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AAV–sBTLA facilitates HSP70 vaccine-triggered prophylactic antitumor immunity against a murine melanoma pulmonary metastasis model *in vivo*

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ABSTRACT

Activation of the BTLA–HVEM co-inhibitory signaling pathway impairs antitumor immunity. Our previous study demonstrated that the extracellular domain of murine BTLA (the soluble form of BTLA) can facilitate HSP70 vaccine-triggered antitumor immunity by blocking BTLA–HVEM interactions in a murine TC-1 non-metastatic tumor model. However, it is unknown whether this strategy has beneficial effects on highly malignant metastatic tumors, such as melanoma. To address this question, we expressed the soluble form of BTLA (sBTLA) in combination with HSP70 vaccine and examined the resulting antitumor activity in a melanoma pulmonary metastasis model. A recombinant adeno-associated virus (AAV) vector was used for the sBTLA gene delivery because of its high transfection efficiency and low toxicity. *In vitro* expression of AAV–sBTLA enhanced lymphocyte activation and induced specific cytotoxicity against B16F1 murine melanoma cells, while *in vivo* administration of AAV–sBTLA plus HSP70 vaccine by tail vein injection exerted a limited, late-stage antitumor effect against the existing B16F1 cells. However, the combination treatment generated a potent prophylactic antitumor response in the melanoma lung metastasis model in B6 mice. In this case, most of the metastatic foci were inhibited, and mouse survival was prolonged. Furthermore, the Th1 cytokines IL-2 and IFN-γ were up-regulated, while the negative regulatory molecules IL-10 and TGF-β were down-regulated. The number of regulatory T cells also decreased in the tumor environment. Therefore, AAV–sBTLA plus HSP70 vaccine may have therapeutic potential for the prevention of metastatic melanoma.

Introduction

Metastatic melanoma is an aggressive form of cancer that is highly resistant to traditional forms of therapy, such as chemotherapy and radiotherapy. High recurrence and metastasis rates have been observed after traditional treatments [1,2]. Therefore, immunotherapy has been proposed as a potential treatment for melanoma and other invasive cancers [3–6]. Most melanoma metastases contain lymphocytic infiltrates, including T cells that recognize melanoma antigens and natural killer cells [7]. Manipulating the function of these infiltrating cells may be an effective strategy for metastatic melanoma therapy.

T cells receive antigens and positive or negative secondary signals from antigen-presenting cells (APCs). B and T lymphocyte attenuator (BTLA) is a recognized inhibitory receptor of the CD28 superfamily that is constitutively expressed by naive $CD4^+$ and $CD8^+$ T cells and is further up-regulated upon T cell activation [8,9]. Herpesvirus entry mediator (HVEM), the interaction partner of BTLA, is a member of the tumor necrosis factor receptor (TNFR) superfamily and is predominantly expressed by resting T cells, monocytes, immature dendritic cells and some types of tumor cells [10,11]. BTLA–HVEM signaling may be involved in tumor progression and resistance to the immune response [12,13].

Manipulation of co-inhibitory pathways can regulate antigen-specific T cell responses, and the utilization of antagonists, such as antibodies or extracellular domains targeting distinct T cell

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co-inhibitory molecules, provides a method for triggering an effective antitumor response [14–16]. Indeed, blocking BTLA–HVEM interactions may augment the immunogenicity of vaccines [17]. Moreover, BTLA-mediated inhibition of tumor-specific $CD8^+$ T cells can be partially reversed by vaccination [18]. Our previous study also demonstrated that blockade of the BTLA–HVEM inhibitory pathway potently enhances HSP70 vaccine-induced immune responses against an existing *in situ* tumor in a murine cervical cancer model [15]. Whether blockade of the BTLA–HVEM interaction will contribute to the inhibition of metastatic tumors such as melanoma is not known.

In this study, we sought to analyze whether blockade of this inhibitory pathway in combination with administration of HSP70 vaccine could be an effective therapeutic strategy to benefit immunotherapy for metastatic melanoma. We chose recombinant adeno-associated viral (AAV) vectors as the gene delivery approach because of its advantages of enabling direct long-term transgene expression without giving rise to host toxicity or serious cellular immune responses in various tissues. We found that AAV-mediated sBTLA gene transfer combined with HSP70 vaccine resulted in a better antitumor effect as a prophylactic vaccine than as a therapeutic vaccine against a murine melanoma pulmonary metastasis model *in vivo*. This strategy may represent a promising approach for preventing metastatic melanoma.

Materials and Methods

Animals and cell lines

Six- to eight-week-old female B6 mice were purchased from the Center of Experimental Animals of the Chinese Academy of Medical Science. The animals were maintained in our facilities under pathogen-free conditions. All of the studies involving mice were approved by the Tongji University Animal Care and Use Committee. The hypotriploid human cell line 293 (an AAV-packaging cell line) was purchased from the American Type Culture Collection (Rockville, MD). Chinese hamster ovary (CHO) cells and murine B16F1, B16F1-GFP, and TC-1 cells were purchased from the China Center for Type Culture Collection and were cultured in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 2 mM L-glutamine, 100 mg/ml penicillin, and 100 U/ml streptomycin. All of the cell culture reagents were obtained from Invitrogen Life Technologies.

Construction and generation of the recombinant virus AAV–sBTLA

The sequences of the primers are listed in Table 1. The extracellular domains of murine BTLA (sBTLA) gene fragments were obtained from the splenocytes of B6 mice by reverse transcription polymerase chain reaction (RT-PCR). The PCR products were digested with the restriction enzymes EcoR I and BamH I, then inserted into the compatible enzyme restriction sites of pAAV-IRES-hrGFP (Invitrogen, Carlsbad, CA). After identification by digestion, the positive clones were submitted for sequence confirmation to Shanghai Bioengineering Co. Ltd (China). The three plasmids (pAAV–IRES–sBTLA–hrGFP or the control plasmid pAAV–IRES–hrGFP, pAAV-RC and pHelper), which were extracted with a Plasmid Midi Kit (QIAGEN, Germany), were co-transfected in a molar ratio of 1:1:1 into AAV-293 cells using a calcium phosphate transfection kit (Beyotime, China). The transfection efficiency was determined by fluorescence microscopy after 48 h. The cells were collected after 72 h and purified by chloroform treatment, polyethylene glycol/sodium chloride precipitation, and chloroform extraction as previously described [19]. The purified virus titration was measured by quantitative real-time PCR as previously described [20]. Briefly, recombinant AAV-containing fractions were pre-treated with DNase I and proteinase K. Two microliters of the sample was then used for qPCR. To quantitate the DNA copy number in unknown rAAV samples, the PCR result of the unknown sample was compared to the reference standard curve calculated from the plasmid dilution series. The viral rAAV titer was calculated by multiplying the dilution factor (1:10) by 500 to obtain the titer per milliliter, assuming that one rAAV particle contained one DNA copy and that 1 pg DNA of the 6889 bp vector plasmid corresponded to 1.3×10^5 plasmid molecules.

Table 1
Primers for construction of sBTLA, and for real-time quantitative PCR.

Gene name	Primers
sBTLA (construction)	(+)GTGACGAATTCTGGGAATGAAGACAGTGCC
	(−)GTCAGGATCCATTAGGCATTGGTGGCATCTGHVEM
IL-2	(+)GGAGCAGCTGTTGATGGACCTAC
	(−)AATCCAGAACATGCCGCAGAG
IFN-γ	(+)CGGCACAGTCATTGAAAGCCTA
	(−)GTTGCTGATGGCCTGATTGTC
IL-10	(+)GACCAGCTGGACAACATACTGCTAA
	(−)GATAAGGCTTGGCAACCCAAGTAA
TGF-β1	(+)TTCCGCTGCTACTGCAAGTCA
	(−)GGGTAGCGATCGAGTGTCCA
β-actin	(+)TTCCAGCCTTCCTTCTTGGGTAT
	(−)GTTGGCATAGAGGTCTTTACGG

(+): Forward primers; (−): reverse primers.

Preparation of HSP70-B16F1 peptide complex (HSP70 vaccine)

B16F1 melanoma peptides were prepared as described previously [21]. Briefly, melanoma cells at a concentration of 5×10^7/ml were subjected to two freeze–thaw cycles, mixed with two volumes of distilled water, and centrifuged to remove the cell debris. The supernatant was incubated in boiling water for 10 min, followed by a 30 min incubation on ice, and then centrifuged to remove the denatured high-molecular-weight proteins. Finally, the pH value of the supernatant was adjusted to 7.0 with NaOH solution. Recombinant HSP70 was prepared from the engineered bacteria carrying pMSH70. The purity of the HSP70 proteins was >95%, as confirmed by silver-stained SDS-PAGE analysis. B16F1 peptide and HSP70 protein, at 75 μg/ml and 250 μg/ml, respectively, were mixed and incubated for 2 h at 37 °C in the presence of 1 mM ADP and 1 mM $MgCl_2$ to promote binding. The lipopolysaccharide (LPS) concentration in the HSP70 vaccine preparation was determined by the Limulus amebocyte lysate (LAL) assay and was <1.5 endotoxin units/μg.

Gene transfection in vitro

CHO cells were cultured in 24-well plates and transfected with AAV–sBTLA at $l \times 10^5$ MOI. The expression of green fluorescent protein (GFP) was observed under a fluorescence microscope 24 or 48 h after transfection. The expression of sBTLA was detected by western blot.

Conventional reverse transcription PCR and real-time PCR

Total RNAs were isolated from the cells or tumor marginal tissues of tumor-bearing mice using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was used to determine the relative quantities of mRNA (One Step RT-PCR kit, Qiagen, Valencia, CA). The sequences of the primers used for detecting the expression of various genes are listed in Table 1. The mRNA expression levels were normalized to β-actin and are expressed as the fold change relative to the control samples (calibrator).

Western blot analysis

Seventy-two hours after transfection, the proteins in the culture supernatants were detected by western blot, as described previously [14]. Briefly, cell lysates were denatured in 1× Laemmli sample buffer and resolved by SDS-PAGE. Proteins were blotted onto a PVDF membrane and probed with goat anti-mouse BTLA polyclonal antibody (R&D system) overnight. After 1 h incubation with secondary antibody, the protein bands were visualized with a chemiluminescence kit.

Isolation of tumor-infiltrating cells

Tumor-infiltrating lymphocytes (TILs) were isolated from the tumor tissues as previously described [15]. Briefly, the lung tissues were minced and digested in PBS containing 0.1% collagenase (Sigma-Aldrich), 0.01% hyaluronidase (Sigma-Aldrich), and 0.002% DNase I (Promega) for 90 min at 37 °C. The released cells were filtered through stainless steel mesh screens and washed twice with RPMI 1640 medium containing 5% FBS. The cells were separated on a Percoll (Pharmacia Biotech) density gradient by centrifugation for 30 min at $1500 \times g$ at room temperature. The dense layer containing enriched lymphocytes was collected and washed for additional experiments.

FACS analysis

The mouse splenocytes and TILs were washed with phosphate-buffered saline (PBS) and incubated with PE-labeled anti-mouse CD4 and FITC-labeled anti-mouse CD8 (eBioscience Inc., San Diego, CA). After washing with PBS, the cells were used for flow-cytometric analysis. Cells were acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with Cell Quest Software (BD Biosciences). In some of the experiments, TILs were also incubated with FITC-labeled anti-mouse CD4, APC-labeled anti-mouse CD25, PE-labeled anti-mouse Foxp3, FITC-labeled anti-mouse NK1.1, PE-labeled anti-mouse F4/80, PE-labeled anti-mouse CD69, CD178 (FasL), allophycocyanin-labeled anti-mouse CD314 (NKG2D), FITC-labeled anti-mouse IL-10, FITC-labeled IL-12 and FITC-labeled TNF-α (eBioscience Inc., San Diego,