RESEARCH ARTICLE

Antitumor Activity of Lentivirus-mediated Interleukin -12 Gene Modified Dendritic Cells in Human Lung Cancer *in Vitro*

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Abstract

Objectives: Dendritic cell (DC)-based tumor immunotherapy needs an immunogenic tumor associated antigen (TAA) and an effective approach for its presentation to lymphocytes. In this study we explored whether transduction of DCs with lentiviruses (LVs) expressing the human interleukin-12 gene could stimulate antigen-specific cytotoxic T cells (CTLs) against human lung cancer cells *in vitro*. Methods: Peripheral blood monocyte-derived DCs were transduced with a lentiviral vector encoding human IL-12 gene (LV-12). The anticipated target of the human IL-12 gene was detected by RT-PCR. The concentration of IL-12 in the culture supernatant of DCs was measured by ELISA.Transduction efficiencies and CD83 phenotypes of DCs were assessed by flow cytometry. DCs were pulsed with tumor antigen of lung cancer cells (DC+Ag) and transduced with LV-12 (DC-LV-12+Ag). Stimulation of T lymphocyte proliferation by DCs and activation of cytotoxic T-lymphocytes (CTL) stimulated by LV-12 transduced DCs pulsed with tumor antigen against A549 lung cancer cells were assessed with methyl thiazolyltetrazolium (MTT). Results: A recombinant lentivirus expressing the IL-12 gene was successfully constructed. DC transduced with LV-12 produced higher levels of IL-12 and expressed higher levels of CD83 than non-transduced. The DC modified by interleukin -12 gene and pulsed with tumor antigen demonstrated good stimulation of lymphocyte proliferation, induction of antigen-specific cytotoxic T lymphocytes and anti-tumor effects. Conclusions: Dendritic cells transduced with a lentivirus-mediated interleukin-12 gene have an enhanced ability to kill lung cancer cells through promoting T lymphocyte proliferation and cytotoxicity.

Keywords: Dendritic cell - interleukin-12 - lung cancer cells - lentivirus vector - T lymphocytes

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Introduction

Dendritic cells are powerful antigen –presenting cells that have been under intensive investigation as components of tumor vaccines (Lesterhuis et al., 2004). They play a vital role in the initiation of immune response by presenting exogenous and endogenous antigens to T-cells via major histocompatibility complex molecules (Ragde et al., 2004). Following induction of the T-cell response, the activated T cells can differentiate into cytotoxic T lymphocytes (Song et al., 2012). CTL are the major killers of tumor cells, they achieve the killing with the help of CD4+ T cells, which cans induce potential long-termCD8+ T-cell responses by producing various cytokines. DCs network system became an attractive approach for research in cancer therapy (Janeway and Medzhitov, 2002). DCs loaded with acid-eluted tumor lysate (Cranmer et al., 2004; Garg et al., 2013) or RNA (Song et al., 2012) are also effective in inducing immunity against tumors for which tumor specific peptides have yet to be identified. Studies have demonstrated that DCs pulsed with tumor associated antigen produce significant therapeutic immunity to tumors with low toxicity (Kaech and Ahmed.2003). Efficient gene delivery to human DC has been achieved with a variety of viral vectors, such as adeno-associated virus, adenovirus, onco-retrovirus and lentivirus (Dullaers et al., 2006). lentiviral vectors they do not express viral proteins and have been proven to transduce dividing and non dividing cells in vitro and in vivo. As well as showing efficient gene transfer and sustained long-term expression for the trans gene. Therefore the lentiviral vector delivery system offers considerable promise for gene therapy on cancer (Picanco-Castro et al., 2012; Vannucci et al., 2013). DCs can be generated from multiple cell types using several tissue culture conditions.From peripheral blood, both circulating CD34 hematopoietic progenitor cells and CD14 adherent monocytes have been found to give rise to DCs in presence of granulocyte-macrophage colony-stimulating factor and interleukin-4 (Garg et al., 2013; Hwang et al., 2013). Mature DCs are distinguished by their dendritic oblique morphology and high expression levels of MHC clas1 and class11 as well as CD40/CD80/CD83/CD86 and ICAM-1 (Hansen et al., 2013). IL-12 is a heterodimeric cytokines, produced by monocytes, macrophages, neutrophil and dendritic cells, play central role for the interaction of DC

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and T. cells and has been shown to exert potent immune stimulatory affects on certain helper T cells as well as on cytotoxic T lymphocytes and natural killer cells (Li et al., 2012). It enhances the generation of CTL specific for viral antigens and allo antigens both in vitro and in vivo (Hansen et al., 2013). IL-12 supports the proliferation, differentiation of activated CD8 T cells into CTL effectors and stimulates the cytolytic activity of fully differentiated CTL. While it does not induce the proliferation of resting T cells but can support the growth of T cells that have been activated by mitogens or specific antigens (Li et al., 2012; Hansen et al., 2013). The main biological activity of IL-12 is on cytotoxic T lymphocytes and natural killer cells which are respectively two main effectors of tumor lysis by the innate and adaptive immune response (Gherardi et al., 2000)Viral vectors for IL-12 have consistently high levels of gene transfer efficiency, expression and transfection efficiencies (Vannucci et al., 2013). In this study we constructed a lentiviral vector encoding the IL-12 gene, which was used to infect DCs to stimulate a DC vaccine. Our results showed that the lenti-IL-12 Vector can effectively transfer the IL-12 gene to DCs and can be expressed in DCs. Moreover, the DC vaccine can activate T-cells and shows a noticeable anti-tumor activity.

Materials and Methods

Reagents

Recombinant human granulocyte-macrophage colony stimulation factor (rhGM-CSF) and recombinant human interleukin-4 (rhIL-4) were purchased from peprotech company (USA). Human sandwich ELISA IL-12kit was purchased from senxiong biotech Company (shanghai-china). Attractene transfection reagent was purchased from QIAGEN Company (Germany). Monoclonal antibodies specific for CD83 was purchased from BD Company (USA) Trizol reagent and PrimeScript™ II 1st Strand cDNA Synthesis Kit and PCR mix were purchased from Takara company (Japan). 4-5methyl thiazolyl tetrazolium (MTT) was purchased from sigma company (USA) DMEM, IMEM medium and fetal bovine serum (FBS) were purchased from Invitrogen Company (USA).

Cell lines

Human lung cancer cell line A549 and 293FT cells were provided by department of central laboratory of China-Japan Union Hospital in Jilin University. The cells were cultured in DMEM media supplemented with 10% fetal bovine serum and 100 units of penicillin, extra poly-lysine, 0.1 mM Non-Essential Amino Acids (NEAA), 2 mML-glutamine and 1Mm Sodium Pyruvate were added to 293FT cells and maintained at 37°C in 5 % CO_2 humidified atmosphere.

Lentivirus vector construction:

The human IL-12 gene including p35 and p40 fragments was previously constructed in our lab and transformed into pcDNA3.1 (+)-IL-12 (p35 and p40). We chose the1640 bp as target DNA fragment. The primers containing Mlu I and Spe I sites for PCR were as follows, forward primer 5’AAATATGCGGCCGCTAAGCCACCATGGGTCAC 3; reverse primer: 5’CCGCTCGAGCGTTAGGAAGCATT CAGATAG 3 (Biological Engineering Company-Shanghai-china). PCR was followed by 30 cycles at 95°C for 5 min, 94°C for 40 seconds, 56 °C for 45 seconds and 72°C for 2 min with a final round for 10 min. The PCR product was subjected to 1% agarose gel electrophoresis. The PCR product of IL-12 gene was ligated with pMD18-T vector to form recombinant pMD18-IL-12-MS.This and lentivirus vector PWPXLd were digested respectively by MLuI x SpeI restriction endonuclease. The digestion products were subjected to 1% agarose gel electrophoresis, visualized under UV light, the cut fragments of IL-12 and linear pWPXLd vectors fragments were extracted and ligated to form PWPXLd-IL-12 vector by T4 DNA ligase.

Lentiviral vectors package

293FT cells counting 1 × 10^6/well were incubated into 6-well plates at 37°C with 5% CO_2 in humidified atmosphere. After growth to 70-80% confluence they were transduced with lentiviral transfer vectors pWPXLd, psPAX2 and pMD2.G as a group named LV-GFP and pWPXLd-IL-12, psPAX2 with pMD2.G as a group named LV-12 by using Attractene transfection reagent following the manufacturer’s protocol. The supernatant was collected at 24 and 48 hours post transfection by centrifugation (at 1250 rpm/min for 5min). The cell pellet was resuspended with 2ml serum-free medium. Both groups were diluted 1:10, 1:100 and 1:1000 times to determine virus concentration and stored at -80°C for further use.

Transduction of 293FT cell

In order to determine the infection titre of LV-GFP and LV-12, human 293FT cells were transduced by transient-infection with 1:10, 1:100 and 1:1000 dilutions of lentiviral particles. Briefly cells counting (1 × 10^6 cells/well) before being inoculated were cultured in 6-well plates with 2ml of poly-lysine (10 μg/ml) at 37°C and removed after 4 hours by washing with PBS thrice. On day 2, the infected cells expressing GFP were counted by flow cytometry.

Identification of human IL-12 expression by RT-PCR in 293FT cells

48 hours post transduction of 293FT cells with LV-12 total RNA was extracted using Trizol reagent and converted into cDNA by using reverse transcriptase kit following the manufacturer’s protocol. Quantitative real-time polymerase chain reaction assays were carried out using SYBR Green Super Mix in a 20 μl reaction volume using human IL-12 gene primer. Forward primer was 5’AAATATGCGGCCGCTAAGCCACCATGGGTCAC 3; and reverse primer was : 5’CCGCTCGAGCGTTAGG AAGCATTCAGATAG 3 (Biological Engineering company- shanghai-china) and β-actin .The forward and reverse primer for β-actin were as follows 5’AGTGCGA CGTGGACATCCG 3’and 5’TGGCTCTAACAGTCCG CCTAG3’ respectively and run in a 7500 Real –Time PCR system (Applied Bio system) at 95°C for 5 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 45 seconds and 72°C for 1 minute with final round at 72°C

for 10 min. The expression of 1L-12gene was determined by normalization of the threshold cycle of this gene to that of the control β-actin. The RT-PCR products were subjected to 1% agarose gel electrophoresis containing ethidium bromide.

Generation and transduction of dendritic cell

DCs were generated from monocyte that was isolated from peripheral blood by Ficoll-Hypaque density gradient centrifugation and cultured for 4 days in 10% FCS-IMDM containing 200ng/ml rhGM-CSF and rhIL-4 (50 ng/ml) at 37°C, 5 % CO_2 (Schuler and Romani, 1997). Suspended cells were seeded in 24 –well plate at a concentration of 1×10^6 cells/1ml medium per well containing rhGM-CSF and rhIL-4 and divided into three groups LV-GFP, LV-12 and DCs, 20 μl 10-fold dilution of the virus was transduced to the first two groups'. DCs group plus medium was considered as negative control. Each group contained replicate. The same amount of virus was added on the six day. Cells were harvested on 7, 10 and 14 days from each group by centrifugation at 3000rpm/20 min and stored at -70°C. The morphology of DCs was observed under an inverted microscope.

Treatment of DCs

DCs were pulsed with tumor antigen (DC+Ag). This was prepared by treated human A549 lung cancer cells with the drugs cisplatin, etoposide (12 μg/ml, 60 μg/ml) respectively. After 24 hours the morphology of cells was observed, the necrotic cells were collected and used as antigen. The experimental design was divided into four groups, DC+Ag, DC+Ag and LV-12, DCs transduced with LV-12 and DCs alone were used as control group. The LV-12 virus was added at the 4th and 6th day of DCs culture, human A549 lung cancer cells antigen were mixed with DCs after 24 hours post virus transduction .The next day TNF-α (10ng/ml) was added and continued culture for 48 h, the suspended cells were collected as mature DCs .

Concenteration of human IL-12

The concentrations of IL-12 (after refer to IL-12p70) secreted from dendritic cells was detected by human sandwich ELISA IL-12kit (IL-12p70). While the concentration of IL-12 in 293FT was measured on10 days post transduction with 10 dilution of LV-12.

Flow cytometry analysis

The DCs surface molecule CD83 was detected in LV-GFP and LV-12 transduced DCs group at 7 days post transduction. Briefly DCs cells were washed 2 times with PBS and centrifuged at 1200 rpm/min for 5 min. The supernatant was diluted with 200μl PBS. Cells counting (1×10^6) were incubated with monoclonal antibody CD83-PE marker at 4C in the dark for 30 minutes .Flow cytometric analysis was performed on the FAC scan flowcytometer (Beckman) program. PBS was used as negative control. Enhance green fluorescent protein expression was detected on 48 hours post transduction of 293FT and DC cells with different concentrations of LV-GFP.

Isolation and culture of T-lymphocytes

T-lymphocytes were isolated from peripheral blood (donated by Changchun city blood bank-china) by Ficoll-Hypaque density gradient centrifugation. 1x106 cells were seeded in 24 –well plate and incubated at 37°C, 5 % CO_2 in the presence of phytohemagglutine (45 ug/ml) for two days (Fuss et al., 2009).

T- Lymphocytes proliferation assay

Dendritic and dendritic cells transduced with LV-12 were incubated for one hour at 37°C in complete medium with mitomycin (50 ug/ml) followed by centrifugation and washed thrice in PBS. Serial dilutions of DCs were mixed with a constant number (1×10^4) of lymphocyte in 96-well culture plate at stimulator to reactor (S: R) ratios of 1:5, 1:10 and 1:20 at the same time, T cells alone were used as controls and were incubated at 5% CO_2, 37°C. Each assay was conducted in triplicate. After 5 days, the cells were treated with 10 μl MTT (5 mg /ml). The solubilized Formosan products were quantified for absorbance at a wave length 490 nm (Gately et al., 1991; Mehrotra et al., 1993).

T-lymphocytes cytotoxicity assays

Cytotoxicity of activated T-lymphocytes was assessed by MTT (Gately et al., 1991; Mehrotra et al., 1993). In brief experimental wells containing A549 lung cancer cells adjusted to a concentration of 1×10^4 cells per well were seeded in 96-well culture plate after 24 hours the cells were treated with graded concentrations of stimulated lymphocytes. The ratios of effectors to target cells were 100:1, 10:1, 1:1 and 0.1:1. Each condition was replicated in triplet wells, wells containing A549 lung cancer cell were used as control. After 48 hours 10 μl MTT (5 mg/ml) were added and MTT assay were performed as described above. Kill rate activity (%) = (control group optical density value-kill group optical density value/control group optical density value.

Results

Vector construction

The IL-12 DNA fragments was amplified by PCR and inserted into pWPXLd plasmid to form the recombinant pWPXLd-12 plasmid. This was digested by Mlu1 x Spe1 endonuclease. Digestion product was subjected to 1% agarose gel electrophoresis and showed clear fragments near 1600bp, confirming the anticipated size of IL-12DNA fragment. Our all data demonstrated that we had constructed the recombinant pWPXLd-12 successfully. Figure 1A, B

Identification of human IL-12 gene

293FT cells were transduced with lentiviral vectors LV-12 group diluted 10, 100 and 1000 times. Cell genomic DNA was extracted from the different dilutions and amplified by PCR. The results showed no product at 645 at 1000 times dilution while there was product at 100 and 10 times dilution, indicating that there was positive correlations between transduction ability of virus titration

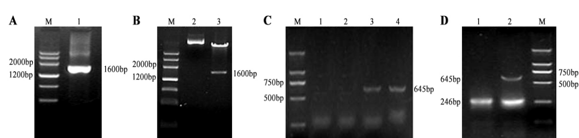

Figure 1. Anticipated Human IL-12 Gene. Fig1-A Lane 1: PCR product of human IL-12 gene. Fig1.B. Lane 2: PWPXLd-IL-12 plasmid, Lane 3: PWPXLd-IL-12 digested by Spe1 and Mlu1. Fig1-C .Detection of hIL-12DNA fragment by PCR in 293FT cells, lane1:293FT Cells, lane 2: IL-12 at 1:1000, lane 3: LV-12 at 1:100, lane 4: at 1:10. Fig1-D.Visualizations of anticipated target of human IL-12 gene in 293FT cells by RT-PCR. Lane 1: 293FT cells, Lane2: 293FT cells transduced with LV-12, Lane M: DNA marker 2000

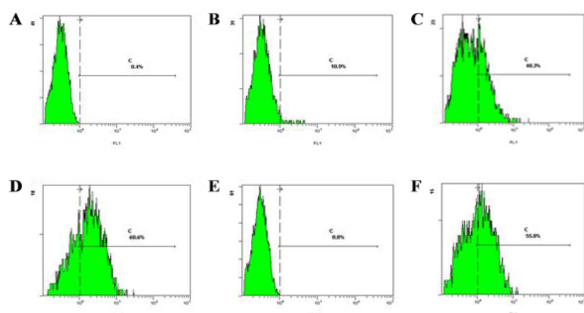

Figure 2. Flow Cytometric Analysis Results of GFP in 293FT and Dendritic Cells Transduced with Different Concentrations of LV-GFP Lentivirus Particles. A: 293FT cell; B: LV-GFP at 1:1000; C: LV-GFP at 1:100; D: LV-GFP at 1:10 , E: dendritic cells , F : dendritic cells transduced with LV-GFP at 10×

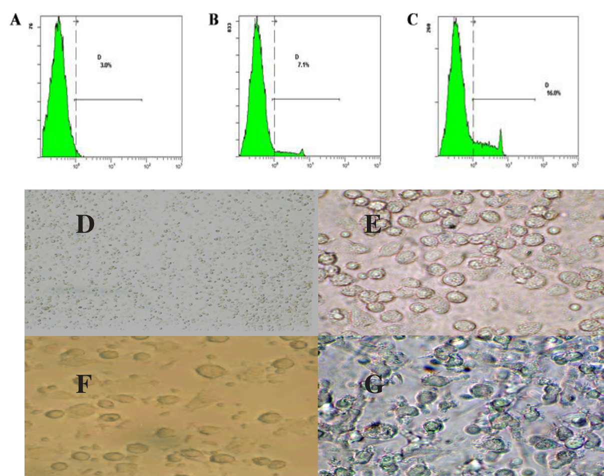

Figure 3. Expression of DCs Surface CD83 Marker by Flow Cytometry and Morphology of DCs during Culture Time(100X) A: DCs , B: DCs transduced with LV-GFP,. C: DCs transduced with LV-12, D: Monocyte, E, F, G; at 5, 7 and 14days respectively

Figure 1C. The mRNA of human IL-12 was detected in transduced and non transduced 293FT cells by RT-PCR, results showed there was expression of human IL-12 in transduced 293FT cells only and B-actin genes were expressed in both transduced and non transduced 293FT cells. Our all results indicate that human IL-12 was well expressed in 293FT cells (Figure 1D).

Flow cytometric analysis results

Flow cytometric analysis showed that no expression of EGFP was seen in non transduced 293FT cells or at 1000 times, while there was an increase in 293FT cells that had been diluted 100 and 10 timesFigure 2-A, B, C, D. For expression of EGFP in DCs, the result showed that about 50% of DCs transduced with 10-fold dilution of the LV-GFP is positive indicating that the concentration of viral particles about 50% (Figure 2E, F). CD83 expression was measured in DCs transduced with LV-GFP and LV-12 groups at 7 days post transduction. The results showed that dendritic cell transduced with LV-12 expressed higher amounts of CD83 than those non- transduced groups (Figure 3A, Table 1) moreover CD83 was measured after 48 hours in DCs loaded with A549 lung cancer cell antigen and LV-12.The results showed that an elevation of CD83 expression in this group (Table 2).

Table 1. Expression of CD83 in DC (±s)* Compared with DCs *p*<0.05

Group	CD83(%)
Dendritic cell	3.6±1.682
Dendritic cell + LV-GFP	6.633±0.416
Dendritic cell +LV-12	17.067±2.386*

Table 2. Expression of DCs Surface Marker CD83 in DCs Transduced with LV-12 and Lung Cancer Lysate (±S)* Compared with DCs *p*<0.05

Group	CD83(%)
Dendritic cell	4.533±0.95
Dendritic cell +A549 cells	6.067±0.378
Dendritic cell +LV-12	29.967±3.075*
Dendritic cell + A549 cells+ LV-12	29.2±2.663*

Table 3. Secretion of IL-12 by 293FT Cell Transduced with LV-12 at 10 Days (±S) * Compared with 293FT *P* <0.05

Group	10d (pg/ml)
293FT	21±0.600 pg/ml
293FT +LV-12	124±10.3 pg/ml*

Observation of DCs morphology

Monocytes were isolated from peripheral blood and incubated for culture. After 3 to 5 days they were observed by an inverted microscope, demonstrating an irregular shape, changed gradually from spindle to round, from adherent to suspended cells with an abundance of cell processes typical for dendritic cell (Figure 3B).

Secretion of IL-12

According to the regression equation of the established standard curve, we found that the IL-12 levels for 10 dilution of LV-12 transduced 293FT cell group after 10 days were 124±10.3 pg/ml and 21±0.600 pg/ml for non-transduced 293FT cell group, there were significant differences between two groups (*P*<0.05) (Table 3). And the IL-12 levels gradually increased in dendritic cell groups that were transduced with LV-12 for 7, 10 and 14 days, while no change in IL-12 was seen in non-transduced dendritic cell or transduced with LV-GFP (Table 4).

100.0
6.3
75.0
56.3
50.0
25.0
31.3
0
Newly diagnosed without treatment

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Table 4. Secretion of IL-12by DCs Transduced with LV-12 or LV-GFP at Different Times* Compared with DCs $p<0.05$; #Compared with LV-GFP $p<0.05$; $Compared with 10d $p<0.05$

Group	7d (pg/ml)	10d (pg/ml)	14d (pg/ml)
Dendritic cell	37.251±1.519	38.652±1.983	41.869±2.124
Dendritic cell +LV-GFP	38.77±1.621	43.876±3.315	43.249±1.852
Dendritic cell +LV-12	39.161±2.271	179.82±41.295*#	260.60±22.264*#$

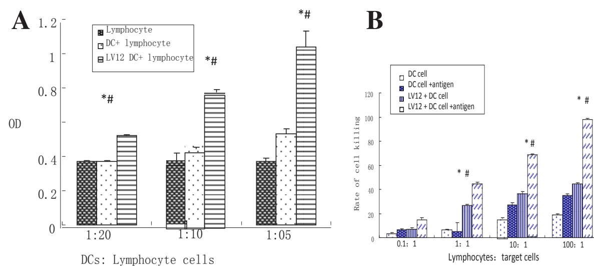

Figure 4. A. Stimulation of lymphopoiesis by DCs transduced with LV-12 *compared with lymphocyte group p<0.05: #compared with DCs group $p<0.05$. B. Effect of stimulated CTL by DCs cell loaded with antigen on inhibition of lung cancer cell *compared with DC+antigen group $p<0.05$: #compared with LV12+DCs group $p<0.05$

Lymphocytes proliferation assay

DCs transduced with LV-12 showed greater increase in their ability to stimulate lymphocyte proliferation than those dendritic cells that had not been transduced with LV-12 as shown in Figure 4A. Furthermore at a 1:05 S/R ratio, displayed strong stimulatory capacities and were statistically significant different ($p<0.05$)

T-lymphocytes cytotoxicity assay

The results demonstrated high response in lymphocyte sensitized by dendritic cell transduced with LV-12, loaded with lung cancer antigen groups and displaying strong stimulatory capacities at a100:1 and 10:1 S/R ratio groups, were significantly different ($P<0.05$) compared with dendritic cell groups (Figure 4B).

Discussion

Lung cancer is the most commonly diagnosed malignancy and the leading cause of mortality among all types of cancers. Every year more than 1 million deaths are contributed by lung cancer worldwide, among which non-small cell lung cancer NSCLC accounts for 85% of cases (Perroud et al., 2011; Koutsokera et al., 2013). Lung tumors appear to have fewer tumor-infiltrating lymphocytes, suggesting that immune response of cancer patients does not properly work to eradicate cancer cells (Perroud et al., 2011). The aim of immunotherapy is to eliminate cancer cells through the activation of host immunity by using biological response modifying polysaccharides, monoclonal antibody and cytokines. (Wang et al., 2012). Interleukin-12 is aheterodimeric cytokine composed of two subunits (p35 and p40) both of which are required for the secretion of the active form of IL-12p70, primarily produced by APCs and exert pleiotropic effects on immune effector cells (Tamandani et al., 2009). Interleukin-12 acts on dendritic cells leading to increased maturation and antigen presentation. It also drives the secretion of IL-12 by DCs, creating appositive feedback mechanism to amplify the response. Once a response stimulated, IL-12 plays an essential role in directing the immune system towards aTh1 cytokine profile, inducing CD4+T cells to secrete IFN- γ and leading to CD8+ cytotoxic T-cell response (Portielje et al., 2003). DCs are essential for stimulating tumor specific cytotoxic T lymphocytes (CTL) and inducing the protective and therapeutic anti-tumor immunity against cancer cells. They have been attempts to produce vaccines based on DCs; including immunizations by DCs pulsed with tumor lysate or apoptotic tumor cells or RNA. DCs have been fused with tumor cells, transduced with peptides to enhance specific anti-tumor immunity (Nouri-Shirazi et al., 2000). Gene therapy provides a new clinic strategy to enhance the immune function of tumor immunity. After activation by stimulated DCs, auxiliary T lymphocytes and CTLs are capable of killing tumors, which is the theoretical basis of tumor vaccines (Wang et al., 2012). DCs based anti tumor immunotherapy have strong ability to stimulate naïve T lymphocytes and induce effective anti- tumor responses (Guermonprez et al., 2002). There were many reports on DC vaccine in the treatment of melanoma, ovarian, colon, breast and gastric cancer (Song et al., 2012). Another study showed that dendritic cells pulsed with lung cancer cell lysate could induce tumor specific immune response and showed promising clinical outcomes in patient with lung cancer (Han et al., 2006). The antitumor activity of recombinant IL-12 was tested in a variety of murine tumor models where it causes tumor regression and prolonged the survival of tumor (Coca et al., 2005). Other clinical studies report that the antitumor effect of IL-12 gene-transduced DC vaccine on melanoma, adenocarcinoma of kidney and neuroglioma (Kikuchi et al., 2001; Marten et al., 2003). Our observation in this work were consistent with other investigations which showed that lentiviral vectors may preserve some advantages compared with other vectors in terms of gene transfer efficiency and long term-expression of the transgene (Seggewiss and Dunbar.2005). In conclusion, the results of our study showed that lentiviral vectors encoding Interleukin-12 can transduce DCs effectively and the level of transgene expression is preserve, promote DCs maturation, secretion of more IL-12. With enhancement of T lymphocyte proliferation and induction of CTL cells to kill lung cancer cells. In addition there are requirements for further investigation in the use of lentiviral vectors encoding Interleukin-12 gene on transduction of DCs in vivo and measuring another mature DCs phenotypes like MHC clas1 and class11 as well as CD40/CD80 /CD86 and ICAM-1.

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