Published in final edited form as:

Cancer Immunol Immunother. 2012 November ; 61(11): . doi:10.1007/s00262-012-1248-y.

Co-delivery of antigen and IL-12 by Venezuelan equine encephalitis virus replicon particles enhances antigen-specific immune responses and anti-tumor effects

Takuya Osada¹, Peter Berglund⁵, Michael A. Morse^{2,4}, Bolyn Hubby⁵, Whitney Lewis⁶, Donna Niedzwiecki⁴, Amy Hobeika¹, Bruce Burnett⁴, Gayathri R. Devi^{1,4}, Timothy M. Clay^{1,3,4}, Jonathan Smith⁵, and H. Kim Lyerly^{1,3,4}

¹Duke University Medical Center, Department of Surgery, Durham, NC 27710, U.S.A

²Duke University Medical Center, Department of Medicine, Durham, NC 27710, U.S.A

³Duke University Medical Center, Department of Immunology, Durham, NC 27710, U.S.A

⁴Duke Comprehensive Cancer Center, Durham, NC 27710, U.S.A

⁵Liquidia Technologies, RTP, NC 27709

⁶Precision Biosciences, Durham, NC 27701, U.S.A

Abstract

We recently demonstrated that Venezuelan equine encephalitis (VEE) virus-based replicon particles (VRP) encoding tumor antigens could break tolerance in the immunomodulatory environment of advanced cancer. We hypothesized that local injection of VRP expressing Interleukin-12 (IL-12) at the site of injections of VRP-based cancer vaccines would enhance the tumor-antigen-specific T cell and antibody responses and anti-tumor efficacy. Mice were immunized with VRP encoding the human tumor-associated antigen, carcinoembryonic antigen (CEA) (VRP-CEA(6D)) and VRP-IL-12 was also administered at the same site or at a distant location. CEA-specific T cell and antibody responses were measured. To determine antitumor activity, mice were implanted with MC38-CEA-2 cells and immunized with VRP-CEA with and without VRP-IL-12 and tumor growth and mouse survival were measured. VRP-IL-12 greatly enhanced CEA-specific T cell and antibody responses when combined with VRP-CEA(6D) vaccination. VRP IL-12 was superior to IL-12 protein at enhancing immune responses. Vaccination with VRP-CEA(6D) plus VRP-IL-12 was superior to VRP-CEA(6D) or VRP-IL-12 alone in inducing anti-tumor activity and prolonging survival in tumor-bearing mice. Importantly, local injection of VRP-IL-12 at the VRP-CEA(6D) injection site provided more potent activation of CEA-specific immune responses than VRP-IL-12 injected at a distant site from the VRP-CEA injections. Together, this study shows that VRP-IL-12 enhances vaccination with VRP-CEA(6D) and was more effective at activating CEA-specific T cell responses when locally expressed at the vaccine site. Clinical trials evaluating the adjuvant effect of VRP-IL-12 at enhancing the immunogenicity of cancer vaccines are warranted.

Corresponding Author: H. Kim Lyerly, MD, Duke University Medical Center, Department of Surgery, Box 2606 MSRB1 Rm 433b Research Dr, Durham, NC 27710, Lyerl001@mc.duke.edu, Tel: 919-684-5613.

Conflict of Interest

Peter Berglund, Bolyn Hubby, Whitney Lewis, and Jonathan Smith were previously employed by Alphavax, Inc. Takuya Osada, Michael A. Morse, Donna Niedzwiecki, Amy Hobeika, Bruce Burnett, Gayathri R. Devi, Timothy M. Clay, and H. Kim Lyerly have no conflict of interest.

Keywords

Interleukin-12; cancer vaccine; CEA; alphavirus

Background

Viral vectors that encode tumor-associated antigens (TAAs) are attractive for cancer immunotherapy because they may be engineered to deliver whole antigens containing multiple CD4⁺ and CD8⁺ T cells epitopes and to produce cytokine (1,2) and immunomodulatory molecules that enhance immune responses. They also infect dendritic cells (DC) and induce DC maturation through both TLR-dependent and -independent pathways, resulting in upregulation of costimulatory molecules and secretion of Th1-inducing cytokines (3,4).

Among the many recombinant viral vectors that have been developed, Venezuelan equine encephalitis (VEE) virus-like replicon particles (VRPs) possess intriguing characteristics including significant expression of the inserted gene in infected cells (6), induction of both humoral and cell mediated immunity (7), potential for repeated immunizations despite induction of vector-specific neutralizing antibody, and potential tropism for DCs. VRP infect both murine and human DC (8,9) and VRP-transduced DCs efficiently process and present VRP-encoded antigens, leading to robust proliferation of antigen-specific T cells and acquisition of effector functions. These in vitro and in vivo effects are thought to be due in part to the maturation of DC by VRP including the up-regulation of costimulatory molecules CD40, CD80, CD86, the maturation marker CD83 (9,10) and the secretion of proinflammatory cytokines including IFN- γ , TNF- α , IL-6, and IL-12p70 (11). We have generated a recombinant VRP expressing a well-characterized human tumor associated antigen (carcinoembryonic antigen (CEA)) (VRP-CEA(6D)) and demonstrated that it was effective in inducing CEA-specific immune responses in cancer patients (12). Nonetheless, the modest magnitude of the immune response and the requirement for multiple immunizations suggested that these vectors could be enhanced.

The use of immunostimulatory cytokines to enhance immune responses has been well established. Many of these were systematically analyzed in the context of gene-modified tumor vaccines, in which the many innate immune stimulatory signals inherent to viral vectors were not present (13). We hypothesized that IL-12, delivered in the context of the potent immune stimulatory signals provided by VRP, might enhance antitumor immune responses when combined with VRP-expressing CEA in murine tumor models. IL-12 plays a key role in the differentiation of type-1 helper T cells (Th1) and enhances CTL responses through its effects on inducing IFN- γ production (14). Indeed, in preliminary studies, we downselected from a series of novel recombinant alphaviral VRP expressing cytokines and growth factors, and identified that VRP expressing IL-12 (VRP-IL-12) was the most potent in enhancing adaptive immune responses to CEA (Peter Bergslund, unpublished observations) when delivered in combination with VRP-expressing CEA. In the current study, we performed a more detailed analysis of the effect of the VRP-IL-12 co-injected with VRP-CEA compared with VRP-IL-12 injected at a distance on enhancing immune responses and anti-tumor immunity in mice harboring CEA-expressing colon cancer.

Materials and Methods

Reagents

Recombinant murine IL-12 protein was purchased from BioVision (Mountain View, CA). Recombinant Influenza Hemagglutinin (HA) protein and carcinoembryonic antigen (CEA(6D)) protein were purchased from Protein Sciences (Meriden, CT).

PE-labeled anti-mouse CD80, anti-mouse CD86, anti-mouse IL-12, FITC-labeled anti-mouse CD14, and APC-labeled anti-mouse I-A/I-E monoclonal antibodies were all purchased from BioLegend (San Diego, CA).

Cell lines

The murine colon adenocarcinoma cell line, MC38-CEA-2, expressing human CEA was kindly provided by Dr. Jeffrey Schlom (National Cancer Institute)(15). Cells were cultured in the complete Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum.

Mice

C57BL/6 mice and BALB/c mice were purchased from Jackson Labs (Bar Harbor, ME) and bred in the Duke Comprehensive Cancer Center Isolation Facility. All animal studies described were approved by the Duke University Medical Center Institutional Animal Care & Use Committee, in accordance with guidelines published by the Commission on Life Sciences of the National Research Council. For in vivo tumor model, C57BL/6 mice were used with MC38-CEA-2 cell line that has C57BL/6 background.

Generation of VRP

Generation of VRP-CEA(6D) (an alphaviral replicon particle (VRP) expressing the tumor antigen CEA(6D) in which an Asn to Asp substitution has been made in the HLA A2-restricted epitope within the CEA gene that results in enhanced T cell receptor recognition was as described elsewhere (12,16,17). The CEA Replicon Map is shown in Supplementary Figure 1A. To generate VRP-IL-12, a replicon plasmid was constructed by cloning the p40 and p35 subunit genes in frame separated by a short linker sequence derived from the elastin gene. This was done by subcloning the p40-p35 gene from mIL-12elasti p40::p35 (Invivogen, San Diego, California) using primers designed to contain flanking EcoRV and AscI restriction sites. The murine IL-12 gene was then cloned into the pERK3 replicon vector as an EcoRV/AscI fragment. Replicon plasmids containing the p40 and p35 genes alone were constructed using the same cloning method. VRP were produced as previously described (16,17).

Infection of cells with VRP-IL-12

Murine dendritic cells were generated from bone marrow of C57BL/6 mice by culturing cells in the presence of murine GM-CSF (10 ng/mL, Invitrogen) and IL-4 (10 ng/mL, Invitrogen) for 10 days. DCs were infected with VRP-IL-12 at moi 10 or moi 100, or empty vector (VRP-Empty) at moi 100. For positive control sample, lipopolysaccharide (1µg/mL, Sigma, St. Louis, MO) was added to the medium. The DCs were incubated for 18–24 h, and DC expression and secretion of IL-12 were analyzed by flow cytometry and ELISA, respectively.

Flow Cytometry

For intracellular staining of IL-12, VRP-IL-12 infected DCs were incubated with Brefeldin A (BD) for 4h and fixed/permeabilized with FACS™ Permeabilizing Solution 2 (BD). The

DCs' Fcγ receptors were blocked with anti-mouse CD16/CD32 (Mouse BD Fc Block, BD) and stained with anti-mouse IL-12, CD14, MHC Class II CD80 and CD86 antibody. The maturation status of DC was assessed by evaluating the CD14-negative/MHC class II-positive DCs for expression of CD80 and CD86 by flow cytometry using a FACSCalibur or LSR II (BD Biosciences, San Jose, CA).

IL-12 ELISA

Culture supernatants from VRP-IL-12 infected DC or VRP-Empty infected DC were analyzed for IL-12 p70 level using Quantikine mouse IL-12 p70 ELISA kit (R&D Systems, Minneapolis, MN).

Vaccination of mice

For vaccine immunogenicity studies, varying doses of VRP-CEA(6D) (and in some experiments, CEA protein) were injected into the footpad of mice (20 microliters per foot pad), twice, three weeks apart. Mice were sacrificed seven days after the second immunization and serum and splenocytes were collected to assess for anti-CEA antibody by ELISA and CEA-specific lymphocytes using an IFN γ ELISPOT assay (Mabtech) as described below. In some experiments, VRP-IL-12 (at various IUs) was co-injected with the VRP-CEA(6D). In other experiments, IL-12 protein (1, 10, 100 ng/mouse) was coinjected.

Comparison of co-injection and contralateral injection of VRP-IL-12 and VRP-CEA(6D)

Mice were vaccinated with VRP-CEA(6D) (5×10^7 I.U) and VRP-IL-12 (5×10^6 I.U) by co-injection of a mixture of these two vectors, or by separate injection of VRP-CEA(6D) and VRP-IL-12 into different limbs (contralateral injection). As controls, mice were vaccinated with VRP-CEA(6D) alone, VRP-IL-12 alone or saline. Two weeks after the vaccination, anti-CEA immune response was analyzed by an IFN γ ELISPOT assay using splenocytes.

ELISA for anti-CEA antibodies

Mouse sera were collected and frozen at -80°C until use. Microtiter plates (Immulon 4HBX, Thermo Scientific, Rochester, NY) were coated with CEA peptide mix or CMVpp65 peptide mix (BD Bioscience) at concentration of 2.6 $\mu\text{g/ml}$. Plates were incubated at 4°C overnight, washed with PBS, and blocked with 1% BSA in PBS for 1 h. Thawed sera were diluted with the titration of 1:40 to 1:320,000 with 1% BSA in PBS, added to each well and incubated for 2 h. Sera from naïve mice (saline injected mice) were used as a negative control. Plates were developed with 100 μl of a 1:2500 dilution of sheep alkaline phosphatase-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 37°C . Plates were washed three times and 50 μl per well of the pNPP substrate solution (5 mg/ml, p-nitrophenyl phosphate, Sigma) were added to each well. The reaction was stopped by adding 50 μl per well of NaOH solution. Absorbance was measured at 405 nm.

ELISpot Analysis

Mouse IFN γ ELISPOT assay (Mabtech Inc., Cincinnati, OH) was performed according to the manufacturer's instructions. Splenocytes (500,000 cells/well) were incubated in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, and CMVpp65 peptide mix or CEA peptide mix (2.6 $\mu\text{g/ml}$; BD Bioscience, San Jose, CA) was used as a stimulating antigen. Peptide mixes consist of 15-mers overlapping by 11 amino acids and cover the entire protein sequence. HIV peptide mix (BD Bioscience) was used as a negative control, and a mixture of PMA (50 ng/ml) and Ionomycin (1 $\mu\text{g/ml}$) as a positive control of the assay.

Effect of VRP-IL-12 Vaccine in MC38 Tumor-bearing Mice

To analyze functional anti-tumor responses *in vivo*, C57BL/6 mice were inoculated with MC38-CEA-2 tumor cells (2.5 M cells/mouse) into the flank and 5 days later mice were randomized into 4 groups (10 mice/group): A: VRP-CEA + VRP-IL-12, B: VRP-Empty + VRP-IL-12, C: VRP-CEA + VRP-Empty, D: VRP-Empty only. The VRP-CEA(6D) and VRP-IL-12 (5×10^7 I.U./mouse) were administered at 5×10^7 I.U./mouse. The total amount of VRP vectors inoculated into each mouse at each injection timepoint was intended to total 1×10^8 I.U., and thus VRP-Empty vector was added to the vaccine inocula which contained only a single vector to adjust to a total number of 1×10^8 I.U. Two weeks after the first vaccination (on day 19), the same combination of vaccine was re-injected into the footpads. Tumor size was measured twice weekly. Mice were euthanized when their tumor size exceeded 2,000 mm³. Survival of mice was also monitored until all the groups had less than 50% survival.

Statistical Analysis

For the ELISpot and ELISA assays, differences in IFN- γ production and antibody binding, respectively, were analyzed using the Student's t test. The differences in tumor volumes for each vaccine strategy compared to control (VRP-Empty only) were assessed by Student's t test at each time point of tumor volume measurements. For the analysis of survival, Kaplan-Meier survival estimates were performed to depict the survival curves by treatment. The Log Rank test was performed to compare the survival between different treatment groups. For all experiments, differences at $P < 0.05$ were considered statistically significant.

Results

Infection of cells with VRP-CEA(6D) induces CEA expression with appropriate translocation and post-translational modification

To test the ability of the VRP-CEA(6D) to infect cells and express CEA, we infected Vero cells with VRP-CEA(6D) and evaluated CEA expression by immunofluorescent staining or flow cytometry with anti-CEA mAb. CEA staining was localized intracellularly as well as on the plasma membrane. The non-cytoplasmic, reticular pattern of the intracellular signal was consistent with biosynthesis through ER-Golgi for secretion (Supplementary Fig. 1B). CEA expression by VRP-CEA infected mouse DC was also demonstrated by flow cytometry (data not shown). We confirmed that full length CEA protein was expressed from the CEA replicon and yielded a shorter fragment consistent with the putative 76.8 kDa molecular weight protein when deglycosylated (Supplementary Fig. 1C). These data demonstrate that CEA is appropriately translocated and post-translationally modified, and transported through the Golgi to the plasma membrane.

Induction of anti-CEA immune response by VRP-CEA(6D) vaccination in animal models

BALB/c were immunized with VRP-CEA(6D) in order to assess the cellular and humoral immune responses. As demonstrated in Figure 1A and 1B, there was a dose/response effect such that at increasing doses of VRP, there was an increasing titer of anti-CEA antibody and anti-CEA-specific T cells. Induction of immune responses was consistent in C57BL/6 mice immunized with a similar protocol (data not shown). These data demonstrate that both antibody and T cell responses are activated by the vector and that the maximum biologic dose was not reached (at 5×10^7 I.U.).

Enhancing VRP immunization by co-delivery of growth factors and cytokines

We generated a VRP construct that expressed IL-12 in the form of a single polypeptide where the p35 and p40 subunits are fused into one open reading frame separated by a short

linker sequence derived from the elastin gene (See Supplementary Fig. 2A for map). Thus, the construct expresses a monomeric form of the p70 IL-12 molecule. Vero cells infected with VRP-IL-12 secreted significant amounts of IL-12 p70 compared to Vero cell lysates spiked with IL-12 protein (data not shown). To confirm the expression of IL-12 p70 in a monomeric form by VRP-IL-12 infected cells, Vero cells at 16 h after infection with VRP-IL-12, were lysed and processed for SDS-PAGE, and Western blot analysis was conducted using an IL-12-specific antibody. Recombinant murine IL-12 (rIL-12) was mixed with cell lysate from uninfected cells, for comparison purpose. rIL-12 migrates as two bands, corresponding to the separation of the p40 and p35 subunits in this denaturing gel, whereas the VRP-expressed monomeric fusion polypeptide appears as a 70 kDa band, as designed (Supplementary Fig. 2B).

VRP-IL-12 infection of DC leads to cytokine secretion and maturation of DC

We have previously shown that IL-12 is a biomarker for DC maturation in a subset of DC (18). Therefore, we analyzed murine bone marrow derived DC infected with VRP-IL-12 for IL-12 expression and for maturation markers. As shown in Figure 2A, VRP-IL-12 infection of DC induced IL-12 secretion greater than DC stimulated with lipopolysaccharide (LPS), while VRP-Empty infection had no effect on IL-12 production by DC. Intracellular staining confirmed IL-12 production by CD14-negative/MHC class II-positive DC after 24 and 48 hours of infection (Fig. 2B). Maturation markers (CD80, CD86) were analyzed by flow cytometry. Interestingly, both VRP-Empty vector (moi 100) and VRP-IL-12 (moi 100) enhanced CD86 expression following infection (Supplementary Fig. 3). These data suggest that VRP vectors upregulate CD86 but only those vectors containing IL-12 caused IL-12 secretion.

Enhanced Antigen-specific cellular immune responses following immunization with VRP vaccines combined with VRP-IL-12

In order to determine if VRP-IL-12 could enhance the immune response to VRP-CEA(6D), we administered either VRP-CEA(6D) (5×10^5 I.U.) or VRP-IL-12 (ranging from 5×10^1 to 5×10^7 I.U.) or a mixture of the two vectors. Study groups receiving more than 5×10^2 I.U. of VRP-IL-12 displayed significantly higher levels of IFN-gamma-secreting antigen-specific lymphocyte responses than mice immunized with VRP-CEA(6D) alone (Fig. 3A). When co-administered with VRP-CEA(6D) (5×10^5 I.U.), the strongest cellular immune responses against CEA were seen in mice receiving 5×10^5 I.U. of VRP-IL-12. The enhancement in cellular immune responses was not due merely to an increase in the VRP-delivered replicons since mock adjuvants consisting of empty VRP (VRP-Empty) did not enhance the CEA-specific responses to the VRP-CEA(6D) vaccine component (Fig. 3A).

Unexpectedly, higher levels of VRP-IL-12 appeared to cause a significant decrease in CEA-specific T cell responses. In order to rule out the possibility that VRP-IL-12 could be immunosuppressive at the highest dose (5×10^7 I.U.), we immunized mice with VRP-CEA(6D) and VRP-IL-12, at escalating doses of both components from 5×10^4 up to 5×10^7 I.U., while keeping the ratio between them constant (Fig. 3B, left side). Dose-dependent increases in the levels of antigen-specific T-cell responses, even up to the highest dosage levels tested (Fig. 3B), were observed. These results suggest that the inhibitory effects of IL-12 only manifest themselves when co-administered together with a significantly lower dose of CEA-VRP.

VRP-IL-12 induces a more potent immune response than IL-12 protein

In order to compare the adjuvant activity of VRP-IL-12 with IL-12 protein, mice were immunized with VRP-CEA(6D) (5×10^5 I.U.) combined with recombinant baculovirus-produced IL-12 protein at escalating doses. The dosage levels ranged from 1 ng to 100 ng

and were chosen because Vero cells (5×10^5) infected with 5×10^5 I.U. of VRP-IL-12 were found to produce approximately 10 ng of IL-12 (data not shown). As seen in Figure 4A, recombinant soluble IL-12 protein did not augment the anti-CEA immune responses at the doses used whereas VRP-IL-12 displayed significant adjuvant activity.

We also carried out experiments to assess the ability of VRP-IL-12 to adjuvant immune responses to recombinant CEA protein. This study showed that 5×10^5 IU of VRP-IL-12 enhanced the humoral as well as cell mediated anti-CEA immune responses, indicating wider applicability of VRP-IL-12 as an adjuvant for vaccine with various platforms (Supplementary Fig. 4).

To determine whether enhancement of antigen-specific immune response induced by VRP-IL-12 is a local effect at the injection site or a systemic effect of generated IL-12, mice were vaccinated with VRP-CEA(6D) and VRP-IL-12 by co-injection or contralateral injection. Although there was a vaccine adjuvant effect from the release of IL-12 systemically as can be seen by contralateral injection of VRP-IL12 and VRP-CEA(6D) (Fig. 4B), co-injection induced a stronger enhancement of CEA-specific T cell responses- than contralateral injection did, suggesting that the local effects of the IL-12 predominate over the systemic effect of IL-12 derived from the VRP-IL-12.

VRP-IL-12 enhances anti-tumor responses against VRP-CEA(6D)

To assess the adjuvant effect of VRP-IL-12 on therapeutic cancer vaccines, we compared VRP-CEA(6D) vaccine with or without VRP-IL-12 in C57BL/6 mice implanted with MC38-CEA-2, a murine colon cancer cell line that expresses human CEA antigen at low level. Mice (10 per group) were inoculated with 2.5×10^6 MC38-CEA-2 cells on day 0, and on day 5 and day 19, vaccination was performed with VRP-CEA(6D), VRP-IL-12, the combination of the two and VRP-empty as a control. No toxicity was observed in these animals. We analyzed the serum level of IL-12 in mice that were treated with a single injection of VRP-IL12 (5×10^7 I.U.) by ELISA. We confirmed significant serum concentrations of IL-12 a week after VRP-IL12 injection (approximately 25 ng/mL on day 1, and 5 ng/mL on day 7).

As expected, ELISPOT analysis of splenocytes and ELISA of serum showed enhanced CEA-specific responses from tumor-bearing mice vaccinated with VRP-CEA(6D)/VRP-IL-12 which was superior to the mice vaccinated with VRP-CEA(6D) alone (Figs. 5A,B). VRP-IL-12 treatment alone did not induce enhanced CEA-specific cellular and humoral responses. These data suggest that the VRP-IL-12 may enhance immune responses in the tumor bearing host.

Regarding antitumor effects in this model of established disease, significantly lower tumor growth was observed when VRP-IL-12 was co-administered with VRP-CEA compared with VRP-CEA alone (Fig. 6A). In addition, there was a trend for a reduction in tumor growth for the combination of VRP-CEA and VRP-IL-12 compared with VRP-IL-12 alone. The experiment was repeated twice, with very similar trend, although the difference between VRP-IL-12 alone and the combination of VRP-CEA(6D) and VRP-IL-12 at time point of day 27 has a p value of 0.15. Survival was monitored until all groups had fewer than 50% mice remaining alive. No metastatic lesions were observed when mice were euthanized. Kaplan-Meier survival estimates of all of the data is shown in Figure 6B. The co-injection of VRP-CEA(6D) and VRP-IL-12 resulted in significantly better survival compared with VRP-CEA alone and VRP-IL-12 alone ($p=0.011$).

Discussion

Viral replicon particles (VRPs) represent a novel strategy to simultaneously deliver both TAAs and a strong maturation signal to antigen-presenting cells such as DCs. Nonetheless, it is likely that this strategy will be applied in cancer patients that may have significant immunoregulatory dysregulation due to their cancer burden, and strategies to augment immune responses may be critical for efficacy. Therefore, an evaluation of additional signals that could enhance immune responses to these recombinant viral vectors was undertaken. We were most interested in IL-12 because IL-12 plays a key role in the differentiation of type-1 helper T cells (Th1) cells from uncommitted T (TH0) cells during an immune response, enhances CTL responses (through its effects on inducing IFN- γ production), and enhances NK cell function (19–22).

In human clinical trials of various malignancies, systemic IL-12 has been administered alone (23–32). Systemic IL-12 has also been utilized in combination with cancer vaccines including a gp100 human melanoma peptide vaccine (33). Although there has been considerable negative data with the use of IL-12 as a vaccine adjuvant, two studies have demonstrated enhancement of immune responses to peptide vaccines in melanoma (34,35).

The use of systemic rhIL-12, however, is limited by toxicity and tachyphylaxis. Toxicity (fatigue, hypotension, hepatic enzyme elevation, hemolytic anemia, gastrointestinal bleeding, lymphopenia, and neutropenia) is dose and schedule dependent, likely related to the rapid elevation of IFN- γ plasma levels. Several deaths were reported during the initial studies with IL-12 given on a more intense schedule (36). Therefore, co-administration along with a vaccine may be preferred to avoid systemic toxicity of IL-12 protein. We observed significant concentrations of IL-12 in mouse sera a week after VRP-IL12 injection; however these serum concentrations of IL-12 are lower than would be expected with systemic treatment with recombinant IL-12 protein (50–70 ng/mL; 5h after subcutaneous injection of 1 μ g IL-12 protein) (37). Furthermore, the VRP-IL-12 vector is expected to induce a slow rise of IL-12 in the serum compared to protein administration, because of the time required for delivery of the transgene and protein production and secretion by the target cells. Thus, toxicities induced by VRP-IL12 are expected to be much less than systemic injection of recombinant IL-12. Indeed, we did not observe significant toxicity in the mice in our studies. In addition, a strategy to prolong IL-12 production at the site of the vaccine could prolong the immune response. In fact, our mouse experiments demonstrated that co-administration of VRP-IL-12 with VRP-CEA(6D) induced significantly stronger anti-CEA immune response than co-injection of recombinant IL-12 protein (Fig. 4A), contralateral injection of these two vectors or vaccination with VRP-CEA(6D) alone (Fig. 4B), suggesting the benefit of local delivery of VRP-IL-12. Co-injection of VRP-IL12 is more efficient than recombinant IL-12 to stimulate CTL induction by VRP-CEA(6D) infected DC at the lymph nodes, probably because ongoing IL-12 production by VRP-IL12-infected cells would have a longer paracrine effect than recombinant IL-12 protein which would diffuse away, lowering the local concentrations over time.

This finding of enhanced immune response with co-delivery of IL-12 was intriguing particularly with the VEE based vaccine, as these particular vectors have been reported to infect and mature DC, but cytokine production by the VEE-infected DC was modest (9). In fact, previously, we noted that only a small subset of DC can produce high levels of IL-12, despite multiple signals (18). Consequently, we examined the use of VRP-IL-12 to confer IL-12 production on human and mouse DC and demonstrated this was vastly superior to VRP infection alone in producing high levels of IL-12.

Regarding our choice of model to test the effects of VRP-IL-12 on anti-tumor immune responses, we used the MC38-CEA-2 cell line as the tumor target, wild-type mice (C57BL/6), and VRP-CEA(6D) as the vaccine. We chose the C57BL/6 mice rather than CEA-transgenic mice because the MC38 cell line was derived from mice with a C57BL/6 background whereas the commercially available CEA-transgenic models are of a BALB/c background. The MC38-CEA-2 cell line expresses CEA at low levels which could explain the low anti-tumor activity of the VRP-CEA(6D) vaccine alone; however we were most interested in the adjuvant effect of IL-12 and therefore wished to use a model with more moderate anti-tumor responses to the vaccine as could be observed in patients with cancer.

We then wished to determine the ideal conditions for combining the VRP-IL-12 with the VRP-CEA(6D). There was a range of ratios of the two vectors that was effective in inducing CEA-specific immune responses (Fig. 3A), and subsequently we found that the inhibitory effects of IL-12 only manifest themselves when co-administered together with a significantly lower dose of vaccine VRP (Fig. 3B). The mechanism for this effect is not clear but might be a result of interferon released in response to the vaccination resulting in silencing of the administered VRP, which would notably affect the VRP present at the lower titer. These results further indicate that a range of ratios between the antigen and the adjuvant VRP components should be tested, and ratios shown to be effective should be used in combination with other vaccines as a potent and promising adjuvant.

Importantly, the combination of VRP-CEA(6D) and VRP-IL-12 significantly enhanced anti-CEA response and anti-tumor response compared to VRP-CEA(6D) alone, and prolonged survival of mice compared to VRP-CEA(6D) vaccine alone or VRP-IL-12 alone. These results clearly demonstrate beneficial adjuvant effect of VRP-IL-12 for cancer vaccines.

In summary, we screened a variety of cytokines and hematopoietic growth factors that were co-delivered in combination with VRP based vaccines, and found that IL-12 was most potent in enhancing antigen specific immune responses and anti-tumor responses. We found that a clinically feasible ratio of IL-12 and antigen delivery could be identified in preclinical studies. Enhancing VEE based anti-cancer vaccines by co-delivery with VEE expressing IL-12 may be an important enhancement of cancer immunotherapy to be tested in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Yang S, Kittlesen D, Slingluff CL, Vervaert CE, Seigler HF, Darrow TL. Dendritic cells infected with a vaccinia vector carrying the human gp100 gene simultaneously present multiple specificities and elicit high affinity T cells reactive to multiple epitopes and restricted by HLA-A2 and -A3. *J Immunol.* 2000; 164:4204–11. [PubMed: 10754316]
2. zum Büschenfelde CM, Metzger J, Hermann C, Nicklisch N, Peschel C, Bernhard H. The generation of both T killer and Th cell clones specific for the tumor-associated antigen HER2 using retrovirally transduced dendritic cells. *J Immunol.* 2001; 167:1712–9. [PubMed: 11466395]
3. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappa B by Toll-like receptor 3. *Nature.* 2001; 413:732–8. [PubMed: 11607032]
4. Diebold SS, Montoya M, Unger H, Alexopoulou L, Roy P, Haswell LE, et al. Viral infection switches non-plasmacytoid dendritic cells into high interferon producers. *Nature.* 2003; 424:324–8. [PubMed: 12819664]

5. Yang Y, Huang CT, Huang X, Pardoll DM. Persistent Toll-like receptor signals are required for reversal of regulatory T cell-mediated CD8 tolerance. *Nat Immunol.* 2004; 5:508–15. [PubMed: 15064759]
6. Pushko P, Parker M, Ludwig GV, Davis NL, Johnston RE, Smith JF. Replicon-helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo. *Virology.* 1997; 239:389–401. [PubMed: 9434729]
7. Caley IJ, Betts MR, Irlbeck DM, Davis NL, Swanstrom R, Frelinger JA, et al. Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. *J Virol.* 1997; 71:3031–8. [PubMed: 9060663]
8. MacDonald GH, Johnston RE. Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. *J Virol.* 2000; 74:914–22. [PubMed: 10623754]
9. Moran TP, Collier M, McKinnon KP, Davis NL, Johnston RE, Serody JS. A novel viral system for generating antigen-specific T cells. *J Immunol.* 2005; 175:3431–8. [PubMed: 16116238]
10. Gardner JP, Frolov I, Perri S, Ji Y, MacKichan ML, zur Megede J, et al. Infection of human dendritic cells by a sindbis virus replicon vector is determined by a single amino acid substitution in the E2 glycoprotein. *J Virol.* 2000; 74:11849–57. [PubMed: 11090185]
11. Mazzoni A, Segal DM. Controlling the Toll road to dendritic cell polarization. *J Leukoc Biol.* 2004; 75:721–30. [PubMed: 14726500]
12. Morse MA, Hobeika A, Osada T, Berglund P, Negri S, Niedzwiecki D, et al. A Novel Recombinant Alphaviral Vector Breaks Tolerance To Self-Antigen In The Setting Of Elevated Regulatory T Cells. *J Clin Invest.* 2010; 120:3234–41. [PubMed: 20679728]
13. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, et al. Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A.* 1993; 90:3539–43. [PubMed: 8097319]
14. Trinchieri G. Proinflammatory and immunoregulatory functions of interleukin-12. *Int Rev Immunol.* 1998; 16:365–96. [PubMed: 9505196]
15. Robbins PF, Kantor JA, Salgaller M, Hand PH, Fernsten PD, Schlom J. Transduction and expression of the human carcinoembryonic antigen gene in a murine colon carcinoma cell line. *Cancer Res.* 1991; 51:3657–62. [PubMed: 1712245]
16. Talarico T, Maughan M, Pancorbo B, Ruiz J, Graham A. Development and manufacture of alphavaccines. *Bioprocessing.* 2006 Fall;8–14.
17. Hubby B, Talarico T, Maughan M, Reap EA, Berglund P, Kamrud KI, et al. Development and preclinical evaluation of an alphavirus replicon vaccine for influenza. *Vaccine.* 2007; 25:8180–9. [PubMed: 17961878]
18. Mosca PJ, Hobeika AC, Clay TM, Nair SK, Thomas EK, Morse MA, et al. A subset of human monocyte-derived dendritic cells expresses high levels of interleukin-12 in response to combined CD40 ligand and interferon-gamma treatment. *Blood.* 2000; 96:3499–504. [PubMed: 11071647]
19. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat Rev Immunol.* 2003; 3:133–46. [PubMed: 12563297]
20. Robertson MJ, Soiffer RJ, Wolf SF, Manley TJ, Donahue C, Young D, et al. Response of human natural killer (NK) cells to NK cell stimulatory factor (NKSF): cytolytic activity and proliferation of NK cells are differentially regulated by NKSF. *J Exp Med.* 1992; 175:779–88. [PubMed: 1346796]
21. Grufman P, Kärre K. Innate and adaptive immunity to tumors: IL-12 is required for optimal responses. *Eur J Immunol.* 2000; 30:1088–93. [PubMed: 10760797]
22. Gherardi MM, Ramírez JC, Esteban M. Towards a new generation of vaccines: the cytokine IL-12 as an adjuvant to enhance cellular immune responses to pathogens during prime-booster vaccination regimens. *Histol Histopathol.* 2001; 16:655–67. [PubMed: 11332721]
23. Little RF, Pluda JM, Wyvill KM, Rodriguez-Chavez IR, Tosato G, Catanzaro AT, et al. Activity of subcutaneous interleukin-12 in AIDS-related Kaposi sarcoma. *Blood.* 2006; 107:4650–7. [PubMed: 16507779]

24. Lenzi R, Rosenblum M, Verschraegen C, Kudelka AP, Kavanagh JJ, Hicks ME, et al. Phase I study of intraperitoneal recombinant human interleukin 12 in patients with Müllerian carcinoma, gastrointestinal primary malignancies, and mesothelioma. *Clin Cancer Res.* 2002; 8:3686–95. [PubMed: 12473577]
25. Younes A, Pro B, Robertson MJ, Flinn IW, Romaguera JE, Hagemester F, et al. Phase II clinical trial of interleukin-12 in patients with relapsed and refractory non-Hodgkin's lymphoma and Hodgkin's disease. *Clin Cancer Res.* 2004; 10:5432–8. [PubMed: 15328181]
26. Robertson MJ, Pelloso D, Abonour R, Hromas RA, Nelson RP Jr, Wood L, et al. Interleukin 12 immunotherapy after autologous stem cell transplantation for hematological malignancies. *Clin Cancer Res.* 2002; 8:3383–93. [PubMed: 12429625]
27. Peeva E, Fishman AD, Goddard G, Wadler S, Barland P. Rheumatoid arthritis exacerbation caused by exogenous interleukin-12. *Arthritis Rheum.* 2000; 43:461–3. [PubMed: 10693889]
28. Wadler S, Levy D, Frederickson HL, Falkson CI, Wang Y, Weller E, et al. A phase II trial of interleukin-12 in patients with advanced cervical cancer: clinical and immunologic correlates. Eastern Cooperative Oncology Group study E1E96. *Gynecol Oncol.* 2004; 92:957–64. [PubMed: 14984966]
29. Ansell SM, Witzig TE, Kurtin PJ, Sloan JA, Jelinek DF, Howell KG, et al. Phase 1 study of interleukin-12 in combination with rituximab in patients with B-cell non-Hodgkin lymphoma. *Blood.* 2002; 99:67–74. [PubMed: 11756154]
30. Parihar R, Nadella P, Lewis A, Jensen R, De Hoff C, Dierksheide JE, et al. A phase I study of interleukin 12 with trastuzumab in patients with human epidermal growth factor receptor-2-overexpressing malignancies: analysis of sustained interferon gamma production in a subset of patients. *Clin Cancer Res.* 2004; 10:5027–37. [PubMed: 15297404]
31. Eisenbeis CF, Lesinski GB, Anghelina M, Parihar R, Valentino D, Liu J, et al. Phase I study of the sequential combination of interleukin-12 and interferon alfa-2b in advanced cancer: evidence for modulation of interferon signaling pathways by interleukin-12. *J Clin Oncol.* 2005; 23:8835–44. [PubMed: 16314644]
32. Alatrash G, Hutson TE, Molto L, Richmond A, Nemec C, Mekhail T, et al. Clinical and immunologic effects of subcutaneously administered interleukin-12 and interferon alfa-2b: phase I trial of patients with metastatic renal cell carcinoma or malignant melanoma. *J Clin Oncol.* 2004; 22:2891–900. [PubMed: 15254058]
33. Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL, et al. Impact of cytokine administration on the generation of antitumor reactivity in patients with metastatic melanoma receiving a peptide vaccine. *J Immunol.* 1999; 163:1690–5. [PubMed: 10415076]
34. Hamid O, Solomon JC, Scotland R, Garcia M, Sian S, Ye W, et al. Alum with interleukin-12 augments immunity to a melanoma peptide vaccine: correlation with time to relapse in patients with resected high-risk disease. *Clin Cancer Res.* 2007; 13:215–22. [PubMed: 17200357]
35. Peterson AC, Harlin H, Gajewski TF. Immunization with Melan-A peptide-pulsed peripheral blood mononuclear cells plus recombinant human interleukin-12 induces clinical activity and T-cell responses in advanced melanoma. *J Clin Oncol.* 2003; 21:2342–8. [PubMed: 12805336]
36. Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood.* 1997; 90:2541–8. [PubMed: 9326219]
37. Rakhit A, Yeon MM, Ferrante J, Fettner S, Nadeau R, Motzer R, et al. Down-regulation of the pharmacokinetic-pharmacodynamic response to interleukin-12 during long-term administration to patients with renal cell carcinoma and evaluation of the mechanism of this “adaptive response” in mice. *Clin Pharmacol Ther.* 1999; 65:615–29. [PubMed: 10391667]

Figure 1. Induction of anti-CEA immune response by VRP-CEA(6D) vaccination in animal models

BALB/c were immunized twice with VRP-CEA(6D) (5×10^4 , 5×10^5 , 5×10^6 , or 5×10^7 I.U.) 3 weeks apart. Immune monitoring assays were performed 7 days after the second immunization. Seven or 8 mice were vaccinated for each group. **(A)** Anti-CEA antibody titers in the sera were analyzed by ELISA. Sera were diluted with the titration of 1:40 to 1:320,000 with 1% BSA in PBS, and incubated for 2 h in CEA protein-coated microtiter plate. Sera from naïve mice (saline injected mice) were used as a negative control. Plates were incubated with alkaline phosphatase-conjugated anti-mouse IgG antibody, and then developed with pNPP substrate solution. The reaction was stopped by NaOH solution, and absorbance was measured at 405 nm. Reciprocal titers of anti-CEA antibody are shown. Bar shows mean values for each group. **(B)** Mouse IFN γ ELISPOT assay was performed with splenocytes (1×10^6 cells/well). CEA peptide mix (2.6 μ g/ml: BD Bioscience, San Jose, CA) was used as a stimulating antigen. Each dot shows the number of IFN- γ -positive spots per 1×10^6 splenocytes for each mouse. Bars shows mean values for each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

2A

2B

Figure 2. VRP-IL-12 infection of DC leads to IL-12 secretion and maturation of DC

Murine dendritic cells were generated from bone marrow of mice by culturing cells in the presence of murine GM-CSF and IL-4 for 10 days. DCs were infected with VRP-IL-12 at MOI 10 or MOI 100, or empty vector (VRP-Empty) at MOI 100, and incubated for 18–24 h. Lipopolysaccharide (1 µg/mL) induced IL-12 secretion served as a positive control. (A) mIL-12 p70 ELISA was performed on culture supernatants. * $p < 0.001$, ** $p < 0.0001$ compared to mock DC. (B) VRP-IL-12 infected DCs (MOI 100) or LPS-treated DCs were incubated with Brefeldin A for 4h, fixed/permeabilized and stained with anti-mouse IL-12 antibody. Percentages of IL-12-positive populations are shown in each dot plot.

Figure 3. Enhanced vaccine effect by co-administration of VRP-IL-12

(A) Mice were co-administered twice with VRP-CEA(6D) (5×10^5 I.U.) and VRP-IL-12 or VRP-Empty (ranging from 5×10^1 to 5×10^7 I.U.). Seven days after the 2nd immunization, splenocytes were harvested and IFN- γ ELISPOT assay was performed. The strongest cellular immune responses were seen in mice receiving 5×10^5 I.U. of IL-12 VRP. * $p < 0.01$, ** $p < 0.0001$ compared to VRP-CEA alone. (B) Mice were co-administered with escalating amounts of VRP-CEA(6D) mixed with VRP-IL-12 at a ratio of 1:1 (ranging from 5×10^4 to 5×10^7 I.U.). In other mice, vaccination was performed with VRP-CEA(6D) (5×10^5 I.U.) and escalating amounts of VRP-IL-12 (ranging from 5×10^4 to 5×10^7 I.U.). IFN- γ ELISPOT assay was performed as in (A). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to VRP-CEA alone.

Figure 4. Co-administration of VRP-IL-12 induces a more potent immune response than IL-12 protein and is applicable to vaccine with CEA protein

(A) Mice were immunized with VRP-CEA(6D) (5×10^5 I.U) combined with VRP-IL-12 (5×10^5 I.U) or with recombinant baculovirus-produced IL-12 protein at escalating doses (1, 10, 100 ng/mouse). Immunization was repeated twice, 3 weeks apart. Immune monitoring assays were performed 7 days after the second immunization. Anti-CEA immune responses were analyzed by IFN γ -ELISPOT assay. Recombinant Influenza Hemagglutinin (HA) protein (100 ng/mouse) was used as a control for IL-12 protein. VRP-IL-12 enhanced anti-CEA response induced by VRP-CEA(6D) significantly stronger than IL-12 protein. * $p < 0.01$, ** $p < 0.0001$

(B) Mice were vaccinated with VRP-CEA(6D) and VRP-IL-12 by co-injection or contralateral injection. Vaccinations with VRP-CEA(6D) alone and with VRP-IL-12 alone served as controls. Two weeks after vaccination, IFN γ ELISPOT assay was performed. Co-injection induced a stronger enhancement of anti-CEA immune response than contralateral injection did. * $p < 0.0001$

Figure 5. Enhanced anti-CEA immune responses by co-administration of VRP-CEA(6D) and VRP-IL-12 in tumor-bearing mice

C57BL/6 mice were inoculated with MC38-CEA-2 tumor cells (2.5×10^5 cells/mouse) into the flank. Five days later mice were randomized into 4 groups (10 mice/group), and were immunized with VRP-CEA(6D) (5×10^7 I.U.) and/or VRP-IL-12 (5×10^7 I.U.) and/or empty vector (VRP-Empty; 5×10^7 I.U.). The total amount of VRP vectors inoculated into each mouse each time was 1×10^8 I.U., adjusted as necessary with VRP-Empty vector.

Vaccination was repeated twice (days 5 and 19). Spleens and blood were collected from mice of each group when they were euthanized. **(A)** IFN γ ELISPOT assay on splenocytes. Mean values of IFN γ positive spots are shown for each group. Combination of VRP-CEA(6D) and VRP-IL-12 showed significantly enhanced anti-CEA cellular response compared to mice vaccinated with VRP-CEA(6D) alone. * $p < 0.001$, ** $p < 0.0001$ **(B)** Mouse sera with serial dilutions in 1% BSA-PBS were analyzed for their titers of anti-CEA antibody by anti-CEA ELISA, using CEA protein-coated 96-well plates. Values of OD415 nm for each titration are shown. Experiments are repeated twice with similar results.

Figure 6. Tumor growth inhibition and prolonged survival by co-administration of VRP-CEA(6D) and VRP-IL-12 in tumor-bearing mice

Tumor implantation and vaccinations were conducted as described in Figure 5 Legend. **(A)** Tumor size was measured twice every week. Mice were euthanized when their tumor size exceeded 2,000 mm³. Mean tumor volumes for each group are shown in the graph. The combination of VRP-CEA(6D) and VRP-IL-12 inhibited tumor growth most significantly. Error bars: SE. * $p < 0.05$ ** $p < 0.01$ compared to control group (VRP-Empty only). **(B)** Survival of mice was also monitored until all the groups had less than 50% survival. Experiments are repeated twice with similar results, and survival data were pooled for analysis. Kaplan-Meier survival estimates by treatment group are shown. The Log Rank test was performed to compare the survival between different treatment groups. The co-injection of VRP-CEA(6D) and VRP-IL-12 resulted in the greatest increase in survival. A significant survival difference was also observed between treatments with VRP-CEA(6D)+VRP-IL-12 and VRP-Empty+VRP-IL-12 (* $p < 0.05$). ** $p < 0.01$, *** $p < 0.0001$ compared to control group (VRP-Empty only).