ORIGINAL ARTICLE

Effects of Dendritic Cell Vaccine Activated with Components of *Lieshmania Major* on Tumor Specific Response

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ABSTRACT

Background: Dendritic cells (DCs) contribute essentially to the outset and course of immune responses. So in patients with malignancy, there have been considerable interests in use of these cells in different interventions. **Objective:** To evaluate the impact of *Leishmania major*'s components on DC maturation and their use as a therapeutic agent against tumor cells. **Methods:** The cancer model was induced by injection of WEHI-164 cells (BALB/c derived fibrosarcoma cell line) subcutaneously in the right flank of animals. Bone-marrow derived DCs (BMDCs) were cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. After 5 days, tumor lysate, *Leishmania major*'s lysate, and Lipopolysaccharide (LPS) were added to the culture and incubated for 2 days. IL-12 production in DCs was measured by ELISA. For Immunotherapy, Mice received DCs subcutaneously around the tumor site. Two weeks after DCs injection, cytotoxicity assay and infiltration of $CD8^+$ lymphocytes were evaluated. **Results:** Our results showed that immunotherapy with dendritic cells exposed to *Leishmania* extract led to producing a higher amount of IL-12, compare to the control group. A considerable increment in specific cytotoxic T cells activity, diminished tumor growth rate and improved survival of immunized animals were seen. **Conclusion:** This study indicates that the use of *Leishmania major* extract, as well as LPS, can enhance the efficiency of DC-based vaccines and provides a basis for the use of *Leishmania major* in DC-targeted clinical therapies.

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Iran.J.Immunol. VOL.16 NO.4 December 2019

268

INTRODUCTION

There exist different protocols for cancer treatment, including surgery, chemotherapy, radiation, hormonal therapy, and immunotherapy. Based on the disease stage, these protocols can be used alone or in combination; However, most of the treatment modalities have been reported to be inefficient in the progression phase of cancer. This is supposedly due to the unfavorable immune response versus the progressive tumors, mainly in the expansion of effector T cell responses. Cancer immunotherapy is a novel approach that eradicates the available tumor cells and creates a stable immunity inhibiting tumor recurrence (1). Considerable evidence shows that DCs play a pivotal role in initiating and directing antitumor immune responses. These cells are recognized as potent antigen-presenting cells (APCs), exclusively sufficient for deriving immunity to recently provided antigen (Ag) (2). Numerous DCs have been generated *in vitro* by the culture of bone marrow cells (PBMCs) with recombinant cytokine interleukin-4 (IL-4), and particularly granulocyte-macrophage colony-stimulating factor (GM-CSF). Such immature DCs are very efficient in antigen uptake by endocytosis pathway; however, they cannot express stimulatory molecules that result in the suboptimal stimulation of T cells (3). Therefore, to attain completely matured DC (mDCs), further signals are necessary. Pro-inflammatory signals or pathogen-derived factors are operated DCs maturation in peripheral tissues. Generally, DCs maturation status is determined by phenotype markers such as the significant expression of CD80 and CD83, and production of cytokines like IL-12 (4). IL-12 has a principle role in the direction of $CD4^+$ T cells into T helper 1 (Th1) cells. Accordingly, mDCs are fully capable of activating Th1 $CD4^+$ cells and fundamentally preparing CD8 cytotoxic T lymphocytes (5). Effective protection against intracellular microorganism infections such as *Listeria Monocytogenes*, *Leishmania Major*, and *Toxoplasma Gondii* requires triggering cell-mediated immune responses (6). Moreover, there is evidence indicating that the type of pathogen-resultant signals and DC activation kinetics probably determine the potency of DC in modulating the quality of T cell response. The capability of DC to generate IL-12 against microbial signals is well documented to be an important factor for the stimulation of a suitable Th1 response (7). DCs uptake parasites and process the antigen in the MHC Types 1 and 2 pathways, there by capably stimulating the $CD4^+$ and $CD8^+$ T cells. It has been shown that DCs area key origin of IL-12 followed by infection with *Leishmania major* (8,9). The crucial role of DC in directing immune responses is hopeful, indicating that DC is probably changed into a natural adjuvant after loading with Antigen. Different investigations have reported that the use of DCs is becoming an auspicious technique for cancer immunotherapy in various animal tumor models and clinical trials (10,11). In the present study, we assessed anti-tumor immune responses triggered by tumor antigen-loaded DCs matured with *Leishmania major* extract in the mouse fibrosarcoma tumor model.

MATERIALS AND METHODS

Mice and Cell Line. 6-8-week-old female BALB/c mice were purchased from Pasteur Institute of Iran. The animal protocols were accepted by the Institutional Animal Care and Use Committee of Tehran University of Medical Sciences. WEHI-164 fibrosarcoma and CT26 colon carcinoma cell lines with BALB/c origin were cultured in RPMI 1640

(Sigma, Steinheim, Germany) supplemented with 10% fetal bovine serum inactivated by heat (Gibco,Grand Island, USA), 2 mM L-glutamine (Sigma, Steinheim, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin in a moistened incubator at 37°C and 5% CO_2.

Parasites and Parasite Lysate. *Leishmania major* (Friedlin strain) was maintained in BALB/c mice *in vivo*. The mice were infected in the footpad with 10^4 amastigote kinds of the parasite newly prepared from the lesions of formerly infected mice. The amastigotes were grown in an insect culture medium of Grace (Life Technologies, Gaithersburg, MD) with 20% FBS inactivated by heat, 2 mM glutamine, and 100 U/ml penicillin. Stationary-phase *L. major* promastigotes were gathered, centrifuged, and rinsed three times in phosphate-buffered saline (PBS). The parasites were exposed to three cycles of thawing and freezing, centrifuged, and passed via a 0.2-μm pore filter. The protein concentration for each lysate was determined through the Bradford technique.

Preparing Tumor Lysate. Tumor lysate was prepared through the subcutaneous injection of 5×10^6 WEHI-164 cells into the mice's right flank while monitoring tumor growth. Three weeks following the injection, tumors were surgically collected from tumor-bearing mice to make a single-cell suspension. The cell lysates were created by 6-7 fast freezing cycles into liquid nitrogen and thawing at37°C. To eliminate cellular debris, the lysate was spun for 10 min at 10,000 rpm. The supernatant was gathered and moved via a 0.2 μm pore filter. The lysate protein concentration was defined via the Brad ford technique.

Preparing DC and Culture. Bone marrow-resultant DCs (BMDCs) were modified through the use of the technique proposed by Inaba *et al.* (12) with certain modifications. In brief , precursor cells were harvested from tibias and femurs of sacrificed BALB/c mice; they were cultured (6×10^5cells/ml) in 24-well, flat-bottom plates in the RPMI-1640 culture medium; the medium was supplemented with 50 μM 2-mercaptoethanol, 10% fetal calf serum, 50 U/ml of recombinant murine IL-4 (Roche, Indianapolis, USA), and 100 U/ml recombinant murine granulocyte-macrophage colony-stimulation factor (GM-CSF) (Bender Med Systems, Austria). On the third day, by removing non-adherent cells, the fresh media were introduced. On the fifth day, 100 μg/ml/10^6 cells of tumor lysate were inserted into immature DCs cultures. After 10 hours, some wells containing immature dendritic cells were encouraged and matured through inserting 70 μg/ml/10^6 cells of *L. major* lysate or 2 μg/ml LPS (cat. no: L6526 Sigma). On the seventh day, mature and immature DCs were gathered and utilized for immunotherapy. In all stages, cells were incubated in a 5% CO_2 incubator.

Flow Cytometry. To determine the phenotype of the cultured DCs, on the fifth and seventh days, cells were stained with PE anti-mouse CD11c, FITC anti-mouse CD80, FITC anti-mouse CD40, FITC anti-mouse CD86, and FITC anti-mouse MHC type 2 (all obtained from BDPharMingen, San Diego, CA); furthermore, analysis was done by a FACS Analysis System (Becton Dickinson). All tests included isotype controls through a proper antibody of the same immunoglobulin (Ig) class or subclass.

Detection of Cytokines by ELISA. To investigate the secretion of IL-12 in the supernatants of DCs culture, supernatants were gathered and maintained frozen at -20ºC. IL-12 concentration was assessed by ELISA Kit (Bender Med system, Germany) based on the instructions of the manufacturer.

Tumor Challenge and Immunization. The tumor model was made by the subcutaneous injection of 0.2 ml of a cell suspension with 10^6 WEHI-164 cells in the

right flanks of syngeneic BALB/c mice. Seven days after tumor challenge, the mice subcutaneously received 10^6 BMDCs matured with antigens of *Leishmania major* (L.m-DCs), immature DCs (Im-DCs), and LPS (LPS-DCs) over the tumor site. For comparison with the DC therapy group, the control mice were injected with PBS. Tumor volume was estimated every two days with calipers spanning the longest and shortest diameters of the surface. Mice were sacrificed upon reaching the tumor diameter of higher than 400 mm^2. The survival of the mice was analyzed using five mice from each group.

Cytotoxicity Assay. Cytotoxicity activity was determined by lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche Applied Science, Germany). Two weeks following DCs treatment, splenocytes were removed from L.m-DC, Im-DC, LPS-DC, and PBS groups; they were utilized as effector cells and CT26 Tumor and WEHI-164 cell lines were employed as target cells. After rinsing the target and effector cells with RPMI1640 supplemented with 1% BSA, the effector cells were co-cultured with target cells at three various ratios (12.5, 25 and 50) in a 96-well round-bottom plate at 37°C. After 6 h, the plates were centrifuged for 5 min at 1000 rpm and the supernatants were relocated to another flat-bottom plate. Four microliter of Triton X-100 was inserted into each of the control wells and mixed properly to ensure the complete destruction of the cell membranes. 100 microliter of LDH mixed detection kit reagent was inserted into each well and incubated at room temperature for 30 min. Absorbance was determined using an ELISA reader at 490 nm. The cell-mediated cytotoxicity percentage was defined as:

Cytotoxicity (%) = (empirical release – spontaneous target release – spontaneous effector release) / (highest target release – spontaneous target release) × 100%.

Immunohistochemistry. Two weeks after DCs treatment, tumors were isolated, rooted in OCT complex (Tissue Tek; Sakura Fine technical, Tokyo, Japan), and frozen in liquid nitrogen. Five-micrometer thick cryostat segments were mounted by thawing on slides, air-dried, and kept at -20°C. The segments were maintained in cold acetone for 2 min, hydrated in PBS, and incubated in a protein-blocking solution (albumin 2%) for 15 min. The endogenous peroxidase activity was blocked with 0.3% H_2O_2. To detect CD8$^+$ T cells in tumor tissues, the sectors were incubated with anti-CD8 mAb (Phar Mingen) for 1 h. After rinsing with PBS, the segments were incubated with biotin-labeled anti-rat IgG; next, they were treated with avidin-biotin-peroxidase complex utilizing a peroxidase kit (PharMingen). The segments were then established with diamino-benzidine tetrachloride (DAB) solution; ultimately, they were counter stained with hematoxylin, mounted, dehydrated, and observed. Light microscopy was used to specify the number of CD8$^+$ cells per 10 high-power fields (magnification, ×400). The CD8-positive cells in each sample were assessed via two separate spectators; the percentage of these cells was determined by the number of stained cells divided by the overall counted cells. The data were stated as the mean ± standard error for each class.

Statistical Analysis. Two groups were compared via a Mann–Whitney U test. One-way ANOVA with Tukey post hoc analysis was used to compare the groups. The log-rank tests were employed to analyze the Kaplan–Meier survival curves. Using GraphPad Prism software (V.5.0), the graphs were statistically analyzed. The differences of $p<0.05$ were regarded as considerable.

RESULTS

Phenotype of DCs.

To determine the maturation state of BMDCs prior to injection, the expression of co-stimulatory (CMs) and MHC-II molecules was examined by flow cytometry. As observed in Figure 1, about 70% of mature DCs expressed mouse-particular DC indicator CD11c. BMDCs exposed to *L.major*'s antigen (L.m-DCs) and LPS up-regulated the surface expression of CD80 (B7.1), CD40, CD86 (B7.2), and MHC Type 2 compared to immature DCs (Im-DCs).
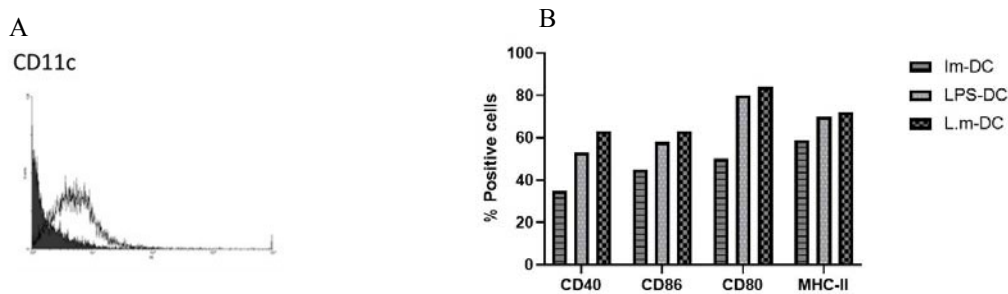
Figure 1. A) Expression of CD11c (gray line) in comparison to isotype control (black line) in mature and immature DC. B) Phenotypic BMDCs alterations against LPS (LPS-DCs) and *Leishmania major*'s antigen (L.m-DCs). Fifth day immature and seventh day mature BMDCs were examined by flowcytometry to express the maturation indicators of DCs. The findings are provided as the percentages of positive cells for each indicator.

Production of IL-12 by DCs.

To assess the capabilities of the DCs to produce IL-12, the supernatants of DC culture were evaluated by cytokine-specific ELISA kit. According to Figure 2, exposure of DCs to *Leishmania major*'s antigen (L.m-DC) and LPS significantly increased IL-12 level compared to the immature DCs and cell culture medium.
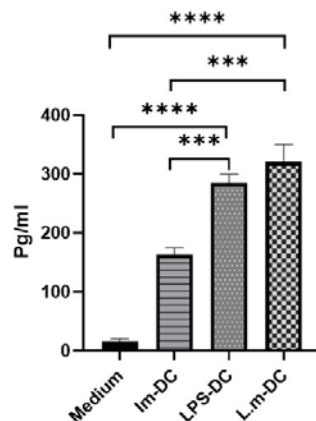
Figure 2. IL-12 production by the DCs matured by *Leishmania major*, LPS, immature dendritic cells, and culture Medium. ELISA was utilized to determine the amount of IL-12 production in the DCs supernatant. Bars show mean ± SEM. The average values from three various tests are shown (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.00001$).

**Immune Response of Tumor-Specific Cytotoxic T Cell (CTL).**
To emphasize the particular anti-tumor immunity induction, we assessed the cytolytic capacity of splenocytes in the mice treated with DCs. Splenocytes were cultured with WEHI 164 or an unrelated tumor cell line (CT26 cells) for 6-8 hours while assessing the LDH release. Intra-tumoral injection of LPS-DCs and L.m-DCs was observed to significantly enhance CTL activity in response to WEHI 164, in comparison to other groups ($p < 0.001$) (Figure 3a). On the contrary, a low CTL activity level versus CT26 cells was found in all groups with no significant differences between the groups (Figure 3b). Our results showed that the cytotoxic activity triggered by treatment with L.m–DCs was specific for WEHI 164 tumor.
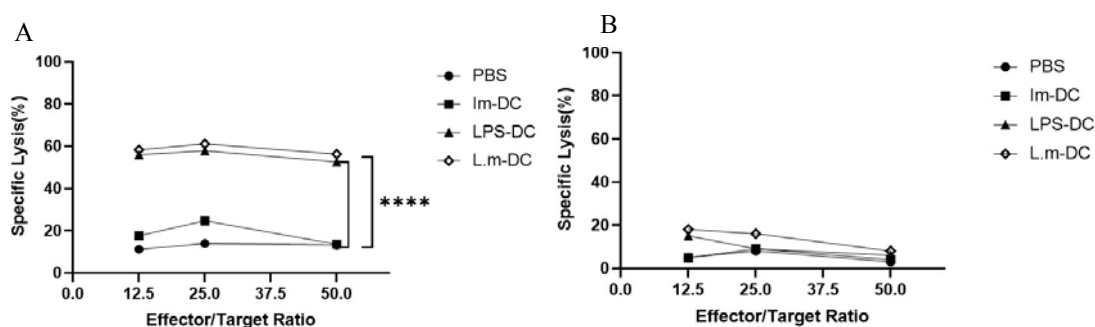
Figure 3. Cytolytic activity in mice receiving DC vaccination. The CD8^{+} T cells capability to recognize WEHI 164 cells as the target cells (a) and non-specific cytotoxicity versus an unconnected tumor cell line (CT26 cells) (b) was assessed in various groups (n=3;* p<0.05, ** p<0.01, *** p<0.001, and **** p<0.00001).

In Vivo **Anti-Tumor Response.**
To determine the antitumor effect of DC vaccination, 1×10^6 L.m-DCs, LPS-DCs, and Im-DCs were inserted in to the WEHI 164 tumors prepared on the seventh day after tumor cell inoculation. Figure 4a shows that LPS-DCs and L.m-DCs diminished the growth rate of DCs treated tumors more significantly than Im-DCs and PBS groups. As shown in Figure 4b, treatment with LPS-DCs and L.m-DCs significantly improved the survival rates compared with Im-DCs and PBS groups.
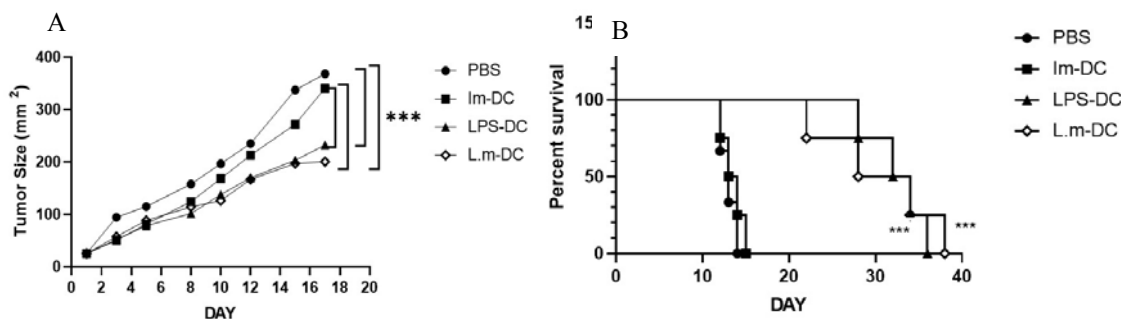
Figure 4. Protective effects of L.m-DCs and Im-DCs in subcutaneous and WEHI 164 tumor models. The means of tumor volume (a) and survival times (b) of each group of mice were monitored (n=5; * p<0.05, ** p<0.01, *** p<0.001, and **** p<0.00001).

Tumor-infiltrating CD8$^+$ T Cells .

Immunohistochemical analysis of the tumor section resulting from treated and control mice represented the infiltration of a larger number of CD8$^+$ T cells in mice receiving LPS-DCs and L.m-DCs in comparison with PBS and Im-DC groups (Figure 5a). As shown in Figure 5b, the percentage of CD8$^+$ cell infiltration was evaluated in all groups. Infiltrating CD8$^+$ T cells in L.m-DCs group was 35% in comparison to almost 5% and 3% in Im-DCs and PBS groups, respectively.

5A

(a) (b) (c)

5B
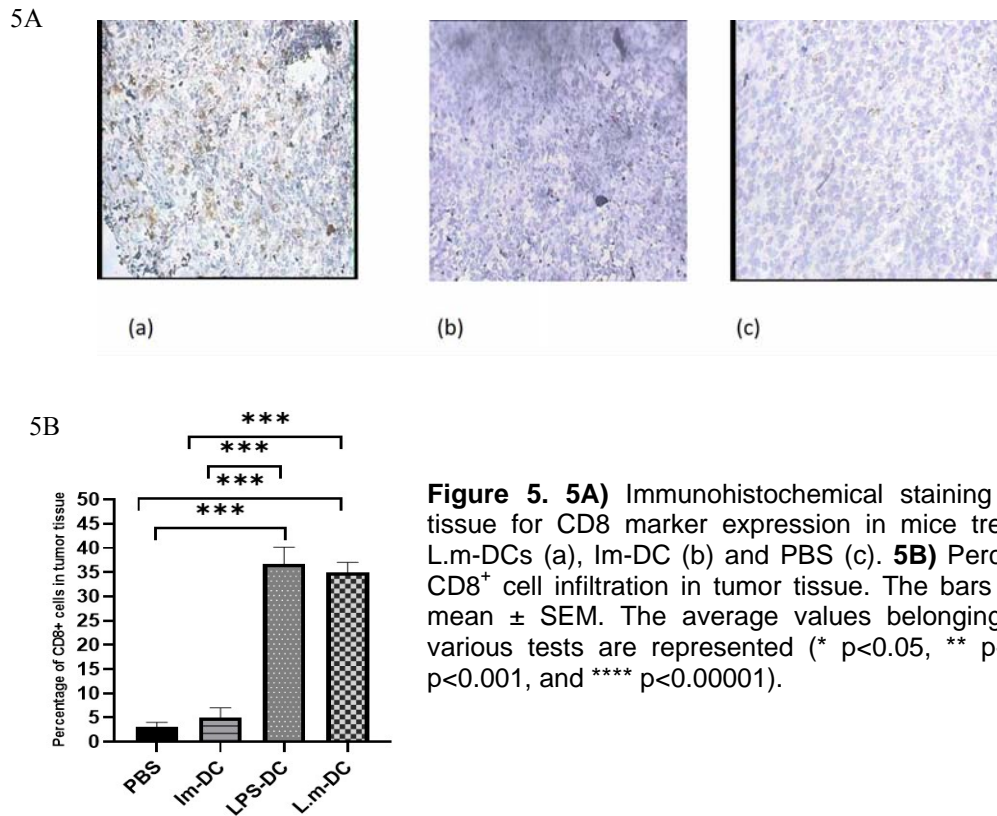
Figure 5. 5A) Immunohistochemical staining of tumor tissue for CD8 marker expression in mice treated with L.m-DCs (a), Im-DC (b) and PBS (c). **5B)** Percentage of CD8$^+$ cell infiltration in tumor tissue. The bars represent mean ± SEM. The average values belonging to three various tests are represented (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.00001$).

DISCUSSION

The tumor micro-environment is the main contributor to prohibiting immunosurveillance and restricting the performance of anti-cancer therapies. DCs are obvious subjects of various cancer microenvironments (13,14). Existence of cytokines such as VEGF (vascular endothelial growth factor), interleukin-10 (IL-10), and prostaglandin E2 (PGE2) can seriously influence the property of DCs. Numerous studies have demonstrated that TA-DCs (tumor-associated DCs) are immunosuppressive and unable to promote a particular immune response or induce regulatory T cell development (15-17). Various approaches have employed DCs to induce immunity in response to tumor Antigen (5,11). The majority of these approaches are based on the activation and maturation of DCs *ex vivo* and their subsequent reinfusion to tumor-bearing subjects followed by a pulse with tumor antigens (18). Additionally, evidence shows that the nature of pathogen-resultant stimulus and DC

Iran.J.Immunol. VOL.16 NO.4 December 2019

274

activation kinetics might significantly affect the ability of DCs to adjust the quality of the developed T cell response (19-22). *Leishmania major* is an intracellular protozoan parasite infecting and proliferating in to macrophages; however, it is able to inoculate other immune cells such as Langerhans cells (LCs), neutrophils, and DCs (23). Murine Langerhans cell-like infected by *L. major* produced TNF-α, IL-6, and IL-12, and expressed MHC class II antigens, CD40, CD54, and CD86 (24). In empirical infection models of *L. major*, a predominant T-helper class I immune response was normally developed by the resistant background (C57BL/6) mice. The attained resistance in this model depended on the activation of the CD4 T cells, leading to the secretion of high gamma interferon levels (IFN-γ) that induced NO-reliant parasite killing by infected macrophages (25,26). In the current research, the immune responses triggered *in vivo* by tumor antigen-loaded on BMDCs matured with *Leishmania major* were assessed and compared with LPS as a powerful stimulator of DCs maturity. DCs influence T-cell responses by producing cytokines. DC-derived IL-12 is the main Th1-provocative mediator (7). It is strongly accepted that Th1 immunity is significantly important for provoking effective cellular immunity (27). Furthermore, the antitumor and anti-metastatic properties of IL-12 have been established indifferent types of animal tumor models, including melanoma, breast, colon, renal carcinoma, and sarcoma. For instance, administrating IL-12 within tumor-bearing mice was able to postpone, diminish, and, in some cases, entirely suppress tumor induction with significant therapeutic effectiveness in some solid tumors and hematological malignancies (28). In this study, *Leishmania Major*-matured DCs, similar to LPS-DCs, generated large amounts of IL-12. During *Leishmania major* infection, IL-12 producing dermal dendritic cells were reported to induce T cell response (24). CTLs play a vital role in tumor immunity, and their preparation requires the activation of DCs (29). Cytotoxicity assay revealed that vaccination with *Leishmania major*-matured dendritic cells promoted a robust particular CTL response versus murine fibrosarcoma tumor model. Meanwhile, infiltrating CD8^{+} T-cell in the immunohistochemical analysis showed a significantly higher level compared with the animals treated with IM-DCs and PBS (controls). These findings emphasize the importance of CD8^{+} T cells in *L. Major*-matured DCs vaccine-induced antitumor activity. In our work, the detailed component of *Leishmania major* was not characterized, leading to a substantial result in DCs. Several reports have documented that Toll-like receptors (TLRs) expressed on APCs are able to recognize *Leishmania major*'s antigens. For example, TLR2 was responsible for recognizing lipophosphoglycan (LPG), known as an important virulence factor, and was able to modulate immune cell activation. This identification resulted in the expression of MHC-II, CD86, and IL-12p70, flagellin, and profilin contributing to IL-12 production identified through TLR5 and TLR11, respectively (30,31). To conclude, our findings indicated that vaccinations with *Leishmania major*-stimulated DCs are protective and result in retarded tumor growth, extended survival of the tumor-bearing mice, and LPS matured-DCs more than the immature DCs. This approach provides the foundation for using *Leishmania major* in DC-targeted clinical treatments.

Iran.J.Immunol. VOL.16 NO.4 December 2019

275

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