Anti-tumor activity of gene transfer of the membrane-stable CD40L mutant into lung cancer cells

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Abstract. Gene transfer of CD40 ligand (CD40L) holds promise as a novel therapy for lymphoid malignancies and a number of solid carcinomas because of its multiple anti-tumor activities. However, membrane-bound CD40L can be cleaved into a soluble form, sCD40L, which contributes to systemic inflammatory and cardiovascular diseases, and induces survival signals in the absence of protein synthesis block, suggesting a deleterious side effect of CD40L gene therapy. We generated a plasmid encoding non-cleavable human CD40L mutant (pcDNA3.1$^+$-CD40L-M) to determine the direct anti-proliferative and pro-apoptotic effects in CD40-positive lung adenocarcinoma cell line A549, to verify activation of immature dentritic cells (DCs) by co-cultivation with the transfected A549 cells and to evaluate the lower expression of sCD40L relative to that of wild-type CD40L (CD40L-WT) transfectant in cell-free supernatants. These studies suggest that gene transfer of the membrane-stable CD40L mutant into CD40-positive cells may provide an efficient and safe method to treat non-small cell lung cancer.

Introduction

Lung cancer remains one of the most prevalent and deadly cancers worldwide despite improvements in diagnostic and therapeutic techniques (1). About 85% of lung cancers are non-small cell lung cancer (NSCLC), and only 15% of people diagnosed with NSCLC survive this disease after 5 years. Current therapeutic strategies of chemotherapy, radiation therapy and cancer-selective biotherapy with the small targeted molecules have only demonstrated, at best, extension in survival by a few months (2). Thus, novel therapeutic strategies are urgently needed to improve the management of this devastating disease.

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Gene therapy approaches have been considered to be the next horizon toward developing a cure for NSCLC. The use of gene therapy with immunostimulatory molecules aiming at prompting the immune system to target and eradicate malignant cells in the host has emerged as novel therapeutic interventions (3). CD40 ligand (CD40L), a type I membrane-bound protein belonging to the tumor necrosis factor (TNF) superfamily, was exploited for cancer therapy by virtue of its ability to stimulate the host anti-tumor immune response, normalize the tumor microenvironment and directly suppress the growth of CD40-positive tumors, which has attracted significant attention among immunologists and oncologists (4). Clinical studies of CD40L gene therapy have been demonstrated in patients with B-cell malignancies (5,6) and multiple solid cancers (7,8). CD40L-related treatment approaches including combination therapies with conventional (e.g. 5-FU) or novel modalities (e.g. oncolytic viruses) also have displayed enhanced efficacy in pre-clinical animal models (9).

CD40L transgene expressing some tumor cells not only become visible to the antigen-presenting cells but also actively ignite their own eradication. However, the potential risk of systemic inflammation remains a concern for CD40L gene therapy. In common with other TNF family ligands, the membrane-bound CD40L has been known to be subsequently cleaved from the cell surface via matrix metalloproteinases (MMPs), releasing the soluble fragment sCD40L that retains its ability to form trimers, to bind CD40, and to deliver biologic signals (10). Studies have reported the increased levels of sCD40L in the plasma of patients with autoimmune diseases (11), metabolic and cardiovascular diseases (12). Moreover, some studies found that sCD40L induced survival signals in some CD40-positive carcinomas when the protein synthesis system was not blocked (13). To circumvent the adverse inflammatory effects and possible anti-apoptotic activity caused by sCD40L, Masuta *et al* (14) designed and engineered a membrane-stable mutant form of human CD40L (CD40L-M) that is resistant to MMPs, then they examined chronic lymphocytic leukemia B cells and dendritic cells (DCs) infected with the mutant construct for the lower expression of sCD40L and unimpaired immunologic function of cell surface CD40L relative to that of wild-type CD40L (CD40L-WT). To further explore the multiple effects of the membrane-stable mutant form of human CD40L in certain solid tumor, we generated plasmid encoding non-cleavable CD40L mutant to determine the direct anti-proliferative and pro-apoptotic

effects in CD40-positive A549 cells, and to verify activation of DCs by co-culture with the generated CD40L-M-transgenic cells with the lower level of sCD40L.

Materials and methods

Cell culture and treatments. The human CD40-positive lung adenocarcinoma cell line A549 (15), human premonocytic cell line THP-1 (16) and embryonic kidney cell (293T) were studied. The cells were maintained at 37˚C in a 5% CO_2 atmosphere in RPMI-1640 media (Sigma) supplemented with 10% fetal calf serum (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin, respectively. To induce immature DCs, the THP-1 cells were harvested by centrifugation, resuspended in RPMI-1640 complete medium at a concentration of $2x10^5$ cells/ml, supplemented with 50 ng/ml rhGM-CSF and 150 ng/ml rhIL-4 (PeproTech). The cells were observed with a binocular inverted microscope every day and half the medium was exchanged every other day. On the fifth day, unstimulated and stimulated cell samples were analysed by flow cytometry.

Construction and identification of plasmids. Full-length cDNA encoding for CD40L was amplified from peripheral blood lymphocytes in healthy volunteers by PCR amplification (Rotor gene) using primers engineered with *Xho*I and *Eco*RI restriction enzyme digestion site. After enzyme digestion and gel purification, the PCR fragment was ligated into the pcDNA3.1$^+$ (Invitrogen) to form a eukaryotic expression vector pcDNA3.1$^+$-CD40L-WT (17). The transgene expression is regulated by the CMV promoter. PCR-amplified CD40L was examined by DNA sequencing to confirm nucleotide sequence with the one in public domain (PubMed). The pcDNA3.1$^+$-CD40L-WT plasmid was manufactured by Invitrogen Biotechnology (Shanghai, China) following the current Good Manufacturing Practice guideline and the entire construct was sequenced to exclude the possibility of any mutated nucleotides. According to the reported mutational site (14), CD40L-M mutant containing 6 substitutions (Gln^{114} to Pro^{114}, Lys^{115} to Arg^{115}, Asp^{117} to Glu^{117}, Gln^{118} to Glu^{118}, Asn^{119} to Asp^{119}, and Pro^{120} to Ser^{120}) was constructed by Invitrogen Biotechnology (Shanghai, China). Then, pcDNA3.1$^+$-CD40L-M was produced and identified as reported above.

Transfection of A549 cells and detection of CD40L expression by real-time PCR. A549 cells were transfected with pcDNA3.1$^+$-CD40L-WT and pcDNA3.1$^+$-CD40L-M by MegaTran 1.0 transfection reagent (Origene). All the procedures were performed according to the guidance of the reagent supplier. The cells transfected with pcDNA3.1$^+$ and untreated served as controls. To demonstrate the expression of CD40L mediated by pcDNA3.1$^+$-CD40L-WT and pcDNA3.1$^+$-CD40L-M vectors, total cellular RNA was extracted from the A549 cells. Synthesis and amplification of CD40L cDNA were performed using the SYBR Green Real-time PCR kit (Toyobo) according to the manufacturer's instructions. Primer sequences used for PCR amplification were as follows: CD40L (GenBank accession no. X67878, position 488 to 620, 132 bp fragment, 53˚C annealing) 5'-TGA GCA ACA ACT TGG TAA CCC TGG-3' (sense) and 5'-CTG GCT ATA AAT GGA GCT TGA CTC G-3' (antisense) (18); ß-actin primers (205 bp fragment) were sense 5'-CAA AAG CCT TCA TAC ATC-3' and antisense 5'-TCA TGT TTG AGA CCT TCA A-3' (Invitrogen).

ELISA for soluble CD40L. The concentrations of soluble CD40L in cell-free supernatants of A549 cells at 24, 48 and 72 h after transfection were measured with a human sCD40L ELISA kit (Bender) according to the manufacturer's instructions. The minimal detection limit for this kit was 7 pg/ml.

Flow cytometric analysis of apoptosis and cell cycle phase. Apoptosis and cell cycle phase were evaluated by flow cytometric analysis. Briefly, cells were fixed in 70% ethanol at 4˚C for 30 min, incubated with 500 μg/ml RNase at 37˚C for 30 min, and then stained with propidium iodide (PI; 50 μg/ml). Sample DNA content was analyzed by flow cytometry (FACS Calibur; BD Biosciences). The proportion of cells in different cell cycle phases and undergoing apoptosis was calculated as percentages from statistics generated using Cell Quest software (BD Immunocytometry Systems).

Co-culture and ELISA for secreted IL-12. To demonstrate activation of DCs by CD40L-transgenic A549 cells, the amount of IL-12 released into the supernatant was determined with a commercial IL-12 p70 immunoassay (Genetimes). A549 cells ($5x10^5$) were transduced with pcDNA3.1$^+$-CD40L-WT, pcDNA3.1$^+$-CD40L-M or pcDNA3.1$^+$ in 6-well plates and dual-chamber transwell plates (19) separated by insertion of a 0.4-μm-pore size semi-permeable membrane (Corning), respectively. After 48 h, $5x10^5$ immature DCs were added for co-cultivation, and release of IL-12 was measured 48 h later. The specificity of CD40L-mediated activation of DCs was confirmed by the reduction of IL-12 levels following the preincubation of the transgenic tumor cells with anti-human CD40L monoclonal antibody (Biolegend). All experiments were performed in triplicate.

Statistical analysis. Unpaired Student's t-test (Microsoft Excel) with two tails was used to determine the significance of results. Data were presented as mean ± standard deviation. A $P<0.05$ was considered as significant.

Results

Evaluation of pcDNA3.1$^+$-CD40L-WT and pcDNA3.1$^+$-CD40L-M. To demonstrate whether the full-length human CD40L-WT-cDNA and CD40L-M-cDNA were inserted into the clone sites of pcDNA3.1$^+$ correctly, pcDNA3.1$^+$-CD40L-WT and pcDNA3.1$^+$-CD40L-M were digested by *Xho*I and *Eco*RI and evaluated with electrophoresis, respectively. Fig. 1A shows that 850 bp fragments corresponding to the CD40L cDNA were amplified. The correct plasmids identified by restriction endonuclease analysis were sequenced. The results of partial sequencing and spatial structures of protein monomer are shown in Fig. 1B and C. It demonstrated that differences in cleavage site were cloned into eukaryotic expression vector, and the mutant did not impact the spatial structures of the CD40L protein monomer (http://swissmodel.expasy.org/).

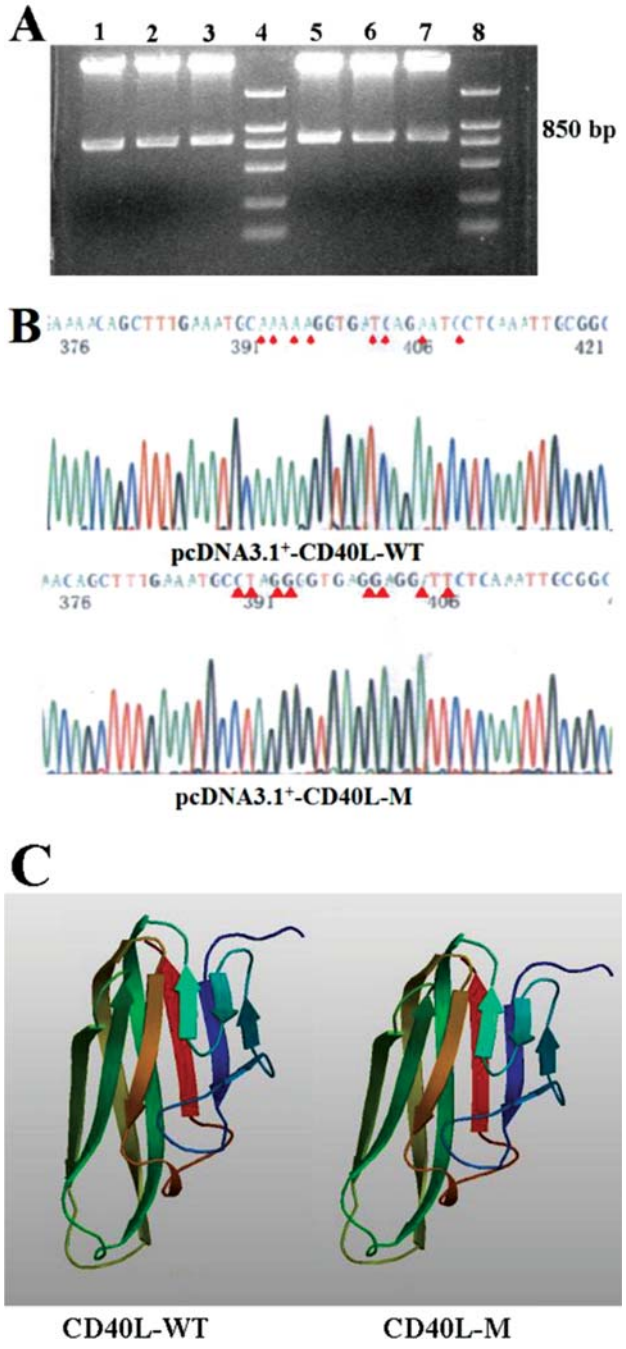

Figure 1. (A) Electrophoresis of recombinant plasmid pcDNA3.1$^+$-CD40L-WT and pcDNA3.1$^+$-CD40L-M. Lanes 1, 2 and 3, pcDNA3.1$^+$-CD40L-WT digested with *Xho*I and *Eco*RI; Lanes 5, 6 and 7, pcDNA3.1$^+$-CD40L-M digested with *Xho*I and *Eco*RI; Lanes 4 and 8, DNA molecular weight marker. (B) The partial sequencing maps of pcDNA3.1$^+$-CD40L-WT and pcDNA3.1$^+$-CD40L-M. The arrows indicate mutation sites. (C) Spatial structures of CD40L-WT and CD40L-M protein monomer.

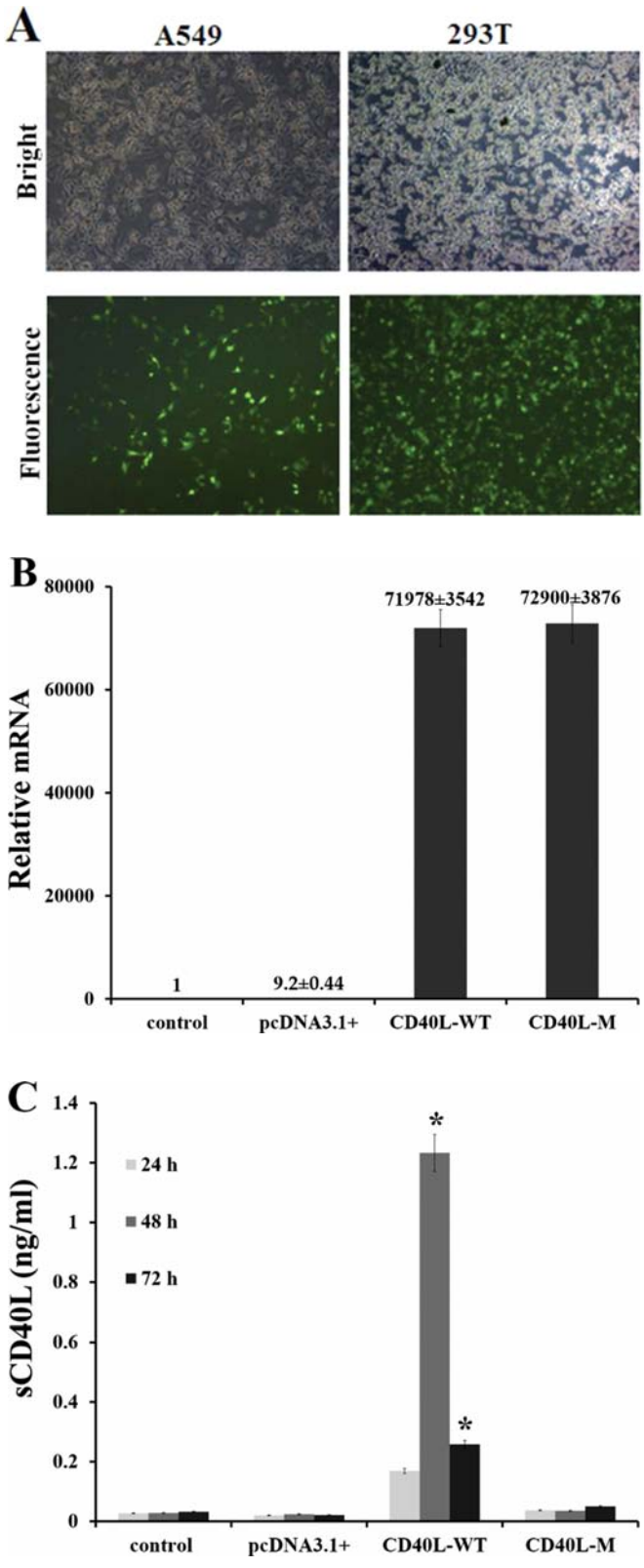

Figure 2. (A) A549 cells were transfected with a standard DNA plasmid carrying full-length cDNA encoding for GFP. Images were taken 48 h post-transfection. Left panel: GFP-transfected A549 cells, right panel: control 293T cells, upper panel: bright field, bottom panel: fluorescence field. (B) Relative CD40L gene transcripts by real-time PCR. A549 cells transfected with an entry vector pcDNA3.1$^+$, pcDNA3.1$^+$-CD40L-WT or pcDNA3.1$^+$-CD40L-M. (C) These transfectants were cultured at the cell concentration of $5x10^5$. Cell-free supernatants were collected and tested at 24, 48 and 72 h after transfection for the presence of sCD40L by ELISA ($^*P<0.01$ vs. control, pcDNA3.1$^+$ and pcDNA3.1$^+$-CD40L-M transfestants). Data represent the mean ± SD of triplicate samples.

CD40L mutant is expressed in A549 but not secreted. We used a standard DNA plasmid carrying a full-length cDNA encoding the GFP marker gene to optimize our transient transfection procedure for A549 cells. Numerous experiments were performed to test various cell handling procedures, DNA and cell concentration and other factors. Fig. 2A shows transient transfection results using GFP plasmids in A549 and normal 293T cells. Transgene GFP expression was observed within 24 h post-transfection.

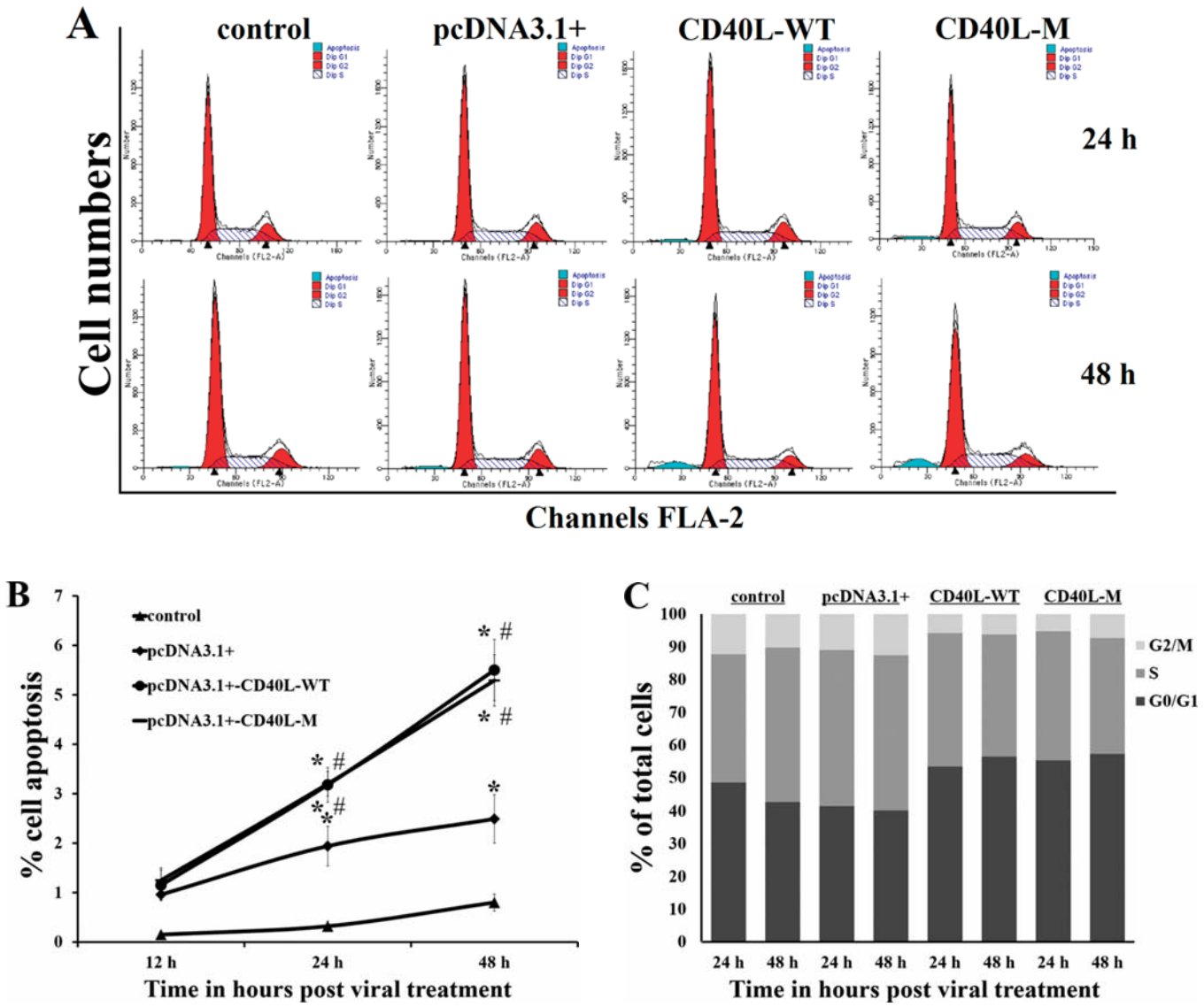

Figure 3. (A) Cell apoposis and cell cycle phase outcome from 24 to 48 h after treatment with pcDNA3.1$^+$-CD40L-WT, pcDNA3.1$^+$-CD40L-M, pcDNA3.1$^+$ and control was determined by flow cytometry after propidium iodide staining. (B) The rate of cell apoposis (*P<0.05 vs. control, $^\#$P<0.05 vs. pcDNA3.1$^+$). (C) Cell cycle distribution. Data represent the mean ± SD of triplicate samples.

After we optimized transfections using the GFP marker gene, we next examined transient transfection of A549 cells using the same plasmid backbone as the one described above, but carrying the full-length cDNA encoding for human CD40L-WT and CD40L-M. Real-time PCR analysis showed that 132 bp fragments corresponding to the CD40L cDNA were amplified with the total cellular RNA from pcDNA3.1$^+$-CD40L-WT-transduced and pcDNA3.1$^+$-CD40L-M-transduced A549 cells (CD40L-WT/A549 and CD40L-M/A549; Fig. 2B).

We next investigated the levels of sCD40L in the culture supernatants of two transfectants. As shown in Fig. 2C, the amount of sCD40L from CD40L-WT/A549 increased significantly at 48 and 72 h after transfection compared with that of control, pcDNA3.1$^+$ and CD40L-M/A549 (P<0.01, respectively). In contrast, negligible levels of sCD40L were detected in the supernatants of CD40L-M/A549 (P>0.05 compared with controls) at 24, 48 and 72 h. The data indicate that the CD40L-M is not cleaved from the cell surface.

Direct anti-tumor activity of the CD40L mutant. As shown in Fig. 3A, the effects of CD40L-induced apoptosis and cell cycle arrest were investigated in untransduced cells as well as cells transfected with pcDNA3.1$^+$-CD40L-WT, pcDNA3.1$^+$-CD40L-M or negative control pcDNA3.1$^+$. The percentage of apoptosis increased significantly in CD40L-WT/A549 and CD40L-M/A549 compared with pcDNA3.1$^+$ group (P<0.05, respectively) at 24 and 48 h after transfection (Fig. 3B). The percentage of G0/G1 phase cells increased in CD40L-WT/A549 and CD40L-M/A549 compared with the entry vector group (P<0.05, respectively) at 24 and 48 h, a corresponding reduction was found in the percentage of G2/M phase cells (P<0.05, respectively) and that of S phase cells (P>0.05, respectively) compared with the entry vector. The difference

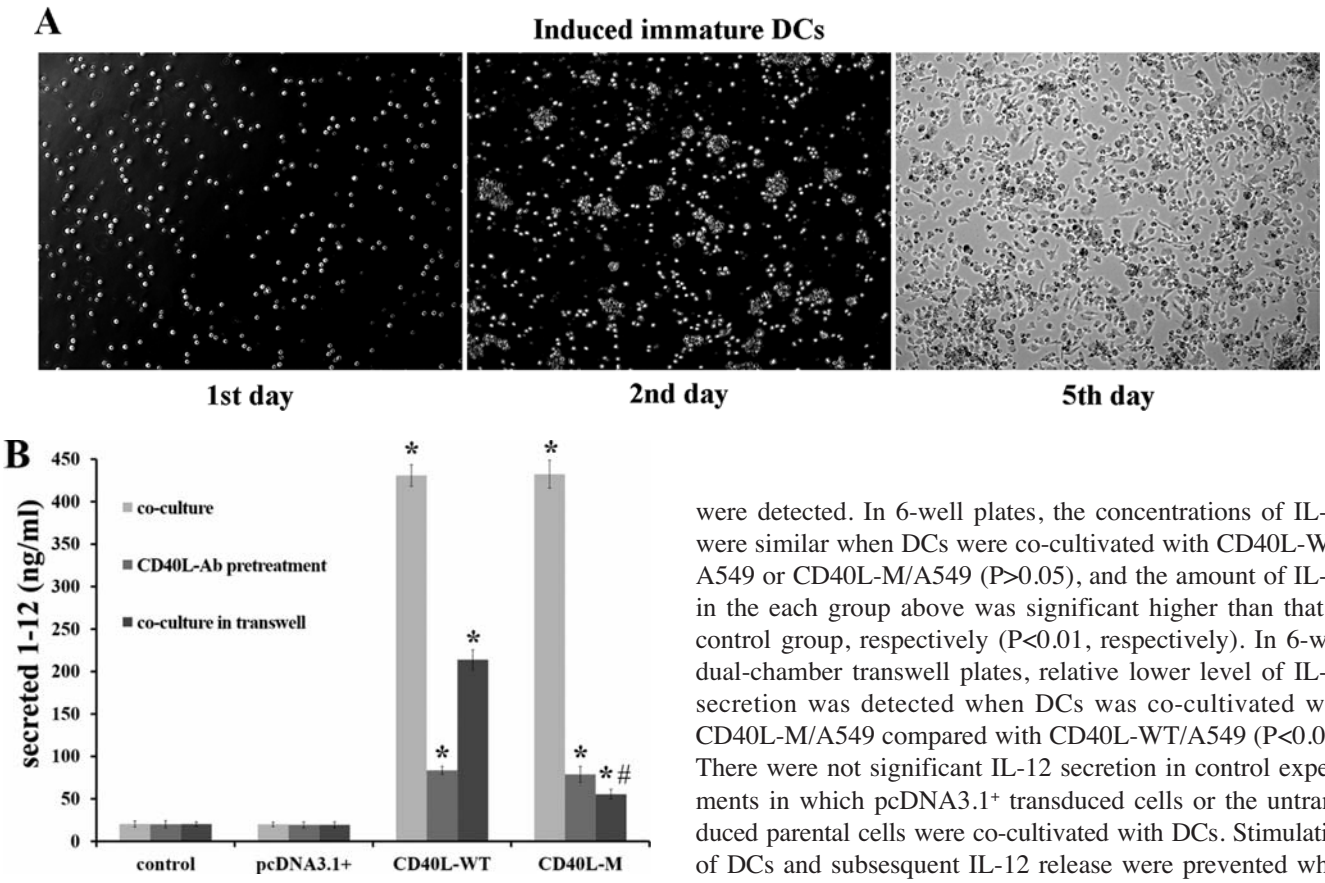

Figure 4. (A) Binocular inverted microscope photos of immature dendritic cells preparing from THP-1 when cultured with rhGM-CSF and rhIL-4 for 1, 2 and 5 days. On the 2nd day of treatment, number of individual small round cells began to gather into clusters, and on the 5th day, the morphology of some cells became irregular. (B) Immature DCs were co-cultivated with transduced cells or controls for 48 h before the amount of interleukin-12 was determined by ELISA. Interleukin-12 production by DCs co-cultivated with transfectant cells and controls (pale bars), co-cultivated with transfectans preincubated by the anti-CD40L (grey bars), and co-culture in dual-chamber transwell plates (black bars) (*P<0.01 vs. control or pcDNA3.1^{+}, $^{\#}$P<0.05 vs. CD40L-WT). Data represent the mean ± SD of triplicate samples.

of apoptosis and cell cycle distribution between CD40L-WT/A549 and CD40L-M/A549 was almost indistinguishable (Fig. 3C).

Activation of DCs by co-cultivation with CD40L-WT/A549 and CD40L-M/A549. We generated DCs essentially according to Berges *et al* (16) who demonstated that THP-1 can be induced to immature DCs when cultured with rhGM-CSF and rhIL-4. The second day after treatments, number of individual small round THP-1 cells began to gather into clusters, and on the fifth day, the morphology of some cells became irregular (Fig. 4A). Up-regulation of the cell surface markers CD1a, CD80, CD83 and CD86, and down regulation of CD14, indicating the conversion of monocyte to immature DCs, was confirmed by flow cytometry before DCs were co-cultivated with CD40L-WT/A549 or CD40L-M/A549. After 48 h co-culture, the levels of IL-12 released to the medium as a result of DCs stimulation with CD40L-WT/A549, CD40L-M/A549 and controls in 6-well plates or dual-chamber transwell plates were detected. In 6-well plates, the concentrations of IL-12 were similar when DCs were co-cultivated with CD40L-WT/A549 or CD40L-M/A549 (P>0.05), and the amount of IL-12 in the each group above was significant higher than that in control group, respectively (P<0.01, respectively). In 6-well dual-chamber transwell plates, relative lower level of IL-12 secretion was detected when DCs was co-cultivated with CD40L-M/A549 compared with CD40L-WT/A549 (P<0.05). There were not significant IL-12 secretion in control experiments in which pcDNA3.1^{+} transduced cells or the untransduced parental cells were co-cultivated with DCs. Stimulation of DCs and subsesquent IL-12 release were prevented when the CD40L-transduced cells were preincubated with an anti-CD40L monoclonal antibody (Fig. 4B).

Discussion

The aims of this study were to generate a membrane-stable mutant of human CD40L (CD40L-M) and to develop efficient and safe strategies for direct anti-tumor therapy, and for the induction of immune responses in NSCLC gene therapy. The method of CD40L gene therapy appears to provide an opportunity to muster different anticancer approaches in one therapy and offers an attractive option for further clinical trials. However, cleaved product from membrane-bound CD40L, soluble CD40L, has been observed to enter into systemic circulation and cause inflammatory diseases (11). Platelet-derived sCD40L is a proatherogenic cytokine and consistently related to obesity, diabetes and increased cardiovascular risk (12). Moreover, sCD40L is observed to induce survival signals in CD40-positive carcinomas unless the protein synthesis machinery is blocked (13), but membrane-bound CD40L is able to directly induce cell death without the requirement for protein synthesis inhibition (20). To reduce the adverse effects caused by sCD40L, we generated human CD40L mutant CD40L-M that was not cleaved by MMPs and remained on the cell surface as a membrane-bound form. CD40L-M mutant contains 6 substitutions which mimic mouse CD40L to replace the cleaved sites (14). There is no obvious spatial structure change in the CD40L protein monomer (http://swissmodel.expasy.org/). Here, we further explored the multi-effect function of the CD40L mutant.

CD40L transgene expression in human breast (9), ovarian (21), cervical (22), prostate (23), hepatic (24), pancreatic (25),

colon (26), bladder (27), non-small cell lung (28), skin (29), and squamous epithelial carcinoma cells (30) was found to produce a direct growth-inhibitory effect through apoptotic induction and/or cell cycle blockage with no overt side effects on their normal counterparts. The pro-apoptotic effects of CD40L are revealed upon disruption of the PI3 kinase/mTOR and ERK signaling pathways or inhibitor of *de novo* protein synthesis (13). Intriguingly, some reports also show that CD40-mediated apoptosis occurs via a TRAF3-JNK pathway that promotes caspase-9 activation (20). In the present study, we found both CD40L-WT and CD40L-M could promote apoptosis and induce G0/G1 phase arrest in A549 cells at the similar level which indicated that a direct anti-tumor effect of the mutant was not impaired even in the absence of any additional immune responses and cells. Recently, Vardouli *et al* (31) found that a replication-defective recombinant adenovirus (RAd) vector expressing CD40L promoted apoptosis of CD40-positive bladder, cervical and ovarian carcinomas and induced accumulation of EJ cells to G0/G1. Taha *et al* (32) generated a RAd expressing another CD40L mutant that is also resistant to MMPs cleavage and showed that the cleavage-resistant form of CD40L was a more potent inducer of apoptosis than wild-type CD40L in CD40-positive bladder and gastric carcinoma cell lines. These findings were not in contradiction with the current results.

It is widely accepted that CD40 stimulation can enhance anti-tumor immune responses by means of DC maturation. Activation of DCs results in their increased survival, secretion of IL-1, IL-6, IL-8, IL-12, TNF-α, and macrophage inflammatory protain-1α. In addition, CD40 activation induces the upregulation of co-stimulatory molecules such as MHC class II, LFA-3, CD80, CD86 and promotes antigen presentation, priming and cross-priming of helper T cells and cytolytic T lymphocytes (CTLs), respectively (33). Using gene delivery of the CD40L gene to DCs or tumor cells has been approached for tumor immunotherapy. Expression of CD40L in a small proportion of tumor cells was enough to activate DCs and generate a long-lasting systemic anti-tumor immune response in mice that was shown to be dependent in CTLs (34). Masuta *et al* (14) have observed that CD40-expressing leukemia B cells transfeced with CD40L-M mutant were highly effective stimulators in the mixed lymphocyte leukemia reaction, and the current studies also demonstrated activation of DCs by CD40L-M-transgenic A549 cells, indicating that CD40L mutant did not lose biologic immunocompetence.

To examine whether the CD40L-M was resistant to cleavage on the cell surface, secretion of sCD40L after transfection was detected in supernatants. As predicted, sCD40L was detected only from CD40L-WT transfectant, not from the CD40L mutant. We also found that IL-12 did not increase when immature DCs co-cultured with CD40L-M-transgenic A549 cells in dual-chamber transwell plates, that is, the gene transfer CD40L-M into A549 was not cleaved from the cell surface and so there were little sCD40L through the semi-permeable membrane to induce the maturation of DCs.

In summary, these studies suggest that gene transfer of the membrane-stable CD40L mutant into CD40-positive cells may provide an efficient and safe method for NSCLC. Although, the feasibility of this approach needs to be further validated by *in vivo* studies, the data shown in this report highlight the potential of exploiting the direct effects of the mutant CD40L gene transfer for lung cancer therapy, and is a valuable addition to the tumor immunotherapy literature.

Acknowledgements

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