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Immunotherapy with tumor cell lysate-pulsed CD8α$^+$ dendritic cells modulates intra-tumor and spleen lymphocyte subpopulations

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Using cellular adjuvants including dendritic cells (DCs) has provided a promising approach in immunotherapy of cancer. Our previous study showed that mice immunization with tumor cell lysate-pulsed DCs (TL-CD8α+DCs) could significantly suppress the tumor growth and increase mice survival. The aim of the present study was to investigate the impact of TL-CD8α+DC vaccine on intra-tumor and spleen lymphocyte subpopulations in tumor-bearing mice. A Balb/c mouse model of fibrosarcoma was used and changes in various lymphocyte subpopulations including CD4$^+$, CD8$^+$ and CD4$^+$CD25$^+$Foxp3$^+$ T cells in mice immunized with TL-CD8α$^+$ DCs were studied. The cytotoxic activity of the lymphocytes and tumor growth inhibitory rate were also measured. Immunotherapy with TL-CD8α$^+$ DCs significantly enhanced both CD4+ and CD8+ lymphocytes, whereas decreased CD4$^+$CD25$^+$ Foxp3$^+$ regulatory T cells as well as the tumor growth rate. There was also a decrease in the ratio of regulatory T cells to CD4$^+$ and to CD8$^+$ lymphocytes in both the tumor and spleen tissues as compared to that in the non-immunized control mice. Immunization with TL-CD8α$^+$ DCs as well as CD8α$^+$ DCs significantly increased the splenocytes cytotoxic activity by 45.1% and 18.2% of control, respectively. In conclusion, the current study indicated that TL-CD8α$^+$ DCs can enhance tumor immunity against the fibrosarcoma by enhancing both the CD4$^+$ and CD8$^+$ lymphocytes and reducing regulatory T cells. This finding suggests the usefulness of TL-CD8α$^+$DCs vaccine for cancer treatment.

Key words: CD8α+ dendritic cells, intra-tumor and spleen lymphocytes, immunotherapy, tumor cell lysate

Induction of anti-tumor immunity using tumor antigens has become a main focus of cancer research in recent years. One promising approach in this regard may be tumor antigen vaccination by means of dendritic cells (DCs) [1-2]. DCs are specialized antigen presenting cells (APCs) that can induce the generation and proliferation of specific cytotoxic T lymphocytes (CTLs) and T helper (Th) cells through antigen presentation by the major histocompatibility complex (MHC) class I and class II molecules, respectively [3-6]. Apart from CTLs that have a pivotal role in antitumor immunity, the Th1 arm of the immune response plays also an important role in battle against malignancies [7]. There are several factors that can influence the development and polarization of immune response toward Th1 cells, among which DCs, their lineage and activation status are the most important. Recent studies have shown that various DC subsets are able to promote different types of immune responses [8-10]. Based on the CD4 and CD8α expression, murine conventional splenic DCs can be divided into three subsets including CD8α$^+$CD4$^-$, CD8α$^-$CD4$^+$ and CD8α$^-$CD4$^-$ [11-12]. It has been recommended that antigen–pulsed CD8α$^+$DCs could induce a Th1 differentiation, whereas CD8α$^-$ DCs drive a Th2 development [10, 13]. Several studies have shown that CD8α$^+$ DCs are the major producers of interleukin (IL)-12 in vitro; CD8α$^+$ DCs (but not CD8α$^-$ DCs) are able to respond to stimulation by secreting IL-12 p70 cytokine [14]. An in vivo study has demonstrated that most of the IL-12 producing DCs in the spleen of mice exposed to soluble extracts from toxoplsama gondii or lipopolysacharide (LPS) belong to the CD8α$^+$ DCs group [15]. In addition, interferon (IFN)-γ production by the CD8α$^+$ DCs has been shown [16].

Abbreviations: DCs, Dendritic cells; TL, Tumor lysate; TL-DCs, Tumor lysate- pulsed DCs; TAAs, Tumor-associated antigens; MACS, Magnetic cell sorting; CTLs, Cytotoxic T-lymphocytes.

Therefore, it is expected that antigen-pulsed CD8α$^+$ DCs could induce Th1 differentiation and CTL immune responses. Use of tumor lysate (TL) allows the sensitization of T cells to multiple tumor associated antigens (TAAs) which is necessary for continued antitumor responses [17-18]. In the present study, the TL-CD8α$^+$ DCs and CD8α$^+$ DCs were used for treatment of fibrosarcoma tumor in mice. We assumed that pulsing with TL might enhance the effect of CD8α$^+$ DCs to stimulate stronger anti-tumor immune responses. The anti-tumor immune response was examined by measuring the intra-tumor and spleen lymphocyte subpopulations, splenocytes cytotoxic activity and tumor growth inhibitory rate in the tumor-bearing mice.

Materials and methods

Animals. Female Balb/c mice (6-8 weeks old) were purchased from the Pasteur Institute, Tehran. Animals were acclimatized to standard laboratory condition for one week before use. The room temperature (22 ± 2° C) and lighting were controlled. The animals were maintained on a commercial chow with water *ad libitum*. All the animal experiments were approved and performed according to the guidelines of the Ethical Committee of Tarbiat Modares University.

Cell culture and preparation of tumor cell lysate. Balb/c mouse-derived fibrosarcoma (WEHI-164) cell line was prepared from the National Cell Bank of Iran, Tehran and maintained by culturing in RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco, USA), 2 mM L-glutamine (Sigma), 100 µg/ml streptomycin and 100 U/ml penicillin in a humidified incubator at 37°C and 5 % CO2. Cultures were allowed to grow until confluent. The cells at the logarithmic phase of growth were collected by detachment from the culture dish using trypsin/EDTA (Gibco) solution and then washed 3 times with phosphate buffered saline (PBS). Tumor cells were disrupted by 5 cycles of freezing and thawing using liquid nitrogen and a 37°C water bath. The large particles were removed from the cell lysate by centrifugation at 3000 rpm for 20 min and then the supernatant was collected and filtered through a 0.2-µm filter.

Isolation of CD8α$^+$ DCs. The mice were sacrificed and their spleens were removed. The spleens were minced into small pieces, pooled and digested with 1mg/ml Collagenase D (Boehringer Mannheim, Germany) in RPMI 1640, containing 10% FCS at 37°C for 10 min. The obtained suspension was filtered through a cell strainer (40µm) and then centrifuged at 1300 rpm at 4°C for 5 min. The cell pellet was resuspended in RPMI 1640 medium and dead cells were depleted by density gradient fractionation on Nycodenz solution. Afterward, CD8α$^+$ DC isolation was performed by magnetic cell sorting (MACS) using antibody-coated microbeads (Miltenyi Biotec, Germany, Cat No: 130-091-169) according to the manufacturer's protocol. The isolation of CD8α$^+$ DCs was performed in a two-step procedure. First, the T cells, B cells and NK cells were magnetically labeled using a cocktail of biotin-conjugated monoclonal anti-mouse antibodies against: CD90 (Thy1.2, isotype: rat IgG2b), CD45R (B220, isotype: rat IgG2a), CD49b (DX5, isotype: rat IgM) and anti-biotin microbeads [microbeads conjugated to monoclonal anti-biotin antibody (clone: Bio3-18E7.2, isotype: mouse IgG1)]. The labeled cells were subsequently depleted by separation over a MACS column. In the second step, the CD8α$^+$ DCs were directly labeled with anti-CD8α conjugated microbeads (Ly-2, isotype: rat IgG2a) and isolated by positive selection from the pre-enriched DCs fraction. To achieve the highest purities, the positively selected cell fraction containing the CD8α$^+$ DCs was passed through a second column. The final purity of the CD8α$^+$ DCs, as accessed by flow cytometric analysis, was more than 96%.

Immunization protocol. The Balb/c mice were injected subcutaneously (S.C.) in the right flank with WEHI-164 fibrosarcoma cell line (1x10^{6}cells/ 100µl) ten days before immunization with the DCs. The isolated CD8α$^+$DCs were cultured in the presence of 100 µg/ml of TL and 1 µg/ml lipopolysaccharide (LPS) for 24 h at 37°C and 5% CO2. The pulsed cells were then harvested and washed in PBS to remove free tumor antigens and resuspended in PBS for using in immunization protocol. After ten days, the tumor-bearing mice were divided into three groups each containing ten mice. They were injected S.C. on days 0, 7 and 14 with PBS (group 1 as control), unpulsed CD8α$^+$ DCs (group 2) and TL- CD8α$^+$ DCs (group 3) in the right flank. A total of 3×10^5 cells in 100µl PBS were used for immunization of the mice in groups 2 and 3.

Analysis of intra-tumor and spleen lymphocyte subpopulations. Two weeks after the last immunization, the mice were sacrificed and their spleen and solid tumors were removed. After measuring the size of the tumors, they were rinsed twice with PBS and then minced into small pieces. The suspensions were passed through 100µm stainless steel mesh and then intra-tumor leukocytes were separated by density gradient centrifugation over ficoll-hypaque. After collecting and washing the cells with medium, their viability was determined by trypan blue exclusion. The splenocytes were also isolated from the mice spleen by passing it through 100µm filters to obtain a single-cell suspension and then lysing erythrocytes at room temperature using ACK lysis buffer (containing NH4Cl, KHCO3, and Na2EDTA). The splenocytes were washed and resuspended in RPMI 1640 medium supplemented with 10% FCS. The freshly prepared cells were analyzed by direct staining using various antibodies as follows: Phycoerythrin (PE)-conjugated anti-CD8 (Serotech, UK), fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Serotech), and CD4$^+$ CD25$^+$ Foxp3$^+$ regulatory T cells kit (eBiosciences, USA). After staining, the cells were washed in washing buffer and fixed with %2 paraformaldehyde and then analyzed by flow cytometry. The percentage of total CD4$^+$and CD8$^+$ cells, and CD25$^+$ Foxp3$^+$ regulatory T cells within the gated CD4$^+$ cells were determined by an EPICS Coulter flow cytometer (USA) and Coulter software .

Evaluation of the cytotoxic activity of splenocytes. The cytotoxic activity of the splenocytes isolated from the immunized

mice (as effector cells) was determined against WEHI-164 tumor cell line (as the target) by LDH cytotoxicity detection kit (Roche Applied Science, Germany). The effector cells were washed with the assay medium (RPMI 1640 supplemented with 1% bovine serum albumin), and then co-cultured with the target cells at a pre-determined ratio (20:1) in a 96-well round bottom plate for 6 h at 37°C. The cell culture plates were then centrifuged and the supernatants were transferred into a flat-bottom enzyme-linked immunosorbent (ELISA) plate. Afterward, 100 µl of LDH detection mixture was added to each well and the plate incubated for 30 minutes at room temperature. Absorbance of the plate wells was measured by an ELISA reader (Pharmacia, Sweden) at 490 nm. The percentage of cell mediated cytotoxicity was determined as follows:

Cytotoxicity (%) = [(test LDH release) – (spontaneous LDH release by the effector and target cells) / (maximum LDH release by the target cells – spontaneous LDH release by the target cells)] × 100. For the controls, the target cells were incubated either in the culture medium alone to determine spontaneous LDH release or in a mixture of 2% triton X-100 to define the maximum LDH release. All the experiments were performed in triplicate.

Evaluation of the tumor growth following immunization. The volume of tumors isolated from mice of all the groups on 30th day of experiment was measured by a vernier caliper and calculated using the following formula [19]. Volume (mm^3) = length ×width^2×π/6. Then, the tumor growth was determined and compared between groups.

Statistical analysis. The results were presented as the mean ± standard deviation (SD). All statistical analyses were conducted with statistical package for the social sciences (SPSS, Abaus Concepts, Berkeley, CA) using One-way ANOVA test. P values less than 0.05 were considered significant.

Results

The effect of TL-CD8α+ DCs immunization on the CD4+ and CD8+ lymphocytes. The effect of TL-CD8α+ DCs on the tumor infiltrated CD4+ and CD8+ subpopulations were measured by flow cytometry. The results indicated a significant increase in the tumor-infiltrated CD4+ cells in mice immunized with TL-CD8α+DCs compared to in those treated with CD8α+DCs or PBS as control (Figure. 1A, p< 0.001). Treatment of mice with CD8α+DCs had no significant effect on increasing the number of tumor-infiltrated CD4+ cells compared with control mice receiving PBS (p=0.081). Analysis of the effect of DCs on the spleen cells showed a significant increase in the CD4+ splenocytes in TL-CD8α+DCs-immunized mice compared to mice receiving no DCs (Figure. 2A, p= 0.02).

With respect to intra-tumor CD8+ cells, a significant increase after treatment of mice with TL-CD8α+ DCs compared to the control was observed (Figure. 1B, (p< 0.001). Administration of CD8α+ DCs also increased the level of tumor-infiltrated CD8+ cells compared to the control but the results was not significant (p=0.058). As shown in Figure. 1C,

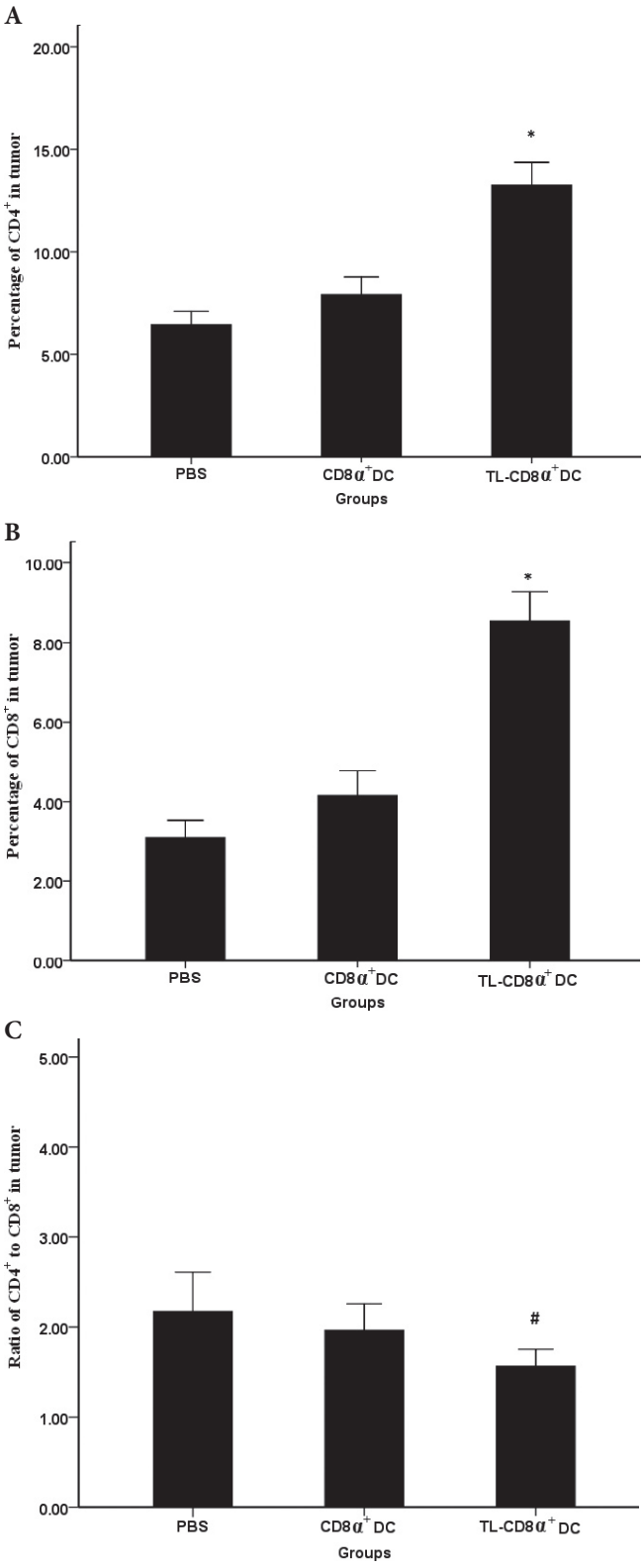

Figure 1. Effects on total intra-tumor CD4+ and CD8+ cells and the resultant CD4+/CD8+ ratio. Data shown are mean ± SD of 10 mice/group.*Value significantly different from unpulsed-CD8α+ DCs and PBS groups (p<0.001). # Value significantly different from PBS group (p=0.017).

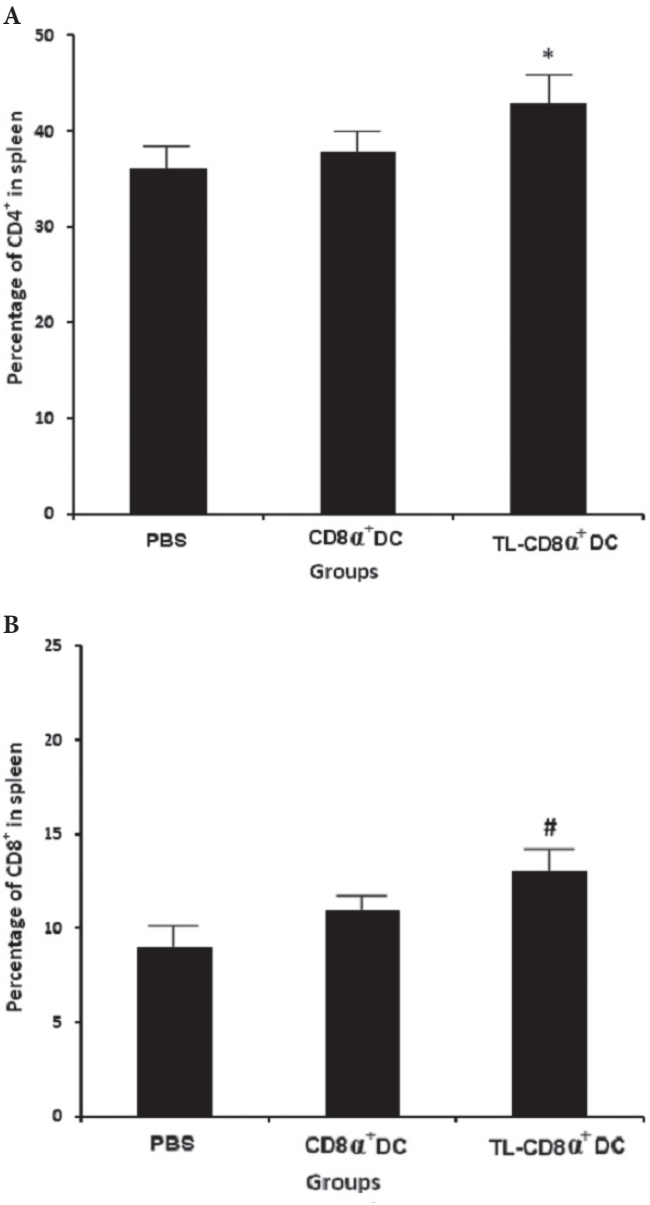

Figure 2. Effects total CD4⁺ (A) and CD8⁺ (B) cells in the spleen
Data shown are mean ± SD of ten mice/group. * Value significantly different from PBS group (p=0.02). # Value significantly different from PBS group (p=0.021).

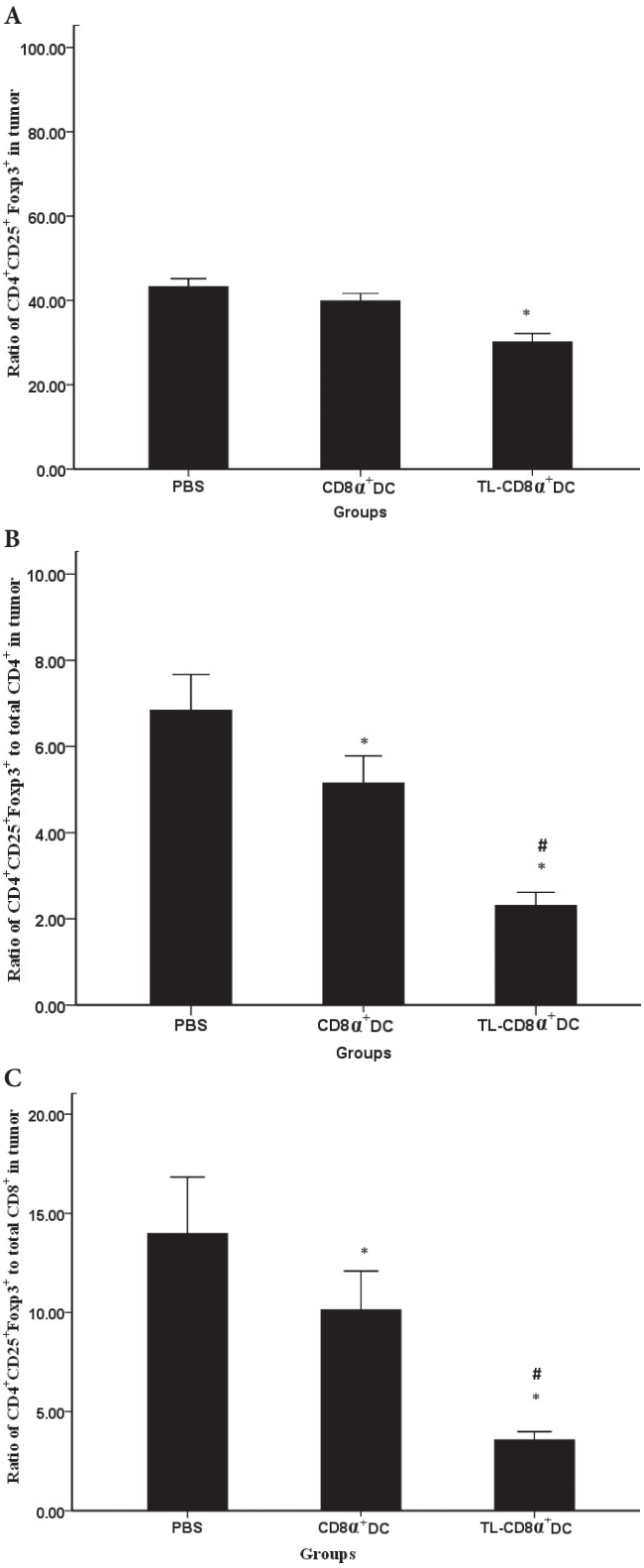

Figure 3. Effects on intra-tumor CD4⁺ CD25⁺ Foxp3⁺ regulatory T lymphocytes (A) and their ratios to CD4⁺ cells (B) and CD8⁺ cells (C). Data shown are mean ± SD of ten mice/group. *Value significantly different from PBS group (p<0.001). # Value significantly different from unpulsed-CD8α⁺ DCs group (p<0.001).

$CD4^+/CD8^+$ ratio in the group of mice treated with TL-CD8α⁺ DCs was significantly lower than the ratio in mice treated with PBS (p=0.017). In addition, the results indicated a significant increase in the $CD8^+$ splenocytes after the immunization with TL-CD8α⁺ DCs compared to PBS (Figure. 2B, p= 0.021).

The effect of TL-CD8α+DCs immunization on CD4+ CD25+ Foxp3+ regulatory T cells. The percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T lymphocytes infiltrated in the tumor was measured in different groups of mice by flow cytometry. As shown in Figure. 3A, immunization with TL-

CD8α+DCs significantly reduced the percentage of intra-tumor regulatory T lymphocytes compared to the control ($p< 0.001$). Treatment with CD8α+ DCs also to a lower extent decreased the number of these cells but the results did not reach to be significant ($p=0.057$). Furthermore, our findings presented in Figure 3B and 3C revealed a significant decrease in the ratio of regulatory T lymphocytes to CD4+ T cells and to CD8+ T cells in mice treated with TL-CD8α+ DCs ($p< 0.001$) and CD8α+ DCs ($p< 0.015$) compared to in those treated with no DCs. In addition, the percentage of CD4+ CD25+ Foxp3+ regulatory T lymphocytes significantly decreased in the spleen tissue of mice treated with TL-CD8α+ DCs compared to in control mice group ($p= 0.038$, Figure. 4). These results indicated that immunization with TL- CD8α+ DCs can reduce intra-tumor and spleen CD4+ CD25+ Foxp3+ regulatory T lymphocytes in fibrosarcoma tumor-bearing mice.

The effect of TL-CD8α+DCs immunization on the splenocytes cytotoxic response. The cytotoxic activity of the slpenocytes in the immunized mice was determined using LDH release assay. The splenocytes were isolated two weeks after the last immunization and co-cultured with the WEHI-164 tumor cells as specific target cells for 6-8 hours. As the results in Figure 5 indicated, the cytotoxic activity of splenocytes isolated from the mice treated with TL- CD8α+ DCs and unpulsed-CD8α+ DCs was significantly more than that in the non-immunized mice ($P<0.001$). Comparison of the cytotoxic activity of splenocytes showed a significant stronger activity of cells from TL- CD8α+ DCs-treated mice than those from unpulsed-CD8α+ DCs-treated ones ($P < 0.001$).

The effect of TL-CD8α+DCs immunization on the tumor growth. The tumor growth was examined by measuring the tumor volume after treatment with TL-CD8α+ DCs, unpulsed CD8α+ DCs, and PBS. The volume of tumors at the time of immunization was between 104 – 149 mm^3. After 30 days, the mean volume of tumors reached to 6135, 5082 and 3597 mm^3 in mice treated with TL-CD8α+ DCs, unpulsed CD8α+ DCs, and PBS (control), respectively. As the results in Figure 6 shows, the tumor volume was significantly decreased in the test groups compared to that in the control group ($p<0.05$).

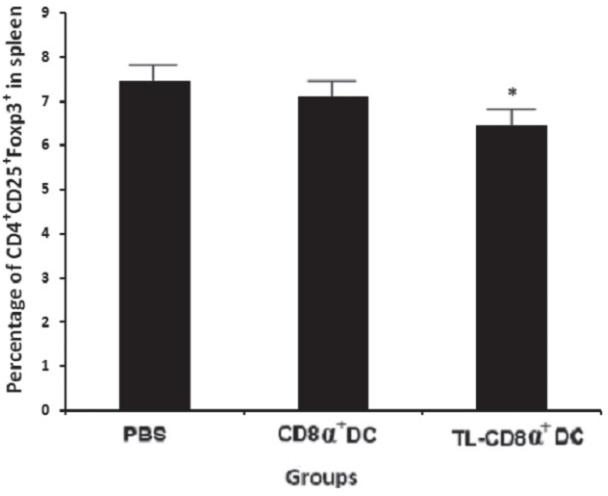

Figure 4. Effects on CD4+CD25+Foxp3+ regulatory T lymphocytes in the spleen. Data shown are mean ± SD of ten mice /group. * Value significantly different from PBS group (p=0.038).

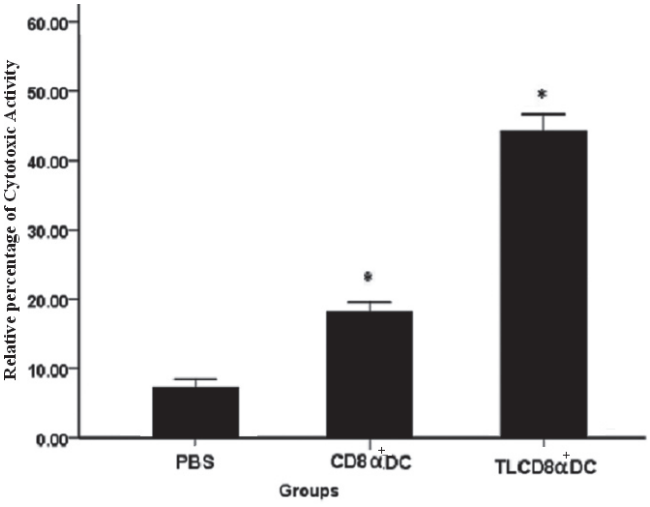

Figure 5. Effects on splenocytes cytotoxic activity
The cytotoxic activity of the splenocytes (as effector) against fibrosarcoma WEHI-164 cells (as target) in different groups of mice treated with phosphate buffered saline (PBS) as control, unpulsed-CD8α+ DCs and TL-CD8α+ DCs determined by LDH release assay. The specific cytotoxic activity in the test groups was significantly higher than in the controls ($P<0.001$). The values are the mean ± SD of triplicate wells.

Discussion

DCs are the most potent APCs mediating the effective in vitro and in vivo immune responses [20, 21]. These cells are used for antigen-specific immunotherapy of cancer because they are the most efficient APCs that can induce primary immune responses [22, 23]. The anti-tumor effect of tumor cell lysate-pulsed DCs was first reported in 1998 [24]. From that time, DCs have been utilized to treat various malignant diseases such as renal cancer, lymphoma, colorectal cancer and other diseases [25-27]. In previous investigations no association with a higher incidence of autoimmunity has been demonstrated in vaccination with tumor lysate-pulsed DCs compared with peptide-pulsed DCs [28] which may suggest the safety of this kind of vaccine.

Among murine splenic DC subsets, CD8α+DCs are known to produce the IL-12p70 cytokine and to have an exceptional capacity for cross-presentation of exogenous antigens [12]. In addition, it has been reported that mouse CD8α+ DCs and human equivalent BDCA3+ DCs are major producers of interferon-lambda (IFN-λ) in response to polyinosinic: polycytidylic acid (poly IC), a synthetic double-stranded RNA, and toll-like receptor (TLR) ligand [29, 30]. IFN-λ has shown

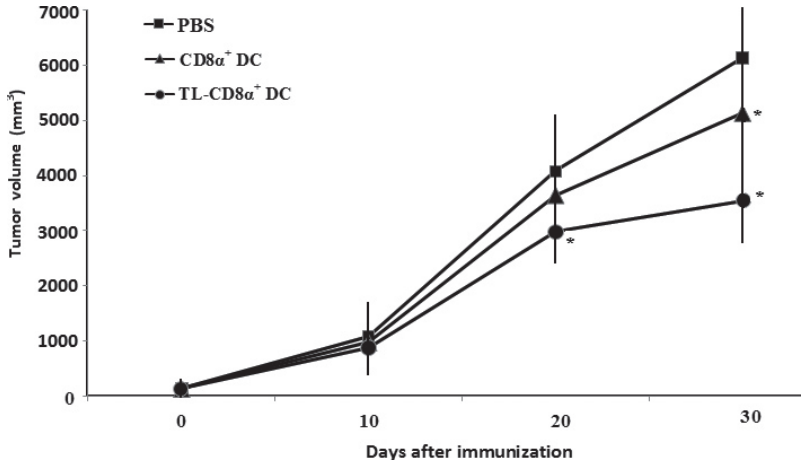

Figure 6. Effects on the tumor growth
Data shown are mean ± SD of ten mice /group. Immunization with TL- CD8α⁺ DC and unpulsed- CD8α⁺ DC in fibrosarcoma-bearing mice significantly inhibited the tumor growth compared with control group (*P<0.05).

anti-tumoral and anti-viral activities. IFN-γ cytokine in the culture medium has increased production of IFN-λ in response to poly IC and TLR ligation [29, 30]. The mechanism of the anti-tumoral activity of IFN-λ produced by CD8α⁺ DCs is not exactly identified and needs further investigations.

Our previous study indicated that immunotherapy with tumor lysate and heat-treated tumor lysate-loaded CD8α⁺ DCs significantly increased lymphocytes proliferation and IFN-γ production by splenocytes [31]. On the other hand, we knew that DCs play a critical role in initiating and activating T cell immunity and are important for the final outcome in cancer immunotherapy. Therefore, in this study we investigated the ability of TL-CD8α⁺ DCs on the modulation of intra-tumor and splenic lymphocyte subpopulations and on the splenocytes cytotoxic activity in the tumor-bearing mice.

It is now well recognized that tumors can aggressively induce conversion of T cells towards $CD4^+CD25^+Foxp3^+$ regulatory T cells [32]. Previous studies have demonstrated that these cells work as the main factor of tumor-mediated immune suppression [33]. In this regard, several studies have focused on the major mechanisms of immune response inhibition by regulator T cells [34-36]. Terabe et al. indicated that either removing regulatory T cells or blocking an immunoregulatory pathway induced by these suppressor cells might improve the efficacy of the tumor immunotherapy [37]. As, it has been previously reported, immunotherapy with DCs in clinical phase could reduce regulatory T cells in peripheral blood of glioblastoma patients and significantly was associated with improving the survival [38]. In addition, in situ vaccination with $CD8^+$DCs and plasmacytoid DCs have been strongly associated with inhibition of $Foxp3^+$ regulatory T cell activity and generation of potent $CD8^+$ CTLs response in melanoma tumor model [39]. A correlation between $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T lymphocytes and tumor size in murine mesotheliomas has also been reported [40]. Our result in this study indicated that immunotherapy with TL-CD8α⁺ DCs significantly increased the intra-tumor and spleen $CD4^+$ and $CD8^+$ lymphocytes whereas decreased regulatory T lymphocytes. The ratio of regulatory T cells to CD4+ and CD8+ cells after immunization with TL-CD8α⁺ DCs and CD8α⁺ DCs was also less than the ratio in mice receiving no DCs. Mehmet and colleagues have demonstrated that activated $CD8^+$ T effector or memory cells can eliminate $CD4^+$ $CD25^+$ $Foxp3^+$ T suppressor cells from tumor site via FasL mediated apoptosis [41]. As the cytotoxic assay in this study showed, splenocytes from the mice immunized with TL-CD8α⁺ DCs as well as unpulsed-CD8α⁺ DCs had more cytotoxicity against WEHI-164 than cells from non-immunized mice. In addition, decrease in tumor volume and increase in the tumor growth inhibitory rate in the immunized mice was observed. These findings suggests that $CD8^+$ CTL activity might be one of the reasons for reduction of the intra-tumor $CD4^+CD25^+Foxp3^+$ suppressor cells after immunotherapy with TL-CD8α⁺ DCs and CD8α⁺ DCs as previously described [41]. Further studies for confirming these findings are needed.

In summary, results of this study showed that immunization with TL-CD8α⁺ DCs in fibrosarcoma tumor-bearing mice can modulate intra-tumor and spleen lymphocytes subpopulations by increasing $CD4^+$ and $CD8^+$ lymphocytes, reducing regulatory T cells and enhancing the cytotoxic activity which would lead to tumor growth retardation. These findings suggest the application of CD8α⁺ DCs as a promising approach for inducing anti-tumor immunity in malignant tumors. Further studies are necessary to find the main mechanisms involved in TL-CD8α⁺ DCs mediated anti-cancer activity.

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