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EWS/FLI-l peptide-pulsed dendritic cells induces the antitumor immunity in a murine Ewing's sarcoma cell model

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ABSTRACT

An increasing number of T-cell epitopes derived from various tumor-associated antigens have been reported, and they proved to play significant roles for tumor rejection both in vivo and in vitro. Over 85% of Ewing's sarcoma family of tumors (ESFTs) express tumor-specific chimeric protein EWS/FLI-1, making it an attractive target for therapeutic cytotoxic T-lymphocyte responses. Here, we identified a novel peptide epitope derived from the EWS/FLI-1 protein and demonstrated that effectors induced by the peptide could specifically secrete IFN-γ and lyse the tumor cell line of EWS/FLI-1-positive and HLA-matched cells. In addition, mice treated with dendritic cells pulsed with the EWS/FLI-1 epitope were able to reject a lethal tumor inoculation of the Ewing's sarcoma A673 cells. Therefore, these data provide evidence for the use of the EWS/FLI-l peptide epitope in T cell-based immunotherapeutic concepts against Ewing's sarcoma cell in vitro and in vivo.

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1. Introduction

Ewing's sarcoma is the second most common malignant bone tumor among children and adolescents and constitutes approximately 1% of all childhood tumors [1–3]. Despite the improvements in adjuvant chemotherapy, radiotherapy, and surgery, 30% to 40% of patients with Ewing's sarcoma experience disease recurrence with only a 23% 5-year overall survival rate [4–6]. The high percentage of relapse, metastasis, and poor prognosis in patients with Ewing's sarcoma led to renewed interest in alternative immunotherapy [7–9].

In recent years, immunotherapy of human tumors has gained much impetus by the finding that CTLs are capable of recognizing and destroying tumor cells that expose peptides that are derived from TAAs and are bound to MHC class I molecules [10–12]. The number of well characterized TAAs has steadily increased during the last years and has led to their classification into different groups [13–15].

Over 85% of Ewing's sarcoma family of tumors (ESFTs) express the tumor-specific chimeric protein EWS/FLI-1 encoded by hybrid genes generated by translocations involving the EWS and FLI-1 genes, and EWS/FLI-1 is essential for the genesis and development of Ewing's sarcoma [16–18]. Therefore, EWS/FLI-1 meets the requirements for an ideal target for Ewing's sarcoma therapy.

In the present study, we investigated whether CD8 + T lymphocytes could be specifically activated against EWS/FLI-1. Here, we demonstrated that monocyte-derived DCs pulsed with a novel predicted EWS/FLI-1-derived peptide proved to elicit a peptide-specific CTL response.

2. Materials and methods

2.1. Cell lines and mice

The human TAP-deficient T2 cell line, BB7.2 cell line producing mAb against HLA-A2 and Ewing's sarcoma A673 cell line were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained in DMEM containing 10% FCS, penicillin (100 U/ml), and streptomycin (100 pg/ml), and were kept at 37 °C in a humidified atmosphere containing 5% CO_2. Male nonobese diabetes/severe combined immunodeficiency (NOD/SCID) and BALB/c (H-2d) mice, 6–10 weeks old at the onset of experiments, were purchased from the Institute of Animal of Beijing Medical University (Beijing, China). Mice were bred and maintained in specific pathogen-free (SPF) facilities. Experiments were conducted in accordance with animal care guidelines approved by the Animal Ethics Committee of the Beijing Military Medical University. And the Institutional Animal Care and Use Committee (IACUC) or ethics committee specifically approved this study.

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2.2. Epitope prediction and synthesis

The EWS/FLI-1 sequence was scanned for HLA-A*0201-binding peptides using the prediction software BIMAS (Section of BioInformatics and Molecular Analysis, National Institutes of Health, Bethesda, MD, USA) and SYFPEITHI (Institute for Immunology, University of Tübingen, Tübingen, Germany) [19,20]. The three 9-mer-peptides that showed the highest scores with both SYFPEITHI and BIMAS were selected for additional evaluation. Peptides were synthesized on a solid-phase simultaneous multiple peptide synthesizer based on the Fmoc strategy. Synthesis products were purified with C18 reverse-phase HPLC and yielded a purity of >95%. Lyophilized peptides were dissolved in DMSO at a concentration of 2 mg/ml and stored at −20 °C.

2.3. Peptide-binding assay

To determine whether the candidate epitopes can bind to HLA-A*0201 molecules, up-regulation of peptide-induced HLA-A*0201 molecules on T2 cells was examined. Briefly, 1×10^6 T2 cells were incubated with 50 μM of the synthesized peptides and 3 μg/ml of human β2-microglobulin (Serotec, Oxford, UK) in serum-free RPMI 1640 medium for 16 h at 37 °C. Expression of HLA-A*0201 on T2 cells was then determined with the FACS Calibur flow cytometer (Becton Dickinson, Franklin lakes, New Jersey, USA), by staining with primary anti-HLA-A2 Ab derived from BB7.2 and FITC-labeled goat-antimouse IgG (BD Biosciences Pharmingen, San Diego, California, USA) secondary antibody. The data were analyzed using CellQuest software (Becton Dickinson, Franklin Lakes, New Jersey, USA). The Fluorescence index (FI) was calculated as follows: FI = (mean FITC fluorescence with the given peptide − mean FITC fluorescence without peptide) ⁄ (mean FITC fluorescence without peptide). Samples were measured in triplicate and then mean FI was calculated. HLA-A2.1-restricted MAGE-2 CTL epitopes KMVELVHFL (amino acid position in MAGE-2; 112–120) and ILLEPVHGV derived from HIV virus (amino acid position in HIVpol; 476–484), served as positive and negative controls, respectively.

2.4. Dendritic cell generation

Briefly, PBMCs were isolated from healthy HLA-A2+ donors by Ficoll-Hypaque density gradient centrifugation and then seeded into culture flasks in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. After monocytes adhered (incubation for 2 h), the nonadherent cells were collected and frozen in freeze medium (60% RPMI-1640 and 30% FBS, and 10% DMSO) for later use in CTL assays. The adherent cells were cultured for 5 days in RPMI-1640 containing 1000 U/ml of GM-CSF (R&D Systems, Minneapolis, MN, USA) and IL-4 (R&D Systems, Minneapolis, MN, USA), and were cultured for an additional 2 days in the presence of 1000 U/ml of tumor necrosis factor α (R&D Systems, Minneapolis, MN, USA) to induce final maturation. After 7 days of culture, the mature DCs were harvested and analyzed for DC typical phenotypes by FACS analysis.

2.5. Induction of peptide-specific CTL

Briefly, DCs were loaded with different EWS/FLI-1 peptides at a final concentration of 100 μg/ml for 4 h and were then irradiated with 20 Gy, which prevented all outgrowths in the control cultures. Autologous T cells were restimulated every 7 days with the previously mentioned peptide-pulsed DCs to generate peptide-specific CTLs (DC:T cell ratio is 1:5). Recombinant interleukin 2 (IL-2) at a concentration of 20 U/ml was added to the culture medium on day 3 after every stimulation. Cytotoxic T lymphocyte activity was then assessed on day 23 by a 4-hour ^{51}Cr release assay. Effectors generated from negative peptide-pulsed DCs were used as controls.

2.6. Cytotoxicity assay

To evaluate levels of CTL activity, a standard 4-h ^{51}Cr-release assay was used. A673 cells (HLA-A2+) were used as target cells. Briefly, target cells were incubated with ^{51}Cr (Beijing, China) (100 μCi per 1×10^6 cells) for 2 h in a 37 °C water bath. After incubation with ^{51}Cr, target cells were washed three times with PBS, resuspended in RPMI-1640 medium, and mixed with effector cells at a 25:1, 50:1 or 100:1 effector to target (E/T) ratio. Assays were performed in triplicate for each sample at each ratio in a 96-well round-bottomed plate. After a 4-hour incubation, the supernatants were harvested, and the amount of released ^{51}Cr was measured with a gamma counter. The percent specific lysis was calculated according to the following formula: Specific lysis = (experimental release − spontaneous release) ⁄ (maximal release − spontaneous release) × 100%.

2.7. Enzyme-linked immunospot assay

IFN-γ secretion of effectors was assayed by enzyme-linked immunospot (ELISPOT). Multiscreen 96-well assay plates (Dakewe, Shenzhen, China) were precoated overnight at 4 °C with anti-IFN-γ antibody according to the manufacturer's instruction. After washing with PBST (PBS-0.05% Tween 20), plates were blocked for 1 h at 37 °C with PBS/1% BSA. Splenocytes were plated in triplicate wells at a density of 1×10^5/100 μl in RPMI-1640 medium. For restimulation, different peptides or medium alone was added. Plates were cultured overnight, washed extensively with PBST, and incubated with anti-IFN-γ mAb for 1 h at 37 °C. After washing, goat antibiotin antibodies (Dakewe, Shenzhen, China) were added, and the plates were incubated for 1 h at 37 °C. Thirty microliters of activator solution (Dakewe, Shenzhen, China) was added to develop spots, and after 10 to 30 min, the plates were washed with distilled water to stop the reaction. After being air-dried, the number of spots in each well was counted using the Bioreader 4000 PRO-X (Bio-Sys; Bad Nenndorf, Germany).

2.8. Preparation of Trimera mice

During the course of experiments, the mice were kept in pathogen free animal facilities with controlled temperature and humidity, under a 12-h light/dark cycle, and with food and water containing cyprofloxacin (20 μg/ml). All animals were acclimated for at least 1 week before the experiments. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. Recipient BALB/c mice received a lethal dose of total body irradiation (i.e., day 0, 3.5 Gy and day 3, 9.5 Gy). On days 4–6, 3×10^6 mixed bone marrow cells (in 0.2 ml PBS) from NOD/SCID mice were transferred into each irradiated recipient by i.v. injection. One day after bone marrow infusion, each recipient mouse was injected (i.p.) with 2×10^8 human PBMCs (HLA-A2). All mice were kept under specific pathogen-free conditions, fed with sterile food and acid water containing cyprofloxacin (20 μg/ml).

2.9. Immunization and tumor cell challenge

Trimera mice were immunized s.c. in the base of the tail with 1×10^6 peptide loaded DCs in 100 μl PBS for three times. Control mice received the same volume of PBS. And 3 days before the first immunization, Trimera mice were challenged with subcutaneous (s.c.) injection of 1×10^6 A673 cells into the left flank to induce a primary tumor model. The tumor volume and mean lifespan of Trimera mice were observed. Tumor volume was measured in two dimensions and calculated as follows: length ⁄ 2 × width^2.

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