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# Artificial antigen–presenting cells plus IL–15 and IL–21 efficiently induce melanoma–specific cytotoxic CD8<sup>+</sup>CD28<sup>+</sup> T lymphocyte responses

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## ABSTRACT

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**Objective:** To develop a novel artificial antigen–presenting system for efficiently inducing melanoma–specific CD8<sup>+</sup>CD28<sup>+</sup> cytotoxic T lymphocyte (CTL) responses. **Methods:** Cell–sized Dynabeads<sup>®</sup> M–450 Epoxy beads coated with H–2K<sup>b</sup>:Ig–TRP2<sub>180–188</sub> and anti–CD28 antibody were used as artificial antigen–presenting cells (aAPCs) to induce melanoma–specific CD8<sup>+</sup>CD28<sup>+</sup> CTL responses with the help of IL–21 and IL–15. Dimer staining, proliferation, ELISPOT, and cytotoxicity experiments were conducted to evaluate the frequency and activity of induced CTLs. **Results:** Dimer staining demonstrated that the new artificial antigen–presenting system efficiently induced melanoma TRP2–specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs. Proliferation and ELISPOT assays indicated that the induced CTLs rapidly proliferate and produce increased IFN– $\gamma$  under the stimulation of H–2K<sup>b</sup>:Ig–TRP2–aAPCs, IL–15, and IL–21. In addition, cytotoxicity experiments showed that induced CTLs have specific killing activity of target cells. **Conclusions:** The new artificial antigen–presenting system including aAPCs plus IL–21 and IL–15 can induce a large number of antigen–specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs against the melanoma. Our study provides evidence for a novel adoptive immunotherapy against tumors.

## 1. Introduction

Adoptive immunotherapy using antigen–specific T cells is

a promising strategy to treat a variety of infectious diseases and cancer. Although adoptive immunotherapy with tumor–specific cytotoxic T lymphocytes (CTLs) has been widely studied and been successfully used to treat certain cancers, obstacles still have to be overcome. Previous studies of adoptive immunotherapy with tumor–specific CTLs have achieved successful results in *in vitro* experiments, but the effectiveness is not as good as *in vivo* experiments and in clinical trials[1–3]. These problems can mainly be explained by the fact that the activities of adoptive CTLs are inhibited in animal and human hosts, preventing the effective clearance of tumor cells[4,5]. Therefore, increasing the anti–tumor activity of tumor–specific CTLs is key to the success of specific adoptive immunotherapy of tumors[6].

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IL-21 and IL-15, members of the common cytokine receptor gamma-chain family, play an important role in the proliferation and activity of CD8<sup>+</sup> T cells. IL-21 is mainly produced by activated CD4<sup>+</sup> T cells and can greatly promote the proliferation and differentiation of CD8<sup>+</sup> T cells[7], and thus enhance the cell toxicity of CTLs[8]. IL-15 can also promote the proliferation of naive and memory CD8<sup>+</sup> T cells[9]. Previous studies reported that the combination of IL-21 and IL-15 synergistically enhanced the biological activity of CD8<sup>+</sup> T cells, and induced memory CD8<sup>+</sup> CTLs with high levels of CD28[10].

It has been shown that artificial antigen-presenting cells (aAPCs) can overcome the shortcomings of the conventional method in expanding antigen-specific CTLs[11]. aAPCs that are constructed by presenting a combination of MHC-peptide complexes and costimulatory molecules on the surface of the carrier mimic the dual signaling mechanisms necessary for T cell activation. aAPCs can effectively activate antigen-specific CTLs and induce CTL responses with high efficiency and feasibility[12,13]. We have previously developed several novel aAPC systems to induce tumor-specific CD8<sup>+</sup> CTLs[12,13], but the biological activity of induced CD8<sup>+</sup> CTLs needs to be further improved to ensure high activity in the human body.

Recent studies have demonstrated that CD8<sup>+</sup> CTLs with high CD28 expression survived longer and had increased proliferation rates[14,15]. In addition, Bloom *et al* reported that the peptide SVDFVWL (TRP2<sub>180-188</sub>) from the TRP2 protein is H-2K<sup>b</sup> restricted and is a mouse melanoma-specific peptide[16]. Thus, in this study, we used the mouse melanoma-specific peptide TRP2<sub>180-188</sub> as a target to develop an artificial antigen presenting system to induce melanoma-specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs for the potential immunotherapy of melanoma. Cell-sized microspheres coated with complexed H-2K<sup>b</sup>:Ig-TRP2 and anti-CD28 antibody were used as aAPCs to induce melanoma-specific and H-2K<sup>b</sup>-restricted CTLs under the stimulation of IL-21 and IL-15. Our study indicates that aAPCs under the stimulation of IL-21 and IL-15 can efficiently induce melanoma-specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs with high tumor-specific activity.

## 2. Materials and methods

### 2.1. Cell lines and cell culture

The B16 melanoma cell line was derived from a spontaneous melanoma in a C57BL/6 mouse and was cultured in our laboratory. The mouse hepatocellular carcinoma cell line H22 was purchased from Shanghai FMGBio Tech Co., Ltd. The dendritic cell line, DC2.4 was cultured in our laboratory. All the cells were cultured in complete RPMI 1640 or Dulbecco's modified Eagle medium (DMEM, HyClone, Thermo) containing 10% Fetal Bovine Serum (FBS) (Hyclone, Thermo), 100 μg/mL streptomycin

and 100 IU/mL penicillin at 37 °C, 5% CO<sub>2</sub>.

### 2.2. Polypeptides

The H-2K<sup>b</sup>-restricted and lung cancer-specific antigenic peptide Mut1 (amino acid: 52–59, N-FEQNTAQP-COOH[17]), and the H-2K<sup>b</sup>-restricted and melanoma-specific antigenic peptide TRP2 (amino acid: 180–188, N-SVYDFFVWL-COOH) were chemically synthesized by Nanjing GenScript Corporation with a purity of greater than 95%.

### 2.3. Antibodies and FACS

Purified hamster anti-mouse CD28 antibody and PE-labeled CD8 monoclonal antibody were purchased from (BD pharmingen, San Jose, USA). FITC-labeled mouse IgG1 monoclonal antibody was purchased from (Biolegend, San Diego, CA, USA). PE-Cy5 labeled mouse CD28 monoclonal antibody was purchased from (eBioscience, CA, USA). Labeled cells were analyzed on a Beckman Coulter flow cytometer and the data was analyzed using EXPO32 analysis software (Beckman Coulter, USA).

### 2.4. Preparation of aAPCs

100 μg/mL of TRP2 antigen or control peptide Mut1 were incubated with 20 μg/mL of recombinant soluble Dimeric mouse H-2K<sup>b</sup>:Ig Fusion protein (BD Bioscience), at 37 °C overnight to generate H-2K<sup>b</sup>:Ig-TRP2 complexes and H-2K<sup>b</sup>:Ig-Mut1 complexes. Artificial beads (Dynabeads<sup>®</sup> M-450 Epoxy, Invitrogen, Norway) were incubated with H-2K<sup>b</sup>:Ig-TRP2 complexes and anti-mouse CD28 monoclonal antibody (2 μg/mL) at 4 °C for 1 h to make H-2K<sup>b</sup>:Ig-TRP2-aAPCs. The control peptide Mut1 was prepared in the same way to make H-2K<sup>b</sup>:Ig-Mut1-aAPCs that were used as a control.

### 2.5. Inducing T cell activation

4–6 weeks female C56B/L mice (H-2K<sup>b</sup>) were purchased from Beijing Unicom Lihua. Mouse splenic lymphocytes were isolated using lymphocyte separation medium (EZ-Sep<sup>™</sup>, DAKWE) and cultured in complete RPMI 1640 at 37 °C under 5% CO<sub>2</sub>. Cells were seeded into 24-well plates at a density of 3×10<sup>6</sup>–4×10<sup>6</sup> cells/mL and co-cultured with H-2K<sup>b</sup>:Ig-TRP2-aAPCs or H-2K<sup>b</sup>:Ig-Mut1-aAPCs (the ratio of cells and aAPCs was 10:1) in the presence of rmIL-21 (25 ng/mL, R & D, USA) and/or rmIL-15 (25 ng/mL, R & D, USA)[18]. The cells were fed every 3–4 days and aAPCs were replaced once every seven days.

### 2.6. Detection of TRP2-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells

Isolated mouse splenic lymphocytes were co-cultured with artificial H-2K<sup>b</sup>:Ig-TRP2-aAPCs or H-2K<sup>b</sup>:Ig-Mut1-aAPCs

in the presence of rmIL-21 and/or rmIL-15 for 1 week. Cells were collected and analyzed by FACS to detect the dimer and CD8<sup>+</sup>CD28<sup>+</sup> cell frequency.

### 2.7. Isolation of CD8<sup>+</sup> T cells and cell proliferation assays

CD8<sup>+</sup> T cells were positively selected from mouse splenic lymphocytes using CD8 antibody coated dynabeads (Invitrogen, Carlsbad, CA, USA) and microbeads (Miltenyi Biotec). The purity of selected CD8<sup>+</sup> T cell populations was > 95%, as measured by FACS analysis. The proliferation of CD8<sup>+</sup> T cells was measured by carboxyfluorescein diacetate succinimidyl ester (CFSE) as described previously<sup>[19,20]</sup>. Briefly, selected CD8<sup>+</sup> T cells were incubated with 5 μmol/L of CFSE at 37 °C for 10min, then washed three times with RPMI 1640 containing 10% FBS. 2×10<sup>6</sup>/mL of CD8<sup>+</sup> T cells were plated into 12-well plates and co-cultured with H-2K<sup>b</sup>:Ig-TRP2-aAPCs at 37 °C with 5% CO<sub>2</sub> in the presence of rmIL-21 and/or rmIL-15. On the third day, cells were analyzed by FACS and the proliferation index was analyzed by ModFit LT software (Beckman Coulter, USA).

### 2.8. ELISPOT assay

The ELISPOT protocol was modified according to the following<sup>[21]</sup>. The CD8<sup>+</sup> T cells were stimulated and co-cultured with H-2K<sup>b</sup>:Ig-TRP2-aAPCs in the presence of rmIL-21 and/or rmIL-15 for one week, then cells were seeded at 1×10<sup>6</sup>/well and co-cultured with H-2K<sup>b</sup>:Ig-TRP2-aAPCs or H-2K<sup>b</sup>:Ig-Mut1-aAPCs in a 96-well plate coated with mouse IFN-γ antibody (DAKEWE), at 37 °C for 16 h. The spots were quantified using an ELISPOT counter, and analysed with the Immunospot count software, according to the manufacturer's instruction (CTL Technologies).

### 2.9. Cytotoxicity assay

The cytotoxicity of TRP2-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells was measured by Proidium Iodide (PI) staining and FACS<sup>[22]</sup>. The target cells B16, DC2.4 and H22 were washed twice with 0.1% BSA-PBS and stained with PKH26 dye (Sigma Aldrich) at room temperature for 4 min, in accordance with the manufacturer's instruction. TRP2-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells (effector cells) and PKH26 positive cells (target cells) were plated in 48-well plates according to the effector:target ratio (40:1, 20:1, 10:1, 5:1) and co-cultured at 37 °C with 5% CO<sub>2</sub> for 6 h. Cells were collected and stained using PI (Roche) at room temperature for 15 min. The stained cells were analyzed by FACS and the data were analyzed with EXPO32 software. The target cell apoptosis rate was defined in PKH26 positive cells. Data are expressed as the percent specific release, calculated as follows: [(experimental release – spontaneous release)/(100 – spontaneous release)] × 100.

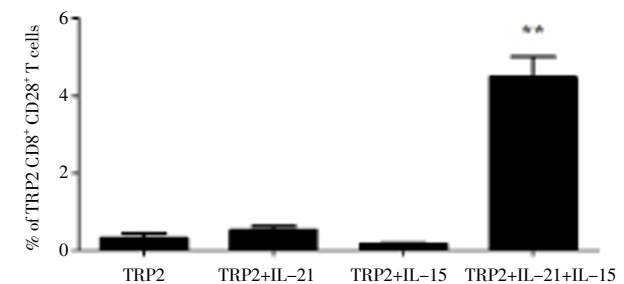
### 2.10. Statistical analysis

The data were showed as mean±standard deviation and analyzed using SPSS 17.0 software (SPSS Inc, Chicago, IL) or the use of GraphPadPrism 5 software (GraphPad, San Diego, CA). Continuous variables were compared by one-way analysis of variance (ANOVA). The value of *P* < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Effect of novel artificial antigen-presenting system on high frequencies of CD8<sup>+</sup>CD28<sup>+</sup> T cells specific to the melanoma peptide TRP2

Dimer staining was used to investigate whether the novel artificial antigen-presenting system generated high frequencies of antigen-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells. As shown in Figure 1, the histograms show the rate of H-2K<sup>b</sup>:Ig-TRP2-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells in the experimental group treated with a combination of H-2K<sup>b</sup>:Ig-TRP2-aAPCs with IL-15 and IL-21 was significantly higher than the other groups (*P* < 0.01). These results indicate that H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 can efficiently induce high frequencies of TRP2-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells.



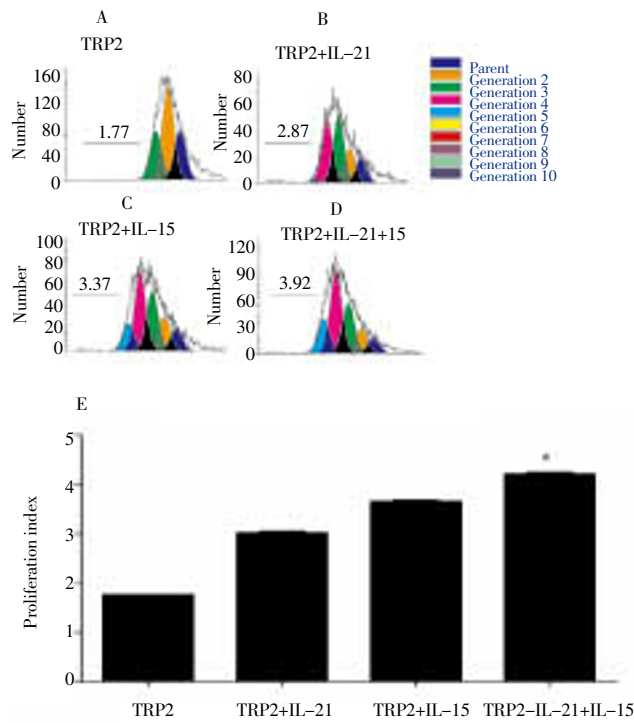
**Figure 1.** Effect of novel artificial antigen-presenting system on high frequencies of CD8<sup>+</sup>CD28<sup>+</sup> T cells specific to the melanoma peptide TRP2.

The rate of H-2K<sup>b</sup>:Ig-TRP2-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells in the experimental group treated with a combination of H-2K<sup>b</sup>:Ig-TRP2-aAPCs with IL-15 and IL-21 was 4.49%.\*\* *P* < 0.01 compared with the control groups.

### 3.2. Effect of induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 on proliferation ability of T cells

CFSE staining was used to evaluate the proliferation ability of T cells induced by different factors including H-2K<sup>b</sup>:Ig-TRP2-aAPCs, H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-15, H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21, and H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15. As shown in Figure 2A–D, T cells induced by the combination of H-2K<sup>b</sup>:Ig-TRP2-aAPCs with IL-15 and IL-21 demonstrated the most proliferation, 3.92, which was significantly higher than proliferation in the

other groups. The Figure 2E histograms show similar results ( $P < 0.05$ ). These results indicate that the induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 proliferated more readily than control groups.

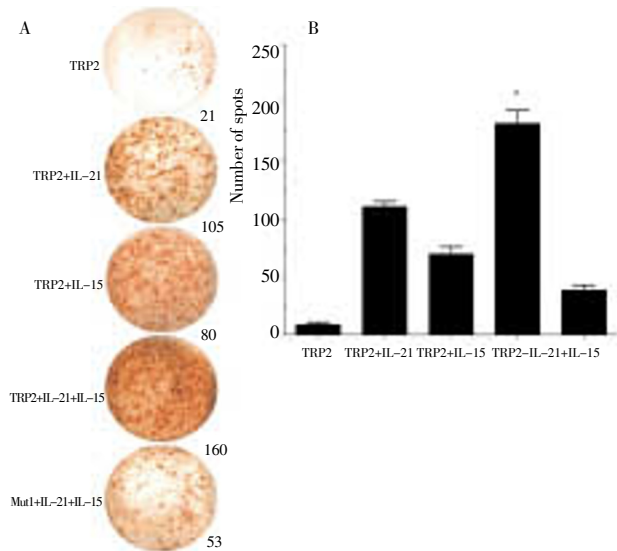


**Figure 2.** Effect of induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 on proliferation ability of T cells.

(A). The proliferation index of the H-2K<sup>b</sup>:Ig-TRP2-aAPCs without cytokines group was 1.77. (B). The proliferation index of the H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 group was 2.87. (C). The proliferation index of the H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-15 was 3.37. (D). The proliferation index of the H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus cytokines IL-15 and IL-21 group was 3.92. \*  $P < 0.05$  compared with the control group.

**3.3. Effect of induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 on levels of IFN- $\gamma$**

We used ELISPOT assays to measure the quantity of the induced-T cells that produced IFN- $\gamma$  under continuous stimulation with H-2K<sup>b</sup>:Ig-TRP2-aAPCs or H-2K<sup>b</sup>:Ig-Mut1-aAPCs. As shown in Figure 3A, the number of the induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 that produced IFN- $\gamma$  under continuous stimulation with H-2K<sup>b</sup>:Ig-TRP2-aAPCs was 160, which was significantly higher than other groups. The histograms (Figure 3B) show the mean and standard deviation of the numbers of the induced-T cells under the stimulation of different molecules and was similar to the results shown in Figure 3A ( $P < 0.05$ ). These results show that the induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 produced IFN- $\gamma$  at high levels under the stimulation of H-2K<sup>b</sup>:Ig-TRP2-aAPCs in the novel artificial antigen presenting system.

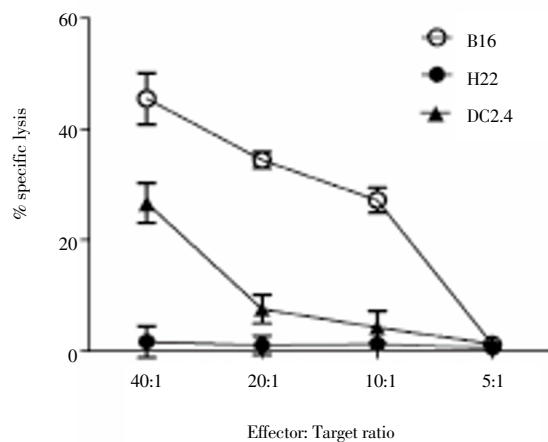


**Figure 3.** Effect of induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 on levels of IFN- $\gamma$ .

(A). Comparison of the quantity of cells producing IFN- $\gamma$  between each of the groups. The number of the induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 producing IFN- $\gamma$  was 180, higher than the other controls (21, 105, 80, 53 respectively). These data are based on triplicate experiments. (B). The mean and standard deviation of the quantity of T cells producing IFN- $\gamma$  from each group. \*  $P < 0.05$  compared to the control group.

**3.4. Specific toxicity activities of induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15**

Cytotoxicity experiments were conducted to test whether the induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 had specific cytotoxic activities. As shown in Figure 4, the antigen-specific ability to kill B16 target cells was significantly higher when compared to control target cells, DC2.4 and H22 ( $P < 0.05$ ). These results indicate that the induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15 have antigen-specific cytotoxic activities.



**Figure 4.** Specific toxicity activities of induced-T cells by H-2K<sup>b</sup>:Ig-TRP2-aAPCs plus IL-21 and IL-15.

The experiments were repeated three times. \*  $P < 0.05$ .



#### 4. Discussion

CD8<sup>+</sup> CTLs play an important role in the adaptive immune response, especially in anti-tumor and anti-infection immunity<sup>[23]</sup>, which makes them a promising candidate for adoptive immunotherapy. However, long-term studies found that adoptive immunotherapy with CD8<sup>+</sup> CTLs is often characterized by poor persistence of the transferred T cells and limited effector responses, which has limited their usage. In this study, we developed a novel artificial antigen-presenting system to induce highly efficient tumor specific CTLs. This artificial antigen-presenting system uses aAPCs together with IL-21 and IL-15 to induce melanoma-specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs as a potential approach for immunotherapy of melanoma.

aAPCs are constructed with artificial materials (such as beads, liposomes, latex microspheres, *etc.*) or gene-transfected cells (such as tumor cells, insect cells, *etc.*) that are used as carriers and coated with molecules that can stimulate T cell activation. T cells can be activated *in vitro* and *in vivo* by aAPCs that mimic T cell activation signals. In addition, aAPCs can be used in adoptive immunotherapy against tumor and infectious diseases by modifying the types and quantity of stimulatory molecules on the surface of aAPCs. They can also be used in the study of antigen-presenting and T cell activation signals. aAPCs can easily be prepared and preserved, and manual regulation of the types and quantity of stimulatory molecules on the surface of aAPCs make them an ideal tool for anti-tumor immunotherapy. In addition, aAPCs can induce and stimulate the proliferation of antigen-specific CTLs *in vitro*, which helps overcome limitations of conventional methods which use peptide-loaded dendritic cells for inducing antigen-specific CTLs<sup>[24]</sup>. Melanoma is one type of cancer that is sensitive to immunotherapy. A large number of melanoma-associated antigen epitopes have been identified and characterized, suggesting aAPCs may be a promising therapeutic tool in adoptive immunotherapy of melanoma. In this study, we successfully developed H-2K<sup>b</sup>-Ig:TRP2-aAPCs, that are specific to the melanoma-specific antigen peptide TRP2<sub>180–188</sub>.

In immune responses, CD28 molecules are highly expressed on the surface of naive T cells. Binding of CD28 with B7 molecules initiates the second signal of T cell activation<sup>[25]</sup>. However, CD28 expression on naive CD8<sup>+</sup> T cells gradually decreases after antigen stimulation, resulting in increased numbers of CD8<sup>+</sup>CD28<sup>-</sup> T cells over time<sup>[18]</sup>. In clinical studies of patients with ulcerative colitis, the number of CD8<sup>+</sup>CD28<sup>-</sup> T cells was higher than CD8<sup>+</sup>CD28<sup>+</sup> T cells<sup>[20]</sup>. In addition, some studies observed that naive T cells in the body decrease with increased age. Due to the absence of CD28 molecules that are critical for starting the second signal, these naive T cells gradually change to become incompetent memory lymphocytes, such as CD8<sup>+</sup>CD28<sup>-</sup> T cells<sup>[18,26,27]</sup>. Recently, studies have shown that high levels of CD28 on CD8<sup>+</sup> CTLs can increase the proliferation and survival of CTLs in the body<sup>[14,15]</sup>. Thus, adoptive immunotherapy targeting CD28 on CD8<sup>+</sup> CTLs might be better than the traditional adoptive immunotherapy based on CD8<sup>+</sup> CTLs alone. In this study, we

confirmed that our novel artificial antigen presenting system efficiently induced melanoma-specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs that specifically killed tumor cells.

IL-21 can induce CD8<sup>+</sup> T cell proliferation and differentiation<sup>[28,29]</sup>, promote the proliferation of memory T cells<sup>[14]</sup>, activate NK cells<sup>[30]</sup>, and enhance CTL toxicity<sup>[10]</sup>. Unlike IL-2, which regulates the function of memory T cells by inducing regulatory T cells (Tregs), IL-21 can inhibit the proliferation of Tregs to ultimately reverse the inhibitory effect of Tregs on CD8<sup>+</sup> T cells. The proliferation of CD8<sup>+</sup> T cells can cause tumor cell-mediated death by CD8<sup>+</sup> T cells<sup>[31,32]</sup>. Therefore, IL-21 may have promising application for cancer immunotherapy. Numerous studies suggest that CTLs and NK cells play an important role in anti-tumor immunity mediated by IL-21. Kaka *et al* confirmed that T cells in which the IL-21 gene was overexpressed were able to promote the proliferation of central memory tumor-specific CD8<sup>+</sup> CTLs and significantly extend the lifespan of the tumor-specific central memory CTLs<sup>[33]</sup>. This study suggests that IL-21 may be associated with the central memory component of the immune system. IL-15 and IL-21 are both members of the common cytokine gamma-chain receptor family. IL-15 has been proven to promote the proliferation of naive CD8<sup>+</sup> T cells and memory CD8<sup>+</sup> T cells, and it can maintain the number and function of naive CD8<sup>+</sup> T cells in humans and mice<sup>[9,20]</sup>. However, IL-15 downregulates the expression of CD28 on CD8<sup>+</sup> T cells<sup>[10,18]</sup>. On the contrary, IL-21 promotes the proliferation of naive CD8<sup>+</sup> T cells and plays an important role in maintaining the long-term activity and function of CD8<sup>+</sup> T cells. One study demonstrated that IL-21 prevented the aging of CD8<sup>+</sup> T cells with increased age in humans<sup>[27]</sup>. In addition, IL-21 prevented the ability of IL-15 to downregulate the expression of CD28 on naive CD8<sup>+</sup> T cells or memory CD8<sup>+</sup> T cells<sup>[18,19]</sup>. Therefore, the combination of IL-15 and IL-21 could significantly increase the proliferation and cytotoxic function of naive or memory CD8<sup>+</sup> T cells. Their combination can also neutralize the negative effects caused by IL-15 regarding the downregulation of CD28. Studies have demonstrated that low doses of IL-15 and IL-21 promote the proliferation of CD28<sup>+</sup> cells<sup>[10,18]</sup>. Our study further confirms that the combination of IL-15 and IL-21 efficiently induced the proliferation of melanoma-specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs mediated by H-2K<sup>b</sup>-Ig:TRP2-aAPCs.

In conclusion, we developed a novel artificial antigen-presenting system that employs melanoma-specific aAPCs plus IL-21 and IL-15 to efficiently induce efficient responses from melanoma-specific CD8<sup>+</sup>CD28<sup>+</sup> T cells. Our results demonstrate that the induced melanoma-specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs express high levels of IFN- $\gamma$  and proliferate at higher rates than regular CTLs. This novel system provides an alternative approach for the generation of tumor-specific CD8<sup>+</sup>CD28<sup>+</sup> CTLs and may be useful for the adoptive immunotherapy against melanoma.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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