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Construction and application of a yeast expression system for thymosin α1

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ABSTRACT: We want to construct a yeast expression system for thymosin α1 (Tα1) to make the orally administered Tα1 preparation possible. The whole Tα1 DNA fragment was obtained by PCR. After being digested with restriction enzymes, it was cloned into pYES2 vector. Sequencing was performed to identify the recombinant. The sequence of Tα1 in recombinant coincided with the original one reported in Genbank. When pYES2-Tα1 plasmid was transformed into yeast, galactose instead of glucose was used to induce Tα1 expression. Western blot was performed to identify the quality of the expressed Tα1. Dried yeast containing pYEST2-Tα1 was fed to Balb/c mice whose immunities were inhibited by cyclophosphamide in advance. Synthesized Tα1 peptide was used as positive control and empty yeast was used as negative control. Compared with the negative control group, both dried yeast containing pYEST2-Tα1 and synthesized Tα1 peptide can significantly increase the CD8+ level (22.74±1.09 and 18.77±4.72 *vs* 7.49±2.14, $p<0.01$), while both of them had little effect on the CD4+ lymphocytes (61.86±6.94 and 65.91±4.78 *vs* 57.93±10.40, $p>0.05$). We concluded that a high effective yeast expression system for Tα1 was constructed successfully and the Tα1 protein expressed by this system can improve CD8+ level in immune inhibited mice.

Introduction

Thymus is a vital immune organ and plays a very important role in the development and maintenance of lymph system. The studies on thymosins were initiated in 1965. Thymosins extracted from animal thymus had been used as an adjunct drug in patients with carcinoma, viral infection and immunodeficiency in clinical practice. However, the extract containing miscellaneous animal proteins may easily lead to allergy in patients. Further investigation implied that the extract from bovine thymus gland can be divided into five groups. Among them, thymosin fraction 5 has the most ability to improve immune response. It turned out that thymosin fraction 5 consisted of a mixture of small polypeptides, such as Tα1, polypeptide β1, prothymosin α, parathymosin, and thymosin β4 and so on. They are biologically important peptides with diverse intracellular and extracellular functions (Hannappel and Huff, 2003). Tα1 is the most potent ingredient in thymosin and the biological activity of pure Tα1 is 10~1000 times over that of total thymus extract.

Tα1, a 28-amino acid peptide, is an immune modifier that has been shown to trigger maturational events in lymphocytes, to augment T-cell function, and to promote reconstitution of immune defects (Ancell *et al.*,

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2001). Tα1 has been shown to promote disease remission and cessation of hepatitis B virus (HBV) replication in patients with HBeAg-positive chronic hepatitis B without significant side effects (Mutchnick *et al.,* 1991; Chien *et al.,* 1998; Saruc *et al.,* 2003). The efficacy and safety of IFN-α 2a and Tα1 combination therapy in patients with chronic hepatitis C were determined. The combination therapy produced a good response rate and minor side effects, especially in naive patients with acceptable safety profile (Kullavanuaya *et al.,* 2001). Moreover, clinical trials using Tα1 in the treatment of patients with immunodeficiency or cancer indicate that this agent is nontoxic, enhances immune responsiveness and augments specific lymphocyte functions, including lymphoproliferative responses to mitogens, maturation of T cells, antibody production, and T cell mediated cytotoxicity. (Moody *et al.,* 2002; Rasi *et al.,* 2003).

Pure chemically synthesized Tα1 has already been used as an immune-mediate factor in clinical practice for the treatment of HBV and hepatitis C virus (HCV) infections, non-small cell lung cancer, hepatocellular carcinoma, AIDS and malignant melanoma. Tα1 is able to potentiate the action of cytokines and also reduce the hematological toxicity of cytotoxic drug therapy (cyclophosphamide-, 5-fluorouracil-, dacarbazine- or ifosfamide-based regimens). It was in phase III trials in the US in combination with PEGylated IFN-α, and it has been launched in Argentina, China, Peru, the Philippines and Singapore for the treatment of chronic HBV infection (Billich, 2002). However, high price and administration by inconvenient intramuscular injection have limited its wide use. A new approach is needed to produce high qualitative, cheap and orally administered Tα1 to satisfy clinical practice. In this study, Tα1 was cloned into a yeast expression vector and then the yeast with its expressed peptides was fed to Balb/c mice, whose immunities were inhibited by cyclophosphamide in advance. The effects of Tα1 on the immune function of the mice were observed.

Material and methods

Construction of Tα1 clone

Obtainment of the whole Tα1 gene: The whole Tα1 single DNA chain and primers were synthesized by Shanghai Boshide Corporation according to the Genbank sequence (E00106). Up stream primer is 5'GGA AGC TTT CTG ATG CTG CTG TTG 3', which contains Hind III restriction enzymatic site, and down stream primer is 5' TAT GGA TCC TAG TTC TCA GCC TCT TCG 3', which contains BamH I restriction enzymatic site. The whole Tα1 double DNA chains were obtained by PCR. Initial denaturation was at 95ºC for 2min followed by 30 cycles at 94ºC (1min), 54ºC (1min) and 72ºC (1min) in an Eppendorf PCR machine. The final elongation step was at 72ºC for 10min. High fidelity Pwo DNA polymerase (Cat. 1644947) was purchased from Roche Corporation.

Digestion: Hind III (Cat. R6041) and BamH I (Cat. R6021) enzymes were purchased from Promega Corporation. Tα1 PCR products were digested with both Hind III and BamH I restriction enzymes at 37ºC for 1h, so was the pYES2 plasmid (ClonTech Corporation, Cat. V825-20). The digested products were reclaimed from agarose gels, and then they were purified with the QIAquick Gel Extraction Kit (Qiagen Corporation, Cat. 28704).

Ligation: The enzymatic digestive products of Tα1 PCR product and pYES2 plasmid were ligated by T4 ligase (Roche Corporation, Cat. 481220) at a weight ratio 1:5 overnight at 15ºC, and then the products of ligation were used to transform the competent TOP 10F *E. coli* strain (Invitrogen Corporation, Cat. 201153). Aliquots of the transformation reaction were plated on LB medium plates containing 50μg/ml Ampicillin and the plates were incubated overnight in a 37°C incubator.

Identification of the Tα1 clone

PCR identification: Ten positive clones were selected randomly and incubated overnight in a shaker at 37°C. After that, 10μl medium of each clone was taken out and added to 10ml LB medium, and then they were incubated in the same shaker for another 4h. Then plasmids were extracted with the QIAprep Spin Miniprep Kit (Qiagen Com, Cat. 27104) and identified by PCR. Initial denaturation was at 95ºC for 2min followed by 30 cycles at 94ºC (1min), 54ºC (1min) and 72ºC (1min) in an Eppendorf PCR machine. The final elongation step was at 72ºC for 10min. The up stream primers and down stream primers were same as mentioned above. Gel electrophoresis was performed to identify the PCR products.

Restriction enzyme digestion: 18μl extracted plasmid, 1μl BamH I restriction enzyme, 1μl Hind III restriction enzyme and 2μl versatile buffer were mixed and incubated in a 37°C water bath for 1h. The product was identified by gel electrophoresis.

Sequencing: The Tα1 recombinant was sequenced by Shanghai Boshide Corporation with T7 forward prim-

ers. The result was compared with the original one reported in Genbank.

Expression and purification of Tα1 protein in INVSc1 yeast

Transformation of yeast: pYES2-Tα1, which was identified by PCR, restriction enzyme digestion and sequencing mentioned above, was extracted and purified with the QIAprep Spin Miniprep Kit. Purified pYES2-Tα1 plasmid was transformed into INVSc1 yeast host strain (Invitrogen Corporation, Cat. 203966) according to a small-scale yeast transformation protocol. Briefly, INVScI cells, which grew in YPD medium with 0.4 OD600, were pelleted at 2,500 rpm. The pellet was resuspended in 1∞TE buffer, pelleted again at 2,500 rpm and resuspended in 1∞LiAc/0.5∞TE. Cells were incubated at room temperature for 10min, and mixed together 1μg purified pYES2-Tα1 plasmid and 100μg denatured sheared salmon sperm DNA with 100μl the yeast suspension. Then, 700μl of 1∞LiAc/40% PEG-3350/1∞TE buffer were added and mixed well, then they were incubated at 30ºC for 30min. 88μl of DMSO were added and mixed well before they were shocked at 42ºC for 7 seconds. The preparation was centrifuged in a microcentrifuge for 10 seconds and the supernatant removed. The cell pellet was resuspended in 1∞TE buffer and plated on a SD-U selective plates. Minimal SD Base (Cat. 8602-1) and Ura DO Supplement (Cat. 8607-1) were purchased from ClonTech Corporation. INVSc1 yeast host strain will not grow in SD minimal medium that is deficient in histidine, leucine, tryptophan, or uracil. The tranformants will exhibit uracil prototrophy. Only INVSc1 yeast containing pYES2-Tα1 can survive in SD-U selective plates.

Expression of Tα1 protein: In INVSc1 yeast, transcription from the GAL1 promoter is repressed in the presence of glucose. Transcription may be induced by galactose instead of glucose as a carbon source. Firstly, cells were maintained in glucose to obtain enough cells, and then transfered to galactose-containing SD-U medium and allowed transcription to be induced.

Western Blot: Yeast was crushed using ultrasound after 4h expression, and then centrifuged at 1,500rpm for 5min at 4ºC. The supernatant was collected and an equal volume of acid-washed glass beads were added. Mixture was vortexed for 30 seconds, and followed by 30 seconds on ice. The procedure was repeated four times, then the preparation was centrifuged in a microcentrifuge for 10min at maximum speed. The supernatant was removed and transfered to a fresh mincrocentrifuge tube, then SDS-PAGE sample buffer was added and the sample was boiled for 5min. Then, 20μg of lysate was loaded onto an SDS-PAGE gel and electrophoresed. Western blot was performed to analyze the protein (Anti-Tα1 antibody was donated by Dr. Zhou Linfu). Synthesized Tα1 peptides (Ac-SDAAVDTSSEITTKDLKEKKEVVEEAEN-OH, Xian Meilian company, China) were used as positive control.

Identification of the biological activity of expressed Tα1

Construction of immune inhibited Balb/c mouse model: Thirty Balb/c mice were injected intra-peritoneally with cyclophosphamide (Jiangsu Henrui Pharmaceutical Corporation, Batch NO. 01101021) (10mg/kg) in the 1st and 2nd day, and the other 6 Balb/c mice were injected intra-peritoneally with normal saline at the same volume as normal control. In the 8th day, 6 mice selected randomly from cyclophosphamide treatment group and 6 normal mice were sacrificed and blood was drawn to quantify T lymphocytes subsets with flow cytometry assay.

Treatment with pYES2-Tα1: The remained 24 cyclophosphamide-treated Balb/c mice received treatment from 9th day. Three groups were divided randomly and evenly: Group A was treated with INVSc1 yeast containing pYES2-Tα1, group C was treated with INVSc1yeast containing empty pYES2, and group B was treated with synthesized Tα1 peptides. From 9th day, each mouse was fed with 0.1ml relevant mixture (containing 600μg of lyophiled INVSc1 yeast containing pYES2-Tα1 plasmids or lyophiled INVSc1 yeast host strain containing empty pYES2 plasmids or 6μg synthesized Tα1 peptides in 0.1ml normal saline, respectively) through gastric tube everyday. The treatment lasted for 7d. All the mice were sacrificed and blood was drawn in the 15th day. Flow cytometry assay was used to determine percentages and absolute numbers of CD4+ and CD8 + T lymphocytes.

Flow cytometry assay: Cell pellets were resuspended in 200μl of staining buffer (phosphate-buffered saline (PBS) containing 2% bovine serum albumin and 0.2% NaN_3). For antibody staining, staining buffer containing 1μg of either FITC-labeled isotype control antibody (BD PharMingen Tech, Cat. 551976) or FITC-labeled anti-CD3+ (BD PharMingen Tech, Cat. 553061) or PE-labeled anti-CD8+ antibody (BD PharMingen Tech, Cat. 553032) or PE-labeled anti-CD4+ antibody (BD PharMingen Tech, Cat. 557308) was used, and cells were stained for 1 h, washed once with staining buffer and resuspended in PBS. CD4+ and CD8+ level were

determined using a Flow cytoMeter (Beckman Coulter EPICS XL). FITC emits green fluorescence and PE emits red fluorescence under laser. Ten thousand cells were collected for every sample. The data were analyzed on computer with related software.

Statistical analysis

All the data were analyzed with SPSS 11.0 software and student t-test was used. A p value less than 0.05 indicated a statistically significant difference.

Results

Identification of Tα1 clone by PCR: To test whether pYES2-Tα1 was constructed successfully, PCR analysis was performed to detect the constructed plasmids extracted and purified with QIAprep Spin Miniprep Kit. Empty pYES2 plasmids were used as negative control. As shown in Fig. 1, positive DNA bands were detected in all of the four clones, while no specific bands were found in the negative control. The PCR results showed that Tα1 was cloned into pYES2 vector successfully.

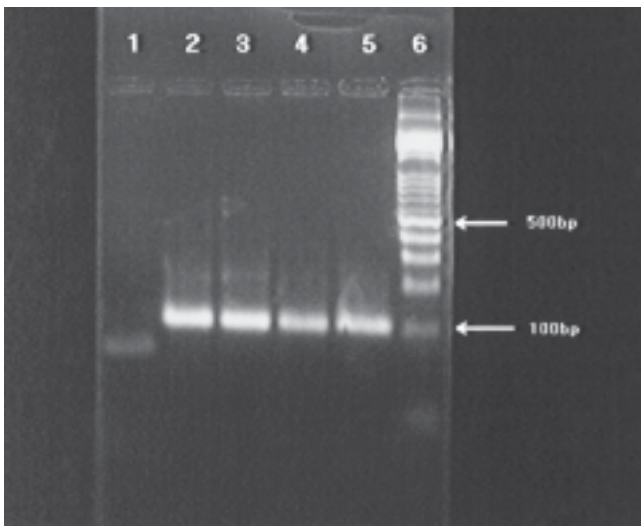

FIGURE 1. PCR results.
Lane 1: empty pYES2, no specific bands were found in the negative control.
Lane 2-5: Tα1-pYES2 clones, positive DNA bands were detected in all of the four clones.
Lane 6: marker.

Identification of pYES2-Tα1 clone by restriction enzymes: To identify the pYES2-Tα1 clone further more, restriction enzymes were used to digest the plasmids, which were identified by PCR assay. Empty pYES2 plasmids were used as negative control. All of them were digested with Hind III and BamH I restriction enzymes. Gel electrophoresis was performed to analyze the products. The PCR products of Tα1 were used as positive control. As shown in Fig. 2, one small fragment at the position of 109bp appeared in the lane of pYES2-Tα1 clone, which was in accord with that of PCR products of Tα1. No small fragment was detected in empty pYES2 plasmid lane. The results of restriction enzymes assay showed that one small fragment, with the same size of Tα1 we designed, had been inserted into the pYES2 vector successfully.

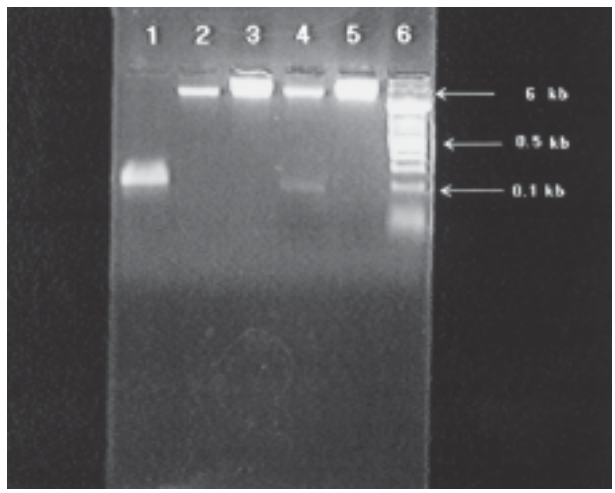

FIGURE 2. Digestion results.
Lane 1: PCR product of Tα1.
Lane 2: empty pYES2 vector after digestion.
Lane 3: empty pYES2 vector before digestion.
Lane 4: Tα1-pYES2 vector after digestion.
Lane 5: Tα1- pYES2 vector before digestion.
Lane 6: marker.

Identification of Tα1 clone by sequencing: To test whether the pYES2-Tα1 clone we got has the correct sequence, sequencing assay was performed to detect the pYES2-Tα1 clone with the T7 forward primers. The results showed that the sequence of the constructed recombinants completely accorded with that reported in GenBank (data not shown). Combined with the results of PCR assay, restriction enzymes assay and sequencing assay, we confirmed that the pYES2-Tα1 clone with correct sequence was constructed finally.

Identification of expressed Tα1 protein by Western blot: To assess whether correct Tα1 protein can be

expressed in yeast, purified pYES2-Tα1 plasmid was transformed into INVSc1 yeast host strain. Since GAL4 promoter in pYES2 vector can be induced in the presence of galactose, Tα1 protein transcription can be detected in the yeast growing in the galactose-containing SD-U medium. As shown in Fig 3, specific bands were detected in the pYES2-Tα1 clone (lane 1 and lane 2) and in the synthesized Tα1 peptides (lane 5). No bands were detected in the empty pYES2 plasmid clone (lane 3 and lane 4).

The immune inhibition effect of cyclophosphamide on Balb/c mice: To assess whether the Tα1 protein expressed in INVSc1 yeast host strain containing pYES2-Tα1 plasmid has bioactitivity effect, immune inhibition mice model was used. CD4+ and CD8+ lymphocytes in cyclophosphamide treated Balb/c mice were significantly lower than those in normal ones (32.50±10.76 *vs* 57.14±9.58, p<0.01, and 12.29±3.49 *vs* 20.14±5.53, $p<0.05$), which showed that the cellular immune function was significantly inhibited. The results showed that immune inhibited mice model had been constructed successfully (Table 1).

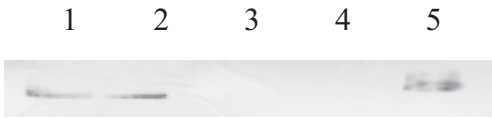

FIGURE 3. Western Blot results.
Lane 1-2: pYES2- Tα1 clone after induction of galactose.
Lane 3-4: empty pYES2 clone.
Lane 5: synthesized Tα1 peptide.

Effect of expressed Tα1 protein on immune inhibited mice: To detect the function of expressed Tα1 protein, lyophilized INVSc1 yeast host strain containing pYES2-Tα1 plasmids were used to treat the immune inhibited mice with the quantity of 600μg to each mouse in normal saline (group A). INVSc1 yeast host strains containing empty pYES2 plasmid were used as negative control with the same concentration (group C), and synthesized Tα1 peptides were used as positive control with the quantity of 6μg to each mouse in normal saline (group B). As showed in Table 2, compared with group C, both group A and B significantly improved the CD8+ lymphocytes (22.74±1.09 and 18.77±4.72 *vs* 7.49±2.14 $p<0.01$), while both group A and group B had little effect on the CD4+ lymphocytes (61.86±6.94 and 65.91±4.78 *vs* 57.93±10.40, $p>0.05$).

TABLE 1.

Effect of cyclophosphamide on the immune function of Balb/c mice.

Group	Number	CD4+ (%)	CD8+ (%)
Normal Saline	6	57.14±9.58	20.14±5.53
100mg/kg Cyclophosphamide	6	32.50±10.76**	12.29±3.49*

**Compared with normal group, P<0.01, *Compared with normal group, $p<0.05$

TABLE 2.

Effect of Tα1 on immune function of Balb/c mice.

Groups	Number	CD4+	CD8+
Group A	8	61.86±6.94	22.74±1.09**
Group B	8	65.91±4.78	18.77±4.72**
Group C	8	57.93±10.40	7.49±2.14

**Compared with group C, P<0.01.

Disscussion

Tα1 plays a lot of vital roles, such as immune regulation, promoting of NK cell activity, enhancing of the anti-infectious ability of the host, promoting of viral clearance, anti-oxidant activity, inhibiting of the growth of cancer cells, *etc.* (Zavaglia *et al.*, 2000). It was used widely in the clinical treatment of hepatitis B, hepatitis C, HIV and some tumors, such as melanoma, lung cancer, leukemia, squamous epithelial cancer, colon cancer, *etc.* (Garaci *et al.*, 2000; Andreone *et al.*, 2001).

There are two kinds of thymus products in clinical practice. One is an artificial chemical synthesized injection. Although it has been approved to have good effect in clinical practice, it is hard to popularize its use for its high price and the inconvenience of every day's injection. It costs about $8,000 by patient in one period of treatment in China. The other thymus product is a kind of thymosin mixture, which was extracted from animal thymus. It has a poorer treatment effect for its lower effective component (only 0.56%~1.00%). It is mingled with animal proteins and easily induces allergy in patients. It is vital to construct a new system, which can produce Tα1 by gene engineering with merits of low cost and high quality.

The results of this study showed that a Tα1 yeast expression system has been constructed successfully. The results of PCR assay, restriction enzyme assay and sequencing assay showed that pYSE2-Tα1 we constructed has correct sequence according with original one which was reported in GenBank (data not shown), and the results of Western blot assay showed that the expressed Tα1 protein can act with the Tα1 antibody.

Yeast strains are proved to be suitable for gene engineering. Yeast can be used as a kind of vector to introduce foreign genes. Whole recombinant yeast heterologously expressing mammalian mutant Ras proteins was used to immunize mice in a carcinogen-induced lung tumor model. Therapeutic immunization with the whole recombinant yeast caused complete regression of established Ras mutation-bearing lung tumors in a dose-dependent, antigen-specific manner (Lu *et al.,* 2004).

It had been identified that *Saccharomyces cerevisiae* can be administrated orally. Yeast strains of the species *Saccharomyces cerevisiae* is currently in use in the production of consumable alcohols, such as beer (Barnett, 1997). Jung *et al.* found out that after being administered *Saccharomyces cerevisiae* beta-glucan orally (50 mg/day/pig) for 3 days before swine influenza virus (SIV) infection, the microscopic lung lesions of pigs induced by SIV infection were significantly minor than those of control ($p < 0.05$). The concentrations of IFN-γ and nitric oxide in bronchoalveolar lavage fluid from these pigs were significantly higher than those of control. These findings support that the *Saccharomyces cerevisiae* beta-glucan can improve the immune function when is orally administered (Jung *et al.,* 2004).

Blanquet *et al.* demonstrated that engineered yeasts secreting compounds can be received directly in the digestive tract. The oral administration of the survival rate and the ability of two recombinant *Saccharomyces cerevisiae* strains (WppV(5)H(6) and WppGSTV(5)H(6)) to initiate the synthesis and secrete either a model peptide (peptide-V(5)H(6), MW: 5.6 kDa) or a model protein (glutathione-S-transferase -V(5) H(6), MW: 31.5 kDa) were studied in a gastric-small intestinal system simulating human digestive conditions. The WppV(5)H(6) and WppGSTV(5)H(6) strains respectively showed 83.1%+/-9.6 (n=3) and 95.3%+/-22.7 (n=4) survival rates in the model upper digestive tract after 270 min of digestion. The secretion products were detected as early as 90min after the yeast intake/gene induction in each compartment of the *in vitro* system, but mostly in the jejunum and ileum. The GST-V(5)H(6) concentrations in the digestive medium reached 15 ng/ml, close to values measured in batch cultures. These results open up new opportunities for the set up of drug delivery systems based on engineered yeasts secreting compounds directly in the digestive tract (Blanquet *et al.,* 2004).

To assess whether INVSc1 yeast host strain containing pYES2-Tα1 plasmids we obtained in present work can affect the immune function with oral administration, immune inhibited mice model was used. Cyclophosphamide is a DNA alkylating agent with striking immunomodulatory properties that are widely exploited in adoptive immunotherapy regimens (Pelaez *et al.,* 2001). Cyclophosphamide treatment is known to induce an immunosuppressed condition. Immunologic effect cells, such as B lymphocytes, T lymphocytes, and natural killer cells, are extremely sensitive to the cytotoxic properties of cyclophosphamide. Cyclophosphamide has also been used by several groups as an immunomodulatory agent against murine and human tumors (Saxton *et al*., 1997; Schiavoni *et al.,* 2000). In the present study, the results showed that CD4+ and CD8+ lymphocytes in those cyclophosphamide treated Balb/c mice were significantly lower than those in normal ones (32.50±10.76 vs 57.14±9.58, $p<0.01$, and 12.29±3.49 vs 20.14±5.53, $p<0.05$). Tα1 expressed by INVSc1 yeast host strain containing pYES2-Tα1 plasmids can improve the CD8+ cell level which was suppressed by cyclophosphamide in advance, the effect

coincided with the synthesized Tα1(22.74±1.09 and 18.77±4.72 vs 7.49±2.14, p<0.01). Interestingly, there was little effect of Tα1 on the CD4+ lymphocytes. The level of CD4+ lymphocytes was similar to normal control, no matter Tα1 expressed by INVSc1 yeast host strain containing pYES2-Tα1 plasmids, or synthesized Tα1 (61.86±6.94 and 65.91±4.78 vs 57.93±10.40, p>0.05). Considering that CD4+ lymphocytes almost reach normal levels at 15th day (57.93±10.40), it is reasonable there was little change in CD4+ lymphocyte level. The reason why CD4+ lymphocytes will return back to normal values is still unknown, it is likely to relate with the cease of cyclophosphamide, which is worth further investigation.

In conclusion, a high effective yeast expression system for Tα1, that is INVSc1 yeast host strain containing pYES2-Tα1 plasmids, was constructed successfully, and the Tα1 expressed by this system can improve the level of CD8+ cells in Balb/c mice treated with cyclophosphamide in advance.

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