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Antigen coated poly α -hydroxy acid based microparticles for heterologous prime-boost adenovirus based vaccinations

Caitlin D. Lemke, Sean M. Geary, Vijaya B. Joshi, and Aliasger K. Salem*

College of Pharmacy, University of Iowa

Abstract

Adenoviruses show promising potential as vectors for cancer vaccines, however, their high immunogenicity can be problematic when it comes to homologous prime-boost strategies. In the studies presented here we show that heterologous prime-boost vaccinations involving ovalbumin (OVA)-antigen coated microparticles as a prime, and adenovirus encoding OVA (Ad-OVA) as a boost, were equally as effective as homologous Ad-OVA prime-boosts at generating OVA-specific CD8+ T cell responses, which translated into effective tumor protection. OVA-coated biodegradable poly α -hydroxy acid based microparticles of varying chemistries, when used as primes in heterologous prime-boost vaccinations, were comparable in terms of promoting OVA-specific CD8+ T cells as well as providing protection against subsequent tumor challenge. These findings auger well for using poly α -hydroxy acid based microparticles in prime-boost viral vaccination strategies geared toward the safer, and potentially more efficient, generation of anti-tumor immunity.

Keywords

poly α -hydroxy acids; microparticles; biodegradable; vaccine; cancer; tumors; prime; antigen; drug delivery; adenovirus; PLGA; PLA; microspheres

Introduction

Activation of tumor-targeting cytotoxic CD8+ T cells is a primary goal of cancer vaccines given the long-recognized key role these cells play in protective anti-tumor responses [1-3]. Both timely initial expansion and generation of memory populations are important outcomes of cancer vaccines since primary malignancy as well as subsequent recurrences would need to be targeted for sustained protection. Many vaccination strategies have been tested for their ability to give rise to T cell activation and memory, but the prime-boost approach is most consistently effective [4-7]. A novel short-interval prime-boost can generate significant CD8+ T cell expansion and rapid memory, demonstrating that the dictates of conventional prime-boost vaccination timing can be manipulated [8]. We have previously focused our attention on tumor antigen (Ag)-encoding recombinant, replication-deficient adenoviruses because they have been successfully used in our mouse tumor models [9-15] and in clinical trials for prostate cancer therapy [16]. However, using viral vectors as cancer vaccines is

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*Corresponding author: S228 PHAR 115 S. Grand Avenue Iowa City, IA 52242 (319) 335-8810 (telephone) (319) 335-9349 (fax) aliasger-salem@uiowa.edu.

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limited by the pre-existence/generation of anti-vector immunity in patients which reduces their effectiveness when applying them in homologous prime-boost vaccination regimens [10]. Adapting our adenovirus cancer vaccine into a heterologous prime-boost system could be advantageous for both the magnitude and kinetics of generating anti-tumor responses.

Heterologous (or diversified) prime-boost vaccinations, wherein the prime consists of a different agent/formulation from the boost, have shown promise in various pre-clinical cancer models [17-23]. While the majority of pre-clinical heterologous prime-boost cancer vaccines rely on antigen (Ag) delivery directly via plasmid DNA or viral vectors, a strong case is being made for employing biodegradable microparticles as vehicles for protein or peptide Ag-based vaccinations [24-28].

Materials & Methods

Mice and tumor cell lines

Studies involving mice were approved by and performed according to guidelines established by the University of Iowa Institutional Animal Care and Use Committee. Inbred 6-8 week old C57BL/6 and male Balb/c were obtained from Jackson Laboratories and maintained in filtered cages. E.G7-OVA tumor cell lines were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI-1640 (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 1mM sodium pyruvate (GIBCO), 10 mM HEPES (GIBCO), 0.05 mM 2-mercaptoethanol, and 50 µg/mL gentamicin sulfate (Mediatech, Inc., Manassas, VA), with 0.4 mg/mL G418 (GIBCO) for selection maintenance.

Biodegradable particle fabrication

Three polymers were used to prepare particles: polylactic acid (PLA), 50:50 end-capped poly(lactide-co-glycolide) (PLGA) (Birmingham Polymers, Birmingham, AL) and 65:35 PLGA with a free carboxylic acid terminus (PLGA-COOH) (Sigma-Aldrich®, St. Louis, MO). Particles were fabricated using a standard oil-in-water (o/w) single emulsion technique. Briefly, polymer was dissolved in dichloromethane (DCM), followed by sonication into 1% polyvinyl alcohol. This solution was then mixed for 2hrs to allow for evaporation of DCM; after this, particles with an average size of 1-3µm were collected by differential centrifugation and lyophilized.

Particle size and zeta potential analysis

Comparative particle size and surface charge (zeta potential) measurements were conducted using the Zetasizer Nano ZS (Malvern, Southborough, MA), as previously described [29]. Briefly, lyophilized particles were suspended in deionized water at a concentration of 1 mg/mL. The size measurements were performed at 25°C with a 173° scattering angle. The mean hydrodynamic diameter was determined by cumulative analysis. Average diameter measurements were also confirmed by scanning electron microscopic (SEM) examination of particle preparations. Zeta potential determinations were based on electrophoretic mobility of the particles in the aqueous medium, which were performed using folded capillary cells in automatic mode.

Tumor Ag coating of particles

PLA, PLGA or PLGA-COOH particles were coated with full-length ovalbumin (OVA) or MHC Class I PSA peptide (HPQKVTKFML188-197) by passive adsorption. Briefly, lyophilized particles were resuspended in a 1 mg/mL solution of either OVA or PSA peptide dissolved in sterile 1X PBS. This mixture was then rotated continuously overnight at room temperature. After allowing for adsorption, the particles were centrifuged and resuspended

in 1X PBS immediately prior to use. The extent of OVA protein and PSA peptide coating was analyzed by comparative zeta potential measurement (see above), and furthermore was confirmed by detecting fluorescence after absorption of OVA-FITC onto particles by flow cytometry.

In vitro stimulation of BMDCs with biodegradable microparticles

Primary murine bone marrow-derived dendritic cells (BMDC) were generated as previously described [30]. Briefly, bone marrow cells were harvested from C57BL/6 or Balb/c mouse femurs by flushing with complete culture medium. To enrich for BMDCs, isolated cells were grown in complete DMEM culture medium supplemented with 20 ng/mL of recombinant murine GM-CSF (PeproTech, Rocky Hill, NJ) for 9-13 days. Resulting cells (~90% DCs as determined by CD11c staining) were then seeded at 10^6 /well in complete DMEM culture medium and particle preparations added in varying concentrations for 24hrs. BMDC activation after culturing with microparticles was measured by mAb staining for surface CD86 (eBioscience).

Short-interval prime-boost vaccination

Mice received subcutaneous prime or boost immunizations spaced 7 days apart. Prime immunizations consisted of either PBS, 2.5mg of biodegradable particles with surface adsorbed tumor Ag, or 10^8 pfu of adenovirus encoding tumor Ag (OVA). Boost immunizations consisted of either PBS or 10^8 pfu of adenovirus encoding tumor Ag. OVA-encoding, replication-deficient adenovirus serotype 5 (AdOVA) was obtained from the University of Iowa Gene Transfer Vector Core, as previously described [14, 15].

Analysis of Ag-specific CD8⁺ T cell frequency

For analysis of Ag-specific circulating T cells, peripheral blood leukocytes (PBL) were isolated from a small volume of blood obtained by submandibular bleeding at the indicated timepoints. The H-2K^b SIINFEKL Class I iTagTM MHC Tetramer (K^b-OVA₂₅₇) (Beckman Coulter, Fullerton, CA) was used as previously described [14]. For general staining, PBLs were incubated with Fc block (2.4G2; BD Biosciences, San Diego, CA), followed by the appropriate peptide:MHC class I tetramer, anti-CD8a (53-6.7; eBioscience, San Diego, CA) and anti-CD3 (145-2C11; eBioscience). Samples were acquired on a FACScanTM flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and data analyzed with FlowJo software (TreeStar, Ashland, OR).

In vivo tumor challenge

For tumor challenge, C57BL/6 mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mix, at a final concentration of 87.5 mg/kg ketamine and 2.5 mg/kg xylazine (provided by the Office of Animal Resources, University of Iowa). Each mouse was challenged subcutaneously in the right flank with 10^6 (E.G7-OVA) tumor cells. Tumor outgrowth, determined by size as a function of time, was measured three times a week. Tumor volume was calculated as previously described [12] and mice were sacrificed when tumor diameter became greater than 20mm.

Statistical analysis

Statistical analysis of CD86 expression levels was performed using two-way ANOVA with a Bonferroni post test. Comparative analysis of Ag-specific CD8⁺ T cell levels was performed using one-way ANOVA with a Tukey post test. Comparison of survival curves was performed using the Log-rank (Mantel-Cox) test. The confidence interval for all tests was set at 95% and a *P* value of <0.05 was accepted as significant (*); *P*<0.01 (**); *P*<0.001

(***). All analyses were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Effect of microparticle chemistry on DC activation

Preparations of particles from either PLA, PLGA 50:50 or PLGA-COOH 65:35 yielded microparticles that were an average of 2-3 μm in diameter and spherical in nature (Fig. 1A-B). All three types of particles were highly negatively charged prior to full-length OVA adsorption (-34 , -34.5 , -34.3mV , respectively) and became almost neutral after adsorption (-2.9 , -3.5 , -3.4mV , respectively) (Fig. 1C). To confirm that the loss of surface negativity was directly related to protein coating we adsorbed PLA, PLGA and PLGA-COOH microparticles with OVA conjugated to FITC (OVA-FITC) and detected fluorescent particles by flow cytometry (Fig. 1D). We consistently observed that PLA particles had the highest proportion that was fluorescent and the greatest reduction in surface negativity after adsorption. Furthermore, net loss of surface negativity was seen after adsorption of PSA peptide onto all three particle preparations (data not shown), which demonstrated that surface coating of microparticles could be achieved regardless of protein length or source.

Biodegradable microparticles are readily phagocytosed by APCs, particularly DCs, which has been shown to induce their activation [31-35]. Because the hydrophobic nature of microparticles can influence their immunostimulatory capacity and uptake by phagocytic cells [36, 37], we wanted to comparatively examine the impact of PLA, PLGA and PLGA-COOH microparticles on murine BMDCs (mBMDC). Incubation of C57BL/6 mBMDCs with PLGA-COOH microparticles resulted in a slightly higher, but not significant, upregulation of CD86 as compared to PLA or PLGA microparticles (Fig. 2A). The effect on Balb/c mBMDCs, however, was not altered by polymer chemistry (Fig. 2B). Overall, we observed a similarly dose-dependent immunostimulatory effect on BMDCs, regardless of polymer.

CD8+ T cell responses to microparticle prime-adenovirus boosts

To determine if particle polymer chemistry impacts CD8+ T cell priming in a heterologous prime-boost system, mice were primed with either OVA adsorbed onto PLA, PLGA or PLGA-COOH microparticles followed by boosting with Ad5OVA. OVA-specific CD8+ T cell responses detected in PBL were not significantly different from naïve mice in any of the groups 7 days after priming. One week after the Ad5OVA boost (d14) PLA-OVA (Fig. 3A) and PLGA-OVA (Fig. 3B) primed mice had significantly higher responses than naïve mice, whereas Ad5OVA-primed mice did not develop a significantly higher response as compared to naïve until two weeks after the boost (d21) (Fig. 3D). Interestingly, PLGACOOH-OVA-primed mice also developed higher responses compared to naïve mice, but the difference was not significant at any of the timepoints measured (Fig. 3B). The peak response for all of the heterologous particle-primed groups was on day 14 (7 days after boost), which was followed by a gradual contraction phase over the next 21 days. In contrast, the homologous Ad5OVA-primed group's response did not peak until day 28 (21 days after boost) and was then followed by a rapid contraction over the next 7 days. Despite the slightly varied kinetics and magnitudes of the early effector phase responses, all of the prime-boost groups had comparable levels of OVA-specific CD8+ T cells during the late memory phase on day 100 (93 days after boost).

Microparticle prime-adenoviral boosts as prophylactic tumor vaccines

It has previously been shown that short interval heterologous prime-boost is effective at inducing protective CD8+ T cell responses in mouse models of infectious diseases [8].

Because CD8⁺ T cells can potently mediate anti-tumor responses in mice after delivery of adenovirus cancer vaccines [9-12, 14-16, 38], we wanted to examine how effective our various heterologous prime-boost formulations were at prophylactically stimulating protective anti-tumor immunity. Mice were primed on day 0, boosted on day 7 and challenged subcutaneously with EG.7-OVA tumor cells on day 14. Solid tumors developed rapidly in the control naïve group of mice and resulted in no survival beyond day 19 post challenge (Fig. 4A). Homologous Ad5OVA prime-boost mice (positive control for protection) developed no tumors and had 100% survival to day 60 post challenge. This level of protection was also observed in the PLGA-OVA and PLGA-COOH-OVA-primed mice, whereas the PLA-OVA-primed mice had a slightly lower survival of 75% to day 60. At day 60 the remaining tumor-free mice were subjected to a re-challenge with EG.7-OVA tumor cells in order to reveal the protective capabilities of the CD8⁺ T cell memory response. Interestingly, the heterologous PLA-OVA prime-Ad5OVA boost mice had 100% survival upon re-challenge, while the PLGA-COOH-OVA prime-Ad5OVA boost mice had 75% survival and the homologous Ad5OVA prime-boost mice and the heterologous PLGA-OVA prime-Ad5OVA boost mice only had 50% survival (Fig. 4B).

Effectiveness of microparticle prime-adenovirus boosts as tumor therapy

After vetting the short-interval prime-boost vaccinations in a prophylactic setting where they were given prior to tumor challenge, we wanted to examine the therapeutic benefit when prime-boost was performed after a tumor challenge. To do this, mice were challenged with EG.7-OVA tumor cells on day 0, followed by priming on day 3 and boosting on day 9. We observed no significant difference in therapeutic protection conferred by any of the three heterologous microparticle prime-Ad5OVA boost groups as compared to naïve mice (Fig. 5). The homologous Ad5OVA prime-boost was the only treatment that resulted in significantly enhanced survival compared to naïve ($p=0.0013$), with 1/8 mice surviving to day 60 post challenge. However, this was not significantly different to survival in mice treated with the heterologous microparticle prime-Ad5OVA boost.

Discussion

Adenoviral vectors possess many characteristics that make them attractive candidates for stimulating cellular immune responses against malignant disease [39]. Multiple adenovirus-based inoculations however may prove problematic in terms of toxicity and immunopotency for patients that generate adenoviral-specific antibodies after the first dose or harbor existing adenoviral-specific antibodies [40, 41]. One way to counter this problem is through heterologous prime-boost vaccinations. Antigen-coated PLGA microparticles have strong potential for priming in heterologous prime-boost vaccination strategies [8]. This approach can generate rapidly protective CD8⁺ T cell immunity against multiple pathogens following various booster regimes. These heterologous vaccine strategies have been shown to generate strong protection in murine models of influenza and malaria. In the studies performed here multiple aims were investigated with the overarching goal of testing a heterologous prime-boost strategy, involving OVA-coated PLGA/PLA microparticles (prime) and AdOVA (boost) against a well-established murine cancer model.

As mentioned, multiple aims were investigated and the first of these was to determine if biodegradable, OVA-coated microparticles with varying chemistries could variably affect: a) *in vitro* DC activation, and/or, b) *in vivo* immune responses when used as a prime in prime-boost vaccinations. It has been well documented that PLGA microparticles, because of their similar size to many pathogens (1 – 10 μm), are readily taken up by murine and human DCs. The surface hydrophobicity of various PLGA particle formulations, as opposed to polystyrene microspheres, does not significantly affect the efficiency of uptake by human DCs [42]. However, there is a paucity of data describing the effect of different PLGA

particle formulations on DC activation/maturation. In terms of the first aim it was apparent that all microparticle formulations were capable of stimulating DCs to a similar extent, as determined through their level of CD86 expression. Therefore the varying chemistries of these particles, such as differences in hydrophobicity, had little impact on activation as measured by this parameter. These results partially conflict with a previously published report which showed that hydrophobic PLA microparticles did not stimulate human monocyte-derived dendritic cells, as determined by CD86 expression, whilst PLGA microparticles were stimulatory (32). One possible explanation for this discrepancy is the nature of the DCs themselves where in our studies murine bone marrow-derived DCs were used as opposed to human monocyte-derived DCs used in the previous study. Nevertheless, our findings highlight the adjuvant potential of these biodegradable polymer formulations, implicating that they could be more than purely vehicles for Ag delivery. Differences in hydrophobicity also had little impact on the OVA-specific CD8⁺ T cell responses observed *in vivo* since both PLA and PLGA microparticles generated similar response profiles (Fig 3A and B). PLGA-COOH microparticles were marginally, but not significantly less effective at generating OVA-specific CD8⁺ T cell responses, which may be explained by these particles retaining OVA less efficiently due to the repulsive forces between carboxylate moieties and the negatively charged OVA (pI 4.6).

The second aim of these studies was to quantitatively compare immune responses generated by: a) heterologous prime-boost vaccinations involving OVA-coated microparticles/AdOVA with b) homologous prime-boost vaccinations using AdOVA/AdOVA. It was demonstrated that the heterologous prime-boost vaccinations, involving PLA/AdOVA or PLGA/AdOVA, had similar response profiles to the homologous prime-boost vaccination AdOVA/AdOVA. Although not statistically significant, the peak immune response to these two heterologous vaccinations was achieved more quickly than for the homologous vaccination (d14 vs. d28).

The third aim of this study was to compare the prophylactic and therapeutic benefit of heterologous particle/AdOVA vaccines with homologous AdOVA/AdOVA vaccines using a well established tumor model system. In a prophylactic setting, both heterologous and homologous prime-boost vaccinations proved to be highly protective. In a therapeutic setting, there was no significant difference in survival between the mice treated with homologous AdOVA/AdOVA vaccinations and the mice treated with heterologous particle/AdOVA vaccinations. This was consistent with the findings that homologous AdOVA/AdOVA and heterologous particle/AdOVA vaccinations generated similar OVA-specific CD8⁺ T cell responses. It is possible that components of the innate immune system, such as NK cells, contributed to a marginal observed therapeutic advantage in the mice treated with the homologous AdOVA/AdOVA vaccinations, since mice receiving the homologous vaccination would have stimulated NK cells earlier and for longer than mice receiving the heterologous vaccinations. However, we reiterate that there was no significant difference between any of the treated groups. That homologous and heterologous adenoviral vaccines alone have only marginal therapeutic potency is not surprising given previous findings in various tumor model systems, where therapeutic vaccination with an antigen-encoding adenovirus showed low cure rates, albeit increased survival time over unvaccinated mice [15, 43]. We believe that therapeutic heterologous prime-boost vaccines can be more effective when they are combined with treatments that: a) dampen immunosuppressive agents such as regulatory T cells, and/or b) decrease tumor burden using radiation or low-dose chemotherapy and these represent research directions that we are currently pursuing.

Conclusion

In summary, homologous and heterologous prime-boost vaccinations are able to stimulate similar Ag-specific CD8⁺ T cell responses and to provide effective prophylactic protection

against tumor challenge. We observed that non-toxic biodegradable microparticles used as a prime can generate equivalent responses to adenovirus based primes. These findings have potentially important implications in terms of prophylactic vaccination strategies, particularly for adenovirus-based vaccines where multiple administrations could be harmful or ultimately ineffective due the generation or presence of virus-specific antibodies. The therapeutic potency of all vaccination strategies tested has the potential to be improved by combining with other therapies.

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References

- [1]. Schlom J. Therapeutic cancer vaccines: current status and moving forward. *J Natl Cancer Inst.* 2012; 104:599–613. [PubMed: 22395641]
- [2]. Morris E, Hart D, Gao L, Tsallios A, Xue SA, Stauss H. Generation of tumor-specific T-cell therapies. *Blood Rev.* 2006; 20:61–9. [PubMed: 15978709]
- [3]. Melief CJ. Tumor eradication by adoptive transfer of cytotoxic T lymphocytes. *Adv Cancer Res.* 1992; 58:143–75. [PubMed: 1532109]
- [4]. Schneider J, Gilbert SC, Hannan CM, Degano P, Prieur E, Sheu EG, et al. Induction of CD8+ T cells using heterologous prime-boost immunisation strategies. *Immunol Rev.* 1999; 170:29–38. [PubMed: 10566139]
- [5]. Ramshaw IA, Ramsay AJ. The prime-boost strategy: exciting prospects for improved vaccination. *Immunol Today.* 2000; 21:163–5. [PubMed: 10740236]
- [6]. Woodland DL. Jump-starting the immune system: prime-boosting comes of age. *Trends Immunol.* 2004; 25:98–104. [PubMed: 15102369]
- [7]. Nolz JC, Harty JT. Strategies and implications for prime-boost vaccination to generate memory CD8 T cells. *Adv Exp Med Biol.* 2011; 780:69–83. [PubMed: 21842366]
- [8]. Pham NL, Pewe LL, Fleenor CJ, Langlois RA, Legge KL, Badovinac VP, et al. Exploiting cross-priming to generate protective CD8 T-cell immunity rapidly. *Proc Natl Acad Sci U S A.* 2010; 107:12198–203. [PubMed: 20616089]
- [9]. Elzey BD, Siemens DR, Ratliff TL, Lubaroff DM. Immunization with type 5 adenovirus recombinant for a tumor antigen in combination with recombinant canarypox virus (ALVAC) cytokine gene delivery induces destruction of established prostate tumors. *Int J Cancer.* 2001; 94:842–9. [PubMed: 11745487]
- [10]. Siemens DR, Elzey BD, Lubaroff DM, Bohlken C, Jensen RJ, Swanson AK, et al. Cutting edge: restoration of the ability to generate CTL in mice immune to adenovirus by delivery of virus in a collagen-based matrix. *J Immunol.* 2001; 166:731–5. [PubMed: 11145643]
- [11]. Lubaroff DM, Karan D, Andrews MP, Acosta A, Abouassaly C, Sharma M, et al. Decreased cytotoxic T cell activity generated by co-administration of PSA vaccine and CpG ODN is associated with increased tumor protection in a mouse model of prostate cancer. *Vaccine.* 2006; 24:6155–62. [PubMed: 16876291]
- [12]. Karan D, Krieg AM, Lubaroff DM. Paradoxical enhancement of CD8 T cell-dependent anti-tumor protection despite reduced CD8 T cell responses with addition of a TLR9 agonist to a tumor vaccine. *Int J Cancer.* 2007; 121:1520–8. [PubMed: 17565748]
- [13]. Lemke CD, Graham JB, Lubaroff DM, Salem AK. Development of an MHC class I L(d)-restricted PSA peptide-loaded tetramer for detection of PSA-specific CD8+ T cells in the mouse. *Prostate Cancer Prostatic Dis.* 2011; 14:118–21. [PubMed: 21263453]
- [14]. Lemke CD, Graham JB, Geary SM, Zamba G, Lubaroff DM, Salem AK. Chitosan Is a Surprising Negative Modulator of Cytotoxic CD8(+) T Cell Responses Elicited by Adenovirus Cancer Vaccines. *Mol Pharm.* 2011; 8:1652–61. [PubMed: 21780831]

- [15]. Geary SM, Lemke CD, Lubaroff DM, Salem AK. Tumor immunotherapy using adenovirus vaccines in combination with intratumoral doses of CpG ODN. *Cancer Immunol Immunother.* 2011; 60:1309–17. [PubMed: 21626029]
- [16]. Lubaroff DM, Konety BR, Link B, Gerstbrein J, Madsen T, Shannon M, et al. Phase I clinical trial of an adenovirus/prostate-specific antigen vaccine for prostate cancer: safety and immunologic results. *Clin Cancer Res.* 2009; 15:7375–80. [PubMed: 19920098]
- [17]. Hodge JW, Poole DJ, Aarts WM, Gomez Yafal A, Gritz L, Schlom J. Modified vaccinia virus ankara recombinants are as potent as vaccinia recombinants in diversified prime and boost vaccine regimens to elicit therapeutic antitumor responses. *Cancer Res.* 2003; 63:7942–9. [PubMed: 14633725]
- [18]. Hodge JW, Higgins J, Schlom J. Harnessing the unique local immunostimulatory properties of modified vaccinia Ankara (MVA) virus to generate superior tumor-specific immune responses and antitumor activity in a diversified prime and boost vaccine regimen. *Vaccine.* 2009; 27:4475–82. [PubMed: 19450631]
- [19]. Krupa M, Canamero M, Gomez CE, Najera JL, Gil J, Esteban M. Immunization with recombinant DNA and modified vaccinia virus Ankara (MVA) vectors delivering PSCA and STEAP1 antigens inhibits prostate cancer progression. *Vaccine.* 2011; 29:1504–13. [PubMed: 21182993]
- [20]. Zhang H, Wang Y, Liu C, Zhang L, Xia Q, Zhang Y, et al. DNA and adenovirus tumor vaccine expressing truncated survivin generates specific immune responses and anti-tumor effects in a murine melanoma model. *Cancer Immunol Immunother.* 2012; 61:1857–67. [PubMed: 22706381]
- [21]. Odunsi K, Matsuzaki J, Karbach J, Neumann A, Mhawech-Fauceglia P, Miller A, et al. Efficacy of vaccination with recombinant vaccinia and fowlpox vectors expressing NY-ESO-1 antigen in ovarian cancer and melanoma patients. *Proc Natl Acad Sci U S A.* 2012; 109:5797–802. [PubMed: 22454499]
- [22]. Dai MS, Vassaux G, Xu M, You RI, Hsieh YF, Ouisse LH, et al. Early Treg suppression by a listeriolysin-O-expressing *E. coli* vaccine in heterologous prime-boost vaccination against cancer. *Vaccine.* 2012; 30:6903–11. [PubMed: 22982404]
- [23]. Osada T, Morse MA, Hobeika A, Lysterly HK. Novel recombinant alphaviral and adenoviral vectors for cancer immunotherapy. *Semin Oncol.* 2012; 39:305–10. [PubMed: 22595053]
- [24]. Audran R, Peter K, Dannull J, Men Y, Scandella E, Groettrup M, et al. Encapsulation of peptides in biodegradable microspheres prolongs their MHC class-I presentation by dendritic cells and macrophages in vitro. *Vaccine.* 2003; 21:1250–5. [PubMed: 12559806]
- [25]. Haining WN, Anderson DG, Little SR, von Bergwelt-Baildon MS, Cardoso AA, Alves P, et al. pH-triggered microparticles for peptide vaccination. *J Immunol.* 2004; 173:2578–85. [PubMed: 15294974]
- [26]. Waeckerle-Men Y, Gander B, Groettrup M. Delivery of tumor antigens to dendritic cells using biodegradable microspheres. *Methods Mol Med.* 2005; 109:35–46. [PubMed: 15585911]
- [27]. D'Souza B, Bhowmik T, Shashidharamurthy R, Oettinger C, Selvaraj P, D'Souza M. Oral microparticulate vaccine for melanoma using M-cell targeting. *J Drug Target.* 2012; 20:166–73. [PubMed: 21981679]
- [28]. Chablani L, Tawde SA, Akalkotkar A, D'Souza C, Selvaraj P, D'Souza MJ. Formulation and evaluation of a particulate oral breast cancer vaccine. *J Pharm Sci.* 2012; 101:3661–71. [PubMed: 22828873]
- [29]. Intra J, Salem AK. Characterization of the transgene expression generated by branched and linear polyethylenimine-plasmid DNA nanoparticles in vitro and after intraperitoneal injection in vivo. *J Control Release.* 2008; 130:129–38. [PubMed: 18538436]
- [30]. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N, et al. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods.* 1999; 223:77–92. [PubMed: 10037236]
- [31]. Lutsiak ME, Robinson DR, Coester C, Kwon GS, Samuel J. Analysis of poly(D,L-lactic-co-glycolic acid) nanosphere uptake by human dendritic cells and macrophages in vitro. *Pharm Res.* 2002; 19:1480–7. [PubMed: 12425465]

- [32]. Jilek S, Ulrich M, Merkle HP, Walter E. Composition and surface charge of DNA-loaded microparticles determine maturation and cytokine secretion in human dendritic cells. *Pharm Res.* 2004; 21:1240–7. [PubMed: 15290866]
- [33]. Zhang XQ, Dahle CE, Baman NK, Rich N, Weiner GJ, Salem AK. Potent antigen-specific immune responses stimulated by codelivery of CpG ODN and antigens in degradable microparticles. *J Immunother.* 2007; 30:469–78. [PubMed: 17589287]
- [34]. Goforth R, Salem AK, Zhu X, Miles S, Zhang XQ, Lee JH, et al. Immune stimulatory antigen loaded particles combined with depletion of regulatory T-cells induce potent tumor specific immunity in a mouse model of melanoma. *Cancer Immunol Immunother.* 2009; 58:517–30. [PubMed: 18719913]
- [35]. Clawson C, Huang CT, Futralan D, Seible DM, Saenz R, Larsson M, et al. Delivery of a peptide via poly(D,L-lactic-co-glycolic) acid nanoparticles enhances its dendritic cell-stimulatory capacity. *Nanomedicine.* 2010; 6:651–61. [PubMed: 20348031]
- [36]. Prior S, Gander B, Blarer N, Merkle HP, Subira ML, Irache JM, et al. In vitro phagocytosis and monocyte-macrophage activation with poly(lactide) and poly(lactide-co-glycolide) microspheres. *Eur J Pharm Sci.* 2002; 15:197–207. [PubMed: 11849917]
- [37]. Thomas C, Rawat A, Hope-Weeks L, Ahsan F. Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to hepatitis B vaccine. *Mol Pharm.* 2011; 8:405–15. [PubMed: 21189035]
- [38]. Lubaroff DM, Karan D. CpG oligonucleotide as an adjuvant for the treatment of prostate cancer. *Adv Drug Deliv Rev.* 2009; 61:268–74. [PubMed: 19166887]
- [39]. Basak SK, Kiertscher SM, Harui A, Roth MD. Modifying adenoviral vectors for use as gene-based cancer vaccines. *Viral Immunol.* 2004; 17:182–96. [PubMed: 15279698]
- [40]. Campos SK, Barry MA. Current advances and future challenges in Adenoviral vector biology and targeting. *Curr Gene Ther.* 2007; 7:189–204. [PubMed: 17584037]
- [41]. Raki M, Sarkioja M, Escutenaire S, Kangasniemi L, Haavisto E, Kanerva A, et al. Switching the fiber knob of oncolytic adenoviruses to avoid neutralizing antibodies in human cancer patients. *J Gene Med.* 2011; 13:253–61. [PubMed: 21520358]
- [42]. Walter E, Dreher D, Kok M, Thiele L, Kiama SG, Gehr P, et al. Hydrophilic poly(DL-lactide-co-glycolide) microspheres for the delivery of DNA to human-derived macrophages and dendritic cells. *J Control Release.* 2001; 76:149–68. [PubMed: 11532321]
- [43]. Sorensen MR, Holst PJ, Pircher H, Christensen JP, Thomsen AR. Vaccination with an adenoviral vector encoding the tumor antigen directly linked to invariant chain induces potent CD4(+) T-cell-independent CD8(+) T-cell-mediated tumor control. *Eur J Immunol.* 2009; 39:2725–36. [PubMed: 19637230]

Figure 1. Characterization of size, morphology, and protein Ag coating of biodegradable microparticles. *A*, Particle diameter of replicate batches was determined using a Zetasizer. *B*, Representative SEM image of microparticles. *C*, Surface charge (zeta potential) of replicate batches of blank and OVA-coated particles. *D*, Detection of FITC-OVA adsorbed onto microparticles.

Figure 2.

Biodegradable microparticles activate DCs in a dose-dependent manner. mBMDCs prepared from C57BL/6 (A) or Balb/c (B) were incubated with increasing concentrations of PLA, PLGA or PLGACOOH microparticles for 24hrs, followed by quantification of surface CD86 (mean $\pm$ SD).

Figure 3.

Short-interval microparticle prime-adenovirus boost generates rapid expansion of Ag-specific CD8+ T cells that results in long-lived memory. C57BL/6 mice were administered a prime and boost, spaced 7 days apart. The kinetics of OVA-specific CD8+ T cell responses (mean $\pm$ SEM, n=4) observed in heterologous (A-C) or homologous (D) prime-boost vaccinated mice (solid circles) were compared to naïve mice (open circles). *, p<0.05; **, p<0.01.

Figure 4.

Prophylactic short-interval heterologous prime-boost vaccination results in varied protection against tumor challenge. Prime and boost vaccination were administered to C57BL/6 mice 7 days apart followed by primary E.G7-OVA tumor challenge 7 days after boost (*A*) or secondary challenge of surviving tumor-free mice 60 days after primary challenge (*B*).

Figure 5.

Heterologous microparticle prime-adenovirus boost vaccination generates therapeutic activity that is equivalent and non-significantly different to homologous adenovirus prime-adenovirus boost vaccinations. C57BL/6 mice were challenged subcutaneously with 10^6 EG. 7-OVA tumor cells followed by prime and boost on day 3 and day 9, respectively. Survival curves comparisons were performed between vaccinated groups and the naïve group using the Log-rank (Mantel-Cox) test ($n=8$); **, $p<0.01$.