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Gastrodin, a traditional Chinese medicine monomer compound, can be used as adjuvant to enhance the immunogenicity of melanoma vaccines

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ABSTRACT

Gastrodin (GAS) is a Chinese medicine with wide application for the treatment of nervous system disease. Previous studies reported that GAS exhibited non-specific immunomodulatory activities. To explore the effects of GAS as a vaccine adjuvant, the expression levels of CD80, CD86, MHCI and MHCII activated markers were detected after GAS treatment in vitro and in vivo, and the expression levels of IL-2 and TNF-α in splenocytes were detected after GAS treatment in vivo. Besides, the expression levels of IL-2 and IFN-γ in $CD4^+$T cells and perforin, TNF-α and IFN-γ in $CD8^+$T cells were detected. The effects of GAS on the survival rate and tumor size of tumor-challenged mice and the effect of cytotoxicity on $CD8^+$T cells were also investigated. Our data showed that GAS ameliorated $CD8^+$T cell mediated immune response and significantly improved protection of tumor-challenged animals. The results demonstrated that GAS is a potential adjuvant contributing to anticancer immunomodulation.

1. Introduction

Melanoma is the most aggressive skin cancer and is characterized with poor prognosis and complex etiology [1–3]. In recent years, the incidence of malignant melanoma has increased world widely, presenting a public health problem in many countries [4,5]. Surgery, radiotherapy, chemotherapy and immunotherapy are the main methods for the treatment of malignant melanoma, but these methods have limitations, including significant trauma, side effects, decreased survival, and low tolerance [6–8]. Better methods to effectively and safely treat melanoma are urgently needed.

Increased understanding of tumor immune mechanisms have led to the idea of tumor immunotherapy. The immunogenicity of tumor antigens, which are expressed by tumor cells, is required for the use of inactivated tumor cells in a tumor vaccine. The immunogenicity of traditional vaccines is typically weak, and insufficient to induce a strong enough immune response to tumor cells. However, this method may become feasible method by the use of an adjuvant to effectively enhance the immunogenicity of inactivated tumor cells [9]. At present, > 100 kinds of adjuvants are used in vaccine research and development, but overall large effects have not been identified, particularly for tumor vaccines [10–12]. Overall, current adjuvants do not effectively increase the immune response of $CD8^+$T cells, a key step of anti-tumor immunity [13–16]. Therefore, it is urgent to identify a new adjuvant with little side effects that can enhance the immunogenicity of tumor antigens.

Traditional Chinese medicine monomer is a compound of traditional Chinese medicine. It can be used in infectious diseases, inflammation and other diseases. Astraodoric is an important natural source of safe anti-tuberculosis and anti-cancer drugs [17]. Nerol is of great significance in the treatment of *Candida albicans* infection [18]. Astrakurkurone enhances the immune efficiency of host cells leading to parasite clearance in vitro and in vivo [19]. GAS is a Chinese medicine with wide application for the treatment of nervous system disease.

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Previous studies reported that GAS exhibited non-specific immunomodulatory activities. According to the *Compendium of Materia Medica*, GAS was found safe in animal and clinical experiments, presenting nearly no side effects at the safe dosage [20,21]. Furthermore, its high solubility in water makes it easier to use [22,23]. In this study, we analyzed its potential usefulness as an adjuvant for B16F10 vaccine in vitro and in vivo.

2. Materials and methods

2.1. Animals, cell lines, and reagents

Female C57BL/6 mice were from the Nanjing Junke Biological Engineering Co. Ltd., Nanjing, China. Mice were aged 8–10 weeks and were kept in Specific pathogen Free (SPF) condition. All animal experiments were conducted with the approval of the Animal Experiments Committee of Qingdao University. RAW264.7 and B16F10 cell lines were obtained from the Shanghai Cell Resource Center (Chinese Academy of Sciences, China), and the DC2.4 cell line was a gift from Professor Bin Wang, Key Laboratory of Medical Molecular Virology/ Ministry of Education And Health, (Fudan University, China). The B16F10 and DC2.4 cell lines were cultured in RPMI1640 medium containing 10% fetal calf serum (FCS). The RAW264.7 cell line was cultured in DMEM medium containing 10% FCS, and cultured in a 37 °C cell incubator containing 5% CO2. LPS was purchased from Sigma. GAS was obtained from Dalian Meilun Biological Technology Co. Ltd. (Liaoning, China), and GAS (purity > 99%) is one of the major bioactive components of Gastrodia elata Blume, the molecular formula is as supplementary figure 1 (Fig. S1). The following antibodies were all obtained from BioLegend (USA): PE anti-mouse H-2Kb (cat. no. 116507PE) anti-mouse I-A/I-E (cat. no. 107607), PE anti-mouse CD80 (cat. no. 104707), PE anti-mouse CD86 (cat. no. 105007), FITC anti-mouse CD4 (cat. no. 100405), PE anti-mouse IL-12 (cat. no. 505203), PE anti-mouse IFN-γ (cat. no. 505807), PE anti-mouse TNF-α (cat. no. 506305) and FITC anti-mouse CD8a (cat. no. 100705). The cell-counting kit-8 assay (CCK-8) was obtained from 7 Sea Pharmatech Co., Ltd.

2.2. Treatment of DC and macrophage cell lines with GAS

DC2.4 and RAW264.7 cells were plated at 1×10^6/ml in 96-well plates. We added GAS and final stimulated concentration was 40 μg/ml. The foundation of GAS dose determination was based on references [24,25]. For the positive control group, We added LPS and final stimulated concentration was 1 μg/ml. For the negative control group, 20 μl RPMI1640 culture medium containing 10% fetal bovine serum was added. After 48 h, the cells were stained by PE anti-CD80, PE anti-CD86, PE anti-I-A/I-E, and PE-anti-H-2Kb in PBS for 30 min at 4 °C, and then analyzed by FACS (Guava Soft).

2.3. Preparation of inactivated tumor antigen and immunization schedule

2.3.1. Preparation of inactivated tumor antigen

After the B16F10 cells had formed a monolayer, the cells were irradiated at 254 nm wavelength for 30 min. The UV dose is 5 mw/cm^2. The cells were collected and the cell concentration was adjusted to 1×10^7/ml.

2.3.2. Immunization schedule

GAS was dissolved in PBS to 12 mg/ml. The C57BL/6 mice were randomly divided into six groups (n = 10 each). Samples containing 100 μl of 1×10^6 inactivated B16F10 cells were formulated with or without GAS and with or without Freud's complete adjuvant (CFA). The samples were injected intramuscularly, with a single injection. Three days after the first immunization, each group of mice was sacrificed for detection of surface proteins and cytokines in innate immunity. Each group was immunized with 100ul of vaccine. The composition of the vaccine is as follows: (1) GAS + Cell: 0.6 mg GAS + 1×10^6 inactivated B16F10 cells, (2) CFA + Cell: 50ul CFA + 1×10^6 inactivated B16F10 cells, (3) Cell: 1×10^6 inactivated B16F10 cells, (4) GAS: 0.6 mg GAS, (5) CFA: 50ul CFA, (6) Naïve: 100ul PBS.

The process of adaptive immunization is as follows: two weeks after the initial immunization, with the same concentration to strengthen the immune once. Seven days later, mice were challenged with 100 μl of 1×10^6/ml B16F10 cells, administered by subcutaneous administration. We monitored tumor growth in tumor-bearing mice and the final survival rate of mice was used to evaluate the immune effect. The process is shown in Fig. 3A.

2.4. Detection of surface protein and cytokine in lymphocytes

Single cell suspensions were prepared from mouse spleen cells after the mice were killed by cervical dislocation. The cells (2×10^6/ml) were separately surface-stained in 96-well plates using PE anti-mouse H-2Kb, PE anti-mouse I-A/I-E, PE anti-mouse CD80, and PE anti-mouse CD86 (1.2 μl/ml) for 30 min at 4 °C. Single cell suspensions were also stained with PE anti-mouse IL-12 and PE anti-mouse TNF-α (1 μl/ml) diluted with 0.2% TritonX-100. Data were acquired and analyzed using BD Accuri C6 software.

2.5. Evaluation of tumor growth and the survival rate of mice

After the growth of measurable (4–5 mm) tumors (between 10 and 15 days after tumor challenge), tumor growth was measured using calipers every 2 days and the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were recorded. Tumor size based on caliper measurements was calculated by the modified ellipsoidal formula: Area = π/4 (length × width). Mice were sacrificed when the tumors reached about 20 mm in average diameter.

2.6. T cell proliferation assay

Single-cell suspensions were prepared and cultured using 96-well plates at 1×10^5/ml in RPMI 1640 medium containing 10% FCS, and re-stimulated with the inactivated antigen (1×10^4/ml) at 37 °C in a 5% CO_2 incubator. After 72 h of culturing, 10 μl CCK-8 was added and the cells were cultured an additional 2 h at 37 °C and 5% CO_2. Cell viability was assessed by cell-counting kit-8 assay and absorbances were read on a spectrophotometer at 450 nm.

2.7. Detection of cytokine in $CD4^+$ T and $CD8^+$ T cells

Single-cell suspensions from tumor-challenged mice spleens were prepared and stimulated with 1×10^4/ml inactivated-tumor cells in the presence of BFA for 10 h at 37 °C and 5% CO_2. Collected cells were surface-stained with FITC anti-mouse CD_4 or FITC anti-mouse CD8a (1.2 μl/ml) for 30 min at 4 °C, and then fixed with 4% paraformaldehyde. The surface-stained FITC $CD4^+$T cells were then stained with PE anti-mouse IL-2, PE anti-mouse IFN-γ, and the FITC $CD8^+$T cells were stained with PE anti-mouse TNF-α or PE anti-Mouse Perforin. Those antibodies were diluted with 0.2% TritonX-100. Samples were analyzed by BD Accuri C6 software in triplicate.

2.8. Cytotoxic assay

Single-cell suspensions from tumor-challenged mice spleens were adjusted to 1×10^7/ml by addition of PBS containing Ca^{2+} and Mg^{2+}. Next, 1×10^7/ml cell suspension was added to 1 μl of the Carboxyfluorescein diacetate succinimidyl ester (CFSE) storage solution (final concentration of 1 μM), and immediately blended at 37 °C for 6 min. These were considered effector cells. Separately, cell suspensions were prepared from logarithmic growing B16F10 cells, labeled with

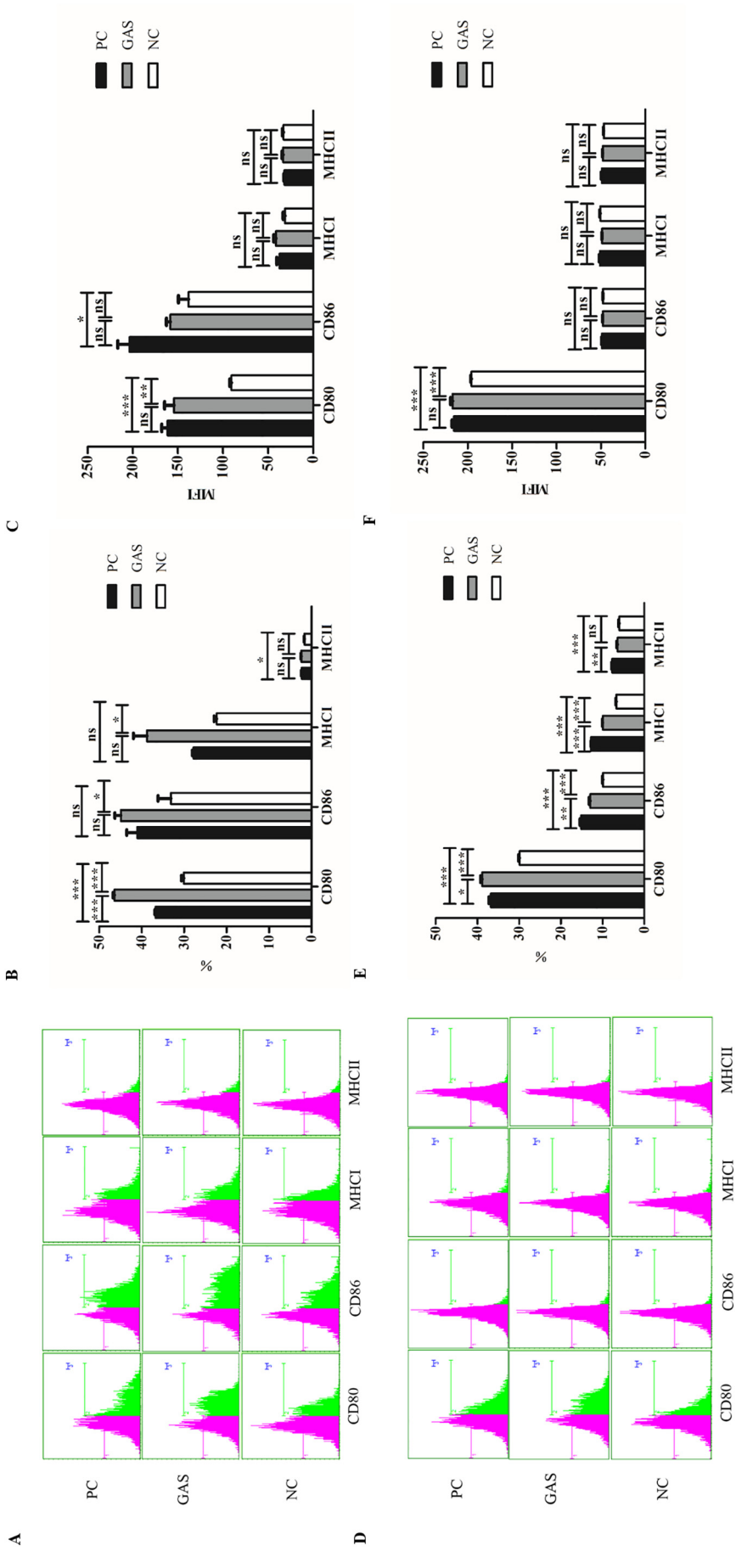

Fig. 1. Effects of GAS on APC activation in vitro. The expression levels of CD80, CD86, MHCI and MHCII in DC2.4 (A) and RAW264.7 (D) were detected by FACS. The percentage of CD80, CD86, MHCI, and MHCII were analyzed in DC2.4 (B) and RAW264.7 (E). Fluorescence intensity of DC cell line and RAW264.7 cell line (C and D). Cell fluorescence was analyzed and all data are presented as mean ± SD. Data shown are representative of three independent experiments, ns (not significant), $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

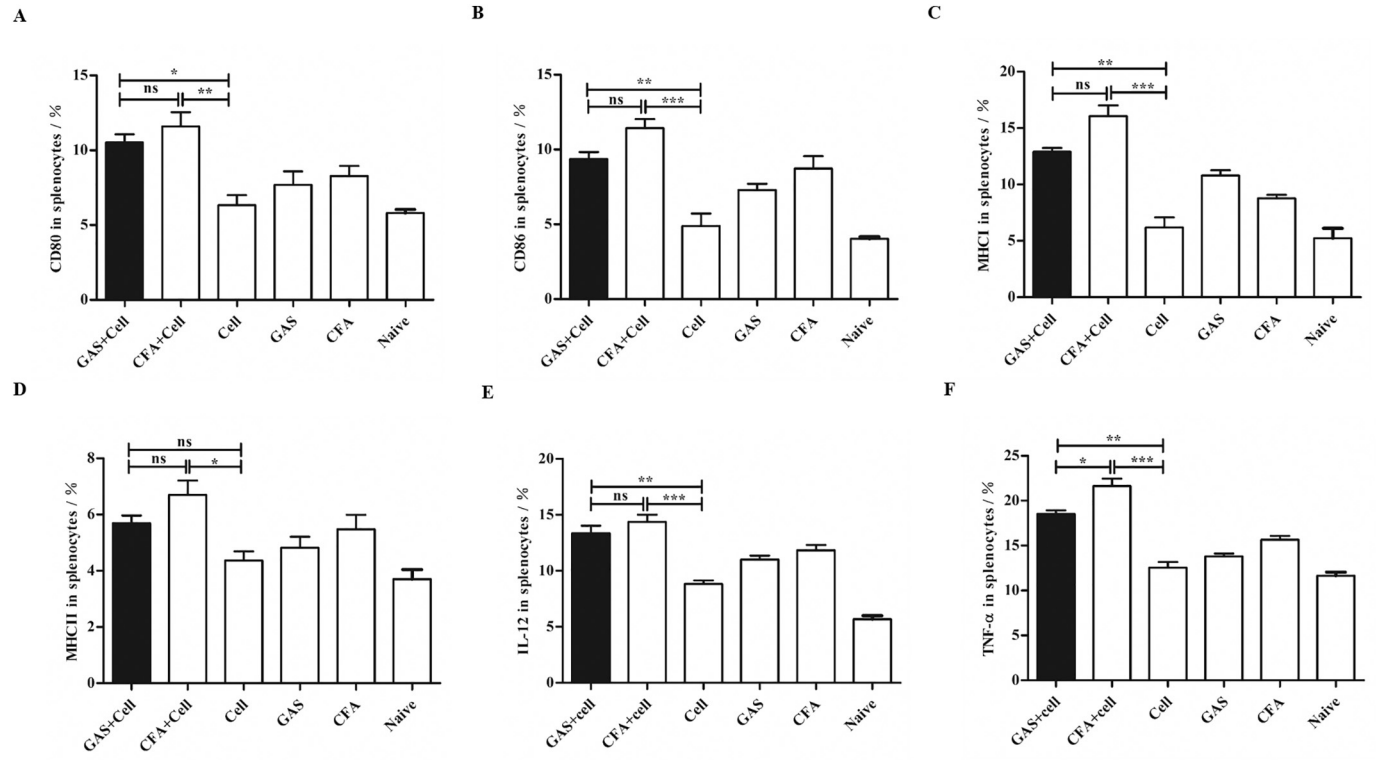

Fig. 2. FACS analysis of splenocytes of C57BL/6 mice after immunization. The expression levels of CD80 (A), CD86 (B), MHCI (C), MHCII (D), IL-12 (E) and TNF-α (F) in splenocytes. All data are presented as mean ± SD, Data shown are representative of three independent experiments, ns (not significant), *P < 0.05, **P < 0.01, ***P < 0.001.

1 umol/l CFSE, mixed immediately, and then placed in a 37 °C cell culture incubator for 15 min in the dark. These cells are considered target cells. In 96-well plates, 50ul 1 × 10^5/ml target cells (B16F10) and 50ul effector cells (effector to target ratios ranging from 1.7:1 to 45:1) were labeled with CFSE in each well. The mixes were cultured for 6 h at 37 °C and 5% CO_2. Samples were analyzed by BD Accuri C6 software in triplicate.

2.9. Statistical analysis

All data are presented as the mean of three measurements ± standard deviation. All data were analyzed by IBM SPSS Statistics software and figures are presented by GraphPad Prism software. Differences between groups were analyzed by ANOVA. Bonferoni test was used for analysis of two groups. Tumor size was analyzed using the two-way analysis of variance. Survival was analyzed using the log-rank test. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. GAS enhanced antigen presenting cells activation in vitro

Antigen presenting cells (APC) activation is essential for vaccines. To determine the effect of GAS on APCs in vitro, the DC cell line DC2.4 and macrophage cell line RAW264.7 were treated with GAS, LPS (positive control) or medium alone (negative control). The dose of GAS was 40 μg/ml, because in the preliminary experiment, we set three concentrations, and the results showed that 40 μg/ml of GAS had the best immunostimulatory effect, as shown in Tables S1 and S2. The expression levels of APC activation markers CD80, CD86, MHCI, and MHCII were detected (Fig. 1A and D). The results showed that CD80, CD86 and MHCI were increased in DC2.4 after treatment with GAS compared to the negative control (Fig. 1B). CD80, CD86, and MHCI were increased in RAW264.7 cells compared to the negative control after treatment with GAS (Fig. 1E). The fluorescence intensities of the DC and RAW264.7 cell lines for detection of the four surface proteins are shown in Fig. 1C and F. Thus, compared with the negative control, GAS enhanced APC activation in vitro.

3.2. GAS enhanced APC activation in vivo

To determine the effect of GAS on APCs in vivo, mice splenocytes were isolated and analyzed by flow cytometry three days after immunization. The dose of GAS was 0.6 mg/mice, because in the preliminary experiment, we set three concentrations, and the results showed that 0.6 mg/mice of GAS had the best immunostimulatory effect, as shown in Tables S3. The results showed that the expression levels of CD80, CD86 and MHCI were increased in the GAS + Cell group compared to inactivated B16F10 cells (Fig. 2A, B and C), the expression levels of MHCII was not significantly increased in the GAS + cell group compared to inactivated B16F10 cells (Fig. 2D). Representative gating strategies for flowcytometric analysis is shown in Fig. S2. Representative flowcytometric analysis of CD80, CD86, MHCI and MHCII are shown in supplementary figure (Fig. S3, S4, S5 and S6). Overall, compared with the negative control, the addition of GAS + Cell stimulated the activity of APCs in vivo.

To determine whether GAS promotes cytokine production, the expression levels of IL-12 and TNF-α were examined by the intracellular staining of splenocytes. The expression levels of IL-12 and TNF-α in both the GAS + Cell group and the CFA + Cell group were up-regulated compared to the inactivated B16F10 cells Representative flowcytometric analysis of IL-12 and TNF-α are shown in supplementary figure (Fig. S7 and S8).

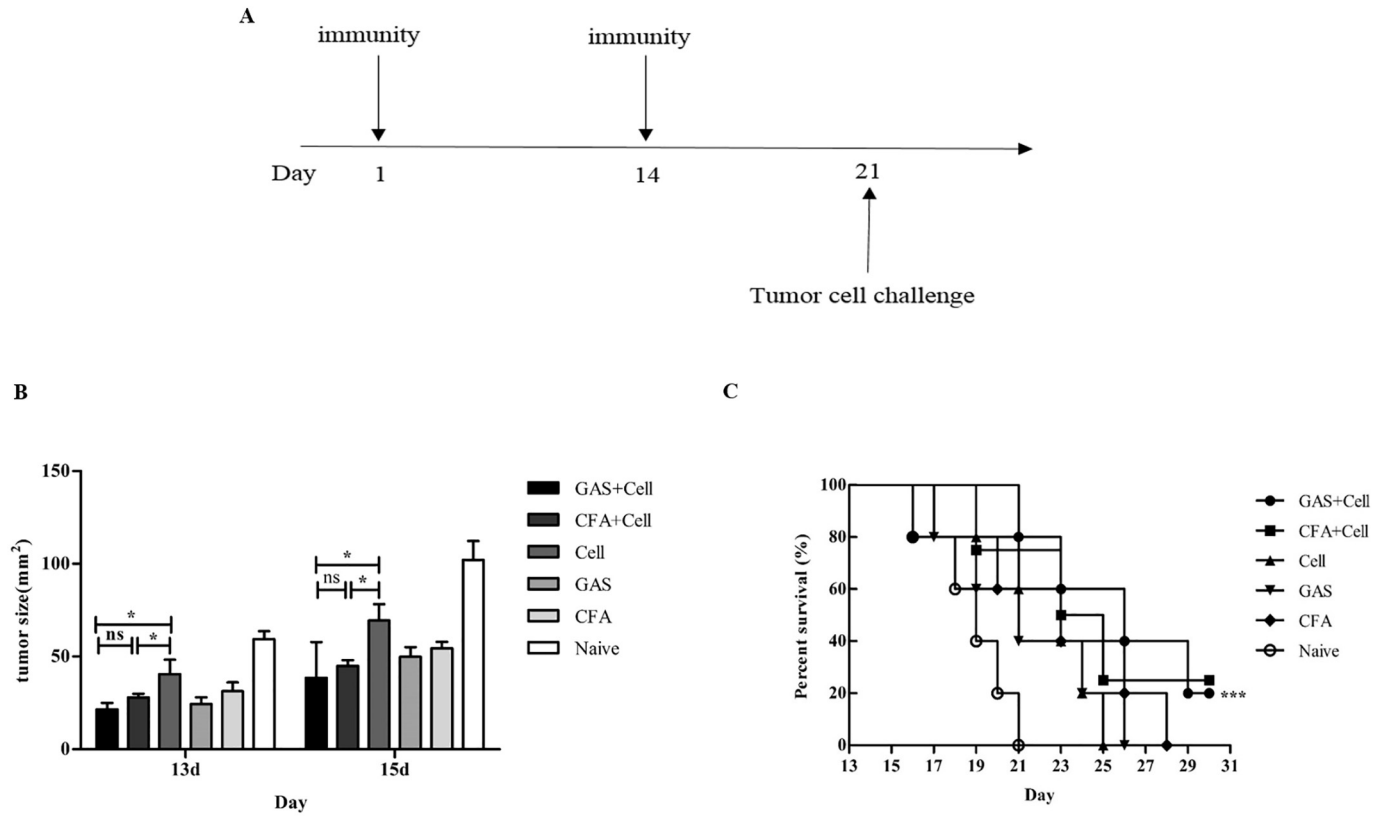

Fig. 3. Inhibition of tumor growth induced by GAS adjuvants in B16F10 melanoma subcutaneous model. Immunization schedule (A). Tumor size (B). Survival curves after B16F10 challenge (C). All data are presented as mean ± SD, Data shown are representative of three independent experiments, **P < 0.01 compared with mice immunized with the inactivated B16F10 cells alone.

3.3. Evaluation of tumor size and survival rate

We next assessed the ability of GAS to serve as an adjuvant in a melanoma vaccine, on the 13th day after modeling, the tumor size was measured every 2 days with vernier calipers and the tumor size was calculated. As shown in Fig. 3B, melanoma size was smaller in the GAS + Cell treatment group compared with the group that only received inactivated B16F10 cells. We also evaluated the mortality of each group of mice. As shown in Fig. 3C, on day 21, the survival rate of mice in the GAS + Cell treatment group was significantly higher than that of the group that only received inactivated B16F10 cells.

3.4. Effects of GAS on T cell mediated responses

We showed above that the use of GAS as an adjuvant can inhibit tumor growth and improve survival rate in mice. So, we next tested the effect of GAS on the proliferation of T cell functions. The results of CCK-8 proliferation assay showed that compared with the inactivated cell group, both the GAS + Cell group and the CFA + Cell group were increased (Fig. 4A). We also examined cytokine expression in $CD4^+$T cells and $CD8^+$T cells. In $CD4^+$T cells, both IL-2 and INF-γ were increased by GAS + Cell or CFA + Cell treatment compared with treatment with inactivated B16F10 cells (Fig. 4B and C). The expression levels of IL-2 and INF-γ in the GAS + Cell group was not significantly different than those for the CFA + Cell group (Fig. 4B and C). Representative gating strategies for flowcytometric analysis is shown in Fig. S9. Representative flowcytometric analysis of IL-2 and INF-γ are shown in supplementary figure Fig. S10 and S11). Cytotoxic $CD8^+$T cells (CTL) are generally considered as the main effectors of immunity against tumors. We next tested whether GAS could augment the cellular response to tumor cells, using CFA + Cell as a positive control. The expression levels of Perforin (Fig. S4D), TNF-α (Fig. S4E), and IFN-γ (Fig. S4F) were increased by GAS + Cell treatment compared with treatment with inactivated B16F10 cells. Perforin and TNF-α expression levels in the GAS + Cell group were higher than in the group immunized with CFA + Cell, the expression level of IFN-γ was not different for the GAS + Cell group (Fig. S4D, E and F). Representative flowcytometric analysis of Perforin, TNF-α and IFN-γ are shown in supplementary Fig. S12, S13 and S14. These results suggest that GAS can promote the responses of $CD8^+$T cells by enhanced T cell proliferation and inflammation cytokine expression.

3.5. GAS effects on cytotoxicity of $CD8^+$ T cells

The above results show that the use of GAS as an adjuvant can improve the survival rate of mice, inhibit tumor growth, and promote $CD8^+$T cell proliferation and cytokine expression. Next, we measured the lethality of $CD8^+$T cells by cytotoxicity testing. As shown in Fig. 5A and B, a ratio of 45:1(E: T) was the most lethal. Cytotoxic lymphocytes (CTLs) were significantly increased in mice immunized with GAS + Cell compared with mice in the inactivated B16F10 cells group.

4. Discussion

Anti-tumor immune response is mainly mediated by cellular immunity. The initiation of cellular immune response requires the presentation of antigens to T cells by APC, which requires initial T cell activation and proliferation for anti-tumor activity [26,27]. The tumor immune process is divided into three steps: 1) APC processing and antigen presentation; 2) specific or nonspecific tumor antigen T cell activation; 3) migration of antigen-specific cytotoxic T cells (CTL) to tumor sites to play specific anti-tumor roles [28,29]. Activated CTL are the most important immune effector cells in the body with powerful anti-tumor immune responses. CTL act by secreting anti-tumor

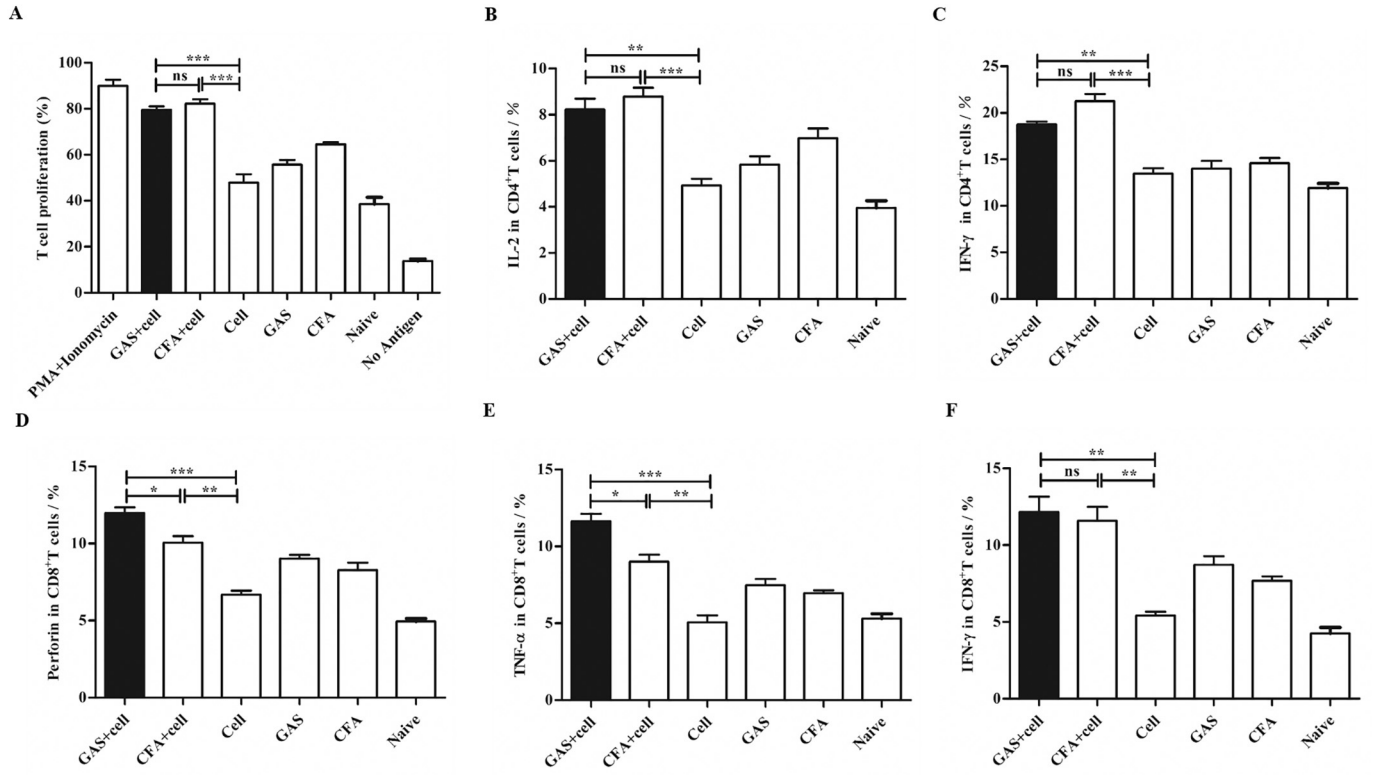

Fig. 4. Effects of GAS on T cell functions. The effect of GAS on the proliferation of T cells and $CD4^+$T and $CD8^+$T cells. CCK-8 absorbance values for different groups (A). The lymphocytes were gated on $CD4^+$T cells then stained for intracellular IL-2 and INF-γ (B, C). Percentages of $CD8^+$T cells positive for perforin, TNF-α, and IFN-γ were analyzed by FCM (D, E, F). All data are presented as mean ± SD, Data shown are representative of three independent experiments, ns (not significant), *P < 0.05, **P < 0.01, ***P < 0.001 compared with inactivated antigen alone.

cytokines such as perforin and granzyme or by killing cells through the Fas-FasL pathway [30,31]. With a continually increasing understanding of tumor immune mechanism, tumor specific immunotherapy has become a more plausible strategy. The induction of long-lasting anticancer T cell responses is a primary objective of cancer immunotherapy. However, both the limited number of tumor antigens and their low immunogenicity present major obstacles in the generation of strong antitumor responses. To more directly activate specific components of the immune system, antigen specific T cell responses have been elicited by mixing antigens and suitable adjuvants [32–35].

Immunological adjuvants are typically targeted to present antigens to modulate cellular and humoral immunity [36]. There are two main classes of adjuvants [37,38]: 1) Immunostimulants, which essentially act on the immune system to enhance the immune response to various antigens. Typical examples are Toll-like receptor ligands, cytokines, saponins, and bacterial exotoxins that stimulate immune responses; 2) Delivery vectors, which deliver antigens to antigen-presenting cells to induce adaptive immune responses and increase antigen specific immune responses [39–43]. Adjuvants are compounds incorporated into vaccines to enhance immunogenicity, and there is growing interest in the development of these molecules. Effective adjuvants may allow dose reduction and rapid, broader, and stronger immune response induction [44]. However, several important obstacles need to be overcome, including comprehensive understanding of adjuvant properties, including side effects and toxicity [45].

In this work, we conducted experiments in vitro and in vivo to investigate whether GAS can enhance the immunogenicity of melanoma vaccines. The in vitro results showed that GAS can up-regulate the

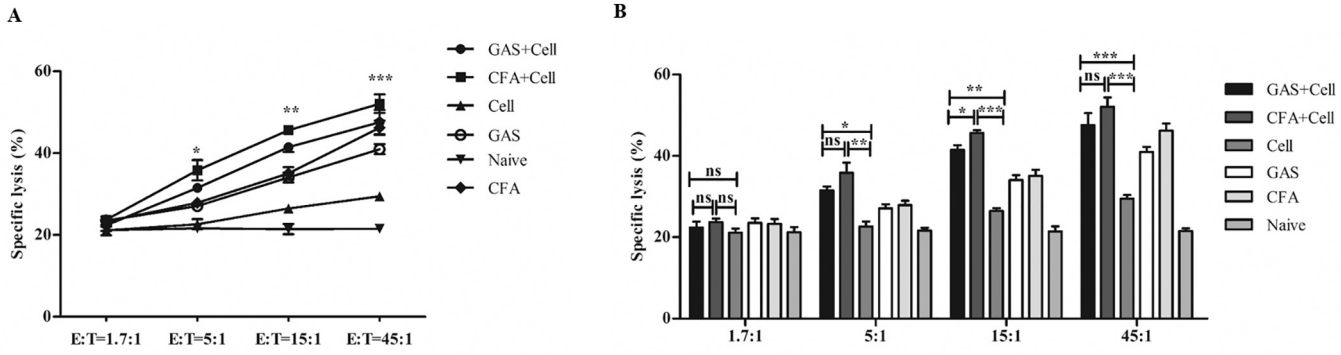

Fig. 5. Results of cytotoxicity tests. In vivo cytotoxic activity was determined by flow cytometry (A, B). E/T: effector to target cell ratio. Experiments were repeated three times. Data shown are representative of three independent experiments, ns (not significant), *P < 0.05, **P < 0.01, ***P < 0.001, compared with inactivated cell group.

expression of surface markers CD80, CD86 and MHCI in RAW264.7 and DC2.4 cells. The use of gastrodin as an adjuvant was further evaluated in vivo. To do this, we examined the expression of surface markers of CD80, CD86, MHCI, and MHCII in APC cells by flow cytometry (Fig. 2A, B, C and D). After confirming changes in APC cell surface markers, we evaluated the expression of inflammatory factors IL-12 and TNF-α in APC cells (Fig. 2E and F). GAS activates APC cells as evident by the increased expression of surface markers and cytokines. Finally, we evaluated the effect of GAS on lymphocyte proliferation (Fig. 4A). GAS promoted lymphocyte proliferation at 72 h, suggesting the potential use of GAS as a tumor cell vaccine adjuvant. TH_1 cells are essential for cellular immunity and CTL responses. Therefore, we examined the levels of cytokines IL-2 and IFN-γ secreted by TH_1 cells (Fig. 4B and C) and the expression of perforin, TNF-α, and IFN-γ in $CD8^+$T cells (Fig. 4D, E and F). Expression of these cytokines are increased indicated that GAS can stimulate $CD8^+$T cells to secrete cytokines. A T cell killing assay demonstrated that the GAS + Cell group significantly increased CTL effects (Fig. 5).

GAS has a low level of toxicity, is easy to obtain, and is inexpensive, making it ideal for use in clinical trials of potential vaccine adjuvants [46]. The in vivo results presented here indicate that GAS may be a promising chemical adjuvant for inactivated tumor cell vaccine development when cellular responses are required.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105699.

Declaration of Competing Interest

Authors has no potential conflicts of interest were disclosed.

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