Immune responses regulation following antitumor dendritic cell-based prophylactic, concurrent, and therapeutic vaccination

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Abstract There is ample evidence in favor of various immunosuppressive mechanisms that weaken antitumor immune responses and affect currently used immunotherapies. Induction of regulatory T cells (Treg) and secretion of indoleamine 2,3-dioxygenase (IDO) by tumor tissue are considered as two main mechanisms of tumor immune escape. However, little is known about the contribution of these mechanisms on the modulation of dendritic cell vaccine-mediated antitumor response. To address this concern, we assessed Treg’s infiltration and the expression of Foxp3 and IDO genes in tumor microenvironment following dendritic cell-based antitumor immunotherapy of mice in different protocols of prophylactic, concurrent, and therapeutic vaccination. According to cytotoxicity assay, the vaccinated mice exposed efficient induction of splenic CTLs in all groups. However, only the mice immunized in prophylactic regimen significantly retarded the growth of tumor cells. Interestingly, the Treg content of tumor samples and transcriptional level of both Foxp3 and IDO genes were reduced in this group, while animals that received the vaccine in concurrent and therapeutic protocols showed increase in tumor-infiltrating Tregs and mRNA levels of Foxp3 and IDO. Accordingly, higher expression of these genes resulted in more inhibition of antitumor response. Our findings indicate that tumor progression may enhance the immunoregulatory response and hence emphasize to the effectiveness of vaccination in early stages of tumor growth for avoiding induction of such regulatory responses.

Keywords Dendritic cell · Regulatory T cell · Foxp3 · IDO · Tumor immunotherapy

Introduction

Dendritic cell-based therapeutic cancer vaccines have for some time been under investigation, and several clinical trials have evaluated their immunological and clinical efficacy. Despite their notable promise, tested vaccine strategies have shown only limited success in clinical settings. There is strong evidence that various immunosuppressive mechanisms considerably reduce antitumor responses and weaken the activity of current immuno-therapeutic regimens. Therefore, assessment of immunoregulatory responses during immunotherapy seems to be essential for DC vaccination strategies [1, 2].

Regulatory T (Treg) cells induce immune tolerance by suppressing host immune responses against self- or non-self-antigens. Hence, they play critical roles in preventing autoimmune diseases but may have unfavorable effects on immunization protocols to cancer and infectious diseases [3]. Thus, Tregs are thought to reduce T-cell immunity against tumor-associated antigens (TAAs), which are
expressed by most tumors and seem to be the main obstacle hampering successful tumor immunotherapy and vaccination [4–6].

Dendritic cells (DCs) have a potential role for the induction of CD4^+CD25^+Tregs. The magnitude of this effect depends on the DC subset, its maturation state, and the nature of DC maturation stimulus [7–10].

Increasing evidence indicates that most tumors express indoleamine 2, 3-dioxygenase (IDO), a tryptophan-catabolizing enzyme that starves T cells from an important amino acid [11] and escape from effective immune responses [11, 12]. However, recent studies show that not only tumor cells but also tumor-infiltrating antigen-presenting cells such as dendritic cells can express IDO [13–15]. IDO-expressing DCs are potent inducers of Tregs [16, 17]. Consequently, DCs are not only involved in the induction of immunity, but can also inhibit immune responses depending on the microenvironment in which their maturation process occurs [18].

In a previous study, we used Listeria monocytogenes bacterial lysate for DC maturation and showed the beneficial effects of vaccination with these matured DCs on the induction of antitumor immune response, which led to the partial retardation of tumor growth, but not to complete tumor rejection [19]. To determine the potential cause of suboptimal efficacy of DC vaccine, herein, we have evaluated the contribution of tumor-infiltrating Tregs and transcriptional levels of Foxp3 and IDO toward the escape of tumor cells following the DC immunization of mice in different vaccination regimens of prophylactic, concurrent, and therapeutic.

**Materials and methods**

**Animal model and cell line**

Female BALB/c mice (6- to 10-week old) were provided from Animal Center, Pasteur Institute of Iran. Mice were handled and tested according to the local guidelines for animal care and ethics. BALB/c-derived fibrosarcoma cell line, WEHI-164, was cultured and maintained in RPMI 1,640 (Gibco, USA), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (Sigma, USA).

**Tumor induction**

1.5 × 10^6 WEHI-164 tumor cells in 200 μl of phosphate-buffered saline (PBS) were injected subcutaneously in the right flank of BALB/c mice. Tumor measurements were performed using calipers spanning the shortest and longest surface diameters. Mice were sacrificed when the tumor size reached 400 mm^2 as end point of the experiment.

**Generation of bone marrow-derived DC**

Bone marrow (BM)-derived DCs (BMDCs) were generated as described by Inaba et al. [20] with slight modifications [19]. Briefly, 10^6 murine BM cells were isolated from femurs and tibias of the sacrificed mice and cultured in 24-well plates. On day 3, non-adherent cells were collected and resuspended in fresh medium. On day five, immature DCs were pulsed with tumor lysate (100 μg/ml). After 10 h, 70 μg/ml Listeria monocytogenes lysate (DC maturation factor) was added to the culture. On day 7 mature, DCs were collected. DC maturation was confirmed by flow cytometry using a panel of monoclonal antibodies against CD40, CD80, CD86, CD11c, and MHC-II (BD PharMingen, CA) and applied for immunotherapy. Listeria monocytogenes and tumor lysates were prepared according to the protocols as previously described [19].

**Immunization protocol**

DCs (10^6/200μl PBS) were injected subcutaneously into the right flank of three groups of 8- to 10-week old female BALB/c mice (n = 6). Vaccines were administered in three different schedules. In the prophylactic protocol, DC vaccination was performed 10 days before tumor challenge. In the concurrent protocol, tumor induction and vaccination were performed simultaneously and in the therapeutic protocol, vaccination was performed 10 days after tumor challenge. A control group received only PBS.

**Cytotoxicity assay**

Twenty days after tumor challenge, mice splenocytes were isolated and used as effector cells against the syngeneic tumor cell lines of WEHI-164 and CT-26 as target cells. As a control, the CT-26 cell line was used to determine the non-specific cytotoxicity response. The effector cells (E) were washed by assay medium (RPMI 1,640 containing 1% BSA) and cocultured with target cells (T) at 3 different E/T ratios (12.5, 25 and 50) in a 96-well round-bottom plate for 6 h at 37°C. The plates were then centrifuged, and supernatants were transferred to a flat-bottom ELISA plate. Cytotoxic activity was measured by LDH cytotoxicity detection kit (Roche Applied Science), according to the manufacturer’s instructions. The percentage of cell-mediated cytotoxicity was determined by the following equation: Cytotoxicity (%) = (experimental release—spontaneous target release—spontaneous effector release)/(maximal target release—spontaneous target release) × 100.
Real-time PCR

Total RNA was extracted from 100 mg of tumor tissue using RNX Plus (Fermentas, Lithuania), according to manufacturer’s instructions. The integrity of RNA was confirmed by gel electrophoresis. To avoid DNA contamination, RNA was treated with RNase-free DNase (Fermentas, Lithuania). One microgram of RNA was used for cDNA synthesis using MMLV reverse transcriptase and random hexamer (Fermentas, Lithuania) in the presence of RNase Inhibitor. cDNA sample was amplified in duplicate (for each primer pairs) by means of a Corbett Research thermocycler (Sydney, Australia) using SYBR® Premix Ex Taq™ kit (TAKARA, Japan) and following primers: Foxp3 (forward 5'-CCCAGGAAGACAGCAACCTT-3' and reverse 5'-TCTCACCCAGGCACTTG-3') [21], IDO (forward 5'-GGCTTCTCCTGTTCTCTATTG-3' and reverse 5'-TGAGCCTCCTGACTGATACT-3') [22], HPRT (forward 5'-GCTTTCCCTGGTTAAGCAGTACA-3' and reverse 5'-CAAACCTGTCTGAATTCCAATC-3') [23]. The target quantity was normalized relative to an endogenous control (HPRT gene), and relative to the non-vaccinated calibrator it was expressed as $2^{-\Delta CT}$ (fold) where $\Delta CT = CT$ of the target gene (Foxp3, IDO)—$CT$ of endogenous control gene (HPRT) and $\Delta CT = CT$ of vaccinated samples for target gene—$CT$ of the non-vaccinated calibrator for the target gene [24].

Flow cytometry analysis of tumor-infiltrating Tregs

Tumors were excised, minced, and treated with 200 IU/ml of type IV collagenase (Sigma, USA) for 2 h at 37°C and 5% CO2. Digested tumors were then passed over a 40-μm nylon mesh. Mononuclear cells were isolated by Ficoll–Hypaque density gradient centrifugation, washed twice, and resuspended in PBS. The isolated tumor-infiltrating mononuclear cells were stained with Anti-Mouse CD4 FITC, Anti-Mouse CD25 PE (eBioscience, CA). After cellular surface staining, cells were fixed and permeabilized with Fix/Perm solution (eBioscience, CA). The cells were then resuspended in Perm buffer and incubated with Anti-Mouse/rat Foxp3 PE-Cy5 (eBioscience, CA) at room temperature in the dark for 30 min. The stained cells were used for flow cytometry analysis (FACSCalibur, BD).

Statistical analyses

SPSS software (Version 13.0, SPSS Inc., Chicago, IL) was used for statistical analyses. Data were analyzed by Student $t$-test, Pearson correlation (r), or one-way ANOVA followed by the post hoc Tukey’s. Results were presented as the mean ± SD per group, and values of $P < 0.01$ were considered statistically significant.

Results

Assessment of antitumor response following DC-based vaccination in prophylactic, concurrent, and therapeutic regimens

After the preparation of *Listeria monocytogenes*-activated DC vaccine [25] and phenotypic analysis of these preparations (data not shown), primarily to evaluate the impact of vaccination timing on induced *in vivo* response, the growth of WEHI-164 tumor cells was measured by the day 20 post-inoculation in mice vaccinated with different protocols. As shown in Fig. 1a, animals that received the vaccine 10 days prior to tumor inoculation (prophylactic regimen) promoted a strong antitumor response that was evidenced by a significant reduction in tumor size, in comparison with the non-vaccinated control group ($P < 0.01$). However, neither the group vaccinated at the same time as tumor inoculation (concurrent regimen) nor the mice that received the vaccine 10 days after tumor inoculation (therapeutic regimen) showed effective responses by significant tumor regressing. This data argued in favor of the superiority of prophylactic strategy over the concurrent or therapeutic approaches.

In the next step, to evaluate the reasons for the observed differences in tumor regression, animals of the same experiment were sacrificed at the day 20 post-tumor inoculation and the cytotoxicity of their splenocytes was individually tested against either WEHI-164 (as the test target) or CT-26 (as the control target) cell lines, for 6–8 h using the LDH release assay (Fig. 1b, c). Interestingly, the specific lysis (%) of WEHI-164 cells in all the groups immunized in either prophylactic or concurrent or therapeutic regimens was significantly higher than that of the control non-vaccinated group ($P < 0.01$). However, as expected no considerable lysis was seen against the CT-26 control targets, indicating the specificity of the induced CTLs. These results propounded that some factor(s) other than CTLs should be searched to as possibly responsible for the observed differences in tumor regression.

Foxp3/IDO gene expression and frequency of tumor-infiltrating Tregs in relation to the antitumor response

According to the obtained results of tumor regression and CTL assays, it appeared to indicate that the inhibition of antitumor response in some vaccinated groups may be due to the expression of Foxp3 and IDO genes. Therefore, the transcription levels of Foxp3 and IDO genes in tumor tissues of the different animal groups were, respectively, analyzed by quantitative real-time RT-PCR.
As shown in Fig. 2a, b, mRNA transcriptional levels of both Foxp3 and IDO genes were diminished in all the vaccinated groups, compared to non-vaccinated control mice; however, it was surprise to observe that the decline was only significant in animals that received the prophylactic vaccine \((P < 0.01)\). This finding suggested that Foxp3 and IDO gene products might be considered as the responsible for lowering the vaccine-induced antitumor immunity.

Based on our data, prophylactic vaccination has beneficial effects on tumor regression and reduction in mRNA level of Foxp3. To confirm these data and also in order to investigate whether there is any difference in tumor-infiltrating Tregs, we assessed the Treg content of tumor samples by flow cytometry following different schedules of vaccination. As shown in Fig. 2c, consistent with real-time PCR results, the percentage of tumor-infiltrating Tregs was significantly reduced in prophylactic regimen compared to the control group \((P < 0.01)\).

Accordingly, to confirm the observed results, the correlation of the transcription of Foxp3 and IDO genes with the tumor size in all animal groups was separately analyzed using the Pearson’s correlation coefficient. The result indicated a positive correlation between tumor growth and transcription levels of Foxp3 \((P < 0.01, r = 0.71)\) and IDO \((P < 0.05, r = 0.47)\) genes (Fig. 3a, b).

Since both Foxp3 and IDO gene transcription may act as inhibitory factors, we evaluated whether there was a correlation between Foxp3 and IDO mRNA levels in the vaccinated groups. Herein, the Pearson’s correlation test again indicated a positive correlation between mRNA levels of Foxp3 and IDO genes (Fig. 3c, \(P < 0.01, r = 0.723\)).

Discussion

Dendritic cell-based cancer vaccines have for some time been under investigation and shown often to be successful in generating elevated numbers of tumor-specific lymphocytes in peripheral blood. However, the tumors usually continue to grow in spite of antitumor immune response induction showing only limited success in clinical settings [1, 25].

Accordingly, our previous studies using dendritic cells activated with *Listeria monocytogenes* showed that these cells can enhance antitumor immune responses [26]. However, repeated doses of the vaccine showed in some cases no significant increase in antitumor immunity, and hence, induction of inhibitory responses following DC vaccination seems plausible [19]. Inhibitory factors from tumor microenvironment are believed to allow the escape of tumor cells from immune surveillance. Thus, cancer cells may escape the immune pressure by secreting soluble factors that can locally paralyze the effector immune cells [27]. Moreover, increasing evidence indicates that Tregs, which play an important role toward the downregulation of antitumor immune response, are synergistically controlled by the same immunosuppressive factors [23].

In the present study, we confirmed that *Listeria monocytogenes*-activated DC vaccination was able to efficiently induce the CTL responses, but had only partial success in tumor regression, especially in therapeutic vaccination regimen. Thus, evaluation of inhibitory responses was
required to clarify the insufficient efficiency of DC vaccination in clinical responses and we analyzed the contribution of Foxp3, as a marker of Treg cells, and IDO, as an immunoinhibitory enzyme, toward the inhibition of vaccine-mediated antitumor response.

Our results indicated that there is a positive correlation between the transcription of Foxp3/IDO gene within the tumor microenvironment and the growth of tumor cells, so that mice vaccinated with prophylactic regimen showed the highest tumor regression and the lowest tumor-infiltrating Treg and Foxp3/IDO transcription level.

In contrary, the highest level of Treg and Foxp3/IDO transcription at tumor sites was seen in the therapeutic-vaccinated group that received the vaccine 10 days after tumor inoculation (Fig. 2). Thus, observation of the inhibitory effects of IDO and Tregs on the induction of antitumor immunity is consistent with other reports that human and mouse tumor cells escape from immune response by the expression of IDO [11] and induction of Foxp3+ regulatory T cells. However, this notion that inhibition of antitumor immune response clearly depends on the time of vaccination before tumor development is a new finding that shows the role of tumor environment for the induction of inhibitory factors. Thus, when the tumor grows in therapeutic-vaccinated group, its immunosuppressive environment finds the required opportunity to sufficiently express the IDO and induces the regulatory T cells. Interestingly, our study also indicated a positive correlation between the transcription of IDO and Foxp3 genes, confirming the idea that IDO-expressing DCs are strong inducers of Tregs [23].

Although DCs strongly stimulate the immune responses, they can obviously also induce unresponsiveness and tolerance to tumor antigens and therefore the application of activated mature DCs for a successful dendritic cell-based vaccination seems to be pivotal [18, 28]. For this reason, we used the strategy of DC activation by means of *Listeria monocytogenes* lysate that has been already demonstrated as a potent activator [26] and as expected this approach led to the efficient induction of tumor-specific CTLs (Fig. 1). However, differences observed in tumor regression efficiency due to different vaccination timing and apparently higher transcription rate of inhibitory factors (IDO and Foxp3) in the therapeutic-vaccinated group, compared to the prophylactic-vaccinated animals, emphasized on the more prominent role of tumor microenvironment than regulatory activity of DC maturation factor. Of note, in recent years, increasing evidence has shown that within the
tumor microenvironment, not only tumor cells but also other infiltrating cells such as dendritic cells, monocytes, and others can be sources of IDO [11]. IDO is overexpressed by antigen-presenting cells in tumor-draining lymph nodes, where it promotes the induction of peripheral immune tolerance to tumor antigens [29].

Induction of IDO can be triggered by various soluble and membrane-bound factors, and several reports have shown that DC maturation in the presence of a cocktail of cytokines and prostaglandin E2 (PGE2) can result in strong upregulation of IDO [13–15, 18]. It has been also reported that administration of celecoxib, a specific inhibitor of cyclooxygenase-2 (COX-2) pathway, has significantly augmented the efficacy of a DC-based cancer vaccine for reducing primary tumor burden, preventing metastasis, and increasing survival [30]. Taken together, these findings point toward the role of tumor-induced regulatory responses and importance of their control for a successful immunotherapy.

In conclusion, our findings suggest that tumor progression proportionally enhances the immunoregulatory response and consequently affects the outcome of tumor immunotherapy. Hence, any positive achievement to tumor immunotherapy primarily depends on the stage of tumor growth and its microenvironmental condition. This notion may be considered for the design and application of antitumor immunotherapeutic vaccines in the future.

Acknowledgments This research has been supported by Tehran University of Medical Sciences and Health Services (Grant number: 86-03-30-6154).

Conflict of interest Authors have no actual or potential conflict of interest.

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