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ORIGINAL ARTICLE

Strengthened tumor antigen immune recognition by inclusion of a recombinant *Eimeria* antigen in therapeutic cancer vaccination

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Abstract The need for novel, effective adjuvants that are capable of eliciting stronger cellular and humoral adaptive immune responses to antigenic targets is well understood in the vaccine development field. Unfortunately, many adjuvants investigated thus far are either too toxic for human application or too weak to induce a substantial response against difficult antigens, such as tumor-associated antigens (TAAs). In spite of this trend, clinical investigations of recombinant *Eimeria* antigen (rEA) have revealed this protein to be a non-toxic immunogenic agent with the ability to trigger a Th1-predominant response in both murine and human subjects. Our past studies have shown that the injection of a rEA-encoding adenovirus (rAd5-rEA) alongside an HIV antigen-encoding adenovirus greatly improves the adaptive immune response against this pathogen-derived transgene. In this report, we investigated whether rAd5-rEA could promote and/or alter cytotoxic memory responses toward carcinoembryonic antigen (CEA), a colorectal cancer-related TAA. We found that the addition of rAd5-rEA to an Ad-based CEA vaccine induced a dose-dependent increase in several anti-CEA T and B cell responses. Moreover, inclusion of rAd5-rEA increased the number of CEA-derived antigenic epitopes that elicited significant cell-mediated and IgG-mediated recognition. These enhanced anti-CEA immune responses also translated into superior CEA-targeted cell killing, as evaluated by an in vivo cytotoxic T lymphocyte assay. Overall, these results suggest that co-administration of rAd5-rEA with a tumor antigen vaccine can substantially boost and broaden the TAA-specific adaptive memory response, thereby validating the potential of rAd5-rEA to be a beneficial adjuvant during therapeutic cancer vaccination.

Keywords rEA · *Eimeria tenella* · Carcinoembryonic antigen · Adenovirus vectors · Vaccine adjuvants · Cancer vaccines

List of symbols

μg	Microgram(s)
μL	Microliter(s)
μM	Micromolar
ANOVA	Analysis of variance
APC	Antigen-presenting cell
CA-125	Cancer antigen 125
cDNA	Complementary deoxyribonucleic acid
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CFSE	Carboxyfluorescein succinimidyl ester
CMI	Cell-mediated immunity
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
dpi	Days post-injection
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallizable

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GFP	Green fluorescent protein
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IL	Interleukin
IFN-γ	Interferon gamma
IM	Intramuscularly
MyD88	Myeloid differentiation primary response gene 88
nm	Nanometer(s)
NK	Natural killer
NKT	Natural killer T
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PRR	Pattern recognition receptor
rAd5	Recombinant adenovirus serotype 5
rEA	Recombinant *Eimeria* antigen
SFC	Spot-forming cell
TAA	Tumor-associated antigen
Th	T helper
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TRIF	TIR-domain-containing adapter-inducing interferon-β
vp	Viral particle(s)

Introduction

Immune responses against cancerous cells are often absent as a result of systemic T cell-mediated tolerance against them and their respective tumor-associated antigens (TAAs). To mount a substantial immune response against malignancies, successful immunotherapy trials have shown that a therapeutic vaccine must be able to initiate and propagate a strong, TAA-specific cytotoxic immune response [1]. Carcinoembryonic antigen (CEA) has been identified as a particularly promising TAA target due to its ubiquitous overexpression in colorectal and other carcinomas, as well as its relatively high level of immunogenicity [2]. Various methods have been employed to use CEA as a vaccine transgene, with several resulting in the induction of CEA-targeted cytotoxic T lymphocyte (CTL) responses and anti-CEA antibody production [3–6]. Recently, a phase I/II clinical trial in which colorectal cancer patients, who had failed an average of three prior chemotherapeutic regimens, were administered adenovirus vectors expressing an enhanced, modified version of CEA (rAd5-CEA) was conducted [7, 8]. This CEA-expressing adenovirus vector multiplied anti-CEA cell-mediated immunity (CMI) responses several fold over patient pre-vaccination baseline. In addition, patient survival rates at 1 year were found to be greater than those previously reported in demographically similar colorectal cancer patient populations [9] and were most improved in the treatment group given the highest dose of rAd5-CEA.

As this and many other cancer immunotherapy trials have revealed, there is a substantial positive correlation between the amount of TAA-directed CMI produced and rates of patient survival. It has also been recognized that even immunotherapy-naïve patient tumor microenvironments often contain TAA-presenting cells, yet they do not readily activate a CTL response against the antigen when present [10, 11]. Therefore, in addition to a TAA vaccination, successful treatment may also require an adjuvant to promote long-term immunity and memory toward the cancer cells.

Several adjuvants have been shown to improve both the degree and breadth of antigen-specific cellular immune responses, particularly those that trigger Toll-like receptor (TLR) and other pattern recognition receptor (PRR)-mediated innate immune responses [12–16]. Recombinant *Eimeria* antigen (rEA) is a notable activator of TLR and non-TLR innate immune signaling pathways. rEA was initially identified within the *Eimeria tenella* protozoan as an inducer of high IL-12 levels in the bovine intestine, an effect that promoted an overall anti-tumorigenic environment [17]. Injection of the rEA protein has since been shown to prolong survival of tumor-carrying mice and induce a safe, cytokine-dependent decrease in the CA-125 tumor marker within advanced malignancy patients [17, 18]. In previous studies, we have created an rEA-expressing recombinant adenovirus vector (rAd5-rEA) and shown that in vivo delivery of this agent can promote a Th1-skewed, pro-inflammatory response greater than rEA protein or a non-specific recombinant adenovirus, as measured by heightened cytokine responses (e.g., IFN-γ, TNF-α, IL-12(p70)), activation of innate immune cells (e.g., NK, NKT, DC), and greater transgene memory responses against a co-injected HIV-derived (HIV-gag) antigen [19]. Moreover, we have found that rEA can directly promote human NK effector cell activation and stimulate human peripheral blood mononuclear cell (PBMC) cytolytic tumor cell killing [20]. Based on these findings, we wished to investigate whether co-administration of rAd5-CEA and rAd5-rEA could further improve anti-CEA immunity. Additionally, we explored the spectrum, quantity, and relationship of T and B cell-facilitated adaptive immune responses that rAd5-rEA introduces to a vaccine regimen targeting a human-relevant TAA.

Methods

Recombinant adenovirus vector construction

Recombinant Ad5 vectors rAd5-CEA [7], rAd5-rEA [19], and rAd5-GFP [21] were built and propagated as previously described. rAd5-GFP was used as a control vector as it was previously confirmed to have no significant impact on measurable adaptive immune responses to co-administered antigens [22]. The cDNA sequence of human CAP1(6D)-modified CEA was produced and generously supplied from Duke University [23]. Vectors underwent recombination and viral propagation as previously described [24]. All vectors underwent direct sequencing to verify correct transgene insertion and were found to be replication-incompetent via E1 gene region deletion by PCR, as previously described [25]. Viral particle (vp) titers were determined by spectrophotometry and SDS-polyacrylamide gel electrophoresis following silver stain or Western blotting.

Animal care and procedures

All animal procedures were conducted under the approval of Michigan State University's Institutional Animal Care and Use Committee. Eight-week-old male C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and injected intramuscularly (IM) into the tibialis anterior of the right hindlimb with 20 μL phosphate-buffered saline (PBS, pH 7.4) solution containing a total of 1×10^8–1×10^{10} vp, including rAd5-CEA and an equivalent vp dose of either rAd5-GFP or rAd5-rEA. Splenocytes and blood plasma were obtained and processed as previously described [26].

Enzyme-linked immunosorbent spot (ELISpot) assay analysis

ELISpot assay analyses were performed using Ready-Set-GO! ELISpot mouse IFN-γ and IL-2 kits (eBioscience, San Diego, CA, USA), as previously described [27]. In brief, splenocytes (5×10^6 cells/well) from individual mice were incubated on capture antibody pre-treated plates with individual CEA peptides or a CEA peptide pool (0.4 μg/well). Individual CEA peptides were 15 aa in size and covered the $CEA_{498–676}$ sequence with a CAP1(6D) modification in 10 aa overlaps using a PepTrack peptide library (JPT Peptide Technologies, Berlin, Germany). The CEA peptide pool of CAP1(6D)-modified CEA sequence peptides was a generous gift from Dr. Michael Morse at Duke Medical Center [8]. Staining of plates was performed as described in manufacturer's protocol. Plate well photography and spot-forming cell (SFC) quantification were performed by an automated ELISpot reader (Cellular Technology, Cleveland, OH).

Cell staining and flow cytometry

Fluorescent intracellular staining was performed as previously described [28]. In brief, 2×10^6 splenocytes were stained with APC-Cy7-CD3, Alexa Fluor700-CD8a, and CD16/32 Fc-block antibodies, fixed using 2 % formaldehyde (Polysciences, Warrington, PA), permeabilized using 0.2 % saponin (Sigma-Aldrich, St. Louis, MO), stained for intracellular cytokines using APC-granzyme B, FITC-IFN-γ, PE-perforin, PE-Cy7-TNF-α (BD Biosciences, San Diego, CA, USA), and PerCpCy5.5-IL-2 (BioLegend, San Diego, CA, USA), and stained for dead cell exclusion using violet fluorescent reactive dye (Invitrogen, Carlsbad, CA, USA). Cells were analyzed, and 2×10^5 events per sample were captured with an LSRII flow cytometer (BD Biosciences) using FlowJo software (Tree Star, San Carlos, CA, USA).

In vivo cytotoxic T lymphocyte (CTL) assay

In vivo CTL analysis was performed similar to previously described studies [27]. Briefly, mice were vaccinated with 5×10^9 vp rAd5-CEA and 5×10^9 vp of either rAd5-GFP or rAd5-rEA. At 21 days post-injection (dpi), syngeneic splenocytes were pulsed with a CEA-specific peptide pool or the irrelevant HIV-gag peptide, AMQ (AMQMLKETI), for 1 h at 37 °C. Carboxyfluorescein succinimidyl ester (CFSE) was used to stain CEA-pulsed splenocytes at a 10 μM concentration ($CFSE^{High}$) and AMQ-pulsed splenocytes at a 1 μM concentration ($CFSE^{Low}$). Immunized and naïve mice were injected in the left retro-orbital sinus with equal amounts of CEA- and AMQ-pulsed splenocytes at a total volume of 8×10^6 cells. Twenty-four hours following splenocyte infusion, mice were killed for splenocyte collection; these cells were washed and analyzed by FACS with a LSRII flow cytometer. FlowJo software was utilized to quantify amounts of CFSE-stained splenocytes. The percentage of specific CEA-pulsed splenocyte killing was determined using the following equation: % specific killing $= 1 - ((\%\ CFSE^{High}/\%\ CFSE^{Low})_{immunized}/(\%\ CFSE^{High}/\%\ CFSE^{Low})_{naive})$.

Anti-CEA and anti-Ad ELISA analysis

ELISAs were performed as previously described [29]. Briefly, 5×10^8 vp of a null Ad5 vector, 0.2 μg CEA peptide pool, or 0.2 μg individual CEA peptide epitopes were plated per well in a high-binding 96-well flat-bottom plate. Following overnight 4 °C incubation, plates were rinsed with washing buffer (PBS containing 0.05 % Tween) and incubated with blocking buffer (PBS containing 3 % bovine serum albumin) for an hour at room temperature. Plasma was plated following 1:1,600 dilution (or pooled and diluted 1:10

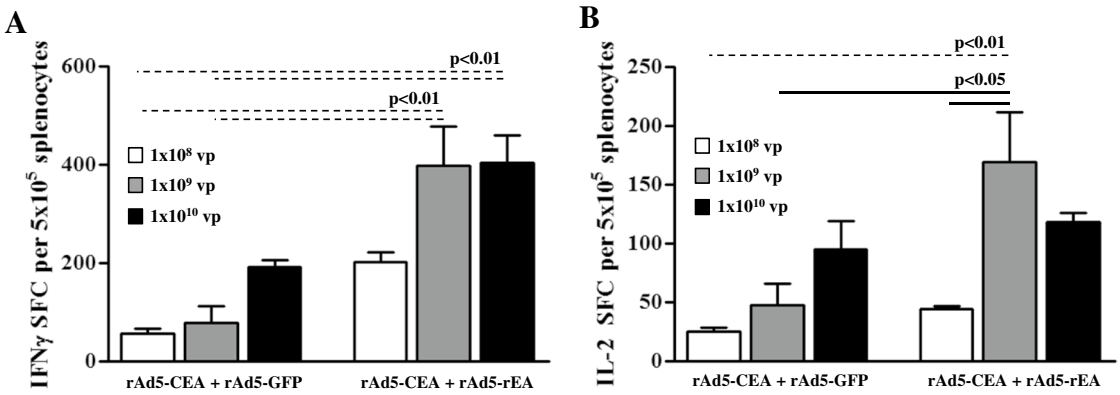

Fig. 1 Strong CEA-specific cell-mediated immunity produced by rAd5-CEA and rAd5-rEA co-vaccination. C57BL/6 mice were IM injected with rAd5-CEA and either rAd5-GFP or rAd5-GFP/rEA at equal viral particles (vp) per vector, totaling 1×10^8 ($n = 3$), 1×10^9 ($n = 3$), or 1×10^{10} vp ($n = 2$). Splenocytes were collected from vaccinated and naïve mice at 14 dpi and examined by IFN-γ **a** and IL-2 **b** ELISpot to count spot-forming cells (SPCs). *Bars* denote mean ± standard error. *Solid* and *dashed lines* denote a significant difference between treatment groups ($p < 0.05$, $p < 0.01$, respectively)

for the measurement of individual CEA epitope IgG binding) in blocking buffer and incubated for an hour at room temperature. Wells were subsequently rinsed with washing buffer and coated with a horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibody (Bio-Rad, Hercules, CA, USA) diluted 1:4,000 in washing buffer. Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) was added to each well to initiate the spectrophotometric reaction, which was stopped with 1 N phosphoric acid. Plates were analyzed using an automatic microplate reader at 450 nm absorbance.

Statistical analysis

Statistical analysis comparing in vivo CTL CEA-specific killing between Ad treatment groups was performed using a Student's *t* test. The association between percent CEA-specific in vivo CTL killing and anti-CEA IgG titers within individual animals was tested with the Pearson correlation coefficient. All other experiment data where statistical significance was determined were analyzed using a one-way ANOVA with a Newman-Keuls post hoc correction. All graphs in this paper are presented as mean ± standard error. Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

Increased rAd5-CEA transgene-specific CMI responses induced in the presence of rAd5-rEA

We and others have verified that rAd5-CEA vaccination of murine and human subjects induces significant CEA-specific CMI [7, 8, 27]. To examine whether the addition of rAd5-rEA to a TAA-expressing vaccine would further promote anti-TAA CMI responses, we treated mice with rAd5-CEA and an equivalent dose of either rAd5-rEA or a GFP-expressing control adenovirus vector (rAd5-GFP). Mice were injected with a total viral dose of either 1×10^8, 1×10^9, or 1×10^{10} vp per mouse. Splenocytes were harvested at 14 dpi, processed, and analyzed for anti-CEA-specific CMI by IFN-γ and IL-2 ELISpot assays. Our data revealed the amount of CEA-specific, IFN-γ-secreting splenocytes to be greater in mice injected with the rAd5-CEA + rAd5-rEA combination rather than those given rAd5-CEA + rAd5-GFP (Fig. 1a). Specifically, both the 1×10^9 and 1×10^{10} vp rAd5-CEA + rAd5-rEA groups yielded statistically more IFN-γ-secreting splenocytes than the 1×10^8 and 1×10^9 vp rAd5-CEA + rAd5-GFP-treated groups ($p < 0.01$). IL-2 ELISpot analysis also revealed a significantly greater CEA-specific CMI response when comparing the 1×10^9 vp dose of rAd5-CEA + rAd5-rEA with the 1×10^8 ($p < 0.01$) and 1×10^9 vp ($p < 0.05$) rAd5-CEA + rAd5-GFP-treated animals, as well as the 1×10^8 vp dose of rAd5-CEA + rAd5-rEA-treated animals ($p < 0.05$), showing a positive relationship between dose and rAd5-rEA-promoted CEA-specific CMI (Fig. 1b). Interestingly, the 1×10^{10} vp dose showed no significant difference in the amount of cells secreting IL-2 in response to CEA stimulation. These findings indicate that co-injection of rAd5-rEA with rAd5-CEA improves anti-CEA CMI in a mainly dose-dependent manner.

Improved cytotoxic T cell responses against CEA following high-dose rAd5-CEA + rAd5-rEA co-administration

During the process of tumor cell killing, effector memory CD8+ T cells produce cytolytic and inflammatory

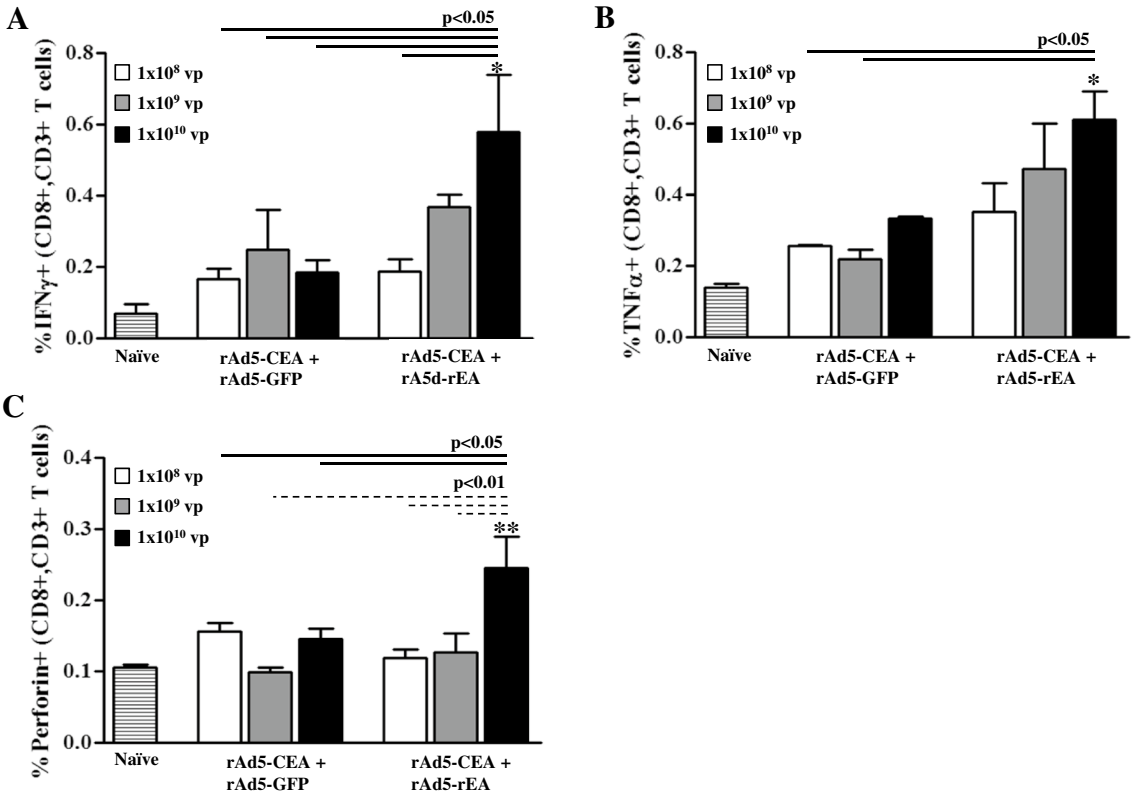

Fig. 2 High-dose rAd5-CEA and rAd5-rEA administration induces a robust, CEA-specific CD8+ cytotoxic T cell response. C57BL/6 mice ($n = 3$) were IM injected with the combination of rAd5-CEA and either rAd5-GFP or rAd5-rEA at equal viral particles (vp) per vector, totaling 1×10^8, 1×10^9, or 1×10^{10} vp. Splenocytes were collected from vaccinated and naïve mice at 14 dpi, permeabilized, and stained for CD8+ T cell IFN-γ **a**, TNF-α **b**, and perforin **c** expression. *Bars* denote mean ± standard error. *Single asterisk* and *double asterisks* denote significant differences between that group and naïve animals ($p < 0.05$, $p < 0.01$, respectively). *Solid* and *dashed lines* denote a significant difference between treatment groups ($p < 0.05$, $p < 0.01$ respectively)

cytokines in response to specific tumor-derived antigens [30, 31]. To quantify the amount of CEA-specific cytokine-producing CD8+ T cells, splenocytes were collected from naïve or vaccinated mice administered rAd5-CEA combined with an equal dose of either rAd5-rEA or rAd5-GFP. Splenocytes were stimulated ex vivo with either a CEA peptide pool or non-specific HIV-gag peptide. Splenocytes were analyzed by multicolor flow cytometry after subsequent fluorescent antibody extracellular staining against CD3 and CD8 and intracellular staining against IFN-γ, TNF-α, IL-2, and perforin.

Upon CEA stimulation, 1×10^{10} vp rAd5-CEA + rAd5-rEA co-injection induced a significantly higher frequency of IFN-γ-expressing CD8+ T cells than those given rAd5-CEA and rAd5-GFP at all treatment doses, rAd5-CEA + rAd5-rEA at the 1×10^8 vp dose, and naïve mice ($p < 0.05$) (Fig. 2a). The highest dose of rAd5-CEA + rAd5-rEA also produced a higher frequency of TNF-α-producing CD8+ T cells than 1×10^8 and 1×10^9 vp rAd5-CEA + rAd5-GFP injected and naive mice ($p < 0.05$) (Fig. 2b). Quantity of intracellular perforin production within CD8+ T cells after CEA stimulation was significantly larger following 1×10^{10} vp rAd5-CEA + rAd5-rEA administration in comparison with all other treatment groups ($p < 0.05$ for 1×10^8 and 1×10^{10} vp rAd5-CEA + rAd5-GFP; $p < 0.01$ for all other groups) (Fig. 2c). No rAd5-treated groups induced significantly greater levels of CEA-specific, IL-2-producing CD8+ T cells than naïve animals (data not shown). Stimulation with the irrelevant HIV-gag peptides also did not produce significantly different amounts of anti-CEA cytokine-producing CD8+ T cells between naïve and rAd5 treatment groups (data not shown). These results suggest that the combination of rAd5-CEA + rAd5-rEA at a 1×10^{10} vp dose induces substantially more CEA-triggered activation of cytotoxic CD8+ T lymphocytes.

To confirm that the improved anti-CEA CTL effects seen with rAd5-CEA + rAd5-rEA treatment were not biased due to unequal levels of CD8+ T cells following vaccination, the frequency of splenic CD8+ T cells was measured via flow cytometry from mice vaccinated with either rAd5-CEA + rAd5-GFP or rAd5-CEA + rAd5-rEA at previously

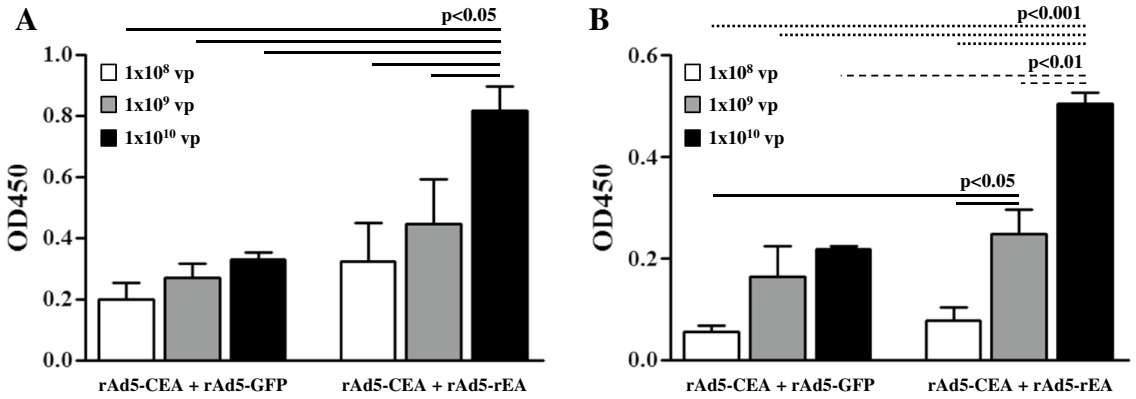

Fig. 3 Anti-CEA IgG production is potently induced by rAd5-CEA and rAd5-rEA co-injection. C57BL/6 mice were IM injected with rAd5-CEA and either rAd5-GFP or rAd5-rEA at equal viral particles (vp) per vector, totaling 1×10^8 ($n = 3$), 1×10^9 ($n = 3$), or 1×10^{10} vp ($n = 2$). Serum was collected at 14 dpi, diluted at a 1:1,600 ratio, and measured for IgG antibody levels against CEA **a** and Ad5 **b** by ELISA with 450 nm OD. *Bars* denote mean ± standard error. *Solid*, *dashed*, and *dotted lines* denote a significant difference between treatment groups ($p < 0.05$, $p < 0.01$, $p < 0.001$ respectively)

utilized doses (1×10^8, 1×10^9, or 1×10^{10} vp). Importantly, no significant differences in the amount of CD8+ T cells were observed between naïve, rAd5-CEA + rAd5-GFP, or rAd5-CEA + rAd-rEA treatment groups (Supplemental Fig. 1).

Dose-dependent increase in anti-CEA IgG production following rAd5-CEA + rAd5-rEA co-administration

In addition to TAA-directed CMI, the generation of TAA-specific IgG antibodies is also an important aspect of effective cancer immunotherapy through participation in antibody-dependent cell-mediated cytotoxicity [23]. Curiously, in our clinical trial using solely rAd5-CEA, we did not find notable changes in patient serum anti-CEA IgG following vaccination [8]. To measure whether the co-administration of rAd5-CEA + rAd5-rEA would induce greater anti-CEA IgG titers than rAd5-CEA + rAd5-GFP, mice were co-injected with either combination at a total viral dose of 1×10^8, 1×10^9, or 1×10^{10} vp. Serum was collected at 14 dpi and used to perform an anti-CEA IgG ELISA. We observed that at the 1×10^{10} vp dose, mice treated with rAd5-CEA + rAd5-rEA had significantly greater anti-CEA IgG titers than all other rAd5-CEA-treated mice ($p < 0.05$) (Fig. 3a). Serum was also used to measure anti-Ad5 IgG titers to evaluate whether vaccinated mice developed an antibody response against the vector itself. As seen with anti-CEA IgG, 1×10^{10} vp rAd5-CEA + rAd5-rEA induced a significantly greater titer of anti-Ad5 IgG than any of the other vaccine-treated mice ($p < 0.01$ for 1×10^{10} vp rAd5-CEA + rAd5-GFP and 1×10^9 vp rAd5-CEA + rAd5-rEA; $p < 0.001$ for all other treatments) (Fig. 3b), suggesting that rEA overexpression enhances B cell responses to all antigenic targets, including Ad-derived antigens. Moreover, the mice in the 1×10^9 vp rAd5-CEA + rAd5-rEA had higher amounts of anti-Ad5 IgG than both groups of 1×10^8 vp dosed mice ($p < 0.05$). This data suggests that high doses of rAd5-CEA + rAd5-rEA successfully induce substantial anti-CEA IgG production, while also producing greater levels of antibodies against the Ad5 vector.

To verify that the variances in antibody responses were not due to uneven amounts of IgG-producing B cells present following vaccination, B cells from mice co-injected with either rAd5-CEA + rAd5-GFP or rAd5-CEA + rAd-rEA at various doses (1×10^8, 1×10^9, or 1×10^{10} vp) were quantified and analyzed via flow cytometry. No significant differences in B cell numbers were found between naïve, rAd5-CEA + rAd5-GFP, and rAd5-CEA + rAd-rEA; independent of viral particle dose (Supplemental Fig. 2).

rAd5-CEA + rAd5-rEA co-injection enhances breadth of T cell-recognized CEA peptide epitopes

While it is important to have a strong memory response against a TAA, worth is also placed on the creation of an epitope-diverse response [32, 33]. Vaccine-induced recognition of a greater number of immunogenic antigen epitopes can possibly allow for superior protection in cases where some epitopes may be bypassed via tumor survival methods and intrinsic HLA haplotype differences [32, 33]. We have previously shown that the addition of rAd5-rEA to an HIV antigen-expressing Ad vaccine increased the breadth and quality of response against HIV antigen epitopes [19]; therefore, we wished to investigate whether rAd5-rEA could also improve the antigen epitope recognition of CEA.

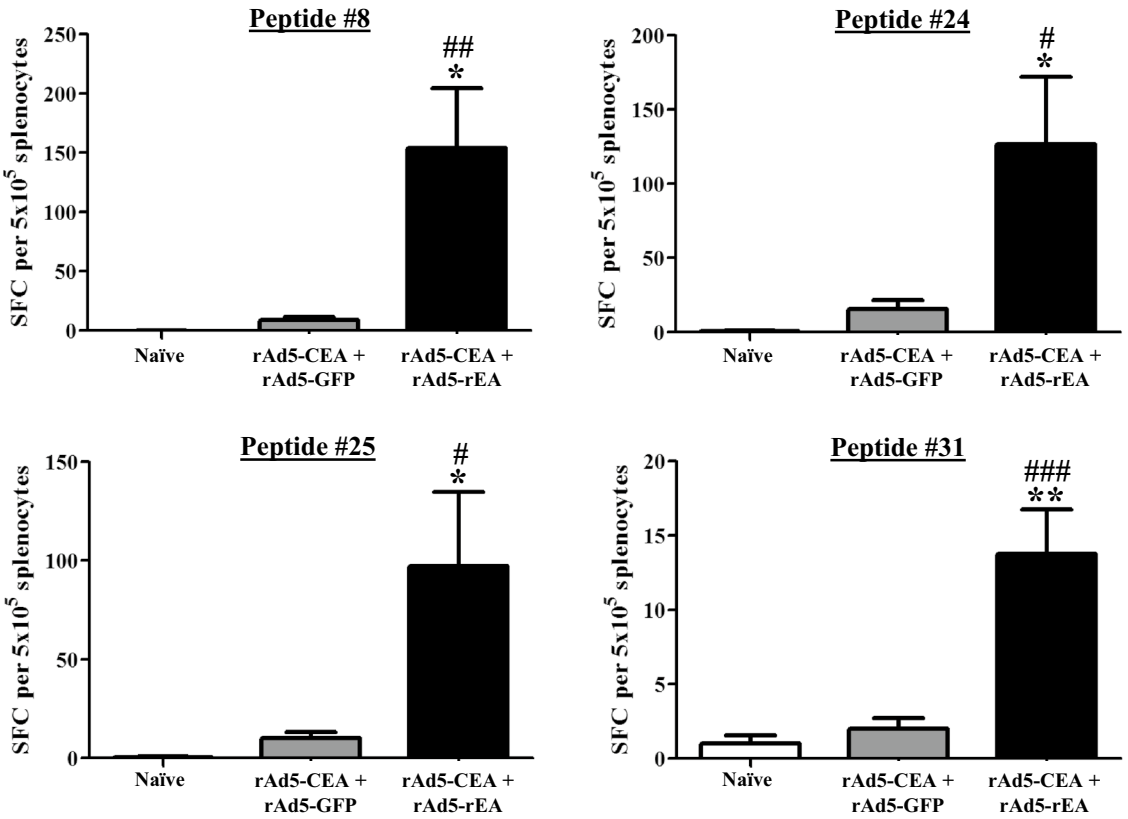

Fig. 4 rAd5-CEA and rAd5-rEA co-vaccination induces CMI against multiple unique CEA peptide epitopes. C57BL/6 mice ($n = 9$) were IM injected with 5×10^9 vp of rAd5-CEA and 5×10^9 vp of either rAd5-GFP or rAd5-rEA. Splenocytes from vaccinated and naïve ($n = 5$) mice were collected at 14 dpi and examined by IFN-γ ELISpot where splenocytes were divided and stimulated by a single CEA-derived peptide epitope per well (see Supp. Table 1 for sequences). ELISpot analysis was completed by quantification of spot-forming cells (SFCs). CEA peptides that induced greater amounts of SFCs in rAd5-CEA-treated animals than naïve animals are shown. *Bars* denote mean ± standard error. *Single asterisk and double asterisks* denote significant difference from naïve group ($p < 0.05$, $p < 0.01$, respectively). *Single hash*, *double hash*, and *triple hash* denote significant difference from rAd5-CEA + rAd5-GFP treatment groups ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively)

To measure the range of CEA immunogenic areas following vaccination, we collected splenocytes isolated from mice injected with 1×10^{10} total vp of either rAd5-CEA + rAd5-rEA or rAd5-CEA + rAd5-GFP. Splenocytes were stimulated ex vivo with individual, 15 aa-long CEA peptides that encompassed the entirety of one of the CEA protein's repeating domains in N- to C-terminal 10 aa overlaps and were numbered subsequently in that order (Supplemental Table 1). Using an IFN-γ ELISpot to measure CMI, we found several areas of epitope recognition following rAd5-CEA + rAd5-rEA vaccination (Fig. 4), as it produced significantly higher levels of IFN-γ-producing cells than naïve ($p < 0.05$ for peptides #8, 24, 25; $p < 0.01$ for peptide #31) and rAd5-CEA + rAd5-GFP vaccinated ($p < 0.05$ for peptides #24 and 25; $p < 0.01$ for peptide #8; $p < 0.001$ for peptide #31) mice following splenocyte stimulation with four distinct peptide epitopes. Independent of the particular CEA epitope, rAd5-CEA + rAd5-GFP was unable to produce significantly higher amounts of IFN-γ-producing cells than the other treatment groups, and for all treatment groups, most CEA epitopes produced SFC counts close to baseline (Supplemental Fig. 3). These results suggest that rAd5-rEA co-administration with rAd5-CEA allows for a diverse subset of CEA epitopes to be recognized by an adaptive CMI response.

rAd5-CEA + rAd5-rEA co-injection enhances diversity of anti-CEA IgG antibody-identified CEA peptide epitopes

While researchers have identified several T cell receptor-recognized CEA epitopes [34–36], there are presently none conclusively verified to be recognized by B cell-based receptors. In addition, previous vaccine prototypes targeting CEA have often been unable to induce measurable amounts of anti-CEA IgG antibodies within patients' blood serum [8, 37]. We wished to see whether the supplementation of rAd5-rEA to a rAd5-CEA injection would allow for a more varied and detectable repertoire of CEA-binding

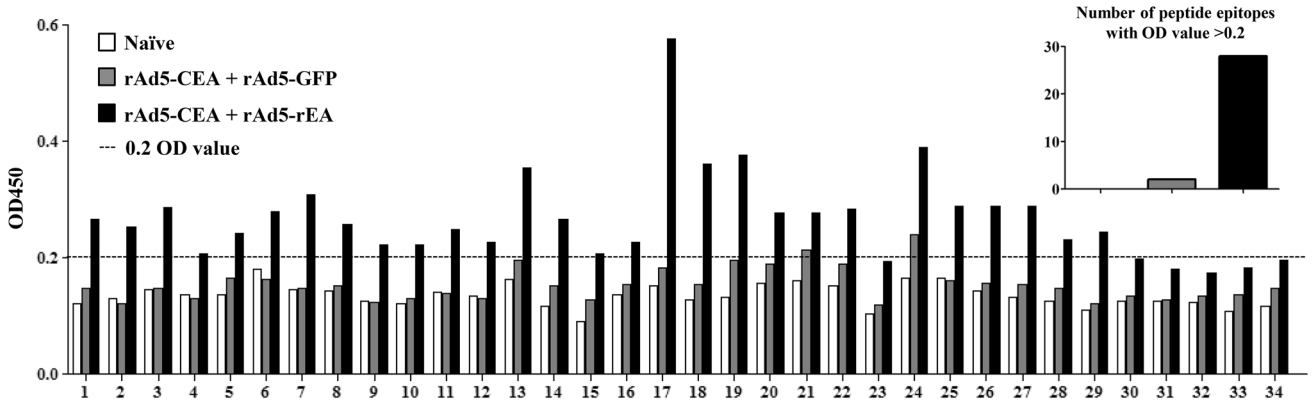

Fig. 5 rAd5-CEA and rAd5-rEA co-injection induces the creation of IgG antibodies against a diverse area of CEA peptide regions. C57BL/6 mice ($n = 7$) were IM injected with 5×10^9 vp rAd5-CEA and 5×10^9 vp rAd5-GFP or rAd5-rEA. Serum from vaccinated and naïve ($n = 5$) mice was collected at 21 days post-injection. Serum dilutions (1:10) were pooled by treatment group and plated on wells containing a single CEA-derived peptide epitope per well (see Supp. Table 1 for sequences). IgG levels against individual CEA epitopes were measured by ELISA at 450 nm OD. The inset graph demonstrates the amount of epitopes in each treatment group that produced an OD value >0.2, as this was the baseline OD measured in wells without serum plated

IgG antibodies. To measure anti-CEA IgG diversity, we collected serum isolated from mice that had been injected with 1×10^{10} total vp of either rAd5-CEA + rAd5-rEA or rAd5-CEA + rAd5-GFP. Serum was pooled within treatment groups and plated on wells that were coated with the aforementioned individual 15mer CEA peptides (Supplemental Table 1). In identifying values above the baseline optical density (OD) of 0.2 (measured value in wells without serum plated), ELISA analysis revealed that both rAd5-CEA + rAd5-rEA and rAd5-CEA + rAd5-GFP treatments induced epitope recognition by anti-CEA IgG (Fig. 5), while naïve mice did not. However, in observing the number of CEA peptide epitopes recognized by rAd5-CEA-vaccinated mouse serum IgG, there were clearly more unique epitopes bound by immunoglobulins derived from mice given rAd5-rEA as compared to rAd5-GFP (28 and 2, respectively). Furthermore, in the instances where rAd5-CEA + rAd5-GFP vaccination did produce a significant amount of epitope-specific anti-CEA IgG, rAd5-CEA + rAd5-rEA vaccination always produced a substantially higher IgG titer against those same epitopes. These findings serve as a novel instance where adjuvant treatment alongside a CEA-targeted vaccine increased the breadth of anti-CEA B cell responses.

Enhanced in vivo CEA-targeted CTL killing following rAd5-CEA + rAd5-rEA co-administration

It is important to verify that the components of a multifaceted, robust memory response can be amplified against a TAA, but these constituents must also be functionally tested to validate their practical anti-tumorigenic actions. To measure the amount of CEA-specific cell killing that could be produced after the use of an adjuvant such as rEA, mice vaccinated 21 days prior with rAd5-CEA + rAd5-rEA or rAd5-CEA + rAd5-GFP, and Ad-naïve mice, were injected with equal amounts of CEA- or AMQ-pulsed syngeneic splenocytes, which had been labeled with high and low amounts of CFSE, respectively. Twenty-four hours following adoptive transfer of peptide-loaded, CFSE-labeled splenocytes, mice were killed and splenocytes were prepared for the measurement of the remaining CEA($CFSE^{High}$)- and AMQ($CFSE^{Low}$)-pulsed cells. Flow cytometry analysis revealed that the ratios of CEA/AMQ cells were no different between naïve (Fig. 6a) and rAd5-CEA + rAd5-GFP (Fig. 6b) co-injected mice. In contrast, splenocytes derived from rAd5-CEA + rAd5-rEA-treated animals possessed a notably smaller ratio of CEA/AMQ cells (Fig. 6), indicating more potent in vivo cCEA-specific CTL activity. Calculation of the percentage of specific CEA cell killing revealed that all animals treated with rAd5-CEA + rAd5-rEA sustained some measurable level of specific cytotoxic lymphocyte killing, while none of the rAd5-CEA + rAd5-GFP co-injected animals could ($p < 0.001$) (Fig. 6d).

To investigate whether the extent of the anti-CEA T cell and B cell responses may be correlated, serum was collected at the same time as splenocyte collection and used to measure anti-CEA IgG by ELISA. Since rAd5-CEA + rAd5-GFP treatment produced no assessable CEA-specific killing, we only compared the killing percentages of rAd5-CEA + rAd5-rEA-treated animals to their respective anti-CEA IgG titers. Linear regression analysis revealed a significant, positive linear correlation ($p = 0.0148$; $r = 0.8523$) between the percent of CEA-specific killing and anti-CEA IgG titers (Fig. 6e). These findings suggest not only that the formulation of rAd5-CEA

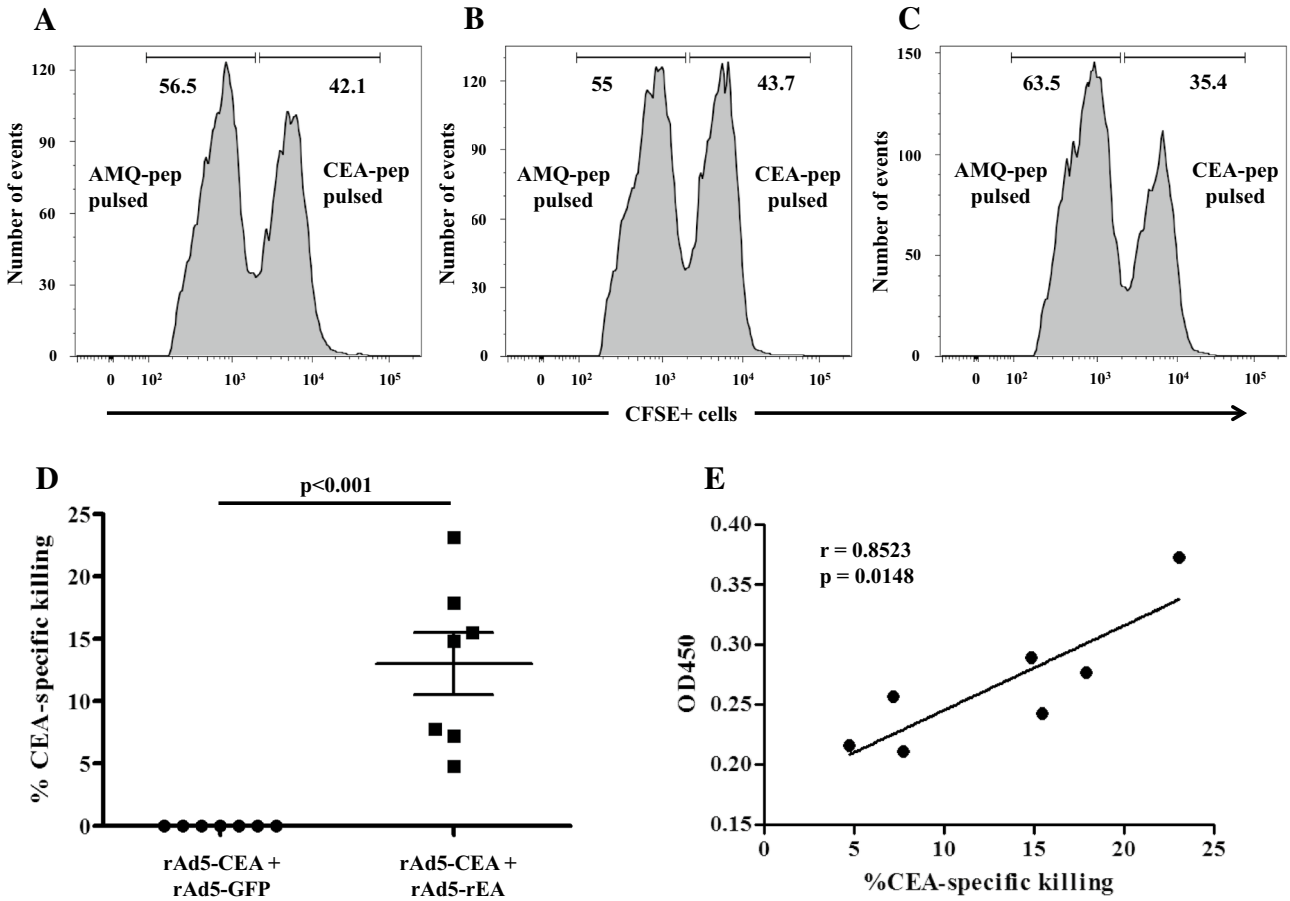

Fig. 6 rAd5-CEA and rAd5-rEA co-injection induces significant CEA-specific in vivo cytolytic T cell killing activity. C57BL/6 mice ($n = 7$) were IM injected with 5×10^9 vp rAd5-CEA and 5×10^9 vp rAd5-GFP or rAd5-rEA. At 21 days post-injection, syngeneic splenocytes were pulsed with CEA-specific peptides or AMQ and labeled with $CFSE^{High}$ (10 μM) or $CFSE^{Low}$ (1 μM), respectively. Immunized and naïve ($n = 5$) mice were injected with equal amounts of CEA- and AMQ-pulsed splenocytes and, 24 h following, mouse splenocytes were collected, washed, and analyzed via FACS. Population percentages of low-CFSE AMQ-pulsed splenocytes and high-CFSE CEA-pulsed splenocytes were compared with naïve **a**, rAd5-CEA + rAd5-GFP **b**, and rAd5-CEA + rAd5-rEA **c** treatment groups. **d** Percentages of CEA-specific CTL killing was compared between vaccine groups by calculating % specific killing $= 1 - ((\%CEA/\%AMQ)_{immunized}/(\%CEA/\%AMQ)_{naive}$. **e** At the time of killing, serum was collected from these mice, diluted (1:100), and performed an anti-CEA ELISA to measure anti-CEA IgG levels. The obtained (450 nm) OD values from rAd5-CEA + rAd5-rEA were plotted against respective percentages of CEA-specific CTL killing from each individual animal. The *line* represents a calculated best-fit linear regression between variables

vaccine with rAd5-rEA allows for measurable, functional CEA-specific cell killing, but also that these actions correspond directly with levels of IgG antibodies against CEA.

Discussion

Creating a strong enough memory response against an often immune-tolerated TAA has been a persistent issue of the cancer immunotherapy field since its inception [38]. Alternatively, researchers have observed that immunogenic treatments that are too aggressive can induce severe systemic side effects, as was seen in the development of an inflammatory colitis following high-dose autologous T cell transfer [31]. Recombinant adenoviral delivery of tumor-associated antigens has become a popular immunotherapy method, leading to the exploration of many antigenic vaccine targets within numerous human clinical trials. This class of vectors serves as a desirable option due to their ability to efficiently and exceptionally promote T cell memory responses against expressed antigens, which is largely due to rAds' ability to trigger multidimensional innate immune responses [16, 26, 39]. Recently, we have reported that the use of an adenovirus vector encoding the gene CEA (rAd5-CEA) can be safely administered to human subjects and may prolong survival of patients with advanced, chemotherapy-resistant colorectal cancers by inducing potent anti-CEA-specific T cell immunity [8].

In this current study, we wished to investigate whether the efficaciousness of this same vaccine could be further improved with the supplementation of a vector expressing the TLR agonist and anti-tumorigenic protein, rEA. The adjuvant properties and human safety profile of rEA have been previously established [17–19]; therefore, we felt that the characterization of rEA's immunomodulatory effects on a TAA-based vaccine was warranted. Generally, our results found that increasing doses of rAd5-CEA + rAd5-rEA resulted in a greater amount of CEA immune recognition, including stronger CMI (as quantified by the amount of IFN-γ- and IL-2-secreting splenocytes), greater amounts of cytotoxic T cell activation (as measured by greater amounts of IFN-γ-, TNF-α,- and perforin-expressing CD8+ T cells), and higher anti-CEA IgG titers.

Interestingly, the amounts of IL-2 expressing CD8+ T cells from vaccinated mice were no greater those from naïve mice, which contrasts with the overall increased amounts of IL-2 expressing splenocytes following rAd5-CEA + rAd5-rEA vaccination. This finding may signify that other non-CD8+ splenocyte populations, such as CD4+ T cells, may be preferential IL-2 expressers in response to CEA recognition, a phenomena that has been observed in previous vaccine studies where broad antigen-specific CTL responses have been induced [40].

It has been observed that T cells from both healthy and tumor-burdened patients can respond to a wide range of CEA-derived antigens; it is a major therapeutic goal that vaccination against CEA may break down immunologic tolerance to these antigens [30, 41]. Vaccine trials where a single CEA epitope was targeted have cited having to exclude many patients who did not meet HLA-matching criteria [42], limiting the vaccine's treatable population. Alternatively, a strong, multiregional repertoire of antigen epitopes could allow for a greater incidence of efficacy across different HLA backgrounds and against multiple variants of CEA-expressing malignancies [32, 33]. We tested whether the adjuvant activities of rAd5-rEA could not only increase the response to individual CEA-derived antigens, but also broaden this type of response to greater numbers of CEA-derived antigens when paired with the rAd5-CEA vaccine. We found that inclusion of a rEA-expressing vector along with a CEA-targeting vaccine induced substantial CMI against several distinct CEA-derived peptides, including four documented epitopes previously shown to be directly recognized by three distinct HLA haplotypes [34–36]. Interestingly, these haplotypes include class I and II HLAs, suggesting that rAd5-CEA + rAd5-rEA co-vaccination may have the potential to activate not only CEA-directed CD8+ T cells, but also effector CD4+ T cells, the numbers of which have been found to be strongly and positively correlated with improved clinical outcomes of advanced malignancy patients [43]. However, verification of these notions will require further study in a humanized murine model or human subjects.

While the importance of anti-TAA IgG development has been well established [2], many CEA-targeting vaccine prototypes have been found to be unable to induce anti-CEA IgG production above pre-treatment levels [8, 37]. In our work, while rAd5-CEA vaccination alone was a weak inducer of anti-CEA humoral immunity [8], the combination of rAd5-CEA with rAd5-rEA was able to create a significant amount of circulating serum anti-CEA IgG to several peptide epitopes of CEA. According to the Immune Epitope Database and Analysis Resource (www.iedb.org), there are over 100 confirmed immune epitopes derived from human CEA protein, but as of yet none have been confirmed by B cell response assay, making further investigation into the humoral promotion that rAd5-CEA + rAd5-rEA could provide even more important. To determine exact MHC-epitope immunogenicity and affinities induced by this vaccine regimen, a cold target inhibition assay would also be helpful to use in forthcoming studies.

Furthermore, rAd5-CEA + rAd5-rEA-vaccinated animals also showed markedly higher levels of CEA-specific killing, with the amount of CEA-specific in vivo CTL killing in rAd5-CEA + rAd5-rEA-vaccinated animals being positively correlated with the amount of anti-CEA IgG detected in their serum. This suggests that induction of humoral- and/or cell-mediated adaptive immune responses against TAAs such as CEA are not mutually exclusive and can be promoted alongside each other using potent adjuvants such as rEA. While none of the mice administered rAd5-CEA + rAd5-GFP were able to induce greater amounts of CEA-specific cell killing over mock-injected mice in this study, previous murine and human experiments using rAd5-CEA have shown it to promote robust killing of CEA-expressing tumor cells [7, 8]. Thus, it may be likely that high levels of CEA-specific cell killing are only initiated when the number of vaccinations administered is increased, as three separate vector inoculations were administered during the rAd5-CEA murine and human trials [7, 8]. Future studies are merited to further explore dosing kinetics of the rAd5-CEA + rAd5-rEA vaccination schema.

The molecular mechanisms by which innate immune modulators, such as rEA, assist in the development of a strong immunologic memory response are still in the process of being understood [44]. Many of the identified characteristics that make an adjuvant effective have been detected in rEA, such as promotion of NK cell activation, increased amounts of DC activation/maturation, induction of pro-inflammatory cytokines (e.g., IL-12(p70), TNF-α), and an overall robust Th1-skewing response [17, 19, 20, 45]. rEA also promotes relatively unique agonist signaling mechanics in that it uses the TLR adapter protein MyD88

as a positive regulator, and yet another TLR adapter, TRIF, as a negative regulator [45]. While there have been previous concerns regarding rEA's immunogenicity as a profilin-like protein to be limited to mammals that express functional TLR11/12, clinical trials and ex vivo human studies have assuaged much of this concern, revealing there to be many TLR11/12-independent mechanisms by which rEA triggers human immunity [17, 18, 20]. Moreover, it has been recently confirmed that human PBMC recognition of profilin is instead dependent on TLR5 [46], thereby suggesting that rEA may be recognized by this PRR as well. We have also previously shown that rEA can directly activate human NK cells [20], a critical subtype of innate immune cells that perform anti-tumor immune activities [47]. Furthermore, rEA has been verified as safe to administer to human patients, even those who may have advanced malignancies with compromised immune systems [17, 18], an attribute that is lacking for many other innate immune agonists [48].

With the knowledge that rEA is a robust innate stimulator, we explored the scope of adaptive immunity that it could affect. Our previous work has revealed that the adjuvant effects rAd5-rEA possesses are target-specific, with injections of rAd5-rEA alone inducing no specific adaptive immune responses without a co-injected rAd5-transgene to direct its boosting properties toward [19]. While we have previously shown rAd5-rEA to promote transgene immunity against the HIV-gag protein [19], this is the first time that co-injection of it with a TAA-expressing Ad has been shown to have similar benefits. Furthermore, the importance of gut microbiota (of which rEA is derived) and innate activation has been shown to be essential in response to several chemotherapy treatments [49, 50], suggesting the use of rAd5-CEA + rAd5-rEA alongside these medications may provide a substantially better clinical outcome without making the treatment regimen more physically rigorous for the patient. As phase II trials have already shown significant preliminary success with the use of rAd5-CEA in advanced colorectal cancer patients, it is exceedingly relevant that an adjuvant which would enhance anti-tumor effect to an even greater extent be considered. All in all, rAd5-rEA has the possibility of serving as a conduit to a much larger and more diverse immune response against CEA and a multitude of other TAA-expressing malignancies.

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Conflict of interest The authors declare that they have no conflict of interest.

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