Original Article

α-MSH inhibits TNF-α-induced maturation of human dendritic cells in vitro through the up-regulation of ANXA1

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α-Melanocytostimulating hormone (α-MSH), an anti-inflammatory and immunomodulatory neuropeptide, has been shown to be effective in the experimental treatment of autoimmune diseases and allograft rejection. However, its regulatory mechanism is still unclear. Mature dendritic cells (DCs) are pivotal initiators of immune response and inflammation. We hypothesized that the regulatory role of α-MSH in DC maturation would contribute to the effects of α-MSH in immune-response-mediated disease models. It was found that α-MSH inhibited tumor necrosis factor-alpha (TNF-α)-induced maturation of human peripheral-monocyte-derived DCs (MoDCs), both phenotypically and functionally. This occurred through the down-regulation of the expression of co-stimulatory molecules CD83 and CD86, the production of IL-12, the promotion of IL-10 secretion, and the MoDC phagocytic activity, suggesting that the inhibition of DC maturation by α-MSH could contribute to the anti-inflammatory effect of this neuropeptide. Furthermore, increased expression of annexin A1 (ANXA1) was found to be responsible for the α-MSH inhibiting effect on TNF-α-induced MoDC maturation, which could be abolished by the treatment of MoDCs with specific, small interfering RNAs targeting ANXA1 (ANXA1-siRNA), suggesting that α-MSH-induced ANXA1 mediates the inhibition. Therefore, α-MSH inhibits TNF-α-induced maturation of human DCs through α-MSH-upregulated ANXA1, suggesting that inhibition of the maturation of DCs by α-MSH could mediate the anti-inflammatory effect of the neuropeptide. Furthermore, ANXA1 could be identified as a new therapeutic drug target based on the role of DCs in immune-mediated inflammatory diseases.

Keywords α-melanocyte stimulating hormone; dendritic cells; annexin A1

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Introduction

α-Melanocyte stimulating hormone (α-MSH) is a neuropeptide with potent anti-inflammatory and immunomodulatory effects [1]. The actions of α-MSH are transmitted via the specific melanocortin receptors [2]. Recent studies showed that melanocortin 1 receptor (MC-1R) was expressed on the surface of immune and inflammatory cells, such as monocytes, macrophages, and dendritic cells (DCs) [3]. α-MSH is able to up-regulate the release of IL-10 by monocytes and down-regulate the production of nitric oxide and pro-inflammatory cytokines by macrophages [4,5]. α-MSH also significantly suppresses the expression of CD86 and CD40 on monocytes and DCs [6]. α-MSH prolongs allograft survival and improves the histopathologic alteration of the allograft in experimental heart transplantation [7]. The anti-inflammatory effects of α-MSH have been confirmed by means of animal models of inflammation, such as irritant and allergic contact dermatitis, cutaneous vasculitis, asthma, inflammatory bowel disease, ocular and brain inflammation, and rheumatoid arthritis [8]. Thus, α-MSH is regarded as an important factor involved in the regulation of inflammation and immune responses. However, its mechanism of action remains unclear.

DCs exert specific functions in innate and adaptive immunity [9]. There is increasing evidence that these cells are crucial regulators of immunity, including the abilities to activate immune response and induce immunotolerance [10]. The functional plasticity of these uniquely well-equipped antigen-presenting cells is exploited to utilize potential targets in the therapeutic strategies of many diseases, including tumors, autoimmune diseases, and transplant rejection [11]. The study of regulatory factors and their effects on the function of DCs is very important, but remains incomplete.

We hypothesized that the regulatory effect of the neuropeptide α-MSH on DCs could contribute to the
anti-inflammatory effects of α-MSH in immune-response-mediated disease models. The primary aim of this study was to investigate the role and mechanism of action of α-MSH in the maturation of DCs. First, we confirmed the inhibitory role of α-MSH in tumor necrosis factor-alpha (TNF-α)-induced maturation of human peripheral-monocyte-derived DCs (MoDCs). Then, we designed a study to determine the molecular mechanism underlying the regulatory effects of the peptide. Using two-dimensional (2D) gel electrophoresis and mass spectrometry in association with western blotting analysis, we found that after α-MSH treatment, annexin A1 (ANXA1) expression was markedly increased in TNF-α-induced MoDCs. Furthermore, specific small interfering RNAs targeting ANXA1 (ANXA1-siRNA) partly reversed the inhibitory role of α-MSH, resulting in the maturation of MoDCs. Our results indicate that α-MSH inhibits the maturation of DCs through ANXA1, at least partially, and suggest that the inhibition of DC maturation by α-MSH could mediate the regulatory effect of the neuropeptide in animal disease models. Furthermore, ANXA1 could be used as a new drug target for treating autoimmune diseases and transplant rejection.

**Materials and Methods**

**Materials**

α-MSH and all the chemicals needed for 2D gel electrophoresis and mass spectrometry were purchased from Sigma (St. Louis, USA). Recombinant human GM-CSF, IL-4, and TNF-α were from PeproTech (Rocky Hill, USA). ELISA kits for IL-12p40 and IL-10 were from R&D Systems (Minneapolis, USA). Fluorescein isothiocyanate (FITC)-Dextran (Mr = 40 kDa) was from Molecular Probes (Carlsbad, USA). FITC-anti-CD1a, Phycoerythrin (PE)-anti-CD86, FITC-anti-CD83, PE-anti-HLA-DR, and isotype-matched control Abs conjugated with FITC or PE were all obtained from eBioscience (San Diego, USA). Anti-ANXA1, anti-GAPDH, and anti-rabbit IgG labeled with HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Geneporter 2 Transfection Reagent was from Genlantis (San Diego, USA). The ANXA1-specific (ANXA1-siRNA, 5′-GCAGCAUAUCUCAGGAATTT-3′) and scrambled control siRNAs were purchased from by GeneChem (Shanghai, China).

**Cell culture**

MoDCs were generated from human peripheral blood CD14+ monocytes by culturing in 10% fetal bovine serum (FBS)-RPMI 1640 medium containing GM-CSF and IL-4, as described previously [12]. On day 6 of the culture, MoDCs were stimulated with 50 ng/ml of TNF-α for 24 h (TNF-α-treated MoDCs). Then, TNF-α-treated MoDCs were exposed to $10^{-14}$–$10^{-6}$ M of α-MSH for 24 h (TNF-α + α-MSH-treated MoDCs). These TNF-α-treated and α-MSH-treated or α-MSH-untreated MoDCs were collected for phenotypic and functional analysis.

**Analysis of cell surface markers**

Cells were stained with the following PE- or FITC-conjugated monoclonal antibodies (mAbs): anti-CD86, anti-CD83, anti-CD1a, and anti-HLA-DR. Isotype-matched antibodies were used as negative controls. MoDCs were collected and resuspended with stain buffer (PBS with 0.2% BSA; pH 7.2). The cells were washed twice in cold stain buffer and pelleted by centrifugation (300 g) at 4°C. Fluorescent antibodies and respective isotype controls were then added at a concentration of 20 μl per 100-μl experimental samples. The cells were incubated at 4°C for 30 min and washed twice with 1 ml of stain buffer and resuspended with 200 μl of stain buffer. The samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, USA) and the data were analyzed with CellQuest software (Becton Dickinson).

**Analysis of DC phagocytic ability**

Mannose-receptor-mediated endocytosis was measured by the cellular uptake of FITC-dextran. MoDCs were resuspended in 10% FBS-RPMI 1640 medium with 25 mM HEPES and then incubated with FITC-dextran (1 mg/ml) at 37°C for 4 h. The cells were washed twice with cold PBS containing 1% FBS and 0.01% NaN₃ and then resuspended in ice-cold PBS for immediate analysis by flow cytometry. MoDCs incubated with FITC-dextran at 4°C were used as negative controls.

**Cytokine assay**

The supernatants of MoDCs were collected for phenotypic and functional analysis. The concentrations of IL-12p40 and IL-10 were detected with ELISA kits according to the manufacturer’s instructions.

**Two-dimensional gel electrophoresis and mass spectrometry**

Cells (4 × 10⁶) were treated with 400 μl lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM dithiothreitol, 5 g/L Bio-Lyte, and 0.001% bromothymol blue. The protein sample (100 μl) was mixed with 400 μl loading buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM dithiothreitol, 2 g/L Bio-Lyte, and 0.001% bromothymol blue) and loaded onto PROTEAN IEF electrophoresis tank (Bio-Rad, Hercules, USA). Two ReadyStrip IPG Strips (17 cm, pH 4.0–7.0) were used. Proteins were separated in two dimensions on 12% SDS–polyacrylamide gels.
gel electrophoresis (PAGE) gels. Gels were stained with silver nitrate as described previously [13].

Image acquisition was performed with a GS-800 device. Image analysis was carried out using Quantity One software. Protein spots of interest were cut from silver-stained 2D gels and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). Peptide mass fingerprint data were searched using a Mascot search within the National Center for Biotechnology Information database.

**Western blot analysis**

Cells (1 x 10^6) were collected and washed with PBS at room temperature. The cells were lysed using ice-cold RIPA buffer with freshly added PMSF on ice for 30 min. The total cell lysate was centrifuged at 10,000 g for 5 min at 4°C and the supernatant fluid was collected. The sample was mixed with an equal volume of 2 x electrophoresis sample buffer and boiled for 5 min. The mixture was loaded into each lane, subjected to 10% SDS–PAGE, and then transferred onto a nitrocellulose membrane according to standard protocols. The membrane was incubated in blocking buffer [1 x TBS, 0.1% Tween-20 (TBST) with 5% nonfat dry milk] for 1 h at room temperature. The membrane was washed three times with TBST and then incubated with the specific primary antibody (1:1000) for 1 h at room temperature. Immunoreactive bands were detected by incubating the membrane with horseradish peroxidase-conjugated secondary antibody (1:2000 in blocking buffer) at room temperature for 45 min. The membrane was washed and developed using ECL plus a chemiluminescent system (Molecular Probes, Carlsbad, USA). Quantification of band intensity was carried out using LabWorks 4.0 Image Acquisition and Analysis Software.

![Figure 1 Inhibitory effects of α-MSH on the expression of CD86 and CD83 in MoDCs stimulated with TNF-α](image1)

MoDCs (5 x 10^5 cells/ml) were stimulated with TNF-α (50 ng/ml) for 24 h and then treated with 10^{-12} M of α-MSH for 24 h. MoDC phenotypes were analyzed by FACS. Numerical values indicate the geometric mean fluorescence intensity (MFI) of each group (for shaded histograms). MoDCs obtained from three donors and data represent one of three experiments with similar results.

![Figure 2 Effects of α-MSH on the secretion of IL-12p40 (A) and IL-10 (B) from TNF-α-stimulated MoDCs](image2)

MoDCs were stimulated with 50 ng/ml of TNF-α at a density of 5 x 10^5 cells/ml for 24 h and then treated with α-MSH (10^{-12} M) for 24 h. The levels of IL-12p40 and IL-10 in the supernatants from these MoDCs were measured by ELISA. The asterisk indicates statistically significant associations (*P < 0.05).
DC transfection by siRNA
The day before transfection, MoDCs (5 × 10^5 cells/well) were seeded in 24-well plates and the medium was replaced so that the cells would be in good condition on the day of transfection. Transient transfections were performed with GenePorter 2 Transfection Reagent according to the manufacturer’s protocol. Briefly, GenePorter 2 reagent (5 μl) was diluted with 20 μl of RPMI 1640 medium. ANXA1-siRNA (100 nM) was diluted with 25 μl of diluent and incubated at room temperature for 5 min. Diluted siRNA was added to the diluted GenePorter 2 reagent. The mixture was incubated at room temperature for 5–10 min to form lipid/siRNA complexes (lipoplexes). The GenePorter 2/siRNA complexes (50 μl) were directly added to MoDCs (5 × 10^5) growing in serum-containing medium at 37°C. After transfection, the cells were cultured for another 36 h before commencing further experiments.

Statistical analysis
Statistical analysis was performed in Microsoft Excel (Microsoft Corporation, Washington, USA) using two-sided Student’s t-tests. A value of P < 0.05 was considered statistically significant.

Results
α-MSH inhibits TNF-α-induced maturation of human DCs, phenotypically and functionally
TNF-α acts as a potent pro-inflammatory cytokine involved in the pathogenesis of autoimmune diseases and transplantation rejection [14]. We generated mature MoDCs using TNF-α, as indicated by the mature phenotype described previously [15,16]. We then added various concentrations of α-MSH (10^{-14} – 10^{-6} M) to investigate the regulatory effect of α-MSH on the phenotype of TNF-α-DCs. The down-regulation of co-stimulatory molecules CD83 and CD86 after α-MSH treatment of TNF-α-DCs was observed for all concentrations (data not shown). We chose one of the concentrations (10^{-12} M), which is the physiological concentration used in previous research [6,17], for further experiment. As shown in Fig. 1, 10^{-12} M of α-MSH could inhibit the expression of CD83 and CD86 on MoDCs stimulated with TNF-α and could significantly decrease IL-12p40 production in MoDCs pretreated with TNF-α (*P < 0.05) [Fig. 2(A)]. In contrast, the secretion of IL-10 from MoDCs pretreated with TNF-α was enhanced when treated with α-MSH (*P < 0.05) [Fig. 2(B)]. The finding that α-MSH induced further phagocytosis of FITC-conjugated dextran by TNF-α-pretreated MoDCs provides further evidence of the inhibitory effect of α-MSH on TNF-α-induced DC functional maturation (Fig. 3).

α-MSH inhibits TNF-α-induced MoDC maturation through the up-regulation of ANXA1
To identify whether increased ANXA1 was related to the inhibition of TNF-α-induced MoDC maturation by α-MSH, we reduced the expression of endogenous ANXA1 using small interfering RNA (siRNA) and tested the effects on TNF-α-DCs. The down-regulation of co-stimulatory molecules CD83 and CD86 after α-MSH treatment of TNF-α-DCs was observed for all concentrations (data not shown). We chose one of the concentrations (10^{-12} M), which is the physiological concentration used in previous research [6,17], for further experiment. As shown in Fig. 1, 10^{-12} M of α-MSH could inhibit the expression of CD83 and CD86 on MoDCs stimulated with TNF-α and could significantly decrease IL-12p40 production in MoDCs pretreated with TNF-α (*P < 0.05) [Fig. 2(A)]. In contrast, the secretion of IL-10 from MoDCs pretreated with TNF-α was enhanced when treated with α-MSH (*P < 0.05) [Fig. 2(B)]. The finding that α-MSH induced further phagocytosis of FITC-conjugated dextran by TNF-α-pretreated MoDCs provides further evidence of the inhibitory effect of α-MSH on TNF-α-induced DC functional maturation (Fig. 3).

ANXA1 expression is increased in TNF-α + α-MSH-treated MoDCs
We prepared total cell lysate from MoDCs treated with either TNF-α and α-MSH, or TNF-α alone, to identify changes in the proteome and search for important signaling molecules. Comparison of 2D electrophoreses, followed by mass spectroscopic analysis, resulted in the identification of ANXA1 as a protein that was significantly induced by α-MSH [Fig. 4(A) and Table 1]. Immunoblotting with anti-ANXA1 [Fig. 4(B)] confirmed the up-regulation of ANXA1 after treatment with α-MSH. These results suggest that ANXA1 is involved in the inhibitory role of α-MSH in the TNF-α-induced maturation of MoDCs.
knockdown (Fig. 8). Further, ANXA1-siRNA resulted in decreased phagocytosis of FITC-conjugated dextran by TNF-α + α-MSH-treated MoDCs (Fig. 9). Taken together, these data indicate that ANXA1 is involved in the inhibitory effects of α-MSH on DC maturation induced by TNF-α.

**Discussion**

MoDCs have been shown to express increased MC-1R with the maturation of DCs, and α-MSH significantly suppresses CD86 and CD40 expression in DCs [6]. In the present study, we confirmed that the maturation markers, CD86 and CD83, on TNF-α-treated MoDCs were down-regulated by α-MSH. An enhanced antigen uptake of MoDCs was observed after α-MSH treatment, which is a feature of immature DCs. Our data also showed that α-MSH inhibited the secretion of the Th1 cytokine IL-12, but promoted the secretion of the Th2 cytokine IL-10 in MoDCs. IL-12 is integral in dictating the adaptive immune response. Higher level of IL-12 from mature DCs possesses T-helper polarizing properties, thus help the maturation of naïve T cells into effector Th1 cells [18]. IL-10 regulates growth and/or differentiation of DCs and plays a key role in differentiation and function of the T regulatory cell, which may figure prominently in the control of immune responses and tolerance in vivo [19]. It has been reported

Table 1 Up-regulated protein spot identified in proteomic analysis of TNF-α + α-MSH-treated MoDCs compared with that of TNF-α-treated MoDCs

<table>
<thead>
<tr>
<th>MOWSE score</th>
<th># (%) Masses matched</th>
<th>Protein MW (Da)/pI</th>
<th>Species</th>
<th>NCBIInr.2.26.2004 Accession No.</th>
<th>Protein name</th>
</tr>
</thead>
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<tr>
<td>1.16e + 005</td>
<td>9/15 (60%)</td>
<td>38714.5/6.57</td>
<td>Homo sapiens</td>
<td>4502101</td>
<td>Annexin A1</td>
</tr>
</tbody>
</table>

Figure 4 Identification of ANXA1 as an up-regulated protein in TNF-α + α-MSH-treated MoDCs

After stimulation with 50 ng/ml of TNF-α, MoDCs were incubated with or without 10⁻¹² M of α-MSH for 24 h. Then, 2D gel electrophoresis and MALDI-TOF-MS were performed as described in the methods. (A) Peptide fingerprinting of ANXA1 and data (Table 1). (B) Up-regulation of ANXA1 was detected by immunoblotting with anti-ANXA1. GAPDH was used as a loading control. The asterisk indicates statistical significance (*P < 0.05). Results are representative of three independent experiments.
that DCs with a distinctive IL-10^+IL-12^- cytokine production profile might be endowed with tolerogenic functions [20]. Therefore, the inhibition of DC maturation by α-MSH may contribute to the suppressive effects of this neuropeptide in immune-mediated inflammation.

Although our data suggest that α-MSH inhibits the maturation of MoDCs, the significance of α-MSH in the maturation of various types of DCs is still controversial. Indeed, other reports show that α-MSH does not play a significant role in aqueous-humor-mediated or corneal-supernatant-mediated DC inhibition [21]. The different immunomodulatory role of α-MSH may be dependent on the microenvironment, in which DCs reside. Despite this evidence, α-MSH has been found to exert multiple anti-inflammatory effects in a variety of in vitro cell-culture systems, such as human lymphocytes, monocytes and keratinocytes [4,22,23]. Results of this study suggest that the inhibition of DC maturation by α-MSH may be one of the anti-inflammatory mechanisms of the neuropeptide in animal disease models [8,24].

To search for the important signaling molecules in effect of α-MSH on DC maturation, we compared the global protein patterns between TNF-α-treated DCs and TNF-α + α-MSH-treated DCs using 2D electrophoreses. Three protein spots that differently expressing in TNF-α + α-MSH-treated DCs were identified as ANXA1, retinoid-acid-receptor-related orphan receptor-alpha (RORα) and gastric cancer multi-drug resistance protein variant (partial data not shown). The up-regulated expression of ANXA1 in TNF-α + α-MSH-treated DCs was successfully detected by western blotting analysis. ANXA1 was first identified as a glucocorticoid (GC)-inducible protein in rat peritoneal macrophages. This 37-kDa protein has calcium- and
phospholipid-binding properties and is actively involved in the inhibition of eicosanoid synthesis and phospholipase A2 (PLA2) [25]. ANXA1 is known as an intra- and intercellular signaling molecule that it is relevant to processes as diverse as cell growth and differentiation, apoptosis, vesicle fusion, endocytosis and exocytosis [26,27]. In view of the findings mentioned above, we chose ANXA1 as a target molecule and used ANXA1-siRNA to further investigate the role of ANXA1 in the modulatory effects of α-MSH on DCs. The results showed that TNF-α-induced mature MoDCs expressed high levels of HLA-DR and lower levels of CD1a, which was consistent with a previous report [15]. Decreased HLA-DR expression and increased CD1a expression were observed in α-MSH-treated MoDCs, whereas ANXA1-siRNA was found to promote HLA-DR expression and reduce CD1a expression in TNF-α + α-MSH-treated MoDCs. HLA-DR is a MHC class II antigen that plays a major role in the cellular interactions that occur during antigen presentation and is also one of the markers of DC maturation. CD1a is traditionally regarded as a differentiation marker of DCs [9]. Our results imply that the regulation of the differentiation marker expressed on DCs by α-MSH is reversed through ANXA1 knockdown. After ANXA1-siRNA transfection, IL-12p40 production was increased markedly, but IL-10 secretion was decreased in TNF-α + α-MSH-treated MoDCs. ANXA1-siRNA suppressed phagocytosis of TNF-α + α-MSH-treated MoDCs. These data further demonstrate that ANXA1 is involved in the α-MSH-triggered signaling pathway that affects DCs. A recent report indicates that ANXA1-deficient DCs acquire an intermediate mature phenotype during differentiation, but they are deficient in terms of acquisition of the full mature phenotype, which is achieved by LPS application [28]. This is partly in line with the data that we present in this study. The functions of DCs can be changed significantly due to their maturation.

![Figure 8](image1.png)

Figure 8 Cytokine secretions in TNF-α + α-MSH-treated MoDCs with reduced ANXA1 expression Control siRNA and ANXA1-siRNA, respectively, were transfected into TNF-α + α-MSH-treated MoDCs with GenePorter2 Reagent. IL-12p40 and IL-10 levels in the supernatants of MoDCs were determined using ELISA. More IL-12p40 (but less IL-10) was secreted from TNF-α + α-MSH-treated MoDCs due to ANXA1 knockdown. Asterisk indicates statistical significance (*P < 0.05).

![Figure 9](image2.png)

Figure 9 Functional changes in TNF-α + α-MSH-treated MoDCs with reduced ANXA1 expression Control siRNA and ANXA1-siRNA, respectively, were transfected into TNF-α + α-MSH-treated MoDCs with GenePorter2 Reagent. Phagocytosis of MoDCs was measured by FACS. Results are representative data of three independent experiments. c-siRNA, control-siRNA; A-siRNA, ANXA1-siRNA.
state, thereby affecting the outcome of immune response. This feature of DCs has been utilized for the research and the treatment of many diseases. The roles of ANXA1 in DC maturation suggest that ANXA1 could be used as a new drug target for DC-based immunotherapy.

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