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A novel system of artificial antigen-presenting cells efficiently stimulates Flu peptide-specific cytotoxic T cells *in vitro*

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ABSTRACT

Therapeutic numbers of antigen-specific cytotoxic T lymphocytes (CTLs) are key effectors in successful adoptive immunotherapy. However, efficient and reproducible methods to meet the qualification remain poor. To address this issue, we designed the artificial antigen-presenting cell (aAPC) system based on poly(lactic-co-glycolic acid) (PLGA). A modified emulsion method was used for the preparation of PLGA particles encapsulating interleukin-2 (IL-2). Biotinylated molecular ligands for recognition and co-stimulation of T cells were attached to the particle surface through the binding of avidin–biotin. These formed the aAPC system. The function of aAPCs in the proliferation of specific CTLs against human Flu antigen was detected by enzyme-linked immunospot assay (ELISPOT) and MTT staining methods. Finally, we successfully prepared this suitable aAPC system. The results show that IL-2 is released from aAPCs in a sustained manner over 30 days. This dramatically improves the stimulatory capacity of this system as compared to the effect of exogenous addition of cytokine. In addition, our aAPCs promote the proliferation of Flu antigen-specific CTLs more effectively than the autologous cellular APCs. Here, this aAPC platform is proved to be suitable for expansion of human antigen-specific T cells.

1. Introduction

Adoptive immunotherapy has been confirmed to be effective in patients with certain types of infection [1], autoimmune disease [2] or cancer [3,4]. The effectiveness depends on the *in vitro* stimulation and proliferation of antigen-specific cytotoxic T lymphocytes (CTLs) and their reinfusion into patients [5].

T cells responses are mediated by recognition signals (peptide-MHC complexes) and co-stimulatory signals (anti-CD28 complexes) from antigen-presenting cells (APCs) [6]. In addition, cytokine (special interleukin-2) signals secreted by activated APCs and other immune cells can impact expansion, survival, effectors function and memory of stimulated T cells [7]. Generally, the generation of APCs includes both cellular technologies and acellular systems. Many data have shown that effective anti-tumor immune responses can be successfully induced by using cellular technologies, notably dendritic cell-based vaccines [8,9]. However, in addition to being expensive, time-consuming and often inefficient for patients [10], these cellular methods may carry the risk of infection or tumorigenicity [11], thereby limiting the general application of this therapy. One alternative to the cellular APCs is acellular systems based on exosomes [12], magnetic beads [13,14], or artificial liposomes [15,16]. Acellular antigen-presenting cells are ready-to-use, applicable to any patient, and stable for storage and carry a low risk of infection. However, these systems lack biocompatibility and cytokine signals for the robust stimulation of T cells. Usually, they need a large dose of exogenous cytokines, which could lead to toxicity in patients [17]. In 2008, a biodegradable artificial antigen-presenting cell (aAPC) system described by Steenblock [18] was shown to be effective for T cells expansion in animals. However, there are still no ideal aAPC technologies in humans that could provide all signals in a format suitable for the safe and effective adoptive immunotherapy.

Based on this fact, we designed an aAPC system using poly (lactic-co-glycolic acid) (PLGA) to target the Flu antigen (Flu p58–66, GILGFVFTL) of humans. PLGA is a polymer that has been approved by the FDA. Due to its characteristics of biocompatibility, controlled release capability and non-toxic degradation products, PLGA can be prepared as microspheres or scaffolds for persistent drug delivery or bone tissue biomaterials [19]. In our study, PLGA particles were fabricated using the emulsion method.

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Interleukin-2 (IL-2) was encapsulated and avidin–palmitic acid conjugates were incorporated on the surface. Through the natural binding of biotin and avidin, biotinylated double signals (MHC-Ig and anti-CD28) were coupled with the particles, forming an aAPCs platform. During the degradation of PLGA particles, the sustained release of IL-2 did not require the traditional approach of adding exogenous cytokine and led to much more T cells expansion. By comparing the action of aAPCs with cellular APCs on the stimulation of T cells, we demonstrate the potential usage of this aAPCs in the proliferation and activation of CTLs against a specific antigen.

2. Materials and methods

2.1. Materials

PLGA, a 50:50 poly(lactide-co-glycolide) copolymer with an inherent viscosity of 0.55–0.75 dL/g and a molecular weight of 8000 was supplied from Birmingham Polymers Inc. Recombinant human IL-2 was purchased from Peprotec Corporation. HLA-A2:Ig dimers and biotinylated CD28-specific antibodies were purchased from BD Biosciences Pharmingen. Palmitic acid-N-hydroxysuccinimide ester (NHS-palmitate), avidin (affinity purified) from egg white, deoxycholate (DOC), PE-labeled biotin (240 kDa) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) were all obtained from Sigma–Aldrich Co.

2.2. Cell line

T_2 cells were kindly provided by associate Prof. Xuewen Pang (Department of Immunology, Peking University Health Science Center, China).

2.3. Preparation of avidin–palmitic acid conjugates

The avidin–palmitic acid conjugates were performed as described by Huang et al. [20] with slight modifications. Briefly, 10 mg/mL of avidin was added to 10-fold (molecular weight) excess of NHS-palmitate in PBS containing 2% DOC buffer. The mixture was gently shaken and incubated at 37 °C for 12 h, then PBS was added until the solution contained 0.5% DOC. The mixture was dialyzed against PBS with 0.15% deoxycholate at room temperature for 2 days to separate it from the excess NHS-palmitate and hydrolyzed palmitic acid.

2.4. Fabrication and surface modification of PLGA microspheres

We used a double emulsion water-in-oil-in-water (w/o/w) technique for producing PLGA microspheres. Through multiple attempts, we optimized the correct conditions for preparation. First, 80 mg of PLGA was dissolved in 5 mL methylene chloride and acetone (4:1, v/v). Then, 100 μL of an aqueous solution containing 1 mg/mL IL-2 (for loaded IL-2 PLGA) or 100 μL PBS (for unloaded PLGA) was added dropwise to the PLGA solution. The mixture was sonicated for 60 s with a sonicator using a microtip probe with an output of 4% duty. This primary emulsion (w/o) was rapidly added to 50 mL deionized water with 4% (w/v) PVA containing avidin–palmitic acid (20 mg avidin). The secondary emulsion was formed by homogenization for 4 h at 5000 rpm on a magnetic stirring apparatus at room temperature to evaporate methylene chloride and acetone. The resultant water-in-oil-in-water (w/o/w) emulsion was centrifuged at 4 °C for 5 min, washed three times with distilled water, and lyophilized.

The microspheres were characterized by scanning electron microscopy (SEM) and optical microscopy. Images from the optical microscope were analyzed with Image Pro Plus software to determine the size distribution of particle diameters by counting at least 150 particles per sample. Scanning electron micrographs were obtained using a Hitachi S-4800 microscope at 5 kV.

2.5. IL-2 loading and controlled release kinetics studies

For determination of IL-2 loading, 10 mg of dried PLGA microspheres was dissolved in 1 mL of 0.1 M NaOH containing 0.5% SDS (w/v). After shaking incubation at 37 °C for 24 h, the mixture was centrifuged at 3500 rpm for 5 min, and the supernatant was removed for IL-2 Micro BCA assay analysis [21].

For controlled release of encapsulated IL-2, 10 mg of dried microspheres was suspended in 1.5 mL round-bottomed tubes containing 1 mL of PBS at pH 7.4. Sample tubes were incubated at 37 °C with continuous agitation on a rotator at 50 rpm to ensure microsphere dispersion. At the indicated time points, samples were centrifuged for 5 min at 11,000*g*, and 1 mL of the supernatant from each sample was removed for assessment. The tubes were replenished with fresh buffer, pre-incubated, and vortexed to resuspend the particles at 37 °C. Micro BCA assays were performed to estimate the amount of IL-2 released from microspheres. All dilutions were made in PBS to within the linear range of the standard curve. On the 24th day of incubation, SEM images of many particles were obtained.

2.6. Detection of surface conjugate on PLGA microspheres

The resultant avidin–palmitate–PLGA conjugate was verified by PE (the red fluorescent protein)-labeled biotin through the natural binding of biotin and avidin. Briefly, 10 mg PLGA particles with or without surface conjugate were washed twice in PBS. The particles were incubated with PE-labeled biotin in PBS on a rotary shaker for 30 min, then centrifuged and washed twice with DI water. Fluorescence microscope was used to detect this binding.

2.7. Peptide

The Flu peptide p58–66: GILGFVFTL used in this study was synthesized by Seajet Scientific Inc. (Bejing, China). It is a short-chain synthetic antigenic peptide derived from the influenza virus A matrix protein and presented by HLA-A2 major histocompatibility complex class I molecules. The modified Flu peptide has been previously shown to have increased binding affinity for HLA-$A2^+$ [22]. The purity (>98%) of the peptide was confirmed by high pressure liquid chromatography and mass-spectral analysis.

For peptide loading, the method used at BD Biosciences Pharmingen involves passive loading of excess peptides in solution with HLA-A2:Ig protein. Peptides of interest were prepared in dimethylsulfoxide (DMSO) and diluted at 2 mg/mL in sterile PBS, pH 7.2. HLA-A2:Ig was added and incubated at 37 °C overnight. The peptide-loaded HLA-A2:Ig can be stored at 4 °C for up to 1 week.

2.8. Generation of aAPCs

The aAPCs were prepared by coupling biotinylated peptide-loaded HLA-A2:Ig dimers and anti-CD28 human monoclonal antibody onto surface-modified PLGA microspheres that were loaded or unloaded with IL-2. Biotinylation of peptide-loaded HLA-A2:Ig was completed by Uscnlife Science & Technology Company. The PLGA particles at 10 mg/mL in PBS were incubated with a one-to-one (molar ratio) mixture of biotinylated peptide-loaded HLA-A2:Ig dimers and anti-human CD28 at 10 μg/mL in borate buffer at room temperature for 30 min. Particles were washed twice with PBS including 1% fetal bovine serum and resuspended in complete RPMI-1640 supplemented with 10% fetal bovine serum.