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Enhancement of fibroblast activation protein α-based vaccines and adenovirus boost immunity by cyclophosphamide through inhibiting IL-10 expression in 4T1 tumor bearing mice

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ABSTRACT

Fibroblast activation protein α (FAPα) is expressed in cancer-associated fibroblasts (CAFs) of more than 90% of malignant epithelia carcinomas. CAFs are the main type of cells in the tumor microenvironment which offer nutrition and protection to the tumor and regulate immunosuppression. To eliminate CAFs, a vaccine targeting FAPα may be used with a heterologous prime-boost strategy to enhance the FAPα-specific cellular immunity. Here, a FAP vaccine using a recombinant adenovirus (rAd) vector was constructed as well as a DNA vaccine reported in our previous work. Although the DNA prime-rAd boost strategy enhanced FAPα-specific immune responses, improvement of anti-tumor immunity effects was not observed. Examination of immunosuppressive factors revealed that high expression of the IL-10 cytokine was considered the main cause of the failure of the prime-boost strategy. However, heterologous vaccination in combination with a low-dose of cyclophosphamide (CY), which was reported to reduce IL-10 production and promote a shift from immunosuppression to immunopotentiation, resulted in enhanced effects in terms of numbers of effector T cells and tumor growth inhibition rates, compared to the CY alone or DNA alone group. Tumor growth was inhibited markedly when the prime-boost strategy was combined with CY in both the prophylactic and therapeutic settings and the survival time of 4T1 tumor bearing mice was also prolonged significantly. With the reduction of IL-10, enhancement of the anti-tumor effect by the prime-boost strategy was observed. These results suggest that FAPα-targeted rAd boosting in combination with CY is an attractive approach to overcoming immunosuppression in cancer vaccines.

1. Introduction

Cancer associated fibroblasts (CAFs), as major components of cancer stromal cells surrounding the outer layer of the tumor tissue, protect tumors and inhibit the entry of anti-tumor drugs and immune cells and factors into tumor tissues [1–3]. CAFs are different from normal fibroblast tissues and can produce stromal cell-derived factor-1(SDF-1), collagen I and other growth factors, cytokines and chemokines to change the extracellular matrix (ECM) to benefit tumor cells [3,4]. Fibroblast activation protein α (FAPα) is a type II membrane-bound serine protease overexpressed on CAFs contributing to ECM remodeling in the tumor microenvironment [5,6]. FAPα as a microenvironment antigen has greater advantages compared with other tumor-associated antigens (TAAs) for immunotherapy [7,8], because it meets two important conditions which are necessary for a successful vaccine. FAPα has been reported to induce high numbers of antigen-specific T cells [9]. Moreover, FAPα can facilitate the entry of T effector cells into the tumor by overcoming the immunosuppression and barriers of the tumor microenvironment [10,11]. Therefore, FAPα is a promising antigen for use in vaccine design.

Currently, DNA vectors are used as ideal vaccines to induce primary immunity [12], and such vaccines including those expressing

Abbreviations: Ad, adenovirus; CAFs, cancer-associated fibroblasts; CpVR-FAP, a recombinant plasmid encoding human FAPα; CY, cyclophosphamide; ECM, extracellular matrix; FAPα, fibroblast activation protein α; HGF, hematopoietic growth factor; qRT-PCR, quantitative real time polymerase chain reaction; rAd, recombinant adenovirus; SDF-1, stromal cell-derived factor-1; Th1, T helper type 1; Tregs, regulatory T cells; VEGFα, vascular endothelial growth factor alpha.

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the antigen FAPα have shown anti-tumor immune effects *in vivo* [10,13–15]. However, the capacity for tumor protection of DNA vaccines is limited. In our previous work, we constructed a DNA vaccine expressing human FAPα which achieved limited anti-tumor effects in the 4T1 tumor model. At present, the recombinant adenovirus (rAd) vector is widely used in clinical trials and gene immunotherapy due to its efficient ability to express exogenous antigens [16], and it can produce robust immune responses by inducing various types of cytokines [17]. To induce better immune responses, a DNA prime-rAd boost strategy is routinely regarded as an effective strategy for vaccine immunotherapy [18].

The tumor microenvironment is the main obstacle to successful immunotherapy, which is often attenuated by immunosuppressive cells and cytokines such as regulatory T cells (Tregs) and interleukin-10 (IL-10) [19,20]. Tregs which express the fork head transcription factor Foxp3 have an immunosuppressive function. IL-10 was initially considered a major immune suppressive factor which is responsible for induction of tolerance through inhibition of T helper type 1 (Th1) immune response and T-cell cytotoxic activity. Specifically, IL-10 was demonstrated to impair the proliferation, cytokine production and migratory capacities of effector T cells which would greatly influence tumor immunotherapy [19].

Therefore, researching methods to block Tregs and IL-10 is worthwhile. Cycyclophosphamide (CY) is a DNA alkylating agent, which is widely used with other chemotherapy drugs in the treatment of various types of cancer [21]. It has been used to modulate immunosuppression in a clinical study based on its functions of inhibiting Tregs and lowering the expression of IL-10 [22]. CY can serve not only as a chemotherapy agent but also to enhance immune responses through blockade of Tregs and IL-10 [23–25]. Thus, CY was chosen in this study to test whether it could control the immunosuppression and enhance the efficacy of our tumor vaccines.

Combining cancer immunotherapies and immunomodulatory agents is a recent trend. Here, a DNA prime-rAd boost strategy was first investigated for its immunogenicity and anti-tumor activities, but it failed to effectively inhibit tumor growth. As the high expression of IL-10 was found in tumors of vaccinated mice, we utilized CY to reduce the immunosuppressive effects of this cytokine. Ultimately, a synergistic effect and improvements of anti-tumor immune responses in the DNA prime-rAd boost group were observed in both prophylactic and therapeutic models in 4T1-bearing mice. More CAFs were targeted and killed with the decrease of FAPα and SDF-1, a stronger immune response was observed because of the decrease of IL-10 expression and immunosuppression by using CY, thus the capacity and superiority of rAd were released.

2. Material and methods

2.1. Construction and preparation of Ad-FAP

The rAd vector expressing full-length human FAP was obtained using the AdMax™ Adenovirus Vector Creation System (Microbix Biosystems). The human FAP fragment was subcloned into the *Bgl* II/*Sal* I sites in the pDC316 shuttle vector. After recombination using pDC316-FAP in HEK293 cells, Ad-FAP was screened and amplified for purification. After verification by PCR and Western blot, Ad-FAP products were desalted and stored at −80 °C in PBS containing 10% glycerol (v/v). Finally, the Reed and Muench method was used to determine the Ad-FAP viral stock titer, which was expressed as the 50% tissue culture infectious dose (TCID50).

2.2. Mice and murine 4T1 tumor model

Female BALB/c mice (6–8 weeks) were purchased from Beijing Huafukang Biology Technology Co., Ltd. The animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Murine 4T1 cells were preserved by the National Engineering Laboratory for AIDS Vaccine, Jilin University. The optimal number of 4T1 cells used for subcutaneous challenge in mice was determined to be 2×10^4 in tumor protection assays, which was verified in past studies. Tumor growth was monitored and measured every two days with a caliper, and the tumor volume was estimated by the formula: (length × width^2)/2 (mm^3).

2.3. Immunization with plasmid DNA, Ad-FAP and CY in BALB/c mice

One hundred micrograms of plasmid or 1×10^8 plaque-forming units (pfu) of Ad-FAP were immunized three times by intramuscular injection into the tibialis anterior muscles of both hind limbs (50 μg each limb) at two-week intervals. CY was injected at 50 mg/kg three times by intraperitoneal administration for each mouse in groups which received CY on day 3, 10 and 17 after tumor challenge according to the experimental schedule. In the therapeutic setting, CY was administered one day before immunization on day 1, 8 and 15 after tumor challenge, related vaccines were immunized one day after CY administration for three times. PBS was administered at 100 μl for each mouse by intramuscular injection as a control.

2.4. Cytotoxicity assay and ELISPOT assay

The procedures for the cytotoxicity assay and ELISPOT assay were previously published [18,26]. In CTL assay, P815 cells (1×10^6 cells/ml) used as target cells were pulsed with or without 5 μg/ml of the MHC-I H-2Db restricted FAP mixed peptides predicted through SYFPEITHI algorithm and PAProC algorithm [27,28]: F105-113 (LSPDRQFVY), F275-283 (VGPQEVPVP) and F542-550 (GGPCSQSVR) in RPMI-1640 medium containing 10%, fetal bovine serum (FBS) for 2 h at 37 °C. Meanwhile, unrelated peptides (AYACNTSTL(80-88), TLPPAWQPF(5-13), DLAQCFFCF(53-61), derived from another tumor-associated antigen survivin) were used as control. Peptide-loaded P815 cells were then labeled with 5, 6-carboxyfluorescein succinimidylester (CFSE) fluorescent dye (5 μM) in RPMI-1640 medium without FBS for 10 min, while unloaded P815 cells were labeled only with CFSE (0.5 μM). CFSE labeling was stopped by the addition of an equal volume of cold FBS for 3 min, and then the cells were washed and counted. CFSE high- and CFSE low-labeled cells were mixed together at a 1:1 ratio and confirmed by flow cytometry. Different numbers of splenocytes from vaccinated mice were then incubated with 5×10^4 of the peptide-loaded or unloaded P815 cells for 8 h at 37 °C, after which the co-cultures were analyzed on a FACS MoFloXDP (Beckman Coulter) for the percentage of CFSE-labeled P815 cells. Specific killing was calculated as follows: % killing = [1 − (peptide-loaded cells/unloaded cells from immunized group)/(peptide-loaded cells/unloaded cells from naive group)] × 100.

In ELISPOT assay, Interferon-γ ELISPOT assays were carried out according to the manufacturer's recommended protocol (BD Biosciences, Franklin Lakes, NJ, USA) to detect and enumerate IFN-γ secreting T cells. Briefly, 96-well filter plates were coated with 5 μg/ml of the purified anti-mouse IFN-γ mAb. After overnight incubation at 4 °C, the wells were washed and blocked for 2 h at room temperature with RPMI-1640 containing 10% FBS. Afterwards, splenocytes were added to the wells (1×10^6 cells/well) with or without stimulator (final concentration: 5 μg/ml). The FAP peptides was chosen to be the stimulator, while the control stimulant was RPMI 1640 medium containing 10% FBS (R10). Each condition was performed in duplicate. Cells were then incubated for at least 24 h at 37 °C, and plates were washed with deionized water and PBS containing 0.05% Tween-20. Plates were then

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