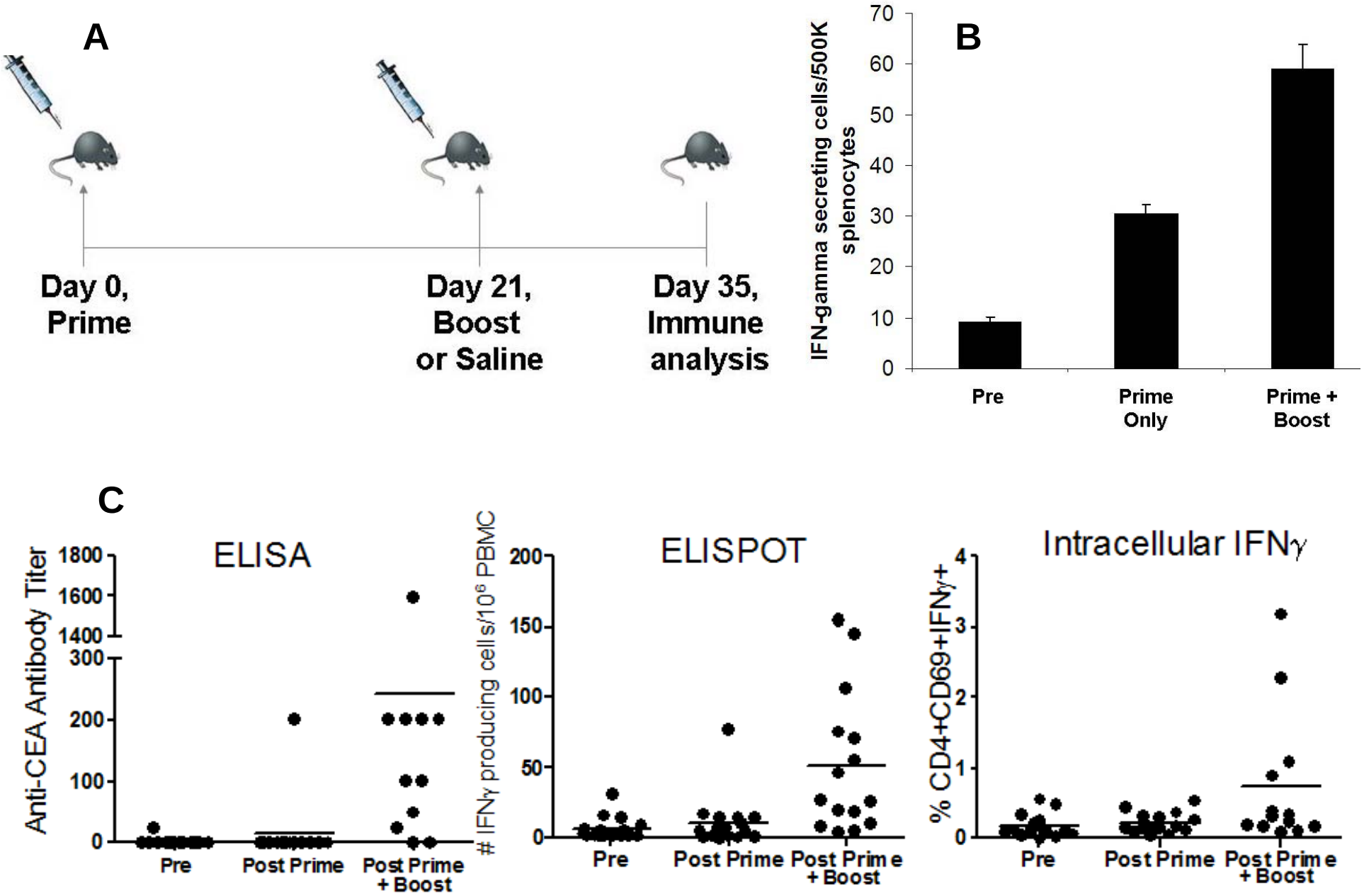
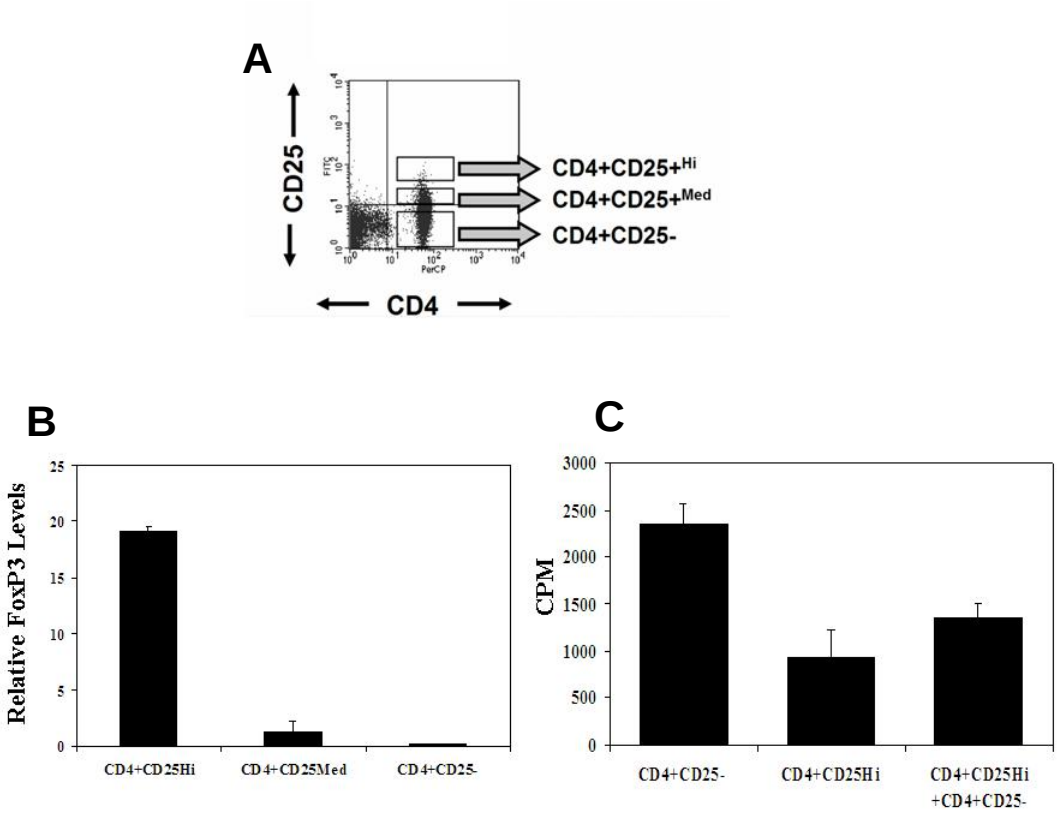
Supplemental Figure 1 (Morse)



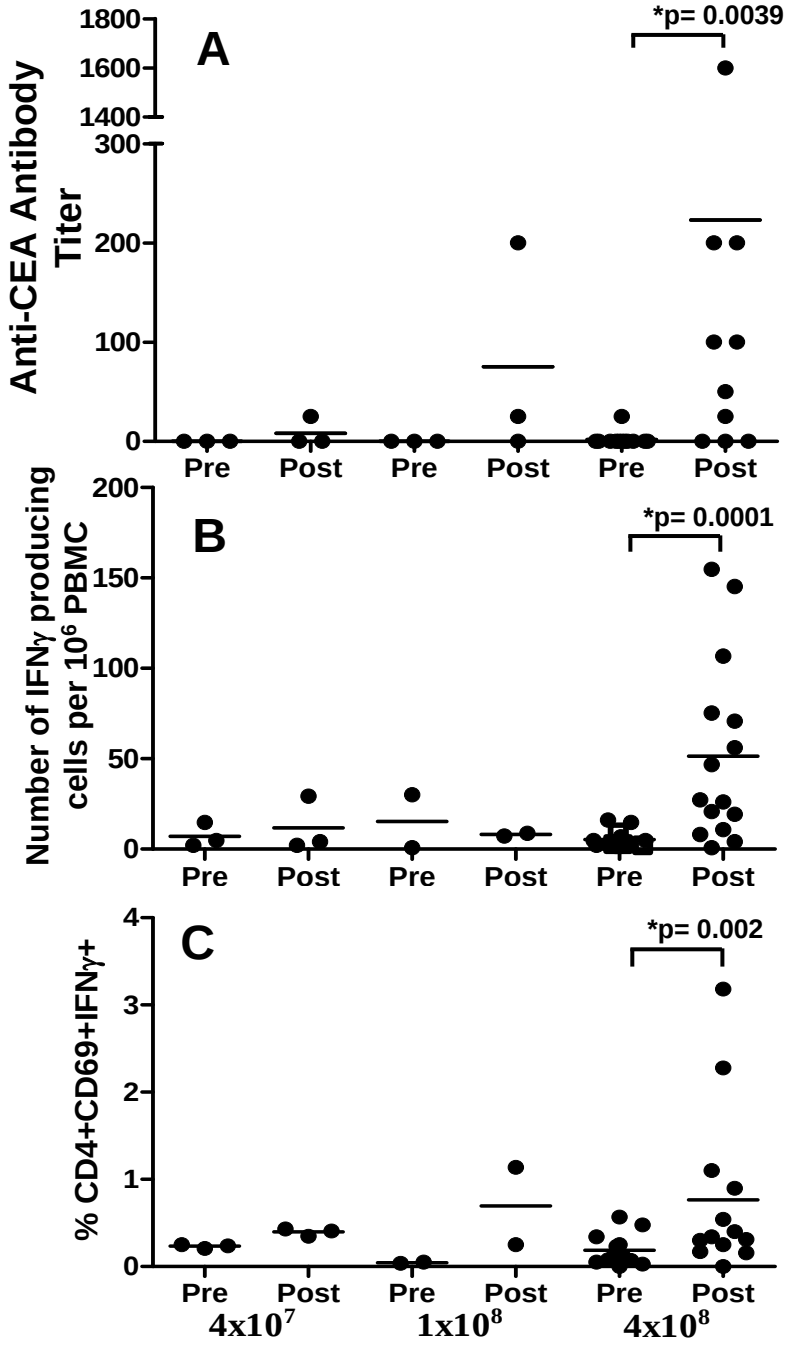
Supplemental Figure 1. Comparison of prime vs. prime plus boost vaccination in mice and humans.
(A, B) A booster immunization enhances the immune response to VRP-CEA in CEA-transgenic mice. (A) Experiment schema. C57BL6-CEA-transgenic mice received bilateral footpad injections on day 0 with VRP-CEA ($1x10^7$ virus particles) and day 21 with either saline or VRP-CEA. Mice were euthanized on day 35 and splenocytes harvested for ELISpot analysis. (B) IFNγ ELISpot responses were measured (4 mice per condition). Mice receiving two vaccinations with VRP-CEA showed enhanced ELISpot responses ($p=0.371$, unpaired T test, one tailed: GraphPad Prism 5.0). Error bars = SE. (C) CEA ELISA, VRP-CEA ELISpot, and Intracellular IFNγ analysis of patient serum or PBMC is compared pre VRP-CEA vaccination, post prime vaccination (1 injection), and post prime + boost vaccinations (2-4 injections). Antibody titer, total number of IFNγ producing cells per 10^6 PBMC, and percent of cells that are CD4+CD69+IFNγ+ represented by a solid circle for each patient vaccination response along with the mean (bar).

Supplemental Figure 2 (Morse)



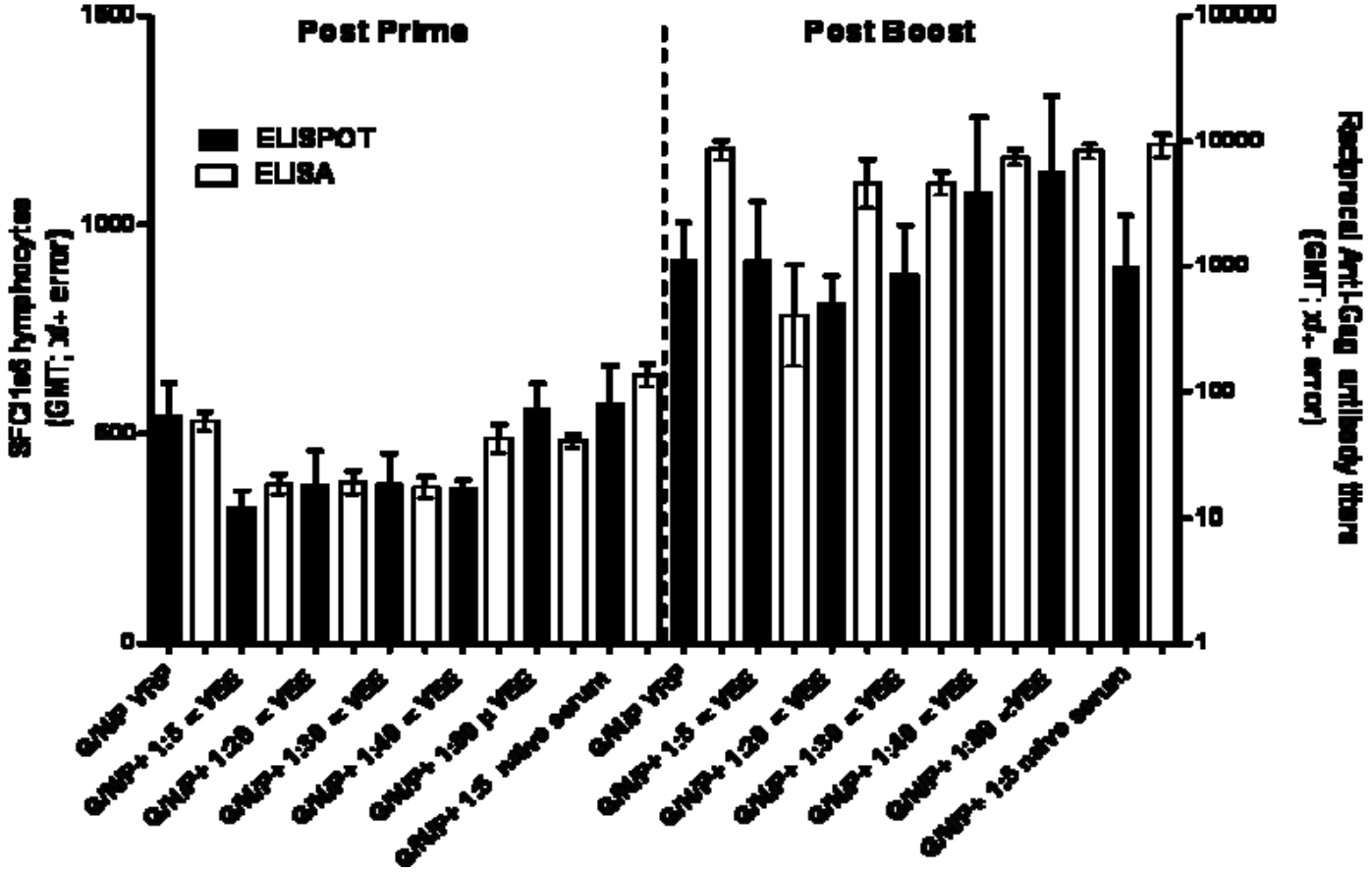
Supplemental Figure 2. CD4+CD25high Treg cell levels and proliferation of PBMCs after co-incubation with Treg. (A) PBMCs from donors were isolated were stained for CD4, CD25, and sorted . (B) Relative FoxP3 levels were determined by RT-PCR. RNA was isolated from ~10^5 sorted cells using the RNeasy mini kit (Qiagen, Carlsbad, CA). Complementary DNA was produced using oligo(dT) primers and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA). cDNA was then used in real-time PCR reaction using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Real time PCR was carried out using an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). The relative quantity of Foxp3 transcripts is expressed relative to HPRT with the value for HPRT=1. (A) Sorted CD4+CD25Hi, CD4+CD25- (5 x 10^4) and CD4+CD25Hi plus CD4+CD25- were stimulated with 10μg/ml OKT3 or media alone in 96 well plates with 10^5 irradiated CD4- cells. Proliferation inhibition was determined by determined by [3H]thymidine incorporation.

Supplemental Figure 3 (Morse)



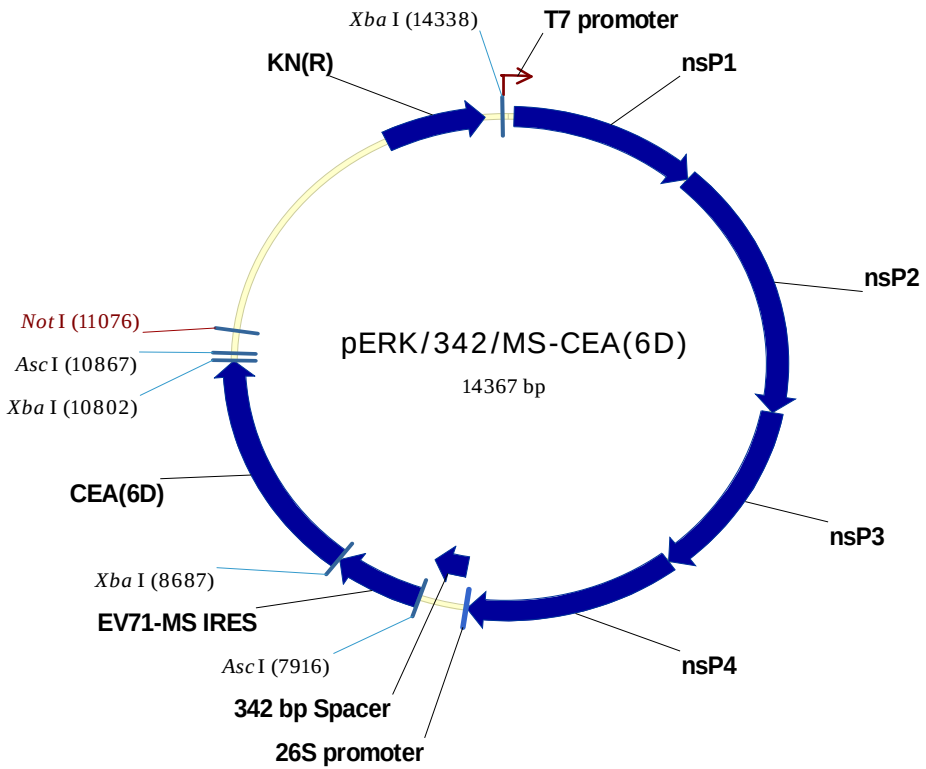
Supplemental Figure 3. Immune response at low, medium and high dose vaccination
Patient serum or PBMC was analyzed pre and post AVX701 immunization for each cohort by (A) CEA ELISA; (B) VRP-CEA ELISpot; and (C) Intracellular IFNγ . (A) Patient sera were tested in against whole CEA protein by ELISA. 96-well plates were coated with whole CEA protein (100 ng/well) and incubated with 100 µl of serum serially diluted 1:10 to 1:31,250. After washing, plates were incubated overnight at 4^{o}C with mouse anti-human IgG AP conjugated antibody (1:25,00 dilution). Plates were developed with PNPP. Reaction was stopped with 3N NaOH and absorbance was measured using ELISA Plate Reader (BioRad). The antibody titer is represented by a solid circle for each patient pre vaccination and best post vaccination response along with the mean (bar) for each vaccine dose. The antibody titer was determined as the mean absorbance that was twice negative control. (B) Patient PBMC were stimulated with VRP-CEA in an ELISpot assay. The total number of IFNγ producing cells per 10^6 PBMC is represented by a solid circle for each patient pre vaccination and best post vaccination response along with the mean (bar) for each vaccine dose. (C) Patient PBMC were stimulated with VRP-CEA in and intracellular cytokine assay. The percent of cells that are CD4+CD69+IFNγ+ is represented by a solid circle for each patient pre vaccination and best post vaccination along with the mean (bar) for each vaccine dose. Cohort 1= $4x10^7$ IU dose; Cohort 2= $1x10^8$ IU dose; Cohort 3/Phase II= $4x10^8$ IU dose. Statistical significance by the Wilcoxon Signed Rank test is noted on each graph.

Supplemental Figure 4 (Morse)



Supplemental Figure 4: Ability of VRP to prime and boost immune response despite pre-incubation with VRP neutralizing antibodies. VRP engineered to express the HIV-1 gag, nef and pol genes (G/N/P VRP) were incubated for one hour with serum from mice previously immunized with irrelevant (non-gag/nef/pol) VRP at dilutions ranging from 1:5 to 1:80, as indicated on the x-axis. Neutralization was verified using an IFA-based infectivity assay in Vero cells (not shown). Balb/c mice were immunized twice three weeks apart subcutaneously by footpad injections, in groups of 12, with neutralized or untreated VRP, or with VRP incubated with normal mouse serum diluted 1:5, as indicated. At three weeks post-prime (left half of graph) or 6 days post-boost (right half of graph), mice were bled and sacrificed. Serum samples were analyzed for anti-gag antibody ELISA titers (white bars, right y-axis). Spleen lymphocytes were prepared and analyzed for cell-mediated immune responses to a pool of overlapping 15-mer peptides corresponding to the gag protein amino acid sequence using an IFNγ ELISpot assay (right bars, left y-axis). As shown in the figure, pre-neutralizing VRP results in significant reduction in both humoral and cellular immune responses to the expressed transgene post-prime. Two immunizations with pre-neutralized VRP resulted in a significant reduction in humoral responses only at the 1:5 dilution. However, pre-neutralization did not inhibit cell-mediated immune responses elicited by two immunizations of VRP vaccine.

Replicon Plasmid Expressing CEA



Supplemental Figure 5. Map of the VRP-CEA.
Starting at the promoter region for T7, used for in vitro transcription for VRP production, and moving clockwise, the solid arrows represent the four VEE viral nonstructural protein genes (nsP1-nsP4), the 342 bp spacer, the EV71-MS IRES, the CEA gene and the kanamycin resistance gene [KN (R)], respectively. The XbaI and AscI restriction sites used for the construction of the plasmid as well as the site for NotI used for plasmid linearization for VRP production are indicated. The CEA gene was modified by site directed mutagenesis to introduce an A to G mutation at ucleotide 1828 resulting in the Asn→Asp mutation at amino acid 610 of the CEA protein [CEA(6D)]. The modified CEA gene was amplified by polymerase chain reaction (PCR) using CEA gene-specific primers which incorporated XbaI restriction sites. The PCR product was subcloned into the pCDNA3.3/MS transfer vector. A region spanning the IRES and the CEA gene was digested from the transfer plasmid using AscI enzyme and cloned into pERK spacer-replicon vectors containing spacers of different sizes. A replicon vector containing a spacer of 342 nucleotides in length was selected based on CEA protein expression and replicon packaging titers.