

# ***In vitro* antitumor cytotoxic T lymphocyte response induced by dendritic cells transduced with $\Delta$ Np73 $\alpha$ recombinant adenovirus**

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**Abstract.**  $\Delta$ Np73 $\alpha$ , the N-terminal truncated form of p73 $\alpha$  is a candidate tumor antigen because of its selective expression in many human cancers and lack of expression in normal tissues. Therefore, we investigated the effects of dendritic cells infected with adenoviral  $\Delta$ Np73 $\alpha$  (DNp73 $\alpha$ ) on breaking immune tolerance and induction of immunity against DNp73 $\alpha$ -expressing (A549 lung cancer, K-562 leukemia) and non-expressing (MCF-7 breast cancer) cell lines. Immature dendritic cells generated in the presence of interleukin-4 and granulocyte/macrophage colony-stimulating factor from a human umbilical cord blood were transduced with a recombinant adenoviral (Ad) vector encoding full-length human DNp73 $\alpha$  cDNA (Ad-DNp73 $\alpha$ ) or a control vector Ad-EGFP, using the centrifugal force method. Induction of DNp73 $\alpha$ -specific CTL response was evaluated by a cytotoxic assay against the three human tumor cell lines with different DNp73 $\alpha$  expression levels. The viability and activation status of transduced dendritic cells were assessed by flow cytometry. The dendrocyte/Ad-DNp73 $\alpha$ -activated cytotoxic T lymphocytes showed significantly higher cytotoxicity against the cell lines A549/DNp73 $\alpha$ , K-562 that expressed DNp73 $\alpha$  than the DNp73 $\alpha$ -null MCF-7 cells. The DCs/Ad-DNp73 $\alpha$  showed higher survival rates than the DCs/Ad-EGFP or

untransduced DCs, presumably due to the inhibition of cell death. These findings, with potential applications for immunotherapy, demonstrate that dendrocytes transduced with Ad-DNp73 $\alpha$  can induce specific and sustained T cell responses against tumors expressing this variant p53-related gene.

## **Introduction**

Lung cancer has emerged as the most common cause of cancer-related deaths among the Chinese people and it is, moreover, the leading cause of cancer-associated mortality in North America (1). Despite advances in radiation, chemotherapy and surgical resection, the prognosis for lung cancer patients remains poor; the overall survival rate is about 11 to 14%. Therefore, development of new treatment modalities is urgently needed. Several reports demonstrated that systemic immunotherapy with dendritic cell (DC)-based vaccines are capable of inducing potent antitumor responses (2), which makes cancer gene therapy and immunotherapy approaches more promising.

The presentation of tumor-associated antigen (TAA) by professional antigen presenting cells (APC), especially DCs and the recognition by cytotoxic T lymphocytes (CTL) play an essential role in the eradication of tumor cells. Many methods for priming the DCs with antigens were tried to induce antitumor immune responses. The most common approaches include the pulsing of DCs with whole tumor cell lysate or tumor-derived MHC class I-restricted peptides. However, whole tumor cell preparations are difficult to standardize and are of limited availability for some tumor cells and the pulsing of DCs with synthetic peptides is limited by the identification of specific HLA-restricted epitopes. Recently, DCs transfected with total TAA cDNA were shown to induce TAA-specific immune responses (3). Gene modification of DCs with the TAA gene(s) confers the potential for presenting various known and unknown TAA epitopes. The endogenous processing and presentation of TAA peptides at the dendritic cell surface may be more efficient than the exogenous loading of synthetic TAA peptides (4). At present, the most efficient method for genetic manipulation of DCs is the use of viral

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*Abbreviations:* CTL, cytotoxic T lymphocytes; DNp73 $\alpha$ ,  $\Delta$ Np73 $\alpha$ ; DC, dendritic cell; Ad, adenoviral; iDCs, immature dendritic cells; mDCs, mature dendritic cells; GFP, green fluorescent protein; LAK, lymphokine-activated killer

*Key words:*  $\Delta$ Np73 $\alpha$ , dendritic cell vaccine, recombinant adenovirus

vectors. In particular, the adenoviral (Ad) vectors were shown to be effective in transducing the DCs (5).

The N-terminal truncated p73 $\alpha$  (DNp73 $\alpha$ ), a protein of the p53 family, was recently identified to be generated from the p73 gene using an alternative promoter in intron 3 and therefore the DNp73 $\alpha$  protein lacks a transactivation domain present in p73 (6). DNp73 $\alpha$  is not expressed in normal tissues but is highly expressed in lung cancer, breast cancer, ovarian cancer, vulval cancer and neuroblastoma; it acts as a potent transdominant inhibitor of the wild-type p53 and the transactivation-competent full-length p73 (7,8). Several studies showed that the positive expression of DNp73 $\alpha$  is a significant poor prognosis indicator for the patient, therefore, this variant can be considered as a molecular target for cancer therapy (9,10). However, few studies reported experimental manipulation targeting the DNp73 $\alpha$ .

Our ongoing studies using specific antisense constructs showed that the down-regulation of DNp73 $\alpha$  expression results in apoptosis of lung cancer cells (not shown). In the present study, we explored the immunotherapy approach for eliciting the DNp73 $\alpha$ -specific immunity via DCs transduced with a recombinant Ad vector expressing the human DNp73 $\alpha$  (Ad-DNp73 $\alpha$ ). The Ad-DNp73 $\alpha$ -modified DCs were tested for surface marker changes over time, cell survival and their ability to induce DNp73 $\alpha$ -specific CTL responses against DNp73 $\alpha$  expressing tumor cells. To our knowledge, this study represents the first report of enforced expression of DNp73 $\alpha$  in DCs and we show specific CTL responses elicited against the tumors expressing DNp73 $\alpha$ .

## Materials and methods

**Cell lines and their culture.** An A549/DNp73 $\alpha$  cell line was derived from the human alveolar epithelial cell line A549 through stable transfection with plasmid pcDNA3.0-DNp73 $\alpha$ -HA under constant G418 selection (1 mg/ml; Bioshop, Canada). The chronic myelogenous leukemia cell line K-562 and the breast cancer cell line MCF-7, which are respectively DNp73 $\alpha$  expression positive and negative, were purchased from the American Type Culture Collection. All cancer cell lines were grown in a complete RPMI-1640 medium supplemented with a 10% heat-inactivated fetal calf serum (FCS, Hyclone), 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin.

**Generation of DCs.** Human umbilical cord blood was obtained from the placentae of normal, full-term infants after the placentae were delivered and separated from the infants, with prior written consent from their mothers. Mononuclear cells were isolated from the umbilical cord blood by Ficoll-Hypaque (d=1.077 g/ml, BDT) density-gradient centrifugation. The cells were suspended in the complete RPMI-1640 medium. After incubation for 2 h at 37°C in 5% CO<sub>2</sub>, the non-adherent cells were removed (for preparation of T lymphocytes, as described below) and the adherent cells were cultured for 7 days in the presence of a granulocyte-macrophage colony stimulating factor (GM-CSF, 100 U/ml, Perprotech) and interleukin-4 (IL-4, 100 U/ml). On day 3 of the culture, 50% of the medium was replaced. On day 6, the non-adherent cells were harvested as immature DCs (iDCs) or were activated with

tumor necrosis factor alpha (TNF- $\alpha$ , 50 ng/ml, Perprotech) to obtain mature DCs (mDCs).

**Preparation of T lymphocytes.** A sterile nylon-wool isolation column was soaked in the complete RPMI-1640 medium for 1 h at 37°C. Then the non-adherent cells which were isolated from the umbilical cord blood mononuclear cells were applied on the column and cultured for an additional 1 h. T lymphocytes were eluted from the column with 10 ml of the complete RPMI-1640 medium and referred to as autologous T cells. Purity of about 90% was obtained with this procedure. Lymphokine-activated killer (LAK) cells were prepared by adding IL-2 (100 U/ml, Perprotech) to the T cells followed by 72 h of culture.

**Adenovirus-mediated gene transduction into DCs.** A recombinant human serotype 5 based replication-deficient (E1A) adenovirus vector carrying an HA tagged full-length human DNp73 $\alpha$  cDNA driven by a CMV promoter was prepared using the AdEasy system and referred to as an Ad-DNp73 $\alpha$ . The same adenoviral vector backbone carrying the marker gene EGFP was constructed; the Ad-EGFP served as the control vector. The two viruses were propagated in 293 cells and titers of the viral stocks were determined with a plaque-forming assay. A maximal titer of 2.5x10<sup>11</sup> pfu/l was obtained for the Ad-DNp73 $\alpha$ .

The adenovirus mediated gene transfer to the immature DCs (iDCs) was performed by the centrifugal force method, as described previously (11). Briefly, 1x10<sup>6</sup> iDCs and the recombinant adenoviral vector were suspended in 500  $\mu$ l of PBS containing 1% serum and mixed. The mixtures of iDCs and viral vectors were centrifuged (2000 x g, at 37°C) for 2 h. The centrifuged DCs were washed twice with PBS and used for subsequent experiments.

**Reverse transcription PCR and real-time PCR.** The expression of DNp73 $\alpha$  mRNA was analyzed by a standard reverse transcription-PCR (RT-PCR) method. The total RNA was isolated from the untreated DCs or DCs 48 h after infection with the Ad-DNp73 $\alpha$  or Ad-EGFP using the standard one-step phenol extraction method. A reverse transcription was performed using an RNA PCR kit (Takara). A regular PCR was then performed with a standard protocol using Taq polymerase. A real-time PCR (SYBR *Premix Ex Taq*, Takara) was adopted to compare the endogenous DNp73 $\alpha$  expression with the A549/DNp73 $\alpha$ , K-562 and MCF-7 cell lines. The real-time PCR primers were: 5'-ACCTCGCCACGGCCCAGTTC-3'-DNp73 $\alpha$  sense; 5'-GGTGGAGCTGGGTTGTGCGTAG-3'-DNp73 $\alpha$  antisense; 5'-ATGCCGACAGGATGCAGA-3'- $\beta$ -actin sense, and 5'-GAGTACTTGCGCTCAGGAGGA-3'- $\beta$ -actin antisense. The  $\beta$ -actin served as an internal control. The amplification cycle consisted of denaturation at 94°C for 30 sec annealing at 60°C for 30 sec and extension at 72°C for 30 sec.

**Western blot analysis.** The DCs, at 48 h after the Ad vector infection, were collected and washed twice in cold PBS. The cells were solubilized in a lysis buffer containing a protease inhibitor cocktail (MC-CellLytics Mammalian Cell Protein Extraction Reagent, Biocolor). The lysis was performed at 4°C

for 20 min and the lysates were centrifuged at 15,000 rpm. The protein levels were standardized by a BCA assay (Shenergy Biocolor). Equal amounts (50  $\mu$ g) of proteins were electrophoresed under reducing conditions on 10% SDS-polyacrylamide gels. The proteins were electrophoretically transferred to Polyvinylidene difluoride transfer membranes and incubated with the primary antibody against the HA tag (mouse monoclonal, Applygen) and then with a peroxidase-linked secondary antibody (goat anti-mouse IgG/HRP, Biosynthesis).

**Analysis of DC markers.** The expression of the DC surface markers at 48 h after the Ad vector transduction was determined by flow cytometry. The DCs were washed once and stained for 30 min at 4°C with the respective primary antibody and then with a PE-conjugated mouse anti-human IgG. After two washings, the cells were analyzed by the FACScan (Bio-Rad), using CELL Quest software. Cells (10,000) were examined for each determination. Anti-CD1a, -CD83 and -HLA-DR (MHC class II; Biolegend, San Diego, CA) were used as the primary antibodies.

**Apoptosis analysis.** An annexin V-propidium iodide apoptosis detection kit (Bender, Austria) was utilized for quantification of the dead DCs and the DCs undergoing programmed cell death at 4, 8 and 12 days after the Ad vector infection. The cells were washed once in PBS and re-suspended in the binding buffer provided by the kit. Annexin V (PE-conjugated) and propidium iodide were added and incubated for 15 min at room temperature in the dark, followed immediately by flow cytometry.

**Mixed lymphocyte reaction (MLR).** MLR was performed with the three types of DCs (Ad-DNp73 $\alpha$ -transduced DCs, Ad-EGFP-transduced DCs and normal DCs control) on 4, 8 and 12 days after transduction. The DCs/Ad-DNp73 $\alpha$  were incubated in a complete RPMI-1640 medium containing 25 ng/ml cytomycin C for 30 min, then were washed by PBS and added as stimulator cells to round-bottom 96-well tissue culture plates at graded doses (reflecting the indicated responder-stimulator ratios). Autologous T cells were used as a source of responder cells and  $1 \times 10^5$  T cells per well were added to the DCs. Stimulations were performed in duplicates or triplicates. The cells were cultured after an additional 5 days in a complete RPMI-1640 medium. During the last 8 h of culture, [ $^3$ H]-thymidine was added (0.5  $\mu$ Ci/well, Isotope Institute of China Institute of Atomic Energy). The cells were harvested onto glass fiber filters and [ $^3$ H]-thymidine incorporation was determined using a flatbed scintillation counter.

**Cytotoxicity assays.** The T cells ( $1 \times 10^6$ ) were co-cultured with the Ad-DNp73 $\alpha$ -modified DCs ( $5 \times 10^4$ ) in a 24-well tissue culture plate in a 1 ml complete RPMI-1640 medium at 37°C in 5% CO<sub>2</sub> for 72 h for the cytotoxic T lymphocytes (CTLs). Then the CTLs were collected and used as the effector cells in CTL assays against A549/DNp73 $\alpha$  cells. The A549/DNp73 $\alpha$  cells, as the target cells, were placed in 96-well tissue culture plates at  $1 \times 10^4$  cells per well and co-cultured with effector cells (CTLs) at the (effect: target) ratio of 1:10, 1:20, 1:40 and 1:80 at 37°C in 5% CO<sub>2</sub>. The cytotoxic activities were determined

by the CytoTox non-radioactive cytotoxicity assay (Promega, USA). Briefly, the cytotoxicity assay plates containing a mixture of CTLs and target cells were incubated at 37°C, 5% CO<sub>2</sub> for at least 4 h. The plates were centrifuged at 250 x g for 4 min, then 50  $\mu$ l aliquots from each well was transferred on to a fresh 96-well flat-bottom plate. The reconstituted substrate mix (50  $\mu$ l/well) was added to the same wells followed by a 30 min incubation at room temperature. Then, 50  $\mu$ l of stop solution was added and the absorbance at 490 nm was read within 1 h. All determinations were carried out in triplicate and repeated four times. The percentage of specific cytotoxicity was calculated as  $([\text{experimental} - \text{effector spontaneous} - \text{target spontaneous}] / [\text{target maximum} - \text{target spontaneous}]) \times 100$ .

**Statistical analysis.** A  $\chi^2$  analysis was performed to evaluate the significance of the differences between the experimental groups when discrete data were presented. For a single comparison of the two groups, the Student's t-test was used. If the data distribution was not normal, the Mann-Whitney rank-sum test was employed for the nonparametric analysis. For all analyses, the level of significance was set at  $p < 0.05$ . All statistical calculations were performed using the Sigma Stat statistical software package SPSS10.0. The data are presented as the mean  $\pm$  SEM.

## Results

**Expression of the adenoviral vector transduced human DNp73 $\alpha$  gene in DCs.** A replication-deficient adenovirus vector carrying a human DNp73 $\alpha$  gene driven by the cytomegalovirus promoter (CMV), Ad-DNp73 $\alpha$ , was constructed and used to infect the immature DCs (iDCs; Fig. 1A) at a multiplicity of infection of 200 by the centrifugal method, which was optimized for maximum transfection efficiency and toxicity (data not shown). A control vector Ad-EGFP was used to infect the DCs in parallel which resulted in a GFP expression in about 90% of the DCs (Fig. 1B), indicating a high transduction efficiency of the Ad vector. The expression of the DNp73 $\alpha$  mRNA was demonstrated by a semi-quantitative RT-PCR assay at 48 h after infection (Fig. 2A). The expression of the DNp73 $\alpha$  (HA tagged) protein was readily detectable in the DCs 48 h after the adenoviral infection by the Western blot analysis using an HA antibody (Fig. 2B). These results suggest that the adenovirus-mediated gene transfer was highly efficient.

**Changes in the phenotype of iDCs following a viral transduction.** The effect of the Ad vector transduction on the expression of the DC surface markers was determined at 48 h after transduction. The DC cells infected with Ad-DNp73 $\alpha$  or Ad-EGFP showed marked up-regulation of the CD83 and HLA-DR expression (Fig. 3). Further exposure of these iDCs to adenoviral particles did not augment the expression of these surface molecules (data not shown).

**Induction of a DNp73 $\alpha$ -specific CTL response against three tumor cell lines.** Three human tumor cell lines, DNp73 $\alpha$  overexpressing A549, K-562 and MCF-7, were selected as targets for assaying DNp73 $\alpha$ -specific CTLs. The status of the

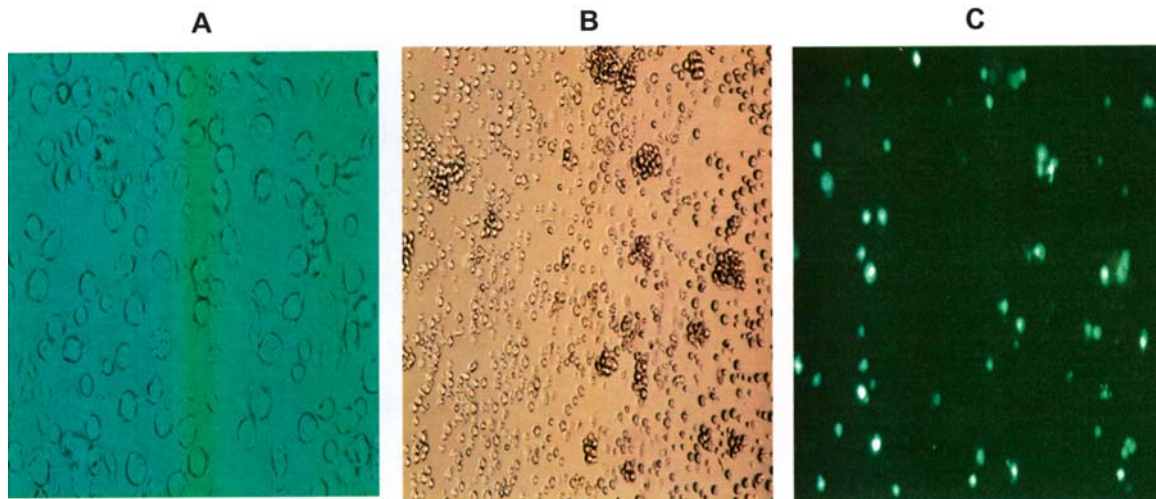


Figure 1. (A) Phase contrast microscopy of DCs used in this study (x400 magnification). Morphology of DCs with characteristic dendritic projections is evident. (B) Morphology of DCs transfected with Ad-DNp73. (C) Strong GFP expression in DCs transfected with the control vector Ad-EGFP (200 MOI for 24 h) as observed by fluorescence microscopy (x100 magnification). The results verified efficient gene expression after adenoviral infection.

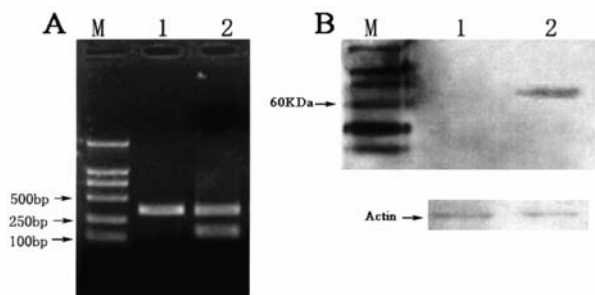


Figure 2. Expression of DNp73 $\alpha$  in dendritic cells. DCs were infected with Ad-DNp73 at 200 MOI. Forty-eight hours later, the expression of DNp73 was analyzed by RT-PCR and Western blot analysis. (A) RT-PCR analysis of cells for the DNp73 mRNA. M, DNA markers, lanes 1, normal DC; 2, DC transfected with Ad/DNp73; (B) Western blot analysis of cells for the DNp73 protein. M, Protein markers; 1, normal DC; 2, DC transfected with Ad/DNp73. The bottom panel is an actin Western blot to show equal protein loading in normal DCs and Ad/DNp73 DCs respectively.

DNp73 $\alpha$  expression in the three cell lines was confirmed by RT-PCR and real-time PCR procedures. As expected, the A549/DNp73 $\alpha$ , stably transfected with the DNp73 $\alpha$  construct, reported higher levels of expression than the human chronic myelogenous leukemia K-562 cell line which naturally expresses DNp73 $\alpha$ , whereas the human breast cancer cell line MCF-7 completely lacked DNp73 $\alpha$  expression (Fig. 4A).

The DNp73 $\alpha$ -specific cytotoxic T lymphocytes (CTL) were generated by using the autologous T cells primed with the Ad-DNp73 $\alpha$ -modified DCs (DCs/d-DNp73 $\alpha$ ) 48 h after transduction. In a non-radioactive cytotoxicity assay, these DCs/Ad-DNp73 $\alpha$ -stimulated effector T cells selectively lysed the DNp73 $\alpha$ -overexpressing A549/DNp73 $\alpha$  cells and K-562 cells but not the DNp73 $\alpha$ -null MCF-7 cells (Fig. 5A).

The specificity of the DCs/Ad-DNp73 $\alpha$ -stimulated CTLs was verified by comparing these T cells with the cytotoxic effect of human lymphokine-activated killer (LAK) cells and untreated T cells against the same cell lines. The untreated T

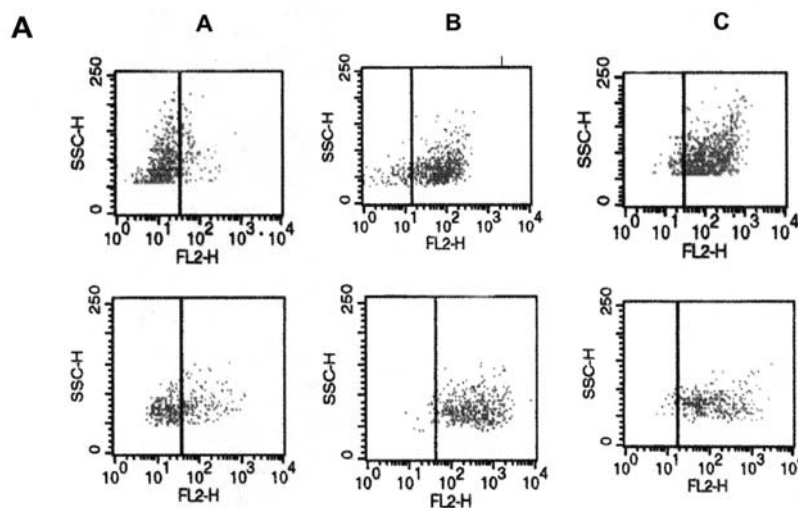


Figure 3. Change in phenotype of iDCs following viral transduction. Forty-eight hours after transduction of iDCs with Ad-DNp73 (MOI 200), the phenotype of the cells was analyzed by flow cytometry for the expression of the surface markers. The results shown are representative of 3 separate experiments. The upper row represents the CD83 expression and the bottom panel HLA-DR expression in: (A) untransduced dendrocytes; (B) Ad-DNp73 $\alpha$ -transduced dendrocytes and (C) Ad-EGFP-transduced dendrocytes.

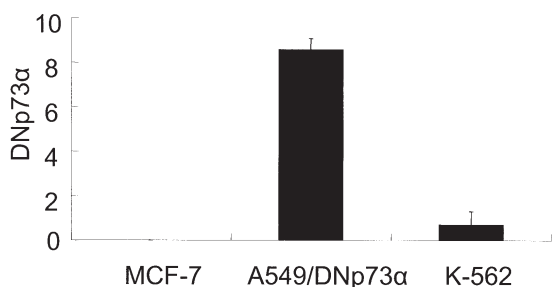


Figure 4. Relative expression levels of DNP73 $\alpha$  mRNA in MCF-7, A549/DNP73 and K-562 cell lines. Results were normalized to  $\beta$ -actin, which had minimal variation in DCs. The relative expression levels were normalized so that the expression level of  $\beta$ -actin equaled 1.00. The relative expression levels of DNP73 in A549 cells infected with Ad-DNP73 (A549/DNP73) and K-562 cell lines were 8.57 and 0.70, respectively.

cells had a minimal effect on the lysis of all the tumor cells. The LAK cells showed comparable lytic activity against the A549/DNP73 $\alpha$  (Fig. 5B) and K-562 cells (Fig. 5C) with those of the DCs/Ad-DNP73 $\alpha$ -stimulated T cells, but demonstrated more effective killing of MCF-7 cells than the DCs/Ad-DNP73 $\alpha$ -stimulated T cells (Fig. 5D).

**Enhanced survival of the transduced DCs.** The DNP73 $\alpha$  was demonstrated to inhibit apoptosis of lung cancer cells, neurons (12) and other tumor cells (13). This observation encouraged us to hypothesize that DNP73 $\alpha$  production in DCs may result in prolonged DC survival. To test this hypothesis,

we evaluated whether a DNP73 $\alpha$  overexpression by a DC may affect their survival in a long-term culture by flow cytometry. The percentage of viable cells among the three types of DCs did not differ on 4 and 8 days after infection. After 12 days of infection, the viable cell percentage of DCs transduced with AdDNP73 $\alpha$  was  $71 \pm 2.83\%$ , which was significantly higher than the DCs transduced with the Ad-EGFP or the uninfected mDCs ( $45.50 \pm 3.54\%$  and  $53.52 \pm 0.71\%$ , respectively). The data suggest that the DC survival was specifically enhanced by the DNP73 $\alpha$  expression.

**Improved proliferation of T cells.** When the Ad-DNP73 $\alpha$ -transduced DCs and Ad-EGFP-transduced DCs on 4 and 8 days after transduction were used to induce T cell proliferation, the two DCs demonstrated identical levels of stimulation, which, however, was higher than normal mDCs (Fig. 6), indicating that the enhancement was related to adenoviral infection and not due to the transgene expression. However, when the ability of DCs to stimulate T cell proliferation 12 days after transduction was compared, we found that the Ad-DNP73 $\alpha$ -transduced DCs showed a stronger T cell response as compared with the Ad-EGFP-transduced DCs (Fig. 6).

## Discussion

In this study, we demonstrated that DCs engineered to express human DNP73 $\alpha$  have the ability to mount a strong and specific CTL response against the DNP73 $\alpha$ -expressing cancer cells. A viral vector-mediated genetic modification of DCs to express tumor antigens was achieved in a number of xenograft

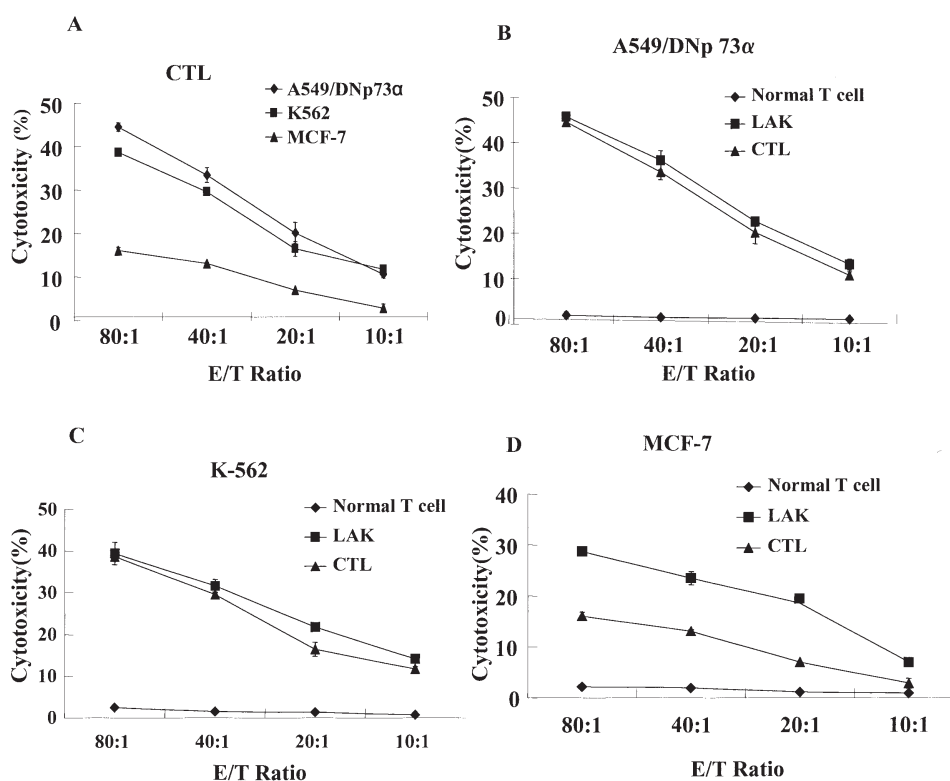


Figure 5. Cytolytic reactivity of cytotoxic lymphocytes (CTLs) in human cancer cell lines with varied DNP73 $\alpha$  expression. (A) Cell lysis activity of CTL stimulated with Ad-DNP73 modified DCs against the A549 cells overexpressing DNP73, marginally expressing K-562 and null MCF-7 cells. CTL activity was assessed by non-radioactive cytotoxicity assay as described in Materials and methods. (B) CTLs were compared with LAK (lymphokine-activated killer) cells and untreated T cells, which served as positive and negative controls, respectively against A549/DNP73 (Panel B), K-562 (Panel C), and MCF-7 (Panel D) human cancer cells.

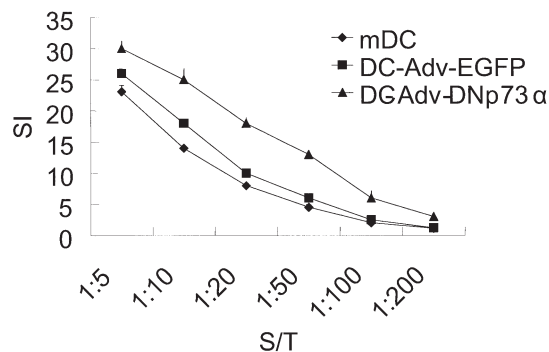


Figure 6. Dendritic cell T cell stimulatory ability in allogeneic mixed lymphocyte reactions (MLR) at varied ratios of DCs to T cells. MLR were performed on day 12 as described in Materials and methods. SI, Stimulation Index.

models for immunotherapy. Diverse transduction methods and different maturational states of DCs were reported (5,14). The level of antigen loading can impact on the capacity for DCs to activate T cell responses. Despite the efficiency attributed to Ad-based antigen loading, DCs do not express coxsackievirus and adenovirus receptors (CAR) and are therefore, inefficiently transduced by the conventional Ad-based approach. The addition of a centrifugal force (2000  $\times$  g) during the DC transduction with the Ad/GFP increased the gene expression by 20-fold (15). Another important factor determining engineered DCs' antigen-presentation was the appropriate maturational state of the DCs. It was reported recently that the DCs exhibited a direct cytotoxic effect on tumor cells and that iDCs undergoing maturation induced a much stronger tumor-specific growth inhibitory effect than iDCs or mature DCs through TNF- $\alpha$ -dependent and TNF- $\alpha$ -independent mechanisms (16). Because iDCs delivered locally at the site of tumors can be expected to ingest adjacent tumor cells and present additional tumor antigens *in vivo*, this process may facilitate direct cell lysis and improved tumor-specific CTL responses (17). This study used the centrifugal force method to achieve a highly efficient transduction and a high level of transgene expression and iDCs were selected for the Ad-DNp73 $\alpha$  infection and then used to stimulate naive T cells. As expected, flow cytometry showed an up-regulation of CD83 and HLA-DR surface markers by the iDCs following transduction with Ad vectors.

In the past, some DC vaccines (18,19) for lung cancer such as the DC vaccine modified with full-length wild-type p53 (wt-p53) gene were attempted (20). Normal cells have very low levels of p53, whereas accumulation of this protein due to mutations or functional inactivation occurs in nearly 50% of human malignancies, including lung cancer. Nikitina *et al* (21) reported that dendritic cells transduced with the full-length wt-p53 gene using an adenovirus vector could generate a specific antitumor immune response. However, over-expression of the wt-p53 gene may itself induce apoptosis of DCs because of its proapoptotic function and this may decrease the constitutive transgene expression and in turn shorten the resulting immune responses. Furthermore, p53 is expressed at low levels in all normal cells and

therefore may not be an appropriate target for immunotherapy (20). In contrast, the DNp73 $\alpha$ , a known antiapoptotic protein, through its absent or limited expression in normal cells and abundance in cancers offers a better choice for immunotherapeutic treatments.

The successful generation of CTLs *in vitro* with Ad-DNp73 $\alpha$ -transduced DCs was verified by the CTL lytic activity against DNp73 $\alpha$ -expressing human tumor cell lines, A549/DNp73 $\alpha$  and K-562 but not in the DNp73 $\alpha$  null MCF-7 cells. In comparison, the lymphokine-activated killer cells effectively lysed all three cell lines, lending additional support that the effector cells for the CTL assay were DNp73 $\alpha$ -specific. However, tumor production of immunosuppressive factors (cytokines, arachidonic acid metabolites, glycosphingolipids, polyamines) with detrimental effects on DC maturation and function can significantly prevent antitumor immune responses (22). It was reported (23,24) that tumor-infiltrating DCs are neither mature nor activated and blood dendritic cells in breast cancer patients express low levels of co-stimulatory molecules and IL-12 along with an impaired capacity to stimulate T-cells. The *in vivo* longevity of DCs was recognized as a necessary factor for priming and maintaining effective antitumor immune responses and one way to achieve this is to prevent the apoptotic pathways in the DCs (25). Transduction of anti-apoptotic genes into DCs is likely to be a fruitful strategy in this regard.

The DNp73 $\alpha$  is capable of suppressing apoptosis in various cell types by counteracting p53/p73-mediated tumor cell death (26). This antiapoptotic effect was also evident in Ad-DNp73 $\alpha$ -transduced DCs which had higher survival rates at the end of 12 days compared with the Ad-EGFP-transduced DCs. Inhibition of apoptosis in DCs can lead to an accumulation of mature DCs, and facilitate an augmented T cell priming and induction of higher T cell proliferation (27). This notion is consistent with a recent observation (28) that CD40 stimulation can induce increased DC survival and IL-12 secretion thereby promoting IFN- $\gamma$  production by T-helper cells and tumor-specific cytotoxic responses. The data strongly support the utility and potential of DNp73 $\alpha$ -modified-DCs for cancer immunotherapy.

Taken together, our results demonstrate that DNp73 $\alpha$ -engineered DCs can induce specific CTL against tumor cells that express DNp73 $\alpha$ . Expression of this antiapoptotic gene enhanced the survival of DCs accompanied by potent and specific antitumor immune responses. Our findings suggest DNp73 $\alpha$ -engineered DCs may have applications as a vaccine against lung cancer and preclinical studies in this direction are underway.

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