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# Administration of sulfosuccinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate conjugated $GP100_{25-33}$ peptide-coupled spleen cells effectively mounts antigen-specific immune response against mouse melanoma

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ABSTRACT

It remains a top research priority to develop immunotherapeutic approaches to induce potent antigen-specific immune responses against tumors. However, in spite of some promising results, most strategies are ineffective because they generate low numbers of tumor-reactive cytotoxic T lymphocytes (CTLs). Here we designed a strategy to enhance antigen-specific immune response via administering sulfosuccinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC)-conjugated melanoma tumor antigen $GP100_{25-33}$ peptide-coupled syngeneic spleen cells in a mouse model of melanoma. We found that infusion of $GP100_{25-33}$ peptide-coupled spleen cells significantly attenuated the growth of melanoma in prophylactic and therapeutic immunizations. Consistent with these findings, the adoptive transfer of spleen cells from immunized mice to naïve syngeneic mice was able to transfer anti-tumor effect, suggesting that $GP100_{25-33}$ peptide-specific immune response was induced. Further studies showed that, CD8+ T cell proliferation and the frequency of interferon (IFN)-γ-producing CD8+ T cells upon ex vivo stimulation by $GP100_{25-33}$ were significantly increased compared to control groups. Tumor antigen, $GP100_{25-23}$ specific immune response was also confirmed by ELISpot and GP100-tetramer assays. This approach is simple, easy-handled, and efficiently delivering antigens to lymphoid tissues. Our study offers an opportunity for clinically translating this approach into tumor immunotherapy.



## 1. Introduction

Melanoma is a malignant tumor derived from the melanocytes, which is highly resistant to many chemotherapeutic regimens and has quite poor prognosis [1]. Immunotherapy may be one of the most important modalities for treating melanoma, which has been a focus for decades. However, only modest improvement was obtained [2,3] in the survival of melanoma patients until recent immune checkpoint blockade was exploited [4–8]. However, for further improving the anti-melanoma immunity, the combination with melanoma tumor antigen vaccine may be needed.

In clinical trials, vaccines have been associated with minimal toxicity while inducing new immune responses or enhancing existing weak immune responses against specific melanoma-associated antigens (MAA) [9]. Such vaccine approaches are expected to be additive or synergistic with, rather than replace existing and emerging immunotherapies. There are numerous vaccine strategies for immunotherapy, which obtained favorable efficacy. It appears that various adjuvants [10–12] and cytokine biological response modifiers [9,13] can enhance antigen-specific immune responses to MAA. The MHC–I binding peptide $GP100_{25-33}$(H-$2B^d$ binding peptide) and $TRP2_{180-188}$ (H-$2K^b$ binding peptide), as melanoma associated antigens, have been extensively studied and are considered to function as immune dominant epitopes recognized by cytotoxic T lymphocytes (CTLs) [14,15]. As a consequence of antigen recognition, CD8 CTLs exert antitumor function via the perforin-granzyme cytolytic pathway or through cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) [16].

In the present study, we aim at designing a peptide

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immunization strategy that can expand antigen-specific CD8+ T cells to mount potent T cell responses against tumors. Recently, we developed a novel approach for inducing antigen-specific immune responses. This approach is to couple protein antigens to murine spleen cells through a crosslinker, sulfosuccinimidyl-4-[N-maleimidomethyl] cyclohexane-1-carboxylate (sulfo-SMCC) or SMCC, and induces antigen-specific immune response by infusion of these antigen-coupled spleen cells. In our previous study, we found that the treatment of ultraviolet-irradiated $MOG_{35-55}$-coupled apoptotic spleen cells significantly prevented experimental autoimmune encephalomyelitis (EAE), or ameliorated ongoing EAE at early or established stage, which was associated with induction of $MOG_{35-55}$-specific Foxp3+ Tregs and suppression of $MOG_{35-55}$-specific Th17 and/or Th1 cells [17]. In the current study, we seek to address the effect of infusion of sulfo-SMCC-mediated MAA, $GP100_{25-33}$-coupled spleen cells on mounting antigen-specific immune response against melanoma tumors. The results are reported herein.

## 2. Materials and methods

### 2.1. Mice

Female C57BL/6 mice, 6–8 weeks old, were obtained from Charles River Animal facility (Beijing, China), and were housed under specific pathogen-free conditions in the animal facility of Capital Medical University, Beijing. Animal uses followed the guidelines of Capital Medical University and Chinese government for the Humane Care and welfare of laboratory animals. Our research protocols were approved by the Capital Medical University Animal Ethics Committee.

### 2.2. B16F1 cell lines and tumor inoculation

The B16F1 melanoma cell lines provided by the Basic Medical Research Institute of Chinese Medical Sciences were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) at 37 °C in a humidified atmosphere containing 5% $CO_2$. Subcutaneous melanomas were generated by subcutaneous injection of $5 \times 10^4$ B16F1 tumor cells in the right hind flank. Tumor volumes were measured every 2–3 days and calculated as: $V = (\text{length} \times \text{width}^2) \times 0.5236$ [15,18], and mice were sacrificed when tumor volume reached 2000 $mm^3$.

### 2.3. Peptide, tetramer and reagents

Synthetic peptides representing the CD8+ T cell epitopes $TRP2_{180-188}$ (SVYDFFVWL; H-2Kb-restricted), $GP100_{25-33}$ (EGSRNQDWL; H-2Db-restricted) were synthesized by SBS Genetech (Beijing, China). The purity (>95%) and identity of peptides were determined by high-performance liquid chromatography and mass spectrometry analysis. H-2Db/GP100 tetramers were purchased from Medical & Biological Laboratories (MBL) Co., Ltd (Nagoya, Japan). Sulfo-SMCC was purchased from Thermo Scientific (Waltham, MA), diluted to 10 mM in phosphate buffer solution (PBS). The following fluorescent antibodies were used: CD4-PerCP (clone RM4-5, BD); CD8a-PE (clone 53-6.7, BD); CD8a-Alexa Fluor 647 (clone 53-6.7, BD); IFN-γ-APC (XMG1.2, BD); Isotype: Rat IgG2a, κ (clone R35–95, BD); Mouse IgG1, κ (clone MOPC-31C, BD). Carboxyfluorescein succinimidyl eater (CFSE) used for cell tracking and T cell proliferation assay was from Life Technology (Grand Island, NY).

### 2.4. Preparation of peptide-coupled spleen cells

Spleen cells were prepared from naïve female mice, the RBCs were lysed using ACK lysis buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2–7.4). The splenocytes ($1 \times 10^7$) were incubated with the mixture of sulfo-SMCC (0.272ug/ml) and $TRP2_{180-188}$ (15ug/ml) or $GP100_{25-33}$ (15ug/ml) for 1 h at room temperature. Thereafter, the cells were washed twice with PBS to eliminate remaining free sulfo-SMCC and possible contamination of soluble GP100 or TRP2, and the viability of coupled spleen cells was around 90% by trypan blue staining.

### 2.5. Melanoma prevention, treatment and adoptive transfer experiment

#### 2.5.1. Prevention experiment

Six weeks old female C57BL/6 mice were injected intravenously with $1 \times 10^7$ $GP100_{25-33}$-coupled spleen cells (S-SMCC-GP100) once a week for two weeks. Two weeks after the primary immunization, all mice were given melanoma inoculation as described above. Tumor growth and animal survival were monitored every other day and recorded.

#### 2.5.2. Treatment experiment

We performed melanoma treatment experiment at early stage in melanoma-bearing mice. Six weeks old female C57BL/6 naïve mice were given subcutaneous inoculation of $5 \times 10^4$ B16F1 tumor cells on day 0, and then all tumor bearing mice in different groups received three therapeutic treatments at day 3, 9 and 15. Tumor growth and animal survival were monitored every other day and recorded.

#### 2.5.3. Adoptive transfer

To study the effects of adoptive immunotherapy, naïve donor mice were immunized for two weeks by infusion of antigen-coupled spleen cells, then splenocytes were prepared from the donor mice, and transferred to naïve recipient mice ($1 \times 10^7$/mouse). Immediately after spleen cells transfer, all recipient mice were inoculated subcutaneously with B16F1 cells ($5 \times 10^4$/mouse). Tumor growth and animal survival were monitored every other day and recorded.

### 2.6. T cells proliferation in vitro and intracellular cytokine staining

Mice of all groups immunized for two weeks were sacrificed. Spleen cells were prepared as mentioned above, and then pre-labeled with CFSE following the instruction from the manufacturer (Invitrogen). CFSE-labeled spleen cells ($5 \times 10^5$/well) were incubated in a round-bottom 96-well plate with peptide $GP100_{25-33}$ (10ug/ml) or cell only at 37 °C in 5% $CO_2$ atmosphere. Triplicated wells were used for each condition. After four days, the cultured cells were harvested, and part of the cells was subjected to flow cytometric analysis for CD8+ T cell proliferation (the dilution of CFSE on CFSE-labeled CD8+ T cells). The rest were stimulated by leukocyte-activation cocktail (LAC, 1ul/ml, BD) before stained with IFN-γ, and the expression of intracellular cytokine was analyzed by a FACS Canto II flow cytometer (BD biosciences) and software Flowjo 7.6.1.

### 2.7. Evaluation of cellular immune responses

Immunological assays for antigen-specific CD8+ T cells were performed as described previously. Briefly, for tetramer staining, cells were stained with APC-CD8a and PE-conjugated tetramers following the staining protocol from the manufacturer (MBL) and