Vaccine 28 (2010) 7837–7843

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Regulation of the activity of an adeno-associated virus vector cancer vaccine administered with synthetic Toll-like receptor agonists

Pierre L. Triozzi[a,*], Wayne Aldrich[a], Selvarangan Ponnazhagan[b]

[a] Taussig Cancer Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA
[b] Department of Pathology and the Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA

ARTICLE INFO

Article history:
Received 18 May 2010
Received in revised form
20 September 2010
Accepted 26 September 2010
Available online 27 October 2010

Keywords:
Melanoma
Trp2
Dendritic cells
Myeloid suppressor cells
Regulatory T cells

ABSTRACT

Recombinant adeno-associated virus (rAAV) is being tested as a vaccine vector, but the cellular immune responses elicited in animal tumor models have not been completely protective. The adjuvant effects of the TLR7 agonist, imiquimod, and the TLR9 agonist, ODN1826, were tested with rAAV expressing the melanoma antigen, Trp2. Mice immunized with rAAV-TRP2 and either TLR agonist alone generated T-helper-1 antitumor immune responses. Antitumor activity in all experiments was still incomplete. Furthermore, antitumor activity was not achieved when the combination of ODN1826 and imiquimod was used as adjuvant. *In vitro*, the combination increased IL-10 production by dendritic cells. *In vivo*, the combination reduced T-helper-1 response and dendritic cell activation and increased myeloid suppressor cells; regulatory T cells were not significantly modulated. Depletion of myeloid derived suppressor cells enhanced the antitumor activity of immunization with rAAV-TRP2 and the imiquimod-ODN1826 combination; depletion of regulatory T cells did not. TLR7 and TLR9 agonists can be used to enhance the immune response to rAAV immunogens, but antagonism can be observed when combined. Suppressor mechanisms, including those mediated by myeloid cells, may negatively regulate the antitumor immune response.

1. Introduction

Viral vectors have been attractive immunogens. Although recombinant adeno-associated virus (rAAV) had been reported to induce little or no immune response to encoded transgene products, several studies have now shown that rAAV vectors can be used to elicit biologically significant immune responses to microbial and other antigens. Many features make rAAV an attractive vector for vaccine approaches [1]. AAV is not known to cause disease in humans. In contrast to other viruses that are currently being tested as vaccine vectors, such as adenovirus and poxvirus, rAAV does not express any viral genes. This may lessen an anti-vector immune response that may confound the immune response to transgene products and may also allow for sustained and higher levels of expression, which may be important in eliciting protective cellular immunity. Nonetheless, compared to other viruses, rAAV vaccines are weakly immunogenic. Cellular immune responses were infrequent in a clinical trial of a rAAV-based vaccine targeting human immunodeficiency virus [2].

rAAV-based immunotherapy has demonstrated activity in animal tumor models [3–5]. We have tested rAAV expressing the tumor-associated carcinoembryonic antigen (CEA) as immunogens. Although rAAV-CEA alone was inactive, a single intramuscular (i.m.) injection of rAAV-CEA in mice followed two weeks later, when CEA expression was optimal, with plasmids that did and did not express immunostimulatory cytokines, elicited CEA-specific T-helper-1 (Th1) associated immune response and partial protection against challenge with CEA-expressing tumors cells [6]. These effects were associated with an infiltration of dendritic cells (DC) *in situ*. Plasmids have been effective in eliciting Th1-associated immunity in part due to their expression of unmethylated CpG motifs found in bacterial but not mammalian DNA. Through interactions with Toll like receptor (TLR) 9, CpG oligodinucleotides (ODN) promote the induction of Th1-associated cytokines and support the maturation/activation of DC [7].

Synthetic TLR agonists are being tested clinically as vaccine adjuvants. Given the effects of plasmids with rAAV immunogens, we evaluated the effects of a synthetic TLR9 agonist, ODN1826, as well as the effects of imiquimod, a synthetic TLR7 agonist that also activates DC and promotes Th1 immune response [8]. The combined effects of these agonists were also tested, as additive and synergistic effects have been reported with TLR agonist combinations [9]. A variety of suppressor mechanisms have been identified that can limit antitumor immune responses, including those medi-

* Corresponding author at: Taussig Cancer Institute, R40, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195, USA. Tel.: +1 216 445 5141; fax: +1 216 636 2498.
E-mail address: triozzp@ccf.org (P.L. Triozzi).

0264-410X/$ – see front matter © 2010 Published by Elsevier Ltd.
doi:10.1016/j.vaccine.2010.09.086

ated by regulatory T cells (Treg cells) and myeloid cells, such as M2-polarized macrophages and myeloid derived suppressor cells (MDSC) [10]. How these influence the activity of rAAV vectored vaccines has not been reported. Trp2, a melanocyte differentiation antigen highly expressed in human and mouse melanoma, was targeted [11]. In contrast to CEA, an alloantigen in mice, an epitope shared by mouse and human Trp2 can be recognized by melanoma-reactive CTL in C57BL/6 mice as well as in humans [12,13]. Furthermore, immunization with human TRP2 plasmids alone, although capable of generating a Trp2-specific immune response, has not elicited significant antitumor activity in mouse B16 melanoma, which mimics observations made in cancer vaccine clinical trials [12]. Our results demonstrate the benefits and limitations of this antigen-specific tumor immunotherapy and support a role for myeloid suppressor cells in its regulation.

2. Materials and methods

2.1. Reagents

CpG ODN1826 was obtained from The Midland Certified Reagent Company, Inc. (Midland, TX) and imiquimod, from InvivoGen (San Diego, CA). A recombinant AAV-2 plasmid encoding mouse TRP2 was constructed by subcloning TRP2 cDNA (kindly provided by Dr. Vincent Hearing (National Institutes of Health, Bethesda, MD) within the AAV terminal repeats in plasmid pSub201 [14]. Expression of the TRP2 was verified by transfecting the AAV-CEA plasmid in 293 cells following which the plasmid was used to package rAAV-TRP2. Packaging of the rAAV-TRP2 into mature virions was done in an adenovirus-free system using AAV serotype 6 capsid helper plasmid as described [15]. Purification of the virions was done in a discontinuous iodixanol gradient centrifugation followed by affinity purification in heparin-agarose columns [16]. The particle titers of the purified virions were determined by quantitative slot blot analysis as described [15].

2.2. Mice and cell line

Mouse B16 melanoma cells (B16.F10) were maintained in Dulbecco's Modified Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Herndon, VA). The cultures were grown at 37 °C in 5% CO_2 to confluence, passaged by treatment with 0.05% trypsin in EDTA at 37 °C, and washed in media before being centrifuged at 200 × *g* for 10 min to form a pellet. Female C57BL/6 mice (age, 4 weeks) were obtained from Taconic Farms (Hudson, NY) and were fed with commercial diet and water *ad libitum*. The animal use and care protocol was approved by the Institutional Animal Use and Care Committee. All animal procedures were performed in accordance with recommendations for the proper care and use of laboratory animals.

2.3. Tumor model

rAAV-TRP2 immunizations were by a single i.m. injection of rAAV-TRP2 in 50 μl normal saline. ODN1826 and imiquimod were injected subcutaneously at the vaccination study in 0.1 ml PBS. Mice were challenged with 3×10^5 B16 cells subcutaneously in the flank. For depletion of Gr-1$^+$ MDSC, 200 μg of anti-Gr-1 monoclonal antibody (mAb) (RB6-8C5; BioExpress, Lebanon, NH) was injected i.p. [17]. For depletion of Treg cells, 0.5 mg of anti-CD25 mAb (PC61; American Type Culture Collection, Manassa, VA) was administrated i.p. [18]. Tumor size was measured at least twice every week with a digital caliper for two-dimensional longest axis (L) and shortest axis (W), and tumor volume calculated using the formula: volume in $mm^3 = (L \times W^2)/2$. When the tumor growth exceeded 2000 mm^3 or tumors became ulcerated, animals were euthanized. Following immunization, mice were also sacrificed, and tumor, lymph nodes, and spleens were dissected for immune response assessment.

2.4. DC generation and separation

Bone marrow cells from C57/BL6 mice were cultured for 7 days with 200 ng/ml Flt3L (PeproTech, Inc., Rocky Hill, NJ). CD11b$^+$ cells were removed by magnetic activated cell sorting (MACS; Mitenyi Biotec, Auburn, CA) and recultured in GM-CSF (PeproTech, Inc.) at 10 ng/ml to produce conventional DC (cDC). CD11b$^-$ cells were re-cultured in 100 ng/ml Flt3L to produce plasmacytoid DC (pDC). MACS (Mitenyi Biotec) was also used to separate CD11c$^+$ DC from lymph nodes.

2.5. Cytokine production

Enzyme linked immunoabsorbent assays (ELISA) were used to assess DC production of IL-10 (R&D Systems, Minneapolis, MN), IL-12p70 (R&D Systems) and IFN-α (IFN-αA, α1, α4, α5, α6, and α9; PBL Biomedical Laboratories, Piscataway, NJ). Single-cell suspensions of splenocytes were prepared by mincing and forcing mouse spleen tissue through a 100 μm sterile nylon strainer in PBS. Erythrocytes were removed by hypotonic lysis and cells cultured in RPMI-1640 medium with 10% fetal calf serum, 4 mM L-glutamine and 12.5 μM β-mercaptoethanol at 1×10^5 cells/well in round bottom 96 well plates. Cells were cultured in the presence of 20 μg/ml purified H2-K^b Trp2 peptide (180–188) or, as a control, a H2-K^b mesothelin peptide (351–358) (Lerner Research Institute Molecular Biotechnology Core, Cleveland, OH). After 3 days, culture supernatants were collected and assayed for IFN-γ by ELISA (R&D Systems) and for IL-2, IL-4, IL-6, IFN-γ, TNF-α, IL-17A, IL-10 using BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (San Jose, CA) according to the manufacturer's instructions.

2.6. Flow cytometry

Cells were washed twice in PBS–1% bovine serum albumin plus 0.05% sodium azide and stained for 30 min on ice with phycoerythrin or fluorescein isothiocyanate conjugated mAb specific for B220, CD8, CD11b, CD11c, CD86 and Gr1 (BD Biosciences, San Jose, CA) and CD4, CD25, and FoxP3 (eBioscience Inc., San Diego, CA). Appropriate isotype controls were used in all experiments. After incubation, cells were washed and fixed with 2% paraformaldehyde. All samples were analyzed using an EPICS Altra flow cytometer (Beckman Coulter, Fullerton, CA).

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

Dissected tumors and lymph nodes treated *in vivo* were placed in RNA Later (Ambion, Austin, TX) and stored at −4 °C. RNA was then extracted with RNeasy method (Qiagen, Valencia, CA) according to the manufacturer's direction and stored at −80 °C. An ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) and pre-standardized primers and TaqMan probes for mouse IL-10 (*IL10*), IL-12(p40) (*IL12B*), interferon regulatory factor 7 (*IRF7*), transforming growth factor (TGF) β (*TGFB1*), arginase (*ARG1*), CD206 (*MRC1*), CXCL10 (*CXCL10*), and FoxP3 (*FOXP3*) were used. Glyceraldehyde-3-phosphate dehydrogenase was used as the endogenous control. The reverse transcription and PCR was accomplished using a one-step protocol and TaqMan Universal Master Mix (Applied Biosystems) according to the recommendations of the manufacturer. C_t values were determined, and the relative number of copies of mRNA (RQ) was calculated using the $\Delta\Delta C_t$ method (Relative Quantitation of Gene Expression, User Bulletin