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ORIGINAL ARTICLE

## Immunization with truncated sequence of Telomerase Reverse Transcriptase induces a specific antitumor response *in vivo*

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### Abstract

To select the MHC-I-binding epitope-rich sequence of mice telomerase reverse transcriptase (mTERT) and study the antitumor immune response induced by truncated TERT through mRNA-transfected dendritic cells (DCs) immunization in mice. The MHC-I-binding epitopes of TERT were predicted using bioinformatics software. The selected sequence of TERT (Truncated mTERT, TERT<sup>t</sup>, mTERT cDNA 1776 bp–2942 bp encoding 584 aa–969 aa) was cloned from B16 mouse melanoma cells and inserted into pBluescriptIIKS(+) plasmid downstream of the T7 promoter. TERT<sup>t</sup> RNA was prepared through *in vitro* transcription. Bone marrow-derived DCs were electroporated with TERT<sup>t</sup> RNA and used to immunize syngeneic naïve mice. The quantity and cytotoxic activity of TERT-specific cytotoxic T lymphocytes (CTLs) in mice spleen were evaluated using IFN- $\gamma$  enzyme-linked immunospot (ELISPOT) and Lactate dehydrogenase release assay. The immunoprophylactic effects against TERT positive tumor induced by TERT<sup>t</sup> RNA transfected DC *in vivo* were evaluated through an immunized-challenged mouse model. TERT<sup>t</sup> was cloned and *in vitro* transcribed into TERT<sup>t</sup> mRNA. As shown in FCM analysis, the efficiency of DC electroporation is 35.1% (29.7–41.2%). After electroporation, a subtle increase of costimulator and MHC-II molecules were expressed on the cell surface. Immunization of TERT<sup>t</sup> mRNA transfected DCs induced IFN- $\gamma$ -secreting CTLs which manifested specific cytotoxic activity against TERT-positive target cells. In a cancer mouse model, vaccination of TERT<sup>t</sup> mRNA-transfected DCs suppressed the growth of TERT positive tumors ( $p=0.001$ ) and prolong the survival time of tumor-bearing animals ( $p=0.029$ ). TERT<sup>t</sup> evokes an antitumor immune response *in vivo* which is targeted to TERT. TERT<sup>t</sup> can be used as an antigenic sequence to produce anti-TERT tumor vaccine.

Telomeres are nucleoprotein complexes which cap the termini of all eukaryotic chromosomes and play essential roles in the maintenance of chromosomal integrity and cell viability. Telomerase, a specialized reverse transcriptase, synthesizes G-rich simple repeat sequences of telomeres *de novo* and maintains telomere length after each cell division cycle [1,2]. Reports of high telomerase activity in immortalized and cancer cells has led to the hypothesis that telomerase reactivation represents a critical step in the neoplastic process [3,4]. Expression of telomerase in human cancer is directly linked to tumor growth and development, whereas inhibition of telomerase in telomerase-positive human tumors leads to growth arrest [5,6]. Telomerase reverse

transcriptase (TERT) is the rate-limiting component of enzyme telomerase. It was reported that hTERT levels are nearly undetectable in normal human cells, but are present in human tumor cell lines at 100-fold higher levels. As TERT amplification plays an important role in malignant transformation [7–10], TERT is seen as an attractive candidate target for tumor therapy.

Vonderheide et al. [11,12] identified the TERT-derived HLA-A2-binding epitope I540 (ILAKF-LHWL) which triggers specific CD8<sup>+</sup> CTLs to kill a wide range of hTERT<sup>+</sup> tumors. He suggested that hTERT may be seen as a universal tumor associated antigen. There are constitutive expressions of telomerase RNA template in APC and the stable

transfection of TERT might activate telomerase in APCs. Nair and his colleagues [13] transfected dendritic cells with whole long TERT RNA and found that immunization of this DC vaccine can stimulate cytotoxic T lymphocytes (CTL) which lysed TERT+ tumor cells and inhibited the growth of TERT+ tumors in animals. As we well know, it is difficult to preserve RNA and transfect DC in a large scale because of the universal presence of RNAase in environment. Can we find an antigenic epitope-rich sequence in TERT? This sequence not only can serve as multivalent antigen but also can be manipulated conveniently in clinical setting. In our present study, using MHC-I Peptide Binding Predictions [14,15], we retrieved the sequence of mTERT and found that there is accumulation of antigenic epitopes in the C-terminal part of TERT. We wanted to determine if immunization with this sequence can lead to immunoprophylactic effects against TERT-positive tumors. In the present study, we cloned truncated TERT (TERT<sup>t</sup>) cDNA into a plasmid vector and then performed *in vitro* transcription to obtain TERT<sup>t</sup> RNA. We transfected bone marrow-derived DC with TERT<sup>t</sup> RNA and studied the immune effects of this TERT sequence.

## Materials and methods

### Epitope prediction

The mouse TERT motif was loaded to predict MHC-I (H-2<sup>d</sup> and H-2<sup>b</sup>) restricted CTL epitopes using [http://bimas.dcrt.nih.gov/molbio/hla\\_bind/](http://bimas.dcrt.nih.gov/molbio/hla_bind/). H-2D<sup>d</sup>, H-2K<sup>d</sup>, H-2D<sup>b</sup> and H-2K<sup>b</sup> were selected as the MHC-I molecule analysis options. Predicted epitopes were limited to nonamer length.

### Mice and cell lines

Six to 8-week-old female BALB/c mice (H-2<sup>d</sup>) and C57BL/6 mice (H-2<sup>b</sup>) were purchased from Laboratory Animal Research Center (LARC) of the Fourth Military Medical University (FMMU, Xi'an) and housed under pathogen-free conditions (License number: SCXK2002-005). All experiments involving the use of mice were performed in accordance with protocols approved by LARC. CT26 is a carcinogen-induced undifferentiated colon adenocarcinoma cell line from BALB/c mice. B16 is a mouse melanoma cell line from C57BL/6. Cells were maintained in RPMI-1640 medium (Gibco, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS; Sijiqin Biotech, HangZhou), 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37°C, 50 ml/l CO<sub>2</sub> and passaged every 2 days.

CT26 cells were transfected with pcDNA3.1(+)-mTERT<sup>t</sup> or pcDNA3.1(+)-hMAGEn by using

Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The transfected CT26 cells were selected with 800 µg/ml G418 (Gibco, Carlsbad, CA) in culture medium and subcloned by limiting dilution assay in 96-well plates. The expanded cell clones were examined by PCR and RT-PCR using mTERT<sup>t</sup> or hMAGEn primers. The selected cell clones CT26-TERT<sup>t</sup> and CT26-hMAGEn were confirmed by immunofluorescence assay with anti-TERT antibody (BA0564, Boster Biotech) and anti-hMAGEn mouse serum, respectively.

### Clone of TERT<sup>t</sup> and *in vitro* transcription assay

RNA was isolated from B16 cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA). Total tumor RNA (1 µg) was reverse transcribed in a final volume of 20 µl using ThermoScript RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was primed with 1 µl of 50 mµ oligo-dT primer and the reaction was incubated at 55°C for 60 min. A total of 2 µl RT product was used to perform PCR reaction with TERT<sup>t</sup> primer: sense 5'-GACGAATTCGACATG-GAGAGAGTGC GGCTACGGGAG-3', antisense 5'-CACGTCGACCTACGACAGGAGCTTGTTCCGCAT-3' (underline indicates the endonuclease cutting sites and start /end codon). The RT-PCR results were analyzed by 1% agarose gel electrophoresis and cloned into pBluescriptIISKS(+) (Promega) between *Eco*RI and *Sal*I to construct pBluescriptIISKS(+)-TERT<sup>t</sup>. The sequence of TERT<sup>t</sup> was confirmed by DNA sequencing (Bioasia Inc.). The *Sac*I-*Not*I fragment containing the EGFP coding region was isolated from pEGFP1 (Clontech, CA) and subcloned into the *Sac*I-*Not*I site of pBluescriptIISKS(+) downstream of the T7 promoter to construct pBluescriptIISKS(+)-EGFP. The *Eco*RI-*Xho*I fragment encoding whole length of human MAGE-n [16] was isolated from pcDNA3.1(+)-hMAGEn and used to construct pBluescriptIISKS(+)-hMAGEn.

The reconstructed plasmids were linearized to serve as DNA templates for *in vitro* transcription (IVT). IVT was performed by using mMessage mMachine T7 Ultra kit (Ambion, USA) according to the manufacturer's instructions. IVT RNA was purified by DNase I digestion followed by LiCl precipitation and 70% ethanol wash. For each experiment, at least three different batches of mRNA were used. RNA quality was confirmed by agarose-formaldehyde gel electrophoresis. RNA concentration was assayed by spectrophotometric analysis at OD<sub>260</sub>. RNA was stored at -80°C in 1 µg/µl aliquots.

#### *Electroporation of murine bone marrow-derived DCs with RNA*

Mouse bone marrow-derived DCs were generated as described by Lutz et al. [17] with minor modifications [18]. The harvest DCs were enumerated and resuspended in PBS at  $5 \times 10^6$  cells/ml. Prior to electroporation, DCs were washed twice with serum-free Opti-MEM and resuspended to a final concentration of  $10^7$  cells/ml in Opti-MEM (Gibco BRL). A total of 0.5 ml of the DC suspension was mixed with 10  $\mu$ g of IVT RNA and electroporated in a 0.4 cm-gap sterile disposable cuvette using the Gene Pulser<sup>®</sup> Electroporation System (Bio-Rad, USA). Cells were transfected with a voltage pulse of 300 V, with a capacitance of 150  $\mu$ F for 6 milliseconds [19]. The BALB/c or C57BL/6 derived DCs were electroporated with no RNA, TERT<sup>t</sup> mRNA or hMAGEn mRNA respectively, so as to generate blank DC, TERT<sup>t</sup>-DC and hMAGEn-DC. DCs were also electroporated with EGFP mRNA to evaluate the transfection efficiency. After electroporation, fresh complete medium was added to the cell suspension immediately and cells were further incubated at 37°C in a humidified atmosphere supplemented with 50 ml/l CO<sub>2</sub> for 24 h. The expression of costimulator CD86 and MHC-I molecule were analyzed by FCM (FACScan, Becton Dickinson) to confirm the phenotypic changes of DCs before and after electroporation. EGFP-transfected cells were checked for EGFP expression 24 h after electroporation by using flow cytometry (FCM) analysis. For EGFP analysis in DC cultures, CD11c positive cells were gated and then evaluated for EGFP expression.

#### *Immunization-challenge assay*

Naïve syngeneic mice were immunized by i.v. DC vaccine (blank DC, TERT<sup>t</sup>-DC, or hMAGEn-DC) through the tail vein at day 0 and day 7 with  $5 \times 10^5$  DCs in 100  $\mu$ l PBS. Seven days after second immunization, splenocytes of immunized mice were harvested and depleted of RBCs with ammonium chloride Tris buffer and were used as the effectors to perform ELISPOT and lactate dehydrogenase release assays [20]. For tumor challenge experiment,  $1 \times 10^4$  B16 cells were inoculated into the back of C57BL/6 mice subcutaneously and every group contained seven mice. The tumor volume was calculated by using the standard formula:  $V = a \times b^2 / 2$ , where 'a' is the longest superficial diameter and 'b' is the shortest superficial diameter. Those were measured with vernier calipers at the 14th day after tumor cell inoculation. The mice were all fed to observe the tumor-bearing survival time.

#### *Immunofluorescence assay*

CT26 cell crawling cover slips were prepared in 6-well cell culture plate. The cells were fixed with cold methanol at -20°C for 5 min and permeabilized with 0.1% TritonX-100 in PBS for 5 min. Cells were blocked with 3% BSA in PBS for 30 min in a humidified chamber. After incubation in primary antibody (diluted 1:50) for 1 hour, the cells were incubated in 1:150 diluted FITC-conjugated secondary antibody for 1 hour. After mounting the coverslips with buffered glycerol, the cells were examined with fluorescence microscopy.

#### *ELISPOT analysis*

The murine interferon-gamma ELISPOT kit (Diaclone, France) was used to determine tumor-specific, IFN- $\gamma$  secreting T cells [21]. 96-well filtration plates (Nunc, Roskilde, Denmark) were coated with 100  $\mu$ l capture antibody (clone DB1). After an overnight incubation at 4°C, the wells were washed and blocked with 2% dry skimmed milk in PBS. A total of  $1 \times 10^6$  splenocytes isolated from the mice were added to the wells and incubated at 37°C, 50 ml/CO<sub>2</sub> for 20 h with  $5 \times 10^4$  target cells (B16, CT26, CT26-TERT<sup>t</sup> and CT26-hMAGEn respectively) which had been pretreated with 50 mg/l mitomycin C in RPMI 1640 for 30 min. Plates were washed and incubated with 100  $\mu$ l biotinylated polyclonal antibody at 37°C for 90 min. After the removal of unbound antibodies, 100  $\mu$ l avidin-alkaline phosphatase was added and plates were incubated for 1 h at 37°C. After washing, spots were developed by adding 100  $\mu$ l of ready-to-use BCIP/NBT buffer and incubated at room temperature for spot formation. The spots were scanned and counted.

#### *Lactate dehydrogenase release assay*

Specific cytolytic activities of murine splenocytes were determined by LDH release assay. The CT26, CT26-TERT, CT26-hMAGEn and B16 were used as target cells respectively. Effectors and target cells were mixed at serial E/T ratios of 5:1, 10:1, 20:1, 40:1 at 0.2 ml/well in 96-well round-bottomed plates (Nunc, Roskilde, Denmark). After incubation for 4 h, cells were centrifuged at 500 r/min and the cell-free supernatant was collected for LDH assay using the CytoTox96 kit (Promega, Madison, WI, USA). The percentage of specific LDH release was calculated by the following formula: % cytotoxicity = [(experimental LDH release) - (spontaneous LDH release by effector or target cells) / (maximum LDH release)  $\times$  (spontaneous LDH release)]  $\times$  100. For

Table I.

| Rank                  | Start position | Subsequence residue listing | Score (Estimate of half time of disassociation of a molecule containing this subsequence) |
|-----------------------|----------------|-----------------------------|-------------------------------------------------------------------------------------------|
| H2-Dd binding epitope |                |                             |                                                                                           |
| 1                     | 760            | LQPYMGQFL                   | 20.000                                                                                    |
| 2                     | 822            | QGIPQGSSL                   | 20.000                                                                                    |
| 3                     | 899            | NFPVEPGTL                   | 20.000                                                                                    |
| 4                     | 826            | QGSSLSTLL                   | 20.000                                                                                    |
| 5                     | 920            | LFPWCGLLL                   | 20.000                                                                                    |
| 6                     | 907            | LGGAAPYQL                   | 20.000                                                                                    |
| H2-Kd binding epitope |                |                             |                                                                                           |
| 1                     | 627            | SYSMGTRAL                   | 2400.000                                                                                  |
| 2                     | 612            | RFIPKPNGL                   | 2304.000                                                                                  |
| 3                     | 862            | DFLLVTPHL                   | 1920.000                                                                                  |
| 4                     | 682            | AFVLRVRAL                   | 1920.000                                                                                  |
| 5                     | 838            | CFGDMENKL                   | 1382.400                                                                                  |
| 6                     | 749            | SFRRQVTTL                   | 960.000                                                                                   |
| 7                     | 643            | HFTQRLKTL                   | 800.000                                                                                   |
| 8                     | 899            | NFPVEPGTL                   | 800.000                                                                                   |
| 9                     | 920            | LFPWCGLLL                   | 800.000                                                                                   |
| 10                    | 798            | LFDFFLHFL                   | 800.000                                                                                   |
| 11                    | 858            | RFVDDFLV                    | 691.200                                                                                   |
| 12                    | 676            | IYRTWRAV                    | 500.000                                                                                   |
| H2-Db binding epitope |                |                             |                                                                                           |
| 1                     | 777            | SALRNSVVI                   | 5588.352                                                                                  |
| 2                     | 668            | SSVLGMNDI                   | 86.619                                                                                    |
| 3                     | 652            | FSMLNYERT                   | 81.331                                                                                    |
| 4                     | 790            | SMNESSSSL                   | 52.557                                                                                    |
| 5                     | 752            | RQVTTLSDL                   | 21.470                                                                                    |
| H2-Kb binding epitope |                |                             |                                                                                           |
| 1                     | 882            | HGVPEYGCM                   | 24.000                                                                                    |
| 2                     | 834            | LCSLCFGDM                   | 11.000                                                                                    |
| 3                     | 937            | CDYSGYAQT                   | 10.000                                                                                    |

the controls, the target cells were incubated either in culture medium alone to determine spontaneous release or in a mixture of 2% Triton X-100 to define the maximum LDH release. The spontaneous release was always <10% of the maximum release. All assays were performed in triplicate.

### Statistical analysis

All experiments were repeated at least three times. Data were expressed as mean  $\pm$  SE. Tumor volume and immune cell yield data were analyzed by one-way analysis of variance (ANOVA). All analyses were conducted with SPSS8.0 software. Values of  $p < 0.05$  were considered statistically significant.

## Results

### MHC-I-binding epitopes of TERT

The nonamer H-2D<sup>d</sup>, H-2K<sup>d</sup>, H-2D<sup>b</sup> and H-2K<sup>b</sup> binding epitopes of C-terminal part of mouse telomerase reverse transcriptase were predicted as shown in Table I.

### Cloning and IVT of TERT<sup>t</sup>

The expression of TERT<sup>t</sup> (mTERT cDNA 1776 bp-2942 bp encoding 584 aa-969 aa) can be detected in B16 cells but not in CT26 cells as shown in Figure 1A. TERT<sup>t</sup> cDNA was cloned from B16 cells using RT-PCR and pBluescriptIIKS(+)-TERT<sup>t</sup> was constructed. Using linearized pBluescriptIIKS(+)-TERT<sup>t</sup> as template, *in vitro* transcription was performed to generate TERT<sup>t</sup> RNA (Figure 1B&C). pBluescriptIIKS(+)-EGFP and pBluescriptIIKS(+)-hMAGEn were also constructed, linearized and the EGFP mRNA and MAGEn mRNA were all generated through *in vitro* transcription. DNA sequencing confirmed that the sequence of TERT<sup>t</sup> completely matches to the corresponding sequence of mouse TERT cDNA (NM 009354).

### RNA electroporation of DC

DC showed higher EGFP expression after EGFP RNA electroporation. FCM analysis directly shows

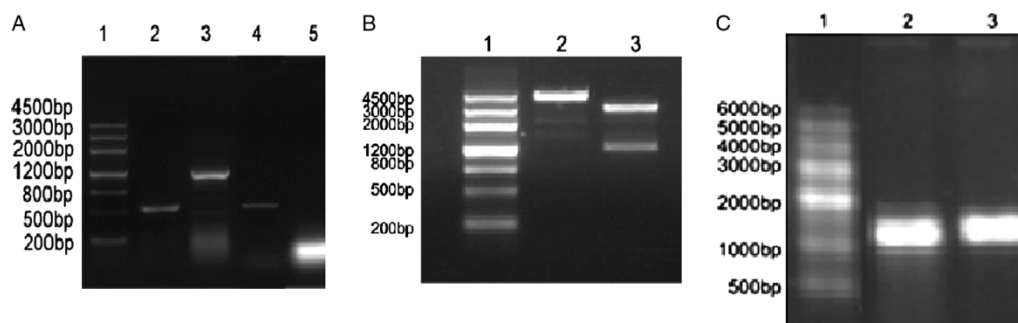


Figure 1. (A) The expression of TERT<sup>t</sup> in B16 and CT26 cells. 1: DNA marker I; 2: RT-PCR product of -actin in B16 cells; 3: RT-PCR product of TERT<sup>t</sup> in B16 cells; 4: RT-PCR product of -actin in CT26 cells; 5: RT-PCR result of CT26 cells using TERT<sup>t</sup> primers. (B) Linearization of pBluescriptIIKS(+)-TERT<sup>t</sup>: 1. DNA marker I 2. Linearized pBluescriptIIKS(+)-TERT<sup>t</sup> template 3. Double enzyme digestion of pBluescriptIIKS(+)-TERT<sup>t</sup>. (C) In vitro transcription of TERT<sup>t</sup> mRNA: 1. RNA marker 2. Capped TERT<sup>t</sup> mRNA 3. Capped TERT<sup>t</sup> mRNA with poly-A tail.

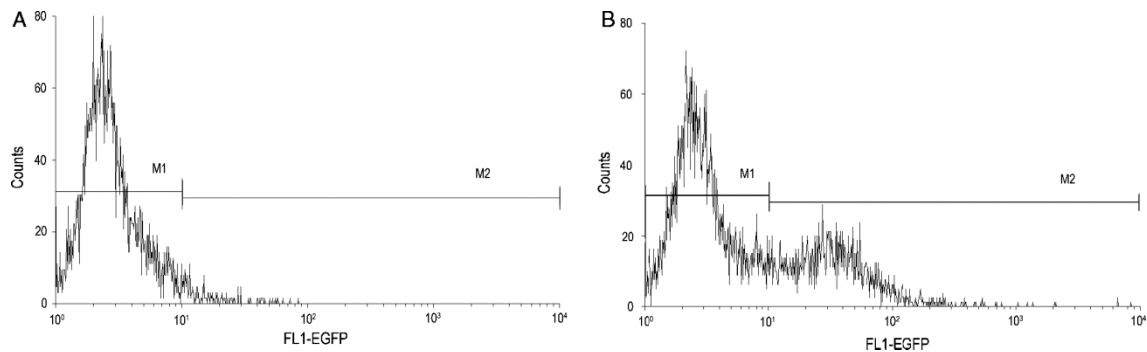


Figure 2. DCs were electroporated with EGFP mRNA to evaluate the transfection efficiency. Post EGFP mRNA electroporation 24h, EGFP expression can be detected in CD11c<sup>+</sup> DCs using FCM analysis. (A) The DCs of 8th day culture system showed no EGFP expression. (B) After EGFP mRNA electroporated for 24h, The DCs of 7th day culture system showed EGFP expression.

that the EGFP RNA transfection efficiency of CD11c<sup>+</sup> DC is as much as 35.1% (29.7–41.2%, five different electroporation) at 24 h post electroporation (Figure 2). DC surface molecules were measured by staining with PE conjugated-anti-mouse CD11c (N418, hamster IgG), FITC conjugated-anti-mouse I-A/I-E (m5/114.15.2, ratIg-G2b), FITC conjugated-anti-mouse CD86 (B7.1, PO3, rat IgG2b) and analyzed by FACS. There was little increase of CD86 and I-A/I-E expression at 24 h after TERT<sup>t</sup>-RNA electroporation. After electroporated with TERT<sup>t</sup> mRNA 24 h, the TERT<sup>t</sup> expression in DCs can be detected using immunofluorescence assay as showed in Image 1.

#### *TERT<sup>t</sup>-DC induced TERT-specific CTL response*

B16 is a natural TERT positive tumor cell line of C57BL/6 mouse. To investigate whether TERT<sup>t</sup>-DC have the potential to induce TERT-targeting anti-

tumor effect, the splenocytes of different DC vaccine immunized mice were isolated to perform an IFN- $\gamma$ ELiSpot experiment. B16 cells can stimulated a large number of IFN- $\gamma$ -secreting T cells in TERT<sup>t</sup>-DC-immunized mice, but not in blank DC or hMAGEN-DC immunized mice. Compared to blank DC and hMAGEN-DC, TERT<sup>t</sup>-DC primed splenocytes displayed the strongest cytotoxic activity against B16 cells (Figure 3).

Primary CT26 cells showed undetectable TERT activity, we further transfected CT26 cells with pcDNA3.1(+)-TERT<sup>t</sup> to generate a CT26-TERT<sup>t</sup> cell line. After stimulated with target cells for 24 h, splenocytes derived from TERT<sup>t</sup>-DC-immunized mice only manifested IFN- $\gamma$ secreting response against CT26-TERT<sup>t</sup> cells but not CT26 or CT26-hMAGEN cells. Furthermore, splenocytes of TERT<sup>t</sup>-DC-immunized mice displayed strong cytolytic activity against CT26-TERT<sup>t</sup> at an E:T ratio of 10:1, 20:1 and 40:1 (Figure 4A&B).



**Image1:** The TERT<sup>t</sup> expression can be detected in Dendritic cells by using immunofluorescence assay 24 hours post TERT<sup>t</sup> mRNA electroporation. (First antibody: rabbit anti mouse TERT; Second antibody: FITC-goat anti rabbit IgG).

#### *Vaccination of TERT<sup>t</sup>-DC suppress TERT positive tumor growth in vivo*

We examined whether TERT<sup>t</sup>-DC could induce TERT-targeting specific immunoprophylactic effects *in vivo* in an immunization-challenge mouse model. After being vaccinated with blank DC, TERT<sup>t</sup>-DC or hMAGEN-DC, respectively twice at an interval of 7 days, the mice received B16 cells inoculation subcutaneously. At day 14 post tumor inoculation, the tumor volume in blank DC vaccination group, TERT<sup>t</sup>-DC vaccination group and hMAGEN-DC vaccination group were  $4456.4 \pm 378.6 \text{ mm}^3$ ,  $2121.4 \pm 381.1 \text{ mm}^3$  and  $3996.0 \pm 243.9 \text{ mm}^3$  respectively. ANOVA analysis showed that there were significant differences in tumor volume among these three groups ( $p=0.001$ ). The B16 tumor volume in the TERT<sup>t</sup>-DC vaccination group is smaller than other two groups. The median

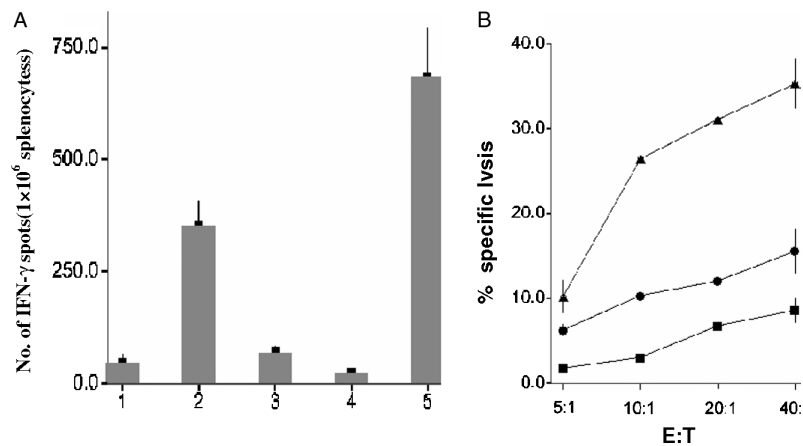


Figure 3. TERT<sup>t</sup> mRNA transfected DCs immunization can induce B16 specific CTLs in C57B/L mice. (A) TERT<sup>t</sup> mRNA transfected DC immunization can induce IFN- $\gamma$  secreting specific Splenocytes responding to Mitomycin C treated B16 cells. 1: Splenocytes of Blank DC immunized mice; 2: Splenocytes of TERT<sup>t</sup>-DC immunized mice; 3: Splenocytes of hMAGEn-DC immunized mice; 4: Negative control (Splenocytes of Blank DC immunized mice, no target). 5: Positive control (Splenocytes of Blank DC immunized mice, no target, 1mg/L PHA stimulated). (B) Splenocytes obtained from TERT<sup>t</sup> mRNA transfected DCs immunized mice showed escalated cytotoxic activity against Mitomycin C treated B16 cells at E:T ratios indicated. ▲: Splenocytes of TERT<sup>t</sup>-DC immunized mice; ●: Splenocytes of Mn-DC immunized mice; ■: Splenocytes of Blank-DC immunized mice. (Values are the mean  $\pm$  SD of triplicate wells).

tumor-bearing survival times of the blank DC, TERT<sup>t</sup>-DC, and hMAGEn-DC vaccination groups were 24 d, 31 d, and 25 d, respectively. Log-rank test showed that the median survival time was different in the three group of mice ( $p=0.029$ ). The mice vaccinated with TERT<sup>t</sup>-DC showed longer survival time than those vaccinated with blank DC or hMAGEn-DC (Figure 5).

## Discussion

In recent years, TERT has been used as a universal tumor-associated antigen to evoke antitumor immune responses. Using a bioinformatic prediction scoring system, we predicted the 9-mer MHC-I binding epitopes of murine TERT and revealed that c-terminal partial sequence of mTERT holds many antigenic epitopes which can be presented through MHC-I pathway (H-2<sup>d</sup> or H-2<sup>b</sup>) by APCs. It's reported that RNA-loaded DC is a rapid and efficient measure to study the potential of tumor associated antigens to stimulate MHC-restricted anti-tumor immunity [22–26]. In our present study, we cloned truncated TERT (TERT<sup>t</sup>) which encodes 584aa–969 aa of mTERT and evaluated the antigenic nature of truncated TERT using TERT<sup>t</sup> mRNA-transfected DC.

We cloned the TERT<sup>t</sup> cDNA and transfected DC with *in vitro* transcribed TERT<sup>t</sup> mRNA using electroporation. Using EGFP as an indicator, RNA electroporation efficiency of DC is estimated as 35.1%. We found a slight increase of MHC-I and CD86 expression of DCs post electroporation, which is similar to a result of Kalady's study. Since tumor-specific CTLs are the most important effec-

tors in the antitumor response, we evaluated the specific recognition and cytolytic characteristics of mice splenic CTLs primed with TERT<sup>t</sup> RNA-transfected DC. After immunizing twice with TERT<sup>t</sup> RNA-transfected DC, the mice splenocytes showed targeting recognition and cytolytic activity to TERT-positive B16 cells. Furthermore, the splenocytes only responded to and lyse TERT<sup>t</sup> transfected CT26 cells but not TERT negative CT26 cells. We also tested the *in vivo* immunoprophylactic effects of TERT<sup>t</sup> RNA-transfected DC using a vaccination-challenge model. We showed that pre-immunization with TERT<sup>t</sup> RNA-transfected DC inhibit the growth of B16 tumor and prolongs the tumor-bearing survival time of B16-challenged mice. Therefore, we conclude TERT<sup>t</sup> can evoke a TERT-targeting antitumor immune response in *in vivo* and TERT<sup>t</sup> might be used as an antigenic sequence to produce anti-TERT tumor vaccine.

It has been exhibited that mice express a higher basal level of telomerase activity in normal tissues compared to humans. Constitutive expression of TERT in mice might trigger a certain degree of immunotolerance to TERT vaccination. In humans, TERT expression is about 100-fold higher in tumor tissue than normal somatic tissue [27]. The absence or lower concentration of basal TERT expression in human tissue may not trigger tolerance as much as in mouse. Thus, truncated TERT may have more potential in stimulating anti-TERT effects in humans. Further studies should be done to determine whether this truncated TERT vaccine could be pursued for humans.

It will be necessary to compare truncated TERT with the residual TERT and whole long TERT for

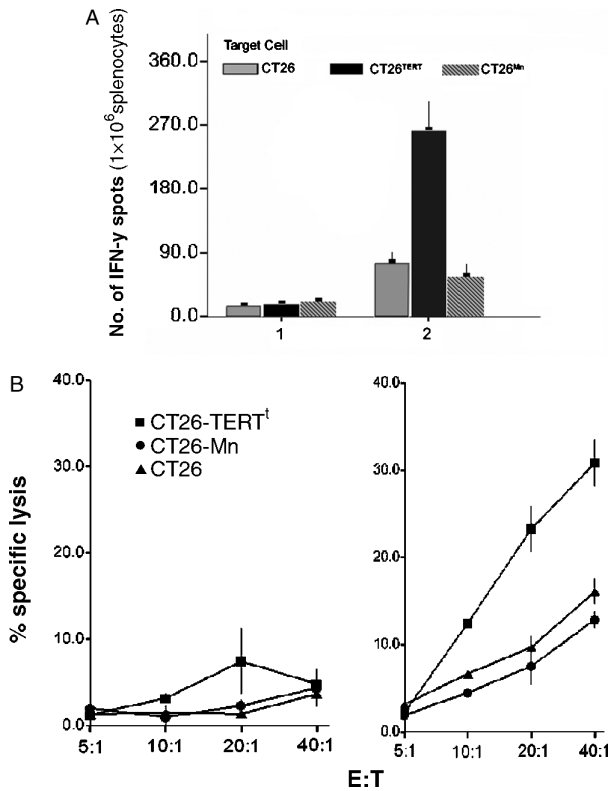


Figure 4. (A) Primary CT26 cells showed undetectable TERT activity. After stimulated with target cells for 24 hours, splenocytes derived from TERT<sup>+</sup>-DC immunized mice only manifested IFN- $\gamma$  secreting response against CT26-TERT<sup>+</sup> cells but not CT26 or CT26-hMAGEn cells. 1: Splenocytes of Blank DC immunized mice; 2: Splenocytes of TERT<sup>+</sup>-DC immunized mice; (Values are the mean  $\pm$  SD of triplicate wells). (B) TERT<sup>+</sup> mRNA transfected DC immunization can generate TERT<sup>+</sup> specific CTLs in BALB/c mice *in vivo*. Splenocytes of TERT<sup>+</sup>-DC immunized mice displayed strong cytolytic activity against CT26-TERT<sup>+</sup> at E:T ratio of 10:1, 20:1 and 40:1. Left: Splenocytes of Blank DC immunized mice; Middle: Splenocytes of TERT<sup>+</sup>-DC immunized mice. (Values are the mean  $\pm$  SD of triplicate wells).

evoking antitumor immune responses. TERT<sup>+</sup> can be conjugated with other immune enhancing sequences such as endosomal targeting motifs to improve presentation by APCs, or co-transfected with other antigenic sequences to extend the anti-tumor spectrum. It can also be loaded to APCs through reconstructed adenovirus transfection to facilitate the MHC-I pathway epitope presentation. Since TERT is activated in proliferating cells such as activated T and B cells, hematopoietic progenitors, germ cells, intestinal and liver cells [28,29], pathological observations should be performed in TERT<sup>+</sup>-DC immunized mice to ensure that no autoimmune responses are present in these tissues and organs.

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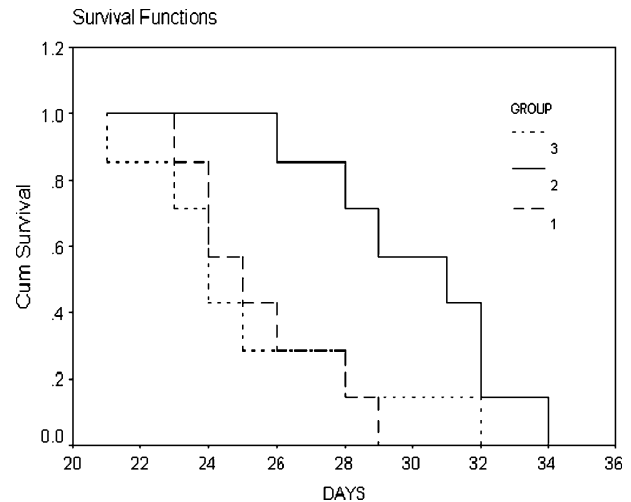


Figure 5. The Survival curve of different mRNA transfected DCs immunized/ B16-challenged mice. Log-rank test showed that the median survival time was different in these three groups of mice ( $p=0.029$ ). The mice vaccinated with TERT<sup>+</sup>-DC showed longer survival time than those vaccinated with Blank DC or hMAGEn-DC. 1: Blank DC immunized mice with B16 challenged; 2: TERT<sup>+</sup>-DC immunized mice with B16 challenged; 3: hMAGEn-DC immunized mice with B16 challenged.

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