Interleukin-4 affects the mature phenotype and function of rat bone marrow-derived dendritic cells

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Abstract. Granulocyte macrophage-colony stimulating factor (GM-CSF), and GM-CSF plus interleukin-4 (GM-CSF + IL-4) are two commonly-used cytokine therapies for the generation of bone marrow-derived dendritic cells (DCs). However, the mechanisms underlying IL-4 involvement in DC generation and maturation remain unclear. In order to investigate the effect of IL-4 on DC generation, DCs from rat bone marrow progenitors were generated using GM-CSF, with and without IL-4. GM-CSF + IL-4 DCs exhibited more mature phenotypes, and the levels of naïve allogeneic T cell stimulation were greater compared with GM-CSF DCs. Phosphorylated signal transducer and activator of transcription 6 (p-STAT6), the active form of STAT6, was expressed in GM-CSF + IL-4 DCs but not in GM-CSF DCs. The present study demonstrated that IL-4 influences DC morphology and immune function, and that this process may be associated with the activation of STAT6.

Introduction

Dendritic cells (DCs) are types of antigen-presenting cells that are involved in the innate and adaptive immune responses (1-3). DCs have been used as target cells in a number of experiments. It is therefore important to develop DCs in larger quantities and of greater purity for use *in vitro* investigations (4,5).

In vitro investigations have demonstrated that cytokines are capable of promoting DC development and proliferation (6,7). Granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) are two commonly-used cytokine therapy protocols for the generation of DCs (8-10). Previous investigations have suggested that GM-CSF promotes the differentiation of bone marrow-derived DCs, while IL-4 may

support DC development and maturation when GM-CSF is absent (11,12). Previous investigations have demonstrated that DCs cultured with GM-CSF alone are functionally immature (13,14). Maturation state of DCs were previously determined by the expression levels of the major histocompatibility complex (MHC) class II molecule as well as co-stimulatory molecules (CD86, CD80, and CD40) (4); the expression of surface molecules, including MHC II, CD80 and CD86, were reported to identify an immature phenotype of DC (14). Furthermore, Lutz *et al* (15) demonstrated that IL-4 promotes DC maturation. However, the molecular mechanisms underlying the effect of IL-4 on DC maturation remain obscure (16).

Signal transducer and activator of transcription 6 (STAT6) is able to translocate to the nucleus and interact with other transcription factors, such as nuclear factor (NF)-κB, to regulate gene transcription (17,18).

In the present study, DCs from rat bone marrow progenitors were cultured with GM-CSF, with or without IL-4. The purpose of the present study was to compare the phenotype and functional properties of DCs generated using the two treatment protocols (GM-CSF and GM-CSF + IL-4). Furthermore, the involvement of IL-4 in DC maturation was examined. The present study also aimed to determine phosphorylated (p-) STAT6 expression in order to elucidate the role of the STAT6 signaling pathway in the DC phenotype.

Materials and methods

Experimental design. F344 rats (weight, 180-200 g; age, 6-8 weeks) were obtained from the Experimental Animal Center of Shanghai Institute for Biological Sciences (Shanghai, China). Sprague-Dawley (SD) rats (weight, 190-210 g; age, 6-8 weeks) were obtained from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). Animals were kept maintained under specific-pathogen-free conditions. Experiments were performed in endotoxin-free conditions. The present study was performed under the authority of a license issued by the Ethics Committee of Nanjing Medical University.

In vitro generation of DCs. DCs were generated according to a previously described method (15). Bone marrow cells were aspirated from F344 rat femurs and suspended in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA). Red blood cells were removed using a lysis buffer (Beyotime, Shanghai, China). DCs

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were cultured in 24-well plates at $6x10^5$ cells/ml in RPMI-1640 medium containing 10% fetal bovine serum (Gibco-BRL), 2 mM glutamine (Gibco-BRL), 100 U/ml penicillin (eBioscience, San Diego, CA, USA) and 100 U/ml streptomycin (eBioscience). One group of cells were treated with recombinant GM-CSF (5 ng/ml; Peprotech, Rocky Hill, NJ, USA) and IL-4 (5 ng/ml; PeproTech, Inc.; GM-CSF + IL-4 group). A second group of cells was treated with 5 ng/ml GM-CSF only (GM-CSF group). Cells were cultured at 37°C with 5% CO_2. Every two days, half of the medium was removed and an equal volume of fresh medium containing GM-CSF + IL-4 or GM-CSF was added. Morphological features of DCs were observed on days two, four and six, using phase-contrast inverted microscopy (IX71-DP30; magnification, x100; Olympus Biological Microscopes, Tokyo, Japan). Following six days of culture and treatment, non-adherent and semi-adherent cells were harvested.

Detection of cell surface molecules. Expression levels of cell surface molecules (MHC II, CD80 and CD86) (14) were determined using a FACScan flow cytometer (BD FACSCanto II™; BD Biosciences, San Jose, CA, USA). Following six days of culture, DCs were harvested and mixed with mouse anti-rat monocloncal fluorescein isothiocyanate (FITC)-conjugated anti-MHC II (1:400; cat. no. 11-0920-82), mouse anti-rat monoclonal phycoerythrin (PE)-conjugated anti-CD80 (1:400; cat. no. 12-0800-82) or mouse anti-rat monoclonal ITC-conjugated anti-CD86 (1:400; cat. no. 11-0860-81) antibodies (eBioscience), as previously described (19). Following 30 min of incubation at 5°C, cells were fixed in ethanol for 10 min and analyzed using flow cytometry (BD FACSCanto™ II; BD Biosciences). In order to investigate the effect of lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) on DC morphology, LPS (1 μg/ml) was added to the medium for 48 h at the end of 6 days of culture treatment. Subsequently, LPS-stimulated DCs were harvested, labeled with antibodies for MCH II, CD90 and CD86 (as above) and analyzed using flow cytometry.

Endocytosis assays. In order to assay endocytic activity, DCs ($5x10^5$) were incubated with FITC-dextran (Sigma-Aldrich) at a final concentration of 1 mg/ml at 37°C for 45 min. Subsequently, cells were collected, resuspended in 250 μl of flow cytometry buffer and analyzed using flow cytometery. Experiments were performed at 4°C.

Pro-inflammatory cytokine measurements. DCs ($2x10^5$ cells/ml) were incubated with or without 1 μg/ml of LPS for 48 h, and supernatants were collected. IL-12p70 and TNF-α concentrations were measured using enzyme-linked immunosorbent assay kits (ELISA; R&D systems™, Minneapolis, MN, USA) according to the manufacturer's instructions. Streptavidin-horseradish peroxidase and 2,2'-azino-bis (3-ethylbenzthiazoline-6- sulfonic acid) (Sigma-Aldrich) were used as enzyme and substrate, respectively. Optical density was measured at 405 nm using an ELISA reader (Bio-Rad 680; Bio-Rad, Hercules, CA, USA).

Mixed lymphatic reaction (MLR). MLR was conducted in order to measure the level of naïve allogeneic T cell stimulation. Cell proliferation was detected using a Cell Counting kit (Dojindo, Kumamoto, Japan) (20). Allogeneic T cells from SD rat splenic cells were isolated as responder cells using a Nylon Fiber Column (Wako, Osaka, Japan). Different numbers of DCs ($2.5x10^3$, $5x10^3$, $10x10^3$ and $20x10^3$) were treated with 25 μg/ml mitomycin C (Sigma-Aldrich) for 30 min at 37°C and added to the allogeneic T cells ($2x10^5$). Following 72 h of incubation, 10 μl WST-8 solution (Dojindo, Osaka, Japan) was added to each well, which contained 100 μl cell suspension in a 96-well plate for 2 h. Absorbance was then measured at 450 nm using the Bio-Rad 680 (Bio-Rad).

Western blotting. DCs were lysed on ice using Triton X-100 buffer, containing a protein inhibitor cocktail (Cell Biolabs, Inc., San Diego, CA, USA). Cytosolic proteins were extracted and separated using 10% SDS-PAGE gel electrophoresis (ZomAnBio, Beijing, China). Separated proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Inc., Pittsburgh, PA, USA). Bovine serum albumin (3%; Pierce, Rockford, IL, USA) was added in order to prevent non-specific binding. Primary mouse anti-rat monoclonal anti-STAT6 (ab130235) or rabbit anti-rat anti-p-STAT6 (ab195701) antibodies (Abcam, San Francisco, CA, USA) were incubated with the membrane for 18 h. The membranes were washed three times and exposed to polyconal goat anti-mouse (ab101063) or goat anti-rabbit (ab102279) immunoglobulin G secondary antibody conjugated to horseradish peroxidase (Absin, Shanghai, China). Bands were then visualized using enhanced chemiluminescence staining (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). β-actin was used as the reference gene.

Statistical analysis. Data are presented as the mean ± standard error. Experiments were repeated three times. An independent samples t-test was performed in order to compare means. SPSS, Inc. (version 11.0, Chicago, IL, USA) was used to analyze the data. P<0.05 was considered to indicate a statistically significant difference. Graphs were produced using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

DC morphology and cell surface molecule expression. On day two, DCs were small, round and adherent to the plate surface. DCs exhibited increased volume, reduced adherence to the plate surface and morphological changes, in a time-dependent manner. On day four, DCs were more irregular in shape, larger in size and were suspended in the medium, compared with cells on day two. On day six, DCs were less adherent to the plate surface compared with those on day four. Morphological changes are shown in Fig. 1A. Higher numbers of GM-CSF + IL-4 DC than GM-CSF DC clusters were observed. Expression of surface molecules in GM-CSF DCs and GM-CSF + IL-4 DCs was detected using flow cytometry. Expression levels of CD80/86 and MHC II surface molecules were significantly greater in GM-CSF + IL-4 DCs than those in GM-CSF DCs (P<0.01; Fig. 1B and C). In order to compare responses to LPS treatment, DCs were treated with LPS for 48 h. Following LPS treatment, GM-CSF + IL-4 DCs expressed significantly higher levels of CD80/86 and MHC II cell surface molecules than GM-CSF DCs (P<0.01; Fig. 2).
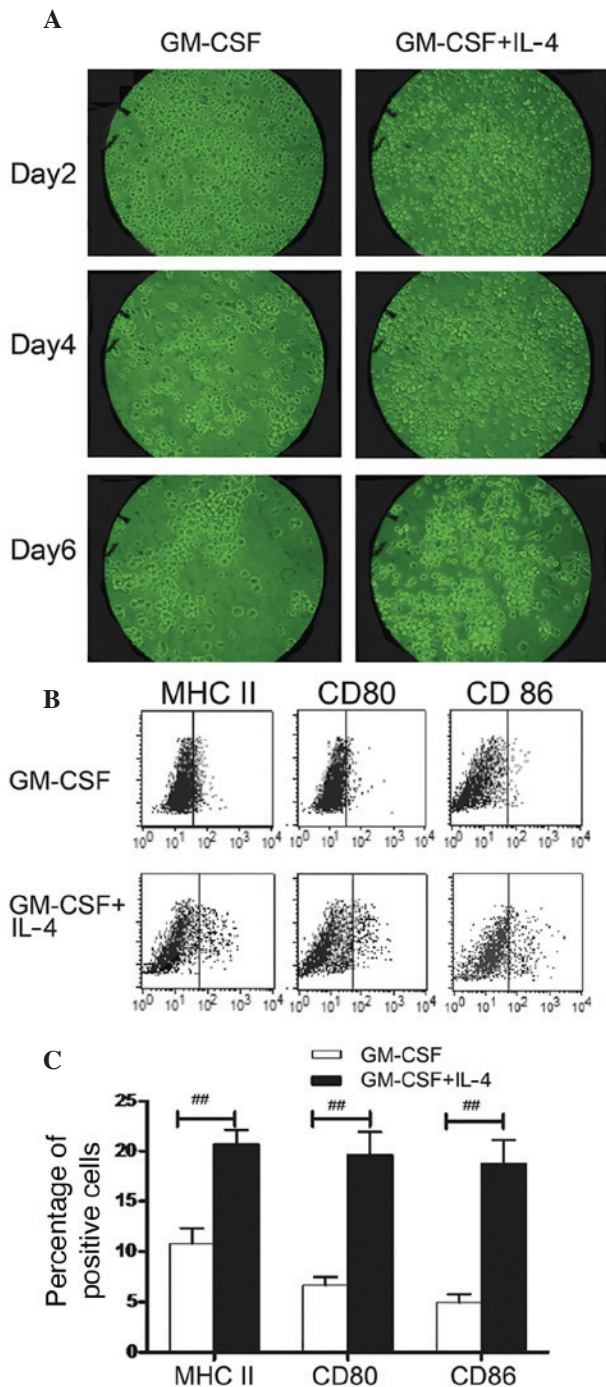
Figure 1. (A) DC morphological features were observed using an inverted phase-contrast microscope (x100). (B) Expression levels of surface molecules were analyzed by flow cytometry. (C) Expression levels of surface molecules. ##P<0.01. MHC, major histocompatibility complex; DC, dendritic cells; GM-CSF, granulocyte macrophage-colony stimulating factor; IL-4, interleukin-4.
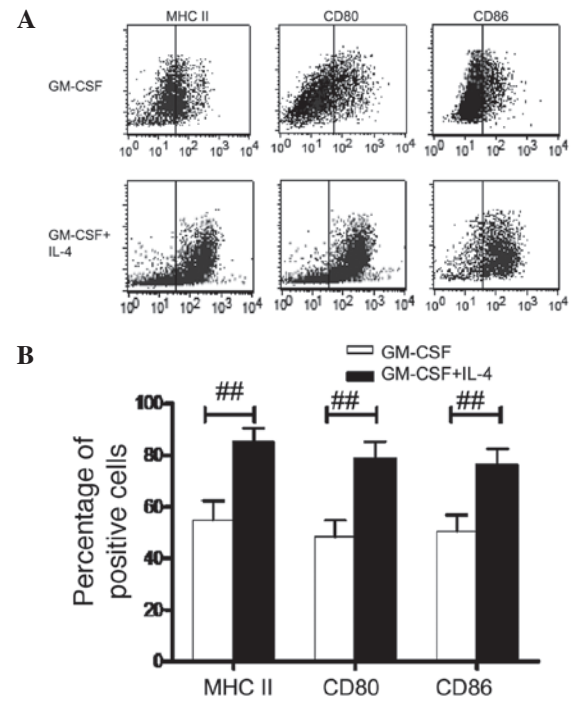

Figure 2. (A) Following LPS treatment of GM-CSF DCs and GM-CSF + IL-4 DCs, expression of surface molecules was analyzed using flow cytometry. (B) Expression of cell surface molecules. ##P<0.01. MHC, major histocompatibility complex; GM-CSF, granulocyte macrophage-colony stimulating factor; IL-4, interleukin-4; DC, dendritic cells.

DC endocytic activity. In order to measure the endocytic activity, DCs were incubated with FITC-dextran and analyzed using flow cytometry. Antigen uptake was significantly higher in GM-CSF DCs compared with that of GM-CSF + IL-4 DCs (P<0.01; Fig. 3A). In order to investigate cytokine profiles, DCs were incubated with or without 1 µg/ml LPS for 48 h. IL-12p70 and TNF-α concentrations were then measured using ELISA. IL-12p70 and TNF-α concentrations were significantly higher in GM-CSF + IL-4 DCs and GM-CSF DCs following LPS treatment compared with those not treated with LPS (P<0.01; P<0.05; Fig. 3B and C).

Naïve allogenic T cell stimulation. The capacity of two DC populations to stimulate allogeneic T cells was investigated using MLR. As shown in Fig. 3D, GM-CSF DCs had a weaker capacity to stimulate the proliferation of allogeneic T cells, while GM-CSF+IL-4 DCs showed enhanced proliferation at all ratios tested, with significantly increased results at $10x10^3$ and $20x10^3$ DCs (P<0.01).

STAT6 expression. Western blotting was performed in order to detect STAT6 expression as well as p-STAT6 expression, which is the active form of STAT6, in the DCs. p-STAT6 was not in GM-CSF DCs; however, it was expressed in GM-CSF + IL-4 DCs (Fig. 4 A and B). Mature DCs were treated with IL-4 and p-STAT6 expression was then detected in GM-CSF DCs without IL-4 (Fig. 4C), GM-CSF DCs with IL-4 (Fig. 4D), GM-CSF + IL-4 DC without IL-4 (Fig. 4E) and GM-CSF + IL-4 DC with IL-4 (Fig. 4F). As p-STAT6 is the active form of STAT6, it was not obviously expressed in the mature state (Fig. 4C and E); however, following the addition of IL-4, p-STAT6 was highly expressed. These observations indicate that IL-4 treatment during DC maturation resulted in the activation of STAT6 signaling (Fig. 4D and F).

Discussion

In the present study, surface molecule expression was significantly higher, antigen uptake was significantly lower and the capacity to stimulate allogeneic T cells was significantly
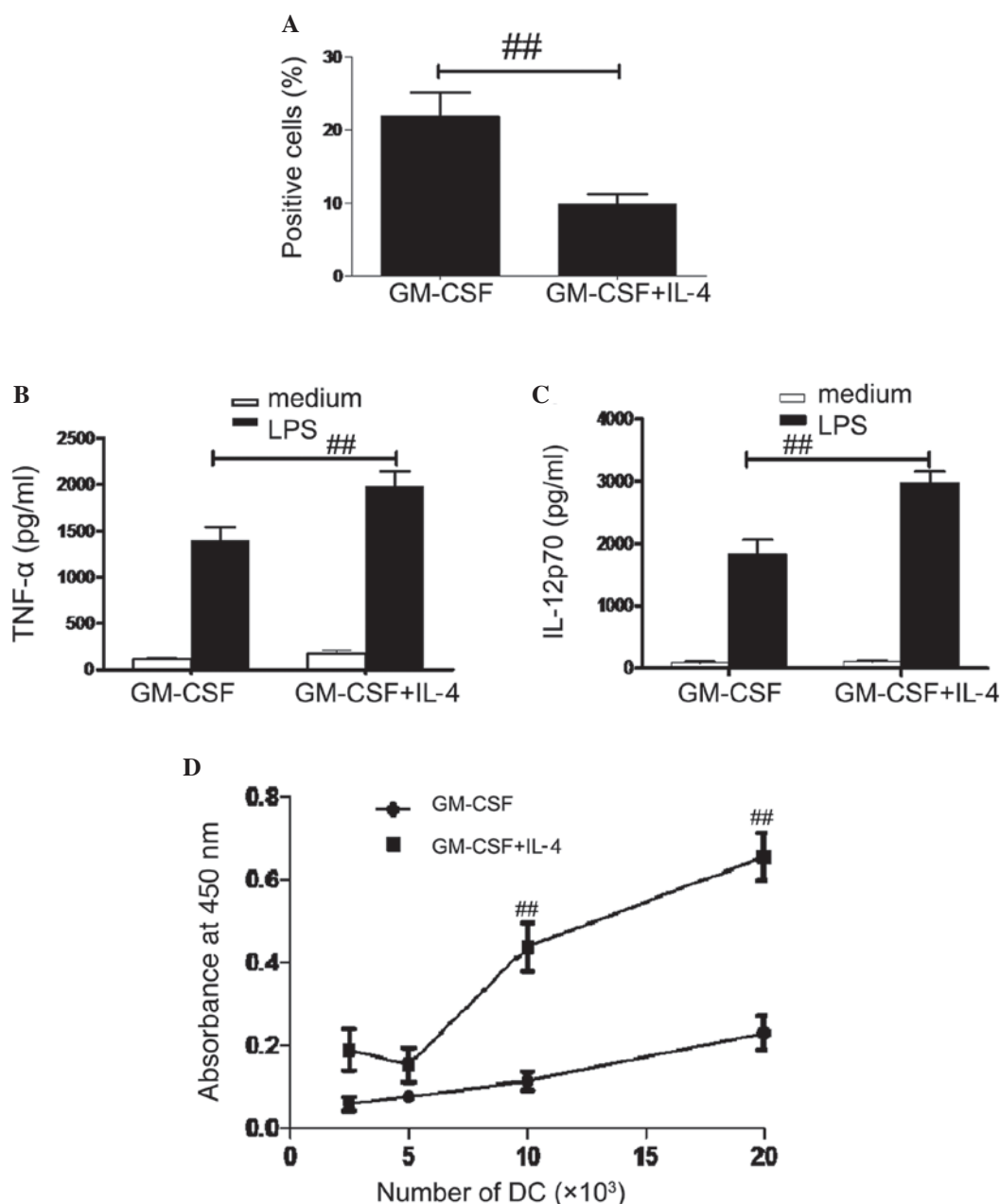
Figure 3. (A) Percentage of positive cells in the GM-CSF and GM-CSF + IL4 groups was analyzed using flow cytometry. (B) ELISA of TNF-α production. (C) ELISA result of IL-12p70 production. (D) Mixed lymphatic reaction result. Allogeneic T cell stimulation was significantly lower in GM-CSF DCs compared with GM-CSF + IL4 DCs. Absorbance was measured at 450 nm. ##P<0.01. DC, dendritic cells; GM-CSF, granulocyte macrophage-colony stimulating factor; IL-4, interleukin-4; medium, culture medium without lipopolysaccharide.
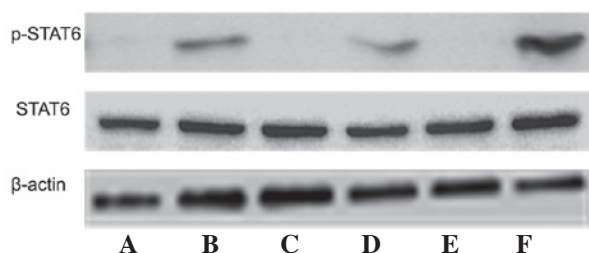

Figure 4. Expression of STAT6 and p-STAT6, analyzed using western blotting. (A) GM-CSF DCs; (B) GM-CSF + IL-4 DCs. Mature DCs were treated with IL-4 and p-STAT6 expression was detected in (C) GM-CSF DCs without IL-4; (D) GM-CSF DCs with IL-4; (E) GM-CSF + IL-4 DC without IL-4; (F) GM-CSF + IL-4 DC with IL-4. p-STAT, phosphorylated-signal transducer and activator or transcription 6; DC, dendritic cells; GM-CSF, granulocyte macrophage-colony stimulating factor; IL-4, interleukin-4.

higher in GM-CSF + IL-4 DCs compared with GM-CSF DCs. Therefore, GM-CSF DCs were phenotypically and functionally immature compared with GM-CSF + IL-4 DCs. Following LPS treatment, GM-CSF + IL-4 DCs matured more quickly and expressed higher levels of pro-inflammatory cytokines compared with GM-CSF DCs.

Wu *et al* (10) cultured bone marrow DCs with GM-CSF and recombinant chicken IL-4. DCs generated using this method exhibited typical DC morphological characteristics, and the presence or absence of the following cell surface molecules was observed: MHC class II (high), CD11c (high), CD40 (moderate), CD1.1 (moderate), CD86 (low), CD83 (not present) and DEC-205 (not present). The presence of these markers indicates whether or not the cells are functionally

mature, thus, GM-CSF + IL4 treatment promotes DC growth and maturation. In the present study, following LPS treatment GM-CSF + IL-4 DCs expressed significantly higher levels of cell surface molecules than GM-CSF DCs. Labeur *et al* (13) demonstrated that DCs cultured with GM-CSF alone were functionally immature, whereas GM-CSF DCs that were subsequently incubated with CD40L or LPS were functionally mature.

Typically, DCs take up extracellular antigens via endocytosis, which they subsequently process and present to T cells (20). During DC maturation, endocytosis is downregulated (21). In the present study, endocytosis levels were significantly lower in GM-CSF + IL-4 DCs compared with those of GM-CSF DCs. Furthermore, the production of pro-inflammatory cytokines was significantly greater in GM-CSF + IL-4 DCs than in GM-CSF DCs. Pro-inflammatory cytokines produced by DCs are important immunomodulatory factors which are capable of influencing immune responses (22).

In the present study, p-STAT6 was expressed in GM-CSF + IL-4 DCs but not in GM-CSF DCs. STAT6 is a downstream transcriptional activator involved in IL-4 signaling (17). Following treatment with IL-4 of the two DC groups (GM-CSF + IL-4 and GM-CSF), STAT6 was shown to be activated. This result indicated that STAT6 signaling is activated when DCs are treated with IL-4 during the maturation period. Once activated, STAT6 translocates into the nucleus and interacts with other transcription factors, such as NF-κB, which regulates gene transcription (18). Therefore, IL-4 treatment during the DC maturation phase resulted in the activation of IL-4 signaling and STAT6.

In conclusion, the present study demonstrates that IL-4 affects DC phenotype and function, and that it may be associated with the activation of STAT6.

Acknowledgements

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