Melanoma Stem Cell Vaccine Modified with IL-33 Induces Anti-Tumor Immunity by Activating CD8⁺T Cells

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Research

Abstract

Background: Melanoma stem cells (MSCs)-based vaccine strategies have been a potent immunotherapeutic approach for melanoma treatment, which aimed at inducing specific anti-tumor immunity and targeting cancer stem-like cells. To boost anti-melanoma activity induced by B16F10 CD44$^+$CD133$^+$ MSCs (B16F10 MSCs) vaccine, we generated a novel vaccine expressing IL-33. Tumor growth and pulmonary metastasis were assessed to estimate the effectiveness of the vaccine.

Methods: The antitumor effect of the vaccine was observed in this study. The mechanism of inducing anti-tumor immunity was detected by flow cytometric assays, cytotoxicity assays, and ELISA, including expression of CD8$^+$T cells surface and intracellular molecules, the cytotoxic activity of splenocytes in the immunized mice and secretion of serum cytokines.

Results: We found that MSCs vaccine expressing IL-33 significantly inhibited melanoma growth and reduced the lung melanoma nodules. Mechanistic investigations established that the vaccine-primed CD8$^+$T cells could selectively target MSCs and confer anti-tumor immunity, which included promoting the proliferation of CD8$^+$T cells, inhibiting the differentiated depletion of CD8$^+$T cells in vivo, inducing the formation of memory CD8$^+$T cells, and activating specific cytotoxic T lymphocyte (CTL) immune response.

Conclusions: MSCs vaccine expressing IL-33 is able to initiate anti-tumor specific immune response by activating CD8$^+$T cells.

1. Background

Melanoma is one of the most sensitive tumors to immune modulation [1, 2]. In the past decade, immunotherapy has greatly changed the treatment prospects of melanoma [1, 3]. In particular, immune checkpoint inhibitors (ICIs) targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1) have achieved apparent clinical effect [1, 4, 5]. As an important branch in the field of immunotherapy, vaccines have been rarely studied in melanoma. Gp100 peptide vaccines could produce high levels of circulating T cells in vitro, recognize and kill melanoma cells, but the clinical efficacy was still not ideal [6]. Therefore, effective tumor vaccines can be an important supplement in the aspect of melanoma immunotherapy.

As melanoma stem cells (MSCs) were discovered, the possible mechanism of the malignant transformation of normal melanocytes and melanoma cells acquiring tumorigenicity was better understood. [7−9]. According to MSCs theory, targeted MSCs therapy could inhibit the growth of melanoma and reduce the risk of tumor recurrence and metastasis [10−12]. Therefore, tumor vaccine targeting MSCs will improve the efficacy of current immunotherapy. At present, the research of MSCs vaccine in melanoma was mostly to induce protective anti-tumor immunity by sensitizing dendritic cells (DCs) with MSCs [12−14]. In addition, immune adjuvant could enhance the anti-tumor immune response of

MSCs vaccine [15, 16]. Immune adjuvant IL-21 gene-modified MSCs enhanced complement-dependent cytotoxic activity and natural killer cell (NK) cytotoxic activity in mice [17]. As a pleiotropic immune effector, IL-33 played an important role in activating anti-tumor immunity, including stimulating DCs maturation, enhancing the killing effect of CD8+T cells and NK cells [18, 19], gaining the immune response of antigen-specific effector T cells and memory T cells in vivo [15, 20, 21], and inhibiting tumor growth and lung metastasis in mice [22, 23]. IL-33 can act as an effective immune adjuvant. In this study, the IL-33 modified MSCs vaccine was assessed in its inhibitory effect on melanoma growth and metastasis and tested its anti-tumor immune mechanism through activating CD8+T cells in direct and indirect ways.

2. Methods

2.1. Cell lines

B16F10 murine melanoma cells line was syngeneic with C57BL/6 mice. These cells were ordered from the iCell Bioscience of China in Shanghai and were cultured at 37°C in 5% CO_2 atmosphere in complete media consisting of RPMI 1640 and 10% fetal bovine serum.

2.2. Mice

C57BL/6 mice of 5-6 weeks of age were ordered from the Liaoning Changsheng animal experimental center of China. All mice were housed under the pathogen-free condition. All the experiments were performed in compliance with the guidelines of the Animal Research Ethics Board of Jilin University.

2.3. Isolation of B16F10-CD133+CD44+ cells and preparation of MSCs vaccine

The CD44+CD133+ double-positive cells were isolated from B16F10 cells line with immunomagnetic bead technology (eBioscience Company, USA) using the CD44 and CD133 monoclonal antibodies (eBioscience Company, USA) following the manufacturer's instructions. The isolated CD44+CD133+ double-positive cells were labeled 'B16F10-CD44+CD133+' cells. And then, B16F10-CD44+CD133+ cells were infected by IL-33 overexpression gene and enhanced green fluorescent protein (EGFP) overexpression control adenovirus. Followed by mitomycin C (MMC) 50 μg/ml was used to inactivate the cells. Finally, we could get B16F10-CD44+CD133+ cells which inhibited cell proliferation but still secreted IL-33. According to this method, we can prepare melanoma stem cell vaccine (vaccine group) and inactivate other control cells in the same way.

2.4. Immunization protocol

100 mice (female, weight: 18-20g and age between 6 and 7 weeks) were randomly divided into five groups of equal size (twenty per group): the B16F10-IL-33-CD44+CD133+ (vaccine group), the B16F10-EGFP-CD44+CD133+ group, B16F10- CD44+CD133+ group, the B16F10 wild type cells group, and PBS group. The mice received subcutaneous vaccination in the right groin with 5×10^5 different cell vaccines

inactivated with MMC (50 μg/ml) for 4 h, a total of three times immunizations with an interval of 7 days between the immunizations. Fifteen vaccinated mice were challenged subcutaneously with 1×10^5 B16F10 cells 7 days after final vaccination, and five vaccinated mice were challenged intravenously with 1×10^5 B16F10 cells. Tumor generation in each mouse was monitored every two days by taking 2-dimensional measurements of individual tumors. The pulmonary metastatic nodules were observed weekly after 3 weeks for the last immunization.

2.5. Indirect co-culture of DCs and tumor vaccine

The bone marrow cells were extracted from femur and tibia of mice, and these cells were induced to immature DCs by granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml) and IL-4 (10 ng/ml). Then, the induced DCs were seeded in 6-well plates with a cell density of 5×10^5/ml. Meanwhile, the inactivated tumor vaccine of different groups (including equal volume PBS) was seed in suspension cell culture chamber (Millicell, Germany); each well was added with 2 ml 10% FBS 1640; the cell density was 5×10^5/ml, and suspended in 6-well plate for indirect co-culture with DCs. After 4 days of indirect co-culture, DCs were collected. The expression of mature markers in DCs was determined by flow cytometry with monoclonal antibodies (eBioscience Company, USA) CD11c, CD80, CD86 and major histocompatibility complex (MHC) II (see below).

2.6. Direct co-culture of DCs and mouse spleen lymphocytes

The spleen lymphocytes were isolated from healthy mice using mouse lymphocyte separation medium (DAKEWEI, China) following the manufacturer's instructions, and these cells were labeled with CFSE (Biolegend, USA). DCs, co-cultured with the inactivated tumor vaccine of different groups (including equal volume PBS) for 4 days, were collected, and then the DCs and CFSE-labeled mouse spleen lymphocytes were co-cultured directly. Briefly, the DCs 1×10^5 were mixed with CFSE-labeled splenic lymphocytes in a ratio of 1:10 and co-cultured in 6-well plate for 6 days. After that, the proliferation of $CD8^+T$ cells was detected by flow cytometry (see below) with anti-CD8 monoclonal antibody (eBioscience Company, USA). Meanwhile, cell culture supernatant from different groups was collected for ELISA to detect the levels of IFN-γ and TNF-α.

2.7. Flow cytometry analysis

The mouse spleen lymphocytes were isolated from different groups using mouse lymphocyte separation medium following the manufacturer's instructions after 3 weeks for the last immunization. Immunofluorescence surface staining was performed as previously described [24]. The FITC-labeled mAbs against CD3, CD8, CD44, CD133 and PE-labeled CTLA-4 were purchased from Biolegend, APC-labeled CD69, PD-1, Tim-3, CD62L, and CD127 Ab was purchased from BD Biosciences.

Intracellular staining was performed as previously described [24]. PE-conjugated or Pacific Blue-conjugated mAbs against IFN-γ and granzyme B (GzmB) were purchased from Biolegend. After staining,

cell samples were analyzed using a Beckman CytoFLEX Flow cytometry (Germany).

2.8. Cytotoxicity assay

The mouse spleen lymphocytes were isolated from different groups after 3 weeks for the last immunization. After counting, 1×10^7 splenic lymphocytes were taken from each group as effector cells, B16F10, were labeled with Calcein-AM (Biolegend, USA), were used as target cell. Effector cells were co-cultured with target cells at different ratios (200:1, 100:1, and 50:1) for 8 h. To examine the specific cytotoxicity of the splenic lymphocytes, the spleen lymphocytes of mice in vaccine group were used as effector cells, B16F10, B16F10-CD44$^-$CD133$^-$ and B16F10-CD44$^+$CD133$^+$ were taken as target cells. Then, the cytotoxicity effect of splenic lymphocytes on B16F10 was determined by Calcein AM release assay.

2.9. Preparation of single cell from mouse tumor mass

In the tumor bearing mice model, tumor cells were collected from different groups after 3 weeks for the last immunization. Briefly, the melanoma tissues were taken out from the tumor bearing mice under aseptic conditions and washed with sterile PBS. Then, tumor tissue was cut into a paste by scissors and digested into single cells with 0.25% trypsin for 60 min. The single cell suspension was obtained after filtration with filter. Finally, the expression of CD44 and CD133 in these was determined by flow cytometry with monoclonal antibodies (eBioscience Company, USA).

2.10. ELISA for cytokines

The IFN-γ and TNF-α concentrations in culture supernatants from direct co-culture of DCs and mouse spleen lymphocytes were detected by ELISA assay using ELISA kit (mlbio, China) following the manufacturer's instructions. In addition, the serum of mice in different groups was collected after 3 weeks for the last immunization. The IFN-α, IL-2, TNF-α and IFN-γ in the serum of mice were detected by ELISA assay.

2.11. Statistical analysis

Numerical data in this study were reported using means ± standard deviation (SD). Data analyses were performed using paired t tests with the GraphPad Prism 8 software. Statistical differences were assessed at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

3. Results

3.1. Inhibitory effect of MSCs vaccine on melanoma growth and lung metastasis

Tumor size was measured at 2, 3 and 4 weeks after tumor bearing. The tumor size in vaccine group was significantly smaller than that in negative control B16F10 group and blank control PBS group ($P < 0.05$);

while compared with B16F10-CD44$^+$CD133$^+$ and B16F10-EGFP CD44$^+$CD133$^+$ groups, the result showed no significant difference ($P > 0.05$). In addition, the tumor size in B16F10-CD44$^+$CD133$^+$ and B16F10-EGFP CD44$^+$CD133$^+$ groups was also smaller than that in PBS group (Fig. 1a).

The lung metastases were observed at 3, 4 and 5 weeks after the tail vein inoculation. The number of lung metastatic nodules in the vaccine group was significantly less than that in other control groups ($P < 0.05$) (Fig. 1b). These results indicated that MSCs vaccine could inhibit melanoma growth and lung metastasis.

3.2. MSCs vaccine enhances the cytotoxicity of splenic lymphocytes in mice

The mouse spleen lymphocytes, acted as effector cells, were isolated from different groups after 3 weeks for the last immunization. B16F10 cells cultured in vitro were used as target cells. The cytotoxicity of splenic lymphocytes in different groups was determined by Calcein AM release assay. The cytotoxicity of splenic lymphocytes in vaccine group was stronger than that in other control groups (Fig. 2a).

The spleen lymphocytes of mice in vaccine group were served as effector cells, and B16F10, B16F10-CD44$^-$CD133$^-$ and B16F10-CD44$^+$CD133$^+$ cells were used as target cells. The cytotoxicity of spleen lymphocytes on B16F10-CD44$^+$CD133$^+$ cells in vaccine group was significantly stronger than that on B16F10 and B16F10-CD44$^-$CD133$^-$ cells (Fig. 2b). The results showed that the vaccine could enhance the ability of splenic lymphocytes of mice to kill tumor cells, and could kill specifically B16F10-CD44$^+$CD133$^+$ cells. That is that it had targeted killing effect on melanoma stem cells.

3.3. MSCs vaccine reduces the expression of CD44 and CD133 in tumor cells of tumor-bearing mice

The tumor cells in the tumor-bearing mice were collected from different groups after 3 weeks for the last immunization. The expression of CD44 and CD133 detected by flow cytometry in vaccine group was lower than that in B16F10 and PBS group. These results indicate that the vaccine can kill specifically CD44$^+$CD133$^+$ melanoma cells, and then inhibit the progression of melanoma (Fig. 3).

3.4. Maturation of DCs, proliferation and activationof CD8$^+$T cells stimulated by MSCs vaccine

DCs are the most effective antigen presenting cells (APC) and play an important role in immune responses. Mature DCs are characterized by cell-specific surface markers, CD80, CD86 and MHC II. Mice bone marrow-derived DCs were stimulated by different experimental groups of inactivated tumor cells (including equal volume PBS). The expression of CD80, CD86 and MHC II was detected by flow cytometry. The level of CD80 in the vaccine group was significantly higher than that in other control groups; the levels of CD86 and MHC II in the vaccine group were higher than that in B16F10 and PBS group (Fig. 4a).

As DCs matured, CD8$^+$T cells were stimulated to proliferate. After the inactivated tumor cells (including equal volume PBS) of different experimental groups were indirectly co-cultured with DCs, the stimulated DCs were directly co-cultured with CFSE-labeled mouse spleen lymphocytes through mixed lymphocyte reaction for 6 days. Non co-cultured CFSE-labeled splenic lymphocytes (Tc group) were used as blank control for flow cytometry. The expression of CFSE was measured by CD8 gating to detect the proliferation of CD8$^+$T cells. The proliferation of CD8$^+$T cells in vaccine group was significantly higher than that in other control groups (Fig. 4b).

DCs could activate CD8$^+$T cells to secreted IFN-γ and TNF-α, which could inhibit proliferation of cancer cells. The levels of IFN-γ and TNF-α was determined by ELISA. The level of IFN-γ secreted by CD8$^+$T cells in vaccine group was significantly higher than that in B16F10, PBS and Tc group; the level of IFN-γ secreted by CD8$^+$T cells in negative control group was higher than that in Tc group. In addition, the level of TNF-α secreted by CD8$^+$T cells in vaccine group was significantly higher than that in other control groups; the level of TNF-α secreted by negative control and PBS group was significantly higher than that in Tc group (Fig. 4c). These results demonstrated that MSCs vaccine could promote DCs maturation, which stimulate the proliferation and activation of CD8$^+$T cells in MSCs vaccine indirectly co-cultured with DCs.

3.5. Effect of MSCs vaccine on the proportion and activity of CD8$^+$T cells in spleen of mice

To further evaluate the effect of MSCs vaccine to CD8$^+$T cells in vivo, the spleen lymphocytes were isolated from different groups mice after 3 weeks for the last immunization. The expression of CD3, CD8 and CD69 in splenocytes of different groups was detected by flow cytometry. The proportion of double positive expression of CD3 and CD8 in vaccine group was significantly higher than that in B16F10 and PBS groups (Fig. 5a); the expression of CD69, the surface activation marker of CD8$^+$T cells, was higher than that in other control groups. In addition, the expression of CD69 in CD8$^+$T cells of B16F10-CD44$^+$CD133$^+$ and B16F10-EGFP CD44$^+$CD133$^+$ groups was higher than that of PBS group (Fig. 5b). The above results indicated that the percentage of CD8$^+$T cells increased and the activation of CD8$^+$T cells enhanced in mice splenocytes by MSCs vaccine.

3.6. Effect of immune checkpoint expression and formation of CD8$^+$ central memory T cells in the spleen by MSCs vaccine

The expressions of PD-1, CTLA-4 and Tim-3 on CD8$^+$T cells in spleen of mice in different groups were detected by flow cytometry. The expression of PD-1 and CTLA-4 on CD8$^+$T cells in vaccine group was significantly lower than that in other control groups; the expression of Tim-3 was significantly lower than that in B16F10 and PBS groups (Fig. 6a). The results showed that the vaccine could inhibit the expression of immune checkpoint on CD8$^+$T cells, especially on PD-1 and CTLA-4.

Then, we assessed the markers of central memory CD8$^+$T cells, CD44, CD62L and CD127, by flow cytometry. The expression of them in vaccine group was significantly higher than that in B16F10 and

PBS group (Fig. 6b). The results indicated that MSCs vaccine trigger central memory CD8^{+}T cells against melanoma.

3.7. Increase of anti-tumor cytokines by MSCs vaccine in spleen CD8^{+}T cells and mice serum

The expression of IFN-γ and GzmB in CD8^{+}T cells was determined by intracellular flow cytometry. The expression of IFN-γ on CD8^{+}T cells in vaccine group was significantly higher than that in other control groups, while the expression of GzmB was significantly higher than that in B16F10 and PBS group (Fig. 7a).

The serum of mice in different groups was collected after 3 weeks for the last immunization. The levels of IL-2, IFN-α, TNF-α, and IFN-γ in serum of mice were detected by ELISA. The levels of IL-2, IFN-α and IFN-γ in vaccine group were higher than those in B16F10 and PBS group; the level of TNF-α in vaccine group was higher than those in other control groups (Fig. 7b). The results showed that the vaccine induced the expression of IFN-γ and GzmB in spleen CD8^{+}T cells and promotde the secretion of IL-2, IFN-α, TNF-α, and IFN-γ in mice serum.

4. Discussion

Cancer stem cells (CSCs) have been proved to exist in many solid tumors, and expressed specific markers [25–27]. In melanoma, CD44 and CD133 can be served as potential markers of MSCs [28, 29]. In preclinical studies, CSCs-based immunotherapy has been tried in mice and humans, and achieved good results. DCs-CSCs vaccine can induce humoral and cellular immune responses against CSCs, thus producing efficient anti-tumor immunity [8, 30, 31]. Here, the B16F10-D44^{+}CD133^{+} cells were separated by immunomagnetic beads, and IL-33 overexpression gene was used to modify the sorted melanoma stem cells to prepare anti-tumor vaccine (MSCs vaccine). Prophylactic immunization of mice showed that the MSCs vaccine could activate the anti-tumor immune response and significantly inhibit tumor progression and lung metastasis.

In previous studies, the vaccine, DCs loaded with lysate of MSCs (ALDEFLUOR^{+} D5 cells), could significantly inhibit melanoma growth and lung metastasis in immunized mice. The mechanism is that the vaccine activated antibodies and T cells to selectively target MSCs and induce protective anti-tumor immunity [8, 14]. IL-33 modified MSCs (B16F10-CD44^{+}CD133^{+}) vaccine in this study could also play an anti-tumor role by selectively targeting B16F10-CD44^{+}CD133^{+} cells. Importantly, the anti-tumor immune mechanism of the MSCs vaccine is through activating CD8^{+}T cells in direct and indirect ways. In the process of tumor immune response, the transformation of DCs from immature to mature is very important to initiate antigen-specific T cells [32]. As the most sensitive activator to stimulate T cells, DCs need a very small amount of antigen to stimulate T cell proliferation [32, 33]. Moreover, as an immune effector, IL-33 can also stimulate DCs maturation and induce DCs recruitment [34, 35]. Here, the MSCs

vaccine activated CD8$^+$T cells by stimulating DCs maturation in vitro, including induced the proliferation of CD8$^+$T cells and initiated the secretion of cytokines by CD8$^+$ T cells. In addition, we found that the MSCs vaccine could increase the ratio and activity of CD8$^+$ T cells in spleen, enhance the cytotoxicity of spleen lymphocytes and promote the secretion of serum cytokines through in vivo experiments, which is consistent with the previous research results of anti-tumor vaccine [7, 8, 14, 36].

Anti-tumor vaccine aims to expand high affinity CD8$^+$T cells, make them differentiate into CTLs that can kill specifically cancer cells, and produce long-lived memory CD8$^+$ T cells [37]. CTLs response is the most effective reaction to induce potential protective immunity against almost all tumors [38, 39]. The MSCs vaccine in our study increased the expression of IFN-γ and GzmB on CD8$^+$ T cells, and induced the activation of specific CTLs. In addition, the formation of malignant tumor presents a chronic process, which creates conditions for the formation of memory CD8$^+$ T cells [40], the vaccination is able to induce the formation of long-term memory CD8$^+$ T cells, which can prevent tumor recurrence [37]. In this study, the MSCs vaccine can promote the formation of central memory CD8$^+$ T cells in spleen. These cells have an ability to proliferation and self-renewal ability [41], when encountering antigen again, they have a high proliferation potential and can differentiate into effector T cells [42, 43].

The anti-tumor immune mechanism of the MSCs vaccine in this study is not entirely caused by the sensitization of DCs. Although IL-33 has no direct effect on naive T cells, it can directly activate DCs to express costimulatory molecules in vivo [35], and inhibit CD8$^+$ T cell differentiation depletion by down-regulating the expression of PD-1 and 2B4 [15]. Furthermore, IL-33 induces memory phenotype of tumor antigen-specific CD8$^+$ T cells in vitro [34]. Here, we found that the negative control group of MSCs vaccine also has a certain role in inducing anti-tumor immunity, including stimulating the maturation of DCs, inducing the memory phenotype of CD8$^+$ T cells and the expression of anti-tumor effector molecules, etc.. However, the MSCs vaccine carrying IL-33 has a more significant effect on activating anti-tumor immunity, which also fully indicates that IL-33 plays an important role in the anti-tumor immunity of melanoma stem cell vaccine.

5. Conclusion

Taken together, this study confirms that the MSCs vaccine expressing IL-33 significantly inhibited the melanoma growth and reduced the lung melanoma nodules. The anti-tumor immune mechanism is through activating CD8$^+$T cells in direct and indirect ways. Importantly, our findings lay a theoretical and experimental foundation for the application of melanoma stem cell vaccine in the immunotherapy of melanoma, and provide ideas for the exploration of new therapeutic schemes for melanoma.

Abbreviations

MSCs: Melanoma stem cells; CTL: cytotoxic T lymphocyte; ICIs: immune checkpoint inhibitors; CTLA-4: cytotoxic T-lymphocyte antigen-4; PD-1: programmed cell death protein-1; DCs: dendritic cells; NK: natural

killer cell; EGFP: enhanced green fluorescent protein; MMC: mitomycin C; GM-CSF: granulocyte-macrophage colony-stimulating factor; MHC: major histocompatibility complex; GzmB: granzyme B; APC: antigen presenting cells; CSCs: Cancer stem cells

Declarations

Acknowledgements

Not applicable

Author's contributions

QY and NX: conception and design, collection and/or assembly of data, data analysis and interpretation, and manuscript writing. HJ and WL: conception and design, supervision of the study, and final approval of manuscript. NZ, YC: collection and/or assembly of data, data analysis and manuscript writing. MD, MX, WX: collection and/or assembly of data. All authors read and approved the final manuscript.

Funding

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The use of human tissue was approved by the ethics committee of the First Hospital of Jilin University, China (EK Nr. 985/2016, 25 January 2018), and the donor gave written consent.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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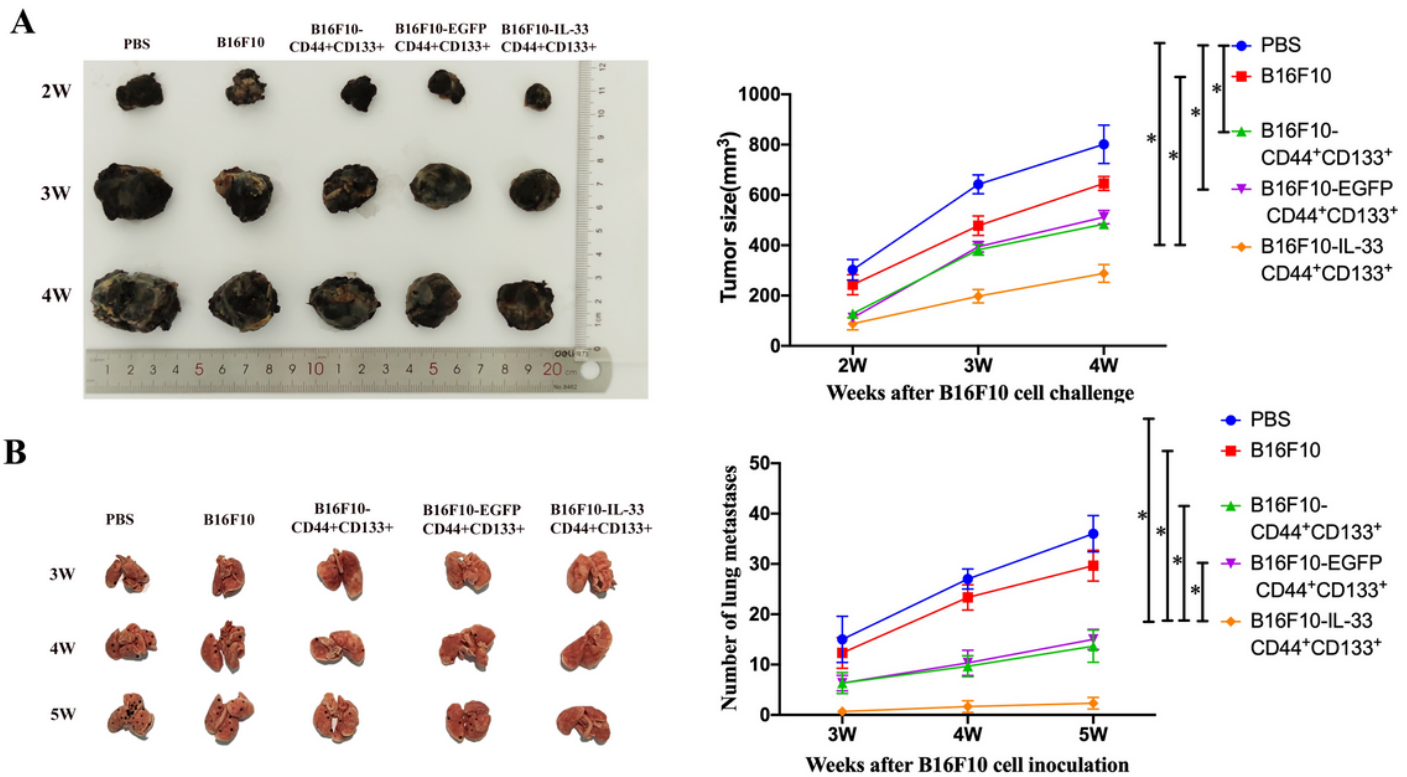
Figures

Figure 1

(A) Tumor size is measured at 2 weeks, 3 weeks and 4 weeks after tumor inoculation, including the maximum and minimum diameter of tumor. The tumor size of mice in vaccine group is significantly smaller than that in PBS and B16F10 group. (B) At 3, 4, and 5 weeks after the tumor cells are injected into the tail vein of mice, the lung tissues are taken out and the number of metastatic nodules is counted. The number of pulmonary metastatic nodules in the vaccine group is significantly less than that in other control groups (n= 3; *P< 0.05).
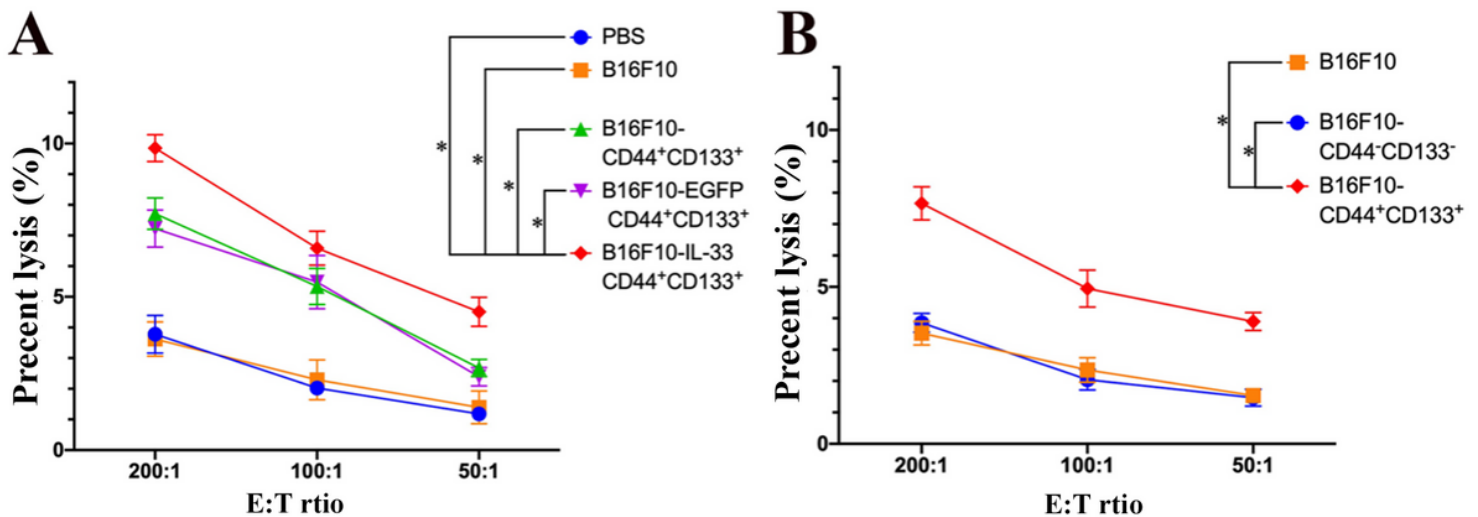
Figure 2

(A) The cytotoxicity ability of spleen lymphocytes in the vaccine group was stronger than that in other control groups. (B) The cytotoxicity effect of spleen lymphocytes in the vaccine group on B16F10-CD44+ CD133+ was stronger than that on B16F10 and B16F10-CD44- CD133- cells, (n= 3，*P< 0.05).
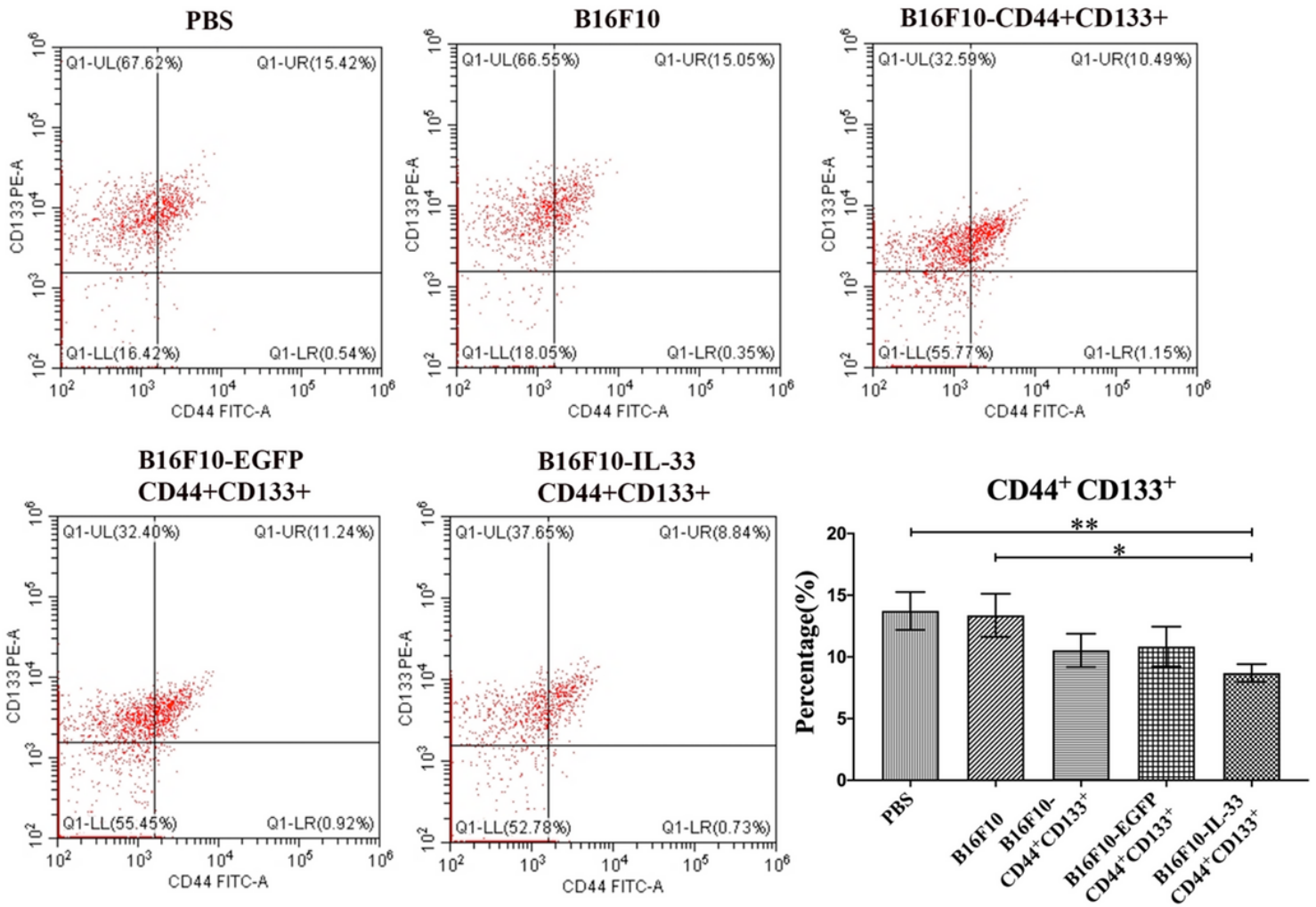

Figure 3

The expression of CD44 and CD133 in tumor cells of different groups was determined by flow cytometry. The expression of CD44 and CD133 in tumor cells of vaccine group was lower than that of B16F10 and PBS group, (n= 3，*P< 0.05，**P< 0.01).
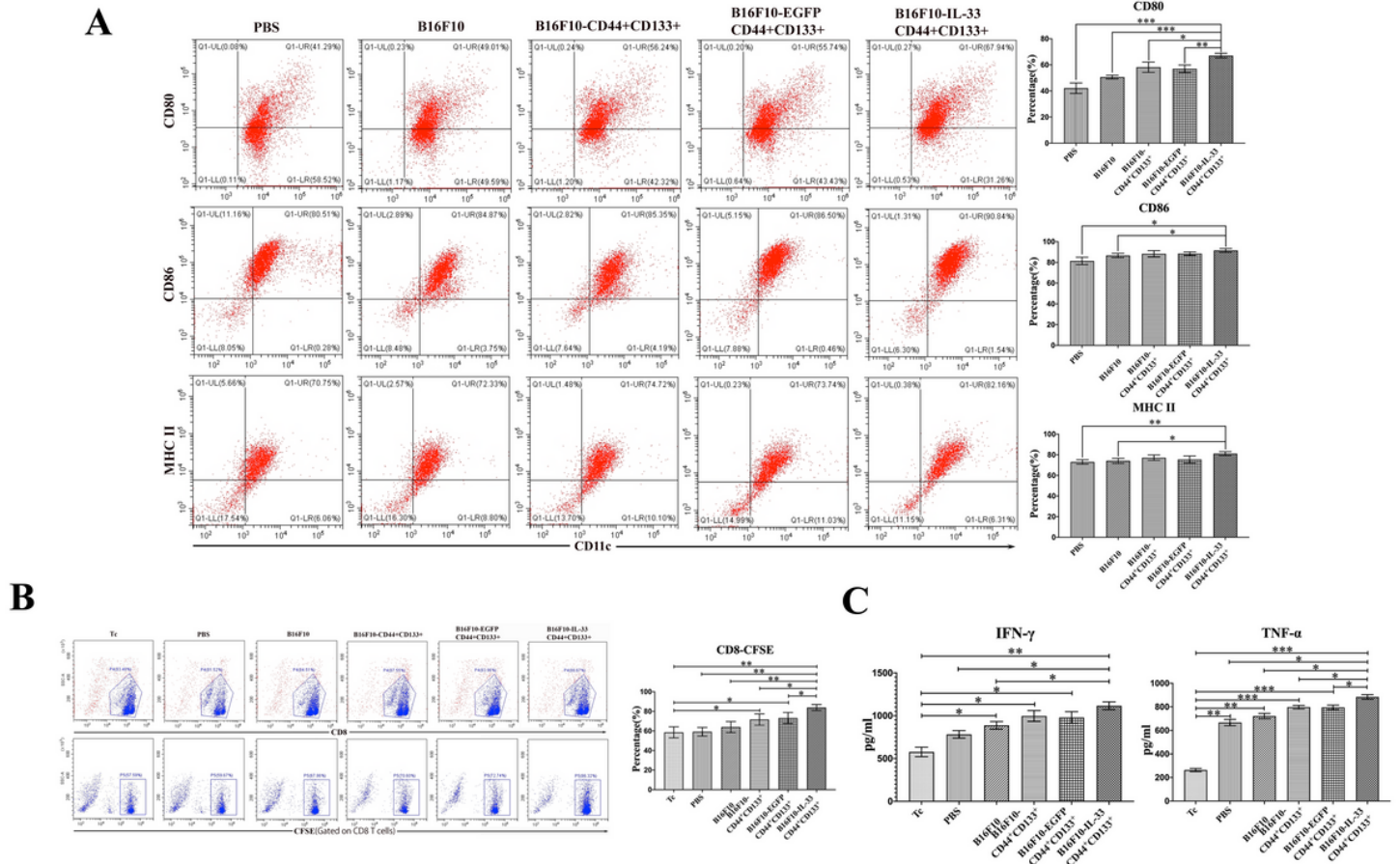
Figure 4

(A) The expression of CD80, CD86 and MHC II is detected by flow cytometry. The expression of CD80 in DCs stimulated by vaccine group is significantly higher than that in other control groups. The expression of CD86 and MHC II is higher than that in B16F10 and PBS groups, while the expression of CD80 is higher than that in PBS group. (B) The proliferation of CD8+T cells is measured by flow cytometry, and CD8, served as the door, is used to measure the expression of lymphocyte labeled CFSE. The proliferation degree of CD8+T cells in vaccine group is significantly higher than that in other control groups. (C) The secretion of IFN-γ and TNF-α is determined by ELISA. The level of IFN-γ secreted by CD8+T cells in vaccine group is significantly higher than that in B16F10 group, PBS group and Tc group. In addition, the level of TNF-α secreted by CD8+T cells in vaccine group is significantly higher than that in other control groups, (n= 3，*P< 0.05，**P< 0.01，***P< 0.001).
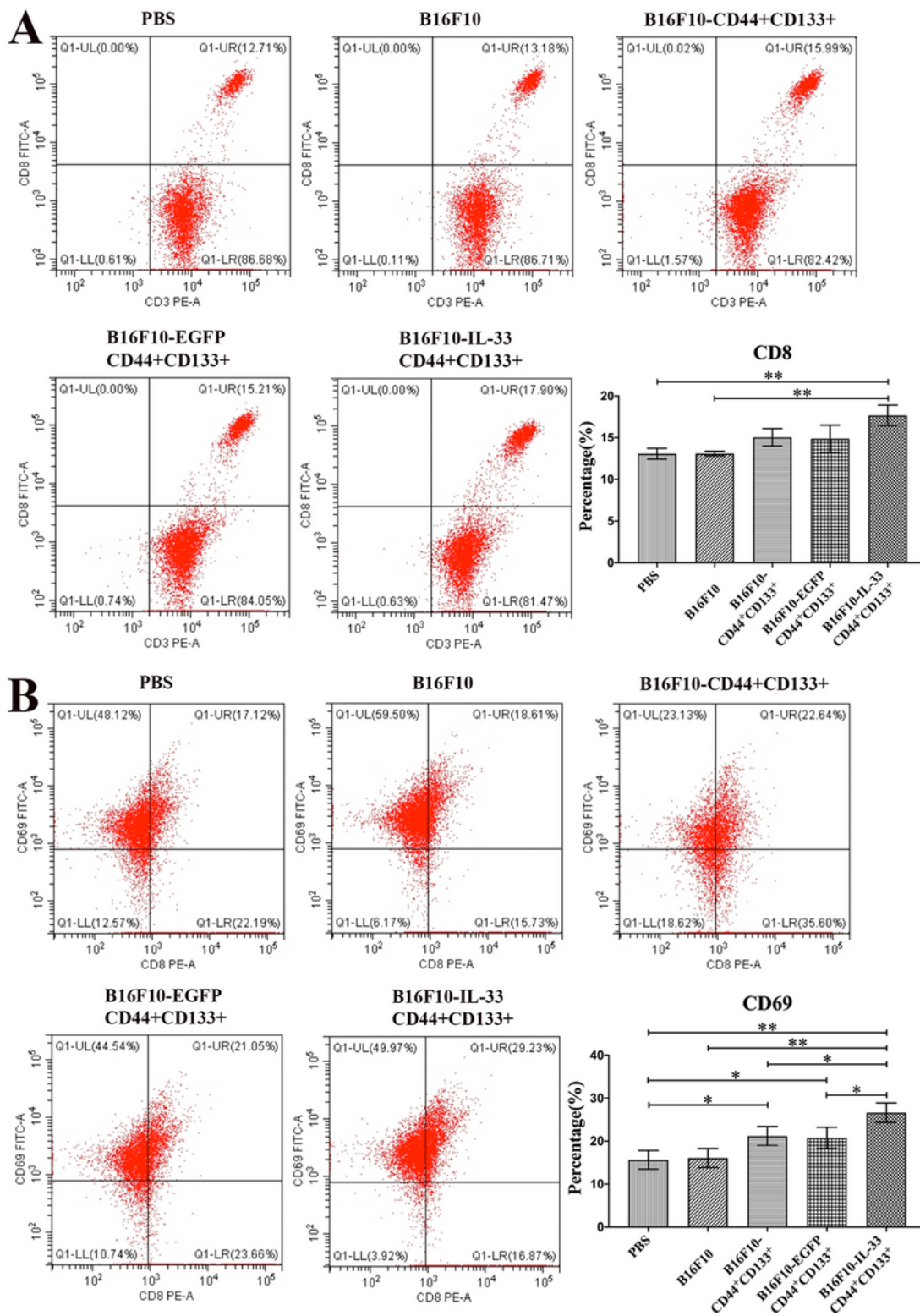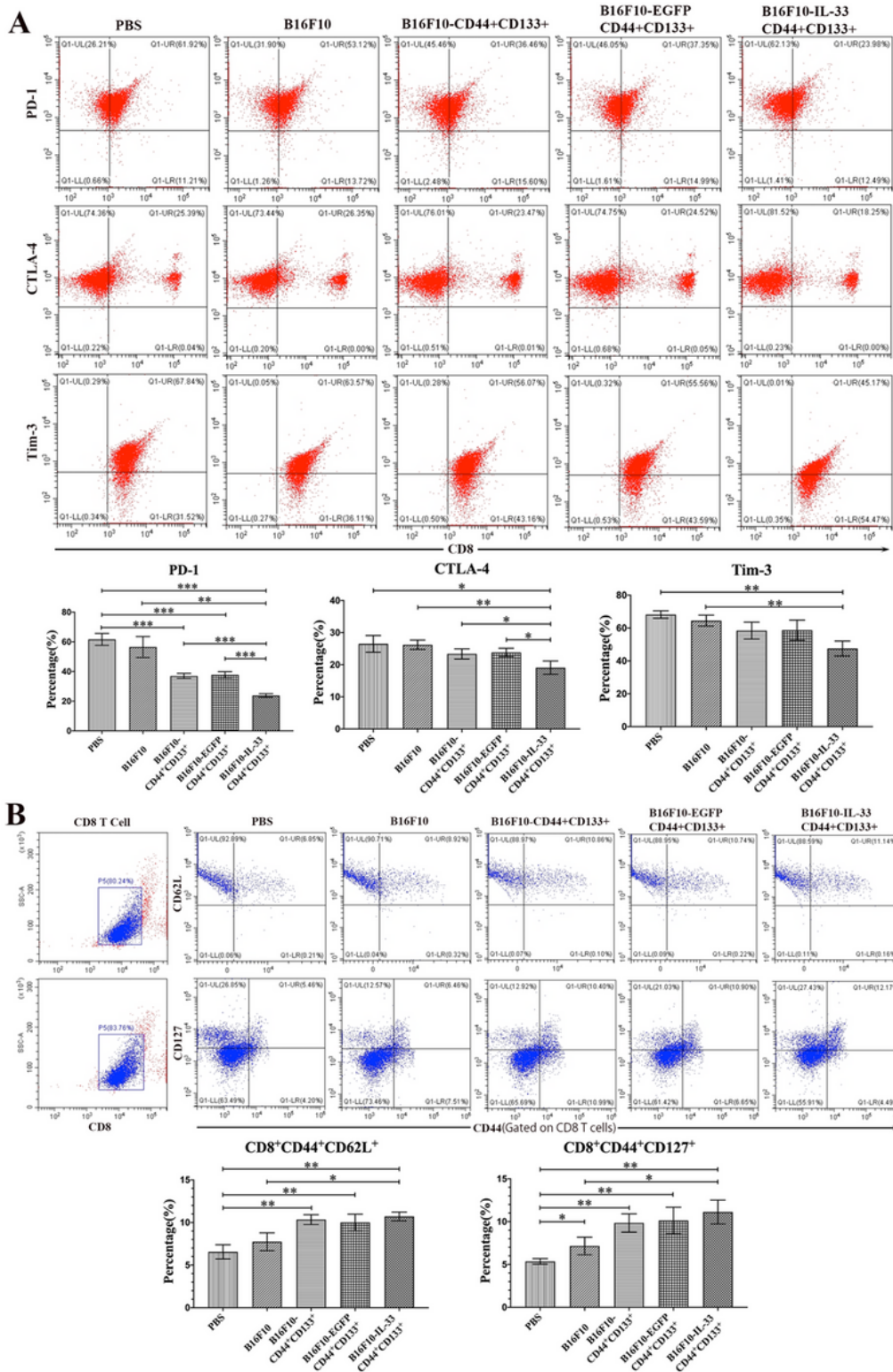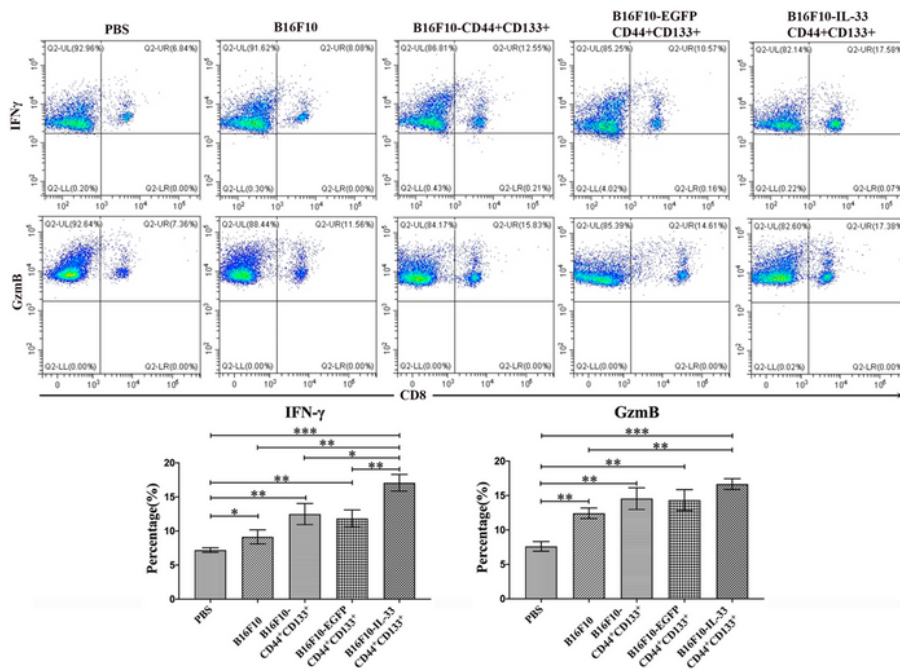
Figure 5

The expression of CD3, CD8 and CD69 in splenic lymphocytes of mice in different groups is determined by flow cytometry. The double positive expression of CD3 and CD8 in splenic lymphocytes of mice in vaccine group is significantly higher than that in B16F10 and PBS groups, and the expression of CD69 in CD8+T cells is higher than that in other control groups, (n= 3，*P< 0.05, **P< 0.01).

Figure 6

(A)The expression of PD-1, CTLA-4 and Tim-3 on CD8+ T cells in different groups was determined by flow cytometry. The expression of PD-1 and CTLA-4 on CD8+ T cells in vaccine group was significantly lower than that in other control groups, and the expression of Tim-3 was significantly lower than that in B16F10 and PBS groups. (B) The expression of CD44, CD62L and CD127 on CD8+T cells in different groups was determined by flow cytometry. The expression of CD44, CD62L and CD127 on CD8+ T cells in vaccine

group was significantly higher than those in B16F10 and PBS groups, (n= 3，*P< 0.05，**P< 0.01, ***P< 0.001).
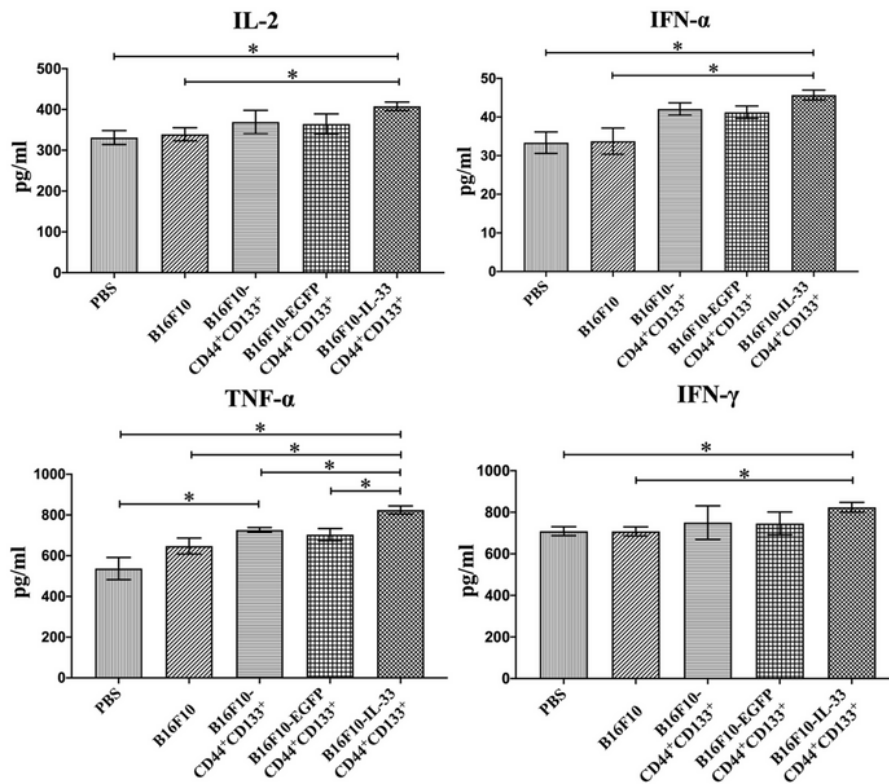

Figure 7

The expression of IFN-γ and GzmB in CD8+T cells was determined by flow cytometry. The expression of IFN-γ on CD8+T cells in vaccine group was significantly higher than that in other control groups, while the expression of GzmB was significantly higher than that in B16F10 and PBS groups. (B) The serum levels

of IL-2, IFN-α, TNF-α, IFN-γ and IL-33 were detected in different groups by ELISA. The secretion levels of IL-2, IFN-α and IFN-γ in vaccine group were higher than those in B16F10 group and PBS group, while the levels of TNF-α and IL-33 in vaccine group are higher than those in other control groups, (n= 3; *P< 0.05, **P< 0.01, ***P< 0.001).