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TNF-alpha-treated DC exacerbates disease in a murine tumor metastasis model

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Abstract

Due to the pivotal role that dendritic cells (DC) play in eliciting functional anti-tumor T cell responses, immunotherapeutic approaches utilizing DC-based vaccines have readily been exploited. It has been argued that, in the setting of immunotherapy, mature DC will be more efficient at T cell priming and, therefore, required for effective vaccination. As TNF-alpha is commonly used as a DC maturation factor, we have examined the efficacy of treatment with DC matured with TNF-alpha (DC-TNF) in a murine model of melanoma. We have now shown that treatment with DC-TNF leads to an increase in the number of lung metastases as compared to mice treated with immature DC. No differences in the number of CD4⁺CD25⁺ T-regulatory cells were measured in the lungs of DC-TNF-treated mice. On examination of the infiltrating lymphocytes, an enhanced secretion of IL-10 and a higher percentage of CD4⁺IL-10⁺ T cells were measured in the lungs of DC-TNF-treated mice. However, treatment with DC-TNF did not enhance the number of melanoma lesions in the lungs of IL-10 knockout mice or in mice depleted of CD4⁺ T cells. Together, these studies indicate that treatment of melanoma-bearing mice with DC treated with TNF-alpha can induce IL-10 production by resident cells at the tumor site, leading to immune tolerance and exacerbation of disease.

Keywords

Dendritic cells; Melanoma; TNF-alpha; Immunotherapy

Introduction

Dendritic cells (DC) are the most potent antigen presenting cells, capable of eliciting both primary and secondary immune responses [1]. While DC are primarily used to induce antigen-specific immune responses in immunotherapy protocols, it has been established that DC serve a dual role in immunity. In this regard, DC can both induce strong antigen-specific T cell responses as well as regulate autoimmune reactions by inducing T cell tolerance to self-antigens [2-4].

There is increasing evidence that the maturation state of DC determine their ability to induce either immunity or tolerance [5-7]. Immature bone marrow-derived DC are characterized by low level expression of costimulatory molecules, such as CD80, CD86 and CD40, and do not produce pro-inflammatory cytokines such as IL-12 [8]. Treatment with DC pulsed with tumor-associated peptides or lysates has been shown to induce anti-tumor T cell responses in both rodents and humans [9-13]. DC maturation, induced by bacterial products such as CpG or LPS or by cell signaling molecules such as CD40L, results in the enhanced ability of DC to interact with naïve T lymphocytes [14,15]. Mature DC demonstrate high level expression of MHC class II molecules, CD80, CD86 and CD40, as well as secretion of IL-12 [16]. Treatment of tumor-bearing mice with DC pulsed with peptides or lysates has been shown to induce superior anti-tumor T cell immunity on maturation [17,18].

Bone marrow-derived DC treated with TNF- α demonstrate a “semi-mature” phenotype [19, 20]. These DC display upregulated costimulatory molecules, but do not produce pro-inflammatory cytokines and resemble migrating steady-state DC required for the induction of peripheral tolerance to self-antigens in vivo [21,22]. In a murine model of autoimmune disease, treatment with DC matured with TNF- α led to the induction of immune tolerance. Intravenous injection with TNF- α -treated DC pulsed with an auto-antigenic peptide resulted in protection against experimental autoimmune encephalomyelitis [23]. Similarly, injection of TNF- α -treated DC pulsed with thyroglobulin antigen was able to inhibit autoimmune thyroiditis in a murine model [24]. In another study, antigen-loaded, TNF- α -treated DC were able to delay the onset of collagen-induced arthritis [25]. In this study, we report an increase in the number of melanoma lesions in the lungs of mice following the administration of DC matured with TNF- α . The mechanism of suppression induced by vaccination with TNF- α -treated DC was examined.

Materials and methods

Animals

Female C57BL/6 mice (6–8 weeks old) were purchased from Harlan Laboratories (Indianapolis IN). C57BL/6-IL-10 knockout mice were purchased from Jackson Laboratories (Bar Harbor, ME). The mice were housed at the Animal Research Facility of the H Lee Moffitt Cancer Center and Research Institute. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida.

Culture medium and tumor cell lines

Complete media (CM) consisted of RPMI 1640 supplemented with 10% heat inactivated FCS, 0.1 mM nonessential amino acids, 1 μ M sodium pyruvate, 2 mM fresh L-glutamine, 100 μ g/ml streptomycin, 100 units/ml penicillin, 50 μ g/ml gentamicin, 0.5 μ g/ml fungizone (all from Life Technologies, Inc. Rockville, MD) and 0.05 mM 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO.). The B16 melanoma is a tumor of spontaneous origin. A poorly immunogenic, highly metastatic subclone of B16, denoted B16-D5, has been characterized previously. B16-D5 expresses a low level of MHC class I molecules and no detectable MHC

class II molecules [26]. B16 cells were maintained by serial in vitro passage in CM. The MCA-207 tumor is a weakly immunogenic, 3-methylcholanthrene (MCA)-induced fibrosarcoma of C57BL/6 origin [27]. MCA-207 tumors were maintained by in vivo serial s.c. transplantation in C57BL/6 mice and used within the third transplantation generation. Tumor cell suspensions were prepared from solid tumors by enzymatic digestion in HBSS (Life Technologies, Inc.) containing 1 mg/ml collagenase, 0.1 mg/ml DNase I and 2.5 units/ml of hyaluronidase (all from Sigma) with constant stirring for 2 h at room temperature. The resulting suspension was passed through a 70 μ m cell strainer. All tumor cells were washed extensively in PBS (Life Technologies, Inc.) prior to administration to mice.

Generation of DC

BM cells were harvested from flushed marrow cavities of femurs and tibiae of mice under aseptic conditions and were cultured in CM supplemented with 20 ng/ml GM-CSF and 20 ng/ml IL-4 at 1×10^6 cells/ml (R&D Systems, Minneapolis, MN) as described previously [9]. DC were harvested from day 5 cultures and enriched by OPTI-prep density gradient separation (Axis-Shield, Norton, MA). The low-density interface was collected. DC were matured by culturing in CM containing 20 ng/ml TNF- α for 24 h. For antigen loading, DC were pulsed with 10 μ g/ml trp-2_{180–188} peptide for 24 h. DC preparations were washed three times in PBS and resuspended at 3×10^6 cells/0.25 ml PBS for i.v. injection.

Treatment of lung lesions

C57BL/6 mice were injected i.v. with 3×10^5 viable B16-D5 (B16) or MCA-207 cells. Three days later, mice were treated i.v. with 3×10^6 DC or DC-TNF. Mice received additional treatments of DC on days 5 and 7 after tumor challenge. To deplete CD4⁺ T cells, mice were treated with 200 μ l of purified GK1.5 ascites i.p., beginning on day 1 after B16 injection and continuing every 3–4 days thereafter. Lungs were harvested on days 21–28. Pulmonary lesions were enumerated in a blinded, coded fashion after insufflation of the lungs with Fekettes solution for B16 lung lesions or insufflation of MCA-207-bearing lungs with India ink and fixation with Fekette's solution, as described previously [27]. Data are reported as the mean number of tumor nodules $\pm$ SEM for five or more mice per group.

Isolation of lung-infiltrating lymphocytes

For analysis of lung-infiltrating lymphocytes, lungs containing tumors were excised 21–28 days after treatment with PBS, DC or DC-TNF. Lungs and tumors were minced and digested for 2 h at room temperature as described above. Digested tumors were then passed over a 70 μ m nylon mesh, washed once with HBSS and resuspended in PBS + 3% BSA to a concentration of 1×10^6 cells/ml for flow cytometric analysis.

Antibodies and flow cytometric analysis

Splenocytes or lung-infiltrating lymphocytes were stained with the following monoclonal antibodies for flow cytometric analysis: purified anti-CD16/CD32 (Fc Block) followed by FITC-conjugated anti-mouse CD4 or CD8, or PE-conjugated anti-mouse CD25. For intracellular cytokine stains, lung-infiltrating lymphocytes were restimulated for 4 h with lymphocyte activation cocktail (BD Pharmingen, San Diego, CA) with BD GolgiPlug (BD Pharmingen). Cells were then stained with APC-conjugated anti-mouse CD4 or CD8 monoclonal antibodies, permeabilized and fixed using an intracellular cytokine stain kit (BD Pharmingen), then stained with FITC-conjugated anti-mouse IL-10. All antibodies were purchased from BD Pharmingen. Data were acquired on a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed using CellQuest Pro software (BD Biosciences).

ELISA

Splenocytes or TIL were plated at $2 \times 10^6/\text{ml}$ in a 12-well plate and co-cultured with $2 \times 10^5/\text{ml}$ irradiated B16 cells. Supernatants were harvested after 48 h to assay for IFN- γ and IL-10. Cytokine concentrations in supernatants were evaluated using OptEIA murine ELISA sets (Pharmingen, San Diego, CA).

Statistical analysis

A Mann-Whitney test (unpaired) or a Student's paired t test was used to compare between two treatment groups. All statistical evaluations of data were performed using GraphPad Prism software. Statistical significance was achieved at $p < 0.05$.

Results

Increased number of tumor metastases following the administration of TNF- α -treated DC

To test the therapeutic efficacy of direct DC injection on the growth of tumor lesions in the lung, we first injected B16 tumor cells i.v. into C57BL/6 mice. Mice were then treated on days 3, 5 and 7 with unpulsed or trp-2-peptidepulsed immature DC (DC) or TNF- α -treated DC (DC-TNF). As shown in Fig. 1a, the administration of immature DC pulsed with trp-2 peptide resulted in a reduction in the number of lung nodules. In contrast, treatment with unpulsed DC-TNF led to an enhancement in the number of lung lesions (169 ± 18 ; $p < 0.05$, compared to DC alone 81 ± 16). As shown in Fig. 1b, treatment with DC-TNF significantly enhanced the number of B16 tumor nodules in the lungs of mice. To test whether this effect was restricted to the B16 melanoma tumor model, we injected mice i.v. with MCA-207 sarcoma cells and treated with PBS, immature DC or DC-TNF on days 3, 5 and 7. As shown in Fig. 2, treatment with DC-TNF also led to increased numbers of MCA-207 lesions in the lungs (169 ± 48) compared to immature DC alone (81 ± 31 , $p < 0.05$). We observed that treatment with DC-TNF exacerbated the growth of tumors in the lungs after injection of either melanoma or sarcoma cells. Hence, these results indicate that DC-TNF-dependent tumor enhancement is not restricted to solely a melanoma model.

Examination of lung-infiltrating lymphocytes

Next, we examined the types of lymphocytes that were localized in the lungs of B16-bearing mice treated with DC-TNF. As shown in Table 1, higher numbers of CD4 $^{+}$ and CD4 $^{+}$ CD25 $^{+}$ T cells were detected in immature DC and DC-TNF-treated lungs compared to lungs of PBS-treated mice. Overall, there were no differences measured in the percentage of CD8 $^{+}$ T cells, CD4 $^{+}$ T cells or CD4 $^{+}$ CD25 $^{+}$ regulatory T cells in the lungs of immature DC or DC-TNF-treated B16 tumor-bearing mice. However, a significant increase in the percentage of CD4 $^{+}$ T cells producing IL-10 was measured in the lungs of mice treated with DC-TNF ($p < 0.05$, compared to DC). Representative dot plots as well as total CD4 $^{+}$ IL-10 $^{+}$ cell numbers in the lung-infiltrating lymphocytes are shown in Fig. 3.

We then measured cytokine secretion from lymphocytes isolated from the lungs of B16 tumor-bearing mice treated with PBS, immature DC or DC-TNF. Lung lymphocytes of mice treated with DC-TNF produced higher amounts of IFN- γ ($3,056 \pm 205$) compared to immature DC-treated mice ($1,339 \pm 57$, $p < 0.001$; Fig. 4a). A low amount of IL-10 was detected in the lung lymphocytes from mice treated with immature DC (Fig. 4b, 762 ± 80 pg/ml). In contrast, the lung lymphocytes of DC-TNF-treated mice produced $2,715 \pm 78$ pg/ml IL-10 ($p < 0.001$ compared to DC-treated mice). Similar findings were observed in the splenocytes of mice treated with DC-TNF (data not shown). No TGF- β secretion was detected in the lung lymphocytes of mice treated with immature DC or DC-TNF (data not shown). IL-10 was not secreted by immature DC or DC-TNF prior to injection (data not shown). This study

demonstrates that a higher amount of IL-10 is produced in the lungs of B16 tumor-bearing mice treated with DC-TNF.

CD4⁺ T cell depletion

To determine whether CD4⁺ T cells were responsible for the tumor-enhancing effect of DC-TNF administration, we repeated our studies in wild-type mice depleted of CD4⁺ T cells. C57BL/6 mice were injected i.v. with B16 cells and treated with PBS, DC or DC-TNF on days 3, 5 and 7. To deplete CD4⁺ T cells, mice received GK1.5 antibodies beginning on day 1 after tumor injection and continuing every 3–4 days until the end of the experiment. As expected from our previous observations, DC-TNF treatment of wild-type C57BL/6 mice harboring B16 lung tumors led to an increase in the number of lung lesions (184 ± 14 , compared to 94 ± 18 ; $p < 0.01$; Fig. 5a). In contrast, mice treated with DC-TNF, but depleted of CD4⁺ T cells, had a decrease in the number of lung lesions (36 ± 7 , $p < 0.01$ compared to PBS treated), indicating that CD4⁺ T cells contributed to the growth of lung lesions in mice treated with DC-TNF.

IL-10 knockout mice

To determine whether IL-10 contributed to the tumor-enhancing effect of DC-TNF administration, we repeated our studies in IL-10 knockout mice. IL-10 knockout mice were injected i.v. with B16 cells and treated with PBS, immature DC or DC-TNF on days 3, 5 and 7. Unlike treatment in wild-type mice, no significant difference was measured in IL-10 knockout mice treated with PBS or DC-TNF (69 ± 20 and 49 ± 12 , respectively, Fig. 5b). The DC injected into the IL-10 knockout mice were harvested from wild-type mice, indicating that it was IL-10 production by endogenous cells that contributed to the tumor-enhancing effect of DC-TNF.

Discussion

DC matured with TNF- α have been described as semimature DC [28]. While these DC display upregulation of MHC class II and costimulatory molecules, they do not secrete pro-inflammatory cytokines, such as IL-12, which is indicative of full DC maturation. In a murine model of autoimmunity, three intravenous (i.v.) injections of TNF- α -treated DC pulsed with an auto-antigenic peptide led to protection against experimental autoimmune encephalomyelitis (EAE) mediated by the induction of CD4⁺ Tr1-regulatory T cells producing IL-10. Injection of peptide-pulsed DC fully matured with LPS led to stimulation of auto-reactive T cells and enhanced disease [23]. In our model of B16 melanoma lung metastases, mice treated i.v. with TNF- α -treated DC (DC-TNF) demonstrated enhanced disease progression with an increase in the number of melanoma lesions compared to mice treated with immature DC. Our experimental evidence suggests that DC-TNF treatment leads to the induction of IL-10, producing Tr1 regulatory T cells. Infiltrating lymphocytes in the lungs of DC-TNF-treated mice produced high levels of IL-10 (Fig. 3b) with a higher percentage of CD4⁺IL-10⁺ T cells (Table 1), and depletion of CD4⁺ T cells abolished the effects of DC-TNF (Fig. 5a). It is possible that other cells at the tumor site may have also been producing IL-10. It has been demonstrated that pulmonary dendritic cells are capable of producing IL-10 after exposure to respiratory antigens to mediate tolerance against the antigens [29]. As treatment of B16 melanoma with wild-type DC matured with TNF- α did not enhance the growth of lesions in the lungs of IL-10 knockout mice, we can eliminate the injected DC-TNF cells as the source of IL-10. Further studies to determine whether other cell types produce IL-10 in the lungs of DC-TNF-treated mice are currently ongoing.

In murine models of autoimmunity, the tolerogenic effects of TNF- α -treated DC required peptide pulsing of DC prior to intravenous injection. Injection of DC-TNF alone could not induce the prevention of EAE [23]. In our model, while injection of DC-TNF pulsed with the

melanoma-specific peptide trp-2 did not ameliorate disease, it did not enhance the progression of B16 melanoma in the lungs. Only injection of unpulsed, TNF- α -treated DC enhanced B16 melanoma growth. TNF- α -treated DC have been compared to “steady-state” DC. In vivo, naturally arising steady-state DC migrate spontaneously from tissues into peripheral lymph nodes. These DC, expressing upregulated MHC and costimulatory molecules, travel to the draining lymph nodes where tissue antigens are processed and presented for the induction of peripheral T cell tolerance [30,31]. In our model, it is possible that i.v.-injected TNF- α -treated DC act as steady-state DC and are capable of picking up antigen at the tumor site. These antigens may be processed by the TNF- α -treated DC and presented to T cells leading to the activation of IL-10⁺ producing regulatory CD4⁺ T cells.

The route of DC injection has been shown to have an effect on the type of immune response elicited. In autoimmune models, i.v. injection of peptide-pulsed DC-TNF led to protection against EAE. Injection of these cells s.c. was shown to reverse the tolerogenic capacity of DC-TNF resulting in EAE disease progression [32]. In our model, injection of peptide-pulsed DC-TNF s.c. was less effective than peptide-pulsed DC alone in reducing the number of B16 lesions in the lungs of mice (data not shown). In contrast to autoimmune models, i.v. injection of immature DC has been shown to be an effective means of inducing anti-tumor immunity in both murine models and human clinical trials [33-35]. For treatment of B16 melanoma, i.v. injection of DC was superior to s.c. injection in reducing the number of lung lesions [36]. Together, our studies suggest that treatment with DC-TNF, s.c. or i.v., is an ineffective means of inducing anti-tumor immunity and can lead to induction of tolerance against tumors in the lungs of mice.

In this study, we found that infiltrating lymphocytes produced high levels of both IL-10 and IFN- γ in the lungs of DC-TNF-treated mice. It has been shown that IL-10 and IFN- γ together can suppress the ability of DC to induce CD4⁺ T cell responses [37]. IL-10 production by T cells can initiate a negative feedback loop to downregulate Th1-type responses [38,39]. One study has shown that TNF- α matured DC, pulsed with both an MHC class I restricted- and MHC class II restricted-peptide could simultaneously induce CD8⁺ T cell immunity and CD4⁺ T cell tolerance [40]. In humans, a subset of regulatory CD4⁺ T cells producing high levels of both IFN- γ and IL-10 has been identified. These cells were capable of suppressing both naïve and memory T cell responses [41]. In our model, it is possible that DC-TNF treatment induces tumor-specific immunity, indicated by IFN- γ production in the lung lymphocytes, and also IL-10-producing CD4⁺ T cells that are capable of tolerizing activated T cells.

In patients with cancer, the maturation state of DC has been shown to have a clinical impact, with antigen-pulsed mature DC being superior to immature DC for the induction of anti-tumor immunity and clinical responses [42]. Regardless of the maturation state of DC being used in cancer patients, many immunotherapeutic trials examining DC-based vaccines resulted in induction of measurable anti-tumor CD8⁺ T cell responses, but demonstrated little clinical efficacy overall. It has recently been shown that human DC matured with TNF- α are highly deficient at inducing helper T cell responses [43]. Many clinical trials in cancer patients utilized TNF- α to mature DC and measured IFN- γ production by tumor-specific CD8⁺ T cells as the indicator of vaccine efficacy. Therefore, it is possible that IL-10 producing regulatory or tolerogenic CD4⁺ T cells were also induced, limiting the effectiveness of tumor-specific T cell responses. These studies indicate that the methods to mature DC must be optimized and suggest that broader immunological readouts must be performed to enhance DC-based immunotherapeutic approaches for the treatment of cancer.

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Fig. 1.

Treatment of B16 lung metastases with DC matured with $\text{TNF-}\alpha$ exacerbates disease. Mice ($n = 6$) were injected i.v. with B16 cells. On days 3, 5 and 7, mice received i.v. injections of unpulsed or trp-2 peptide-pulsed immature DC or $\text{TNF-}\alpha$ -treated DC (DC-TNF). Control mice received PBS. Lungs were collected on days 21–28. **a** Total number of lung metastases. Data presented as mean number of lung lesions $\pm$ SEM. Two experiments were performed. $*p < 0.05$ compared to the PBS-treated mice. **b** Representative lungs from B16-bearing mice treated with PBS, DC or DC-TNF

Fig. 2.

Treatment of MCA-207 lung metastases with DC treated with $\text{TNF-}\alpha$ exacerbates disease. Mice ($n = 7$) were injected i.v. with MCA-207 cells and treated with PBS, immature DC or DC matured with $\text{TNF-}\alpha$ on days 3, 5 and 7. Lungs were collected on day 21 and the number of lung lesions was counted. $*p < 0.05$ compared to those treated with DC

Fig. 3.

Increased numbers of CD4⁺IL-10⁺ T cells are measured in the lung-infiltrating lymphocytes of DC-TNF-treated mice. **a** Representative dot plots. Cells were gated on the infiltrating lymphocyte population. **b** Total number of CD4⁺IL-10⁺ T cells in the lungs of mice treated with PBS, immature DC or DC-TNF. Data presented as mean number of cells $\pm$ SEM. * $p < 0.05$ compared to immature treated DC

Fig. 4.

TIL cells produce IL-10 and IFN- γ . Mice were injected i.v. with B16 cells and treated with PBS, DC or DC matured with TNF- α on days 3, 5 and 7. Lungs were collected on day 21. TIL were isolated as described in "Materials and methods". TIL were restimulated in the presence of B16 cells and supernatants were collected at 48 h. **a** IFN- γ production. **b** IL-10 production. The mean $\pm$ SEM of four mice per group is shown. * $p < 0.001$ compared to DC-treated mice

Fig. 5.

Treatment of B16 lung metastases with DC matured with $\text{TNF-}\alpha$ exacerbates disease in wild-type, but not CD4^+ depleted or IL-10 knockout, mice. **a** Wild-type C57BL/6 mice ($n = 7$) were injected i.v. with PBS or DC-TNF on days 3, 5 and 7. CD4^+ T cells were depleted by i.p. injection of GK1.5 ascites beginning on day 1 after tumor injection and continuing every 3–4 days. Lungs were collected on days 21–24 and the number of lung lesions were counted. **b** IL-10 knockout C57BL/6 mice ($n = 7$) were injected i.v. with B16 cells and treated with PBS, DC or DC matured with $\text{TNF-}\alpha$ on days 3, 5 and 7. Lungs were collected on days 21–24 and the number of lung lesions was counted. Experiment was repeated two times with similar results. * $p < 0.01$ compared to DC-treated mice. ** $p < 0.01$ compared to PBS

Table 1

Characterization of lung-infiltrating lymphocytes

Treatment	CD8 ⁺	CD4 ⁺	CD4 ⁺ CD25 ⁺	CD4 ⁺ L10 ⁺
PBS	6.4 ± 1.42 ^a	7.4 ± 2.0	1.0 ± 0.3	0.34 ± 0.1
DC	5.8 ± 0.6	16.5 ± 2.7	3.1 ± 0.1	0.64 ± 0.2
DC-TNF	6.0 ± 1.0	15.3 ± 0.7	2.6 ± 0.1	1.76 ± 0.5 [*]

Lungs were collected on day 21. Cells were gated on the lymphocyte fraction in lungs

^aThe mean percentage ± SEM of four mice per group is shown^{*}
p < 0.05, compared to PBS or DC