Original Article Mechanism and antitumor effect of combined treatment with gene modification in situ and adoptive T cell therapy in malignant melanoma

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Abstract: Immunotherapy is an important method to treat the malignant melanoma nowadays. But the immune tolerance of patients to tumor was formed from immune system in traditional immunotherapy. Our previous experiments found that using the activated vaccine can avoid the immune tolerance and its immune effects are better than the inactivated one. However, transplanted tumor might be formed by using the activated vaccine. In this report, we recombined adenovirus vector rAV-Interleukin 21/Early secretory antigenic target of 6 kD (ESAT-6) and modified the immunogenicity of activated vaccine combined with adoptive transfer of ESAT-6 specific T cells to tumor in situ. Using this method, we provided experimental evidence that it can develop enhanced antitumor effects and suppress transplanted tumor formed. Collectively, our data suggest that combined treatment with gene modification in situ and adoptive T cell therapy has great potential in melanoma therapy in clinical.

Keywords: Malignant melanoma, B16F10 cell, ESAT-6, interleukin 21, adenovirus vector

Introduction

Malignant melanoma (MM) is the most invasive skin melanoma. It is formed by malignant melanocyte locating in the epidermal basal part. It is also one of the most malignancies to treat effectively because of rapid progression, early metastasis and insensitivity to radiotherapy and chemotherapy. Therefore, it is necessary to exploit and apply new therapeutic method to treat this deadly disease [1]. Among that, adoptive immunotherapy is emerging as an important progress during oncotherapy. Active immunotherapy is a very important method of immune therapy [2]. Application of tumor vaccine can stimulate the immune system to produce specific antitumor immune response, kill the tumor cells, and prevent tumor growth, metastasis and relapse [3, 4].

Based on the immunogenicity of MM and its sensitivity to immunotherapy, it's one of the hot spots in the field to improve the therapeutic effect through active immunotherapy [5]. But sometimes, the body is unable to identify tumor antigens because of immune tolerance. Using tumor vaccine can break the immune tolerance of endogenous tumor antigen and it is an important method to increase the immunogenicity of tumor. Early secretory antigenic target of 6 kD (ESAT-6) is an important target protein in the study of tuberculosis vaccine, which is an immunoadjuvant and it is safe for human body. ESAT-6, locating on T, B cell epitopes, has strong immunoreactivity and immunogenicity [6, 7]. Previously, we generated mouse melanoma B16F10-ESAT-6-gpi/Interleukin 21 (IL-21) vaccine by fusing IL-21 and ESAT-6 modified by GPI into B16F10 cells and studied the mechanism and the effect of antitumor induced by the vaccine. We found that, as a heterologous antigen, ESAT-6 can break the immune tolerance to melanoma and increase the immunogenicity of B16F10-ESAT-6-gpi/IL-21 vaccine. Besides, as an effective adjuvant molecular, IL-21 can enhance the effect of tumor vaccine. B16F10-ESAT-6-gpi/IL-21 live vaccine, inoculated into C57BL/6 mice, can stimulate the immune mice to produce effective anti-melanoma immune response and resist the attack of wild type

B16F10 cells effectively. The effects are better than those in the inactivated vaccine [8-10].

Because of the possibility of transplanted tumor occurrence caused by the live tumor vaccine in vivo, its application is limited in clinical application. One of the solutions is changing the host tumor cells into tumor vaccine in situ, expressing heterologous antigen ESAT-6 and cytokine IL-21 through intratumoral injecting the tumor antigen and/or cytokine gene by the carrier. Adenovirus vector is easy to be prepared and purified. It can meet the requirements of clinical gene therapy because the virus can reach high titer and achieve high transfection efficiency after infecting target cells without overt clinical symptoms [11]. Whether tumor vaccine can play a role or not is affected by tumor immune tolerance and tumor microenvironment. With immunomodulatory cytokines secreted by tumor cells, the cytokine modified tumor vaccine can increase the concentration of local tumor specific cytokines, alter the microenvironment maintained by tumor cells, and facilitate the immune suppression. Then it can induce immune response against tumor cells and stimulate the generation of a number of known or unknown tumor antigen [12]. Several cytokines, such as IL-2, GM-CSF, IL-12, IFN-y and IL-21, have been used in the research of immunotherapy of tumor [13-17].

In this study, we aimed to recombine adenovirus vector rAV-IL-21/ESAT-6 and to change the immunogenicity of tumor vaccine by modifying the melanoma gene in situ. The adoptive transfer of ESAT-6 specific T cells to the tumor in situ was combined to test whether tumor vaccine exerts better effect of immunotherapy in this way.

Materials and methods

Construction of ESAT-6 and IL-21 shuttle vectors

The full length of ESAT-6 and IL-21 was synthesized and subcloned into p-shuttle vector respectively. For construction of the shuttle vector co-expressing of these two targets, the ORF of ESAT-6 and IL-21 were linked through IRES and subcloned into p-shuttle vector. The inserts were confirmed by Sanger sequencing. The recombinant shuttle vectors were prepared by Qiagen plasmid maxi prep kit (Shanghai, China).

Generation of recombinant adenoviral vector

First, the adenoviral backbone vector was prepared. Defrost the frozen E.coli cells containing adenoviral backbone vector pAD-Easy from -80°C by spreading on a LB plate and incubate the plate in a 37°C incubator overnight. Pick up a single colony from the LB plate and incubate the E.coli cells in 5 mL liquid LB culture medium on an orbital shaker at 180 RPM for 8 hr, transfer the E.coli suspension to 100 mL LB medium, and shake at 180 RPM for 12 hr. Then harvest the E.coli cells by centrifugation at 4000 RPM for 15 min. Extract the pAD-Easy vector by using Qiagen maxi prep kit and determine the concentration of the linearized pAD-Easy vector. Prepare three PCR tubes, add 100 ng linearized pAD-Easy vector into each tube and then add 100 ng p-shuttle-ESAT-6 vector, pshuttle-IL-21 vector and p-shuttle-ESAT-6-IRES-IL-21 vector respectively. Mix thoroughly by pipetting up and down and incubate the tubes at 25°C overnight. Thaw three vials of DH5α competent E.coli cells on ice and add 10 µL ligation products into each tube respectively. Keep the E.coli cells on ice for 30 min followed by heat shock at 42°C for 45 s. Then spread the E.coli cells on a LB plate and incubate at 37°C overnight. Randomly pick up several colonies and shake in 5 mL LB culture medium at 180 RPM overnight. Extract the plasmid using Qiagen mini prep kit. Sequence the recombinant adenoviral vector with universal CMV F primer to confirm the recombinant vector. Linearize the pAD-Easy vector with Pacl enzyme followed by purification with ethanol precipitation.

Generation of primary adenovirus stock

Thaw one vial of 293A cells from liquid nitrogen in a 37°C water bath, and maintain the cells in DMEM, 10% FBS culture medium overnight. Routinely passage 293A cells twice and seed the cells into a 10 cm culture dish overnight. Prepare three 10 cm dishes in total. Warm the PEI stock to room temperature. After thoroughly mixed by pipetting up and down, for each transfection, prepare the PEI/DNA complex as following: added 2 mL PBS, 10 µg recombinant adenoviral plasmid into one well of a 6-well plate, then added 12 μ L 100 μ M PEI and mixed immediately. Incubate at room temperature for 15 min. Add the PEI/DNA complex into a 10 cm culture dish dropwise and gently shake the dish back and forth. Culture the dish for 6-8 hr in 37°C incubator and refresh the culture medium. Continuously culture the cells for 5 to 7 days until the cytotoxic pathogenesis effect (CPE) appears. Harvest the cells using a cell scraper and resuspend the cell pellet with 5 mL PBS. Lysate the cell pellet by repeatedly froze and thaw for three cycles and centrifuge the suspension at $2000 \times g$ for 15 min, and transfer the supernatant containing virus to a new tube. Store the primary adenovirus stock at -80°C.

Propagation of adenovirus

Thaw one vial of 293A cells from liquid nitrogen in a 37°C water bath, and maintain the cells in DMEM, 10% FBS culture medium overnight. Expand the 293A cells routinely and prepare twenty 15 cm dishes for each virus propagation. Adjust the cell confluency to 70% prior to transduction with primary adenovirus stock. Use 5 MOI primary adenovirus stock to transduce the 293A cells. Drop the virus to culture medium and gently shake the culture dish back and forth to diffuse the virus evenly. Continuously maintain the cells for extra 2 to 4 days until the CPE appears. Harvest the cells using a cell scraper and centrifuge at 800× g for 10 min at room temperature. Resuspend the cell pellet with 10 mL PBS and lysate the cells by freezing and thawing cycles. Centrifuge the suspension at 2000× g for 15 min at 4°C and transfer the supernatant to a new tube.

Purification of adenovirus

Transfer the virus supernatant into an ultracentrifuge tube and centrifuge at 4000× RPM for 5 min at 4°C. Repeatedly centrifuge the tube until the remaining virus volume was approximately 1.5 mL, then add PBS buffer into the tube and centrifuge at 4000× RPM for 5 min at 4°C. Repeatedly add PBS and centrifuge for five times to exchange the buffer. Aliquot the concentrated adenovirus and store at -80°C.

Titration of adenovirus

Seed 1 ml of healthy, log-phase HEK 293 cells $(5 \times 10^5 \text{ cells/ml})$ in each well of a 12-well plate. Using PBS as diluent, prepare 10-fold serial dilutions of adenovirus from 10^{-2} to 10^{-6} ml. Add 100 µl of viral dilution dropwise to each well and incubate cells at 37°C in 5% CO₂ for 48 hr. Then get rid of medium and allow cells to dry in hood. Fix the cells by gently adding 1 ml ice-cold 100% methanol into each well. Then incubate the plate at -20°C for 10 min. Aspirate methanol. Gently rinse the wells three times

with 1 ml PBS containing 1% BSA. Dilute Mouse Anti-Hexon Antibody (1:1000) in PBS containing 1% BSA. Aspirate final rinse from the wells. Then add 0.5 ml of Anti-Hexon Antibody dilution in each well. Incubate 1 hr at 37°C. Prior to removing the Rat Anti-Mouse Antibody (HRP conjugate), prepare DAB working solution by diluting 10× DAB substrate 1:10 with stable peroxidase buffer. Allow the 1× DAB working solution to come to room temperature. Aspirate Rat Anti-Mouse Antibody (HRP conjugate) dilution. Gently rinse each well three times with 1 mL PBS containing 1% BSA. After removing the final PBS with 1% BSA rinse, add 500 µl DAB working solution to each well. Incubate it at room temperature for 10 min. Then aspirate DAB and add 1 mL PBS to each well. Count a minimum of three fields of brown/black positive cells using a microscope with a 20× objective, and calculate the mean number of positive cells in each well.

Transduction of B16F10 cells with adenovirus

Recover B16F10 cells from liquid nitrogen and routinely passage the cells until exponential phase. Inoculate 3×10^5 cells per well into a 6well-plate and supplement 3 mL culture medium. Maintain the cells in a 37° C and 5% CO₂ incubator overnight. Use MOI = 5, 20, 50, 100, 200, and 500 to do pilot transduction. The amount of either IL-21-expressing adenovirus or GFP-expressing adenovirus was calculated per the formula below. Drop the adenovirus into the 6-well-plate and mix by shake the plate back and forth gently. Continuously culture the cells for 48 hr prior to doing downstream assay.

Isolation of mouse DC cells

Mouse DC was isolated using a Pan Dendritic Cell Isolation Kit from Miltenyi Biotec (China). The procedures were briefly introduced below. Sacrifice the C57BL/6 mice by cervical dislocation and isolate the spleen. Place isolated spleen in a 6 cm petri-dish with sufficient Collagenase D solution to completely cover the bottom of the dish (5 mL/spleen). Inject mouse spleen with 500 µL of Collagenase D solution per spleen using a 1 mL syringe and a 25G needle, then cut the tissues into smaller pieces using sharp scissors. Incubate the spleen pieces in Collagenase D solution for 30 minutes at 37°C. Pass the whole material gently through a 70 µm cellstrainer using a plunger. Collect all cells in a 15 mL tube and wash the cells by adding buffer to a final volume of 14 mL. Determine cell number and centrifuge cell suspension at 300× g for 10 minutes. Aspirate supernatant completely. Resuspend cell pellet in 350 µL of buffer per 10⁸ total cells. Add 50 µL of FcR Blocking Reagent per 10⁸ total cells. Add 100 uL of Pan Dendritic Cell Biotin-Antibody Cocktailper 10⁸ cells. The final labeling volume is 500 µL per 10⁸ cells. Mix well and incubate for 10 minutes in the refrigerator (2-8°C). Wash cells by adding 5-10 mL of buffer per 10⁸ cells and centrifuge at 300× g for 10 minutes. Aspirate supernatant completely. Resuspend cell pellet in 800 µL of buffer. Add 200 µL of Anti-Biotin MicroBeads per 10⁸ cells. Mix well and incubate for 10 minutes in the refrigerator (2-8°C). Place column in the magnetic field of a suitable MACS Separator. Prepare column by rinsing with 3 mL of buffer. Apply cell suspension onto the column. Allow the cells to pass through and collect effluent as fraction with unlabeled cells, representing the enriched dendritic cell fraction. Wash column with 2-3 mL of buffer. Perform washing steps by adding buffer two times. Only add new buffer when the column reservoir is empty. Collect entire effluent in the same tube as before. This fraction represents the enriched dendritic cells.

Transduction of mouse DC cells with ESAT-6expressing adenovirus

Inoculate about 5×10^8 DC cells into one well of a 6-well-plate and maintained in X-vivo15 culture medium with 10 ng/ml GM-CSF (R&D systems, USA) and 10 ng/ml IL-4 (R&D systems, USA). Use MOI = 200 to transduce the DC cells. The amount of ESAT-6-expressing adenovirus was calculated per the formula below. Drop the adenovirus into the 6-well-plate and mix by shake the plate back and forth gently. Continuously culture the cells for 48 hr prior to priming T cells.

Isolation of mouse T cells

Harvest mouse peripheral blood and isolated the PBMC cells. Resuspend PBMC pellet in 40 μ L of buffer per 10⁷ total cells. Add 10 μ L of Biotin-Antibody Cocktail per 10⁷ total cells. Mix well and incubate for 5 minutes in the refrigerator (2-8°C). Add 30 μ L of buffer per 10⁷ total cells. Add 20 μ L of Anti-Biotin MicroBeads per 10⁷ total cells. Mix well and incubate for 10 minutes in the refrigerator (2-8°C). Place LS Column in the magnetic field of a suitable MACS Separator. Prepare column by rinsing with 3 mL of buffer. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells, representing the enriched T cells. Wash column with buffer. Collect unlabeled cells that pass through, representing the enriched T cells, and combine with the effluent as before.

T cell priming assay with ESAT-6 loaded DC

Calculate the number of naïve T cells and DC. mix naïve T cells with ESAT-6-loaded DC at the ratio of 10:1. Wash the cell mixture once and resuspend the cells in X-Vivo15 medium with 5% mouse serum and 1 µg/mL Anti-CD28. Incubate the cells at 37°C, 5% CO₂ for two days. After two days cultivation, add 200 µL of X-vivo15 with 5% serum (Gibco, USA), 60 ng/mL IL-7 (R&D systems, USA) and 60 ng/mL IL-15 (R&D systems, USA). And continuously maintain the cells for extra two days. Add 800 µL of X-vivo15 with 5% serum and 250 IU/mL IL-2 (R&D systems, USA) and culture the cells for extra two days. Then discard 1 mL supernatant from one well and add 1 mL X-Vivo 15 with 5% serum, 10 ng/mL IL-7, 10 ng/mL IL-15 and 100 IU/mL recombinant IL-2, and continuously culture the cells for extra 6 days. Harvest all the primed T cells for assay.

Lysis of target cells by CAR-T

Use the ESAT-6, IL-21 or ESAT-6/IL-21 transduced B16F10 cells as the target cells. Adjust cell density to 5×10⁵ cells/mL and inoculate 100 µL/well into a 96-well plate, seat the plate in a 5% CO, 37°C incubator overnight. Resuspend the ESAT-6-primed T cells with X-vivo15 medium, and add the primed T cells into the 96-well plate according the E/T ratio as indicated in the figures. Take the plate back to incubator and maintain for 8 hr. After cultivation, add Lysis buffer into the "Maxi lysis" well and centrifuge at 1200× g for 5 min. Transfer 50 µL supernatant to a new 96-well plate and add LDH substrate. Incubate the plate for 15 min and read the OD value using a microplate reader. Aliquot the rest supernatant for ELISA assay to determine the secretion of cytokines. Use the formula below to calculate percent lysis ×100%.

B16F10-derived Xenograft (CDX) mouse models

For evaluating the anti-tumor effects of ESAT-6primed T cells *in vivo*, a mouse melanoma cell



Figure 1. Adenovirus-mediated transduction of B16F10 cells. (A-D) Titration of adenovrius by immunocytochemical method. Representative figures of each adenovirus-transduced HEK293 cells at 10⁻³ dilution under 10×10 fields: (A) EGFP, (B) B16F10-ESAT-6, (C) B16F10-IL-21 and (D) B16F10-ESAT-6-IL-21; Scale bar: 200 µm. (E) GFP-expressing adenovirus transduced B16F10 cells with different MOI; Scale bar: 50 µm.

line B16F10 was subcutaneously inoculated into C57BL/6 mouse. The procedures for conducting the in vivo study were briefly introduced below. Twenty male mice at six-week old were kept in a separate ventilated cage with constant temperature and humidity, keeping the room temperature at 22-23°C and humidity at 56-60%, 10-20 times per hour ventilation. The mouse body weight ranged between 18 g and 23.5 g at the day of inoculation. B16F10 cells were maintained in DMEM culture medium supplemented with 10% FBS and were routinely expanded every 3 days until the cells were ready for inoculation. All mice were subcutaneously inoculated with 1×10⁶ B16F10 cells in 100 µL PBS. After inoculation, the animals were raised as normal and the growth of tumor cells were monitored daily. Approximately seven days after inoculation when palpable tumor formed and the average tumor volume reached about 50 mm³, all mice were grouped randomly according to the average body weight, each group consisted of five mice and there were four groups in total. The tumor volume was estimated as $(width)^2 \times (length)/2$. Primary mouse T cells were isolated, primed and expanded in vitro as described before. Each mouse bearing tumors in all groups was intravenously injected with 5×10⁶ primed T cells by tail vein. Tumors were injected intratumorally using an insulin syringe in a volume of 50 µL with the appropriate concentration of adenoviruses carrying IL-21, ESAT-6, IL-21-ESAT-6 or GFP. Tumors were measured in three dimensions using calipers every seven days after intratumoral injection and tumor volume was calculated assuming a prolate spheroid. The serum samples from each mouse were harvested one day before intratumoral injection of adenovirus and every 7 days after the first intratumoral injection. Serum samples of one group harvested at the same time point were pooled together for determination of the serum level of interleukin 2 and interferon gamma by ELISA assay (R&D systems, USA).

Statistical analysis

All values are expressed as mean \pm SDs of at least 3 individual experiments. The statistical comparisons between the groups were performed using the Student's t-test. Statistical analyses were performed by using SPSS 13.0 statistical package software (SPSS, Chicago, IL, USA) or GraphPad Prism (GraphPad Software Inc., San Diego, CA). *P* value less than 0.05 were considered statistically significant.

Results

Adenovirus-mediated transduction of B16F10 cells

All the adenoviruses produced in the present research were titrated by immunocytochemical method using anti-Hexon antibody after purification with ultracentrifuge tube. **Figure 1A** showed the representative figures of each ade-





Figure 2. Lysis of target cells by ESAT-6-primed T cells. Target cells: (A) B16F10-IL-21, (B) B16F10-ESAT-6 and (C) B16F10-ESAT-6-IL-21. The statistical comparisons between the groups were performed using the Student's t-test. *P < 0.05; **P < 0.01; ***P < 0.01.

novirus-transduced HEK293 cells at 10^{-3} dilution under 10×10 fields. Data showed that the titer of adenovirus was above 10^{10} PFU/mL. The B16F10 cell line was transduced with GFP-expressing adenovirus to determine the optimal MOI for transduction. MOI of 5, 20, 100, 200 and 500 was applied to B16F10 cells, and 48 hr post transduction, the expression of GFP was visualized under fluorescent microscope. As shown in **Figure 1B**, adenovirus at MOI below 20 could not transduce B16F10 cells. When the MOI reached up to 200, almost all B16F10 cells showed GFP expression.

Lysis of B16F10-ESAT-6, B16F10-IL-21 and B16F10-ESAT-6-IL-21 cells with in vitro primed T cells

Three different recombinant B16F10 cells were used as target cells and cocultured with ESAT-6-primed T cells at different E/T ratio respectively. After 8 hr cocultivation, the supernatant was harvested for determination of the amount of LDH released by killed target cells. As a result, ESAT-6-primed T cells could efficiently target the recombinant B16F10 cells bearing ESAT-6 expression while only showed minor unspecific lysis effects on the B16F10-IL-21 cells (**Figure 2**).

Secretion of interleukin 2 and interferon gamma by primed T after engagement with target cells

After coculturing with the three target cells for 8 hr, the supernatant was harvested for determine the secretion of cytokines. Data showed that ESAT-6-primed T cells robustly produced both IL-2 and IFN- γ when being engaged with target tumor cells regardless of the E:T ratio. The secretion level of IL-2 and IFN- γ kept enhancing along with the increase of E:T ratio (**Figure 3**).

Tumor regression followed by intratumoral injection of adenovirus

The day that mouse received intratumoral injection of adenovirus was designated as Day 0. The average tumor volume was around 51.6



Figure 3. Secretion of interleukin 2 and interferon gamma by primed T after engagement with target cells. (A, B) Cytokines released in ESAT-6-primed T cells cocultivation with B16F10-IL-21 cells. Serum levels of IFN- γ (A) and IL-2 (B) in indicated groups were measured by ELISA. (C, D) Cytokines released in ESAT-6-primed T cells cocultivation with B16F10-ESAT-6 cells. Serum levels of IFN- γ (C) and IL-2 (D) in indicated groups were measured by ELISA. (E, F) Cytokines released in ESAT-6-primed T cells cocultivation with B16F10-ESAT-6-IL-21 cells. Serum levels of IFN- γ (E) and IL-2 (F) in indicated groups were measured by ELISA. The statistical comparisons between the groups were performed using the Student's t-test. Control T vs. ESAT6-primed T; *P < 0.05; **P < 0.01; ***P < 0.01.

mm³. The tumor cells grew fast in the mice injected with control GFP adenovirus when compared with all other three groups receiving IL-21, ESAT-6 or the combination of them. Taken GFP-adenovirus-injected mice as control, intratumoral injection of IL-21 solely could induce tumor regression to some extent in all treated mice, while injection of IL-21 plus ESAT-6 or ESAT-6 almost resulted in complete tumor regression. This result indicated that IL-21 had anti-tumor effects and it could exert synergistic effects when combined with the tumor specific antigen-primed T cells. After intratumoral injection 22 days, the mice injected with GFP adenovirus gradually died while those mice received IL-21 or ESAT-6 survived longer time although the tumor volume kept growing slowly (**Figure 4A**).



Figure 4. Tumor regression followed by intratumoral injection of adenovirus. (A) Tumor volume changed by intratumoral injection of different adenovirus in indicated groups (Student's t-test, compared with AD-GFP, comparison was performed at day 22, *P < 0.05, **P < 0.01). (B, C) IL-2 and IFN- γ levels in serum after intratumoral injection. Serum levels of IL-2 (B) and IFN- γ (C) in indicated groups were measured by ELISA (Student's t-test, compared with AD-GFP, compared with AD-GFP, comparison was performed at day 29, **P < 0.01, ***P < 0.001). (D) Immunohistological analysis of CD3⁺ T cells in xenograft tumor tissues in indicated groups. (E) Comparison of survival rate among indicated groups. Scale bar: 100 µm.

Then mouse serum was harvested by tail vein bleeding every seven days after intratumoral injection. All serum samples were kept at -80°C after preparation until to the 29th day. The serum level of both IFN-y and IL-2 were determined by ELISA kits. The results showed that the overall serum level of cytokines in the mice injected with IL-21 & ESAT-6 was much higher than those in other treated mice throughout the assay process. Serum IFN-y secretion kept increasing after injection with adenovirus regardless whether an exogenous gene was loaded or not, this might be caused by the innate immunogenecity of adenovirus; however, the adenovirus carrying IL-21 or ESAT-6 triggered a more robust immune response based on the serum level of IL-2 (Figure 4B) and IFN-γ (Figure 4C). The serum level of IL-2 decreased dramatically after 22 days of intratumoral injection.

Moreover, the xenograft tumor tissues were removed from all experimental animals at the end of the experiment. Paraffin-imbedded sections were histochemically stained with Anti-

mouse CD3 antibody. The representative images were showed in Figure 4D. The tumor tissues from mice injected with GFP adenovirus or IL-21 adenovirus were slightly stained with CD3 antibody; while the tissues from mice received IL-21 and ESAT-6 injection were strongly stained with CD3 antibody, indicating that coexpression of both IL-21 and ESAT-6 could recruit ESAT-6specific T cells migrating into tumor tissues. During the whole experimental period of fifty days, the mice received adenovirus carrying both IL-21 and ESAT-6 kept alive and no death was observed; while among other groups the mice gradually died from 20 days after inoculation of B16F10 cells (Figure 4E), indicating that the injection of ESAT-6-primed T cells by tail vein could inhibit the proliferation of tumor cells bearing ESAT-6 expression.

Discussion

The effect of the current treatment of malignant melanoma is poor, such as surgery, radiotherapy and chemotherapy [18, 19]. Immunotherapy is noticeable gradually in the treatment while the effect is not so ideal [20]. It is necessary to find a more effective way to improve the efficacy of immunotherapy. Compared with inactivated vaccine, the immune effect in C57BL/6 mice immunized with B16F10-ESAT-6-IL-21 live tumor vaccine is better in the previous study. It can stimulate the immunized mice to produce anti-melanoma immune response effectively and resist the attack of wild type B16F10 cells.

Previously, Zhao et al generated the B16F10-ESAT-6-gpi/IL-21 CD133⁺CD44⁺ cell stem cell vaccine, which demonstrated to enhance antimelanoma efficacy as revealed by decreased tumor growth and increased survival time of tumor-bearing mice. Furthermore, the anti-tumor effects were also associated with increased serum level of anti-ESAT-6 and IFN-y as well as enhanced cytotoxic activities of NK cells, splenocytes, and complement dependent cytotoxicity. Meanwhile, the B16F10-ESAT-6-gpi/IL-21 CD133⁺CD44⁺ cell stem cell vaccine also suppressed melanoma lung metastasis through regulating the epithelial mesenchymal transition process [21]. Meanwhile, He et al demonstrated that the anti-ESAT-6 antibody induced by the ESAT-6 DNA vaccine activates a complement classical pathway and contributed to the B16F10 tumor cell lysis and apoptosis, which subsequently served as a trigger to elicit an initiation of anti-melanoma immunity [8, 9].

In this experiment, we injecting ESAT-6 and IL-21 into melanoma cells in vivo through constructing the recombinant adenovirus vector first. Adenovirus cannot be transfected into B16F10 cells when MOI was less than 20, while almost all of the B16F10 cells were GFP positive when MOI was 200. Combined with different genes, B16F10 cells were cultured with ESAT-T cells in different proportion of E/T, the LDH release was much more than that in B16-F10 cells cultured with T cells. It showed that the effect of antitumor of T cells combined with ESAT-6 was much better. The results also showed that the LDH release was much more in B16F10-ESAT-6-IL-21 than that in B16F10-IL-21, suggesting that the immunogenicity and the anti-tumor effect of B16F10-ESAT-6-IL-2 are better. Regardless of E/T proportion, the levels of IL-21 and IFN-y in B16F10-IL-21, B16-F10-ESAT and B16F10-ESAT-6-IL-21 transfered with ESAT-6-T cells were higher than those in control groups. The levels of IL-21 and IFN-y were increased along with the increased E/T proportion. Data showed that IL-21 can lead to IFN-y increase and then the activity of NK cells and CTL cells are strengthened. NK cells and CTL cells can promote the IFN-y production in reverse and enhance antitumor effect of lymphocyte [22]. Therefore, the more IL-21 and IFN-y, the better antitumor effect is. As shown in Figure 4A, tumor shrank was more prominent in the B16F10-ESAT-6-IL-21 tumor vaccine than that in other tumors, the results suggested that the stronger the immunogenicity, the better the anti-tumor effect. Compared with B16F10-GFP tumor vaccine, tumor immunogenicity was stronger after transfusion of ESAT-T cell. The tumor growth was slower and the antitumor effect was also better. As the stronger immunogenicity of tumor, the releases of IL-21 and IFN-y are higher and these results also contribute to the better anti-tumor effect.

In this study, heterologous antigen ESAT-6 plays a "fire" effect through mediating effect of high immunogenicity [21]. With nonspecific and common used tumor gene modified in situ with adoptive ESAT-6 specific T cell transfusion, targeting specific antitumor immune response in the body was activated cascade and immune tolerance of tumor was broken. The immune response induced by tumor vaccine can attack cells vaccined and primary or metastatic tumor cells, exert immune therapeutic effect, and solve the problem of efficiency of tumor active immunotherapy [23, 24]. This method can not only play the advantages of immune effect of live tumor vaccine, but also can avoid the restriction of the use of tumor vaccine in the clinical application. Collectively, our study provides an advanced technology solutions for the immunotherapy of melanoma.

Disclosure of conflict of interest

None.

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