Article

Ex Vivo Stimulation of Tumor-Draining Lymph Node Cells from Lung Cancer Patients: A Potential Resource for Adoptive Immunotherapy

Baoen Shan[1, 2], Qiliang Li[1], Ming He[1] and Yutong He[1]

To find a feasible method for the stimulation of tumor-draining lymph node (TDLN) cells in preparation for use in the clinic, the CTL activity of TDLN cells induced by different stimuli (IL-2 alone, IL-2 + autologous tumor antigen (atAg), IL-2 + GM-CSF + IL-4 + atAg) was measured by maximal LDH enzyme release. The mechanisms were explored by the observation of morphology and the detection of $CD83^+$ TDLN cells. The expansion of TDLN cells by IL-2 + GM-CSF + IL-4 + atAg was significantly higher than that by IL-2 alone or IL-2 + atAg ($p < 0.01$). Antitumor CTL activity of TDLN cells induced by IL-2 + GM-CSF + IL-4 + atAg was significantly higher than those of other groups. The number of $CD83^+$ cells within the TDLN population treated with IL-2 + GM-CSF + IL-4 + atAg was significantly elevated. The method of stimulating TDLN cells by IL-2 + GM-CSF + IL-4 + atAg was better than the stimulation with IL-2 or IL-2 + atAg. TDLN cells induced by IL-2 + GM-CSF + IL-4 + atAg produced more dendritic cells (DCs). In our study, we established a system that T cells and DCs were stimulated together *ex vivo*, which was easy to conduct and produce promising results. It provided a new method for improving TDLN cell antitumor activity which might be used in the clinical cancer therapy. *Cellular & Molecular Immunology*. 2008;5(4):307-313.

Key Words: tumor-draining lymph node cell, dendritic cell, antitumor CTL activity

Introduction

Metastasis and recurrence of malignant tumor is the main cause of mortality in cancer patients. Radiotherapy and chemotherapy may be used instead of surgery to reduce the risk of metastasis; however, the therapeutic efficacy (in terms of patient survival) is poorer. Radiotherapy and chemotherapy cannot prevent metastasis and the recurrence arises due to residual malignant cells in most patients. In addition, some patients become even much worse after high dose chemotherapy and radiotherapy due to immune function failure (1). In contrast, adoptive immunotherapy has the potential to prevent micrometastasis through killing tumor cells at disparate sites, and can also enhance the function of normal immune cells effectively (2, 3). Tumor-draining lymph nodes (TDLNs) are lymph nodes in which tumor cells drain and meet cells of the immune system and have active cellular immunological function. Li et al. reported that after adoptive transfer of *ex vivo* IL-12 and IL-18 stimulated TDLN cells into mice bearing pulmonary metastases, the mean number of metastases was significantly reduced (4). Previous studies also showed that the transferred TDLN cells could accumulate in plexus vasculosus of alveolus, all the lymph nodes in the body and spleen, proliferate, and migrate into cancer tissue to inhibit tumor growth (5).

Adoptive immunotherapy using LAK cells and cytokine induced killer (CIK) cells has been applied clinically for the treatment of cancers and it has been shown to have good therapeutic effects (6). However, both LAK and CIK belong to a family of non-specific killer cells and they retain the non-specific killing function after being transferred back into the body. Other techniques of adoptive therapy used in clinic included tumor infiltrating lymphocyte (TIL), NDV-modified autologous tumor vaccine, and so on (7, 8). Compared with these cells, TDLN cells can be regarded as an important resource of specific antitumor cells (9, 10). It has been shown that lymphocytes from TDLNs were sensitized to the tumor antigen *in vivo*. Moreover, after activated *in vitro*, these cells

[1]Hebei Cancer Research Center, the Fourth Hospital of Hebei Medical University, Shijiazhuang 050011, China;

[2]Corresponding to: Dr. Baoen Shan, Hebei Cancer Research Center, The Fourth Hospital of Hebei Medical University, Shijiazhuang 050011, China. Tel: +86-311-8609-5283, Fax: +86-311-8699-2004, E-mail: baoenshan@yahoo.com.cn

Received Apr 20, 2008. Accepted Aug 1, 2008.

Abbreviations: APC, antigen presenting cell; atAg, autologous tumor antigen; DC, dendritic cell; NO, nitrous oxide; rIL, recombinant interleukin.

could be utilized for adoptive immunotherapy. TDLNs comprise lymph node cells which have been soaked in tumor tissue and have been shown to activate cellular immunity (11). They are composed of antigen presenting cells (APCs) and immunologically active cells, such as T cells, NK cells and CTLs. Their killing function on tumor cells is specific because they are continuously in contact with tumor cells (12). It was shown that TDLN cells could lyse autologous tumor cells *in vitro* and that they had more active killing function than CIK and LAK cells (11). In addition, they could probably decrease the chance of tumor metastasis (13). However, TDLN cells were often not very effective in killing tumor cells *in vivo* due to the lack of costimulation. If TDLN cells can be readily sensitized and expanded, they could be used in the clinic to treat tumors. In this study, we investigated the effects of different stimuli (autologous antigen and cytokines) on the cytokine secretion of TDLN cells and the subsequent capacity of these cells to stimulate T cells that led to specific killing of tumor cells. The aim of this study was to provide preclinical data for the potential of *ex vivo* expansion and stimulation of TDLN cells that may be used in the clinical setting for adoptive immunotherapy protocols.

Materials and Methods

Reagents

Recombinant interleukin-4 (rIL-4) was purchased from R&D (Lille, France); rIL-2 from Peprotech (London, UK); rGM-CSF was kindly provided by North China Pharmaceutical Group Corporation. RPMI 1640 medium was purchased from Gibco (Carlsbad, USA); heat-inactivated fetal bovine serum from MD Systems (USA); glutamine, HEPES and MTT from Sigma (St. Louis, USA); β-mercaptoethanol from Amresco (Ohio, USA); Ficoll (d = 1.077) from Tianjin Genomapping Technology Co. Ltd. (Tianjin, China); FITC-anti-CD83 and PE-anti-CD3 monoclonal antibodies from eBioscience (San Diego, USA); LDH enzyme kit and nitrous oxide (NO) test kit from Promega (Madison, USA); human IL-12p70, IFN-γ and TNF-α ELISA test kits from Jingmei Biotech Co. Ltd. (Shengzhen, China).

Preparation of the single cell suspension of TDLN cells from lung cancer patients

Samples were collected from six patients with lung cancer treated at the Fourth Hospital of Hebei Medical University from November 2004 to March 2005. Four patients were male, and two were female, with average age of 53. The samples were collected following local ethical permission and informed consent. During the operation, the tumor tissue and two to four peripheral lymph nodes were taken. These patients were shown to have metastasis by pathology. The samples were washed by sterilized D-Hank's (Sigma, St. Louis, USA) to remove serum, connective tissue and fat, then cut into 1 mm^3 cubes using scissors and filtered through a 200 gauge steel mesh to prepare single cell suspension. Cell fractions were isolated by Ficoll centrifugation using standard procedures.

Preparation of lung cancer cell antigen

Tumor tissue was prepared as above described and the 1 mm^3 of tumor tissue was placed in RPMI 1640 medium with 0.05% collagen enzyme (Sigma, St. Louis, USA), 0.02% DNase (Promega, USA), 0.01% hyaluronidase (Sigma, St. Louis, USA) and 10% fetal bovine serum. The sample was mixed overnight at 4°C, and filtered through a 200 gauge steel mesh to obtain single cell suspension. Ficoll was used to separate tumor cells and the cells were resuspended at 5×10^6 cells/ml. Lung cancer cell lysates (from approximately 10^7 cells) were prepared by three-time freeze-thaw cycles (-140°C/37°C) in 1 ml PBS, with removal of cell debris by centrifugation at 3,000× g for 10 min. The suspension was subsequently referred as autologous tumor antigen (atAg).

Activation of TDLN cells

TDLN cell suspensions from patients (1×10^6 cells/ml) were seeded into 24-well plates (1 ml/well) (Costar Co. Ltd., USA) and stimulated with 1) rIL-2 (175 U/ml), 2) rIL-2 (175 U/ml) + atAg, 3) IL-2 (175 U/ml) + rGM-CSF (500 U/ml) + IL-4 (100 U/ml) + atAg, respectively. Cells were cultured at 37°C with 5% CO_2 for 7 days. The change in morphology of the TDLN cells was observed using an optical microscope (OLYMPUS LH50A, Japan). The expressions of CD83 and CD3 were detected with direct immunofluorescence assay using FITC-anti-CD83 and PE-anti-CD3 mAbs by a fluorescence microscope. $CD83^+$ cells are shown to fluoresce in yellow-green and $CD3^+$ cells fluoresce in hyacinth.

Proliferation of TDLN cells

TDLN cells were prepared, resuspended at 1×10^5 cells/ml and seeded into 96-well culture plates (Costar Co. Ltd., USA), and stimulated as described above. After incubation for 1-7 days, 10 μl MTT was added and incubated for further 4 h at 37°C. Samples were centrifuged at 1,500 × g for 10 min and the supernatant was removed. DMSO (150 μl) was added to each well and shaken for 10 min. The value of OD_{490} was determined by the plate reader (HT2, Anthos Co. Ltd, Austria). A standard curve for TDLN cell proliferation was determined.

Phenotypic analysis of TDLN cells

TDLN cells were incubated with different stimuli for 14 days at 37°C, 5% CO_2. Both FITC-anti-CD83 and PE-anti-CD3 mAbs were added to the TDLN cells and co-incubated at room temperature for 30 min. Then the cells were washed with PBS twice and analyzed by flow cytometry (EPICS-XL, Beckman Coulter Co., USA).

Tumor specific cytolytic activity of TDLN cells

Autologous cancer tissue cells (to atAg) were prepared as described above and cultured to the logarithmic phase. These target cells were collected and resuspended at 2×10^5 cells/ml in RPMI 1640 containing 5% FCS. The effector cells induced from TDLN cells were prepared and resuspended at 2×10^7 cells/ml in RPMI 1640 containing 5% FCS. The target cells and effector cells (each in 50 μl final volume) were placed into 96-well round bottom plates at the ratio 1:10,

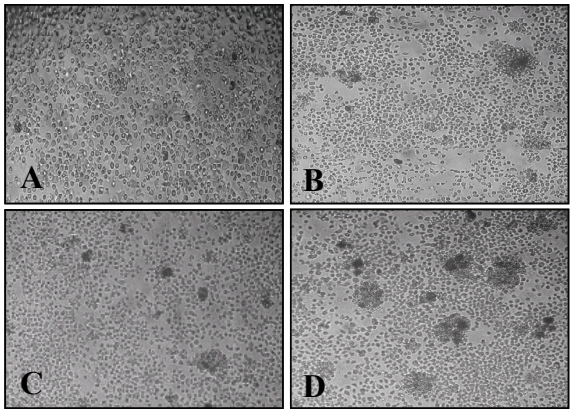

Figure 1. The morphology of TDLN cells treated with different stimuli. TDLN cells (1×10^6 cells/ml) were seeded into 24-well plates (1 ml/well) without stimulation (A), activated with rIL-2 (175 U/ml) (B), rIL-2 (175 U/ml) + atAg (C), or IL-2 (175 U/ml) + rGM-CSF (500 U/ml) + IL-4 (100 U/ml) + atAg (D). Cells were cultured at 37°C with 5% CO_2 for 7 days. The morphology of TDLN cells was observed using an optical microscope (×100).

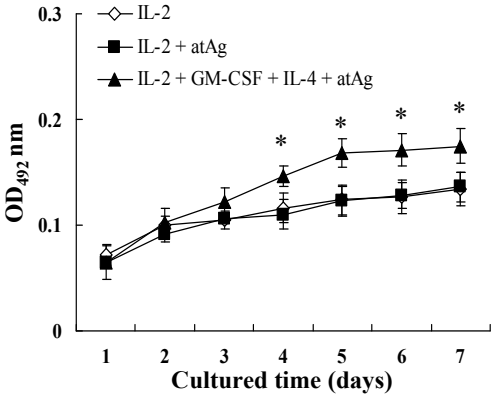

Figure 2. Proliferation curve of TDLN cells induced by different stimuli. MTT method was used to study the proliferation of TDLN cells following stimulation with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg as described in Materials and Methods. Data were representative of 3 independent experiments which were performed on all six patient samples.

respectively. In addition, wells were set up to determine effector cell spontaneous lysis, target cell spontaneous lysis, target cell maximal release, medium alone, volume control and negative control wells (without stimulation). Each well was prepared at the same time and every test consisted of 3 wells. The cytolytic activity of TDLN cells was detected by LDH cytotoxicity assay kit according to the manufacturer's instructions. The percentage lysis of targets was calculated as follows: lysis ratio (%) = $[(OD_{E+T} - OD_{Espontaneity} - OD_{Tspontaneity}) / (OD_{Tmaximum} - OD_{Tspontaneity})] \times 100\%$. OD_{E+T}, OD of well with effector and target cells - OD of well with medium alone; $OD_{Espontaneity}$, OD of well containing effector cells and measuring spontaneous release - OD of wells with medium alone; $OD_{Tspontaneity}$, OD of spontaneous release from target cells - OD of well with medium alone; $OD_{Tmaximum}$, OD of well in which target cell lysis is maximal - OD of the wells with volume control (mediun alone).

Cytokine assay

TDLN cells (2×10^5) were co-cultured with different stimuli in 24-well culture plates at 37°C with 5% CO_2 and the supernatants were collected at days 7, 14 and 21 to analyze the secretion of IL-12p70, IFN-γ and TNF-α by ELISA. The amount of NO was detected by Griess.

Statistical analysis

Data were analyzed with SPSS13.0 software and presented as the mean ± SE. Statistical significance was determined by One Way ANOVA. A p value of < 0.05 was considered statistically significant.

Results

The change in morphology of TDLN cells following treatment with different stimuli

After cultured with IL-2, IL-2 + atAg and IL-2 + GM-CSF + IL-4 + atAg for 7 days, TDLN cells formed clumps and were in close contact with each other. The number of cells stimulated with IL-2 + GM-CSF + IL-4 + atAg was much larger than those with IL-2 and IL-2 + atAg observed under the optical microscope ($p < 0.01$, Figure 1).

The effect of different stimuli on the proliferation of TDLN cells

TDLN cells proliferated after treated with IL-2, IL-2 + atAg or IL-2 + GM-CSF + IL-4 + atAg. TDLN cell proliferation was significantly enhanced by IL-2 + GM-CSF + IL-4 + atAg in comparison with IL-2 and IL-2 + atAg ($p < 0.01$, Figure 2).

The effect of different stimuli on the immunophenotype of TDLN cells

After stimulation with IL-2, $CD83^+$ TDLN cells formed clusters with $CD3^+$ T cells. The number of $CD83^+$ cells was increased, and the volume of the T cell colonies were larger when TDLN cells were stimulated with IL-2 alone (Figure 3A) than that with IL-2 and atAg (Figure 3B). The number of $CD83^+$ TDLN cells treated with IL-2 + GM-CSF + IL-4 + atAg was significantly increased and $CD83^+$ cells formed large clusters with the T cells (Figure 3C). The percentages of $CD83^+$ cells within the TDLN cells cultured with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg were 20.40 ± 4.92%, 24.89 ± 7.31% and 30.87 ± 2.25%, respectively, and showed significant differences ($p < 0.05$, Figures 3D-3E).

The effect of each stimulus on CTL activity of TDLN cells

The CTL activities of TDLN cells following treatment with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg were 41.67 ± 6.28%, 57.76 ± 9.51% and 70.78 ± 6.06%, respectively, much higher than that of the control group (8.02 ± 1.88%, p

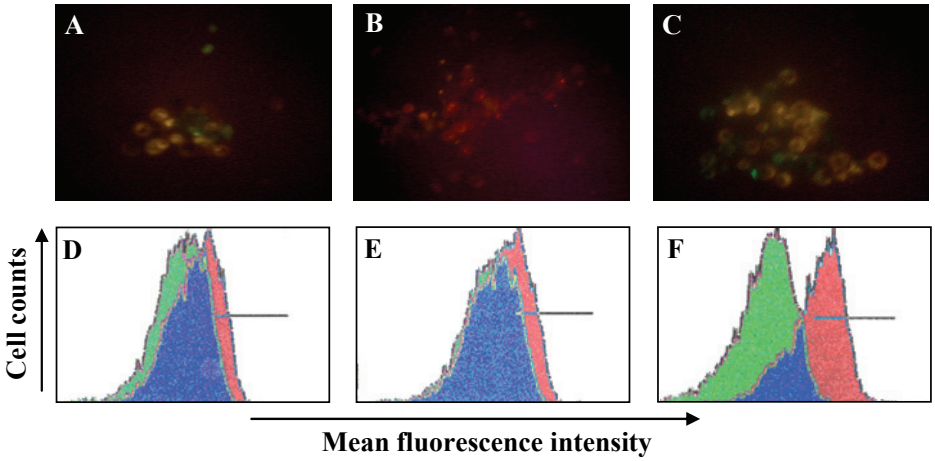

Figure 3. Expressions of CD83 and CD3 on TDLN cells induced by different stimuli. TDLN cells (1×10^6) were cultured with (A, D) IL-2 (175 U/ml); (B, E) IL-2 (175 U/ml) + atAg, or (C, F) IL-2 (175 U/ml) + rGM-CSF (500 U/ml) + IL-4 (100 U/ml) + atAg at 37°C with 5% CO_2 for 7 or 14 days. (A-C) Expressions of CD83 and CD3 were detected with direct immunofluorescence assay using FITC-labeled anti-CD83 and PE-labled anti-CD3 mAbs by a fluorescence microscope (×400). $CD83^+$ cells were shown to fluoresce in yellow-green and $CD3^+$ cells were shown to fluoresce in hyacinth. (D-F) Detection of CD83 expression on TDLN cells by flow cytometry. Green histograms showed the cells stained with isotype control and red histograms indicated the cells stained with FITC-conjugated anti-CD83.

< 0.01). There were significant differences between the three groups ($p < 0.05$, Figure 4).

The effect of different stimuli on the secretion of IL-12p70 by TDLN cells

The production of IL-12p70 by TDLN cells stimulated with IL-2 + GM-CSF + IL-4 + atAg was higher than that with IL-2 or IL-2 + atAg ($p < 0.05$, Figure 5). There was no statistical difference in the secretion of IL-12p70 by TDLN cells stimulated with IL-2 and IL-2 + atAg. The production of IL-12p70 by stimulated TDLN cells peaked on day 14, as compared with that on day 7 or day 21 ($p < 0.01$, Figure 5).

The effect of different stimuli on the production of IFN-γ by TDLN cells

As shown in Figure 6, the secretion of IFN-γ by TDLN cells stimulated with IL-2 + GM-CSF + IL-4 + atAg was higher than that with IL-2 or IL-2 + atAg ($p < 0.05$). There was no statistical difference in IFN-γ secretion by TDLN cells stimulated with either IL-2 alone or IL-2 + atAg. The peak of

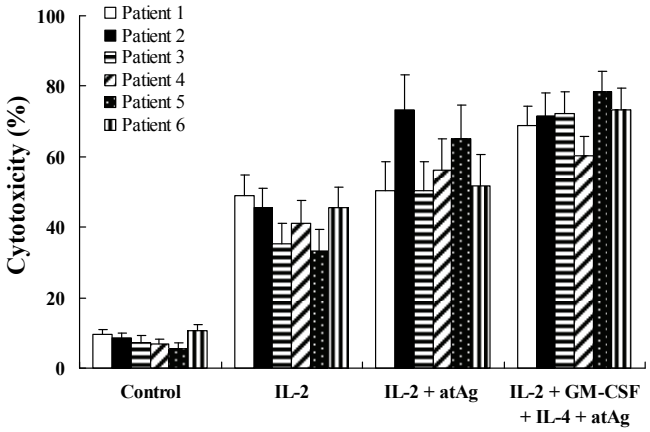

Figure 4. Tumor specific cytotoxic activity of TDLN cells from lung cancer patients with different stimulations. TDLN cells (effectors) were treated with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg and incubated with autologous tumor cells (targets). CTL activity was measured by LDH cytotoxicity assay. Patients 1-6 represented 6 different patient samples. Data were representative of 3 independent experiments.

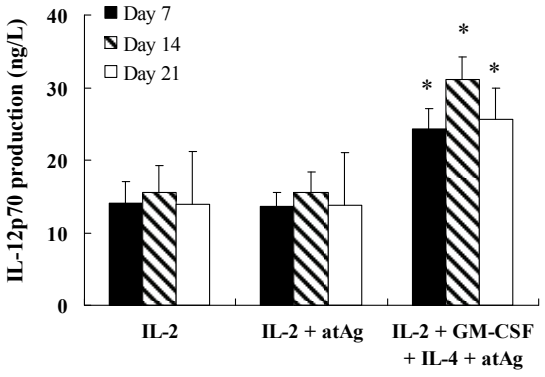

Figure 5. The effect of different stimuli on secretion of IL-12p70 by TDLN cells. TDLN cells (2×10^5) were co-cultured with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg at 37°C with 5% CO_2 and the supernatants were collected on days 7, 14 and 21 to analyze the secretion of IL-12p70 by ELISA. The production of IL-12p70 by TDLN cells stimulated with IL-2 + GM-CSF + IL-4 + atAg was higher than that with IL-2 or IL-2 + atAg ($p < 0.05$). The production of IL-12p70 in each group peaked on day 14 compared with day 7 or 21 ($p < 0.01$).

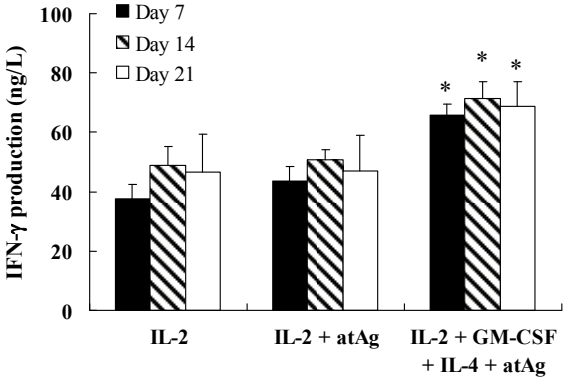

Figure 6. The effect of different stimuli on secretion of IFN-γ by TDLN cells. TDLN cells (2×10^5) were co-cultured with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg at 37°C with 5% CO_2 and the supernatants were collected on days 7, 14 and 21 to analyze the amounts of IFN-γ by ELISA. The production of IFN-γ by TDLN cells stimulated with IL-2 + GM-CSF + IL-4 + atAg was higher than that with IL-2 or IL-2 + atAg ($p < 0.05$). IFN-γ secretion in each group peaked on day 14 rather than day 7 or 21 ($p < 0.01$).

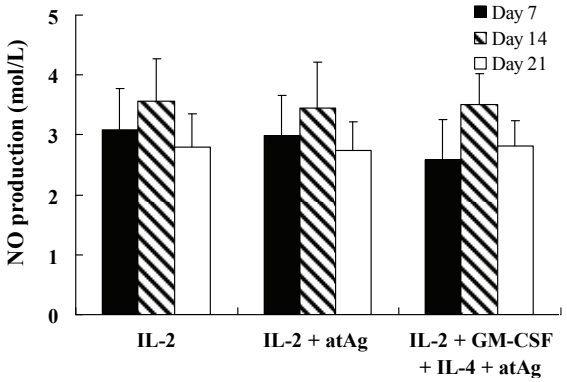

Figure 8. The effect of different stimuli on secretion of NO by TDLN cells. TDLN cells (2×10^5) were co-cultured with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg at 37°C with 5% CO_2 and the supernatants were collected on days 7, 14 and 21 to analyze the secretion of NO by Griess. The production peak of NO by stimulated TDLN cells was on day 14, rather than day 7 or 21 ($p < 0.01$).

IFN-γ production by stimulated TDLN cells occurred on day 14 rather than on day 7 or 21 ($p < 0.01$).

The effect of different stimuli on TNF-α secretion by TDLN cells

The production of TNF-α by TDLN cells treated with IL-2 + GM-CSF + IL-4 + atAg was higher than those with IL-2 alone and IL-2 + atAg ($p < 0.05$). There was no statistical difference in the secretion of TNF-α by TDLN cells treated with IL-2 alone compared with stimulation with IL-2 + atAg. Although the production peaked on day 14, there was no statistically significant difference in the TNF-α production at each time point (days 7, 14 or 21) (Figure 7).

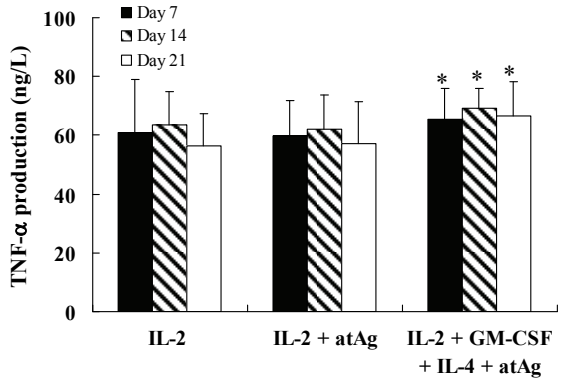

Figure 7. The effect of different stimuli on secretion of TNF-α by TDLN cells. TDLN cells (2×10^5) were co-cultured with IL-2, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg at 37°C with 5% CO_2 and the supernatants were collected on days 7, 14 and 21 to analyze the secretion of TNF-α by ELISA. The production of TNF-α by TDLN cells treated with IL-2 + GM-CSF + IL-4 + atAg was higher than those with IL-2 alone or IL-2 + atAg ($p < 0.05$).

The effect of different stimuli on the production of NO by TDLN cells

There was no difference in the levels of NO secretion by TDLN cells stimulated with IL-2 alone, IL-2 + atAg, or IL-2 + GM-CSF + IL-4 + atAg. The production of NO peaked on day 14, instead of on day 7 or 21 ($p < 0.01$, Figure 8).

Discussion

Lung cancer is one of the most common malignant tumors of respiratory system. It is progressive and the prognosis is poor. The patients always die of micrometastasis after therapy due to the spillage of a small dose of cancer cells in the peripheral blood. It is of note that about 40% of lung cancer patients have lung cancer cells in their peripheral blood at diagnoses (14). With the development of molecular biology and cellular biology techniques, the immunotherapeutic treatment of tumors can support traditional therapies such as surgery, radiotherapy and chemotherapy.

TDLN provides an important resource of cells with a tumor specific killing function which can be used for adoptive immunotherapy (9, 10). However, these cells are often anergic and can not generate antitumor immune responses without additional stimuli (15). There are many traditional approaches to activate TDLN cells, such as stimulation with low dose of IL-2 plus tumor antigen (9), or only with low-dose IL-2 (10, 12). In this study, we activated TDLN cells using above mentioned approaches and compared their effects with stimulation with IL-2 + GM-CSF + IL-4 + atAg. The cytotoxicity of TDLN cells stimulated with IL-2 + GM-CSF + IL-4 + atAg was higher than that with IL-2 alone or IL-2 + atAg, and the T cell response in terms of proliferation and volume was significantly greater. After the

activation by IL-2 + GM-CSF + IL-4 + atAg, the frequency of $CD83^+$ cells in the TDLN population was higher than that stimulated with IL-2 alone or IL-2 + atAg. CD83 is a surface marker specific for DCs, and this result suggested that the stimulus increased the DC population.

In normal lymph nodes, there are predominantly inactive T cells, B cells and APCs. However, TDLN is composed mainly of APCs and immunologically active cells, because it has been sensitized by local tumor tissue and has already experienced some tumor antigens (12). After tumor antigen recognition by immune cells, the histology of TDLN changes greatly, and the proliferations of the inter cortex, cortex follicle and tissue of sinus are observed (16). The proliferation involves a large quantity of immune cells such as T cells, B cells and monocytes (including macrophages, veiled cells, interdigitating cells and DCs) (16). It has been previously reported that the number of DCs in the peripheral blood of lung cancer patients has a positive relationship with prognosis (17-19). In the TDLN, about 1% TDLN cells are mature DCs (20). The maturation of DCs is associated with many factors including stimulation of antigen, the expression of co-stimulation molecules, the effect of chemokines and their receptors (21-23). There are many factors that can be used to mature DCs, such as LPS, TNF-α and CD40L. In this study, we focused on stimulating the killing potential of TDLN cells through the use of both GM-CSF and IL-4. Numerous studies showed that GM-CSF and IL-4 could drive monocytes into DCs, and increase the expression of CD83 on DCs (24). Rouard's research indicated that CD83 molecule was not expressed on DCs in early culture but presented when DCs matured (25), when their capacity to activate T cells was most effective (26, 27). However the role of CD83 on mature DCs and T cell activation remains unclear.

We compared the capacity of IL-2 or IL-2 + atAg, and IL-2 + GM-CSF + IL-4 + atAg to stimulate TDLN cells. The results showed that TDLN cell stimulation with IL-2 + GM-CSF + IL-4 + atAg induced the highest levels of IL-12p70, IFN-γ and TNF-α secretion compared with IL-2 alone or IL-2 + atAg. GM-CSF and IL-4 are factors which can induce DC proliferation and differentiation. They have been shown to induce large quantities of DCs with typical phenotype and function. So the differences in the secretion of cytokines by TDLN cells in response to the various stimuli may be a reflection of the number of DCs in TDLN population (26, 27). It was reported that the application of mature rather than immature DCs in patients was more effective in adoptive immunotherapy (26).

The CTLs in TDLN cells have been sensitized to tumor antigens in the body and have high sensitivity and specificity. However, immune inhibitors secreted by tumor cells suppress the transduction of stimulatory signals, and inhibit CTL cytotoxicity against tumor cells. In addition to the presentation of tumor antigens, DCs can also secrete a series of cytokines and provide co-stimulating signals stimulating T cells and inducing antigen specific CTL response. This leads to protective immunity and prevents tumor formation (16, 28). This is just one of the mechanisms by which DCs provide antitumor activity. DCs can secrete IL-12p70 and induce T cells to secrete cytokines such as IFN-γ and TNF-α, which have antitumor activaties (20, 29). IFN-γ can induce MHC class II expression by monocytes, macrophages and DCs, and enhance antigen presentation and specific immunity. IFN-γ can also improve IL-2R expression on T cells, induce the expression of MHC class I molecules on tumor cells, increase CTL cytotoxcity, induce the synthesis of acute proteins and cause the differentiation of the pulp cells. TNF-α can inhibit tumor cells directly. By the immunomodulatory effect, these cytokines improve the killing of tumor cells by T cells, monocytes/macrophages, and dendritic cells. In addition, they can injure endothelial cells of the blood vessel or lead to the functional disorder of the blood vessel, destroy blood vessel in the tumor tissue, form thrombi, interrupt the local blood stream, and induce bleeding, anoxia and necrosis of tumor tissue. DCs can also produce NO, but the outcome of this experiment showed that the level of the NO induced by various stimulating factors had no obvious difference.

Our data indicated that the cooperation of IL-2, GM-CSF, IL-4 and atAg in different concentrations could further increase the killing activity of TDLN cells compared with IL-2 alone or IL-2 plus atAg. In providing this combination of cytokines and antigen it showed that we could expand a population of $CD83^+$ cells, induce T cell secretion of cytokines relevant to antitumor activity and improve CTL activity against the autologous tumor cells. Furthermore, other methods involved the separation and isolation of DCs and T cells from the peripheral blood or lymph tissue and their preparation for readministration. In this study we established a system in which T cells and DCs were stimulated together *ex vivo*, which was easy to conduct and obtain promising results. It provided a new concept in improving TDLN cell antitumor activity which might be used in the clinical cancer therapy setting.

References

1. Saykin AJ, Ahles TA, McDonald BC. Mechanisms of chemotherapy-induced cognitive disorders: neuropsychological, pathophysiological, and neuroimaging perspectives. Semin Clin Neuropsychiatry. 2003;8:201-216.
2. Catchpole B, Gould SM, Kellett-Gregory LM, Dobson JM. Immunosuppressive cytokines in the regional lymph node of a dog suffering oral malignant melanoma. J Small Anim Pract. 2002;43:464-467.
3. Takenoyama M, Yasumoto K, Harada M, Sugimachi K, Nomoto K. Antitumor response of regional lymph node lymphocytes in human lung cancer. Cancer Immunol Immunother. 1998;47:213-220.
4. Li Q, Carr AL, Donald EJ, et al. Synergistic effects of IL-12 and IL-18 in skewing tumor-reactive T-cell responses towards a type 1 pattern. Cancer Res. 2005;65:1063-1070.
5. Lores B, Garcia-Estevez JM, Arias C. Lymph nodes and human tumors. Int J Mol Med. 1998;1:729-733.
6. Bordignon C, Carlo-Stella C, Colombo MP, et al. Cell therapy: achievements and perspectives. Haematologica. 1999;84:1110-1149.
7. Jewett A, Head C, Cacalano NA. Emerging mechanisms of

immunosuppression in oral cancers. J Dent Res. 2006;85:1061-1073.
8. Stevenson FK. Tumor vaccines. FASEB J. 1991;5:2250-2257.
9. Sameshima S, Sakai K, Nagawa H, Tsuno N, Kitayama J, Muto T. Correlation of the therapeutic effect of activated tumor-draining lymph node cells with specific interferon-γ production. Surg Today. 1999;29:55-62.
10. Santin AD. Lymph node metastases: the importance of the microenvironment. Cancer. 2000;88:175-179.
11. Munn DH, Mellor AL. The tumor-draining lymph node as an immune-privileged site. Immunol Rev. 2006;213:146-158.
12. Tuttle TM, Inge TH, McCrady CM, Bethke KP, Bear HD. Activation of $CD8^+$ murine T cells from tumor-draining lymph nodes by phorbol dibutyrate plus calcium ionophore. J Immunother. 1992;12:32-40.
13. Ruttinger D, Li R, Urba WJ, Fox BA, Hu HM. Regression of bone metastases following adoptive transfer of anti-CD3 activated and IL-2-expanded tumor vaccine draining lymph node cells. Clin Exp Metastasis. 2004;21:305-312.
14. Peck K, Sher YP, Shih JY, Roffler SR, Wu CW, Yang PC. Detection and quantitation of circulating cancer cells in the peripheral blood of lung cancer patients. Cancer Res. 1998;58: 2761-2765.
15. Cohen PA, Peng L, Kjaergaard J, et al. T-cell adoptive therapy of tumors: mechanisms of improved therapeutic performance. Crit Rev Immunol. 2001;21:215-248.
16. Xiu FM. The research evolution on the immunologic property and antitumor mechanism of tumor-draining lymph node cells. Guo Wai Yi Xue Mian Yi Xue Fen Ce. 1995;18:170-174.
17. Zeid NA, Muller HK. S100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. Pathology. 1993;25:338-343.
18. Imoto H, Osaki T, Taga S, Ohgami A, Ichiyoshi Y, Yasumoto K. Vascular endothelial growth factor expression in non-small-cell lung cancer: prognostic significl carcinoma. J Thorac Cardiovasc Surg. 1998;115:1007-1014.
19. Johnson SK, Kerr KM, Chapman AD, et al. Immune cell infiltrates and prognosis in primary carcinoma of the lung. Lung Cancer. 2000;27:27-35.
20. Tanigawa K, Craig RA, Stoolman LM, Chang AE. Effects of tumor necrosis factor-α on the maturation of tumor-reactive effector T cells. J Immunother. 2000;23:528-535.
21. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392:245-252.
22. De Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M. Effect of interleukin-10 on dendritic cell maturation and function. Eur J Immunol. 1997;27:1229-1235.
23. Geissmann F, Revy P, Regnault A, et al. TGF-β1 prevents the noncognate maturation of human dendritic Langerhans cells. J Immunol. 1999;162:4567-4575.
24. Fattorossi A, Battaglia A, Ferrandina G, et a1. Lymphocyte composition of tumor draining lymph nodes from cervical and endometrial cancer patients. Gynecol Onco1. 2004;92:106-115.
25. Rouard H, Leon A, Klonjkowski B, et al. Adenoviral transduction of human 'clinical grade' immature dendritic cells enhances costimulatory molecule expression and T-cell stimulatory capacity. J Immunol Methods. 2000;241:69-81.
26. Kimura H, Dobrenkov K, Iida T, et al. Tumor-draining lymph nodes of primary lung cancer patients: a potent source of tumor-specific killer cells and dendritic cells. Anticancer Res. 2005;25:85-94.
27. Vuylsteke RJ, van Leeuwen PA, Meijer S, et al. Sampling tumor-draining lymph nodes for phenotypic and functional analysis of dendritic cells and T cells. Am J Pathol. 2002; 161:19-26.
28. Caux C, Liu YJ, Banchereau J. Recent advances in the study of dendritic cells and follicular dendritic cells. Immunol Today. 1995;16:2-4.
29. Nakayama H, Kitayama J, Muto T, Nagawa H. Characterization of intercellular cytokine profile of $CD4^+$ T cells in peripheral blood and tumor-draining lymph node of patients with gastrointestinal cancer. Jpn J Clin Oncol. 2000;30:301-305.