A study on anti-tumor immunity induced by gene-modified melanoma B16 cells

LIJUAN FANG[1*], KEMIN WANG[1*], XIA LIU[1,3], LEILEI ZHANG[1,2], XINGQIAN ZHANG[1], LI QIAN[1], JIAN LU[1], GUANXIANG QIAN[1] and SHENGFANG GE[1,2]

[1]Research Center for Human Gene Therapy, Department of Biochemistry and Molecular Biology, School of Medicine, Shanghai Jiao Tong University; [2]Institutes of Medical Sciences, Shanghai Jiao Tong University School of Medicine; [3]Department of Biochemistry, Shanghai Institute of Health Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China

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Abstract. T cell-mediated cell immunity is the main anti-tumor immunity in which the effector T cells need specific antigen and costimulatory signals. One of the vaccines applied in tumor immunotherapy is the gene-modified tumor cell vaccine. One potential method to increase cell epitope density is to link the antigen with the major histcompatibility complex subunit ß2m. Our previous research indicated that the strategy of epitope fusion gene OVA-linker-ß2m can promote the formation of specific compounds on the tumor cell surface *in vitro*. In this study, we constructed two coexpression vectors pGL3-CD80-OVA-linker-ß2m and pGL3-IL21-OVA-linker-ß2m, in order to explore the cooperative action of CD80 or interleukin-21 (IL21) with the epitope fusion gene in anti-tumor immunity. Results showed that gene-modified B16 cells (B16/OVA, B16/CD80-OVA and B16/IL21-OVA) grew slower than B16 cells *in vitro* and *in vivo*, especially the B16/IL21-OVA subline, which illustrated that such gene modification decreased oncogenicity of malignant tumor cells. On the other hand, gene-modified tumor cell subline immunization can induce effective long-term anti-tumor immunity defending tumor cell attacks. IL21 played a more cooperative role with the OVA-linker-ß2m than CD80 in this study. This strategy might lay foundations for the research of a new type of tumor vaccine.

Correspondence to: Dr Shengfang Ge, Research Center for Human Gene Therapy, Department of Biochemistry and Molecular Biology, School of Medicine, Shanghai Jiao Tong University, No. 280 Chongqing Road South, Shanghai 200025, P.R. China
E-mail: geshengfang@sjtu.edu.cn

[*]Contributed equally

Abbreviations: IL21, interleukin-21; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; CTL, cytotoxic T lymphocyte; NK cell, natural killer cell; ELISA, enzyme linked immunosorbent assay; ICAM-I, intercellular adhesion molecule-I; AICD, activation-induced cell death; PBMC, peripheral blood mononuclear cells; HBSS, hank's balanced salt solution

Key words: melanoma, epitope fusion gene, CD80, interleukin-21, immunogene therapy

Introduction

Recent research indicates that the possible mechanisms that allow tumor cells to escape from immune system attacks are tumor antigen modulation, weak immunogenicity, the low-expression or lack of MHC molecule, the low expression of a costimulatory molecule and lack of local cytokines. Many factors eventually cause the local immune-suppressive state of tumors and the immune effector cells cannot stimulate the effective anti-tumor immune response. On the other hand, a tumor can also edit the immune system, which means that long-term, existent and chronic stimulation of a tumor leads to immune system exhaustion even tumor-specific T cell clonal deletion (1). Therefore, the main purpose of tumor immunotherapy at present is to try to reverse the local immune-suppressive state, enhance the original weak tumor immunogenicity and improve the quantity of local activated immune effector cells so as to stimulate a strong anti-tumor immunity response.

One of the vaccines applied in tumor immunotherapy is the gene-modified tumor cell vaccine, which can enhance tumor cell immunogenicity and degrade tumorigenesis through gene modification. One potential method is to link a specific epitope with ß2 microglobulin (ß2m) molecules in order to enhance tumor cell epitope density. ß2m is irrelevant with the antigenicity of the MHC-I molecule, though it is necessary for the correct expression and normal function of the MHC-I heavy chain (2,3). We previously constructed an OVA-linker-ß2m fusion vector, which served as an immunogen and successfully induced the specific CTL cells. A previous study has verified that the OVA-linker-ß2m fusion gene can promote the formation of a specific compound on the cell surface which accordingly enhances the tumor cells' immunogenicity (4,5).

Based on previous work, we wished to investigate whether the method of fusion gene can induce effective anti-tumor immunity in the weak immunogenicity tumor model. The weak immunogenicity of tumor cells is partly due to the

low expression of costimulatory molecules. Without a costimulatory signal, T cells cannot be activated thoroughly and can even be in an anergy condition (6,7). The co-stimulatory molecule, CD80, plays an important role in the process of T cell activation (8). In addition, the lack of cytokines is one of the reasons for the tumor's escape from the immune system. IL21 is a new member of the IL-2 family, mainly produced by the activated $CD4^+$ T cells. It has extensive biological activities, especially in enhancing the functions of the effector T cell and activated NK cell, which can effectively strengthen the innate immunity and specific immunity and promote the tumor-bearing animal to produce anti-tumor immunity (9,10). In contrast with IL2 treatment, which induces activation-induced cell death (AICD), IL21 sustains the number of $CD8^+$ T cells as a result of increased survival (11). So we constructed two new vectors, which respectively coexpress the OVA-linker-ß2m fusion protein and CD80, or IL21, in order to study whether combining gene-modification of tumor cells can induce a more intense anti-tumor immunological effect.

Materials and methods

Mice and cell lines. Female C57BL/6 mice, 6-8 weeks of age, were treated according to the NIH Guidelines for Animal Care in the Experimental Animal Center of Shanghai Jiao Tong University School of Medicine. Melanoma B16 cells of mice were obtained from the cell bank of Academia Sinica and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone), 2 M pyruvate, 50 μM 2-mercaptoethanol, penicillin (200 U/ml) and streptomycin (200 μg/ml) at 37˚C in a humidified atmosphere containing 5% CO_2. For gene-modified cells, Geneticin (500 μg/ml) (Invitrogen) was added to the medium.

Expression vector construct. The following plasmid vectors were constructed: i) pCDNA3-OVA-linker-ß2m, ii) pGL3-CD80-OVA-linker-ß2m, iii) pGL3-IL21-OVA-linker-ß2m.

The plasmid pCDNA3-OVA-linker-ß2m was constructed (4). The epitope $OVA_{256-264}$ is the sequence of nine peptides which has high affinity with $H\text{-}2K^b$. The epitope sequence is linked with human ß2m by a short linker sequence mainly made up of glycine and serine (GGGSGGGGS). To construct the plasmid pGL3-CD80-OVA-linker-ß2m, the plasmid pCDNA3-OVA-linker-ß2m and pGL3-CD80-P1A were digested with BglII and SmaI enzymes, the 450 bp BglII-SmaI OVA fusion gene was cloned into the same enzyme digested plasmid backbone pGL3-CD80. The resulting vector was named pGL3-CD80-OVA-linker-ß2m. To construct a plasmid pGL3-IL21-OVA-linker-ß2m, we cloned the mouse IL21 gene from a mouse peripheral blood mononuclear (PBMC) with a 5' primer ccc aag ctt ctc ctg gag act cag ttc tgg and a 3' primer tgc tct aga tca gga atc ttc ggg tcc ta (9). The upstream primer incorporated a HindIII site at the 5' end and the downstream primer incorporated an XbaI site. Then the PCR product of IL21 was digested with HindIII and XbaI and cloned into the same enzyme-digested vector pGL3-promoter. After constructing the plasmid pGL3-IL21, the 450 bp BglII-SmaI OVA fusion gene was cloned into the BglII and SmaI digested pGL3-IL21 vector similarly. The final vector was named pGL3-IL21-OVA-linker-ß2m. All of these constructs were confirmed by the enzyme digestion and sequence analyses.

Cell transfection. Tumor cells were transfected with plasmid DNA using liposome Lipofectamin 2000 according to the manufacturer's manual (Invitrogen). Briefly, B16 cells were plated at a concentration of $2x10^5$ cells per well in a 2 ml medium in a 6-well plate before transfection and grew to 60-70% confluency. As the two new plasmids have no selective gene Neo, the plasmid pSV40-neo is needed for cotransfection. The plasmid/liposome complexes were prepared 30 min before transfection. Cells were incubated with plasmid/liposome complexes for 6 h. Then the medium was replaced with a fresh culture medium. At the same time, the transfection efficiency was determined by simultaneous transfection of plasmid pCDNA3-EGFP. While selecting stable transgene clones, the tumor cells were grown in 500 μg/ml Geneticin containing medium 48 h after transfection and continued to select monoclones by limiting dilution in 96-well plates. The expression of the transgene was confirmed by the reverse transcription PCR. Tumor cell surface expression of CD80 was determined by flow cytometry. Cytokine IL21 expression was detected by an enzyme-linked immunosorbent assay (ELISA).

RT-PCR reaction. The total RNA of either wild-type B16 cells or the stable transfected tumor cells was isolated by using Trizol agent (Invitrogen). The RNA samples were reverse transcribed according to the manufacturer's instructions using reverse transcriptase (Invitrogen), random hexamers and dNTP. The synthesized cDNAs were amplified by PCR (both 30 cycles for OVA, CD80, IL21 and ß-actin) with Taq DNA polymerase in the presence of both dNTP and an appropriate pair of primers. Table I shows the sense and anti-sense primers used.

Measurement of CD80 on transfected B16 tumor cells. The CD80 expression on the cell surface was determined by flow cytometry. All labeling steps were carried out at 4˚C. Wild-type tumor cells and gene-modified cells were collected and incubated for 30 min with a FITC-anti-mouse CD80 antibody (eBioscience), then washed twice and suspended in fixing solution. Stained cells were analyzed by FACS.

IL21 expression assays. The cytokine IL21 expression was analyzed by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D System). Briefly, a 96-well plate was incubated with a goat anti-mouse IL21 antibody and blocked with blocking buffer. After washing, a standardized IL21 solution and 24 and 48 h cell culture supernatants were then added to the wells. Following 2 h of incubation, the plate was washed three times. Biotin-labeled detection antibody and HRP-conjugated antibody were added respectively. After another hour of incubation and washing, the substrate solution was added and the plate was read at 450 nm.

Tumorigenicity of the gene-modified tumor cells. Confluent cultures of gene-modified and wild-type tumor cells were

Table I. The sense and anti-sense primers.

OVA-linker-ß2m sense	5' CAG CAT ATG TCC ATA ATC AAC TTT GAA AAA CTC GGA AGG AGG ATC CGA GGT GGC AGC ATC CAG CGT ACT CCA AAG-3'
OVA-linker-ß2m anti-sense	5' CAACTCGAGCATGTCTCGATCCCAC-3'
CD80 sense	5' CAAAGCATCTGAAACCATGGCT-3'
CD80 anti-sense	5' CTAAAGGAAGACGGTC-3'
IL21 sense	5' CCCAAGCTTCTCCTGGAGACTCAGTTCTGG-3'
IL21 anti-sense	5' TGCTCTAGATCAGGAATCTTCGGGTCCTA-3'
ß-actin sense	5' TCGACAACGGCTCCGGCAT-3'
ß-actin anti-sense	5' CACAATCCACCAGCCAGCCTCA-3'

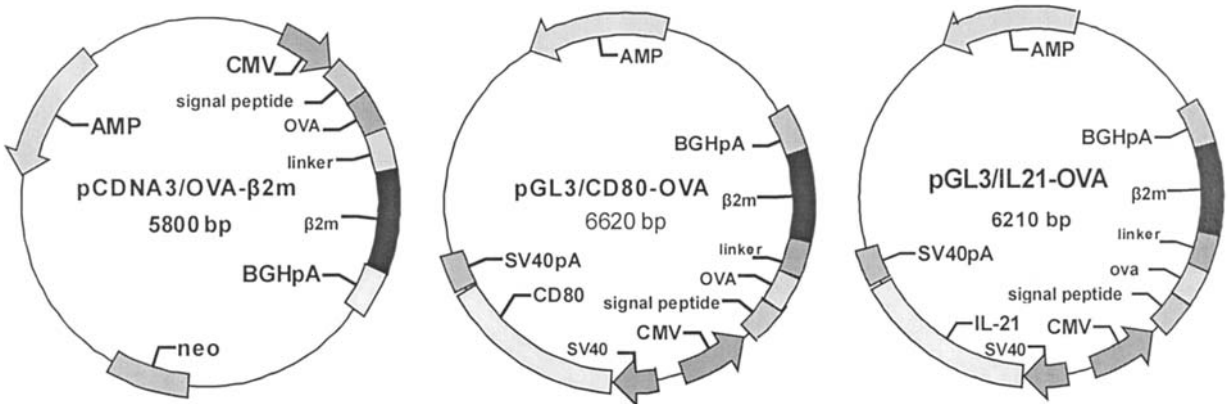

Figure 1. A schematic diagram describing the different OVA-linker-ß2m expression vectors. pCDNA3-OVA-ß2m: CMV promoter driving the expression of the epitope fusion gene, in which the encoding gene of antigen epitope OVA is linked with the human ß2 microglobulin gene by a short linker sequence (GGGSGGGGS). PGL3-CD80-OVA-ß2m: The original Neo gene was replaced by the mouse CD80 gene. The SV40 promoter is driving the expression of mouse CD80, while the CMV promoter is driving the expression of the OVA-linker-ß2m fusion protein. PGL3-IL21-OVA-ß2m: The original Neo gene was replaced by the mouse IL21 gene. The SV40 promoter is driving the expression of mouse IL21, while the CMV promoter is driving the expression of the OVA-linker-ß2m fusion protein.

harvested and inoculated subcutaneously in the right flank of syngenic C57BL/6 mice (six animals per group) with $1x10^5$ viable tumor cells in 0.1 ml of HBSS. Tumors were measured every 3 days with calipers once the tumors became palpable. The tumor volume was calculated using the following formula: length x width $^2/2$.

Immunization with genetically modified tumor cells. C57BL/6 mice were immunized s.c. with either $1x10^6$ irradiated wild-type B16 cells or gene-modified B16 cells, which were B16/OVA, B16/CD80-OVA and B16/IL21-OVA. The immunization procedure was repeated 7 days later. One week after the final immunization, mice of four groups were challenged s.c. with 10^5 viable tumor cells. Then, the animals were monitored for more than 60 days and the tumor load was measured in each group.

Results

Assay of fusion gene expression in B16 cells through RT-PCR. After transfection and Geneticin selection, the stable transfectants were referred to as B16/OVA, B16/CD80-OVA and B16/IL21-OVA, respectively. RT-PCR results showed that transfectants had significantly up-regulated the expression of OVA-linker-ß2m, CD80 and IL21 mRNA (Fig. 2). In contrast, B16 cells showed no expression of these genes.

The expression of the CD80 molecule on the surface of G418 selected. The wild-type B16 tumor cells and the stable transfected cell lines B16/CD80-OVA were evaluated by flow cytometry for the expression of CD80. The data showed that B16 cells were almost negative for CD80 expression while B16/CD80-OVA cells were 70.62% positive for CD80 expression 2-3 weeks following transfection and subsequent culture in a medium containing G418 as shown in Fig. 3.

ELISA analysis of IL21 secreted by the G418 selected cells. Culture supernatants from different cell lines (B16, B16/OVA and B16/IL21-OVA) were collected and assayed for IL21 by ELISA. Culture supernatants from B16, B16/OVA contained hardly detectable levels of IL21, while supernatants from stable transfected B16/IL21-OVA contained high levels of IL21 48 h after culture in the medium (Fig. 4). There is an obvious difference between B16, B16/OVA and B16/IL21-OVA.

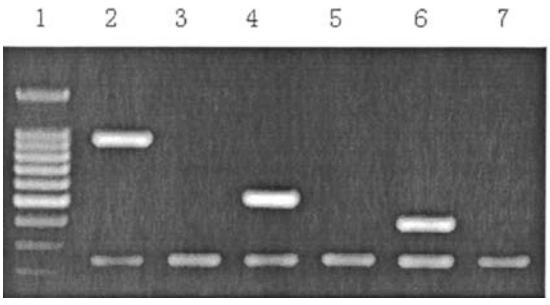

Figure 2. Assay of fusion gene expression in B16 cells through RT-PCR. Lane 1 is the 100 bp DNA ladder marker. Lane 2 is the 920 bp RT-PCR result of mouse CD80 gene from B16/CD80-OVA cell total RNA, while CD80 expression is almost negative in B16 RNA in lane 3. Similarly, lane 4 is the 508 bp RT-PCR result of the mouse IL21 gene from B16/IL21-OVA cell total RNA and its expression is negative in the B16 control group in lane 5. Lane 6 is the 450 bp RT-PCR result of the OVA fusion gene from B16/OVA RNA and there is no expression in the B16 control group in lane 7. All the tumor cells show 239 bp ß-actin gene expression.

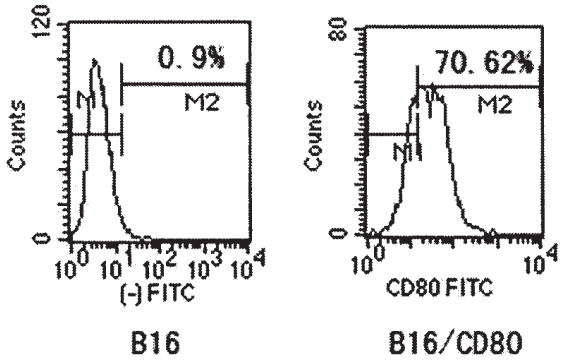

Figure 3. The expression of the CD80 molecule on the surface of G418 selected transfectants. After transfection and subsequent culture in a medium containing G418, the cultured B16 and B16/CD80 cells were collected and the expression of CD80 was detected by FACS. B16/CD80 cell surface shows a high level of expression of CD80 (~70.62%), while it is almost negative on the B16 cell surface. The result is relevant with the RT-PCR analysis.

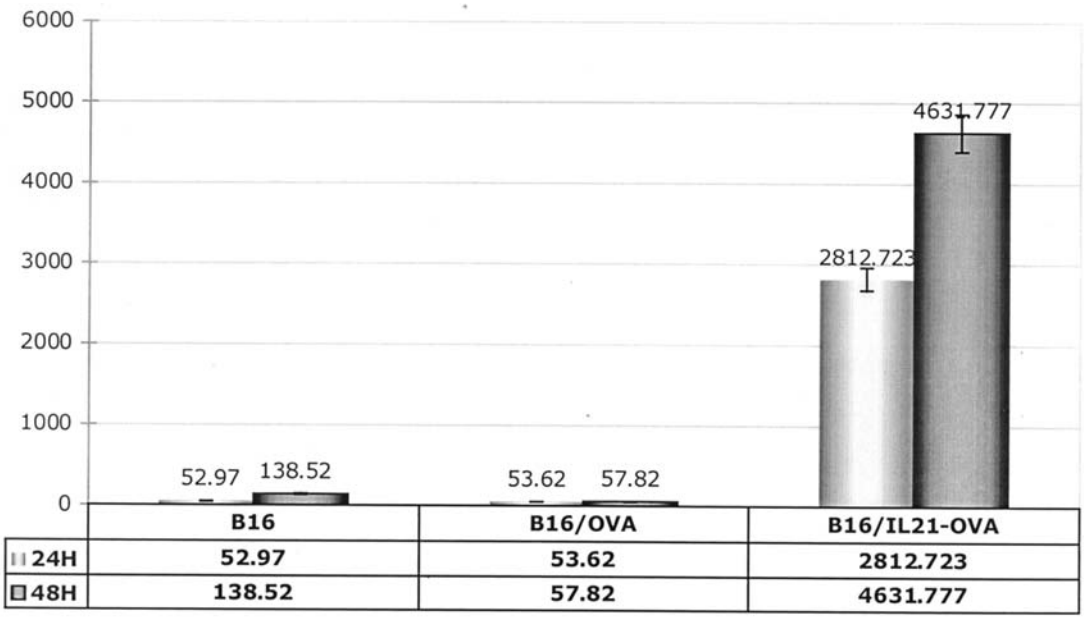

Figure 4. ELISA analysis of IL21 secreted by the G418 selected cells. Twenty-four and 48 h culture supernatants from B16, B16/OVA and B16/IL21-OVA were collected and analyzed by ELISA, respectively. B16 and B16/OVA cell culture supernatants showed hardly detectable levels of IL21. However, the B16/IL21-OVA cell secretes high levels of IL21, that is 2812.72 pg/ml and 4631.77 pg/ml after 24 and 48 h of culture, respectively.

Cell growth curves of gene-modified B16 cells. An *in vitro* cell proliferation assay showed that the growth tendency curves of stable transfectants were different from the control B16 cells. B16/OVA, B16/CD80-OVA and B16/IL21-OVA sublines grew significantly slower than the B16 cells ($P<0.05$), especially the B16/IL21-OVA cell lines (Fig. 5).

Assay of the tumorigenicity of gene-modified B16 cells. C57BL/6 mice (six mice in each group) were injected subcutaneously with $1x10^5$ wild-type B16 cells or the different stable B16 sublines. Tumors were monitored every 3 days once the tumor became palpable. Results suggested that the antigen epitope fused with the ß2m strategy resulted in decreased tumorigenicity and tumors derived from epitope and IL21 expressing B16 sublines grew significantly slower than the wild-type B16 cell group ($P<0.05$). However, there was no obvious difference between the B16/CD80-OVA subline group and the B16 cell group ($P>0.05$) (Table II).

The anti-tumor protective immunity induced by gene-modified B16 immunization. We then explored the possibility that immunization with gene-modified B16 cells might afford protection against tumor cell attacks. Four groups of C57BL/6 mice (five mice in each group) were immunized with irradiated B16, B16/OVA, B16/CD80-OVA or B16/IL21-OVA cells, respectively. Following immunization, mice were challenged s.c. with different transfected B16 cells and monitored for tumor load. Mice immunized with B16 cells rapidly developed aggressive tumors and showed no evidence of protection from tumor challenge. On the other hand, mice

Table II. Average tumor volume of each group (unit: cm^3).

Days	10	14	17	21
B16	0.220±0.008	0.222±0.032	0.596±0.027	1.697±0.342
B16/OVA	0.048±0.013	0.109±0.047	0.381±0.027	0.852±0.670
B16/CD80-OVA	0.091±0.033	0.165±0.101	0.454±0.290	1.206±0.702
B16/IL21-OVA	0.018±0.010	0.081±0.093	0.266±0.084	0.817±0.295

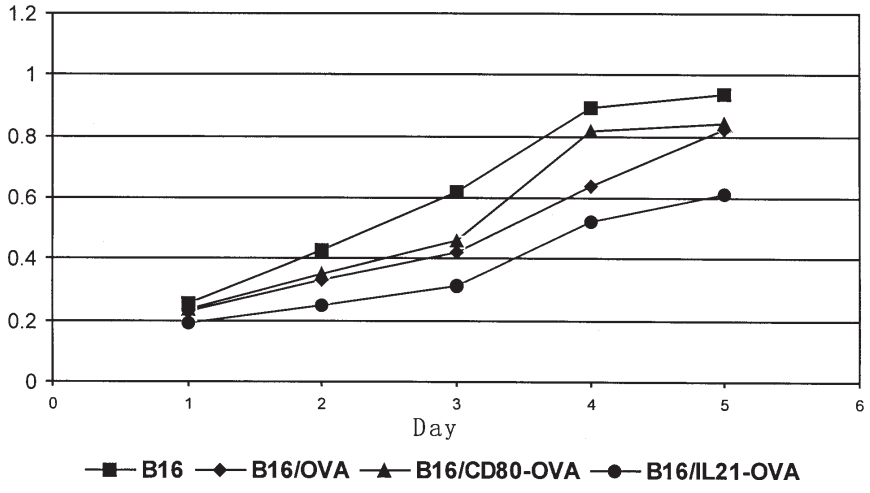

Figure 5. Cell growth curves of gene-modified B16 cells. Gene-modified B16 cells and B16 cells were plated at the concentration of 1,000 cells per well in a 100 μl medium in 96-well plates, respectively. Twenty μl of MTS was added into each well from days 1 to 7. After 2 h of culture, the results were read under a 490 nm wave length. The gene-modified B16 cells grew significantly slower than the control B16 cells. B16/IL21-OVA cells grew the slowest.

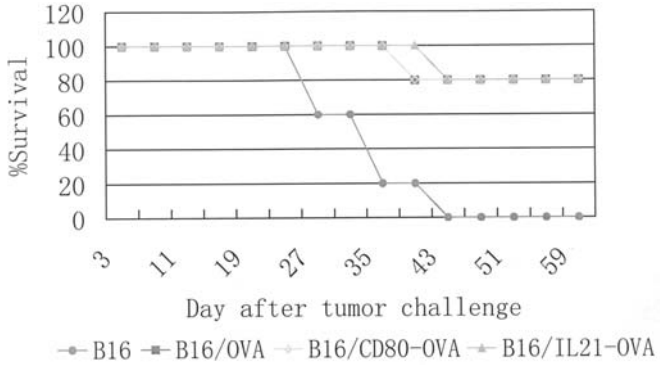

Figure 6. The anti-tumor protective immunity induced by gene-modified B16 immunization. Four groups of C57BL/6 mice were immunized with $1x10^6$ irradiated different gene-modified B16 cells and control B16 cells, respectively. After immunization, each mouse was challenged with $1x10^5$ viable transfected B16 cells and control B16 cells. By day 42, five mice in the B16 group all died. In contrast, only one mouse in the other three groups died, while the remaining four mice in each group were in a tumor-free condition for more than 60 days.

immunized with gene-modified B16 cells showed strong protection and tumors grew much less aggressively. By day 42, mice immunized with B16 cells all died, while 80% of mice in each of the other three groups were still alive under a tumor-free condition for more than 60 days. By day 64, the mice that survived were challenged again with tumor cells and monitored for tumor load. Notably, only 1/4 of the mice in each group developed tumors and grew much less aggressive than before (data not shown). This suggests that animals immunized with gene-modified B16 cells show strong protection against tumor attack and gain a long-term immune response.

Discussion

T cell-mediated cell immunity is one of the anti-tumor immunity mechanisms. The activation of T cells needs two signals: one is the signal provided by the T cell receptor and MHC-peptide compound recognition; another is the co-stimulatory signal provided by the costimulatory molecules. The MHC/peptide compound density on the antigen presenting cell (APC) surface directly determines the intensity of a T cell response (12). The expression of MHC on melanoma cell surface is very low, resulting in a weak first signal and is unable to induce thorough the activation of T cells (13-15).

It has been reported that a potential method to enhance cell epitope density is to link the specific epitope with the MHC-I heavy chain, or to link an antigen epitope with an exogenous ß2 microglobulin molecule (16). The construction strategy of the ova-linker-ß2m fusion gene vector is verified to facilitate the formation and stability of the MHC/peptide

compound, which served as an effective immunogen and successfully induced the specific CTL cells. In addition, a mechanism that prevents antigen-specific T cells from activation and causing local immunity tolerance in the tumor microenvironment is the low expression of the costimulatory molecule on the tumor cell surface (17,18). CD80, as a costimulatory molecule, plays an important role in T cell activation (8). The role of cytokines in T cell activation is also vital. It has been named the 'third signal' (19). Previous research indicated that the microenvironment of cytokines is an important link in determining the differentiation of T cells and the subsequent characteristics of the immune response (20). That is to say, we can co-transfect CD80 and some cytokines in order to stabilize the cooperation of the micro-environment of T cells in the anti-tumor immunity responses.

Interleukin (IL), especially the IL-2 family plays an important role in anti-tumor immunity or adjunctive therapy. IL21 is noted for its strong immunological regulation function. We constructed two coexpression vectors, which coexpress the OVA fusion protein and CD80, or OVA fusion protein and IL21 respectively, in order to gain an intense anti-tumor effect. The data of the *in vitro* proliferation assay indicated that gene-modified B16 cells B16/OVA, B16/CD80-OVA and B16/IL21-OVA grew significantly slower than wild-type B16 cells, especially the B16/IL21-OVA cells ($P<0.05$). The possibility is that cytokine IL21 autocrined by B16/IL21-OVA cells directly inhibited the tumor cell growth, which is also verified by the result of ELISA. Oncogenicity tests suggested that the B16/OVA and B16/IL21-OVA sublines grew slower *in vivo* than B16 cells. However, there was no obvious growth difference between the B16/CD80-OVA sublines and B16 cells ($P>0.05$). A report by Chen *et al* (21) pointed out that the immunogenicity of tumor cells decides the costimulation effect of CD80 in T cell-mediated anti-tumor immunity. That is, oncogenicity of immunogenic tumor cells (P815, EL4) degraded when transfected with CD80 gene. However, transfection with the CD80 gene did not influence the oncogenicity of non-immunogenic tumor cells, such as B16 cells. It was also suggested that the introduction of the CD80 gene can enhance the oncogenicity of B16 cells, though the effect of CD80 was not as strong as it was on immunogenic tumor cells. This phenomenon might be relevant with the expression levels of ICAM-I or MHC molecules on the tumor cell surface (22-24). This may be one of the reasons that illustrate why the coexpression of the CD80 molecule did not significantly degrade the oncogenicity of B16 cells *in vivo*.

In a further exploration of the immunoprotection assay, we challenged the experimental group of mice with the same modified B16 cells instead of the wild-type B16 cell after immunization. The results showed that, compared with the control group, gene-modified B16 cell vaccines can induce strong protection against tumor cell attacks and also gain a long-term immunity response, which illustrated the activation of the *in vivo* immune system and the strengthening of the function of effector immune cells while the control group cannot induce an effective immune response. A gene-modified tumor vaccine might activate an adaptive immunity mechanism that gains long-term immunity. It was reported by Guo *et al* (25,26) that gene-modified tumor cell vaccines can elicit tumor-specific anti-tumor immunity and the activated effector CTLs can kill not only gene-modified tumor cells, but also parental tumor cells because of the *in vivo* immune system activation and gene-modified tumor cell growth retardation. Apoptosis can release more specific tumor antigen in order to induce effective immunity. It can be said that immunization of gene-modified B16 cells elicit such a strong tumor-specific immunity that it can also protect mice from parental B16 cell attacks through direct CTLs or indirect cytokine function.

Based on the above, we can conclude that the epitope fusion gene OVA-linker-ß2m modified melanoma cells grow slower than wild-type melanoma cells both *in vitro* and *in vivo* and its cell immunization induces effective anti-tumor immunity. Enhancement of the second signal provided by coexpression of CD80 does not significantly degrade the melanoma cell oncogenicity, although its immunization is affected. However, by increasing the amount of cytokine IL21 in a tumor micro-enviroment does strengthen the epitope fusion protein's effect on melanoma cells *in vitro* and *in vivo*. These results contribute to the research and development of new types of tumor vaccines which have latent applied values.

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