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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

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Abstract

Recombinant poxviruses expressing tumor-associated antigens (TAA) are currently being evaluated in clinical trials as an approach to treat various cancers. We have previously generated poxviral vectors expressing a TAA and a TRIad of COstimulatory Molecules (B7-1, ICAM-1, and LFA-3; TRICOM) as transgenes, including replication competent recombinant vaccinia (rV) or replication-defective modified vaccinia Ankara (MVA), to prime tumor-specific immune responses, and a replication-defective recombinant fowlpox (rF) to boost these responses. MVA is a potentially safer, replication-defective form of vaccinia virus with unique immunostimulatory properties that could make it a superior priming vaccine. Here, an MVA vector encoding a tumor antigen (CEA) and TRICOM was utilized (rMVA). A single rMVA-CEA/TRICOM vaccination induced greater expression of several serum cytokines associated with enhanced T-cell immunity than that seen with vaccinia. We hypothesized that this effect might “precondition” the vaccination site for a more effective boost. An rMVA-CEA/TRICOM prime followed 7 days later (but not 30 days later) by an rF-CEA/TRICOM boost at the same injection site (but not at a distal site) induced more potent CEA-specific T-cell responses, and superior CEA-specific immunity and antitumor activity, than rV-CEA/TRICOM followed by rF-CEA/TRICOM. This preconditioning effect was also observed using a heterologous antigen model, where priming with rMVA-CEA/TRICOM followed 7 days later by rF-LacZ/TRICOM enhanced β -gal-specific immunity compared to rF-LacZ/TRICOM only. The studies reported here show for the first time that priming with rMVA followed 7 days later by an rF boost at the same injection site, versus a distal site, generates superior tumor-specific immunity and antitumor activity.

Keywords

Modified vaccinia Ankara (MVA); Carcinoembryonic antigen (CEA); Vaccinia; Costimulation

1. Introduction

One current approach to vaccine design includes the insertion of antigens of interest into a wide array of vectors to generate immunity; this is being evaluated in the treatment or prevention of diseases such as malaria, HIV, tuberculosis, influenza, and cancer [1–5].

Vectors used in recombinant cancer vaccines are generally based on DNA, bacteria, viruses,

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or yeast [6–9]. One approach to cancer vaccine design is to identify potentially immunogenic tumor-associated antigens (TAAs) and optimize epitopes of these antigens to elicit a high-avidity CD8⁺ T-cell based antitumor response [10]. However, of equal importance is the selection of a vaccine vector that can also induce an innate immune response, and thus potentiate a Th1 immune response. This preclinical study focuses on the use of poxviruses to induce tumor-specific T-cell immunity and antitumor responses, while capitalizing on both the vaccine vectors' innate and adaptive immunostimulatory characteristics.

We and other groups have previously demonstrated (a) that replication-defective attenuated modified vaccinia Ankara (MVA) can be used to deliver various TAAs expressing costimulatory molecules or cytokines to generate tumor-specific immunity [11–15]; (b) generation of host immune responses against MVA or vaccinia virus vectors limits the efficacy of multiple vaccinations [11, 16, 17]; and (c) the advantages of a diversified strategy employing recombinant MVA (rMVA) or recombinant vaccinia (rV) as a prime, followed by replication-deficient recombinant fowlpox (rF) as a boost [18–21]. Previous studies have also demonstrated that priming with an rMVA vector expressing carcinoembryonic antigen (CEA) and a TRIad of COstimulatory Molecules (B7-1, ICAM-1, and LFA-3, designated TRICOM) as transgenes, then boosting with an rF-CEA/TRICOM vector, more effectively induced CEA-specific immunity in human CEA-transgenic (CEA-Tg) mice, compared to a similar rV prime and rF boost [11]. The reasons for this however were unclear.

Previous studies have demonstrated that infecting dendritic cells (DCs) with MVA, but not with vaccinia, induces production of cytokines such as IFN- α by DCs, leading to DC maturation and enhanced antigen presentation, and thereby initiating potent antigen-specific CD8⁺ T-cell responses [22–25]. We hypothesized that using rMVA to initiate a local innate immune response leading to production of Th1 supportive cytokines and DC maturation at the draining lymph node might “precondition” the vaccination site for a more effective boost. By examining the temporal and spatial relationship of the prime and boost vaccinations, we found that a vaccination strategy of an rMVA prime, followed 7 days later by a fowlpox boost at the same local injection site, harnessed unique immunostimulatory properties of rMVA, prompting the generation of superior tumor-specific immunity and reduced tumor burden.

2. Materials and methods

2.1 Recombinant poxviruses

MVA was generated from a stock (graciously provided by Dr. A. Mayr, Ludwig-Maximilians University, Munich, Germany) through 3 successive rounds of plaque purification in chicken embryo dermal primary cultures, and amplified further in these cells to produce research stocks. rMVA-CEA/B7-1/ICAM-1/LFA-3 (designated rMVA-CEA/TRICOM) is the recombinant rMVA-TRICOM construct containing the human CEA gene under control of the 40k promoter [11]. The rV and rF viruses containing the human CEA gene and the murine B7-1, ICAM-1, and LFA-3 genes (designated rV-CEA/TRICOM and rF-CEA/TRICOM, respectively) have been previously described [26]. The rF virus containing the gene for murine granulocyte-macrophage colony-stimulating factor (GM-CSF) under control of the 40k promoter has also been described [27]. rF-LacZ-B7-1/ICAM-1/LFA-3 (designated rF-LacZ/TRICOM) has been previously described [28].

2.2 Animals and cells

Female C57BL/6 mice were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). Mice were housed and maintained in pathogen-free

conditions in microisolator cages until used for experiments at 6–8 weeks of age. CEA-Tg C57BL/6 mice were obtained from a breeding pair provided by Dr. John Thompson (Institute of Immunobiology, University of Freiburg, Freiburg, Germany). The generation and characterization of CEA-Tg mice have been previously described [29]. These studies used murine colon adenocarcinoma cells expressing human CEA (MC-38-CEA) [30]. Before transplantation to mice, the cells were trypsinized, dispersed through a 70- μ m cell strainer (Falcon; Becton Dickinson, Franklin Lakes, NJ), and washed twice in HBSS before final suspension in HBSS.

2.3 Efficacy of MVA or vaccinia to prime CEA-specific immune responses

CEA-Tg mice (3 per group) were primed s.c. with 1×10^8 pfu rMVA-CEA/TRICOM or rV-CEA/TRICOM, then boosted s.c. 1, 2, or 3 times at 7- or 30-day intervals with rF-CEA/TRICOM. Control mice received HBSS buffer. Three to 4 weeks after the last vaccination, spleens were removed, dispersed into single-cell suspensions, pooled, and immune responses were analyzed. Purified splenic CD4⁺ T cells were isolated and tested for reactivity to CEA or β -galactosidase (β -gal) protein in an in vitro lymphoproliferation assay, as previously described [9]. Briefly, purified CD4⁺ T cells were cultured with irradiated antigen-presenting cells (APCs) and CEA or β -gal protein for 5 days. [³H] thymidine was added to the wells (1 μ Ci/well) for the last 24 h; proliferation was assayed by measuring incorporated radioactivity. Results from triplicate wells were averaged and reported as mean CPM $\pm$ one standard error (SE). To evaluate CD8⁺ T-cell responses, pooled splenocytes were coincubated with 10 μ g/ml of the H-2D^b-restricted 8-mer peptide CEA_{526–533} (EAQNTTYL) [31] for 6 days. Bulk lymphocytes were recovered by centrifugation through a Ficoll-Hypaque gradient. T cells were restimulated with fresh, irradiated, naive splenocytes and 10 μ g/ml of either CEA peptide or VSV-N_{52–59} peptide (vesicular stomatitis virus, RGYVYQGL) [32] for 24 h. Supernatant was collected and analyzed for murine IFN- γ by cytometric bead array (BD PharMingen, San Jose, CA) according to the manufacturer's instructions.

2.4 Serum cytokine studies

C57BL/6 mice were vaccinated s.c. once with 1×10^8 pfu of either rMVA-CEA/TRICOM or rV-CEA/TRICOM; serum was collected at 0, 2, 3, 4, or 7 days following vaccination. Murine IFN- γ was measured using ELISA (Biosource; Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Serum CXCL-1, G-CSF, GM-CSF, IL-6, IL-12p70, and MCP-1 levels were determined by LICNOplex analysis (Millipore, Billerica, MA).

2.5 Ability of MVA priming to enhance LacZ-specific immune responses from a rF-LacZ boost

CEA-Tg mice (3 per group) were vaccinated s.c. with 1×10^8 pfu of either rMVA-CEA/TRICOM or HBSS, and vaccinated s.c. 7 days later with 1×10^8 pfu rF-LacZ/TRICOM. Four weeks after the last vaccination, immune responses were analyzed. Purified splenic T cells were tested for reactivity to β -gal protein in an in vitro lymphoproliferation assay, as described above. For tetramer analysis, 1×10^6 cells were incubated on ice with FITC-labeled anti-CD8 (BD Biosciences, San Jose, CA) and PE-labeled H-2K^b β -gal tetramer (Tetramer Core Facility, National Institutes of Health, Bethesda, MD) for 30–45 min, washed twice, then analyzed on a FACSCalibur (BD Biosciences). Background staining was assessed by isotype control antibodies (BD Biosciences).

2.6 Tumor therapy studies

Before transplantation to CEA-Tg mice, MC38-CEA⁺ cells were trypsinized, dispersed through a 70- μ m pore size cell strainer (Falcon; Becton Dickinson), and washed twice in

HBSS before final suspension in HBSS. Mice were injected s.c. in the right flank with 3×10^5 MC38-CEA⁺ tumor cells. Four days following tumor transplant, mice were primed s.c. with 1×10^8 pfu of either rMVA-CEA/TRICOM or rV-CEA/TRICOM admixed with 1×10^7 pfu rF-GM-CSF, then boosted 7 and 14 days later with 1×10^8 pfu rF-CEA/TRICOM admixed with 1×10^7 pfu rF-GM-CSF. Additional groups of mice were injected with 1×10^8 pfu of either control vector rMVA-TRICOM or rV-TRICOM admixed with 1×10^7 pfu rF-GM-CSF, then boosted 7 and 14 days later with 1×10^8 pfu rF-TRICOM admixed with 1×10^7 pfu rF-GM-CSF.

2.7 Statistical analysis

Results of tests of significance are reported as P values, derived from Student's t test using a 2-tailed distribution. P values were calculated at 95%. For graphical representation of data, Y-axis error bars representing ± 1 SE are shown. In some cases, the variation is such that error bars are obscured by the plot symbol.

3. Results

3.1 Recombinant MVA provides superior priming for induction of CEA-specific immunity in CEA-Tg mice

We previously demonstrated that after 2 vaccinations with either rMVA-CEA/TRICOM or rV-CEA/TRICOM, additional homologous vaccinations did not increase CEA-specific immunity due to vector-neutralizing antibodies [11]. Thus, we determined that a diversified prime (rMVA or rV) and boost (rF) strategy was most effective in producing CEA-specific immune responses. To further analyze the differences between rMVA and vaccinia vaccines, CEA-Tg mice were primed with rMVA-CEA/TRICOM or rV-CEA/TRICOM, followed by 0, 1, or 2 weekly rF-CEA/TRICOM boosts (Fig. 1). Priming with rV-CEA/TRICOM followed by a single rF-CEA/TRICOM boost demonstrated only a minor difference in CEA-specific CD4⁺ proliferation compared to rV-CEA/TRICOM only (Fig. 1A). Priming with rV-CEA/TRICOM followed by 2 boosts with rF-CEA/TRICOM significantly increased CD4⁺ proliferation compared to a single boost (Fig. 1A; $P = 0.008$). In contrast, priming with rMVA-CEA/TRICOM followed by a single rF-CEA/TRICOM boost significantly increased CEA-specific CD4⁺ proliferation compared to rMVA-CEA/TRICOM only (Fig. 1B; $P = 0.002$). An additional boost with rF-CEA/TRICOM further significantly increased CEA-specific CD4⁺ proliferation compared to priming with rMVA-CEA/TRICOM followed by a single rF-CEA/TRICOM boost (Fig. 1B; $P = 0.003$). Additionally, rMVA-CEA/TRICOM followed by 2 boosts with rF-CEA/TRICOM significantly increased CEA-specific CD4⁺ proliferation compared to rV-CEA/TRICOM followed by 2 boosts with rF-CEA/TRICOM (Fig. 1A and 1B; $P = 0.011$).

We also examined the effect of rMVA or rV vaccines on CEA-specific CD8⁺ immunity and found a similar trend. Priming with rV-CEA/TRICOM or rMVA-CEA/TRICOM followed by a single rF-CEA/TRICOM boost had little effect on the induction of IFN- γ from CEA-specific CD8⁺ T cells (Fig. 1C and 1D). However, priming with rV-CEA/TRICOM followed by 2 boosts with rF-CEA/TRICOM increased IFN- γ production 7.5-fold over an rV-CEA/TRICOM prime with no boost. Priming with rMVA-CEA/TRICOM and then boosting with rF-CEA/TRICOM once or twice increased IFN- γ production 6-fold and 16-fold respectively over an rMVA-CEA/TRICOM prime with no boost (Fig. 1D). Further, rMVA-CEA/TRICOM followed by a single boost with rF-CEA/TRICOM increased IFN- γ production 3-fold over priming with rV-CEA/TRICOM followed by rF-CEA/TRICOM (Fig. 1C and 1D). Also, rMVA-CEA/TRICOM followed by 2 boosts with rF-CEA/TRICOM increased IFN- γ production 1.6-fold over priming with rV-CEA/TRICOM followed by 2 boosts with rF-CEA/TRICOM. Overall, rMVA-CEA/TRICOM followed by 1 or 2 boosts with rF-CEA/

TRICOM increased generation of CEA-specific CD4⁺ and CD8⁺ immunity compared to priming with rV-CEA/TRICOM followed by boosting with rF-CEA/TRICOM (Fig. 1).

3.2 MVA enhances serum levels of proinflammatory cytokines

Studies were conducted to help define why priming with rMVA was superior to the use of rV. Previous studies have found that DCs infected in vitro with MVA produce proinflammatory cytokines such as MCP-1, TNF, IFN- α , IL-2, IL-6, and IL-12 within 24 h of infection [33–35]. To confirm and to extend these findings in vivo, C57BL/6 mice were vaccinated with rMVA-CEA/TRICOM or rV-CEA/TRICOM, and serum cytokines were measured at 0, 2, 3, 4, or 7 days (Fig. 2). Control mice received buffer only. Elevated serum IFN- γ was observed at 3 days post-vaccination with rV-CEA/TRICOM and returned to near baseline at 4 days post-vaccination (Fig. 2A). In contrast, mice vaccinated with rMVA-CEA/TRICOM had significantly higher levels of circulating IFN- γ at 2, 3, 4, and 7 days post-vaccination (day 2: $P = 0.018$; day 3: $P = 0.024$; days 4 and 7: $P < 0.01$) compared to mice vaccinated with rV-CEA/TRICOM. Monitoring of additional serum cytokines showed that in mice vaccinated with rMVA-CEA/TRICOM, CXCL1, G-CSF, and MCP-1 were elevated > 2 -fold at 2 or 4 days post-vaccination (Fig. 2B). These same cytokines were not elevated in mice vaccinated with rV-CEA/TRICOM (Fig. 2B). Additionally, levels of GM-CSF, IL-6, and IL-12p70 increased at greater levels in rMVA-CEA/TRICOM-vaccinated mice compared to rV-CEA/TRICOM-vaccinated mice (Fig. 2B).

3.3 Employing a shorter interval between MVA prime and fowlpox boost improves the generation of antigen specific T cells

Studies were undertaken to determine if timing of vaccine prime versus boost doses could optimize immune response. As described above, rMVA-CEA/TRICOM elevated serum IFN- γ levels at 7 days post-vaccination (Fig. 2). To take advantage of this induction of cytokine to optimize the vaccination schedule, we primed CEA-Tg mice with rMVA-CEA/TRICOM or rV-CEA/TRICOM and boosted 7 versus 30 days later with rF-CEA/TRICOM (Fig. 3). In contrast with rV-CEA/TRICOM priming, priming with rMVA-CEA/TRICOM and boosting 7 days later with rF-CEA/TRICOM significantly increased CEA-specific CD4⁺ proliferation compared to priming with rMVA-CEA/TRICOM and boosting 30 days later with rF-CEA/TRICOM (Fig. 3B; $P = 0.004$). Additionally, priming with rMVA-CEA/TRICOM and boosting 7 days later with rF-CEA/TRICOM significantly increased CEA-specific CD4⁺ proliferation compared to priming with rV-CEA/TRICOM and boosting 7 days later with rF-CEA/TRICOM (Fig. 3A and 3B; $P = 0.019$). Overall, these data demonstrate that employing a shorter interval (7 days versus 30 days) between the rMVA prime and the fowlpox boost optimizes generation of CEA-specific immunity.

3.4 Same-site fowlpox boost is required to enhance CEA-specific T-cell responses induced by recombinant MVA prime

Based on the finding that 7 days is superior to 30 days between rMVA prime and fowlpox boost (Fig. 3), studies were initiated to determine if the observed systemic cytokine responses to the rMVA prime (Fig. 2) were enhanced locally, and whether subsequent boosting in the same site versus a distal site would generate superior CEA-specific immunity. CEA-Tg mice were primed with rV-CEA/TRICOM or rMVA-CEA/TRICOM on the left side and boosted with rF-CEA/TRICOM on the same or contralateral side 7 days later (Table 1). Priming mice with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM on the same side significantly increased CEA-specific CD4⁺ proliferation 1.7-fold as compared to mice primed and boosted on contralateral sides (Table 1; $P = 0.006$). Priming with rMVA-CEA/TRICOM and boosting with rF-CEA/TRICOM on the same side significantly increased CEA-specific CD4⁺ proliferation 3.5-fold as compared to mice primed and boosted on contralateral sides (Table 1; $P < 0.001$). Further, priming mice with

rMVA-CEA/TRICOM and boosting with rF-CEA/TRICOM on the same side significantly increased CEA-specific CD4⁺ proliferation compared to priming with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM on the same side (Table 1; $P < 0.001$).

Similar trends were observed in CEA-specific CD8⁺ T-cell responses. Priming mice with rV-CEA/TRICOM and boosting with rF-CEA/TRICOM on the same side increased CEA-specific CD8⁺ IFN- γ production 2.5-fold compared to mice primed and boosted with the same vectors on contralateral sides (Table 1). Priming with rMVA-CEA/TRICOM and boosting with rF-CEA/TRICOM on the same side increased CEA-specific CD8⁺ IFN- γ production > 10 -fold compared to mice primed and boosted with the same vectors on contralateral sides (Table 1). Additionally, mice primed with rMVA-CEA/TRICOM and boosted with rF-CEA/TRICOM on the same side demonstrated a 5-fold increase in CEA-specific CD8⁺ IFN- γ production compared to mice primed with rV-CEA/TRICOM and boosted with rF-CEA/TRICOM on the same side (Table 1). Overall, these data demonstrate that an rMVA prime followed by a fowlpox boost at the same injection site enhances the generation of CEA-specific immunity compared to priming and boosting on contralateral sides (Table 1).

3.5 Pretreatment of the injection site with recombinant MVA enhances immunity to a heterologous antigen encoded by a recombinant fowlpox

To determine if pretreatment of the injection site with an rMVA-CEA/TRICOM prime would enhance immunity to a heterologous antigen carried by a recombinant fowlpox, CEA-Tg mice were primed with rMVA-CEA/TRICOM or buffer and boosted 7 days later with rF-LacZ/TRICOM. After 4 weeks, β -gal-specific immune responses were measured (Fig. 4). A sham prime with buffer followed by an rF-LacZ/TRICOM boost significantly increased β -gal-specific CD4⁺ proliferation compared to buffer only (Fig. 4A; $P = 0.004$). Priming with rMVA-CEA/TRICOM followed by an rF-LacZ/TRICOM boost significantly increased β -gal-specific CD4⁺ proliferation compared to rF-LacZ/TRICOM only (Fig. 4A; $P = 0.04$). We also measured splenic β -gal tetramer⁺ CD8⁺ cells in vaccinated mice and found that buffer followed by a boost with rF-LacZ/TRICOM increased the number of β -gal tetramer⁺ CD8⁺ cells 2-fold compared to buffer only (Fig. 4B). Additionally, priming with rMVA-CEA/TRICOM increased the number of splenic β -gal tetramer⁺ CD8⁺ cells 5-fold compared to buffer only, and 2-fold compared to vaccination with rF-LacZ/TRICOM only (Fig. 4B). These data demonstrate that pretreating the injection site with rMVA enhances immunity to a heterologous antigen (β -gal) delivered by a recombinant fowlpox vector.

3.6 rMVA prime followed by fowlpox boost reduces volume of 4-day established MC38-CEA⁺ tumors

CEA-Tg mice bearing MC38-CEA⁺ tumors were primed with rV-TRICOM, rMVA-TRICOM, rV-CEA/TRICOM, or rMVA-CEA/TRICOM 4 days post-tumor transplant and boosted at the same site with rF-TRICOM or rF-CEA/TRICOM on days 11 and 18 (Fig. 5). Priming with rV-CEA/TRICOM and boosting twice with rF-CEA/TRICOM significantly decreased MC38-CEA⁺ tumor volumes compared to control (Fig. 5A and 5D; $P < 0.001$). Further, an rMVA-CEA/TRICOM prime followed by 2 boosts with rF-CEA/TRICOM significantly delayed tumor growth compared to control (Fig. 5A and 5E; $P < 0.001$) or rV-CEA/TRICOM prime (Fig. 5D and 5E; $P < 0.0027$). Tumors in untreated mice grew rapidly, exceeding a volume of 500 mm³ by day 21 (Fig. 5A). Control vectors lacking the CEA transgene were unable to reduce tumor burden and tumors progressed as in untreated mice (Fig. 5B and 5C). These data demonstrate the enhanced antitumor activity of an rMVA prime compared to an rV prime.

4. Discussion

Recombinant poxvirus-based vaccines are currently being examined in clinical trials to determine therapeutic or prophylactic efficacy in diseases such as malaria, HIV, tuberculosis, influenza, and cancer [1–5]. The safety profile of replication-defective MVA when used as a small pox vaccine, along with its ability to deliver TAAs and costimulatory molecules to APCs, makes it a very suitable vaccine vector [11, 36–39]. However, in the clinical administration of recombinant MVA vaccines, issues related to vaccination schedule, timing, and location have not been clearly defined. Here these studies report for the first time that (a) priming with rMVA-CEA/TRICOM and boosting 7 days later with rF-CEA/TRICOM rather than boosting 30 days later generates superior tumor-specific immunity (Fig. 3); (b) priming with rMVA-CEA/TRICOM and boosting with rF-CEA/TRICOM at the same injection site versus a distal site generates superior tumor-specific immunity (Table 1); (c) priming with rMVA-CEA/TRICOM can enhance immunity to a heterologous antigen (Fig. 4); and (d) a vaccination strategy of priming with rMVA-CEA/TRICOM rather than rV-CEA/TRICOM and boosting 7 days later with rF-CEA/TRICOM at the same site generates superior antitumor activity (Fig. 5). This vaccination strategy of priming with MVA followed 7 days later with a fowlpox boost at the same injection site could be evaluated in patients to determine effects on clinical outcomes.

The enhanced generation of tumor-specific T-cell immunity when priming with MVA as compared to vaccinia, and when boosting at the same injection site within 7 days suggest activation of the innate immune system by MVA leading to enhancement of adaptive immunity. Previous studies have demonstrated how MVA can more potently activate the innate immune system than vaccinia. Specifically, vaccination with MVA leads to activation and maturation of DCs, and enhanced production of various proinflammatory cytokines by DCs, such as MCP-1, TNF, IFN- α , IL-2, IL-6, and IL-12 within 24 h in vitro [22, 33–35, 40, 41]. This activation of the innate immune system could then enhance the generation of tumor specific adaptive immunity and potentiate antitumor activity. Specifically, MVA has been shown to preferentially target plasmacytoid DCs (pDC), leading to the production of IFN- α following MVA infection [22]. In contrast to vaccinia, MVA does not express TNF, IFN- α/β , and IFN- γ cytokine receptor homologs, thus pDCs could produce cytokines such as IFN- α in response to MVA infection without inhibition from cytokine receptor homologs [42]. This production of IFN- α can activate and mature myeloid derived DCs (mDC) leading to enhanced antigen presentation to adaptive immune cells such as T cells [43, 44]. The activation of innate immunity by priming with MVA (Fig. 2) leading to improved mDC antigen presentation could explain the enhanced generation of tumor specific immunity as compared to vaccinia (Fig. 1), since vaccinia abortively infects DCs, does not induce DC maturation, and produces lower levels of proinflammatory cytokines than MVA (Fig. 2) [40]. Further, the initial activation of pDCs by priming with MVA could then enhance the ability of nearby mDCs to uptake and present antigen delivered by a fowlpox boost at the same local injection site, together leading to enhanced generation of systemic tumor specific T-cell immunity and antitumor activity (Fig. 3; Table 1; Fig. 5). This same process of IFN- α activating nearby DCs could also explain the ability of MVA to enhance the generation of immunity to a heterologous antigen delivered by a fowlpox boost (Fig. 4). Overall, vaccination with MVA likely leads to activation of innate immunity, leading to the production of supportive cytokines that mature mDCs, and thus enhance the generation of tumor specific T-cell immunity and antitumor activity as demonstrated by these present studies.

In several reported phase II trials, recombinant MVA vaccines were well tolerated, induced tumor-specific immunity through CD8⁺ T-cell or antibody responses, and produced clinical responses when combined with chemotherapy or supportive cytokine therapy [12–14, 45,

46]. However, issues related to vaccination schedule, timing, and location have not been clearly defined. One clinical study by Dreicer et al. of recombinant MVA-MUC1-IL2 vaccine in prostate cancer patients with evidence of PSA progression compared the temporal separation of prime and boost vaccinations [45]. Patients in Arm 1 received 6 weekly vaccinations, and then were vaccinated once every 3 weeks; patients in Arm 2 were vaccinated once every 3 weeks. A significant improvement in PSA doubling time was observed in Arm 1 that received initial vaccinations weekly compared to patients vaccinated every 3 weeks in Arm 2. Our findings support these data and suggest superior generation of tumor-specific immunity by priming with MVA and boosting with fowlpox 7 days later. Interestingly, in that study MVA-MUC1-IL2 was the only vaccine utilized and thus was administered multiple times. Previous preclinical studies have demonstrated that multiple injections of MVA generate vector-specific neutralizing antibodies which limit the ability of recombinant MVA vectors to stimulate immunity to inserted transgenes [11, 16]. Thus, priming with MVA followed by boosting with fowlpox could improve current clinical approaches using recombinant MVA vaccines. Notably, in an ongoing phase III trial comparing MVA-5T4 or placebo plus standard of care for locally advanced or metastatic clear cell renal cancer, vaccinations were discontinued as the trial was not expected to meet its primary survival endpoint [47]. The trial design involved the use of an MVA vector alone, with 2- to 8-week intervals between vaccinations. The data presented in the present studies, and the Dreicer et al. clinical study, suggest that MVA vaccination schedules can be further optimized by separating the initial MVA prime and the subsequent boost by only 7 days in order to improve clinical outcomes (Figs. 1 and 3) [45]. Other preclinical studies have demonstrated that using poxviruses to deliver TAAs in combination with costimulatory molecules can enhance cytotoxic T-cell avidity 100-fold, and is significantly more effective at treating established tumors than using poxviruses to deliver TAAs without costimulation [48, 49]. Together, these current studies and previous studies suggest that the design of MVA-based cancer vaccine clinical trials could be improved by using recombinant MVA-based vaccines that incorporate a TAA and multiple costimulatory molecules as a prime, followed 7 days later at the same injection site with a similar recombinant fowlpox vector.

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Fig. 1.

Priming with recombinant MVA and boosting with recombinant fowlpox enhanced induction of CEA-specific immunity. CEA-Tg mice were vaccinated with rV-CEA/TRICOM (V) or rMVA-CEA/TRICOM (M) followed by 0, 1, or 2 weekly boosts with rF-CEA/TRICOM (F). Control mice were given buffer (HBSS). Three weeks after the last vaccination, spleens were harvested and splenocytes were assayed. (A) and (B) CD4⁺ cell proliferation. Purified CD4⁺ T cells were isolated from pooled splenocytes and cultured with irradiated APCs and CEA protein for 5 days. [³H] thymidine (1 μ Ci/well) was added to the wells for the last 24 h and proliferation was assayed by measuring incorporated radioactivity. (C) and (D) CD8⁺ IFN- γ production. Splenocytes from a single group were dispersed into single-cell suspensions, pooled, and coincubated with 10 μ g/ml of the H-2D^b-restricted 8-mer peptide CEA₅₂₆₋₅₃₃ for 6 days. Bulk lymphocytes were recovered and restimulated with 10 μ g/ml of either CEA₅₂₆₋₅₃₃ peptide or VSV-N₅₂₋₅₉ negative control peptide for 24 h. Supernatant fluid was collected and analyzed for murine IFN- γ by cytometric bead array.

Fig. 2.

Recombinant MVA- versus recombinant vaccinia-based vaccines differentially affect serum cytokine levels. C57BL/6 mice were vaccinated with rMVA-CEA/TRICOM (closed circles) or rV-CEA/TRICOM (closed squares). Control mice were given HBSS buffer (open triangles). Serum samples were tested for cytokine levels 0, 2, 3, 4, or 7 days after vaccination.

Fig. 3.

Employing a shorter interval between MVA prime and fowlpox boost improves the generation of antigen specific T cells. CEA-Tg mice were vaccinated with (A) rV-CEA/TRICOM (V) followed 7 days (closed squares) or 30 days (open squares) later by rF-CEA/TRICOM (F) or (B) rMVA-CEA/TRICOM (M) followed by rF-CEA/TRICOM 7 days (closed circles) or 30 days (open circles) later. Control mice were given buffer (open triangles). Four weeks after the last vaccination, spleens were harvested and splenocytes were used for CD4⁺ cell proliferation assay. Purified CD4⁺ T cells were isolated from pooled splenocytes and cultured with irradiated APCs and CEA protein for 5 days. [³H] thymidine (1 μCi/well) was added to the wells for the last 24 h and proliferation was assayed by measuring incorporated radioactivity.

Fig. 4.

Pretreatment of the injection with recombinant MVA enhances immunity to a disparate antigen encoded by a recombinant fowlpox. CEA-Tg mice were vaccinated with rMVA-CEA/TRICOM (closed circles) or HBSS buffer (closed triangle), followed 7 days later by rF-LacZ/TRICOM on the same side. Control mice were given HBSS (open triangles). Three weeks after the last vaccination, spleens were harvested and splenocytes were assayed. (A) Purified CD4 $^+$ T cells were isolated from pooled splenocytes and cultured with irradiated APCs and β -gal protein for 5 days. [^3H] thymidine (1 $\mu\text{Ci/well}$) was added to the wells for the last 24 h and proliferation was assayed by measuring incorporated radioactivity. (B) Splenocytes were stained with anti-CD8 mAb and β -gal tetramer.

Fig. 5.

Optimized vaccine regimen comprised of recombinant MVA priming followed by recombinant fowlpox boosting as the same site within 7 days results in superior antitumor activity. CEA-Tg mice (5 per group) were injected with MC38-CEA⁺ tumors s.c. on day 0, primed on day 4, and boosted on days 11 and 18; all vaccinations were given in combination with rF-GM-CSF. Tumor volumes were monitored weekly. (A) Untreated mice. (B) Mice primed with rV-TRICOM and boosted with rF-TRICOM. (C) Mice primed with rMVA-TRICOM and boosted with rF-TRICOM. (D) Mice primed with rV-CEA/TRICOM and boosted with rF-CEA/TRICOM. (E) Mice primed with rMVA-CEA/TRICOM and boosted with rF-CEA/TRICOM.

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Table 1
Prime and boost vaccination at the same site versus a distal site boost enhanced induction of antigen-specific immunity.

Prime Vaccination Site ¹	Boost Vaccination Site	CEA-Specific CD4 ⁺ Proliferation (CPM) ²	CEA-Specific CD8 ⁺ IFN- γ Production (pg/ml) ³
rV-CEA/TRICOM	rF-CEA/TRICOM		
None	None	200 $\pm$ 98	50
Left	None	2423 $\pm$ 198	80
Left	Right	2598 $\pm$ 204	1x
Left	Left	4507 $\pm$ 310 *	2.5x
rMVA-CEA/TRICOM	rF-CEA/TRICOM		
None	None	200 $\pm$ 98	50
Left	None	698 $\pm$ 204	100
Left	Right	2450 $\pm$ 202	1x
Left	Left	8608 $\pm$ 303 **	11.6x

^{*} Significantly greater ($P < 0.01$) CEA-specific proliferation than other groups primed with rV-CEA/TRICOM.

^{**} Significantly greater ($P < 0.01$) CEA-specific proliferation than all other groups primed with rV-CEA/TRICOM or rMVA-CEA/TRICOM

¹ CEA-Tg mice were vaccinated with rV-CEA/TRICOM or MVA-CEA/TRICOM followed by rF-CEA/TRICOM 7 days later on the same (left) or contralateral (right) side.

² Three weeks after the last vaccination, spleens were harvested and splenocytes were assayed for CEA-specific CD4⁺ T-cell proliferation. Purified CD4⁺ T cells were cultured with irradiated APCs and CEA protein for 5 days. [³H] thymidine (1 μ Ci/well) was added to the wells for the last 24 h and proliferation was assayed by measuring incorporated radioactivity.

³ Three weeks after the last vaccination, spleens were harvested and splenocytes were assayed for CEA-specific CD8⁺ T-cell IFN- γ production. Splenocytes from a single group were dispersed into single-cell suspensions, pooled, and coincubated with 10 μ g/ml of the H-2D^b-restricted 8-mer peptide CEA526-533 for 6 days. Bulk lymphocytes were recovered and restimulated with 10 μ g/ml of either CEA526-533 peptide or VSV-N52-59 negative control peptide for 24 h. Supernatant fluid was collected and analyzed for murine IFN- γ by cytometric bead array.