

Enhancement of dendritic cell-tumor fusion vaccine potency by indoleamine-pyrrole 2,3-dioxygenase inhibitor, 1-MT

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Abstract

Purpose Dendritic cell (DC)-based cancer vaccines are currently being evaluated as novel anti-tumor vaccination strategies, but in some cases, they are demonstrated to have poor clinical efficacies than anticipated. A potential reason is immune tolerance due to the immunosuppressive enzyme, indoleamine-pyrrole 2,3-dioxygenase (IDO). The aim of this study was to determine whether blocking the activity of IDO might improve the anti-tumor efficacy of DC/Lewis lung carcinoma (LLC) fusion vaccine applied to the mouse LLC model.

Methods To prepare the DC/LLC fusion vaccine, DCs were fused with LLC using polyethylene glycol (PEG) as described. The IDO expression in the DC/LLC fusion vaccine and in the vaccinated mice was detected by western blot (WB) and/or immunohistochemical (IHC) analysis. This fusion vaccine, as a single agent or in combination with 1-methyl-tryptophan (1-MT, an IDO inhibitor), was administered to LLC mice. The anti-tumor efficacy in different treatment was determined by regular observation of tumor development and the level of splenic cytotoxic T lymphocyte (CTL) response, which was examined by lactate dehydrogenase (LDH) release.

Results In the LLC mice, we observed that IDO-positive cells were extensively accumulated in tumor draining lymph nodes (TDLNs). Furthermore, WB and IHC analysis results showed that vaccination with fusion DC/LLC cells alone caused significant up-regulation of IDO in spleens. 1-MT enhanced the anti-tumor efficacy elicited by DC/LLC fusion vaccine via delaying the tumor development and inducing stronger splenic CTL responses.

Conclusions Our results indicate an IDO-mediated immunosuppressive mechanism might be involved in weakening the anti-tumor efficacy elicited by DC/LLC fusion vaccine, and specific inhibition of IDO activity might be required for development of cancer vaccines.

Keywords Indoleamine-pyrrole 2,3-dioxygenase · 1-methyl-tryptophan · Dendritic cells · Cancer vaccines

Introduction

Immunotherapeutic strategies designed to prime anti-tumor T cells are currently under active clinical or pre-clinical development. Among them, dendritic cell (DC)-based cancer vaccine has been paid much attention as a new and cancer cell-specific therapeutic in the last decade. DCs, as the most potent antigen presenting cells (APCs), play a crucial role in triggering antigen-specific T cell responses because they are specifically equipped to engulf and process antigens and to present them to T cells (Banchereau and Steinman 1998; Shurin 1996). Several methods have been used for loading tumor antigens on DCs, including transduction of DCs with tumor antigen genes (Okada et al. 2005; Kirk et al. 2001; Wan et al. 1999), pulsing DCs with peptide antigen (Breckpot et al. 2004; Kono et al. 2002), and generation of DCs-tumor cell hybrids (Siders et al.

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2003; Kao et al. 2003). However, the clinical outcome of most conventional DC vaccines tested to date has been less than anticipated (Vermij and Frei 2004; Shibata et al. 2006). One of the essential reasons for this is that the tumor-specific cytotoxic T lymphocytes (CTLs) in response to cancer vaccines become functionally inactive when they enter the tumor microenvironment (Steinbrink et al. 1999; Mukherjee et al. 2004). Immune escape, the ability of tumor cells to avoid destruction by the host immune system, is a major obstacle that must be addressed in designing and delivering a successful cancer vaccine (Basu et al. 2006).

An immune escape mechanism for tumors mediated by indoleamine-pyrrole 2,3-dioxygenase (IDO) has been recently discovered. IDO catalyses the initial and rate limiting step in the metabolism of an essential amino acid, tryptophan, along the kynurenine pathway (Takikawa et al. 1986). By depleting tryptophan, IDO blocks proliferation and activation of T lymphocytes, which are particularly sensitive to loss of tryptophan (Frumento et al. 2001; Mellor et al. 2002). Thus, IDO expression is involved in T cell suppression and induction of immune tolerance to tumors.

It has been reported that IDO is overexpressed in various tumor cells and APCs in response to the tumor (Munn and Mellor 2004; Uyttenhove et al. 2003; Hwu et al. 2000; Friberg et al. 2002), and that treatment with the IDO competitive inhibitor 1-methyl-tryptophan (1-MT) shows significant effects in reducing the incidence and progression of tumors in both preventive and therapeutic treatment models (Uyttenhove et al. 2003; Friberg et al. 2002; Muller et al. 2005a, b). However, since IDO is certainly not the only mechanism participating in these processes of tumor tolerance, simply inhibiting IDO is not likely to be sufficient (Munn 2006).

Emerging evidence suggests that IDO might be not only induce by the tumor, but also constitute a significant counter-regulatory mechanism induced by clinically relevant pro-inflammatory signals, such as IFN- γ , IFN- α , CpG oligodeoxynucleotides, and 4-1BB ligation (Speiser et al. 2005; Seo et al. 2004; Nam et al. 2005; Mellor et al. 2005). More recently, Marion Wobser et al. (2007) reports that IDO is up-regulated in mature human DCs used in cancer immunotherapy, and predicts that IDO might contribute to the systemic immunological unresponsiveness and the failure of DC-based cancer immune therapies. These published data suggest that specific inhibition of IDO activity might be required for development of conventional or novel immune-based therapies.

In the present study, we sought to evaluate the anti-tumor efficacy of the DC/Lewis lung carcinoma (LLC) fusion vaccine applied to the mouse LLC model, a poorly immunogenic murine lung cancer model, as a single agent

or in combination with 1-MT. The results demonstrated that the combination treatment was more effective than DC/LLC fusion vaccine alone in reducing tumor growth and inducing stronger CTL responses.

Materials and methods

Animals and cell lines

Four- to 5-week-old male C57BL/6 mice were purchased from Sun Yat-sen University (Guangzhou, China), and housed in a specific pathogen-free facility. All animal studies were conducted in accordance with institutional guidelines for the care and use of experimental animals. The LLC cell line, which was originally derived from a C57BL/6 mouse, was cultured in complete medium (CM) consisting of Dulbecco's Modified Eagle's Medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotics.

Western blot (WB) analysis

Ten days after C57BL/6 mice were subcutaneously injected with LLC cells (1×10^6), tumors and lymphoid organs were homogenized in 0.5 ml of lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM PMSF, 2 mM sodium vanadate, 20 mg/ml aprotinin and 5 mg/ml leupeptin as described (Du et al. 2004). Insoluble material was removed by centrifugation (12,000 rpm 30 min, 4°C). The soluble cell lysates were separated on 10% SDS-PAGE, and blotted onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). After nonspecific binding was blocked by incubating with 5% non-fat dry milk, the membrane was incubated with the anti-IDO monoclonal antibody (1:1,000, Chemicon, Temecula, CA, USA) at 4°C overnight and then with a HRP-conjugated anti-mouse IgG (1:5,000, Boster, China). The resulting signal was detected by ECL Western blot method (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the loading control was β -actin (1:5,000, Cell Signaling Technology, Beverly, MA, USA).

Immunohistochemical (IHC) analysis for IDO expression

The tumors and lymphoid organs were fixed in formalin, embedded in paraffin, and the paraffin blocks were sectioned. Sections were deparaffinized and then incubated with anti-IDO monoclonal antibody (1:50; Chemicon) overnight at 4°C. An isotype-matched irrelevant antibody (1:50, Santa Cruz) was used as the negative control. After three washes with PBS, slides were incubated with the

HRP-labeled polymer conjugated secondary antibody (EnVision+; DAKO, Copenhagen, Denmark), and DAB (DAKO) was applied as a substrate. The slides were rinsed in distilled water and counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO, USA).

Generation of bone marrow-derived DCs

Erythrocyte-depleted murine bone marrow cells obtained from the femur were cultured in CM supplemented with rmGM-CSF (10 ng/ml; PeproTech, London, UK) at 1×10^6 cells/ml (Kao et al. 2003). The cytokines were replenished on day 3. On days 6–8, non-adherent DCs were harvested by vigorous pipetting for further use.

DC/tumor fusion cell preparation

Bone marrow-derived DCs were fused with tumor cells at a DC-to-tumor cell ratio of 3–1 using 50% polyethylene glycol (PEG, MW 1,450)/DMSO solution (Sigma-Aldrich), as described (Wang et al. 1998). Briefly, the DC/tumor cell suspension was washed twice with RPMI-1640 (pre-warmed to 37°C). PEG (50%, 0.5 ml, pre-warmed to 37°C) was added over 1 min and the suspension was stirred gently for 1 min. Pre-warmed RPMI-1640 (1 ml) was then added over 1 min and the suspension was stirred. An additional 3 ml of RPMI-1640 was added over 3 min, after which 10 ml of RPMI-1640 was added slowly with consistent stirring. After 5 min of incubation at 37°C, the resultant cell mixture was pelleted and grown overnight in CM with 10 ng/ml GM-CSF. The fusion cells were irradiated (10,000 rads) to render them non-proliferative before being injected into mice. Co-cultured groups were identically prepared except that PEG was omitted.

Immunofluorescence analysis

For phenotype analysis, cells were washed with PBS and incubated with mAb anti-CD40, mAb anti-CD80, mAb anti-CD86 (BioLegend, San Diego, CA, USA), or mAb anti-MHC class II (eBioscience, San Diego, CA, USA) for 30 min on ice. After washing with PBS, cells were analyzed by flowcytometry.

To evaluate the fusion efficiency, tumor cells were identified by staining 10^6 /ml cells with 2 µg/ml 3,3'-diiodoacetyl-oxacarbocyanine perchlorate (DiOC18; Invitrogen Life Technologies, Carlsbad, CA, USA) for 30 min at 37°C in PBS as previously described (Wang et al. 1998). The labeled cells were washed three times with PBS and fused with DCs as described above. Fusion preparations were further stained with FITC-conjugated anti-CD11c antibody (BioLegend). After three washes with PBS, cells were analyzed by flowcytometry.

Character of DC/LLC fusion vaccine and its effect on IDO induction in mice

To determine whether the DC vaccine affects IDO activity, 1×10^6 DCs, DC/LLC fusion cells, and DC + LLC mixture cells lysed, respectively and subjected to WB analysis with monoclonal IDO antibody as described above. Additionally, 1×10^6 non-proliferative DC/LLC fusion cells and DC + LLC mixture cells (fusion mock control) were s.c. injected into mice respectively twice within a 7-day interval. One week after the second injection, spleens from the vaccinated mice were removed and screened for IDO expression by WB and IHC analysis.

Treatment of mice with DC/LLC fusion vaccine and IDO inhibitor 1-MT

Four- to 5-week-old male C57BL/6 mice were randomized into the following four groups: Group 1 ($n = 12$): PBS treated controls; Group 2 ($n = 12$): vaccinated with DC/LLC fusion cells (1×10^6 non-proliferative cells per s.c. injection twice within a 7-day interval); Group 3 ($n = 12$): treatment with 1-MT (Sigma-Aldrich; begun 1 week prior to tumor implantation; twice a day by mouth for 2 weeks; 400 mg/kg, dissolved in 1% Tween 80/0.5% methylcellulose (TMC) and stirred continuously to get a fine suspension); Group 4 ($n = 12$): treated with DC/tumor fusion cells and 1-MT. A schematic of the treatment regimen used is depicted in Fig. 5a. One week after the second DC vaccination, 1×10^6 LLC cells were injected s.c. into the flanks of the mice. Animals were checked for tumors twice weekly. Tumor size was measured in two perpendicular dimensions with a pair of vernier calipers. Tumor volume was determined by the following formula: $\text{volume} = (a \times b^2)/2$, where a is the largest diameter of the tumor and b is the smallest diameter.

Cytotoxicity assays

One week after the second vaccination, spleens were removed from two mice randomly selected from each group for preparation of single-cell suspensions by pressing against fine nylon mesh. Red cells were lysed using 0.84% ammonium chloride. Spleen lymphocytes were co-cultured with mitomycin C (50 µg/ml)-treated LLC cells at 25:1 in RPMI 1640 plus 10% FBS. Five days later, cytotoxicity assay was conducted using non-radioactive lactate dehydrogenase (LDH) release using a cytotoxicity detection kit (CytoTox 96, Promega, Madison, WI, USA) as the manufacturer's instructions. Spontaneous release and maximum release were determined by incubating target cells without effector cells in medium alone or in 0.5% NP40, respectively. The percent cytotoxicity was calculated as follows: $(\text{experimental release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100\%$.

Statistical analysis

Student *t*-test was used to evaluate the significance of differences between experimental groups. Between-group differences in percentage of tumor-free individuals were evaluated using the Log-Rank test. Statistical tests were two-sided. *P* values less than 0.05 were considered to be statistically significant.

Results

IDO is expressed by mononuclear cells within tumor draining lymph nodes and spleens

Western blot and IHC analyses were carried out to determine whether the immunosuppressive enzyme IDO is expressed within LLC tumors or tumor draining lymph nodes (TDLNs). The tumors and TDLNs were removed from the inguinal region and divided into two aliquots: one for WB and one for IHC analysis. The distant lymph nodes (DLNs), the axillary lymph nodes, were also isolated. As shown in Fig. 1a, 10 days after tumor cell injection, IDO was strongly expressed in the TDLNs, weakly expressed in the tumor parenchyma, and not detectable in the DLNs. To check whether this tumor cell line can express IDO, LLC were treated with rm-IFN- γ (PeproTech), at a concentration well known to induce expression of IDO. WB analysis of the resultant LLC lysates found no IDO expression (Fig. 1b). Thus, tumor cells directly or indirectly induce IDO expression in other cells but not in themselves. Additionally, IHC analysis showed that IDO-positive cells were extensively accumulated in reticular region of TDLNs (Fig. 2d), while scattered and sparse within the tumor parenchyma (Fig. 2b), but not in DLNs from the same mouse (Fig. 2c). Together, these results indicated that non-

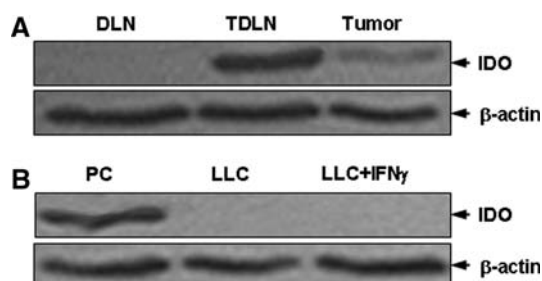


Fig. 1 IDO expression in TDLNs but not LLC cells. **a** Tumor tissues (*Tumor*), tumor-draining lymph nodes (*TDLNs*) and distant lymph nodes (*DLNs*) were removed 10 days after mice were s.c. injected 1×10^6 LLC, and were detected for IDO expression by WB analysis with an anti-IDO antibody. **b** LLC tumor cell line was cultured for 2 days with or without mouse IFN- γ (50 ng/ml) in the final 18 h culture. The cell lysates of tumor cells were analyzed for IDO expression and TDLN described above was used as positive control (*PC*)

malignant cells rather than tumor cells themselves produced IDO.

Immunofluorescence analysis of DC/LLC fusion cells

Lewis lung carcinoma cells were fused with bone marrow-derived DCs to enhance the T lymphocyte-mediated immunity to the weakly immunogenic tumor. The DCs were stained with FITC-conjugated anti-mouse CD11c (a green membrane marker) and LLC cells were labeled with DiOC18 (a red membrane marker). Flow cytometry found 22.1% double-positive cells after DC/LLC fusion (Fig. 3a). This is consistent with the reported fusion efficiencies of 10–30% (Jantscheff et al. 2002; Xia et al. 2005). In contrast to the tumor cells, DCs expressed MHCII and costimulatory molecules (CD80 and CD86) (Fig. 3b), as did the DC/LLC fusion products.

DC/LLC fusion cells did not express IDO, but induced IDO up-regulation in the spleens of vaccinated mice

To explore whether vaccination with DC/LLC fusion cells affects IDO-mediated immunosuppression, IDO expression was measured in DCs, DC/LLC fusion cells, and the immune organs of vaccinated mice. As shown in Fig. 4a, IDO was not expressed in bone-marrow derived DCs cultured in vitro, DC/LLC fusion cells, or DC + LLC mixed cells (Fig. 4a). However, vaccination with fusion cells up-regulated IDO expression in the spleens of the vaccinated mice (Fig. 4a). IHC identified these cells as non-malignant mononuclear cells (Fig. 4b).

The synergistic effect of DC/LLC fusion cells combined with 1-MT on delaying tumor growth

Since we hypothesized that expression of IDO by mononuclear cells infiltrating the tumors and immune organs might prevent immune-mediated rejection of LLC tumors, the combination of DC/LLC fusion cells and IDO specific inhibitor, 1-MT, was tested to determine whether it was more effective than its component parts. C57BL/6 mice were randomly assigned to four groups as described in “Materials and methods”. One week after the final vaccination, mice were challenged by local s.c. injection of 1×10^6 LLC tumor cells, and the growth was assessed at regular intervals by a blinded observer to determine therapeutic efficacy. Tumors developed in the first week and grew progressively in 60% of mice immunized with PBS (Fig. 5b). Tumor growth was evidently decreased in mice immunized with DC/LLC fusion cells ($P < 0.05$) or 1-MT ($P < 0.05$) in contrast to the PBS control group in day 27 after tumor challenge. However, mice treated with combination showed a significant reduction ($P < 0.001$) in tumor development.

Fig. 2 IDO is expressed by mononuclear cells infiltrating LLC tumors and tumor draining lymph nodes. Tissue sections from LLC tumor (a, b), distant lymph node (c), and tumor draining lymph node (d) were detected for IDO expression by IHC with a specific anti-IDO antibody. The slides were rinsed in distilled water and counterstained with haematoxylin. The insets in (b, d) show a higher magnification (400×) of a representative area in the panel

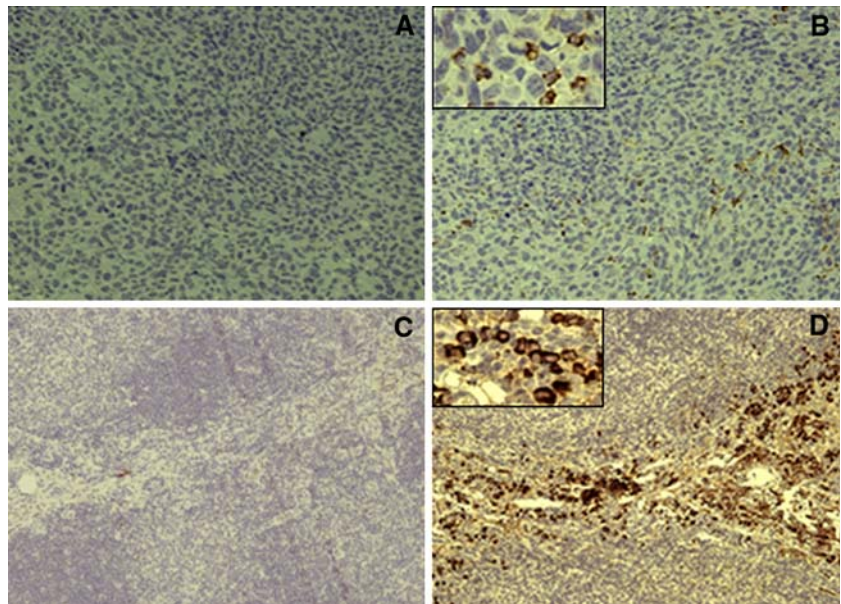
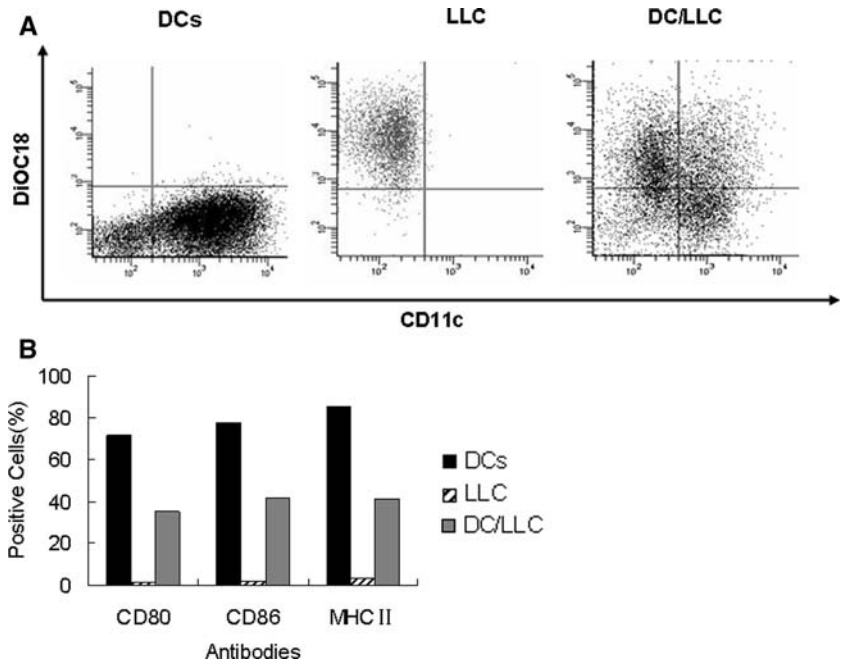


Fig. 3 Flowcytometer analysis of DCs, LLC cells, fusion cells and their fusion rates. **a** Prior to PEG treatment, expression of CD11c on DCs and DiOC18 fluorescence on LLC cells were detected. The fusion rate was determined by double staining of CD11c with DiOC18 on DCs and LLC fusion cells (DC/LLC). The double-positive cells were 22.1%. **b** Analysis of surface phenotypes of DCs, LLC and DC/LLC. The cells were analyzed using flowcytometry. The histograms show cells stained with the indicated molecule specific mAbs



Compared with the mice vaccinated with DC/LLC fusion vaccine alone, the combination treatment was also able to delay the tumor outgrowth ($P < 0.05$) (Fig. 5c). These results demonstrated that 1-MT could enhance DC/LLC fusion vaccine-induced protective immunity against tumor challenge.

1-MT with DC/LLC fusion vaccine induced cytotoxic T lymphocyte responses

To determine the effect of the combination on host T cell responses, CTL activity analysis was performed. LDH release assays showed that the combination treatment elic-

ited stronger CTL activity ($29.23 \pm 2.37\%$, E:T = 50:1) than DC/LLC treatment alone ($19.33 \pm 2.37\%$, $P < 0.01$) and the PBS control treatment ($6.53 \pm 2.00\%$, $P < 0.001$) (Fig. 6). A separate experiment found similar results. Thus, the combination treatment induced stronger CTL responses against LLC cells.

Discussions

Tumors suppress anti-tumor immunity through several pro-tolerogenic mechanisms. Among them, IDO has recently been identified as an impediment to successful cancer

Fig. 4 DC/LLC fusion cells did not express IDO, but induced IDO up-regulation in the spleens of vaccinated mice. **a** DCs, DC + LLC mixture (fusion mock) and DC/LLC fusion cells were lysed in a lysis buffer, and subjected to WB analysis with a specific anti-IDO antibody. **b** After second vaccination with or without DC/LLC fusion cells for 1 week, the spleens were removed and subjected to IHC analysis with a specific anti-IDO antibody. 1 Spleen from unvaccinated mice as a control; 2 Spleen from vaccinated mice with DC + LLC cell mixture; 3 Spleen from vaccinated mice with DC/LLC fusion cells. The *inset* in **b** shows a higher magnification (400 \times) of a representative area in the panel

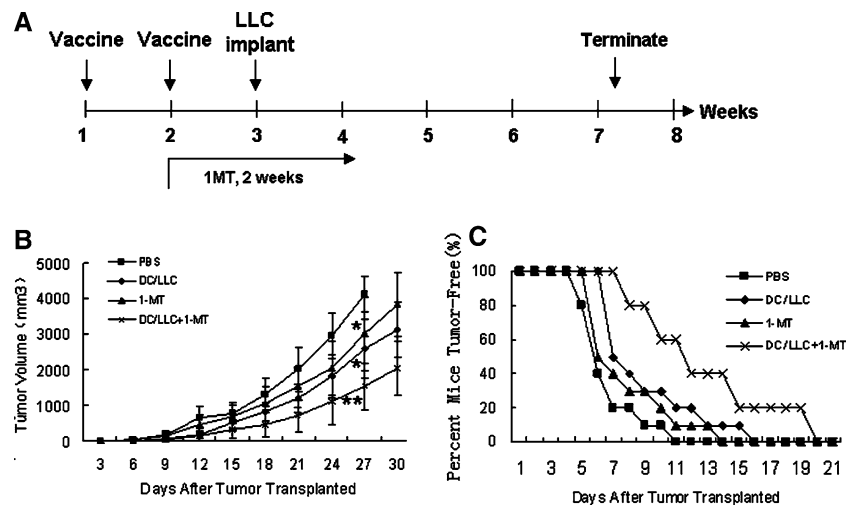
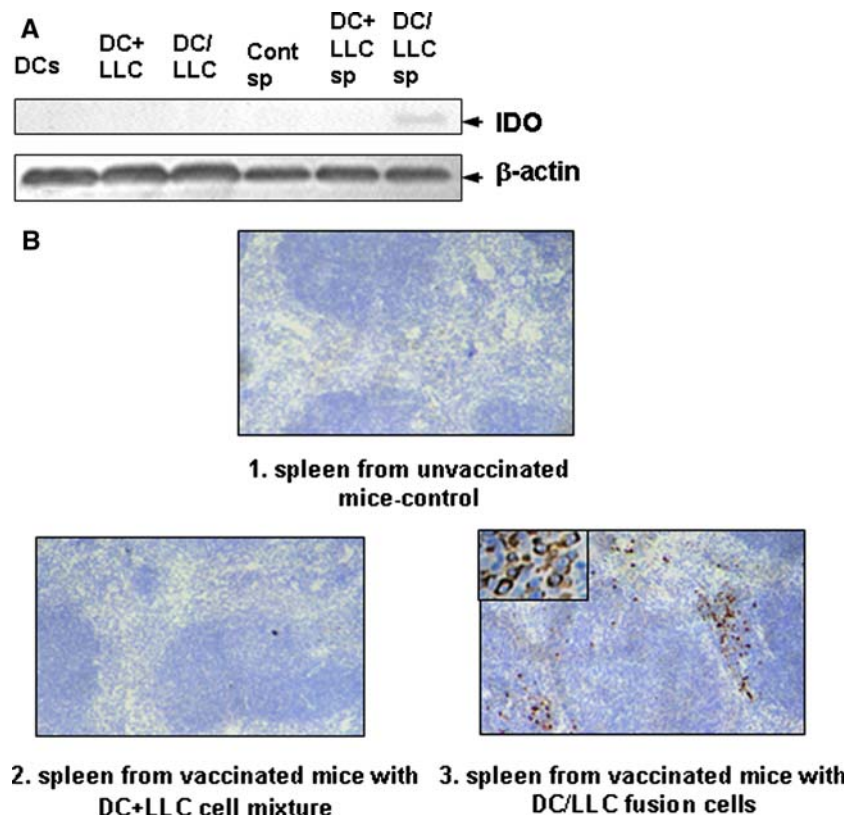


Fig. 5 Combination treatment with 1-MT promotes DC/LLC fusion vaccine-elicited host resistance against LLC tumor challenge. Mice were s.c. immunized twice with DC/LLC fusion vaccine at a 7-day interval as a single agent or in combination with 1-MT, the PBS was as a control. One week after the final immunization, mice were challenged by s.c. injection of 10^6 LLC tumor cells. Tumor growth and tumor-free time in each group of mice were recorded. **a** Schematic representation of the treatment schedule in the LLC mice. **b** Average

tumor growth over time for indicated treatment groups. *Asterisks* and *double asterisks* are an indication that significantly different from control on day 27 after tumor implantation ($*P < 0.05$; $**P < 0.001$, by Student's *t*-test). **c** The percentage of mice that remained tumor-free over time for indicated treatment groups. The difference between the combination and the DC/LLC fusion vaccine alone treated groups was shown to be statistically evident using the Log-Rank test ($P < 0.05$)

immunotherapy. IDO can be expressed by tumor cells and by other non-malignant cells in response to the tumor (Uyttenhove et al. 2003; Friberg et al. 2002). Abnormal expression of IDO in tumor cells has been shown to be inducible

by IFN- γ and inhibit local T-cell responses (Taylor and Feng 1991), and tumor cells transfected with IDO become resistant to immunologic rejection, even in mice that have been pre-immunized against the tumor (Uyttenhove et al.

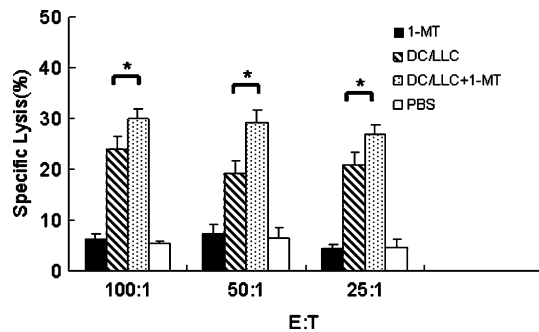


Fig. 6 1-MT enhances DC/LLC fusion vaccine-induced tumor specific CTL responses. Splenocytes were collected on day 7 after second DC/LLC immunization and incubated at indicated E:T ratio. CTL activity was determined by LDH releasing assay. All of the experiments were performed in triplicated, and the results were calculated as the mean \pm SD. Significantly different between DC/LLC fusion vaccine group and combination treatment group was determined by Student's *t*-test (* $P < 0.01$)

2003). The induction of IDO could also occur in nonmalignant cells either in direct proximity or at more distal sites, such as the DCs in the TDLNs (Friberg et al. 2002), probably via B7 ligation by regulatory T cells (Treg) (Fallarino et al. 2003). Since TDLNs are the primary sites for immune system to encounter tumor antigens (Spitotto et al. 2002), it has been argued that this might even be the principal means of establishing tolerance (Munn and Mellor 2004).

The preceding discussion has focused on IDO expression related to the tumor, however, the IDO expression relevant to the anti-tumor immune response provoked by clinical immunotherapy is still not elucidated well. In this report, we used the LLC mouse model, in which two remarkable features were observed. Firstly, IDO-positive cells were extensively accumulated in TDLNs, but scattered and sparse within the tumor parenchyma in the tumor bearing mice. These cells were most likely APCs such as DCs or macrophages (Muller et al. 2005a, b). More importantly, prior to tumor implantation, vaccination with DC/LLC fusion cells alone also caused significant up-regulation of IDO in spleens, though IDO was undetectable in vitro both in mature bone marrow-derived DCs or in DC/LLC fusion cells. It is well known that IDO could be induced by IFN- α and IFN- γ , which are secreted by activated T cells in response to any successful tumor-immunotherapy strategy, while expression of IDO might be an important event for altering T cell clonal expansion environment, causing T cell anergy. Therefore, in the present study, this IDO up-regulation in spleens is supposed to be an inflammation-induced counter-regulatory responses, which are utilized to limit the immune reactions of activated T cells elicited by the DC/LLC fusion vaccine.

Counter-regulatory responses are important in the immune system and they help to limit the intensity and extent of strong immune responses, which otherwise could

result in dangerous collateral damage to the host (Munn 2006). However, when it comes to tumor immunotherapy, this negative regulation might have the potential to antagonize the effects of other immunotherapy approaches desired to prime anti-tumor T cells.

Thus, to improve the anti-tumor efficacy of tumor immunotherapy, it should be helpful to add an IDO inhibitor to the conventional immune treatment scheme to overcome IDO-mediated immune tolerance. In our study, 1-MT enhanced reduction of tumor growth and induction of splenic CTLs in response to the DC/LLC fusion vaccine alone. There are two reasons: (1) 1-MT inhibited IDO-mediated immune suppressive microenvironment, in the tumor tissue and TDLNs and (2) 1-MT blocked the IDO-mediated counter-regulatory responses induced by the DC/LLC fusion vaccine. Thus, the anti-tumor T cells activated by the DC vaccine were allowed to clonally expand and became more effective once migrating into the tumor parenchyma.

It is indubitable that the efforts to improve the immunotherapeutic strategies should be focused on more efficient T cell activation and better generation of tumor-specific killer T cells (Lee 2002; Hatakeyama et al. 2006). However, the status of microenvironment for activated T cells to take effects is also should be concerned on. Ineffective cancer vaccines in clinical trial (Rosenberg et al. 2004; Gajewski et al. 2006) suggest that CTLs directed against the tumor might become functionally inactive when they enter the tumor microenvironment. Malignant tumors have been shown to evade T cell-mediated rejection by producing a variety of immunosuppressive proteins including IL-10, TGF- β and Fas ligand (Chouaib et al. 1997), while in the present work, we proved the IDO expression in non-malignant cells in immune organs, caused by the tumor as well as some clinical immunotherapy, could also contribute to the suppression of anti-tumor T cell response via the tryptophan depletion.

Additionally, in this experiment, we also found some inflammation at mucosal surfaces in oral cavity in mice, where encounter with non-pathogenic bacteria and non-harmful foreign antigens was frequent (unpublished data). Some other researchers reported that blocking IDO activity in either autoimmune colitis (Gurtner et al. 2003) or autoimmune asthma (Hayashi et al. 2004), significantly worsened the severity of disease. Besides, Munn and Mellor reported that pregnant mice exposed to IDO inhibitor exhibited dramatically increased tendencies to lose conceptuses in allogeneic but not syngeneic mating combinations (Munn et al. 1998; Mellor and Munn 2000; Mellor et al. 2001). These data suggest that IDO might play a physiological role in regulating immune responses and the specific route of administration of 1-MT remains to be developed.

In summary, our results suggest that 1-MT could enhance the anti-tumor efficacies of other immunotherapeutic

approaches by helping to break tumor tolerance and removing undesired counter-regulation.

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