Effect of thymosin α_1 on the phenotypic and functional maturation of dendritic cells from children with acute lymphoblastic leukemia

XUERONG LI, XIAODAN LIU, YANXIA ZHAO, REN ZHONG, AIQIN SONG and LIRONG SUN

Department of Pediatric Hematology and Oncology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266003, P.R. China

Received October 21, 2014; Accepted June 15, 2015

DOI: 10.3892/mmr.2015.4153

Abstract. To determine the effect of thymosin α_1 (Tα1) on the phenotypic and functional maturation of HL-60 cells, freeze-thaw antigen-loaded dendritic cells (DCs) were derived from peripheral blood mononuclear cells (PBMCs) of children with acute lymphoblastic leukemia (ALL). The DCs were generated from the PBMC samples that were collected from the PB of 10 consecutive ALL children. On day 3 of culturing, the cells in the antigen + no Tα1 (AN) and antigen + Tα1 (AT) groups were incubated with 100 μl lysates obtained from freeze-thaw cycling. After 5 days of incubation, the AT group was administered with 100 ng/ml Tα1. On day 8, the DCs were stained with fluorescein isothiocyanate-conjugated cluster of differentiation (CD)1a, CD83 and HLA-DR antibodies and analyzed by flow cytometry. In addition, the killing activity of cytotoxic T lymphocytes (CTLs) from the different groups on wild-type leukemia cells was measured. The DCs in the AT group exhibited more apparent, characteristic dendritic morphologies than the control and AN group DCs. Furthermore, the lowest expression level of CD1a, and the highest expression of CD83 and HLA-DR were observed in the AT group when compared with the AN and control groups ($P<0.05$). The lactate dehydrogenase release assay demonstrated that the killing rate of CTL in the AT group was significantly higher than that in the control and AN groups ($P<0.01$). Thus, Tα1 may markedly promote the phenotypic and functional maturation of DCs, and may serve as a suitable immunomodulator of DC-based immunotherapy for treatment of hematological malignancies.

Correspondence to: Dr Xuerong Li, Department of Pediatric Haematology and Oncology, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao, Shandong 266003, P.R. China
E-mail: lxr0060@163.com

Key words: thymosin α_1, acute lymphoblastic leukemia, dendritic cell vaccine, maturation

Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood malignant disease, and accounts for the greatest percent of malignancies in children newly diagnosed with cancer in the USA (1,2). Following chemotherapy and hematopoietic stem cell transplantation, novel therapeutic strategies have been developed to improve the complete remission (CR) rate and overall survival of ALL patients (3,4). However, significant toxicity, relapse due to a state of minimal residual disease (MRD) and transplant-associated mortality limit the efficacy of allogeneic stem cell transplantation (5). Therefore, the development of additional immunotherapeutic strategies that selectively recognize and destroy leukaemia cells is required, with the aim of reducing relapse rates. In previous years, dendritic cell (DC)-based immunotherapy has presented as a promising strategy for the elimination of MRD in patients with ALL (6-8).

DCs are professional antigen-presenting cells (APC), and are critical in the induction of cellular and humoral immunity (9). Various studies have reported that the injection of tumor antigen-loaded DCs induces tumor-specific cytotoxic T lymphocyte (CTL) responses and leukemia resistance (6,10-12). However, the primary obstacles to the introduction of this therapeutic strategy in clinical practice include insufficient numbers of DCs and insufficient production of cytokines. Therefore, DC vaccine therapy relies on either the generation of sufficient numbers of DCs, to prime CTLs, or administration of immunomodulatory agents to overcome deficiencies in DC and CTL function (5). Previous studies that focused on an *in vitro* methodology have revealed that it is possible to derive DCs from peripheral blood mononuclear cells (PBMCs) using cytokines, which can be used to harvest sufficient numbers of DCs for use in vaccine therapy (13,14). In addition, specific immunomodulatory agents that induce DC maturation may improve DC vaccine therapy for the treatment of leukaemia (12).

Thymosin α_1 (Tα1) is a naturally occurring thymic peptide, consisting of 28 amino acid residues, that is widely distributed in numerous tissues and cells (15,16). Tα1 is administered worldwide for the treatment of certain immunodeficiencies, malignancies and infections (17). For example, Tα1 was shown to induce apoptosis and inhibit proliferation in human leukemia cell lines, suggesting its potential therapeutic effects in

leukemia (18). Additionally, Tα1 exerts immunomodulatory effects on T cells, natural killer cells and macrophages, and has an important role in the modulation of differentiation, maturation and the function of DCs. However, it remains unclear as to whether Tα1 affects the functional maturation of DCs that are derived from PBMCs of ALL patients.

In the present study, the influence of Tα1 on functional maturation of PBMC-derived DCs was investigated by analysis of the morphology, phenotype and CTL cytotoxicity in HL-60 cells. It was hypothesized that the immunomodulatory agent, Tα1 may be an effective adjuvant to DC vaccine therapy for the treatment of hematological malignancies.

Materials and methods

Patients. A total of 10 consecutive patients (males, n=5; females, n=5) with ALL (mean age, 5.5 years) were enrolled at the Department of Pediatric Hematology at The Affiliated Hospital of Qingdao University (Qingdao, China). The patients had achieved CR for ≥6 months and had not been administered with immunomodulators for ≥4 weeks. Informed consent to participate in the current study was obtained from their guardians and the study was approved by the Ethical Committee of The Affiliated Hospital of Qingdao University.

HL-60 cell culture and antigen preparation. The acute promyelocytic leukemia cell line HL-60 cells were obtained from Shanghai Institutes for Biological Sciences of Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (Gibco-BRL, Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco-BRL) and 1% penicillin-streptomycin solution (100 units/ml; Gibco-BRL) under a humidified 5% CO_2 atmosphere at 37˚C, as previously described (19). A half-medium exchange was performed every 2 days with fresh RPMI-1640 medium and the cells were subcultured under the same conditions.

Approximately $1.0x10^7$ cells/ml were collected by centrifugation (at 3,000 xg for 15 min) during the logarithmic growth phase and the cells were subjected to five rapid repetitive freeze-thaw cycles (-140˚C to 37˚C) to obtain the whole-cell (HL-60) tumor lysates. Removal of cell debris was conducted by centrifugation, leaving the tumor lysate-containing supernatant, which was stored at -20˚C for the subsequent loading of DCs with antigens.

Generation of PBMC-derived DCs and Tα1 treatment. PB samples (20 ml) from the ALL patients were obtained by venipuncture and maintained in sterile heparinized tubes; the blood samples were subjected to Ficoll-Hypaque density gradient centrifugation at 400 xg for 30 min (Ficoll-Hypaque, GE Healthcare, Little Chalfont, UK) (20) at room temperature. The PBMCs were collected from the interface and washed three times using Hank's buffer (Gibco-BRL). The DCs were prepared according to a previously described method (21). Briefly, the cell density was adjusted to $2.0x10^6$/ml with RPMI-1640 medium and incubated at 37˚C in 24-well culture plates (Sino-American Biotechnology, Shanghai, China) for 3 h. Nonadherent cells were gently removed by washing twice with RPMI-1640 medium and the adherent monocytes were cultured in the RPMI-1640 medium supplemented with 100 ng/ml recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ, USA) and 75 ng/ml interleukin (IL)-4 (Peprotech) at 37˚C to differentiate into immature DCs. On the third day of culture, the medium was aspirated and the cells were divided into three groups, in triplicate (n=3 wells in each group): i) Control; ii) AN (antigen + no Tα1); and iii) AT (antigen + Tα1; Patheon Italia S.P.A, Italy). The groups were incubated with fresh medium supplemented with 100 ng/ml GM-CSF and 75 ng/ml IL-4. Additionally, 100 μl freeze-thaw antigens were added to the AN and AT groups. Following 5 days of incubation, the new medium containing recombinant human tumor necrosis factor-α (TNF-α; 50 ng/ml) was added to each well, in addition to the GM-CSF and IL-4, to induce DC maturation. The AT group was then treated with 100 ng/ml Tα1: A Tα1 dose-dependent curve had previously been generated (data not shown), and optimal changes could be observed at a concentration of 100 ng/ml (21). After an additional 3 days, the DCs were harvested and used for *in vitro* analysis.

Morphological analysis. Morphological characteristics of PBMCs cultured for 3, 5, and 7 days in the presence of GM-CSF, IL-4 and TNF-α were analyzed on an inverted light microscope (magnification, x1,000; SZH-ILLB; Olympus, Tokyo, Japan). On day 8 of the culture, the morphology of mature DCs in each group was analyzed.

Phenotypic analysis of DCs by flow cytometry. Cultured DCs were harvested and washed twice with phosphate-buffered saline (PBS; Shanghai Threebio Technology Co., Ltd, Shanghai, China). Subsequently, the cells were incubated for 1 h at 4˚C with 1 μl of the following DC marker-specific antibodies: Mouse anti-human CD1a monoclonal antibody (cat no. 300102; BioLegend), mouse anti-human CD83 monoclonal antibody (cat no. 305305, BioLegend) and mouse anti-human HLA-DR monoclonal antibody (cat no. 307602; BioLegend). After washing with PBS, the stained DCs were incubated with fluorescein isothiocyanate (FITC)-conjugated Alexa Fluor 488F secondary antibody (mouse anti-human monoclonal antibody; cat no. A-10631; Invitrogen Life Technologies) for 30 min at room temperature. Isotype controls comprising mouse anti-human immunoglobulin G1 FITC-conjugated antibodies were included. After washing twice with PBS, 10,000 scatter-gated cells in each sample were analyzed with a FACScan flow cytometer using CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA).

Cytotoxicity assays. T lymphocytes isolated from PB were purified using a nylon wool column filtration method, as previously described (22). The autologous cells ($1.0x10^6$/ml) were cultured in the 6-well culture plate (2 ml/well) with the RPMI-1640-containing medium, 10% FBS and 100 μl IL-2 (20 ng/ml), at 37˚C in a 5% CO_2 atmosphere for 7 days. Half of the medium was exchanged for fresh culture medium supplemented with IL-2 (100 IU/ml) every other day. On day 8, the T lymphocytes were co-cultured with DCs from the different groups at a ratio of 10:1 in 24-well culture plates, which contained the medium of RPMI-1640 with 10% FBS, IL-2 and 25 μg/ml mitomycin C (Roche Diagnostics, Basel, Switzerland) for an additional 4 days to obtain the CTLs.

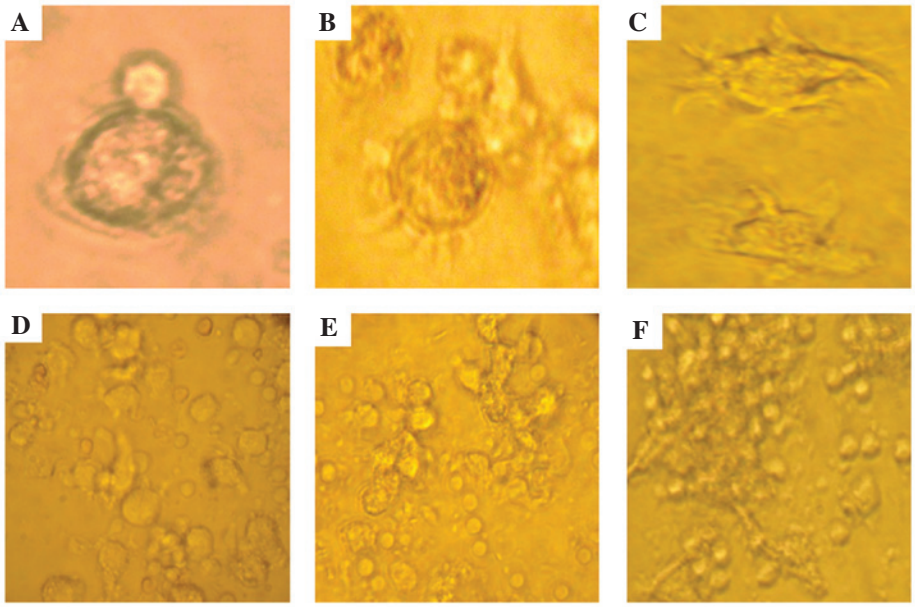

Figure 1. Morphology of peripheral blood mononuclear cell-derived DCs observed under an inverted light microscope. DCs following (A) 3; (B) 5; and (C) 7 days of culture (magnification, x1,000). (D-F) Following 8 days of culture: (D) Control group; (E) antigen + no Tα1 group (treatment with 100 μl freeze-thaw antigen); and (F) antigen + Tα1 group (treatment with 100 μl freeze-thaw antigen and 100 ng/ml Tα1) (magnification, x300). DC, dendritic cell; Tα1, thymosin α_1.

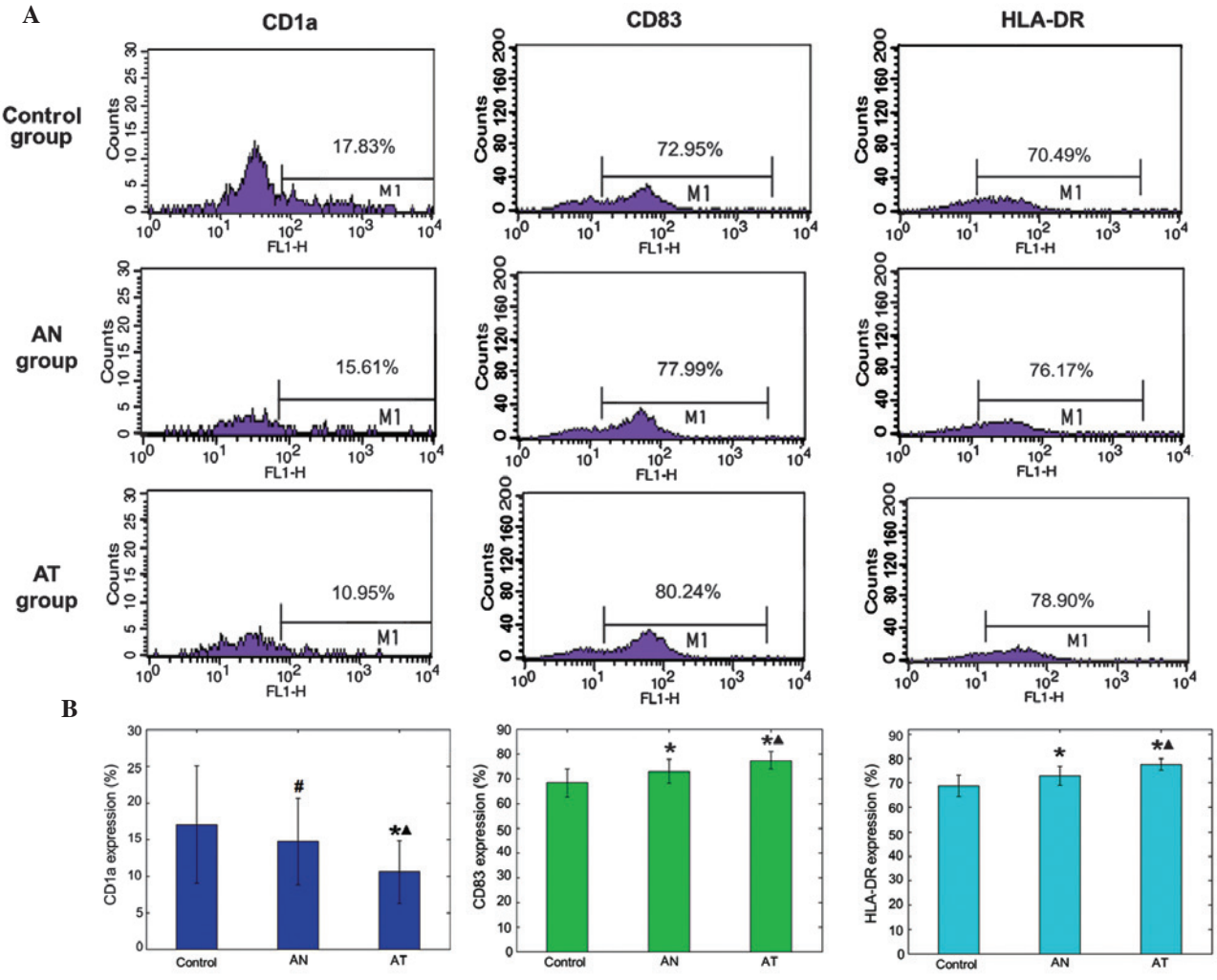

Figure 2. Expression of surface antigens of peripheral blood mononuclear cell-derived dendritic cells following an 8-day culture. (A) Flow cytometric analysis of phenotypic markers for CD1a, CD83 and HLA-DR. (B) Bar charts demonstrating the percentage of marker-stained positive cells. The data are expressed as means ± standard error of the mean (n=10). *P<0.05 vs. the control group; ▲P<0.05 vs. the AN group; #P>0.05 vs. the control group. CD1a, cluster of differentiation 1a; AN, antigen + no Tα1; AT, antigen + Tα1; Tα1, thymosin α_1.

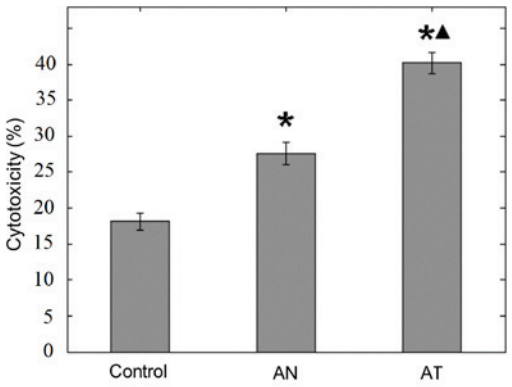

Figure 3. T-lymphocyte cytotoxicity in the control, AN and AT groups assessed by lactate dehydrogenase release method. Values are expressed as the mean ± standard error of the mean. *P<0.01 vs. the control group; ▲P<0.01 vs. the AN group. AN, antigen + no Tα1; AT, antigen + Tα1; Tα1, thymosin α_1.

The CTLs, which served as effector cells (E), continued to co-culture with the wild-type HL-60 cells, which were regarded as target cells (T), at a ratio of 20:1 (E:T) for 4 h at 37˚C. The cytotoxicity was measured using the lactate dehydrogenase (LDH) Cytotoxicity Assay kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The optical density (OD) was measured at 490 nm using a microplate reader (model MR 5000, Dynatech, Paris, France) and the cytotoxicity of CTL cells was calculated according to the following formula: Cytotoxicity (%) = (experimental OD value - natural release OD value)/(target maximum OD value - natural release OD value) x 100.

Statistical analysis. The results are expressed as the mean ± standard error of the mean. Statistical analysis was performed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The different groups were compared by analysis of variance and P<0.05 was considered to indicate a statistically significant difference.

Results

Alterations in DC morphology. DC morphology was observed under an inverted light microscope every other day (Fig. 1). On the third day of culture, the cells displayed smooth round contours without apparent dendrites, which is the characteristic morphology of immature DCs (Fig. 1A). On day 5, the DCs had increased in size, and most of the nonadherent cells had acquired a typical dendritic morphology (Fig. 1B). After 7 days, the DCs revealed a typical mature dendritic cell morphology (Fig. 1C). Furthermore, following 8 days of culture, numerous clusters of cells with long dendrites were apparent in the control, AN and AT groups. DCs in the AT group exhibited looser adherence and more dendritic-like cytoplasmic projections when compared with the other groups (Fig. 1D-F).

Immunophenotypic characteristics of DCs. No significant difference was noted in CD1a expression between the AN and control group (14.68±5.86 vs. 17.01±7.99; P>0.05), while the AN group demonstrated higher levels of CD83 (72.85±4.79 vs. 68.23±5.65 and HLA-DR (72.91±3.92 vs. 68.81±4.4) compared with the control group (Fig. 2; P<0.05). Compared with the control and AN groups, the AT group expressed significantly lower CD1a levels, and significantly higher levels of CD83 and HLA-DR as a result of treatment with freeze-thaw antigens and Tα1: 10.55±4.25, 77.31±3.51 and 77.51±2.40%, respectively (Fig. 2; P<0.05).

CTL cytotoxicity. The CTL cytotoxicity assay response to the wild-type HL-60 cells was measured using the LDH release method according to the manufacturer's instructions. The lowest cytotoxicity (18.15±1.20%) was observed in the control group, while the AT group exhibited the highest cytotoxicity (40.20±1.48%) among the three groups (Fig. 3; P<0.01).

Discussion

Immunotherapy of malignant diseases that is mediated by a tumor antigen-loaded DC vaccine is considered to be a promising novel therapeutic strategy for the treatment of malignancies. (11,23-25). Previous studies have revealed that Tα1 exerts immunomodulatory effects on the maturation, differentiation and function of DCs from murine bone marrow (18,26) and healthy human PB (21). However, there are few published studies on the effects of Tα1 on DC-based vaccines for ALL. In the present study, via analysis of morphology, phenotype and CTL cytotoxicity in HL-60 cells, Tα1 was shown to induce the phenotypic and functional maturation of DCs with the capacity to induce antitumor immunity toward leukemic cells.

It is generally accepted that the degree of maturity of DCs correlates with the level of cytotoxicity and it is considered to be a critical factor that requires investigation to allow the DC vaccination to be improved (27,28). Furthermore, the ability of therapeutic DCs to migrate to the lymph nodes is influenced by the DC maturation stage and is considered to be important (5,29). A previous study regarding melanoma demonstrated that mature DCs loaded with melanoma antigens were superior to immature DCs loaded with melanoma antigens in the induction of immunological and clinical responses (30). The maturation of DCs is characterized by the altered expression of the DC markers, CD1a, CD80, CD86, DC-specific intercellular adhesion molecule-3-grabbing non-integrin and HLA-DR (31,32). CD1 molecules efficiently present antigens in immature DCs, therefore, a high CD1a level indicates an immature DC status (33,34). The surface expression of CD1a is downregulated during DC maturation (35). CD83, one of the primary markers of mature DCs, is upregulated concurrently with DC maturation (36,37). In addition, the major histocompatibility complex class II receptor, HLA-DR is also an important maturation marker for DCs, and the upregulated expression of it is apparent during DC maturation (38). In the current study, the DCs treated with lysates obtained by freeze-thaw cycling exhibited apparent dendritic morphology and markedly increased expression levels of CD83 and HLA-DR, when compared with the control group. However, the combined treatment, with lysates and Tα1, induced a reduced expression level of CD1a and increased levels of CD83 and HLA-DR expression in the DC surface phenotype, when compared with the lysate-alone treatment. Thus, Tα1 appears to promote the phenotypic maturation of leukaemia cell-derived antigen-loaded DCs.

In addition, an LDH release assay was used to measure the killing activity of CTLs (39). In the present study, the killing

rate of CTL in the group of leukemic cell lysates + Tα1 (the AT group) was higher than that of the lysate-alone (the AN group) and the control, indicating that Tα1 significantly improves the antigen presentation capacity of DCs and ultimately enhances the killing activity of CTL on leukemia cells.

In conclusion, the present study demonstrates that Tα1 promotes the phenotypic and functional maturation of DCs, thus inducing enhanced CTL killing activity on leukemia cells. These findings may provide a basis to further evaluate Tα1 as a potential immunomodulator for DC-directed vaccines and therapeutic strategies for the treatment of children with ALL.

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