Article

cAMP Modulates Macrophage Development by Suppressing M-CSF-Induced MAPKs Activation

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M-CSF is a key cytokine in macrophage development by inducing MAPKs activation, and cAMP can inhibit MAPKs activation induced by inflammatory stimuli. To explore the effects of cAMP on M-CSF-induced MAPKs activation and on macrophage development, the model of bone marrow-derived murine macrophages (BMMs) was used. The effects of cAMP on M-CSF-induced MAPKs activation were analyzed by Western blotting assay, and the effects of cAMP on CD14 and F4/80 expression during macrophage development were examined by FACS analysis. Macrophage morphology showed the successful establishment of the model of macrophage development. Western blotting assay revealed that M-CSF activated ERK, JNK and p38 in both mature and immature macrophages, and cAMP inhibited M-CSF-induced ERK, JNK and p38 activation in a time-dependent manner. FACS analysis revealed that macrophage development was impaired with cAMP pretreatment. In conclusion, cAMP modulates macrophage development by suppressing M-CSF-induced MAPKs activation. *Cellular & Molecular Immunology*. 2008;5(2):153-157.

Key Words: macrophage development, M-CSF, cAMP, MAPKs

Introduction

Macrophages are key players in the immune response to foreign invaders such as infectious microorganisms. Macrophages help destroy bacteria, protozoa, and tumor cells. They release substances that stimulate other cells of the immune system. And they also act as a lynch pin between innate and adaptive immunity as antigen presenting cells (APCs). To do this, they carry the antigen on their surface and present it to T cells. Blood monocytes migrate into the tissues of the body where they differentiate (evolve) into macrophages. Macrophage-colony-stimulating factor (M-CSF or CSF-1) is a key cytokine involved in macrophage lineage development from bone marrow precursors by contributing to proliferation and differentiation (1).

The second messenger cyclic adenosine monophosphate (cAMP) is produced from ATP by adenylyl cyclases (AC) and can be degraded to 5'-AMP by phosphodiesterases (2, 3). AC is stimulated by a variety of extracellular stimuli, such as hormones, growth factors and neurotransmitters through G-protein (Gs)-coupled membrane receptors (3). In addition, AC can be activated by pharmacological agents, such as forskolin (FSK), which is a direct activator of AC (4). cAMP regulates many cellular activities, from proliferation to apoptosis, in a cell type-dependent manner (5-9). The biological functions of cAMP are mediated by its downstream effectors such as protein kinase A (PKA) (10, 11).

There are three major groups of mitogen-activated protein kinases (MAPKs) in mammalian cells, the extra-cellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38. These kinases are activated by distinct upstream MAPK kinases (MKKs) which phosphorylate the residues within a tripeptide motif (Thr-X-Tyr). Once activated, MAPKs, in turn, phosphorylate a variety of intracellular substrates, including certain transcription factors (12). These MAPKs are activated by many proinflammatory stimuli and play important roles in the inflammatory process.

Previous studies have revealed that second messenger cAMP inhibits MAPKs (ERK, JNK and p38) activation induced by inflammatory stimuli, thereby contributing to immunosuppression and anti-inflammatory action (7, 13-15). Recent work from several laboratories has shown that M-CSF

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activates ERK and JNK during macrophage development, and ERK and JNK play important roles during macrophage development (16-18). It remains unknown whether cAMP inhibits M-CSF-induced MAPKs activation, thereby modulates macrophage development. To answer the questions, we conducted the following studies.

Materials and Methods

Animals

Female C57BL/6 mice, 4-6 weeks, purchased from Experimental Center, Academy of Military Medical Sciences, were the source of bone marrow cells.

Reagents

RPMI1640 was purchased from Invitrogen Gibco (Carlsbad, CA, USA). Fetal bovine serum was purchased from the Biotechnology Institute of Beijing Yuan Heng Sheng Ma (Beijing, China). Recombinant human M-CSF was purchased from Cetus Corp. (Emeryville, CA, USA). Antibodies against phospho-ERK, phospho-JNK and phospho-p38 were from Cell Signaling Technology (Beverly, MA, USA). Goat anti-rabbit IgG-HRP was from Jackson Laboratory (Bar harbor, ME, USA). Antibody against actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL Chemi-luminescence Kit was obtained from Amersham (Arlington Heights, IL, USA). Forskolin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in DMSO. Rat IgG-isotype control-FITC/PE, CD14-PE (clone Sa2-8), and F4/80-FITC (clone BM8) were obtained from eBioscience (San Diego, CA, USA).

Collection of mouse bone marrow cells and cell culture

Bone marrow cells from C57BL/6 mice were collected as previously described (19). If not noted otherwise, the non-adherent bone marrow cells (5×10^5/1.5 ml/well) were then cultured in 6-well plates at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium containing 15% (v/v) FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercapto-ethanol and 100 ng/ml recombinant human M-CSF.

Giemsa staining

On day 7 of M-CSF-dependent differentiation, the super-natant was drawn off and BMMs were washed with PBS. After methanol fixation for 1 min, the cells were immersed in Giemsa staining buffer (1 ml) for 5 min. After washing with water, the morphology of BMMs was observed under microscope.

Western blotting assay

BMMs were washed with PBS and harvested with a cell scraper (Costars, Cambridge, MA, USA) in ice-cold lysis buffer (0.5% NP-40, 20 mM Tris-Cl, pH 7.6, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM sodium orthovanadate, 1 mM DTT, 10 mM PNPP, 10 μg/ml aprotinin). Cell lysates were resolved by SDS-PAGE before transferred to nitrocellulose membranes. Nitrocellulose membranes were then incubated with 5% (w/v) nonfat dry milk in washing buffer (20 mM Tris-Cl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 1 h at 37°C to block nonspecific protein binding. Primary antibodies (1:1,000) were diluted in washing buffer containing 3% BSA and applied to the membranes for overnight at 4°C. After extensive washing, the membranes were incubated with goat anti-rabbit IgG-HRP (diluted up to 1:2,500 in washing buffer containing 5% (w/v) nonfat dry milk) for 1 h at room temperature. Following washing, immunoreactive bands were visualized by the ECL Chemiluminescence Kit.

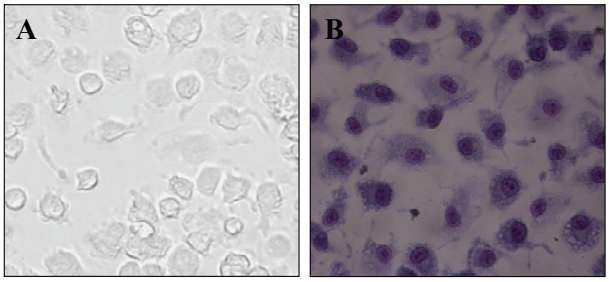

Figure 1. Observation of murine macrophages under microscope (40×). Mouse non-adherent bone marrow cells were cultured with M-CSF for 7 days. The cells appeared typical macrophage morphology. (A) The result from phase contrast microscope. (B) The result from Giemsa staining.

FACS analysis

The non-adherent bone marrow cells pretreated with forskolin or DMSO of equal volume were cultured in the presence of M-CSF. On day 4, the supernatant was drawn off. BMMs were washed with PBS and harvested with trypsin digestion. The cells were washed with FACS washing buffer (2% FBS, 0.1% NaN_3 in PBS) twice, then incubated with CD14-PE and F4/80-FITC for 25 min at 4°C, isotype antibodies were included as negative control. After washing with FACS buffer, the cells were fixed with 1% (w/v) paraformaldehyde in PBS and preserved at 4°C. Flow cytometry was carried out on the Becton Dickinson FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA).

Statistical analysis

The data were shown as mean ± standard deviations (SD). The Student's *t* test was used to compare the difference between the two groups. The difference was considered statistically significant when $p < 0.05$.

Results

The establishment of the model of macrophage development

In the presence of M-CSF, non-adherent bone marrow cells containing macrophage progenitor cells began to proliferate, with a fraction of them being gradually transformed into adherent cells with monocyte/macrophage morphology. On

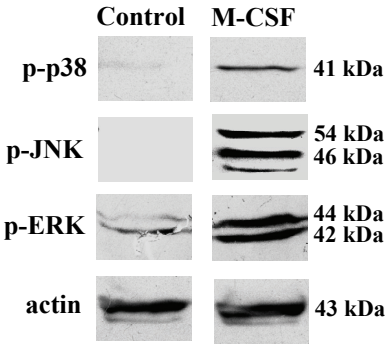

Figure 2. Western blotting analysis of the effects of M-CSF on the activation of MAPKs. M-CSF-starved mature macrophages were stimulated with M-CSF for 15 min or untreated. Then the cell lysates were prepared. After 12% SDS-PAGE, the bands of each group were transferred to nitrocellulose membranes, then the membranes were incubated with antibodies against phospho-ERK (p-ERK), phospho-JNK (p-JNK), phospho-p38 (p-p38), actin and the second antibody sequentially. Immunoreactive bands were visualized by the ECL Chemiluminescence Kit.

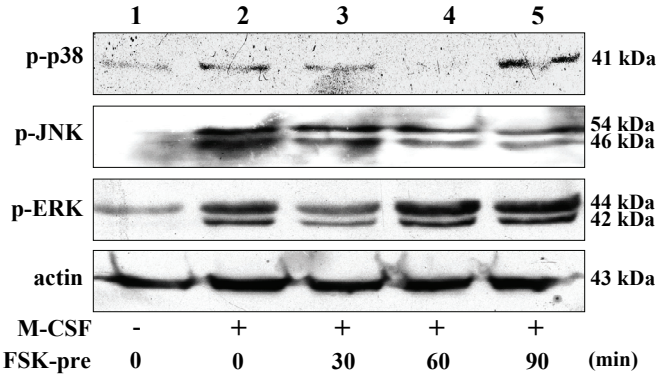

Figure 3. Western blotting analysis of the effects of cAMP on M-CSF-induced MAPKs activation. M-CSF-starved immature macrophages were pretreated with forskolin for various time periods (Lanes 3-5), then immature macrophages were stimulated with M-CSF for 15 min (Lanes 2-5) or untreated (Lane 1). The cell lysates were prepared and Western blotting assay was done as described in Figure 2. FSK-pre: forskolin pretreatment.

day 7, more than 98% of the cells in the culture were differentiated into mature macrophages. Under phase contrast microscope, the cells were large, with a lot of vacuoles in cytoplasm (Figure 1A). After Giemsa staining, the cells exhibited typical macrophage morphology under inverted microscope. They were elongated spindle-shaped and large. Cytoplasm was rich. Vacuoles phenomenon was obvious. A kidney-shaped nuclei tended edge (Figure 1B). The above results showed that the model of macrophage differentiation and development was established successfully.

M-CSF activated ERK, JNK and p38 in both mature and immature macrophages, and cAMP inhibited M-CSF-induced ERK, JNK and p38 activation in a time-dependent manner

Mature macrophages induced with M-CSF for 7 days were washed extensively. The macrophages were incubated with M-CSF-free RPMI 1640 medium for 12 h, and stimulated with M-CSF for 15 min. The effects of M-CSF on the activation of MAPKs were analyzed *via* Western blotting. Consistent with previous studies, our results showed that M-CSF activated ERK and JNK. But we also found that M-CSF activated p38 (Figure 2).

These findings led us to investigate whether cAMP inhibits M-CSF-induced MAPKs activation. On day 4 of M-CSF-dependent differentiation, immature macrophages were washed extensively. As stated above, the immature macrophages were incubated with M-CSF-free RPMI 1640 medium for 12 h. After pretreated with forskolin for various time periods, immature macrophages were stimulated with M-CSF for 15 min. Western blotting assay revealed that M-CSF also activated ERK, JNK and p38 in immature macrophages. cAMP inhibited M-CSF-induced MAPKs activation in immature macrophages. For ERK, the inhibition occurred with forskolin pretreatment for 30 min. For p38, the inhibition occurred with forskolin pretreatment for 30 to 60 min, and the maximal inhibition occurred after 60-min forskolin pretreatment. JNK activation was inhibited in a similar pattern to p38, however, the maximal inhibition lasted up to 90 min of forskolin pretreatment (Figure 3). Taken together, our data indicated that M-CSF activated ERK, JNK and p38 during macrophage development, and cAMP inhibited M-CSF-induced ERK, JNK and p38 activation in a time-dependent manner.

Macrophage development was impaired with cAMP pretreatment

cAMP as the second messenger inhibited M-CSF-induced ERK, JNK and p38 activation in immature macrophages. The previous reports have shown that ERK and JNK play an important role during the development of macrophages (16-18). Our unpublished data suggest that p38 is also involved in macrophage differentiation. So we hypothesized that cAMP might also modulate macrophage development. After pretreatment with forskolin or DMSO of equal volume for 30 min, mouse non-adherent bone marrow cells were cultured in RPMI 1640 medium containing 100 ng/ml M-CSF for 4 days. With antibodies specific for monocyte/ macrophage markers, CD14 and F4/80, the effects of cAMP on macrophage development were examined. The results showed that forskolin pretreatment led to declined percentage of $CD14^{+}F4/80^{+}$ double positive cells. Furthermore, the fluorescence mean for CD14 and F4/80 also significantly decreased with forskolin pretreatment (Figure 4). Therefore, cAMP modulated macrophage development.

Discussion

Our results showed that M-CSF activated ERK, JNK and p38 during M-CSF-induced macrophage differentiation and development. The previous reports suggest that ERK and

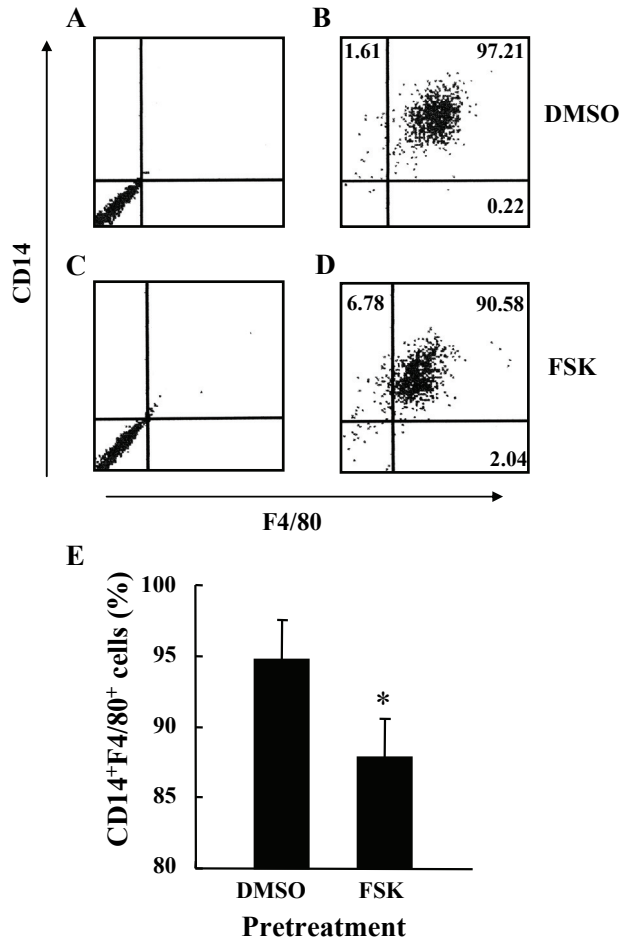

Figure 4. FACS analysis of the effects of cAMP on CD14 and F4/80 expression during macrophage development. Mouse non-adherent bone marrow cells pretreated with or DMSO (A, B) or forskolin (C, D) of equal volume were cultured in the presence of M-CSF. After 4 days, BMMs were collected for F4/80 and CD14 staining. (A, C) The cells were stained with isotype Abs as negative control; (B, D) the cells were stained with PE-labeled rat anti-mouse CD14 and FITC-labeled rat anti-mouse F4/80. (E) Statistical analysis of the percentages of $CD14^+F4/80^+$ cells in control macrophages with DMSO pretreatment or in macrophages with forskolin pretreatment. Data were shown as mean ± SD, n = 3. *$p < 0.05$. FSK, forskolin.

JNK play an important role during the macrophage development (16-18). Our unpublished data suggest that p38 is also involved in the macrophage differentiation. In this work, our data suggest that cAMP as the second messenger modulates macrophage development through inhibiting M-CSF-induced MAPKs activation. Thus we provide a new mechanism for explaining why cAMP has immuno-suppressive and anti-inflammatory effects.

cAMP inhibited M-CSF-induced ERK, JNK, and p38 activation in a time-dependent manner. cAMP inhibited ERK activation only when the cells were pretreated with forskolin for 30 min. Then the inhibition disappeared. For JNK and p38, the inhibition occurred with forskolin pretreatment for 30 min. The maximal inhibition occurred with forskolin pretreatment for 60 min. The inhibition of p38 activation diminished if the cells were pretreated with forskolin for longer time. However, the inhibition of JNK activation lasted even when the cells were pretreated with forskolin for 90 min. These data are consistent with the notion that the mechanisms by which cAMP inhibited ERK, JNK and p38 activation are different (20). In our previous work, we have clarified that cAMP inhibited p38 activation *via* CREB-induced dynein light chain (20). Interestingly, the *de novo* protein synthesis was required for cAMP-mediated inhibition of JNK activation but not for the inhibition of ERK activation (20 and our unpublished data). Furthermore, the inhibition of JNK by cAMP also required CREB, similar to the inhibition of p38 by cAMP (20 and our unpublished data). However, dynein light chain was not required for the inhibition of JNK by cAMP (20 and our unpublished data), suggesting that another CREB target gene(s) is involved. Thus, cAMP inhibits MAPKs through different mechanisms.

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