

Study on the functions and mechanism of immune functions of human telomerase reverse transcriptase regulating dendritic cells treating sepsis

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Abstract. – **OBJECTIVE:** We analyzed the functions and mechanisms of immune functions of human telomerase reverse transcriptase regulating dendritic cells (DC) treating sepsis of mice models.

MATERIALS AND METHODS: Eighty clean grade Balb/c animals aged from 6 to 8 weeks, weighted from 18 g to 22 g were selected for this study. The DC cells were harvested from the animals and cultivated to transfect with the recombinant eukaryotic expression plasmid hTERT-IRES2-EGFP construct. The LPS (E. coli 0111:B4, 5 mg/kg) was injected into the abdominal cavity of mice to establish sepsis models. Afterwards, animals were divided randomly into the sepsis group (A group), the group of hTERT transfecting DC (B group), the group of DC un-transfected (C group) with 25 mice in each group. 5 mice were in the normal control group (D group), without any treatment. An equivalent volume of normal saline was injected into the abdominal cavity of A group. Subsequently, 1 ml of cell suspension (105/ml) was transfected into B and C groups respectively. Five animals from A, B, C groups and one animal from group D were sacrificed after 24h, 48h, 72d, 7d and 10d respectively.

RESULTS: It was found that median survival time of the group of hTERT transfecting DC was remarkably higher than that of the untransfected group and the sepsis group. The average scores of the pathology of kidney and intestine at each time were significantly lower than that of the other two groups ($p < 0.05$). At each time point, in the group of hTERT transfecting DC, levels of CRP and Cr were remarkably lower than that of the other two groups; HLA-DR, CD40 of immune phenotype and the expression level of peripheral blood T cells MHC-II molecules were significantly higher than that of the other two groups; the expression level of IL-12 and TNF- α were sig-

nificantly lower than that of the other two groups; apoptosis rate of DC were significantly lower than that of the other two groups; the content and activity of NF- κ B were significantly higher than that of the other two groups ($p < 0.05$).

CONCLUSIONS: The telomerase reverse transcriptase gene can raise the expression and maturity of DC, reduce apoptosis, induce cytokine secretion, reduce the inflammatory response and increase the survival time.

Key Words:

Telomerase, Reverse transcriptase gene, Dendritic cells, Sepsis.

Introduction

Sepsis is characterized by dangerous disease condition, high mortality rate and expensive treatment cost, whose incidence is 700-2400/100 thousand every year. It is increasing every year, and it is estimated that by 2020 there will increase newly one million cases of sepsis¹. Currently, our understanding of the mechanisms of sepsis has changed from simple “excessive inflammatory response” to more “excessive immune suppression” to participate in the composition², in which the apoptosis of dendritic cells (DC) may play a role of middle bridge in sepsis immune disorders³. DC is the antigen presenting cells with the strongest function in the body, which activates the initial type of T cells as well. DC is the key factor to start and adjust the innate and acquired immune response⁴. Many studies confirmed^{5,6} that the process of DC decreasing synchronizes with the oc-

currence of sepsis and has continuous influence, which is closely related to mortality rate. Then, exogenous injections of DC can increase the immune level and decrease mortality rate⁷. Therefore, it is reasonable to believe that the prevention of apoptosis of DC can reverse the process of immune suppression in sepsis and improve the immune function of the body, which may become a new feasible method for the prevention and treatment of sepsis.

The discovery of telomeres and telomerase provides a promising prospect for cell immortality; the discovery has important significance for raising and keeping telomerase activity of the cells, increasing cell replication *in vitro*, expanding ability and preventing cell apoptosis⁸. Based on it, this study explored the hTERT gene modifying DC targeting the therapy for sepsis and observed its influence on immune function.

Materials and Methods

Materials

Eighty clean grade Balb/c male mice aging from 6 to 8 weeks old and weighing from 18 to 22 g, were purchased from Shanghai S & P-Bikai Laboratory Animal Co. Ltd, China. The animals were kept in an environment of room temperature of 22±0.5°C, relative humidity of 50±5%, with 12h light and 12h dark cycles, with unlimited access to food and water.

Main Reagents and Instruments

Sugar fatty LPS (*E. coli* 0111:B4) purchased from Sigma-Aldrich (St. Louis, MO, USA), mouse anti-human HLA-DR labeled by FITC, CD40 monoclonal antibody (Biolegend, San Diego, CA, USA), CD11c antibody of anti-mouse labeled by PE and MHC-II of anti-mouse labeled by CY5 purchased from BD Biosciences (Franklin Lakes, NJ, USA), in situ cell apoptosis detection kit (S7111; Chemicon International, Billerica, MA, USA), TUNEL in situ cell apoptosis detection kit labeled by FITC (Chemicon International, Billerica, MA, USA), EMSA kit (Pierce, Rockford, IL, USA), biotin labeled the NF-κB nucleotide probe (5'-AGTTGAGGGGACTTTCCAGGC-3' 5'-GCCTGGGAAAGTCCCCTCAACT-3') purchased from Shanghai Yingjun Company, China. High speed centrifuge (Heraeus, Hanan, Germany), MiniMACS magnetization cell sorter (Miltenyi Biotec GmbH Company, Bergisch Gladbach, Germany), FACS Calibur flow cytome-

try instrument (BD Biosciences; Franklin Lakes, NJ, USA), fluorescence microscope (Olympus, Tokyo, Japan).

Separation of DC *in Vitro*, Construction and Transfection of Recombinant Plasmid

Isolation and cultivation of DC: The peripheral anticoagulant was diluted and placed in lymphocyte separation medium to centrifuge at 1800 rpm for 15 min. The peripheral blood mononuclear cells (PMBC) were separated by density gradient centrifugation. The immunomagnetic beads were used to connect monoclonal antibody M2DC8 (Miltenyi Biotec GmbH Company, Bergisch Gladbach, Germany) and were used to centrifuged DC precursor cell from PBMC. RPMI-1640 culture medium (Beijing Tianrunshanda Biotech Company, Beijing, China) was used to cultivate cells and IFN-α (Invivogen, San Diego, CA, USA) was adopted to promote the maturity of DC.

Construction of recombinant eukaryotic expression plasmid hTERT-IRES2-EGFP: The pGRN145 plasmid (Beijing Tianrunshanda Biotech Company, Beijing, China) was digested with EcoRI enzyme and hTERT fragments were retrieved. T4 DNA ligase was used to connect and transfer hTERT (human telomerase reverse transcriptase) colonies for obtaining recombinant plasmid hTERT-IRES2-EGFP, which was identified by enzyme digestion and confirmed by sequencing.

Plasmid transfected DC *in vitro*: The liposome mediated transfection method was adopted to import constructed recombinant eukaryotic expression plasmid into cultured human epidermal stem cells. The G418 was used for the filter, the amplification, and the batches. The fluorescence microscope (Olympus, Tokyo, Japan) was used to test whether there was green fluorescence in positive clones or not. Further, PCR was adopted to test whether there was the integration of exogenous NeoR gene in positive clones or not.

Sepsis Models and Experimental Grouping

LPS (5 mg/kg) was injected for one time into the abdominal cavity to prepare models of mouse sepsis. After the development of sepsis mouse model, the animals were divided randomly into the sepsis group (A group), the group of hTERT transfecting DC (B group), the group of DC untransfected (C group), with 25 mice in each group. 5 mice were in the normal control group (D group) without any treatment. An equivalent

volume of normal saline was injected into the abdominal cavity of A group. 1 ml of cell suspension ($10^5/\text{ml}$) was transfused into B and C groups respectively. 5 mice from A, B, C groups were sacrificed after 24h, 48h, 72d, 7d and 10d, and only a mouse was sacrificed from group D as control.

Observation Index and Test Methods

Following indices were observed (1) Median survival time; (2) Histopathological changes of organ kidney and intestinal lesion.

Immunohistochemistry

The tissue sections were stained with H & E, to observe under an optical microscope (Olympus, Tokyo, Japan) ranging from 0 to 4 scores, the higher score was corresponding to the more serious the inflammatory response.

Levels of Selective Biomarkers

The levels of CPR and Cr was tested with an automatic biochemical analyzer (Hitachi Ltd, Tokyo, Japan). Further, HLA-DR, CD40 level of DC immune phenotype by flow cytometry and peripheral blood T cells MHC-II levels by Western blotting. PBS was used to wash the DC suspension, and later cells were centrifuged for 10 min at 1000 rpm. The phosphate buffered saline (PBS) was added to the cells at a concentration of $1 \times 10^5/\text{ml}$. The cell suspension of 100 μl was added into the flow cytometry tube. Subsequently, 10 μl of labeled antibody was mixed evenly and place inside the refrigerator of 4 °C (Haier, Qingdao, China) to incubate for 30 min. The cells were centrifuged for 10 min at 1000 rpm, and after washing with pre-cooled PBS the cells were suspended and analyzed. ELISA was used to analyze the expression levels of serum cytokine IL-12, TNF- α (InvivoGen Company, San Diego, CA, USA).

Detection of Dendritic Cells (DC) Apoptosis

It was performed according to situ hybridization. The DC cell suspension of kidney and tissue of intestine was prepared and adjusted to $1 \times 10^5/\text{ml}$ concentration. Immediately proper FITC labeled TUNEL dye mixtures (positive sorting of apoptotic cells), PE-labeled CD11C fluorescent antibody and PE-Cy5 labeled MHC-II of anti-mouse fluorescent antibody (sorted mature DC) was added.

The cells were incubated for 30 min on ice in the dark, and later centrifuged for 20 min

at 2000 rpm. The excess dye was washed off and finally cells were resuspended in 1% bovine serum albumin (BSA)-PBS. The flow cytometry was used to detect the expression of TUNEL+CD11C+ apoptosis DC, TUNEL+C-D11C+ MHC-II+for mDC. According to the instruction of in situ cell apoptosis detection kit, the TUNEL dye was used to label apoptotic cells for observing under a fluorescent microscope. CD11C+TUNEL+ marker was used to estimate apoptosis in DC.

EMSA Electrophoretic Mobility Shift Assay Test

It was conducted to determine the content and activity of NF- κB in the nucleus of DC. After mixing equivalent single stranded probe, the deionized water was added to denature for 10 mins at 95°C, slowly cooled to 20°C, and then diluted with deionized water to 1 $\mu\text{mol/L}$. The 6% polyacrylamide gel was used to establish probe binding protein reaction system (total 20 L). After even mixing, the samples were incubated for 20 min at room temperature and 5 μl of sample buffer was mixed to each of the sample. The sample was loaded on gel for electrophoresis (voltage 100V, electrophoresis buffer 0.5 $\times$ TBE), the electrophoresis was stopped once bromine phenol blue got closer to the bottom. Subsequently, 3 filter papers and a nylon film were soaked in TBE (Tris Borate Edta) buffer for 3 min. A sandwich of 3 filter papers- nylon film- gel-3 filter papers was prepared from the positive to negative, and was placed neatly in the splint of the wet transfer film instrument. A glass rod was used to remove any air bubbles. The film was transferred for 45 min at the 380 mA constant current in a buffer of 0.5 $\times$ TBE. The nylon film was taken to cross-link for 3 min under ultraviolet cross linker, and immune chemiluminescence method was to test DNA fragments labeled biotin.

Statistical Analysis

SPSS20.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were presented by $\bar{x} \pm s$. A single factor ANOVA analysis was used to compare the normal distribution data between two groups. The data was represented by the number of cases or percentage (%). While comparing between groups, chi-square test 2 was used. The K-M method was adopted to analyze median survival time. The difference with $p < 0.05$ was considered as of statistical significance.

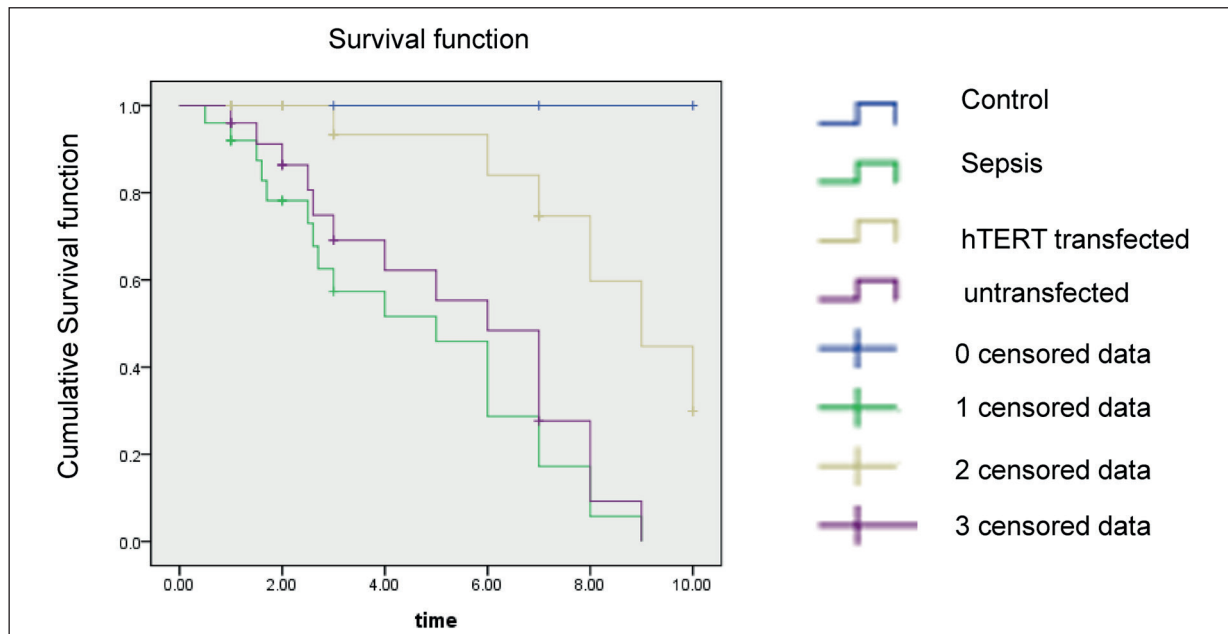


Figure 1. Comparisons of time and survival rate.

Results

Comparisons of Time and Survival Rate

The median survival time of the group of hTERT transfecting DC was (9.0 ± 1.2) d, and it was remarkably higher than the untransfected group which was (6.0 ± 0.9) d and the sepsis group which was (5.0 ± 1.2) d; the differences were of statistical significance (Log-Rank test: $\chi^2=21.219$, $p<0.001$) (Figure 1).

Comparisons of Tissue Pathology Scoring and Levels of CRP and Cr

At each time, in the group of hTERT (human telomerase reverse transcriptase) transfecting DC, average scores of pathology of kidney and intestine were significantly lower than that of other two groups ($p<0.05$); compare average

scores of pathology of kidney and intestine among groups, and the differences were of no statistical significance ($p>0.05$) (Figure 2).

At each time, in the group of hTERT transfecting DC, the levels of CRP and Cr were significantly lower than that of the other two groups ($p<0.05$) (Table I).

Comparisons of Immune Phenotype of HLA-DR, CD40 and Molecular Level of MHC-II

At each time, in the group of hTERT transfecting DC, HLA-DR and CD40 of immune phenotype and the expression level of peripheral blood T-cells MHC-II molecules were significantly higher than that of the other two groups ($p<0.05$) (Figure 3 (7d flow chart) and Tables II-III).

Table I. Comparison of levels of CRP and Cr.

Group	CRP (mg/L)					Cr (mg/dl)				
	1d	2	3	7	10	1d	2	3	7	10
A	1.5 $\pm$ 0.2	2.2 $\pm$ 0.4	3.4 $\pm$ 0.6	4.1 $\pm$ 0.8	4.0 $\pm$ 0.5	1.6 $\pm$ 0.3	1.8 $\pm$ 0.4	2.2 $\pm$ 0.5	2.5 $\pm$ 0.6	2.3 $\pm$ 0.5
B	1.2 $\pm$ 0.3	1.6 $\pm$ 0.4	1.9 $\pm$ 0.5	1.8 $\pm$ 0.4	1.7 $\pm$ 0.5	1.1 $\pm$ 0.3	1.3 $\pm$ 0.3	1.5 $\pm$ 0.3	1.4 $\pm$ 0.3	1.4 $\pm$ 0.3
C	1.4 $\pm$ 0.3	2.0 $\pm$ 0.6	2.7 $\pm$ 0.8	3.5 $\pm$ 0.9	3.4 $\pm$ 0.8	1.4 $\pm$ 0.5	1.6 $\pm$ 0.4	1.8 $\pm$ 0.6	2.2 $\pm$ 0.7	2.1 $\pm$ 0.8
F	4.628	4.935	5.250	5.634	5.769	4.857	5.201	5.634	5.967	6.231
p	0.037	0.035	0.031	0.026	0.023	0.035	0.031	0.026	0.017	0.014

Note: A group, the sepsis group; B group, the group of hTERT transfecting DC; C group, the group of untransfected DC

Table II. Comparisons of levels of HLA - DR and CD40 of immune phenotype (%).

Group	HLA-DR					CD40				
	1d	2	3	7	10	1d	2	3	7	10
A	36.7±6.8	46.8±9.2	53.5±11.4	69.4±12.3	72.1±12.5	7.8±3.2	9.2±3.6	12.4±4.5	16.6±5.2	22.7±6.2
B	53.6±8.2	64.5±10.3	82.7±12.4	96.6±11.5	98.2±13.3	20.4±5.6	32.3±5.9	42.5±6.3	53.1±7.4	56.7±7.7
C	42.4±7.5	56.5±12.5	67.4±13.2	77.8±15.6	82.4±16.4	13.2±4.2	18.7±4.6	24.4±5.7	28.9±5.5	33.3±6.2
F	5.124	5.362	5.648	5.967	6.235	5.236	5.525	5.768	6.235	6.627
p	0.032	0.027	0.025	0.017	0.013	0.025	0.022	0.018	0.013	0.011

Comparisons of Expression Levels of Cell Factors IL-12, TNF- α

At each time, in the group of hTERT transfecting DC, HLA-DR, the expression levels of IL-12 and TNF- α were significantly lower than that of the other two groups ($p<0.05$) (Table IV).

Comparisons of Apoptosis Rate of DC

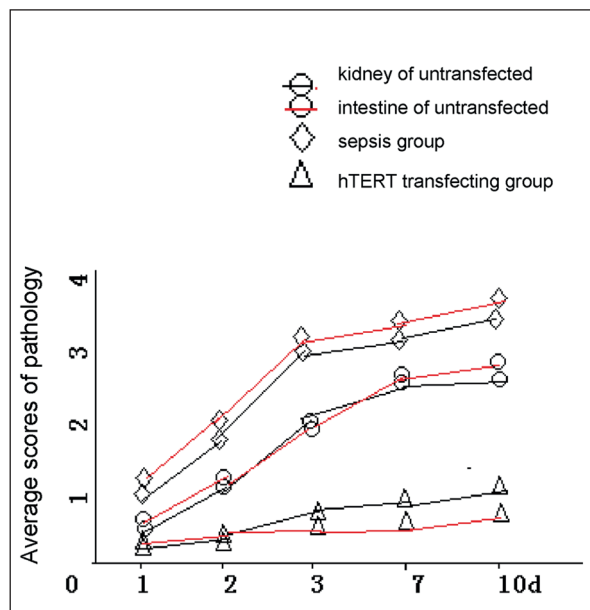
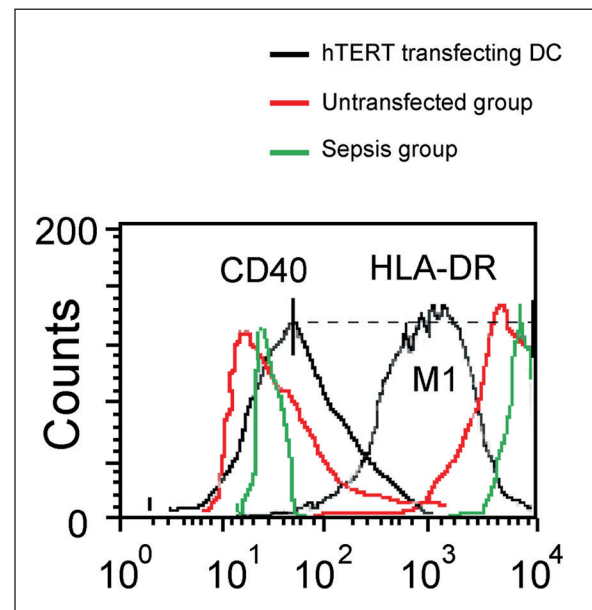
At each time, in the group of hTERT transfecting DC, apoptosis rate was significantly lower than that of the other two groups ($p<0.05$) [Figure 4 (7d flow cell chart) and Table V].

Comparisons of Content and Activity of NF- κ B

At each time, in the group of hTERT transfecting DC, content and activity of NF- κ B were significantly higher than that of the other two groups ($p<0.05$) (Figure 5).

Discussion

A telomere is the repeat sequence of TTAGGG which exists at the end of the eukaryotic linear chromosome. Due to the terminal replication of normal somatic cells, the telomere is shortened with each cell division. Every time the cells divides, the telomeres shortened by about 5-200 bp such a function of molecular erosion has biological constraints to the times of cell division. Therefore, the length of telomere reflects the history and potential of replication of cells, which is called "mitosis clock" of cell lifetime⁹. Telomerase is a kind of RNA protease that can extend the ends of telomeres and keep length of telomere¹⁰. It can use itself RNA as a template for synthesis of telomere DNA and add it to the ends of chromosomes; complete telomere DNA lost in the chromosome replication to prolong length of the telomere so as to prolong

**Figure 2.** Comparisons of tissue pathology scoring and levels of CRP and Cr.**Figure 3.** Comparisons of immune phenotype of HLA-DR, CD40

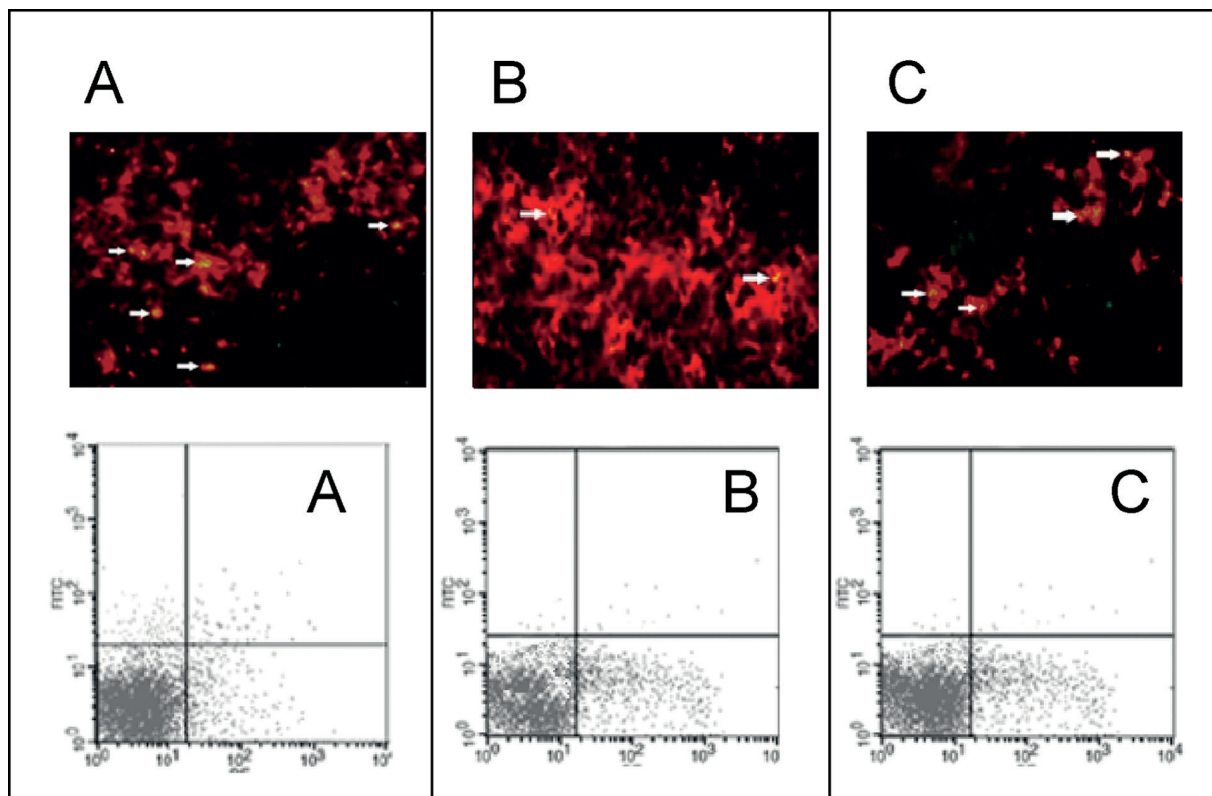


Figure 4. Flow chart showing DC apoptosis (A is the sepsis group, B is transfected group, C is un-transfected group).

the cell lifetime and even make it immortal¹¹. Telomerase consisted of one RNA subunit complementary to telomere DNA, catalytic subunit with reverse transcription activity (human telomerase reverse transcriptase, hTERT) and other relevant proteins (TPI). The expression and transcription of hTERT genes are the main rate limiting step to determine telomerase activity¹². Sequence analysis showed that the promoter region of the hTERT gene contains several transcription factors binding sites including c-Myc, p53, Spl, Madl, etc. These factors act on the promoter region of hTERT gene singly or cooperatively and participate the various degree of transcriptional regulation of hTERT gene¹³. In the human body, telomerase activity exists in stem cells, germ cells, some of the somatic cells

with regenerative capacity and the vast majority of malignant tumor tissues¹⁴. Its high activity expression is one important condition for the malignant proliferation of tumor cells, but its proper expression has the function of prolonging cell lifetime. Raising and keeping telomerase activity of cells will have important significance on increasing cell replication in vitro, amplifying ability and preventing cell apoptosis¹⁵.

The study showed that the median survival time of the group of hTERT transfecting DC was remarkably higher than the un-transfected group and the sepsis group, and was close to the normal control group, suggesting that hTERT regulating DC was closely related to improving the prognosis of sepsis. At each time, average scores of pa-

Table III. Comparisons of levels of HLA - DR and CD40 of immune phenotype (%).

Group	1d	2	3	7	10
A	13.4±6.7	23.6±8.9	32.4±12.3	42.1±13.3	53.2±15.2
B	42.5±10.2	63.8±11.4	85.7±11.6	96.5±12.2	98.4±15.4
C	26.3±12.4	42.1±13.6	53.6±14.5	76.9±15.2	83.2±15.3
F	5.634	5.967	6.230	6.435	6.769
p	0.025	0.017	0.013	0.006	0.003

Table IV. Comparisons of expression levels of cell factors IL-12, TNF- α (pg/ml).

Group	IL-12					TNF- α				
	1d	2	3	7	10	1d	2	3	7	10
A	63.2 $\pm$ 10.3	85.4 $\pm$ 15.4	102.7 $\pm$ 20.8	136.5 $\pm$ 26.5	152.4 $\pm$ 33.2	156.2 $\pm$ 23.5	236.4 $\pm$ 32.6	289.7 $\pm$ 45.5	356.4 $\pm$ 56.3	376.2 $\pm$ 62.5
B	26.4 $\pm$ 10.2	38.7 $\pm$ 13.2	46.9 $\pm$ 16.4	65.8 $\pm$ 23.5	73.5 $\pm$ 26.9	46.3 $\pm$ 13.2	53.2 $\pm$ 16.5	64.5 $\pm$ 23.4	73.2 $\pm$ 27.5	82.2 $\pm$ 30.6
C	45.5 $\pm$ 13.6	63.3 $\pm$ 18.5	82.4 $\pm$ 23.4	96.7 $\pm$ 25.7	112.7 $\pm$ 34.5	86.5 $\pm$ 20.1	123.5 $\pm$ 32.6	157.4 $\pm$ 38.4	196.5 $\pm$ 45.2	235.5 $\pm$ 53.6
F	5.468	5.762	6.354	6.649	6.968	6.230	6.458	6.695	7.203	7.625
<i>p</i>	0.024	0.018	0.013	0.005	0.001	0.015	0.013	0.006	0.001	0.001

Table V. Comparisons of apoptosis rate of DC (%).

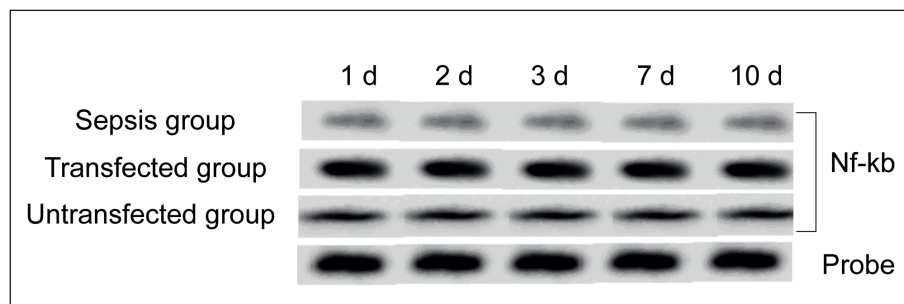
Group	1d	2	3	7	10
A	35.6 $\pm$ 10.5	53.2 $\pm$ 13.4	67.8 $\pm$ 17.8	82.1 $\pm$ 23.2	89.2 $\pm$ 25.8
B	10.5 $\pm$ 4.2	16.2 $\pm$ 4.6	21.3 $\pm$ 4.9	26.7 $\pm$ 5.3	30.2 $\pm$ 5.5
C	18.2 $\pm$ 4.6	26.4 $\pm$ 5.2	32.4 $\pm$ 5.8	45.8 $\pm$ 6.4	49.2 $\pm$ 6.6
F	5.754	5.825	6.165	6.352	6.645
<i>p</i>	0.018	0.017	0.015	0.012	0.007

thology of kidney and intestine were remarkably lower than that of the other two groups. The comparison of average scores for kidney pathology and intestine among groups, showed no statistical significance. It was suggested that kidney and intestine in sepsis might be the first to be involved, and the involvement is of more weight. The hTERT regulating DC could decrease noticeably inflammatory response of tissue and time of duration was rather long (still effective 10d after the study). At each time in the group of hTERT transfecting DC, the levels of CRP, Cr, IL-12 and TNF- α were remarkably lower than that of the other two groups. It suggests that hTERT regulate DC and it could downregulate the level of inflammatory responses in vivo. At each time point, the expression of HLA-DR, CD40 and the level of peripheral blood T cells MHC-II molecules were remarkably higher than that of the other

two groups. It was suggested that hTERT could raise the expression of DC, promote maturity, induce the T lymphocyte activation and enhance immune functions. At each time, apoptosis rate of DC was remarkably lower than that of the other two groups; the content and activity of NF- κ B were remarkably higher than that of the other two groups. It was suggested that hTERT could reduce apoptosis of DC and might be related with the survival of telomerase.

Conclusions

The telomerase reverse transcriptase gene can raise the expression and maturity of DC, reduce the apoptosis, induce cytokine secretion, reduce the inflammatory response and increase the survival time.

**Figure 5.** Western blotting for expression of MHC-II among different groups.

Acknowledgements

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Conflict of Interests

The Authors declare that they have no conflict of interests.

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