001$ compared with the control sample.

(**Fig. 1A**). After treatment with PMA, THP-1 cells became adherent and aggregated in clusters. The adherent cells displayed hypertrophy, developed vacuoles in the cytoplasm, and extended pseudopodia (**Fig. 1B**). F-actin reorganization was also investigated using FITC-labeled phalloidin. In the cytoplasm of untreated cells, F-actin was distributed in a diffuse pattern with concentrations in membrane ruffles, delineating the cell boundaries (**Fig. 1C**). After stimulated by PMA, more intense F-actin stress fibers arranged in spike-like protrusions resembling microvilli-like structures and protruded the cell membrane at the leading edging to form cell motile structures such as lamellipodia and filopodia (**Fig. 1D**). All these morphological characteristics are similar to the monocyte-derived macrophages. In control cells, the percentage of $CD14^+$ THP-1 cells was $5.5\% \pm 0.9\%$ (**Fig. 1E**). After treatment with PMA, the expression of CD14 was increased significantly: the percentage of $CD14^+$ cells was increased up to $55.7\% \pm 4.3\%$ (**Fig. 1F**), and the level of CD14 expression, measured as the relative mean fluorescence intensity (MFI), was increased up to 53.0 ± 2.5 (compared with 1.8 ± 0.3 in control cells) (**Fig. 1G**). These data indicated that THP-1 cells had differentiated into mature macrophages.

Alterations of CD44 during monocyte differentiation

Flow cytometry was used to analyze the CD44 expression. The results revealed that CD44 was highly expressed in THP-1 cells: the percentage of $CD44^+$ cells was $>95\%$ (**Fig. 2A**), and the MFI value was 179.6 ± 10.2 (**Fig. 2C**). After treatment with PMA for 48 h, there was no significant change in the percentage of $CD44^+$ cells (**Fig. 2B**), but the level of CD44 expression was increased significantly: the MFI value was increased up to 642.2 ± 26.3 (**Fig. 2C**).

FL-HA was used to study the binding of surface CD44 to soluble HA. In the flow cytometry analysis, the percentage of FL-HA-positive THP-1 cells was $7.9\% \pm 2.5\%$, indicating that CD44 on the THP-1 cell surface was in a relatively quiescent state (**Fig. 2D**); however, after treatment with PMA, the percentage of FL-HA-positive THP-1 cells was increased up to $84.7\% \pm 9.6\%$ (**Fig. 2E**), and the MFI value was increased from 15.4 ± 2.6 up to 58.9 ± 3.0 (**Fig. 2F**), which revealed that FL-HA binding can be induced by PMA. Immunocytochemistry analysis was also used to detect FL-HA binding of THP-1 cells. The fluorescence intensity (green) of PMA-treated THP-1 cells was increased significantly compared with that of the control cells (**Fig. 2G,H**), which confirmed the results of flow cytometry analysis.

Chemical cross-linking experiment was used to assess CD44 clustering on cell surface of THP-1 cells. Without BS^3 treatment, CD44 had an apparent molecular mass of 85 kDa in control cells (**Fig. 3A**). PMA significantly increased the level of CD44 expression, which was consistent with the results of flow cytometry analysis (**Fig. 2A**), but did not change the apparent molecular weight of CD44. However, after subsequent BS^3 treatment, in addition to the 85 kDa protein, a higher molecular weight protein of 170 kDa was also detected in PMA-treated THP-1 cells, indicating that PMA could stimulate the clustering of CD44 on the THP-1 cell surface.

Involvement of CD44 in monocyte differentiation

We examined whether anti-CD44 mAb can specifically inhibit PMA-stimulated clustering of CD44. Cells were incubated with PMA in the presence of anti-CD44 mAb (50 μg/ml) or non-immune mouse IgG (50 μg/ml), and subsequently treated with BS^3. As shown in **Fig. 3B**, when cells were incubated with PMA plus anti-CD44 mAb for 72 h, the intensity of the 85 and 170 kDa bands decreased markedly. Non-immune mouse IgG (50 μg/ml) had no effect on PMA-induced CD44 clustering.

Next, we examined whether PMA-induced CD14 expression would be inhibited by the addition of anti-CD44 mAb (**Fig. 4**). When THP-1 cells were incubated with anti-CD44 mAb (50 μg/ml), the ability of PMA to stimulate the expression of CD14 was inhibited ~30% compared with PMA-treated cells. Non-immune mouse IgG (50 μg/ml) did not abrogate PMA-induced CD14 expression. Thus, incubation of the cells with anti-CD44 mAb had a specifically suppressive effect on the ability of PMA to stimulate THP-1 differentiation.

Association of ERK1/2 signal pathway in CD44-induced monocyte differentiation

To further understand the intracellular mechanism through which PMA induced THP-1 cell differentiation, we also studied one of the important cellular signal pathways, ERK1/2, which has been regarded as a crucial regulator of cell differentiation and also has been reported to interact with CD44 directly. Western blot analysis revealed that the phosphorylation level of ERK1/2 was significantly increased after PMA treatment compared with control cells (**Fig. 5**). In addition, the up-regulated level of p-ERK1/2 by PMA was decreased after the blockage of CD44 by anti-CD44 mAb.

Discussion

In this study, we treated THP-1 cells with PMA to establish a monocyte differentiation model [19,20]. The results revealed that the morphology of THP-1 cells converted to macrophage-like, with significantly increased CD14 expression, indicating that the model of monocyte differentiation *in vitro* was successfully established. During differentiation, the alterations of CD44, including its expression, distribution, and HA binding ability were detected. The results showed that CD44 expression and binding capacity towards

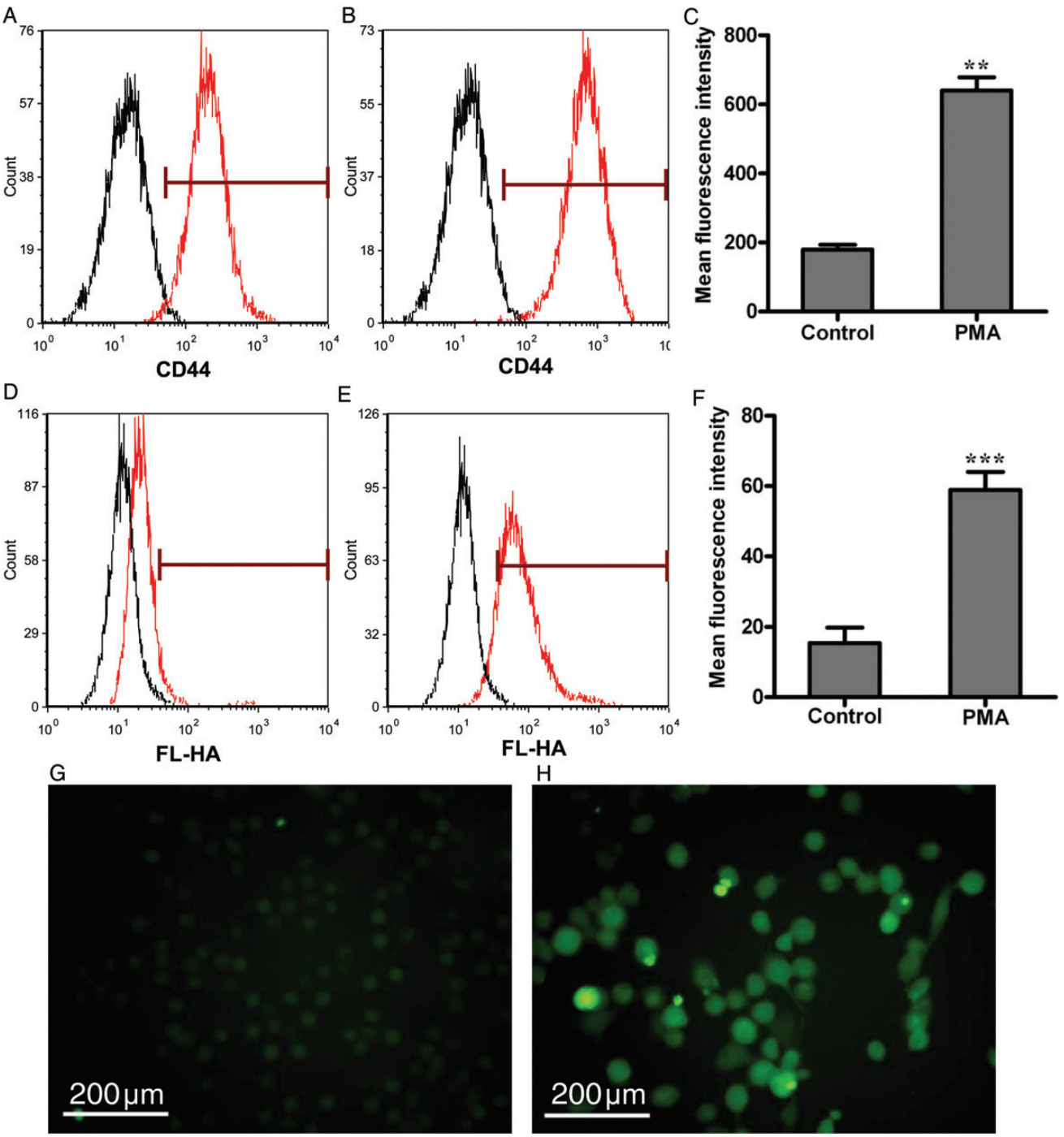

Figure 2. Effects of PMA on CD44 (A–C) Expression of CD44 on THP-1 cells. Cell surface expression of CD44 in THP-1 cells was assessed by flow cytometry. (A) Control cells. (B) PMA treated for 48 h. (C) MFI of cells stained with anti-CD44 mAb (isotype controls subtracted). $^{**}P < 0.01$ compared with the control sample. (D–H) Binding of exogenous FL-HA by THP-1 cells. The capacity of THP-1 cells to bind exogenous FL-HA was assessed by flow cytometry (D–F) and immunocytochemistry analysis (G,H). (D,G) Control cells. (E,H) PMA treated for 48 h. (F) MFI of cells incubated with FL-HA (blank controls subtracted). Images shown are representative of three independent experiments with similar results. $^{***}P < 0.001$ compared with the control sample.

HA were increased significantly. The distribution of CD44 was converted into clusters after differentiation. For the mechanism experiment, we co-treated PMA-stimulated THP-1 cells with anti-CD44 mAb and found that anti-CD44 mAb suppressed the PMA-induced CD44 clustering and CD44 high expression, which resulted in inhibition of CD14 expression. PMA-induced phosphorylation of ERK1/2 signal was also suppressed by pre-treatment of anti-CD44 mAb. Our results suggested that CD44 was involved in monocyte differentiation. The mechanisms of monocyte

differentiation following CD44 activation may include CD44 high expression and clustering which in turn lead to phosphorylation of ERK1/2 signal.

CD44, a cell surface receptor for the glycosaminoglycan HA, is expressed on many different types of cells. However, not all CD44-positive cells can bind HA, and the binding of HA by CD44 is highly regulated [22,23]. Three activation states for CD44/HA-binding ability have been defined: inactive, inducible, and constitutively active [24]. When freshly isolated from peripheral blood, monocytes do not have the ability to bind HA [16,17]. On the contrary, CD44 on macrophages derived from monocytes can bind HA constitutively [16]. This phenomenon indicates that CD44 on monocytes is inducible and can be activated during differentiation. In this study, we detected the expression of CD44 and HA-binding capacity on THP-1 cells before and after differentiation. It was found that CD44 was highly expressed on THP-1 cells, but could not bind HA. After differentiated by PMA, the HA-binding ability was enhanced significantly, suggesting that CD44 on THP-1 cells was activated during the differentiation. In addition, the expression level of CD44 was also increased significantly during THP-1 differentiation, which may be one of the mechanisms of CD44 activation [25]. However, the HA-binding ability of CD44 cannot be solely explained by the densities of CD44 on the cell surface [26]. Some type of qualitative/functional change would be essential for the induction of binding of CD44 to HA as reported previously, such as clustering, glycosylation, and phosphorylation of CD44 [27,28]. To further investigate the mechanism of CD44 activation, chemical cross-linking experiment was used to assess CD44 clustering on cell surface of THP-1 cells. The result showed that CD44 was distributed into clusters in THP-1 cells differentiated by PMA. Clustering of CD44 on the cell surface has been proposed as a possible mechanism of modulating the HA-binding function of CD44 [29]. Our findings suggested that CD44 on monocytes was activated during differentiation into macrophages, and the increased expression and clustering of CD44 may contribute to the underlying mechanisms.

CD14, a molecule marker of mature macrophages, was used to assess the monocyte differentiation level. After stimulation by PMA, CD14 expression was increased significantly, indicating that THP-1 cells were differentiated into macrophages. To identify whether CD44 participates in

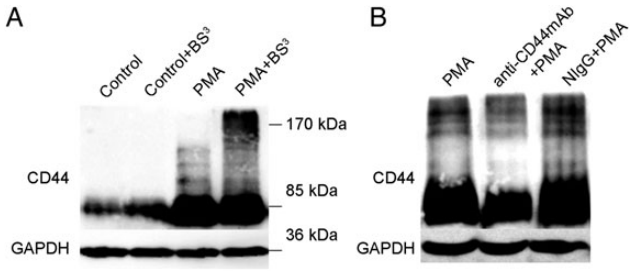

Figure 3. Chemical cross-linking analysis of CD44 clustering in THP-1 cells (A) THP-1 cells were treated with or without PMA (320 nM) for 72 h, then all cells were incubated with or without BS^3 protein cross-linker (2 mM) for 1 h at 4°C. The cross-linked CD44 on cell surface was detected by western blot analysis. (B) Before treatment with PMA for 72 h, THP-1 cells were pretreated with RPMI 1640 (Control), anti-CD44 mAb (50 μg/ml), or non-immune mouse IgG (NIgG) for 2 h at 37°C, then all cells were incubated with BS^3 protein cross-linker (2 mM) for 1 h at 4°C. The cross-linked CD44 on cell surface was detected by western blot analysis. Images are representative of three independent experiments with similar results.

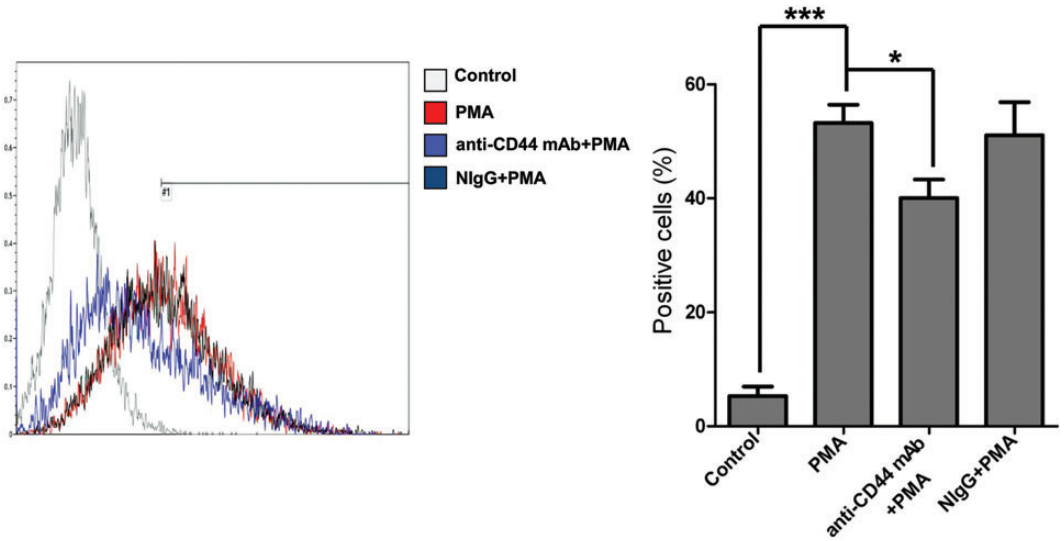

Figure 4. The effect of anti-CD44 mAb on PMA-induced CD14 expression THP-1 cells were pretreated with RPMI 1640, anti-CD44 mAb (50 μg/ml), or non-immune mouse IgG (NIgG) for 2 h at 37°C, then all cells were treated with PMA (320 nM) for 72 h. CD14 expression was analyzed by flow cytometry, and unstimulated THP-1 cells were used as negative control. Statistical significance was examined by one-way ANOVA with *post hoc* test. $*P < 0.05$ vs. PMA-treated cells; $***P < 0.001$ vs. control.

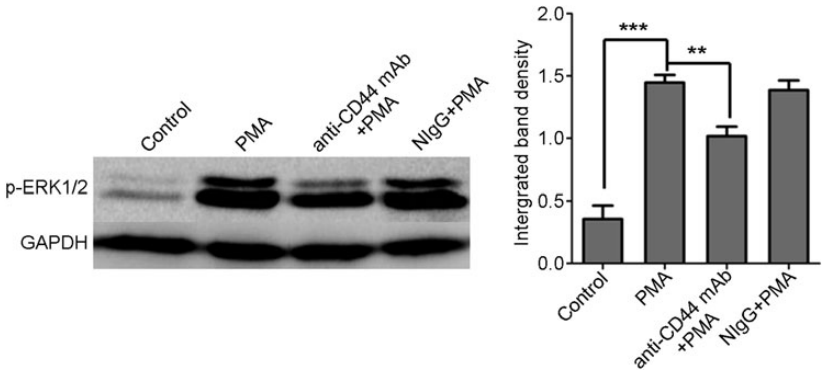

Figure 5. The effect of PMA on ERK1/2 phosphorylation THP-1 cells were pretreated with or without anti-CD44 mAb (50 μg/ml) for 2 h and then treated with PMA (320 nM) for 15 min. Cells treated with RPMI 1640 were used as control. Non-immune mouse IgG (NIgG) was used as negative control. The phosphorylated ERK1/2 (p-ERK1/2) was detected by western blot analysis. The pictures showed the representative blots of three independent experiments with similar results. Statistical significance was examined by one-way ANOVA with *post hoc* test. $^{**}P < 0.01$ vs. PMA-treated cells; $^{***}P < 0.001$ vs. control.

monocyte differentiation, anti-CD44 mAb was used to block CD44 during differentiation. The data showed that anti-CD44 mAb markedly inhibited the PMA-mediated CD14 expression [30], suggesting that CD44 was involved in monocyte differentiation. To further understand how CD44 participates in monocyte differentiation, the effect of anti-CD44 mAb on PMA-induced CD44 clustering was investigated. The result showed that PMA-induced CD44 high expression and CD44 clustering were suppressed by anti-CD44 mb. So we propose that CD44 may be involved in monocyte differentiation through its increased expression level and change in distribution.

To further explore the intracellular mechanism of PMA-induced THP-1 differentiation, the phosphorylation of ERK1/2 was analyzed. ERK1/2 is one member of the mitogen-activated protein kinase family, and has been proved to play an important role in the regulation of both monocytic differentiation and induction of cytokine synthesis by activated macrophages [31,32]. It was found that ERK1/2 phosphorylation was significantly enhanced in THP-1 cells after PMA stimulation. Furthermore, the up-regulated level of p-ERK1/2 by PMA was decreased after the blockage of CD44 by anti-CD44 mAb. It has been reported that CD44 could interact directly with ERK1/2 and enhance ERK1/2 phosphorylation [33]. Based on our results and the others, we propose that the stimulating effect of PMA on ERK1/2 signaling may be partially mediated through the activation of CD44, and this pathway may play an important role in the monocyte differentiation.

In summary, our data suggested that CD44 was activated during monocyte differentiation, and the underlying mechanisms may include its increased expression and clustering. The alterations of CD44 may in turn stimulate monocyte differentiation via its influence on ERK1/2 pathway. Our study only preliminarily investigated the role of CD44 in monocyte differentiation on a differentiation model *in vitro*. Further studies are needed to fully understand the role of CD44 in monocyte differentiation *in vivo*.

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