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Original Article

Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on human non-small cell lung cancer through EGFR signaling pathway

Bin Wang, Enhai Cui, Huadong Lu, Xiaoyong Li

Department of Respiratory Medicine, Central Hospital of Huzhou, Huzhou, Zhejiang Province, China

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Abstract: The death rates of lung cancer have ranked first in the world. Many patients are at advanced stage when diagnosed; therefore, they miss optimal operative period. And some patients cannot endure radiotherapy or chemotherapy. The present study investigated the antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on human non small cell lung cancer (NSCLC) and the potential underlying mechanisms. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells suppressed the cell proliferation, increased the cytotoxicity, induced the apoptosis and promoted caspase-3/9 activities in NSCLC. Meanwhile, the effects of cocultured dendritic cells and cytokine-induced killer cells suppressed the protein expression of TGF-β, inhibited IL-4 activity, enhanced IL-10 and PGE-2 activities and activated the protein expression of IFN-γ and suppressed EGFR protein expression of NSCLC. This study provides a theoretical and experimental basis for clinical immunotherapy using the effect of cocultured dendritic cells and cytokine-induced killer cells on NSCLC through activating of caspases, IL-10 activity, PGE-2, IFN-γ and EGFR expression, and suppression of IL-4 activity and TGF-β protein expression.

Keyword: Cocultured dendritic cells, cytokine-induced killer cells, human non small cell lung cancer

Introduction

With increasing morbidity and fatality rates year by year, lung cancer is the major cause of malignant cancer death in China [1]. It can be classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2]. The latter form occupies about 75% and patients of this form are generally advanced when diagnosed and accompanied by malignant pleural effusion [3]. Traditional therapeutic methods have some limitations [4]. For patients with NSCLC, only 25%-30% can be administrated with operative therapy with low survival rates [5]. Radiotherapy, by using high-power electron beams such as γ and χ to destroy chromosome, is an essential therapy for tumor. It would stop cellular growth and has lethal effects to tumor cells [6]. However, it would cause various side reactions such as radiodermatitis, radiation esophagitis, loss of appetite, vomiting and stomachache [7]. As patients are not endurable, therapeutic effects are reduced. For routine chemotherapy, it is to kill tumor cells through cellular toxic effects of drugs. Meanwhile, normal cells would be destroyed [8]. It is studied that therapeutic failure would occur because of drug resistance owing to the existence of genes such as multidrug resistance gene, lung resistance related protein and glutathione S-transferaseπ [7]. Only one third of NSCLC patients can benefit from chemotherapy [9].

Bio-chemotherapy is a new comprehensive therapy model by combing bio-therapy with chemotherapy, which becomes more and more important in treating malignant tumor [10]. Biochemotherapy can kill tumor cells as well as reduce toxic and side effects caused by chemotherapeutics [11]. In spite of these achievements, methods and sequence of combined application of bio-therapy and chemotherapy are in need of further study [11].

As an immuno-biological cancer therapy, cytokine-induced killer cells (CIK) induced by dendritic cells (DC) and cytokines has been widely applied to clinic treatment [12]. Its combination with chemical treatment has achieved certain

effects. DC and CIK applied to clinic treatment are acquired by in vitro culture of precursor cells extracted from peripheral blood and bone barrow [13]. In 1997, Gjomarkaj et al. reported that they found DC in malignant pleural effusion and later studies proved that mature DC can be induced and generated by malignant pleural effusion [14].

In recent years, molecular targeting treatment has gained increasing attention and widely applied to clinic. As a member of epidermal growth factor receptor family, EGFR is closely related with occurrence and progression of tumor and participates in the regulation of tumor cell growth, proliferation, vasculogenesis and diffusion [15]. By now, EGFR inhibitors have become an important target spot of molecular targeting treatment.

Materials and methods

Culture and amplification of CIK cells and DCs in vitro

Human experiments were performed in accordance with the ethical standards provided by the responsible committee of the institution and in accordance with the Central Hospital of Huzhou. Healthy blood donors were collected from Healthy volunteer in Central Hospital of Huzhou, which was used to collect peripheral blood. Mononuclear cells were separated by centrifugation using a lymphocyte separating medium (Sigma-Aldrich), which incubated at 37°C in 5% CO_2 for 2 h in a humidified incubator. Non-adherent cells (3×10^6/mL) were collected and cultivated with RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in 5% CO_2. 10 U/mL of rhIFN-γ was added to the culture and incubated at 37°C in 5% CO_2 for 24 h. Then, the medium was replaced with fresh medium comprising CD3 monoclonal antibody (50 ng/mL, R&D Systems, Minneapolis, MN, USA) and rhIL-2 (300 U/mL, R&D Systems, Minneapolis, MN, USA) every 3 days. Meanwhile, adherent cells (1×10^6/mL) was incubated with RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with rhGM-CSF (1000 U/mL, R&D Systems, Minneapolis, MN, USA) and rhIL4 (500 U/mL, R&D Systems, Minneapolis, MN, USA).

DC-CIK cells coculture and human non-small cell lung cancer cell culture

DCs were co-cultured with CIKs at a ratio of 1:5 with CIK cell culture medium and incubated with fresh medium comprising CD3 monoclonal antibody (50 ng/mL, R&D Systems, Minneapolis, MN, USA) and rhIL-2 (300 U/mL, R&D Systems, Minneapolis, MN, USA) every 3 days at 37°C in 5% CO_2. Human non small cell lung cancer A549 cell line were cultured with RPMI1640 medium comprising 10% FBS in a humidified incubator at 37°C with 5% CO_2 atmosphere.

Analysis of cell proliferation and cytotoxicity

DC-CIK cells and A549 cell were inoculated onto the culture plate at ratios of 2.5:1, 5:1, 10:1, and 20:1, and were placed at 37°C in 5% CO_2 for 48 h. 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen Life Technologies, Carlsbad, CA, USA) was added to each well and incubated for 4 h. Then, 100 μL dimethyl sulfoxide (DMSO, Invitrogen Life Technologies, Carlsbad, CA, USA) was added to each sample and subsequently stirring sufficiently. Cell proliferation was measured using enzyme mark instrument at 490 nm. Meanwhile, maximal release of LDH (Invitrogen Life Technologies, Carlsbad, CA, USA) was performed to analyze cytotoxicity using enzyme mark instrument at 570 nm.

Analysis of apoptosis

DC-CIK cells and A549 cell were inoculated onto the culture plate at ratios of 2.5:1, 5:1, 10:1, and 20:1, and were placed at 37°C in 5% CO_2 for 48 h. Coculture cell was collected by centrifugation and washed twice with ice-cold PBS and were then resuspended with 200 μL of binding buffer (KeyGEN BioTECH, Nanjing, China). Then, Coculture cell was incubated with 5 μL each of Annexin V-FITC for 30 min in the dark, at room temperature, and then incubated with propidium iodide (PI) for 15 min in the dark, at room temperature. Apoptosis rate was analyzed immediately after staining, using a FACScan flow cytometer (Becton-Dickinson).

Analysis of caspase-3, caspase-9, IL-4, IL-10 and PGE-2 activities

DC-CIK cells and A549 cell were inoculated onto the culture plate at ratios of 2.5:1, 5:1,

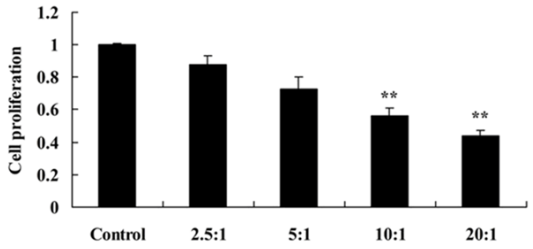

Figure 1. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on cell proliferation of A549 cell. **P<0.05 compared with control day.

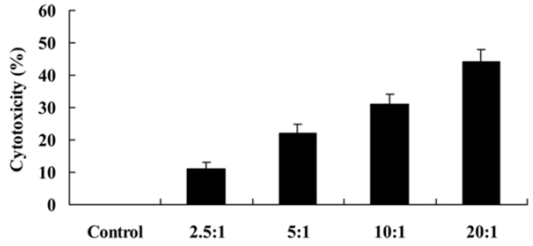

Figure 2. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on cytotoxicity of A549 cell.

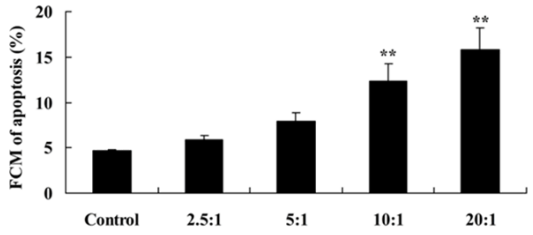

Figure 3. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on apoptosis of A549 cell. **P<0.05 compared with control day.

10:1, and 20:1, and were placed at 37°C in 5% CO_2 for 48 h. Caspase-3, caspase-9, IL-4 and IL-10 activities was analyzed using ELISA assay kits, in according with the manufacturer's protocol.

Western blot analysis

DC-CIK cells and A549 cell were inoculated onto the culture plate at ratios of 2.5:1, 5:1, 10:1, and 20:1, and were placed at 37°C in 5% CO_2 for 48 h. Coculture cell was washed twice with PBS and lysed in ice-cold lysis buffer for 30 min with occasional rocking. Supernatant was collected at 12,000 rpm, for 5 min at 4°C and determined using protein concentration using the BCA kit (Cwbiotech, Beijing, China). Protein (60 μg) was separated on 10-12% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked using 5% non-fat milk in TBST for 2 h at room temperature, and incubated overnight at 4°C with anti-IFN-γ (1:3000, Cell Signaling, Beverly, MA, USA), anti-TGF-β (1:4000, Cell Signaling, Beverly, MA, USA), anti-EGFR (1:4000, Cell Signaling, Beverly, MA, USA) and β-actin (1:5000, Beyotime, Shanghai, China). The membrane was washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:4000, Cell Signaling, Beverly, MA, USA) for 1 h at room temperature.

Statistical analysis

Values are expressed as the mean ± standard error of the mean. And the original data were tested via homogeneity of variance, and then used for t-test and variance analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Antitumor effects of DC-CIK cells on cell proliferation of A549 cell

A549 cell added by DC-CIK cells was analyzed the cell proliferation of A549 cell using MTT assay. At 48 h after co-cultured, the antitumor effects of DC-CIK cells suppressed the cell proliferation of A549 cell in dose-dependence (**Figure 1**). However, DC-CIK cells: A549 cell was 10:1 or 20:1 significantly inhibited cell proliferation of A549 cell (**Figure 1**).

Antitumor effects of DC-CIK cells on cytotoxicity of A549 cell

To explore the antitumor effects of DC-CIK cells on cytotoxicity of A549 cell, cytotoxicity was measured using LDH assay. When cultured for 2 days, we found that cytotoxicity of A549 cell was enhanced in dose-dependence (**Figure 2**).

Antitumor effects of DC-CIK cells on apoptosis of A549 cell

When A549 cell were co-cultured with DC-CIK cells, we examined the antitumor effects of DC-CIK cells on apoptosis of A549 cell. As showed in **Figure 3**, the apoptosis rate of A549 cell was activated by co-cultured with DC-CIK cells (10:1 or 20:1).

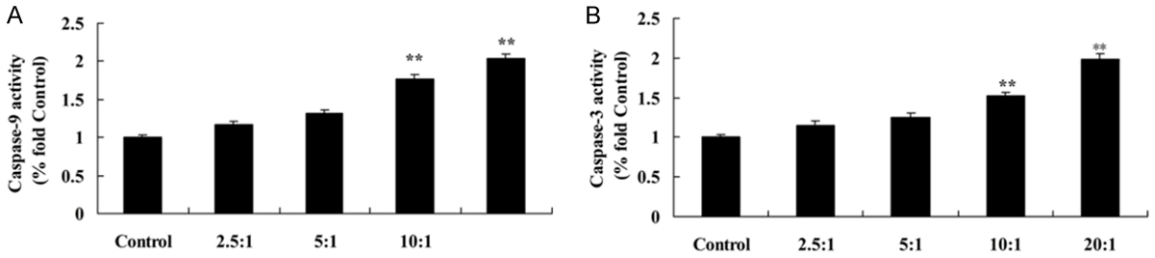

Figure 4. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on caspase-3 and caspase-9 activities of A549 cell. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on caspase-3 (A) and caspase-9 (B) activities of A549 cell. **P<0.05 compared with control day.

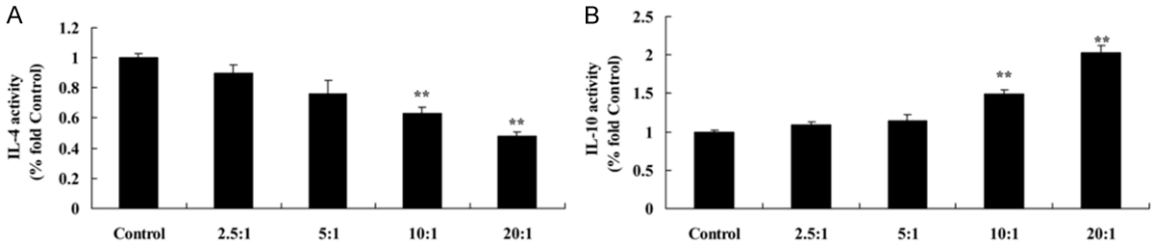

Figure 5. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on IL-4 and IL-10 activities of A549 cell. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on IL-4 (A) and IL-10 (B) activities of A549 cell. **P<0.05 compared with control day.

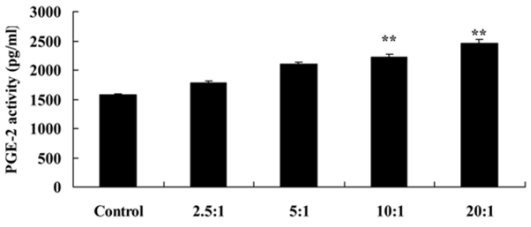

Figure 6. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on PGE-2 activity of A549 cell. **P<0.05 compared with control day.

Antitumor effects of DC-CIK cells on caspase-3 and caspase-9 activities of A549 cell

To inspect the antitumor effects of DC-CIK cells on apoptotic mechanism of A549 cell, caspase-3 and caspase-9 activities of A549 cell were detected using apoptotic ELIST kits. As showed in **Figure 4**, 10:1 or 20:1 of DC-CIK cells significantly increased the caspase-3 and caspase-9 activities of A549 cell.

Antitumor effects of DC-CIK cells on IL-4 and IL-10 activities of A549 cell

To inspect the apoptotic mechanism of DC-CIK cells on A549 cell, IL-4 and IL-10 activities were detected as an important index. As showed in **Figure 5**, IL-4 activity was significantly decreased and IL-10 activity significantly increased by 10:1 or 20:1 of DC-CIK cells in A549 cell.

Antitumor effects of DC-CIK cells on PGE-2 activity of A549 cell

We examined the antitumor effects of DC-CIK cells on PGE-2 activity of A549 cell using ELIST Kits. As showed in **Figure 6**, PGE-2 activity of A549 cell was significantly promoted by 10:1 or 20:1 of DC-CIK cells.

Antitumor effects of DC-CIK cells on IFN-γ protein of A549 cell

To explore the antitumor effects of DC-CIK cells on IFN-γ protein expression of A549 cell, IFN-γ protein expression was measured using Western blot analysis. **Figure 7** from Western blot analysis showed that there is significant increase IFN-γ protein expression of 10:1 or 20:1 of DC-CIK cells group in comparison with control group.

Antitumor effects of DC-CIK cells on EGFR protein of A549 cell

To further explore the antitumor effects of DC-CIK cells on EGFR protein expression of

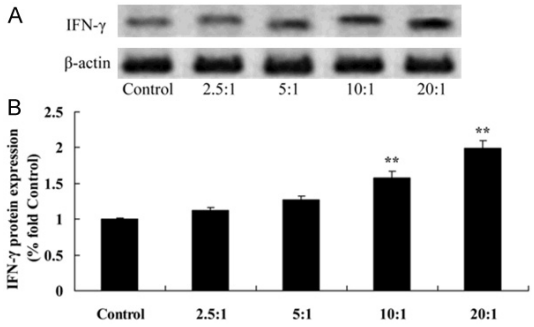

Figure 7. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on IFN-γ protein of A549 cell. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on IFN-γ protein using western blotting analysis and statistical analysis of IFN-γ protein of A549 cell. **P<0.05 compared with control day.

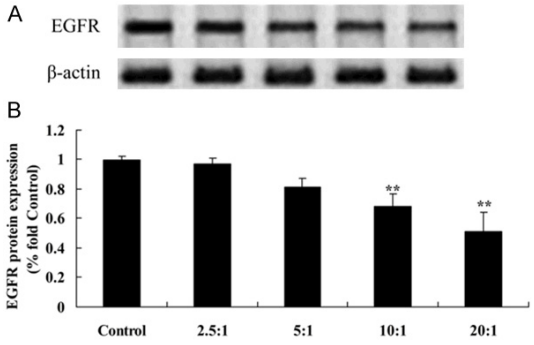

Figure 8. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on EGFR protein of A549 cell. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on IFN-γ protein using western blotting analysis and statistical analysis of EGFR protein of A549 cell. **P<0.05 compared with control day.

A549 cell, EGFR protein expression was measured using Western blot analysis. These results from Western blot analysis showed that EGFR protein expression of A549 cell was significantly suppressed by 10:1 or 20:1 of DC-CIK cells (**Figure 8**).

Antitumor effects of DC-CIK cells on TGF-β protein of A549 cell

Next, we explored the antitumor effects of DC-CIK cells on TGF-β protein expression of A549 cell using Western blot analysis. As showed in **Figure 9**, 10:1 or 20:1 of DC-CIK cells significantly suppressed the protein expression of TGF-β protein expression in A549 cell.

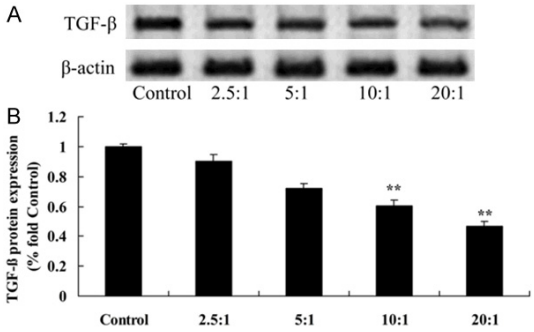

Figure 9. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on TGF-β protein of A549 cell. Antitumor effects of cocultured dendritic cells and cytokine-induced killer cells on IFN-γ protein using western blotting analysis and statistical analysis of TGF-β protein of A549 cell. **P<0.05 compared with control day.

Discussion

Lung cancer is the most common malignant cancer ranking first in the world. Statistics showed that new lung cancer cases in 2002 reached about 1.35 million with 1.18 million of death cases [16]. Most of patients with lung cancer have reached middle and advanced stage accompanied by malignant pleural effusion [17]. With further development of lung cancer, therapeutic methods have been improved. 1980s, Oldham proposed the theory of biological response modifier which suggested that the occurrence and progression of tumor was caused by unbalance of defensive system and tumor cells [18]. The present study identified the antitumor effects of DC-CIK cells suppressed cell growth and increased cytotoxicity of human non-small cell lung cancer A549 cell.

As a form of biotherapy, adoptive cellular immunotherapy is a fundamental cancer therapy to strengthen immunologic functions by input of effector cells with anti-cancer activities [19]. According to inducing methods and sources, it can be grouped as tumor infiltrating lymphocyte, lymphokine-activated killer cells, anti-CD3+ monoclonal antibody activated killer cells, CIK and DC [20]. The latter two have become hot spots for present research. Furthermore, we found that identified the antitumor effects of DC-CIK cells induced apoptosis and promoted caspase-3/9 activities of human non small cell lung cancer A549 cell. Wang et al. indicated that DCs- glypican 3 gene

-CIKs significant induced tumor growth inhibition of HepG2 cell in nude mice [21].

DC was first discovered by Steinman and Cohn in 1973. It is antigen-presenting cell with strongest functions. It is named because of the dendritic protuberance. It is induced by stimulation of antigen [22]. Under the stimulation of microorganism and inflammatory factors as well as the combined action of cytokines, DC precursor cells such as CD34+, CD14+ and peripheral blood CD14+ can differentiate into mature DC with strong phagocytosis and processing ability of antigens [23]. Under the influence of antigens and inflammatory factors, it starts to transfer into peripheral lymphoid organs [24]. During the metastasis process, DC is becoming more and more mature. In this study, we found that DC-CIK cells significantly promoted PGE-2 activity of human non small cell lung cancer A549 cell. Tateosian et al. identify dendritic cells promote the generation of CD4(+) FOXP3(+) regulatory T cells through TGF-β in vitro [25].

In 1991, Schmidt-Wolf in Stanford University gave a classic definition that peripheral blood mononuclear cells were a kind of specific cell groups cultured by stimulation of in vitro cytokines [26]. They have the characteristics of anti-tumor activity of T lymphocyte and limited tumor-cytotoxicity of NK cells [27]. Their main effector cells are CD3+CD56+ cells, which can secrete various cytokines including IL-4 and IFN-γ, characterized by rapid proliferation, strong tumor-killing effects and wide anti-tumor spectra [27]. The results of this study revealed that DC-CIK cells significantly decreased IL-4 activity, increased IL-10 activity and induced IFN-γ protein expression of human non small cell lung cancer A549 cell. Shekhar et al. demonstrate that lung dendritic cell mediated Th1/Th17 immunity through IL-4 and IFN-γ during intracellular bacterial infection [28].

It was reported that as DC has functional defects, thus it cannot give full play to its antitumor effects [29]. Numbers of DC is negative correlated with surrounding invasion. DC can prevent from tumor metastasis and relapse [30]. Therefore, improvement of its sources and culture technique is an issue urgently needed. In the present study, DC-CIK cells significantly suppressed the protein expression of TGF-β protein expression in A549 cell.

EGFR is possibly related with the formation of non-small cell lung cancer [31]. Therefore, it was predicted that EGFR inhibitors may be effective to non-small cell lung cancer. EGFR inhibitors can be grouped into EGFR exhibitor and tyrosine kinase inhibitor [31]. Further studies found that the effective rates of EGFR inhibitors for non-small cell lung cancer were about 20% [15]. Finally, we found that DC-CIK cells significantly suppressed EGFR protein expression of A549 cell. Hayashi et al. reported that DC-CIK cells inhibit IL-4 and activated EGFR in tumor cells [32].

In conclusion, the results demonstrate that, the antitumor effects of DC-CIK cells suppressed cell growth, increased cytotoxicity, and induced apoptosis of human non small cell lung cancer through activating of caspases, IL-10 activity, PGE-2, IFN-γ and EGFR expressions, and suppression of IL-4 activity and TGF-β protein expressions, which provides a experimental basis for the application of DC-CIK cells co-cultured to the immunotherapy of human non small cell lung cancer in clinical applications.

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Disclosure of conflict of interest

None.

Address correspondence to: Enhai Cui, Department of Respiratory Medicine, Central Hospital of Huzhou, Huzhou 313000, Zhejiang Province, China. Tel: +86-572-2555775; E-mail: chizon484@yeah.net

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