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The *in vivo* immunomodulatory and synergistic anti-tumor activity of thymosin α1–thymopentin fusion peptide and its binding to TLR2

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ABSTRACT

In the present study, the immunomodulatory and synergistic anti-tumor activity of thymosin α1–thymopentin fusion peptide (Tα1–TP5) was investigated *in vivo*. In addition, the potential receptor of Tα1–TP5 was investigated by surface plasmon resonance (SPR) binding studies. It was found that Tα1–TP5 (305 μg/kg) alleviated immunosuppression induced by hydrocortisone (HC). Tα1–TP5 (305 μg/kg) combined with cyclophosphamide (CY) had a better tumor growth inhibitory effect than CY alone. Furthermore, Tα1–TP5 had a higher affinity (K_D = 6.84 μmol/L) to toll-like receptor 2 (TLR2) than Tα1 (K_D = 35.4 μmol/L), but its affinity was not significantly different from that of TP5. The results of our present work indicate that Tα1–TP5 can possibly be developed as a new immunomodulatory agent.

1. Introduction

The thymus is considered as an important organ for T-lymphocyte maturation and differentiation. Along with forming the appropriate microenvironment for lymphocyte activity, thymic epithelial cells are capable of secreting many peptides into the circulation: these peptides can regulate the development of different phenotypic markers and lymphocyte functions [1]. Thymopentin (TP5) and thymosin alpha 1 (Tα1) are two thymic peptides with similar immunoregulatory activities that are being used in the clinic for the treatment of some immunodeficiencies, malignancies, and infections [2–6].

TP5, a chemically synthesized pentapeptide, was able to restore the thymic atrophy induced by immunosuppressants [7] and induce a clinical response in patients with cutaneous and subcutaneous melanoma metastases [3]. Tα1, as a biological response modifier (BRM), can enhance the immune response, promote specific lymphocyte functions, and stimulate the production of lymphokines such as IFN-γ, IFN-α, IL-2, and macrophage migration inhibitory factor (MIF) [8]. Tα1 can also antagonize the dexamethasone (DEX)-induced apoptosis of $CD4^+CD8^+$ thymocytes [9] and the hydrocortisone (HC)-induced decreases in the thymus index and spleen index values [10]. In addition, Tα1 alone or combined with other BRMs or chemotherapeutic agents displays good effects in reducing tumor burden and progression [11–15].

Innate and/or acquired immunity have the ability to recognize and destroy spontaneously arising tumors. Natural killer (NK) cells represent "the first line of defense" against pathogens. Dendritic cells (DCs) acquire and present antigen(s) to cytotoxic T lymphocytes (CTLs), which boost the effective antitumor response. Similarly to other immunomodulators [16,17], Tα1 was found to augmented the immune response of tumor-bearing mice. The depletion of immune cells, using either total-body sub-lethal irradiation (400 rads) or antibodies directed against T-cell or NK-cell (anti-asialo GM1) populations, abolished the anti-tumor response [13], which indicates the important role of immunoregulation in anticancer therapy.

Although TP5 and Tα1 have potential immunoregulatory effects, the action mechanism on the immune system is not clear. Early in 1985, Rinaldi Garaci et al. found that exogenous Tα1 localized to the surface of cortical thymocytes [18]. It was then shown that Tα1 activates DCs for antifungal T-cell helper 1 (Th1) resistance through toll-like receptor (TLR) signaling and that Tα1

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strongly activates the expression of toll-like receptor 2 (TLR2) on murine DCs [19]. Peng et al. found that Tα1-activated immune cells produce cytokines via the IRAK4/1/TRAF6/PKCζ/IKK/NF-κB and TRAF6/MAPK/AP-1 pathways [20], which are downstream of TLR signaling. It is believed that TLR2 is a receptor of Tα1. Whether TP5 acts on the same receptor as Tα1 remains unknown.

The half-life ($t_{1/2}$) of TP5 is very short in human plasma due to its low molecular weight. To prolong the $t_{1/2}$ of TP5, a Tα1–TP5 fusion peptide was designed and expressed in *Pichia pastoris* by our research team [21]. A previous study showed that Tα1–TP5 had higher activity than TP5 in inducing T-lymphocyte proliferation *in vitro*. In the *in vivo* carbon clearance test, the abilities of Tα1–TP5 to promote the phagocytic capability of macrophages as well as the secretion of IL-2 in the peripheral blood of mice were also higher than those of TP5 and Tα1. In addition, Tα1–TP5 had a longer *in vitro* plasma $t_{1/2}$ than TP5 and Tα1 [21]. To easily obtain a large quantity of active Tα1–TP5, a new intein-fused expression and purification strategy for the production of the Tα1–TP5 fusion peptide was investigated [22]. The self-cleavage property of intein allowed us to avoid using chemical reagents or proteases, which simplified the purification procedure and reduced the cost. However, the *in vivo* immunomodulatory effect of Tα1–TP5 and the mechanism of action have not been intensively studied.

HC is a glucocorticoid (GC) that leads to reduced thymus index and spleen index [10], lowered developing $CD4^+CD8^+$ thymocytes [23] and disturbed T cell cytokine profile [24], which induce many disorders and symptoms. In this work, we present evidence that Tα1–TP5 can alleviate the immunosuppression induced by HC, inhibit melanoma growth when combined with cyclophosphamide (CY) and reduce the side effects caused by CY. Furthermore, Tα1–TP5 had a higher affinity to TLR2 than Tα1, but its affinity was not significantly different from that of TP5. Tα1–TP5 may mediate the immune response through TLR2 signaling.

2. Materials and methods

2.1. Reagents

Tα1–TP5 was prepared as described previously [22]. TP5 and Tα1 were synthesized by Chinapeptides Co., Ltd. (China). Hydrocortisone sodium succinate (HC) was obtained from Tianjin Biochem Pharmaceutical Co., Ltd. (China); the FITC-CD4 antibody was from BD Pharmingen (USA); the PE-CD8 antibody was purchased from eBioscience (USA); CY and fluorescein isothiocyanate (FITC) were from Sigma (USA); anti-CD8 antibody, anti-CD86 antibody and anti-MHC Class 1 H2 Db antibody were obtained from Abcam (USA); and the interferon γ enzyme-linked immunoassay kit was purchased from Ying U.S. Technology Co., Ltd. (Beijing); DiI and Hoechst 33342 were obtained from Beyotime Institute of Biotechnology (China); N-hydroxysuccinimide (NHS), ethanolamine, polysorbate 20 (P-20) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden); and the recombinant mouse TLR2 Fc chimera was obtained from R&D Systems (USA). Other chemicals and reagents were of analytical grade.

2.2. Animals and cell line

Male BALB/c mice (6–8 weeks old, 18–20 g) were purchased from the Experimental Animal Center of Shandong University (China). Wild-type male C57BL/6 mice (5–6 weeks old, 16–18 g) were obtained from the Laboratory Animal Center, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (China). Mice were housed under controlled temperature and 12 h light/dark cycle conditions, with food and water freely available. The experimental design and procedures were approved by the Institutional Ethical Committee for Animal Care and Use of Shandong University, People's Republic of China. The mouse melanoma cell line B16 was maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin–streptomycin (100 IU/mL–100 μg/mL), 2 mmol/L glutamine and 10 mmol/L HEPES.

2.3. Induction of immunosuppression and treatment with peptides in mice

Male BALB/c mice were divided randomly into five groups. Animals in the normal control group ($n = 10$) were given 0.1 mL of physiological saline (NS) via subcutaneous (s.c.) injection once daily for 16 d consecutively. Animals in the model group ($n = 13$), the TP5 group ($n = 10$), the Tα1 group ($n = 10$) and the Tα1–TP5 group ($n = 10$) were intraperitoneally injected with 250 mg/kg of HC once daily for two consecutive days. Then, the mice in the model group were subcutaneously given 0.1 mL of NS once daily for 14 d. The mice in the peptide treatment groups were subcutaneously administered TP5, Tα1 or Tα1–TP5 at a dose of 55, 250, 305 μg/kg (the same molar dosage) once daily for 14 d.

2.4. Thymus index, spleen index and histomorphological analyses

Two weeks after peptide treatment, the mice were weighed, blood was collected from the posterior orbital venous plexus for IFN-γ analysis under ether anesthesia, and the mice were euthanized. The thymus and spleen of each mouse were removed immediately and were washed and weighed to assess the changes in the immune organs. The organ indices of the thymus and spleen were calculated according to the formula: organ index (%) = organ weight/body weight × 100%. Three thymuses from each group were immediately excised and fixed in 10% neutral buffered formalin and embedded in paraffin. The tissues were then sectioned to generate 4 μm sections and stained with hematoxylin and eosin to examine the morphology of thymus. In the model group, three mice were randomly chosen for euthanization at one day and seven days after HC injection, and their thymuses were excised and stained for thymus morphological observation.

2.5. Flow cytometric analysis of thymocyte subsets

The thymuses ($n = 3$) of each group were dissociated to single cells and the thymocytes were then harvested, blocked with rat serum and stained with FITC-CD4 antibody and PE-CD8 antibody for 1 h at 4 °C. Cells were washed three times with cold PBS and analyzed by flow cytometry (FACSCalibur, USA). In the model group, thymocytes ($n = 3$) were collected seven days after HC injection and stained for flow cytometric analysis of thymocyte subsets.

2.6. ELISA detection of IFN-γ in peripheral blood

The serum was separated by centrifuging the blood sample at 4 °C and was then stored at −80 °C until measuring the IFN-γ serum concentration by ELISA. ELISA was performed according to the manufacturer's protocols. All samples were analyzed on a microplate reader (Bio-Rad, 680).

2.7. Tumor inoculation and treatment with CY and peptides

C57BL/6 mice were inoculated subcutaneously with 2×10^5 B16 melanoma cells/mouse in the forelimb armpit. Five days after inoculation, the mice were divided into six groups according to tumor size. Mice in the model control group ($n = 9$) were given s.c. injections of 0.1 mL NS once daily for 16 d consecutively. Mice in the CY group ($n = 9$), TP5 group ($n = 9$), Tα1 group ($n = 9$), Tα1–TP5 group ($n = 9$) and TP5 + Tα1 mixture group ($n = 9$) were intraperitoneally administered 200 mg/kg of CY once daily for 2 d. Mice in the CY group were given 0.1 mL of NS once daily for 14 d consecutively. Mice in the peptide treatment groups were subcutaneously administered TP5, Tα1, Tα1–TP5 or TP5 + Tα1 at a dose of 55, 250, 305, 55 + 250 μg/kg, respectively, once daily for 14 d.

2.8. Tumor size and weight measurement

Tumor size was measured every two days and calculated according to the following formula: Tumor size (cm^3) = $\pi \times$ length × width^2/6. The mice were sacrificed on the 15th d after peptide treatment and the tumor tissues were weighed and then stored at −80 °C for further analysis.

2.9. White blood cell analysis

Peripheral blood was collected from the mouse tail vein on the 0, 4th, 9th and 14th d after CY injection. 200 μL of blood was placed in a sterile EDTA-2 K-anticoagulated tube and counted by CA800 hemocytometer (SANKYO, Japan).

2.10. Flow cytometry analysis of MHC I expression

Tumors were recovered and minced into DMEM medium. Tumor cells were collected, washed and incubated with anti-MHC I antibody (0.5 μg/mL) or an isotype control. Cell-absorbed mAb was revealed by incubation with FITC-labeled goat anti-mouse IgG (2.5 μg/mL, ZSGB-BIO Co., Ltd., Beijing). After the tumor cells were washed twice with PBS, they were then analyzed by flow cytometry (FACSCalibur, USA).

2.11. Immunohistochemical analysis of CD8 and CD86 expressions

For immunohistochemical analysis, the tumors cryo-sections, 6-μm thick, were fixed in ice-cold acetone for 10 min and washed three times with PBS. The sections were incubated in H_2O_2 (3%) for 10 min at room temperature to block the

endogenous peroxidase activity. Subsequently, the sections were incubated with rat anti-mouse CD8 monoclonal antibody (Abcam) or rat anti-mouse CD86 monoclonal antibody (Abcam) overnight at 4 °C. HRP-tagged goat anti-rat IgG (ZSGB-BIO Co., Ltd., Beijing) was added as a secondary antibody for 30 min at room temperature. Finally, the sections were treated with 3,3-diaminobenzidine (DAB) as a peroxidase substrate and stained with hematoxylin. The sections were then dehydrated, cleared, and mounted with neutral gum.

2.12. Peptides labeling, bone marrow-derived macrophages (BMDMs) isolation and confocal microscope analysis of membrane binding

The covalent binding of FITC to TP5, Tα1 and Tα1–TP5 was carried out according to the methods described in a previous report [25]. Briefly, 1 mL of peptide solution (0.5 mg/mL TP5, 1 mg/mL Tα1 or 1 mg/mL Tα1–TP5) was mixed with 1 mg/mL FITC (dissolved in 0.1 mol/L carbonate buffer, pH 9.5) and incubated in the dark at 4 °C for 8 h. For TP5, the unbound FITC was separated from FITC-TP5 by filtration through a Biogel P10 column with 50 mmol/L NH_4HCO_3 solution as the eluent. The fractions containing FITC-TP5 were lyophilized for use. For Tα1 and Tα1–TP5, the unbound FITC was removed by dialysis against a 1000 Da dialysis membrane. The preparation of BMDMs was performed as previously described [26]. BALB/c mice were sacrificed by cervical dislocation and the bone marrow cells were then flushed from the femoral shafts with cold PBS. Cells were collected, incubated in RPMI 1640 medium for 2 h at 37 °C in the presence of 5% CO_2 in a CO_2 incubator to remove the adherent macrophages. Nonadherent cells were further cultured in RPMI 1640 medium containing 100 ng/mL M-CSF (PeproTech, USA) to induce BMDMs proliferation and maturation. On the 7th day of cell culture, the BMDMs were incubated with 200 μL of FITC-labeled peptides for 2 h at 37 °C in the presence of 5% CO_2. After the cells were washed with PBS, they were then fixed with freshly prepared 4.0% paraformaldehyde at room temperature for 10 min. The cells were then incubated with Hoechst 33342 for 10 min at room temperature to stain the cell nuclei, followed by cell membrane staining with DiI for 20 min. The BMDMs were washed three times and preserved in blocking solution (glycerol-PBS, 1:1, v/v) in preparation for confocal microscopy (Carl Zeiss) observation.

2.13. SPR analysis of the interaction between Tα1–TP5 and TLR2

Interactions between Tα1–TP5 and TLR2 were examined using a Biacore 3000 (GE Healthcare, Sweden). TLR2 was immobilized by standard amine coupling using an amine coupling kit. The chip surface was activated by EDC/NHS, and then the TLR2 (25 μg/mL) in sodium acetate solution (10 mmol/L, pH 4.8) was passed over the chip surface until a ligand density of 25,000 resonance units (RU) was achieved. The remaining active esters were quenched with an ethanolamine solution (1.0 mol/L, pH 8.5). The control flow cell was treated as above in the absence of TLR2. HBS-EP (10 mmol/L HEPES, 150 mmol/L NaCl, and 3 mmol/L EDTA, 0.005% v/v P-20) was used as the running buffer for the immobilization and kinetic studies. The peptides were dissolved in running buffer, and a flow rate of 30 μL/min was employed for association and dissociation at a constant. The chips were regenerated after each cycle by injecting 15 μL of Gly–HCl (pH 2.5). In the kinetics analysis, the 1:1 Langmuir binding fit model was used to determine the equilibrium dissociation constant (K_D), association (k_{on}) and dissociation (k_{off}) rate constants. The fitting results were evaluated and judged by the χ^2 test, a statistical parameter in the surface plasmon resonance (SPR) assay.

2.14. Statistical analysis

All data were expressed as mean ± SEM. The significant differences were analyzed by one-way analysis of variance. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Tα1–TP5 increased thymus index and spleen index values in immunosuppressed mice

To investigate the immunomodulatory activity of Tα1–TP5 *in vivo*, an immunosuppression model was established by the intraperitoneal injection of HC and the thymus index and spleen index were calculated. We observed that the thymus index in the HC group was 0.444 ± 0.407, which was almost 74% lower than that of the normal control group after two weeks (Table 1). However, this reduction was completely reversed by the administration of Tα1–TP5. The thymus index of the Tα1–TP5 treated group was 2.253 ± 0.427, which was much higher than that of the HC group ($p < 0.01$) and also higher than that of the TP5 group and the Tα1 group ($p < 0.05$). This result demonstrated that Tα1–TP5 had a better effect with respect to improving the immune conditions of the immunosuppressed mice than did TP5 and Tα1 alone. The spleen index was found to be decreased in HC-treated mice compared to that of the normal control mice ($p < 0.05$). However, Tα1–TP5 had little effect on the restoration of spleen atrophy.

Table 1
The thymus index and spleen index of immunosuppressed mice following s.c. injection of TP5, Tα1 or Tα1–TP5 for 14 consecutive days.

Treatment	Thymus index (mg/g)	Spleen index (mg/g)
Normal control	1.734 ± 0.167[a]	5.504 ± 0.668[a]
HC	0.444 ± 0.407	4.106 ± 0.53
HC + TP5	1.814 ± 0.579[a]	4.441 ± 0.578
HC + Tα1	1.745 ± 0.131[a]	4.906 ± 0.6
HC + Tα1–TP5	2.253 ± 0.427[a,b,c]	4.473 ± 0.242

Each value represents the mean ± SD ($n = 10$).
[a] Compared with HC group, $p < 0.05$.
[b] Compared with TP5 group, $p < 0.05$.
[c] Compared with Tα1 group, $p < 0.05$.

3.2. Tα1–TP5 raised the percentage of $CD4^+CD8^+$ thymocytes

The effect of Tα1–TP5 on the $CD4^+CD8^+$ thymocyte population was examined by flow cytometry. As shown in Fig. 1A, mice in the normal control group had a normal $CD4^+CD8^+$ thymocyte percentage (81.07%). Compared with the normal control group, a significant reduction in the percentage of $CD4^+CD8^+$ thymocytes, with a concomitant increase in single-positive $CD4^+CD8^-$ and $CD4^-CD8^+$ cells, was observed seven days after HC injection (24.24% vs. 81.07%, $p < 0.05$). After treatment with HC for 14 days, the percentage of $CD4^+CD8^+$ thymocytes slowly increased, but it was still lower than the normal level ($p < 0.05$). After the injection of Tα1–TP5, $CD4^+CD8^+$ thymocytes reached 88.5 ± 0.27%, which was higher than that of the HC-treated group. This result proved that Tα1–TP5 can prevent the loss of thymocytes by rescuing $CD4^+CD8^+$ thymocytes from HC-induced death. Treatments with TP5 and Tα1 alone was also able to prevent the reduction of $CD4^+CD8^+$ thymocytes (compared with the HC group, $p < 0.05$). Tα1–TP5 demonstrated a better effectiveness than TP5 and Tα1 alone in increasing $CD4^+CD8^+$ thymocyte subsets.

3.3. Tα1–TP5 antagonized the thymic atrophy induced by HC

The histomorphology of the thymus was examined with an optical microscope. The thymuses of normal control mice displayed massive closely arranged and deeply stained thymocytes in the cortical region (Fig. 1B). One day after HC injection, the thymuses of the model group appeared slightly atrophied, leaving partially unoccupied zones (white area). In addition, a thinner cortical region and a decreased number of thymocytes were observed. Seven days after HC injection, a rapid and dramatic atrophy of the thymuses was observed and the tissue architecture in these thymuses was completely damaged, as evidenced by the observed cellular margin isolation or rupture. Two weeks after HC injection, atrophic thymuses and reduced thymocytes were still observed, suggesting a profound effect of HC on the thymus. In contrast, this form of tissue disruption was alleviated in the thymuses of mice treated with Tα1–TP5, as evidenced by the thickened cortex region and the increased thymocytes. The thickened thymus cortex and the increased thymocytes in the Tα1 group were also observed, although unoccupied zones in the thymuses could still be observed. The thymuses in the TP5 group presented morphologies similar to as those of the Tα1 group.

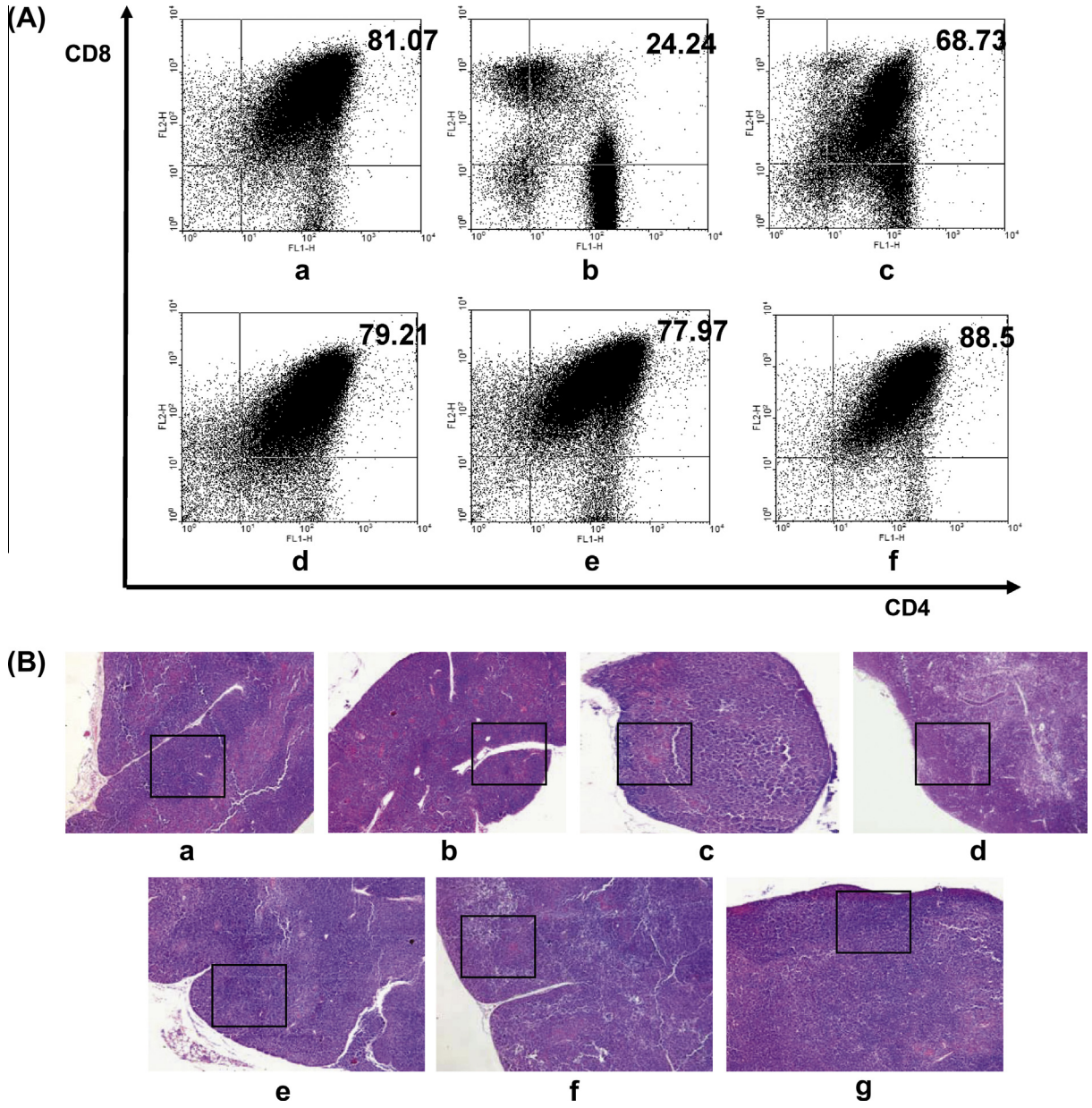

Fig. 1. Tα1–TP5 reversed the against immunosuppression induced by HC. Mice were injected with HC for two consecutive days and treated with peptides for 14 days consecutively. The thymuses ($n = 3$) of each group were dissociated to single cells for thymocyte staining. The percentage of $CD4^+CD8^+$ thymocytes was determined by flow cytometry after staining with FITC-CD4 and PE-CD8 mAb. The thymuses ($n = 3$) of each group were fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin. (A) Thymocyte subpopulation distribution of mice: (a) normal control; (b) HC day 7; (c) HC day 14; (d) HC + TP5; (e) HC + Tα1; and (f) HC + Tα1–TP5. (B) HE staining of mouse thymuses: (a) normal control; (b) HC day 1; (c) HC day 7; (d) HC day 14; (e) HC + TP5; (f) HC + Tα1; and (g) HC + Tα1–TP5. The area of difference is circled in each of the seven panels. The regions containing a large number of thymocytes are dyed dark blue, and the regions with few thymocytes are dyed pink. Images were captured at 200× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Tα1–TP5 enhanced the concentration of IFN-γ in the peripheral blood

The level of IFN-γ in the peripheral blood was examined as a representative of the cytokines produced in mice that were treated with HC. ELISA analysis showed that the serum level of IFN-γ was decreased by HC injection (179.87 ± 7.56 vs. 288.34 ± 9.54, compared with the normal control group, $p < 0.05$), whereas Tα1–TP5 administration protected the IFN-γ reduction induced by HC. Although the concentration of IFN-γ in the Tα1-TP5 group did not return to normal levels, no significant difference was observed compared with the normal control group (261.58 ± 8.9 vs. 288.34 ± 9.54, $p > 0.05$). Tα1–TP5 promoted a higher level of IFN-γ secretion into the peripheral blood than TP5 and Tα1 alone (261.58 ± 8.9 vs. 225.79 ± 16.1 or 213.46 ± 9.8, $p < 0.05$). Our findings indicated that the injection of Tα1–TP5 could help allow promote the mice to recover from immunosuppression.

3.5. Tα1–TP5 combined with CY had a better tumor growth inhibitory effect than CY alone

To determine the antitumor effect of Tα1–TP5 combined with CY, the mice bearing B16 melanoma were treated with the direct s.c. administration of NS, TP5, Tα1, Tα1–TP5 and TP5 + Tα1. Five days after tumor inoculation, all mice developed tumors with a diameter of 2–3 mm. The mice in the control group (treated with

NS) had regular diet and activities, and the tumors in this group increased significantly to an average tumor size of 4.769 ± 0.84 cm^3 at the end of the experiment. In comparison, all mice except the control mice showed fur piloerection and reduced food and water intake one day after the first injection of CY, and especially four days after CY injection, after which they recovered slowly. During the 7–15 days after tumor inoculation, the tumors of each group grew slowly except those of the control group. During days 15–17, the tumor volume in the CY group increased significantly from 0.15 ± 0.094 cm^3 to 0.32 ± 0.087 cm^3. The tumor growth rates in the polypeptide treatment groups were less than that of the CY group. During days 17–19, the tumor volume in the CY group was approximately 0.95 ± 0.097 cm^3, but the tumor volume in the CY + Tα1–TP5 group was only 0.115 ± 0.03 cm^3, demonstrating a significant tumor growth inhibition of the combined treatment ($p < 0.05$) (Fig. 2a). Similarly, the tumor weight was more significantly reduced by the treatment of Tα1–TP5 and CY compared to the other treatment groups ($p < 0.05$ vs. CY; $p < 0.05$ vs. CY + TP5; $p < 0.05$ vs. CY + Tα1) (Fig. 2b). At the end of the experiment, the tumors in the control group were grayish black in color, with irregular tumor edges; however, the tumors in the CY group and peptide treatment groups were black and wrapped in a layer of integral membrane. Compared to the CY group, the tumor weight inhibition rates of the TP5, Tα1, Tα1–TP5 and TP5 + Tα1 groups were 66.0%, 59.7%, 88.1% and 77.6%, respectively (Fig. 2c).

3.6. Tα1–TP5 increased leukocyte number lowered by CY treatment

The number of leukocytes in the peripheral blood of tumor-bearing mice was detected during the treatment. Before CY administration, little difference was observed among the different groups (Table 2). Four days after CY injection, the peripheral blood leukocytes in the control group were 8.63 × 10^9/L, whereas the leukocytes in the other four groups decreased by 67%, 66%, 72%, 69% and 71%, respectively. Nine days after CY injection, the number of leukocytes in all groups except the control group gradually increased; however, the growth rate of the CY group was the slowest. Fourteen days after CY injection, the number of leukocytes in the CY group reached approximately 5.37 × 10^9/L, which was still less than that of the control group ($p < 0.05$). The number of leukocytes in the CY + Tα1–TP5 group and the CY + TP5 + Tα1 group returned to normal levels (compared with the CY group, $p < 0.05$). These results indicated that treatment with Tα1–TP5 caused a statistically significant attenuation of the reduction of the number of leukocytes in the peripheral blood of immunosuppressed mice.

Table 2
Effect of Tα1–TP5 on leukocytes in C57BL/6 mice suppressed by CY.

Treatment	Leukocyte number (10^9/L) after CY injection			
	0 d	4 d	9 d	14 d
Control	7.97 ± 1.05	8.63 ± 1.71	8.93 ± 1.05	9.40 ± 1.39
CY	7.16 ± 1.58	2.81 ± 0.72	3.64 ± 1.81	5.37 ± 0.64
CY + Tα1	7.87 ± 0.76	2.38 ± 1.23	4.87 ± 1.51	6.52 ± 1.78
CY + TP5	7.46 ± 1.42	2.96 ± 1.45	4.29 ± 2.05	7.48 ± 1.49
CY + Tα1–TP5	7.24 ± 0.96	2.68 ± 0.80	5.09 ± 1.41	9.24 ± 0.98[a]
CY + TP5 + Tα1	7.53 ± 0.74	2.49 ± 0.69	4.23 ± 1.42	8.05 ± 1.20[a]

Each value represents the mean ± SD ($n = 10$).
[a] Compared with CY, $p < 0.05$.

3.7. Tα1–TP5 combined with CY upregulated MHC I expression in tumor tissue

MHC I expression in tumors was examined using flow cytometry. As shown in Fig. 3, cells extracted from the tumor tissue of the

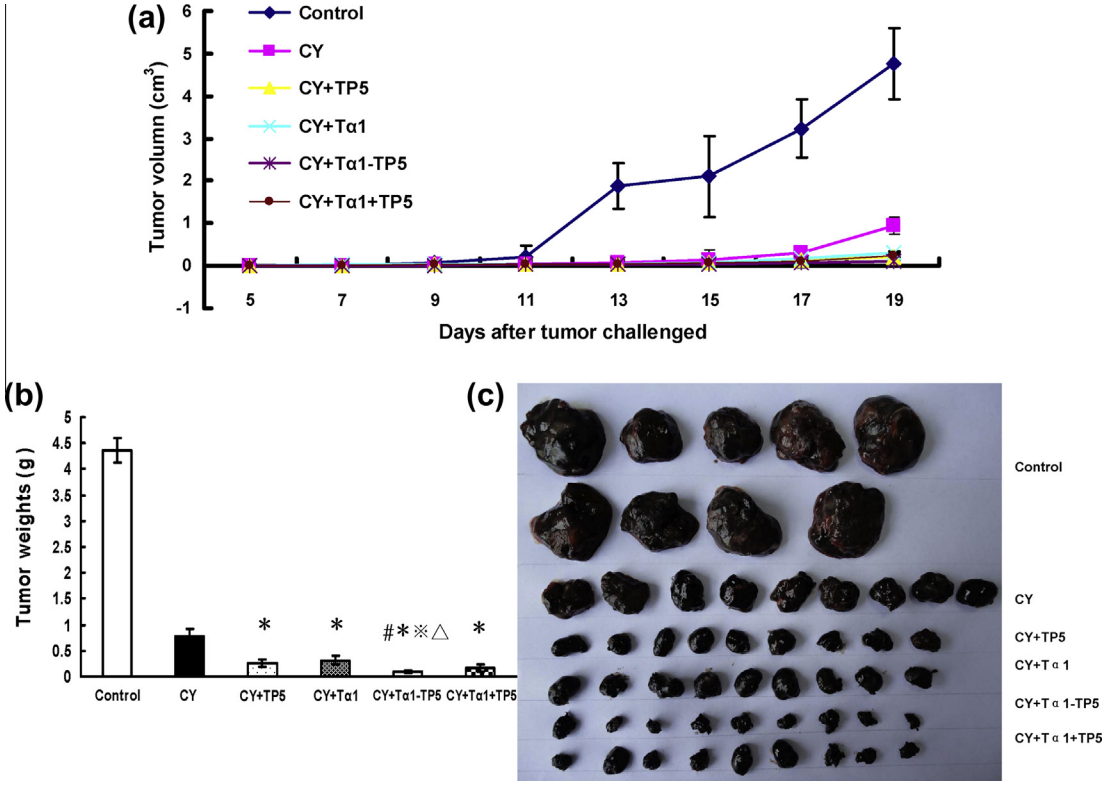

Fig. 2. Tα1–TP5 combined with CY resulted in an improved tumor growth inhibitory effect than CY alone. C57BL/6 mice were injected s.c. with B16 melanoma cells in the forelimb armpit for 5 days. Then, CY was injected for two consecutive days, followed by the administration of peptides for 14 days. Two weeks later, the mice were sacrificed and tumor tissues were separated and weighed. (a) Tumor volume measurement. (b) Tumor weights of different groups, (* Compared with the CY group, $p < 0.05$. # Compared with the TP5 group, $p < 0.05$. ※ Compared with the Tα1 group, $p < 0.05$. △ Compared with the Tα1 + TP5 group, $p < 0.05$). (c) Picture of isolated tumors.

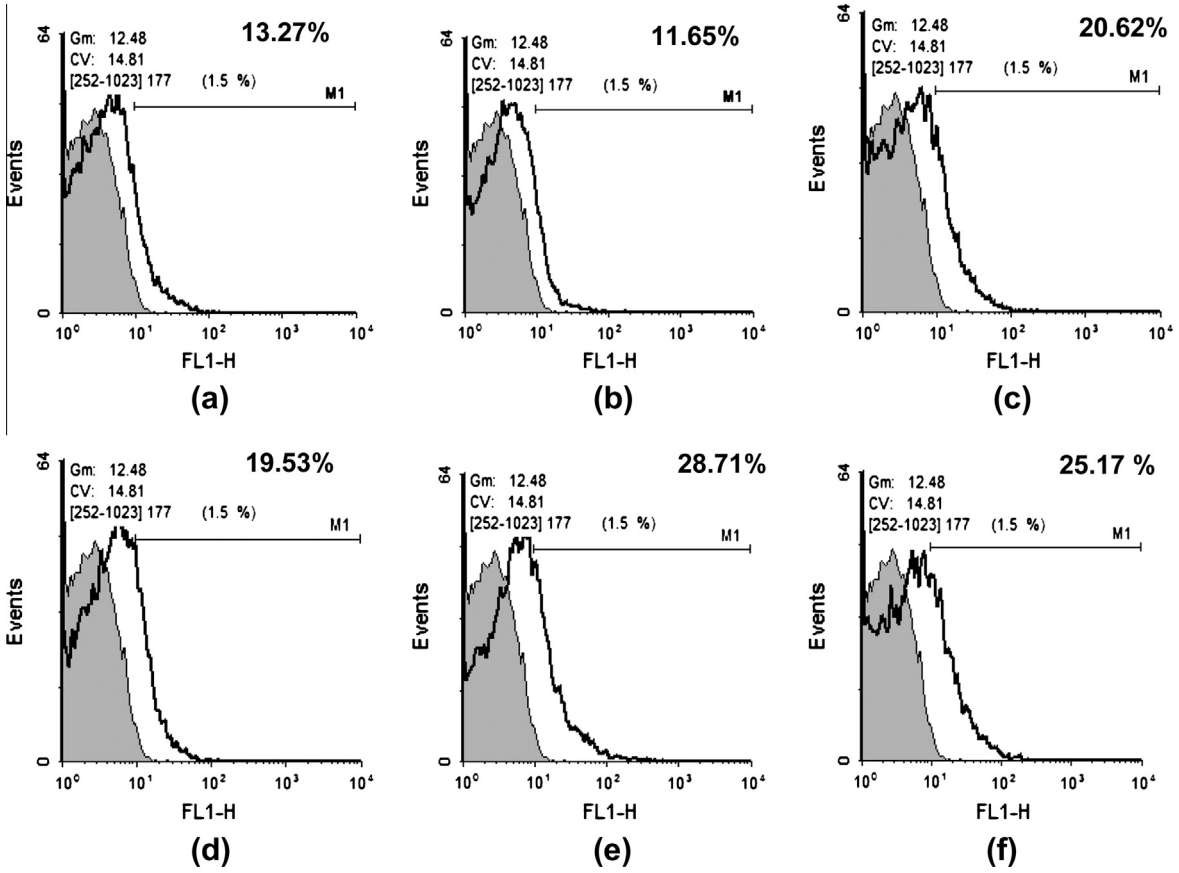

Fig. 3. Tα1–TP5 combined with CY upregulated MHC I in tumor tissue. Tumors were recovered and minced into DMEM medium. Cell suspensions were labeled with anti-H2-Db antibody, followed by incubation with FITC-labeled goat anti-mouse secondary antibody, and were detected by flow cytometry. Data were analyzed using the WinMDI29 software. (a) Control; (b) CY; (c) CY + TP5; (d) CY + Tα1; (e) CY + Tα1–TP5; and (f) CY + TP5 + Tα1.

control group displayed a detectable level of MHC I molecules (13.27%). The MHC I expression level in the CY group was 11.65%, indicating that CY did not cause a decreased or increased expression of MHC I in tumor tissue. An increase in the MHCI level was detected after CY + Tα1–TP5 treatment (28.71% vs. 11.65%, $p < 0.05$). These results show that Tα1–TP5 has the ability to promote the expression of MHC I in tumor tissues and that the effect was higher than that of TP5 or Tα1 alone.

3.8. Tα1–TP5 promoted lymphocyte infiltration and CD86 expression in tumor tissues

Immunohistochemical analysis was carried out to study lymphocyte infiltration and CD86 expression in tumor tissues using anti-CD8 and anti-CD86 monoclonal antibodies. As seen in Fig. 4, no $CD8^+$ T cell infiltration was observed in the group treated with NS. Similarly, the CY group did not exhibit any dark brown-stained regions. Mild $CD8^+$ T cell infiltration was observed in the tumor tissue of the CY + TP5 group and the CY + Tα1 group. The number of $CD8^+$ T cells was substantially higher in the TP5- and Tα1-treated animals compared to the NS- or CY-treated animals. A greater abundance of $CD8^+$ T cells was observed in the tumor tissues treated with Tα1–TP5 compared with the control, CY only, CY + TP5 and CY + Tα1 treatment groups, indicating that a higher number of cytotoxic T cells were recruited to the tumor tissues. To further examine whether the coadministration of Tα1–TP5 and CY could stimulate the expression of CD86 *in situ*, the expression of CD86 in tumor tissues was analyzed. As shown in Fig. 5, a greater number of $CD86^+$ stained cells was observed in the CY + Tα1–TP5 group compared with the CY + TP5 group and the CY + Tα1 group. Tα1–TP5 also had a greater ability to recruit immunologically competent cells into the tumor tissues than did the TP5 + Tα1 combined treatment.

3.9. Tα1–TP5 can bind to TLR2

As seen in Fig. 6, the blue fluorescence staining (corresponding to the cell nuclei) was delimited by the green fluorescence (corresponding to the FITC-labeled peptides). Red fluorescence (corresponding to the cell membrane) overlapped with the green fluorescence, implying that Tα1-TP5 was located on the cell membrane of the BMDMs. TP5 and Tα1 were also located on the cell membrane of the BMDMs. To further investigate if Tα1–TP5 can interact with TLR2, a Biacore 3000 biosensor system was used. All of the kinetic parameters and the sensorgrams are listed in Table 3 and Fig. 7. Experiments at 25 °C showed that TLR2 was immobilized on the CM5 chip with a coupling capacity of approximately 25,000 RU. Tα1 showed binding affinity towards TLR2 with a K_D value of 35.4×10^{-6} mol/L, which agrees with the hypothesis that TLR2 acts as a receptor of Tα1 which was presented in the previous report [20]. The K_D of Tα1–TP5 for TLR2 was 6.84×10^{-6} mol/L, which is much smaller than that of Tα1, indicating that Tα1–TP5 has a higher affinity to TLR2 than does Tα1. Compared with TP5, the affinity of Tα1–TP5 to TLR2 was slightly lower, but this difference was not statistically significant. Thus, the receptor of the three peptides was first confirmed to be TLR2.

4. Discussion

In this study, the administration of 250 mg/kg HC once daily for 2 consecutive days reduced the thymus and the spleen indices as

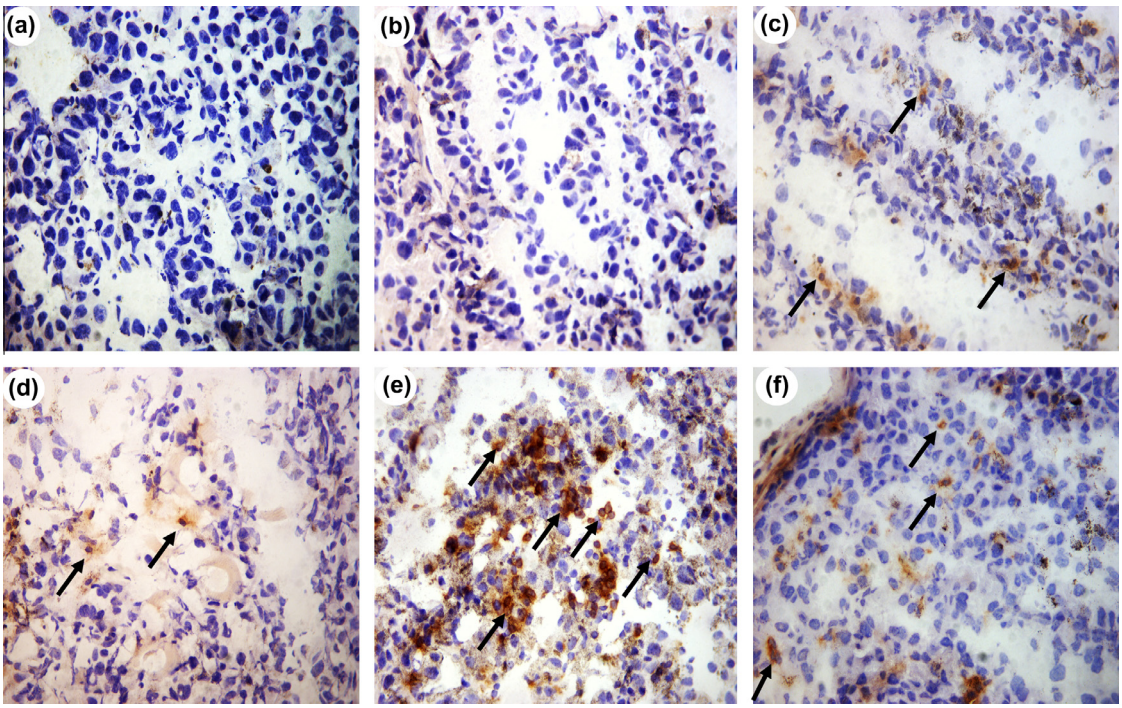

Fig. 4. Tα1–TP5 promoted lymphocyte infiltration into tumor tissues. Tumors from B16 melanoma-bearing mice were harvested, sectioned and stained with anti-CD8 antibody, followed by incubation with HRP-tagged goat anti-rat IgG. (a) Control; (b) CY; (c) CY + TP5; (d) CY + Tα1; (e) CY + Tα1–TP5; and (f) CY + TP5 + Tα1. Arrows indicate $CD8^+$ T cell infiltration in tumor tissues. Images were captured at 400× magnification.

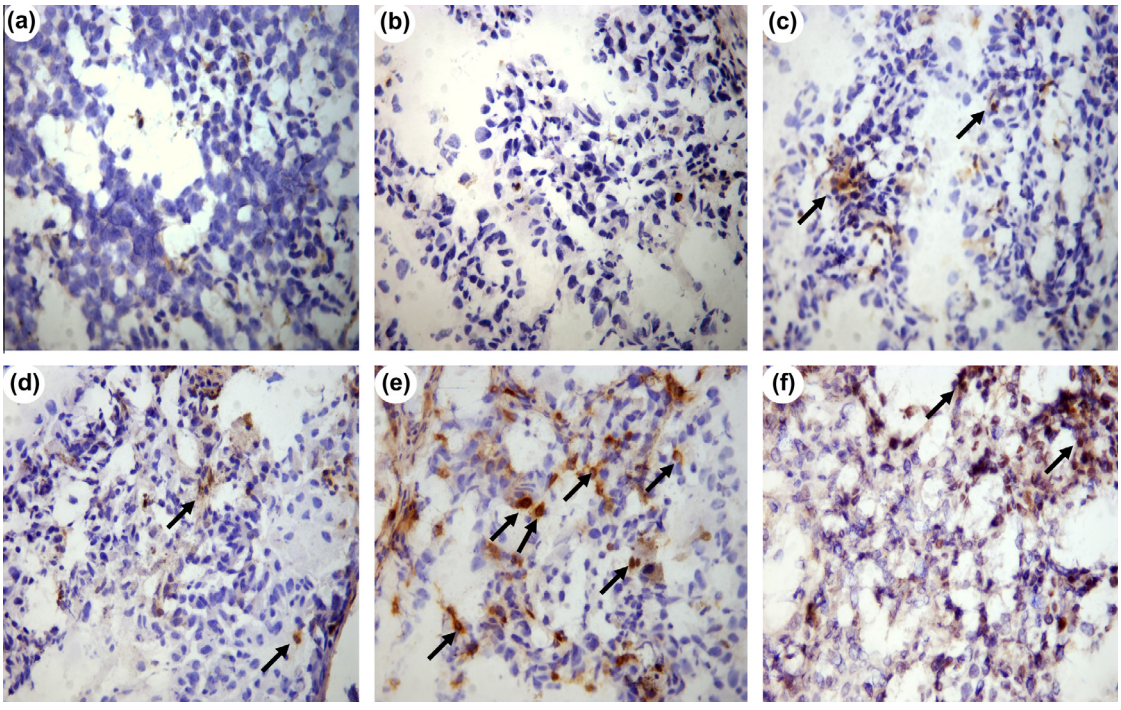

Fig. 5. Tα1–TP5 promoted CD86 expression in tumor tissues. Tumors from B16 melanoma-bearing mice were harvested, sectioned and stained with anti-CD86 antibody, followed by incubation with HRP-tagged goat anti-rat IgG. (a) Control; (b) CY; (c) CY + TP5; (d) CY + Tα1; (e) CY + Tα1–TP5; and (f) CY + TP5 + Tα1. Arrows indicate CD86 expression in tumor tissues. Images were captured at 400× magnification.

well as the number of $CD4^+CD8^+$ thymocytes. Two weeks after HC withdrawal, the thymus and spleen did not regain their normal size and the $CD4^+CD8^+$ thymocytes did not return to normal levels, which should be more than 70% [27]. These results indicate that the mice cannot totally recover from immunosuppression after two weeks, and the increase of $CD4^+CD8^+$ thymocytes in the HC group should be caused by the immune regulation of the mice. Compared to the thymus, HC treatment resulted in less damage to the spleen, which contained fewer $CD4^+CD8^+$ cells than the thymus. Histomorphological analysis revealed that thymic atrophy

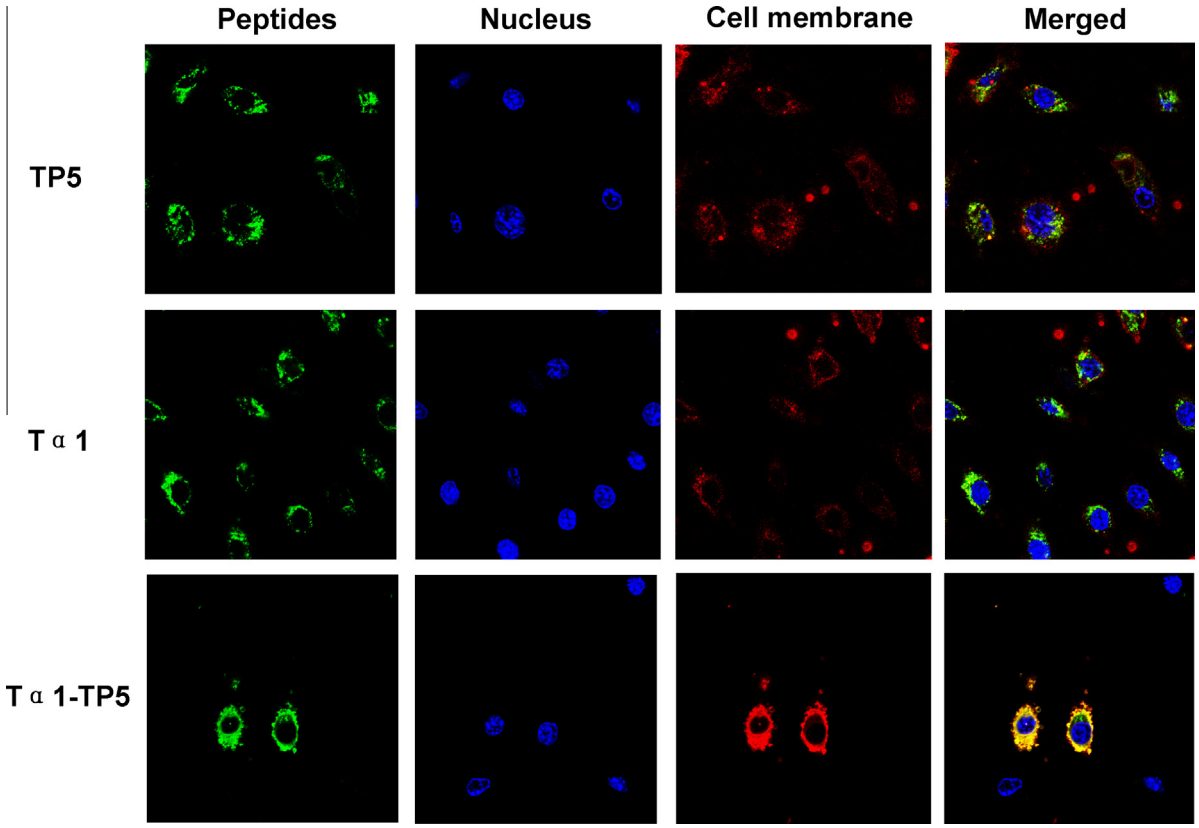

Fig. 6. Confocal microscopic observation of the binding of Tα1–TP5 to BMDMs. BMDMs were incubated with FITC-TP5, FITC-Tα1 and FITC-Tα1–TP5 for 2 h at 37 °C in the presence of 5% CO_2. Cells were then stained with Hoechst 33342 (blue) and DiI (red). After washing three times with PBS, the cells were observed using confocal microscopy. Images were captured at 630× magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Association (k_{on}) and dissociation (k_{off}) rate and equilibrium dissociation (K_D) constants of TP5, Tα1 and Tα1–TP5 binding to the immobilized TLR2 obtained from SPR experiments.

Analyte	k_{on} ($mol^{-1}\ s^{-1}$)	k_{off} (s^{-1})	K_D (mol/L)	χ^2
TP5	771 ± 31	(1.21 ± 0.11) × 10^{-3}	(1.57 ± 0.13) × 10^{-6}	0.425
Tα1	150 ± 1.11	(5.32 ± 0.14) × 10^{-3}	(35.4 ± 0.66) × 10^{-6}	0.106
Tα1–TP5	241 ± 4.82	(1.65 ± 0.04) × 10^{-3}	(6.84 ± 0.05) × 10^{-6}[a]	0.437

Each value represents the mean ± SD (n = 3).
[a] Compared with Tα1, $p < 0.05$.

could not be restored after two weeks. On the contrary, the extent of thymic atrophy was aggravated as time went on. After the individual administration of TP5, Tα1 and Tα1–TP5, a marked accentuation of thymus recovery was observed.

IFN-γ is one of the major cytokines produced by Th1 cells, such as activated $CD4^+$ T cells, $CD8^+$ T cells and NK cells, and contributes to cell-mediated inflammatory immune responses. In addition, IFN-γ can promote the differentiation of Th0 cells to Th1 cells, inhibit the proliferation of Th2 cells, and promote the differentiation of B-cells. The pre-exposure of $CD4^+$ lymphocytes to GCs suppressed the secretion of Th1-type cytokines, such as IFN-γ and TNF-α, and increased the secretion of Th2-type cytokines [24]. GCs suppress the production of the main inducer of Th1 responses, IL-12, both *in vitro* and *ex vivo* by acting through their classic cytoplasmic/nuclear receptors on antigen-presenting cells (APCs) [28]. Our previous studies demonstrated that Tα1–TP5 was able to directly activate spleen lymphocytes to produce a higher level of IFN-γ at the transcriptional level than did TP5 and Tα1 (data not shown). In the present study, the suppressed IFN-γ secretion was restored to almost normal levels following the s.c. administration of Tα1–TP5. Of course, the level of IFN-γ level was not abnormally high, which indicated that Tα1–TP5 had the ability to modulate the Th1/Th2 balance.

CY is a widely used chemotherapeutic agent in cancer therapy, but it induces a marked suppression of the immune response. Daily doses of intraperitoneal CY (50 mg/kg) reduced the peripheral white blood cell count by ~85% in CD-1 mice [29]. Tα1 can significantly promote increased white blood cell density in Fisher rats bearing mammary carcinoma [30]. A low white blood cell count, which is a symptom of low body immunity, results in an increased risk of infection and even death. Therefore, chemotherapeutic agents should be used in combination with immunomodulators to reduce immune system side effects. Combined treatment protocols consisting of the administration of Tα1 and IL-2 after CY or 5-FU in the treatment of Lewis lung carcinoma and colorectal cancer have been reported, and these treatments resulted in a powerful antitumor effect [31,13]. However, the use of IL-2 should be limited because of its relative inefficacy, and its high toxicity, side effects and cost. Hence, the anti-tumor effect of Tα1–TP5 combined with CY was evaluated in this study. Our data showed that the

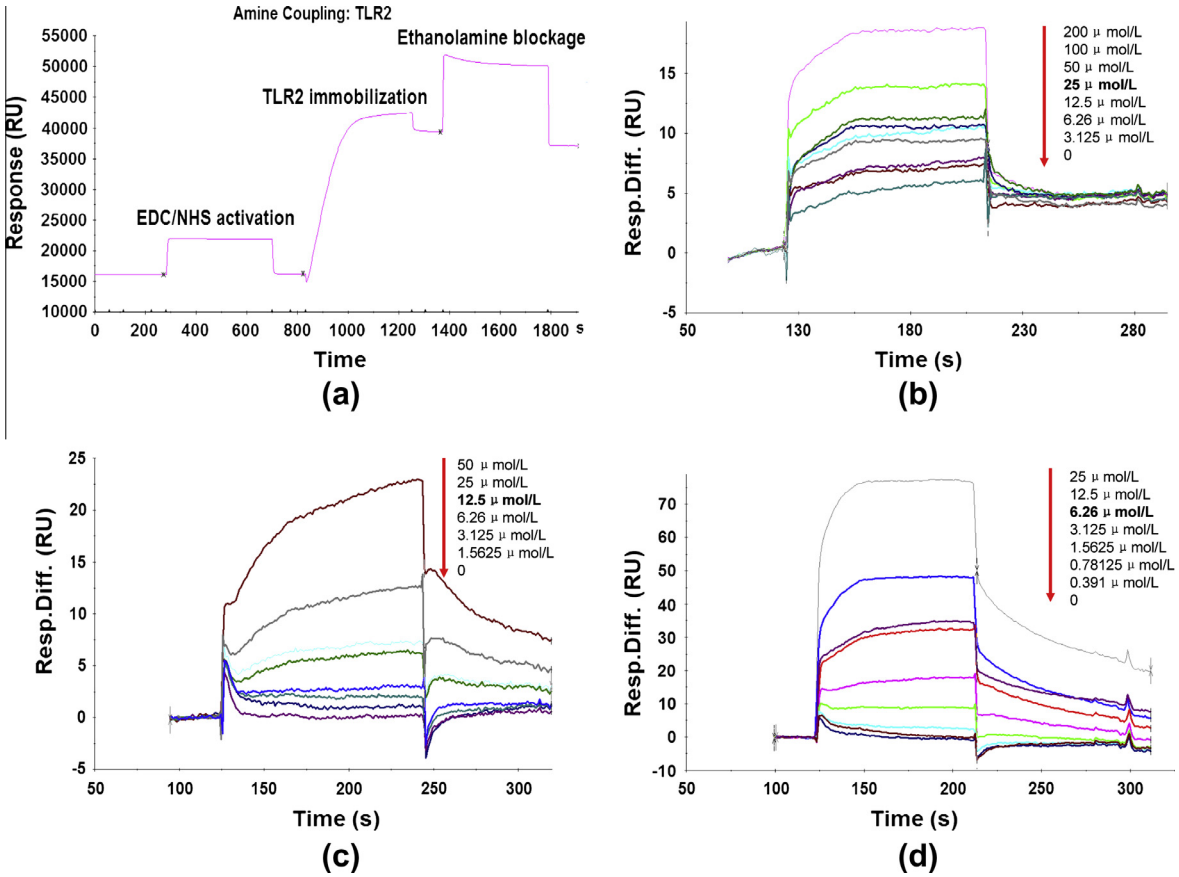

Fig. 7. Tα1–TP5 exhibited high affinity against TLR2 as evaluated by SPR. Six to eight serial dilutions of the three peptides were injected at 30 μL/min for 90 s association periods. The concentrations were shown next to the arrows. Bold figures indicated repeated concentrations. The sensorgrams were obtained, and the K_D, K_{on} and K_{off} were analyzed using a global fit algorithm (BIAevaluation 3.1). (a) TLR2 was immobilized on CM5 sensor chips. (b–d) TP5, Tα1 and Tα1–TP5 kinetic-binding responses to TLR2.

combination therapy not only inhibited tumor growth and reduced tumor volume but also alleviated the reduced of white blood cell count caused by CY. In addition, the effect of Tα1–TP5 was better than that of TP5 or Tα1 alone. In a previous *in vitro* study, it was found that 20 μg/mL of Tα1–TP5 can inhibit the growth of melanoma by only 17.5% after 72 h (data not shown). We supposed that Tα1–TP5 alone had a weak cytotoxic effect on tumor cells and its primary effect when used in the combination therapy was immune system activation.

The expression of MHC I molecules on the tumor cell surface is thought to be an important determinant in the interplay between tumor cells and the immune system [32]. When expressed on the cell surface, MHC I enables the antigen presentation that is required for target cell recognition by the CTLs. B16 cells are considered to be MHC I low-expression cells and are known to be poor spontaneous stimulators of the adaptive response *in vivo* [33], so the cancerous cells can easily escape the attack of CTLs. It was reported that Tα1 can increase the expression of the MHC I surface molecule at the transcriptional level in B16 cells [34]. In this study, Tα1–TP5, makes tumor cells more "visible" to CTLs and less prone to escape from immune surveillance by inducing the increased expression of MHC I in tumor tissue.

$CD8^+$ T cells acquire their cytotoxicity through two stimulating events. First, the T cell receptor (TCR) on $CD8^+$ T cells recognizes tumor antigen, which is presented by MHC I. Second, several costimulators such as CD80 and CD86 are required. After activation, $CD8^+$ T cells act as effector cells that are able to induce the death of tumor cells by two mechanisms. First, target cells can be killed by the release of cytolytic proteins such as perforin and granzyme B [35]. Second, apoptosis is induced by the expression of Fas ligand on CTLs, which bind to Fas on the target tumor cells [35,36]. CD86 is expressed on DCs, monocytes and activated T lymphocytes that provide costimulatory signals necessary for T cell activation and survival. In addition, CD86 plays an important role in inducing the generation and activating the function of CTLs. To establish that the induction of a tumor-specific immune response is responsible for the observed antitumor effect, the expression of CD8 and CD86 in tumor tissue was analyzed by immunohistochemistry. In tumor-bearing mice treated with Tα1–TP5 and CY, significantly higher levels of $CD8^+$ T cells and CD86 were detected in the tumor tissue compared with the NS-treated tumor-bearing mice or those treated with CY alone. These findings imply that the s.c. injection of Tα1–TP5 attracts $CD8^+$ T cells, DCs and macrophages from surrounding tissues and promotes their maturation from hematopoietic precursors; each of these cell types is responsible for the induction of specific antitumor immune responses.

TLR2, an important signal transduction molecule, is expressed on macrophages, DCs and activated $CD4^+$ T cells [37]. The induction of TLR2 signaling by its agonist, a bacterial lipoprotein (BLP), has a general tumor therapeutic effect involving the up regulation of CTL function and the reciprocal down regulation of regulatory T (Treg) cells [38]. Thus far, the action mechanism of Tα1–TP5, TP5 and Tα1 in activating immune effector cells is not yet known. Tα1 was reported to activate DCs for antifungal Th1 resistance through TLR signaling [19], so we supposed that Tα1–TP5 may mediate the immune response by acting with TLR2. Hence, the interaction of Tα1–TP5, TP5 and Tα1 with TLR2 was analyzed using the SPR technique, which has received increasing attention for its utility in examining

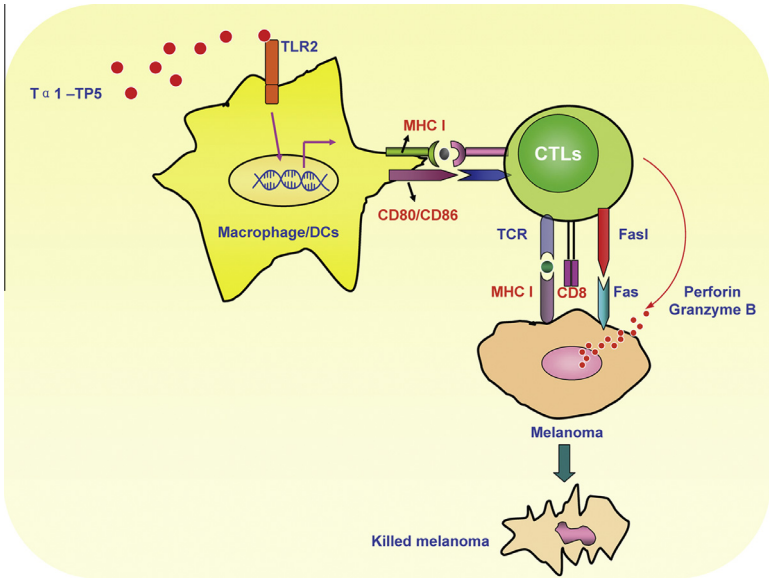

Fig. 8. Schematic diagram of the proposed mechanism of Tα1–TP5 in antitumor immune modulation. The molecules in red have been found to be upregulated by Tα1–TP5 in tumor tissues. Tα1–TP5 may activate DCs and macrophages via TLR2 signaling, which further present antigen to tumor-specific CTLs. CTLs recognize the increased MHC I molecules expressed on melanoma cells and then kill the tumor cells by two mechanisms. First, target cells are killed by the release of cytolytic proteins such as perforin and granzyme B. Second, apoptosis is induced by the expression of Fas ligand on CTLs, which bind to Fas on the target tumor cells.

the affinity and kinetics between protein–protein and protein–peptides interactions [39]. K_D is an important parameter for the measurement of ligand–receptor interactions; the smaller the K_D value the greater the binding capacity. The K_D values of TLR2 and peptidoglycan from gram-positive and gram-negative bacteria were 78 ± 6 and 11 ± 2 μmol, respectively [40]. The K_D for Tα1–TP5 evaluated in this paper was also at the μmol level. Tα1–TP5 may be a ligand of TLR2 that can activate DCs and macrophages via TLR2 signaling. In turn, DCs and macrophages can further present antigen to tumor-specific CTLs. CTLs can recognize increased MHC I expression on melanoma cells and can then kill these tumor cells (Fig. 8). The stronger immunomodulatory activity of Tα1–TP5 may due to its higher affinity to TLR2 than Tα1 and due to its longer $t_{1/2}$ than TP5. It is unclear whether Tα1–TP5 can bind to other receptors on other immune cells; this question requires further investigation.

The present report showed, for the first time, the activity of Tα1–TP5 in alleviating the immunosuppression induced by HC and the effectiveness of Tα1–TP5 combined with CY in inhibiting melanoma growth. This paper is also the first to report that TLR2 is the receptor of Tα1–TP5. These findings have helped provide for a better understanding of the immunologic mechanisms required to both oppose immunosuppression and optimize the efficacy of anticancer therapy.

Conflicts of Interest

None declared.

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