

CD27 stimulation unveils the efficacy of linked class I/II peptide vaccines in poorly immunogenic tumors by orchestrating a coordinated CD4/CD8 T cell response

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ABSTRACT

Despite their promise, tumor-specific peptide vaccines have limited efficacy. CD27 is a costimulatory molecule expressed on CD4⁺ and CD8⁺ T cells that is important in immune activation. Here we determine if a novel CD27 agonist antibody (ahCD27) can enhance the antitumor T cell response and efficacy of peptide vaccines. We evaluated the effects of ahCD27 on the immunogenicity and antitumor efficacy of whole protein, class I-restricted, and class II-restricted peptide vaccines using a transgenic mouse expressing human CD27. We found that ahCD27 preferentially enhances the CD8⁺ T cell response in the setting of vaccines comprised of linked class I and II ovalbumin epitopes (SIINFEKL and TEWTSSNVMEERKIKV, respectively) compared to a peptide vaccine comprised solely of SIINFEKL, resulting in the antitumor efficacy of adjuvant ahCD27 against intracranial B16.OVA tumors when combined with vaccines containing linked class I/II ovalbumin epitopes. Indeed, we demonstrate that this efficacy is both CD8- and CD4-dependent and ahCD27 activity on ovalbumin-specific CD4⁺ T cells is necessary for its adjuvant effect. Importantly for clinical translation, a linked universal CD4⁺ helper epitope (tetanus P30) was sufficient to instill the efficacy of SIINFEKL peptide combined with ahCD27, eliminating the need for a tumor-specific class II-restricted peptide. This approach unveiled the efficacy of a class I-restricted peptide vaccine derived from the tumor-associated Trp2 antigen in mice bearing intracranial B16 tumors. CD27 agonist antibodies combined with peptide vaccines containing linked tumor-specific CD8⁺ epitopes and tumor-specific or universal CD4⁺ epitopes enhance the efficacy of active cancer immunotherapy.

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CD27; tumor immunotherapy; peptide vaccine; adjuvant; monoclonal antibody


Introduction

Tumor immunotherapy has emerged as a promising treatment modality for advanced malignancies. Specifically, peptide vaccines derived from MHC class I-restricted tumor antigens offer the promise of inducing robust tumor-specific CD8⁺ T cell responses^{1,2} to promote effective antitumor immunity.^{3–6} Unfortunately, the efficacy of class I-restricted peptide vaccines has proven to be limited,^{7,8} with overall clinical response rates as low as 3%.⁹ Class II CD4⁺ T cell helper epitopes are also capable of inducing potent antitumor immune responses,^{10–13} but peptide vaccines consisting of co-administered class I/II epitopes have also not yet experienced widespread clinical success.⁷ Novel adjuvant strategies are clearly needed to enhance the clinical utility of peptide vaccines in cancer immunotherapy.

Immunomodulatory antibodies targeting T cell checkpoint molecules have emerged as promising therapies that may be capable of promoting robust tumor-specific immunity in the setting of tumor vaccines that are otherwise ineffective.^{14–16} CD27, a member of the tumor necrosis factor receptor (TNFR) superfamily, is a costimulatory molecule expressed on naïve and activated CD4⁺ and CD8⁺ T cells¹⁷ and is known to be important in T cell activation,¹⁸ maturation,¹⁹ cytokine secretion,²⁰ and survival,²¹ making it a promising target for T cell-based immunomodulation. Recently, a novel, fully human anti-human CD27 monoclonal antibody (ahCD27) was developed which binds with high affinity to induce potent human T cell responses in the context of T cell receptor stimulation.²² Because CD27 stimulation on both CD4⁺ T cells and CD8⁺ T cells can lead to their enhanced effector function and concomitant vaccine-induced CD4⁺ T cell help strengthens CD8⁺ T cell vaccine responses, we hypothesized that ahCD27 could be leveraged as

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an adjuvant for peptide vaccines and that it would provide a therapeutic benefit preferentially in the setting of peptide vaccines comprised of class I and II epitopes.

In this study, we evaluated the therapeutic effect of hCD27 as a vaccine adjuvant in the setting of a highly aggressive intracranial tumor model. We demonstrate that hCD27 enhances the immune response to class I-restricted tumor antigens, and that its adjuvant effect is potentiated in the setting of linked class I- and class II-restricted peptides. Additionally, we show that a universal CD4⁺ epitope is sufficient to enhance the tumor-specific CD8⁺ T cell response and increase the efficacy of tumor-derived class I-restricted peptide vaccines in the setting of adjuvant hCD27, eliminating the need for class II-restricted tumor antigens. Taken together, our data suggest that hCD27 coordinates CD8⁺ and CD4⁺ T cell responses to enhance the antitumor immune response. These findings highlight the potential for CD27 agonist antibodies to improve the clinical benefit of peptide vaccines for cancer immunotherapy.

Materials and methods

Study design

The goal of this study was to characterize the adjuvant activity of a clinically available immunomodulatory agonist anti-CD27 antibody and its therapeutic potential in a mouse model of advanced stage intracranial malignancy. The experimental design involves studies of vaccine-induced immunogenicity and survival of mice bearing intracranial B16 melanoma tumors. All mice were of the C57BL/6 background, aged 6–12 weeks, and female; naïve or tumor-bearing animals were randomized into treatment groups before the start of each experiment. Immunogenicity experiments were performed in groups of 5 mice each, while survival studies were performed with group sizes in excess of 7 mice each. Sample sizes were calculated using F-power analysis ($\alpha = 0.05$) to yield at least 80% power to detect interactions, based on pilot data. For survival studies, pre-defined humane endpoints were used, according to the Duke University Institutional Animal Care and Use Committee (IACUC) guidelines. All experimental protocols and procedures were approved by the Duke University IACUC. All experiments were performed at least three times, and all outliers were included in the data analysis.

Mice and tumor cell lines

All mice were bred and maintained under pathogen-free conditions at Duke University Medical Center (DUMC). C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, NC, USA), and transgenic OT-I and OT-II mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Human CD27 transgenic (hCD27) mice, which express both murine and human CD27 molecules under the native murine CD27 promoter,²³ were obtained from Celldex Therapeutics (Hampton, NJ, USA) and bred at DUMC. Homozygous hCD27 males were bred with C57BL/6, OT-I, or OT-II females to generate heterozygous hCD27, hCD27xOT-I, or hCD27xOT-II mice, respectively, for use in

experiments. All animal experiments were performed according to protocols approved by the Duke University IACUC (Protocol Number: A283–15–11). The tumor cell lines B16, F10 and B16.OVA were a kind gift from Dr. Richard G. Vile at Mayo Clinic.^{24,25} All cell lines used were submitted for cell line authentication (Cell Check) and pathogen testing including mycoplasma testing (IMPACT) at IDEXX BioResearch prior to use. We confirmed species of origin, cell line specific markers and assessed for possible cross-contamination with other cell lines or pathogens

Evaluation of vaccine-induced t cell responses

Whole ovalbumin (Ova) protein was purchased from Sigma Aldrich (St. Louis, MO, USA), and custom peptides were purchased from JPT (Berlin, Germany) (for peptide sequences see Table 1). For whole Ova protein vaccination, hCD27 mice received intraperitoneal (ip) vaccination on day 0 with 2.5 mg Ova resuspended at 10 mg/mL in water. For peptide vaccination, hCD27 mice received intradermal (id) vaccination on day 0 with peptide emulsified in incomplete Freund's adjuvant (IFA) (ThermoFisher, Waltham, MA, USA). As indicated by the experiment, the following peptides were used for vaccination: 50 μ g of H2-Kb class I-restricted (Ova(I)); 100 μ g of H2-IAb class II restricted Ova(II^A); 100 μ g of H2-IAb class II restricted Ova(II^B); 150 μ g of class II-restricted P30; 150 μ g of linked Ova(I-II^A); 200 μ g of linked Ova(I)-P30; 50 μ g of H2-Kb class I-restricted Trp2 (Trp2(I)); or, 200 μ g of linked Trp2-P30. The quantity of Ova-derived peptide used corresponds to the equimolar mass of the peptide in relation to 2.5 mg of whole Ova protein. Administration of hCD27 was performed ip three days prior to (day -3) and on the day of vaccination (day 0) with 100 μ g hCD27 (Celldex Therapeutics) or 100 μ g recombinant human IgG1 Fc isotype control (Bio X Cell, West Lebanon, NH, USA). On day 7 after vaccination, whole blood (50–100 μ L) was collected by retro-orbital puncture for flow cytometric analysis of Ova(I)-specific cells, and spleens were harvested for ELISpot assays.

Flow cytometric analysis of ova(i)-specific CD8⁺ t cells

For the detection of circulating Ova(I)-specific CD8⁺ T cells, 50 μ L whole blood was incubated with rat anti-mouse CD8-FITC (BD Biosciences, San Jose, CA, USA) and H2-Kb (SIINFEKL)-PE murine tetramer (MBL International, Woburn, MA, USA) in 100 μ L PBS in the dark for 30 min at room temperature. Red blood cells (RBCs) were lysed, and

Table 1. Peptide sequences.

Peptide	Residue	Sequence
Ova(I)	Ova(257–264)	SIINFEKL
Ova(II ^A)	Ova(265–280)	TEWTSNNVMEERKIKV
Ova(II ^B)	Ova(323–339)	ISQAVHAAHAEINEAGR
Ova(I-II ^A)	Ova(257–280)	SIINFEKLTEWTSNNVMEERKIKV
P30	TT(948–968)	FNNFTVSFWLRVPKVSASHLE
Ova(I)-P30	Ova(257–264)- Linker-TT(948–968)	SIINFEKLRVKRFNFTVSFWLRVPKVSASHLE
Trp2(I)	Trp2(180–188)	SVYDFVWL
Trp2(I)-P30	Trp2(180–188)- Linker-TT(948–968)	SVYDFVWLRVKRFNFTVSFWLRVPKVSASHLE

cells were fixed with 1 mL 1X FACS Lysing Solution (BD Biosciences) in the dark for 15 min at room temperature and washed in PBS. All samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences); absolute numbers per μL blood were calculated using Flowcount[®] beads (Beckman Coulter, Indianapolis, IN, USA), according to the manufacturer's instructions.

Ifn γ ELISPOT

Vaccine-specific T cell responses were evaluated *ex vivo* by IFN γ ELISPOT. MultiScreen[®] 96-well filter plates (EMD Millipore, Billerica, MA, USA) were coated with 10 $\mu\text{g}/\text{mL}$ anti-mouse IFN γ antibody (Mabtech, Cincinnati, OH, USA) overnight at 4°C. A total of 2.5×10^5 splenocytes/well were incubated in duplicate in RPMI media supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA), 1X non-essential amino acids (Life Technologies, Carlsbad, CA, USA), 1 mM L-glutamine (Life Technologies), and 100 IU/mL penicillin + 100 $\mu\text{g}/\text{mL}$ streptomycin (Life Technologies), in the presence or absence of 1 $\mu\text{g}/\text{mL}$ of the indicated peptide overnight at 37°C in a 5% CO₂ incubator. Spots were developed using 1 $\mu\text{g}/\text{mL}$ biotinylated anti-mouse IFN γ mAb (Mabtech), a VECTASTAIN[®] Elite ABC horseradish peroxidase kit (Vector Laboratories, Burlingame, CA, USA), and AEC substrate chromogen (Sigma); spots were quantified by ZellNet Consulting (Fort Lee, NJ, USA).

Tumor implantation

B16.F10 and B16.OVA cells were grown in DMEM (Life Technologies), 10% FBS and 2 mM L-glutamine at 37°C in 5% CO₂. For intracranial tumor implantation, cells were harvested, resuspended at 3×10^6 cells/mL (B16.OVA) or 2×10^5 cells/mL (B16.F10), mixed 1:1 with 10% methylcellulose in PBS, and loaded into a 250 μL syringe (Hamilton, Reno, NV) with an attached 25-gauge needle. The needle was positioned 2 mm to the right of bregma and 4 mm below the surface of the skull at the coronal suture using a stereotactic frame (Kopf Instruments, Tujunga, CA). A dose of 7,500 cells (B16.OVA) or 500 cells (B16.F10) in a total volume of 5 μL was injected into hCD27 mice. For therapeutic survival studies, tumors were implanted on day 0, followed by 100 μg of ahCD27 or isotype ip on days 3 and 6 after tumor implantation. On day 6, the same day as the second dose of ahCD27, vaccination was administered (either 2.5 mg of ip injected whole Ova protein in water, or the indicated amount of id injected peptide emulsified in IFA). Tumor-bearing mice were monitored daily for morbidity endpoints and survival according to the Duke University IACUC guidelines.

Analysis of tumor-infiltrating lymphocytes

Tumors were harvested at day 14 after implantation and homogenized in a Stomacher[®] 80 Biomaster (Seward, Islandia, NY) in 6 mL digestion buffer [RPMI 1640 supplemented with 100 IU/mL penicillin + 100 $\mu\text{g}/\text{mL}$ streptomycin, 1 mM L-glutamine, 1X non-essential amino acids, 1 mM sodium pyruvate (Life Technologies), 25 μM β -

mercaptoethanol (ThermoFisher), 10% FBS, 133 $\mu\text{g}/\text{mL}$ DNase I (Roche, Indianapolis, IN, USA), and 133 units/mL Type IV collagenase (Life Technologies)] for 20 min at 37°C. The resultant cell suspension was filtered through a 40 μm strainer and washed twice with PBS. The cells were stained with LIVE/DEAD[®] (ThermoFisher), H2-Kb(SIINFEKL) tetramer, and antibodies for CD3, CD4, and CD8 cell surface markers (BD Biosciences), according to the manufacturers' instructions. The cells were resuspended in 150 μL PBS and analyzed on a FACSCalibur flow cytometer.

T cell depletion studies

For immunogenicity studies, mice were depleted of CD4⁺ or CD8⁺ cells in the priming phase by once daily intraperitoneal doses of 200 μg αCD4 (GK1.5, Bio X Cell) or αCD8 (2.43, Bio X Cell), respectively, for three consecutive days prior to vaccine/ahCD27 administration (as previously described), and immune responses were assessed at day 7 after vaccination. For survival studies, CD8⁺ cells were depleted by once daily intraperitoneal administration of 200 μg αCD8 for three consecutive days immediately after intracranial tumor implantation and before Ova/ahCD27 treatment. For CD4 depletion studies in tumor-bearing mice, a tumor challenge model was employed in which mice were implanted with intracranial B16.OVA tumors seven days after vaccination with whole Ova protein and ahCD27; CD4⁺ cells were depleted by once daily intraperitoneal administration of 200 μg αCD4 for three consecutive days prior to Ova/ahCD27 vaccination (priming phase) or for three consecutive days immediately after intracranial tumor implantation (effector phase).

Adoptive lymphocyte transfers

CD4⁺ and CD8⁺ T cells were purified from the spleens of OTII and OTI mice, respectively, by magnetic labeling in an autoMACs[®] Pro Separator (Miltenyi Biotec, San Diego, CA). Briefly, spleens were disaggregated and filtered into single cell suspensions, and RBCs were removed by incubating for 5 min in 1X lysis buffer (BD Biosciences). The splenocytes were then washed once in RPMI media and once in MACs buffer (Miltenyi Biotec) and resuspended at 2.5×10^8 cells per mL. The cells were then subject to magnetic labeling and T cell isolation using CD4⁺ and CD8⁺ T cell isolation kits (Miltenyi Biotec), according to the manufacturer's instructions. The purified CD4⁺ and CD8⁺ T cells were then mixed at a 2:1 ratio in PBS at a total cell concentration of 3×10^7 per mL, and 100 μL of the appropriate cell mixture was injected intravenously in the tail veins of wildtype C57BL/6 mice.

Statistical analysis

Overall survival was computed from the date of tumor implantation to the date of humane endpoint or death. Survival distributions are described using Kaplan-Meier methods, and the Gehan-Breslow-Wilcoxon test was used to compare survival distributions between treatment groups. Student's unpaired t-test was used to compare IFN γ SFU and tetramer values upon adjuvant ahCD27 administration

versus isotype control. One-way ANOVA was used to analyze TIL levels and the effect of T-cell depletion on the ovalbumin vaccine response. Two-way ANOVA was used to assess the magnitude of the effect of α hCD27 on the CD8⁺ T cell response in animals receiving different vaccine types. Associations of class II responses with class I responses were assessed using the Pearson correlation coefficient.

Results

ahcd27 enhances the immune response to a whole protein vaccine and promotes antitumor efficacy

To explore the use of CD27 costimulation as a vaccine adjuvant, we first evaluated the effect of α hCD27 on the CD8⁺ T cell response to vaccination with whole protein using ovalbumin as a model antigen. Groups of hCD27 mice received α hCD27 or hIgG1 3 days before and on the day of vaccination, vaccine responses were evaluated by the frequency (as determined by tetramer staining of peripheral blood leukocytes) and effector function (as determined by *ex vivo* re-stimulation in a IFN γ ELISPOT) of T cells specific for the

immunodominant ovalbumin class I epitope (Ova(I)) (Figure 1). We found that the frequency of peripheral blood Ova(I)-specific CD8⁺ T cells increased from ~0.5% to ~7% in mice that received whole ovalbumin protein combined with α hCD27 compared to human IgG1 (hIgG1) controls (Figure 1A, $P = 0.0035$). These vaccine-induced responses peaked a week after vaccination and consisted largely of a CD44⁺CD62L⁻ effector memory phenotype (Supplemental Figure 1). Additionally, we observed an increase in the level of IFN γ -producing splenic lymphocytes upon *ex vivo* re-stimulation with Ova(I) peptide, from 110 ± 37 IFN γ + spot forming units (SFUs) per 10^6 splenocytes in control mice to $1,656 \pm 139$ SFUs per 10^6 splenocytes in α hCD27-treated mice (Figure 1B, $P < 0.0001$). Both altering the timing of α hCD27 administration or increasing the number of vaccinations did not further enhance vaccine immunogenicity; therefore, our original regimen was continued for all subsequent experiments (Supplemental Figure 2).

We next asked whether adjuvant α hCD27 combined with whole ovalbumin protein would potentiate the tumor immune response and be efficacious against intracranial B16.OVA, a highly aggressive melanoma model that is

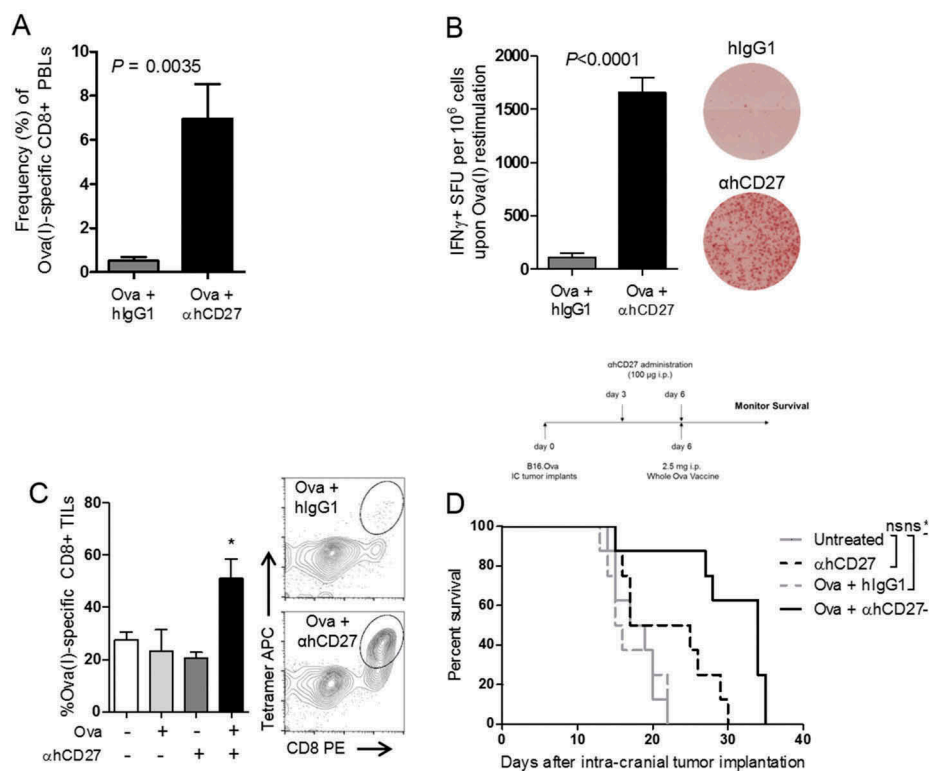


Figure 1. *ahCD27 enhances the ovalbumin protein response and promotes antitumor efficacy.* (A) hCD27 mice ($n = 5$ per group) received ip 2.5 mg whole Ova protein alongside 100 μ g of α hCD27 or hIgG1 isotype control (3 days prior to and on the day of whole Ova protein vaccination). Ova(I)-specific CD8⁺ T cell responses were evaluated by H2-Kb(SIINFEKL)-PE tetramer staining of peripheral blood cells seven days after vaccination. Flow cytometric analysis was done by FWD vs SSC identification of the lymphocyte population, followed by gating on CD3⁺ cells, with subsequent gating on tetramer⁺ CD8⁺ cells. (B) Vaccine-induced CD8⁺ T cell responses were also evaluated by *ex vivo* re-stimulation of splenocytes with Ova(I) peptide in an IFN γ ELISPOT assay. (C) hCD27 mice bearing intracranial B16.OVA tumors received 100 μ g of α hCD27 or hlgG1 isotype control at days 3 and 6 after tumor implantation, with or without whole Ova protein vaccination on day 6 after tumor implantation. Tumors were harvested on day 14 after implantation and analyzed for the frequency of tumor-infiltrating Ova(I)-specific CD8⁺ T cells by H2-Kb(SIINFEKL)-PE tetramer staining. Flow cytometric analysis was done by gating on live CD3⁺ cells, followed by gating on tetramer⁺ CD8⁺ cells. (D) The efficacy of combinatorial whole Ova protein vaccination and α hCD27 administration ($n = 7$ per group) was evaluated in hCD27 mice bearing 3-day established intracranial B16.OVA tumor. Schema on top of the survival curve indicates the experimental design. B16.OVA tumors were implanted on day 0, followed by 100 μ g of α hCD27 or isotype ip on days 3 and 6 after tumor implantation. On day 6, same day after the last dose of α hCD27, vaccination with 2.5 mg of whole Ova protein was given ip and survival was monitored. Statistical analyses were performed using Student's unpaired t-test (A and B), one-way ANOVA with Tukey post-hoc comparisons (C), or the Gehan-Breslow-Wilcoxon test (D). Statistical significance was determined at a * P value < 0.05.

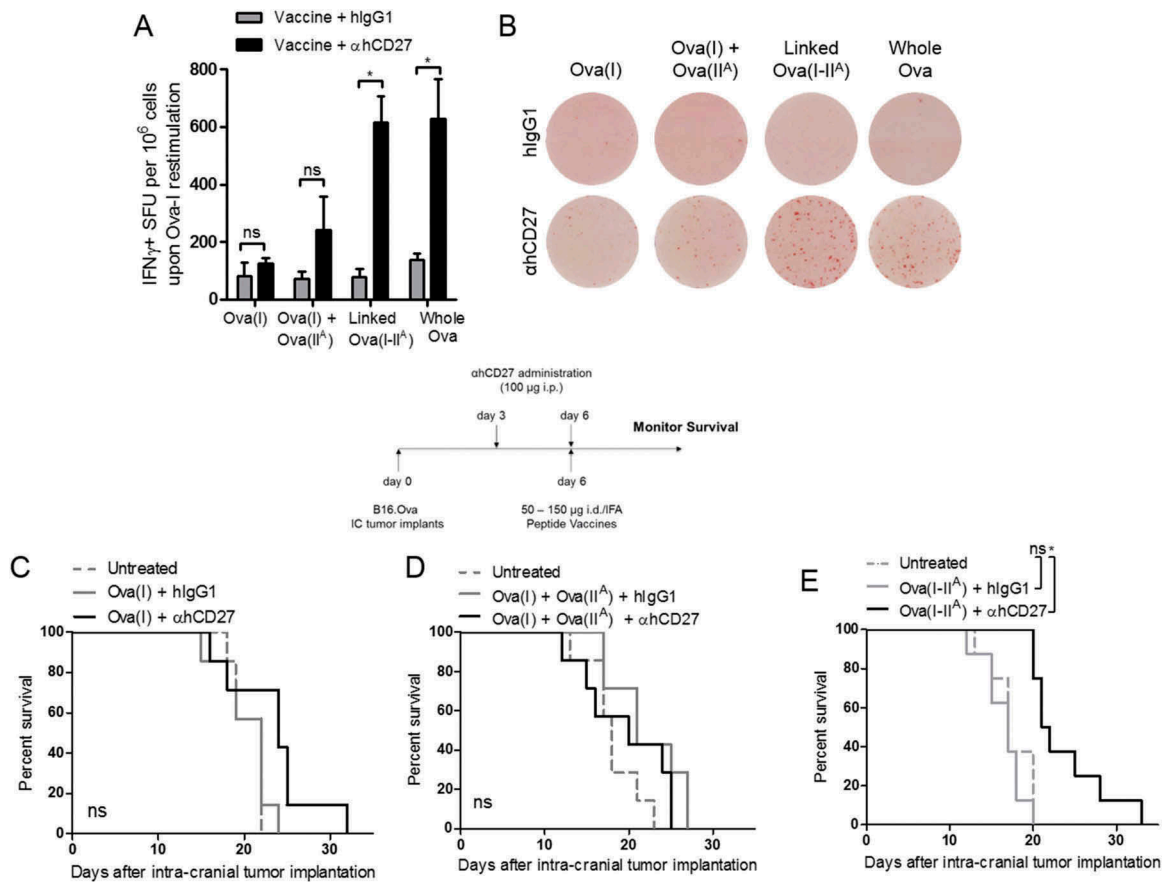


Figure 2. The adjuvant effect of ahCD27 on the class I peptide response is enhanced by a linked class II epitope. (A) hCD27 mice ($n = 5$ per group) were vaccinated with either: 50 μg of Ova(I) peptide, 50 μg Ova(I) and 100 μg Ova(II^A) peptides, 150 μg of the linked Ova(I-II^A) peptide, or 2.5 mg whole Ova protein. Peptide vaccines were emulsified in IFA and given id and whole Ova protein was administered ip in water. 100 μg ahCD27 or hlgG1 isotype control were given ip three days before (day -3) and on the day of vaccination (day 0). CD8⁺ T cell responses were evaluated by *ex-vivo* splenocyte re-stimulation with the Ova(I) peptide in a IFN γ ELISpot (B) representative ELISpot results are shown. (C-E) hCD27 mice ($n = 7$ per group) were implanted with intracranial B16.OVA tumors on day 0, received 100 μg of ip ahCD27 or hlgG1 isotype control 3 days and 6 days after tumor implantation, and received vaccination 6 days after implantation. Schema on top of the survival curves indicates the experimental design. Vaccination consisted off: 50 μg of Ova(I) peptide (C), co-administration of 50 μg Ova(I) and 100 μg of Ova(II^A) peptides (D), or 150 μg linked Ova(I-II^A) peptide (E). Statistical analyses were performed using two-way ANOVA with Tukey post-hoc comparisons (A) or the Gehan-Breslow-Wilcoxon test (C, D, E). Statistical significance was determined at a * P value < 0.05.

consistently resistant to vaccine immunotherapy.²⁶ hCD27 mice bearing 3-day established intracranial B16.OVA tumors and treated with whole ovalbumin protein with adjuvant ahCD27 demonstrated an increased frequency and absolute number of Ova-specific CD8⁺ T cells both peripherally and intratumorally in comparison to mice treated with isotype control (Supplemental Figure 3). Flow cytometric analysis of the tumor-infiltrating lymphocytes (TILs) revealed a marked increase in the level of Ova(I)-specific CD8⁺ T cells, from ~20% of the CD8⁺ TIL population in mice that received ovalbumin alone to ~50% in mice treated with ovalbumin + ahCD27 (Figure 1C, Ova vs Ova + ahCD27: $P = 0.0162$). This enhanced level of tumor-infiltrating CD8⁺ T cells corresponded to an 18-day increase in median survival in mice that received ovalbumin + ahCD27 compared to control animals (Figure 1D, Ova + ahCD27 vs. Ova + hlgG1: $P = 0.0024$).

The adjuvant effect of ahCD27 on the class I peptide response is enhanced by a linked class II epitope

Given the potent therapeutic effect of ahCD27 combined with whole ovalbumin protein and to inform the use of ahCD27 in

combination with peptide vaccines, we next examined which peptide components of whole ovalbumin protein were contributing to the enhanced ovalbumin-specific CD8⁺ T cell response in the setting of adjuvant ahCD27. We compared the adjuvant effect of ahCD27 when combined with four different ovalbumin-derived vaccines (Table 1): 1) the single immunodominant class I-restricted ovalbumin peptide epitope, SIINFEKL (Ova(I)); 2) Ova(I) co-administered with an immunodominant class II-restricted peptide epitope, TEWTTSSNVMEERKIKV (Ova(II^A), located immediately downstream of Ova(I)); 3) a long peptide comprised of the continuous Ova(I) and Ova(II^A) sequences (Ova(I-II^A)); and 4) the whole ovalbumin protein. We administered each of these four vaccines with combined ahCD27 or hlgG1 isotype control and evaluated the CD8⁺ T cell response seven days after vaccination by *ex vivo* restimulation with the Ova(I) peptide in a IFN γ ELISPOT assay. We observed only slight (but statistically insignificant) increases in the Ova(I)-specific CD8⁺ T cell response in the settings of ahCD27 alongside the single Ova(I) peptide vaccine or Ova(I) co-administered with Ova(II^A) (Figures 2A and 2B). In contrast, ahCD27 in the setting of Ova(I-II^A) and whole ovalbumin protein resulted in a 5-fold increase in the level of Ova(I)-specific CD8⁺ T cells

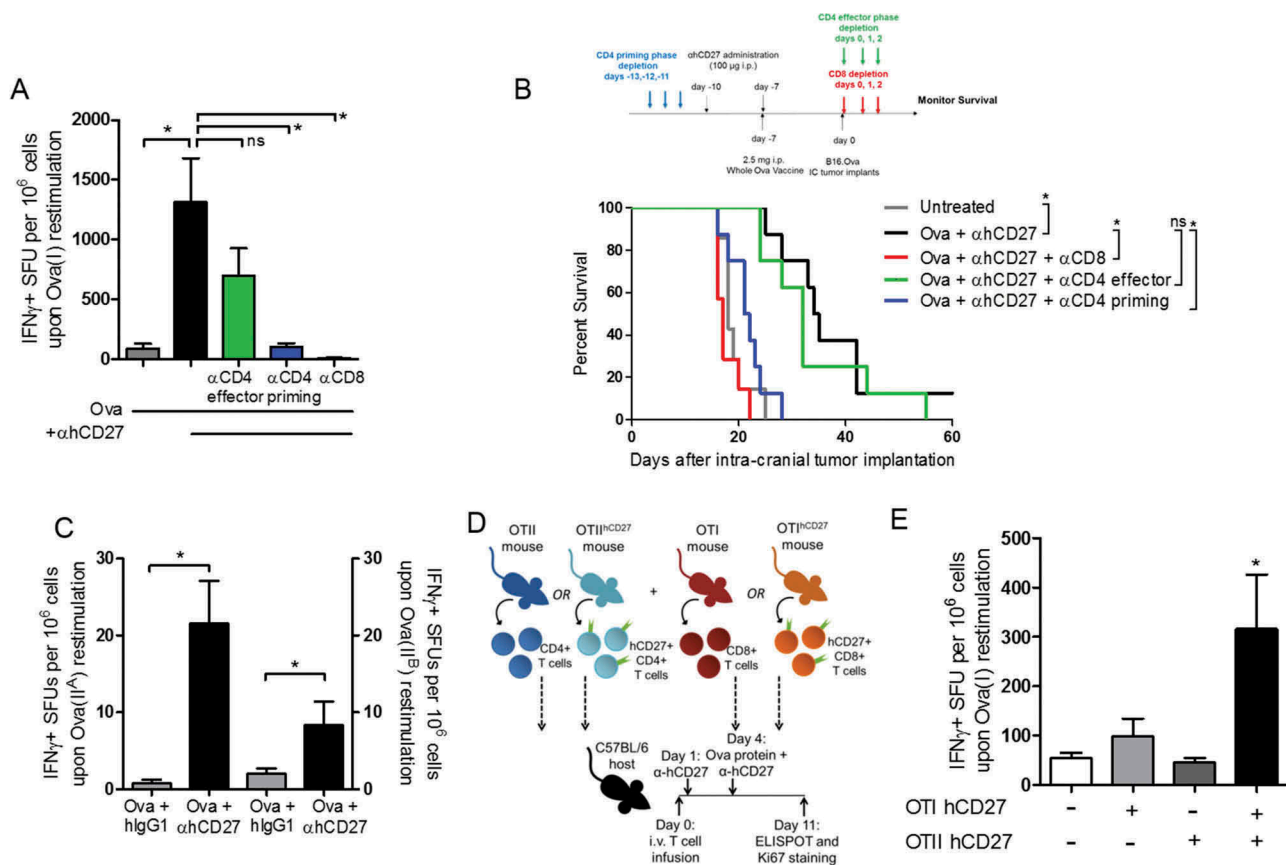


Figure 3. CD8⁺ and CD4⁺ T cells are required for the adjuvant effect and survival benefit of ahCD27. (A) hCD27 mice ($n = 5$ per group) received 2.5 mg whole Ova protein vaccine on day 0 with or without 100 μ g of ahCD27 on days -3 and 0. To assess the impact of T cell depletion, CD8⁺ T cells were depleted by 200 μ g of α CD8 antibody (clone 2.43) administered ip on days -6, -5, and -4 immediately prior to the first ahCD27 administration on day -3. CD4⁺ T cells were depleted by 200 μ g α CD4 antibody (clone GK1.5) administered ip on days -6, -5, and -4 to deplete during the priming phase, or on days 5, 6 and 7 to deplete during the T cell effector phase. CD8⁺ T cell responses were determined 8 days after vaccination by *ex vivo* re-stimulation of splenocytes with Ova(I) peptide in an IFN γ ELISpot. (B) The effect of T cell depletion on antitumor efficacy was evaluated in a tumor challenge (see schema on top of the survival curve). For all experiments, hCD27 mice ($n = 7$ per group) received 2.5 mg of whole Ova protein vaccine on day -7, 100 μ g of ahCD27 on days -10 and -7, and tumor implantation on day 0, survival was monitored. For CD8⁺ T cell depletion, 200 μ g of α CD8 was administered on days 0, 1 and 2 immediately after tumor implantation (red). For CD4⁺ T cell depletion during priming, 200 μ g of α CD4 was administered on days -13, -12, and -11 immediately prior to ahCD27 treatment on day -10 (blue). For CD4⁺ T cell depletion during the effector phase, α CD4 was administered on days 0, 1, and 2 immediately after tumor implantation (green). (C) The immune responses to two class II Ova epitopes, Ova(II^A) and Ova(II^B), were evaluated in hCD27 mice ($n = 5$ per group) vaccinated with 2.5 mg whole Ova protein ip on day 0 and treated with 100 μ g of ahCD27 or hlgG1 isotype control on days -3 and 0. Seven days after vaccination, splenocytes were harvested and T cell responses were evaluated by *ex vivo* re-stimulation of splenocytes with Ova(II^A) or Ova(II^B) peptide in an IFN γ ELISpot. (D-E) hCD27 transgenic mice were crossed with transgenic mice that possess Ova-specific CD8⁺ T cells (OTI) or Ova-specific CD4⁺ T cells (OTII) to generate Ova-specific T cells with hCD27 expression. As described in the methods, C57BL/6 hosts ($n = 5$ per group) received adoptive transfer of one of four combinations of T cells on day 0: OTI and OTII cells (no hCD27), OTI/hCD27 and OTII cells, (hCD27 on CD8) OTI and OTII/hCD27 cells (hCD27 on CD4), or OTI/hCD27 and OTII/hCD27 (hCD27 on CD4 and CD8). ahCD27 was administered ip on days 1 and 4 and whole Ova protein was given on day 4 (D). CD8⁺ T cell responses were measured by IFN γ ELISPOT upon *ex vivo* re-stimulation with Ova(I) peptide (E). Statistical analyses were performed using a one-way ANOVA with Tukey post-hoc comparisons (A), the Gehan-Breslow-Wilcoxon test (B), Student's unpaired t-test (C), and two-way ANOVA with Tukey post-hoc comparisons (E). Statistical significance was determined at a * P value < 0.05.

compared to controls (Figure 2A, linked Ova(I-II^A) + hlgG1 vs linked Ova(I-II^A) + ahCD27: $P = 0.00045$; whole Ova + hlgG1 vs whole Ova + ahCD27: $P = 0.0016$).

These results were mirrored in our tumor efficacy experiments with the three peptide vaccines. hCD27 mice bearing 3-day established intracranial tumors were treated with Ova(I), Ova(I)+Ova(II^A), or Ova(I-II^A), with or without ahCD27. None of these vaccines were therapeutic in the absence of adjuvant ahCD27, and ahCD27 combined with Ova(I) (Figure 2A) or Ova(I)+Ova(II^A) (Figure 2B) had no effect on survival. In contrast, we found that adjuvant ahCD27 in the setting of linked Ova(I-II^A) resulted in a significant therapeutic benefit (Figure 2C, Ova(I-II^A) + hlgG1 vs Ova(I-II^A) + ahCD27: $P = 0.0004$), reminiscent of our previous findings with whole ovalbumin protein. Taken together, these results suggest that linked class I and II epitopes drive the adjuvant

activity of ahCD27 on the CD8⁺ T cell response such that ahCD27 prolongs survival preferentially in the setting of vaccines comprised of linked class I and II epitopes.

CD8⁺ T cells and CD4⁺ T cells during the priming phase of the vaccine response are required for the adjuvant effect of ahCD27

The failure of a class I-restricted peptide vaccine to induce effective antitumor immune responses is consistent with prior clinical experience with peptide vaccines,⁹ but we found that ahCD27 could enhance the tumor-specific CD8⁺ T cell response and resultant antitumor efficacy when combined with vaccines containing linked class I and II epitopes. We thus hypothesized that effector CD8⁺ T cells and CD4⁺ T cells, in either the priming or effector phase, were necessary for the antitumor effect of adjuvant

ahCD27. To test this hypothesis, we first examined the effect of CD4⁺ T cell depletion on the CD8⁺ T cell response to whole ovalbumin protein vaccination, during the priming phase (depletion prior to vaccination) and effector phase (depletion 6 days after vaccination) of the vaccine response. We found that a lack of CD4⁺ T cells in the priming phase significantly reduced the adjuvant effect of ahCD27 on the CD8⁺ T cell response to whole ovalbumin protein vaccination, as determined by *ex vivo* re-stimulation with Ova(I) peptide in a IFN γ ELISPOT (Figure 3A, Ova + ahCD27 vs Ova + ahCD27 + α CD4 priming: $P = 0.00316$), while the depletion of CD4⁺ T cells later in the vaccine response had only a slight but statistically non-significant effect (Figure 3A). Additionally, the depletion of CD8⁺ T cells at the effector phase completely abrogated the *ex vivo* response to Ova(I) peptide re-stimulation (Figure 3A, Ova + ahCD27 vs Ova + ahCD27 + α CD8: $P = 0.00272$).

We next examined the effect of CD8⁺ or CD4⁺ T cell depletion on the antitumor effect of adjuvant ahCD27 in a challenge setting of intracranial B16.OVA, in which mice were pretreated with ahCD27 + ovalbumin seven days prior to tumor implantation. To distinguish between the priming and effector phases of the vaccine response, CD8⁺ T cells were depleted seven days after vaccination, at the time of tumor implantation (effector phase), and CD4⁺ T cells were depleted immediately prior to vaccination (priming phase) or at the time of tumor implantation (effector phase). We found that the depletion of CD8⁺ T cells seven days after vaccination completely abrogated the efficacy of combined ahCD27 + whole ovalbumin protein (Figure 3B, red). In contrast, the depletion of CD4⁺ T cells seven days after vaccination had no significant effect on the efficacy of ahCD27 + ovalbumin (Figure 3B, green); however, CD4⁺ T cell depletion prior to vaccination completely abrogated antitumor efficacy (Figure 3B, blue, Ova + ahCD27 vs Ova + ahCD27 + α CD4 priming: $P = 0.0004$). These data demonstrate that both CD8⁺ and CD4⁺ T cells are necessary for the therapeutic effect of adjuvant ahCD27 but that CD4⁺ T cells contribute to this effect only during the priming phase of the vaccine response. Consistent with our own data and previous reports showing that class II epitopes are integral for robust class I-restricted vaccine responses,¹¹ these results suggest that CD4⁺ T cell help is critical for the adjuvant effect of ahCD27.

Direct CD27 stimulation on vaccine-specific CD4⁺ and CD8⁺ T cells is necessary for the adjuvant effect of ahCD27

Our previous data reveals the necessity of CD4⁺ T cells in the priming phase of the vaccine response for ahCD27 to enhance the immunogenicity and antitumor efficacy of a whole ovalbumin protein vaccine, leading us to further investigate the effect of ahCD27 on the ovalbumin-specific CD4⁺ T cell response. We hypothesized that ahCD27 enhances the CD4⁺ T cell response to ovalbumin class II epitopes and that direct stimulation of CD27 expressed on ovalbumin-specific CD8⁺ and CD4⁺ T cells was necessary for the observed adjuvant effect of ahCD27. To test this hypothesis, we first asked if ahCD27 enhances the CD4⁺ T cell response to class II epitopes in the whole ovalbumin protein vaccine. We measured changes in the immune response to Ova

(II^A) and Ova(II^B), a class II ovalbumin epitope downstream from Ova(II^A) (Table 1), elicited by whole ovalbumin protein vaccination, with or without adjuvant ahCD27. Indeed, we found that the addition of ahCD27 enhanced the T cell response to both of these class II-restricted epitopes, as determined by IFN γ ELISPOT (Figure 3C, Ova(II^A): $P = 0.0056$; Ova(II^B): $P = 0.071$).

Next, to determine if direct engagement of CD27 on antigen-specific CD4⁺ and CD8⁺ T cells was necessary for the enhanced CD8⁺ T cell response to whole ovalbumin protein vaccination, we employed an adoptive transfer wherein wildtype C57BL/6 mice received intravenous infusions of CD8⁺ T cells purified from OT-I or OT-I x hCD27 transgenic mice, whose CD8⁺ T cells are specific for Ova(I), and CD4⁺ T cells purified from OT-II or OT-II x hCD27 transgenic mice, whose CD4⁺ T cells are specific for Ova(II^B). CD8⁺ and CD4⁺ T cells purified from OT-I or OT-II mice express only murine CD27 and so would not be responsive to ahCD27 treatment, while those purified from OT-I x hCD27 or OT-II x hCD27 mice, which express both murine and human CD27, could be responsive to treatment with ahCD27 (Figure 3D). Of note, the cohort of mice receiving OT-I + OT-II T cells, which do not express hCD27, serve as an experimental control for determining the baseline immune response to whole ovalbumin protein vaccination (i.e., in the absence of exogenous CD27 stimulation) in this adoptive transfer setting. We found that vaccine responses were highest in mice that received OT-I x hCD27 CD8⁺ T cells and OT-II x hCD27 CD4⁺ T cells, as measured by *ex vivo* re-stimulation with Ova(I) in a IFN γ ELISPOT (Figure 3E, OTI + OTII vs OTI x hCD27 + OTII x hCD27: $P = 0.028$), indicating that direct CD27 stimulation on both ovalbumin-specific CD8⁺ and CD4⁺ T cells contributes to the immunogenicity of whole ovalbumin protein in the setting of ahCD27. Taken together, our data suggest that ahCD27 enhances both vaccine-induced CD8⁺ and CD4⁺ T cell responses, resulting in increased CD4⁺ T cell helper function to allow for a more pronounced vaccine-induced CD8⁺ effector response.

A universal CD4⁺ T cell helper epitope is sufficient to enhance CD8⁺ tumor-specific vaccine responses in the setting of adjuvant ahCD27

In light of our finding that the therapeutic effect of adjuvant ahCD27 requires vaccine-specific CD4⁺ T cells in the priming phase of the immune response, we wondered if non-specific CD4⁺ T cell help would be sufficient to instill antitumor efficacy in the setting of adjuvant ahCD27. To test this hypothesis, we evaluated the adjuvant activity of ahCD27 when combined with a known CD4⁺ T cell universal class II helper epitope, tetanus toxin (TT) P30 (TT(948–968)).²⁷ We developed a peptide vaccine consisting of Ova(I) covalently linked with the P30 epitope (Ova(I)-P30) (Table 1) and administered it with or without adjuvant ahCD27. A linker sequence consisting of a furin cleavage site²⁸ was included between the Ova(I) and P30 epitopes to ensure that this synthetic long peptide would be processed into two distinct epitopes. Indeed, we found that ahCD27 enhances the vaccine response to Ova(I)-P30 (Figures 4A and 4B, Ova(I)-P30 + hIgG1 vs Ova(I)-P30 + ahCD27: $P = 0.0003$) and that the CD8⁺ T cell response to Ova(I) was greater upon vaccination with Ova(I)-P30 compared to Ova(I) in the setting of adjuvant ahCD27 (Figure 4B, Ova(I) + ahCD27 vs Ova(I)-P30 + ahCD27: $P = 0.0327$). Consistent with

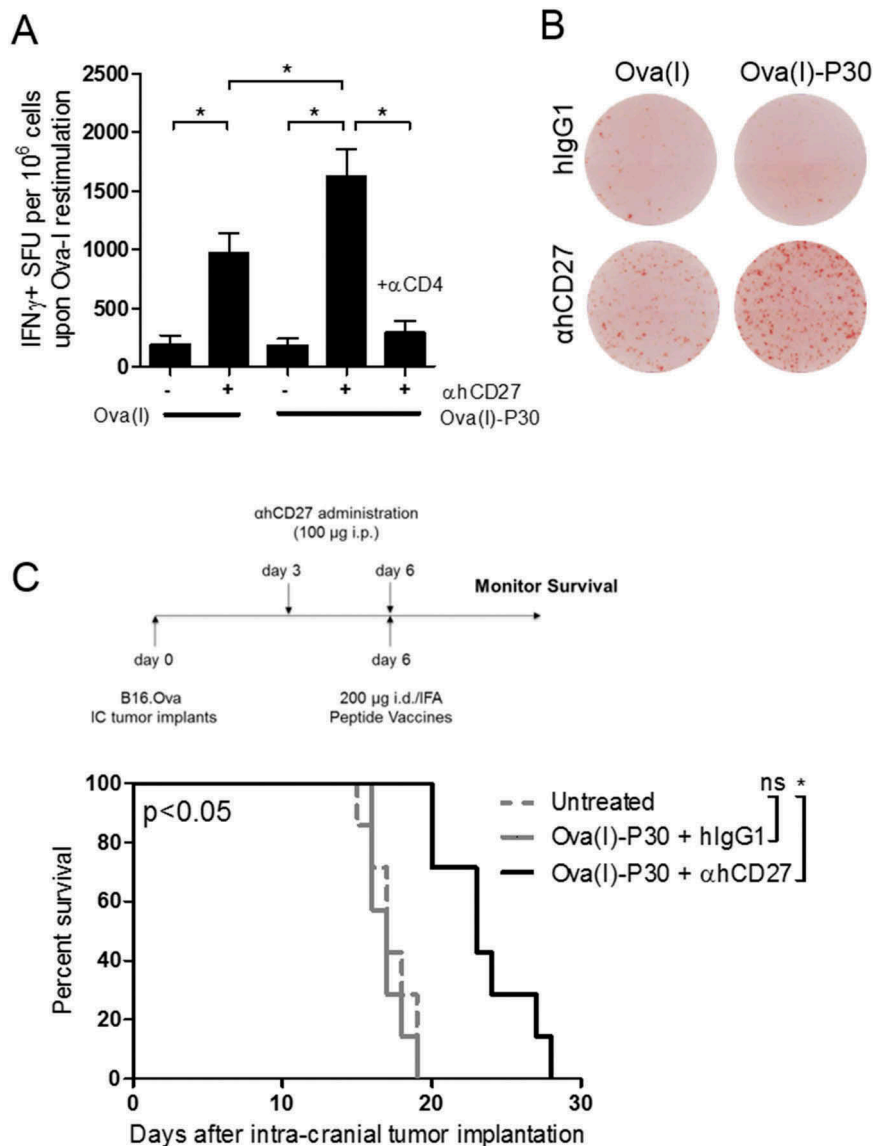


Figure 4. A universal CD4⁺ T cell helper epitope is sufficient to enhance CD8⁺ tumor-specific vaccine responses in the setting of adjuvant ahCD27. (A) CD8⁺ T cell responses were evaluated in mice vaccinated id with 50 μ g of Ova(I) or 200 μ g of Ova(I)-P30 peptides. 100 μ g of ahCD27 or hIgG1 was administered on days -3 and 0 with vaccination on day 0. Seven days after vaccination, splenocytes were harvested and *ex vivo* re-stimulated with Ova(I) peptide in an IFN γ ELISpot. CD4⁺ cells were depleted during vaccine priming by α CD4 antibody (GK1.5) administered ip on days -6, -5, and -4. (B) Representative ELISpot images from mice vaccinated with Ova(I) or Ova(I)-P30 that also received ahCD27 or hIgG1 are shown. (C) For survival analysis, hCD27 mice bearing 3-day established intracranial B16.OVA tumors were vaccinated with Ova(I)-P30 and received ahCD27 or hIgG1 isotype control (n = 7 per group). Schema on top of the survival curves indicates the experimental design. B16.OVA tumors were implanted on day 0, 100 μ g of ahCD27 or isotype was administered ip on days 3 and 6, and 200 μ g of linked Ova(I)-P30 peptide emulsified IFA was given id on day 6 and survival was monitored. The quantity of peptide used correspond to the equimolar mass of the peptides in relation to 2.5 mg of whole Ova protein. Statistical analyses were performed using two-way ANOVA with Tukey post-hoc comparisons (A) or the Gehan-Breslow-Wilcoxon test (C). Statistical significance was determined at a *P value < 0.05.

our previous findings, this enhanced Ova(I) response was reduced when CD4⁺ T cells were depleted prior to vaccination (Figure 4B, Ova(I)-P30 + ahCD27 vs Ova(I)-P30 + ahCD27 + α CD4: $P = 0.0001$). Moreover, ahCD27 combined with Ova(I)-P30 prolonged survival in hCD27 mice bearing intracranial B16.OVA tumors (Figure 4C, Ova(I)-P30 + hIgG1 vs Ova(I)-P30 + ahCD27: $P = 0.0005$). These data indicate that a universal class II helper epitope can be the source of vaccine-induced helper CD4⁺ T cells, thereby broadening the utility of this strategy by eliminating the need for a tumor-derived class II epitope in the vaccine. Importantly, however, the addition of the class II P30 epitope alone was not sufficient to enhance the vaccine-induced immune response and promote antitumor efficacy, while

combined ahCD27 unveiled the efficacy of this synthetic long peptide vaccine, thus highlighting the synergy between CD4⁺ T cell help and adjuvant ahCD27.

CD27 stimulation coordinates CD4⁺ t cell help and vaccine-induced CD8⁺ t cell responses

We show above that vaccination with linked class I and II epitopes increases the capacity for ahCD27 to enhance the vaccine-induced CD8⁺ T cell response. We hypothesized that the adjuvant activity of ahCD27 occurs because the enhanced CD4⁺ T cell response potentiates the CD8⁺ T cell response in the setting of CD27 stimulation. If correct, this hypothesis would predict that the

magnitude of the CD4⁺ T cell response dictates the magnitude of the CD8⁺ T cell response. We thus analyzed the relationship between vaccine-induced CD4⁺ and CD8⁺ T cell responses in the settings of ahCD27 and hIgG1 isotype control. We plotted the class I and class II T cell responses (as determined by IFN γ ELISPOT) of individual mice vaccinated with Ova(I-II^A) or Ova(I)-P30 peptide upon *ex vivo* re-stimulation with Ova(II^A) versus Ova(I) (Figures 5A and 5B) or P30 versus Ova(I) (Figures 5C and 5D), respectively, normalized across cumulative experiments. Indeed, for both of these cases, we found that the class II response positively correlates with the class I response in the presence of adjuvant ahCD27 (Ova(II^A) vs Ova(I) with Ova(I-II^A) vaccination, $P = 0.0156$ and $R = 0.631$; P30 vs Ova(I) with Ova(I)-P30 vaccination, $P = 0.0299$ and $R = 0.6817$) (Figure 5), while no statistically significant relationship between the CD4⁺ and CD8⁺ T cell responses was found in the setting of hIgG1 isotype control. This analysis suggests that a coordinated CD4/CD8 response is orchestrated upon CD27 stimulation, further highlighting the potential of ahCD27 as a vaccine adjuvant for peptide vaccines comprised of linked class I and class II epitopes.

ahcd27 enhances the immune response to a peptide vaccine derived from a tumor-associated antigen

To demonstrate the clinical feasibility of ahCD27 as a peptide vaccine adjuvant, we evaluated the ability of ahCD27 to enhance the vaccine response to a known tumor-associated antigen, tyrosinase-related protein 2 (Trp2).^{29,30} Vaccination with the Trp2 immunodominant class I epitope (Trp2(I))

(Table 1) has been shown to promote robust antigen-specific immune responses in C57BL/6 mice.^{31,32} We found that ahCD27 enhances the vaccine response to both Trp2(I) and a linked Trp2(I)-P30 peptide vaccine (Figures 6A and 6B, Trp2(I) + hIgG1 vs Trp2(I) + ahCD27: $P = 0.0220$; Trp2(I)-P30 + hIgG1 vs Trp2(I)-P30 + ahCD27: $P = 0.0009$). Additionally, similar to our previous findings, ahCD27 enhances the vaccine-induced CD8⁺ T cell response to a greater degree in the setting of Trp2(I)-P30 compared to Trp2(I) alone ($P = 0.00147$), leading to an overall higher vaccine response in the setting of Trp2(I)-P30 (Figures 6A and 6B, Trp2(I) + ahCD27 vs Trp2(I)-P30 + ahCD27: $P = 0.0004$). Furthermore, the magnitude of the P30-specific CD4⁺ T cell response correlates with the magnitude of the Trp2(I)-specific CD8⁺ T cell response in the setting of ahCD27 (Figure 6D, P30 vs Trp2(I), $P = 0.0012$ and $R = 0.867$), but not in the setting of hIgG1 (Figure 6C), indicating the ability for ahCD27 to coordinate CD4⁺ and CD8⁺ T cell responses in this vaccine setting as well. Our immunogenicity results were mirrored in a tumor efficacy experiment, in which ahCD27 combined with Trp2(I)-P30 prolonged survival in mice bearing 3-day established intracranial B16.F10 tumors (Figure 6E, Trp2(I)-P30 + hIgG1 vs Trp2(I)-P30 + ahCD27: $P = 0.0095$), while ahCD27 in combination with Trp2(I) had no effect relative to control groups. Our findings in the setting of ahCD27 combined with a clinically-relevant tumor antigen serve as a proof of principle for the promising translational potential of ahCD27 as a peptide vaccine adjuvant for tumor immunotherapy.

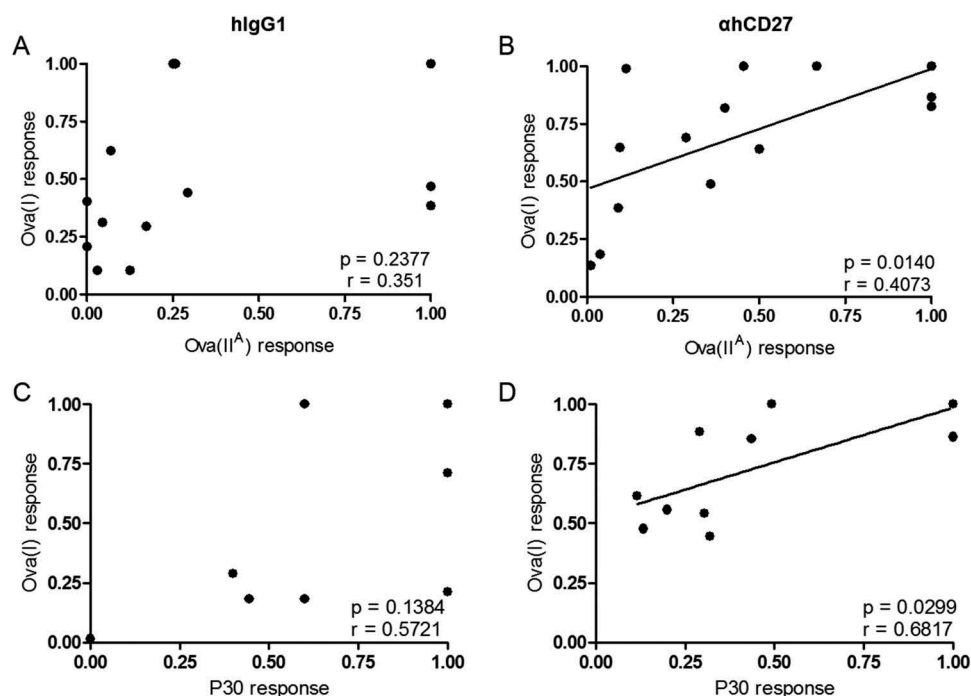


Figure 5. CD27 stimulation coordinates CD4⁺ T cell help and vaccine-induced CD8⁺ T cell responses. hCD27 mice ($n = 5$ per group) were immunized id with 150 μ g of Ova(I-II^A) or 200 μ g of Ova(I)-P30 on day 0, and 100 μ g of ahCD27 or hIgG1 isotype control was given ip on days -3 and 0. The quantity of peptide utilized correspond to equimolar mass of the peptides in relation to 2.5 mg of whole Ova protein. T cell responses to class I and II peptide epitopes were evaluated seven days after vaccination by *ex vivo* re-stimulation of splenocytes in an IFN γ ELISPOT using Ova(I), Ova(II^A), or P30 as indicated. Correlation analyses were performed comparing *ex vivo* responses to peptide re-stimulation of T cells derived from mice that received Ova(I-II^A) vaccination and hlgG1 (A) or ahCD27 (B), and mice that received Ova(I)-P30 vaccination and hlgG1 (C) or ahCD27 (D). Statistical analyses were performed using Pearson correlation analysis, and statistical significance was determined at a * P value < 0.05.

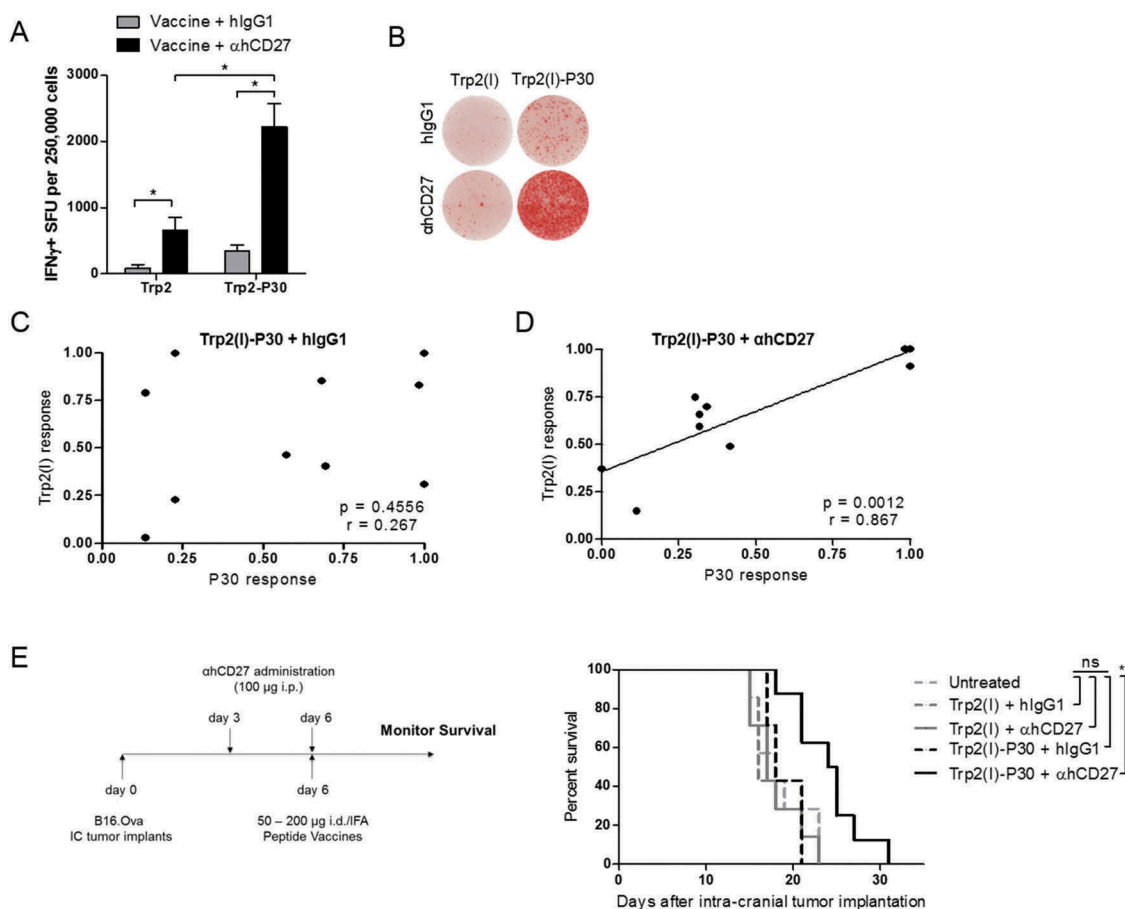


Figure 6: ahCD27 enhances the T cell response to a linked class I/II peptide vaccine derived from Trp2. (A) Mice were vaccinated id with 50 µg of Trp2(I) or 200 µg of Trp2(I)-P30 peptides emulsified in IFA on day 0 with 100 µg of ahCD27 or hlgG1 administered on day –3 or day 0. CD8⁺ T responses were evaluated seven days after vaccination by *ex vivo* re-stimulation of splenocytes with Trp2(I) peptide in an IFN γ ELISpot. (B) Representative ELISpot images are shown. (C-D) Mice were vaccinated with Trp2(I)-P30 peptide (day 0) and received ahCD27 or hlgG1 administration (days –3 and 0). Correlation analyses were performed comparing *ex vivo* responses of T cells derived from vaccinated mice that had received IgG (C) or ahCD27 (D) and were then re-stimulated with Trp2(I) and P30 peptides. (E) For survival analysis, hCD27 mice ($n = 7$ per group) were implanted with intracranial B16.F10 tumors on day 0, were vaccinated with 50 µg of Trp2(I) or 200 µg of Trp2(I)-P30 on day 6, and received 100 µg of ahCD27 or hlgG1 isotype control on days 3 and 6. Survival was monitored and the results of two separate experiments for each vaccine are shown. Schema on the left of the survival curves indicates the experimental design. Statistical analyses were performed using two-way ANOVA with Tukey post-hoc comparisons (A), Pearson correlation analysis (C, D), or the Gehan-Breslow-Wilcoxon test (E). Statistical significance was determined at a * P value < 0.05.

Discussion

In this study, we show that ahCD27 preferentially enhances the T cell response to peptide vaccines containing linked class I/II epitopes. Similar to clinical experiences with single-epitope class I-restricted peptide vaccines,⁹ we found that ahCD27 was not efficacious as a vaccine adjuvant in the setting of single class I-restricted peptides. In contrast, when administered alongside linked class I/II peptide vaccines designed to target both CD8⁺ and CD4⁺ T cells, we show that adjuvant ahCD27 leads to robust vaccine-induced CD8⁺ T cell responses in a CD4-dependent manner. Indeed, we found that CD27 stimulation on both antigen-specific CD4⁺ and CD8⁺ T cells, rather than either T cell compartment alone, is required for the adjuvant activity of ahCD27. As such, we concluded that ahCD27 preferentially enhances vaccine responses in the setting of linked class I/II-epitopes, which distinctively allows for increased tumor-specific CD8⁺ T cell responses and prolonged survival in mice bearing aggressive and poorly immunogenic intracranial tumors. Most importantly for clinical translation, we show that the

linked class II epitope can be a universal helper epitope and does not need to be derived from a tumor-specific antigen to give rise to antitumor efficacy. We demonstrate the clinical potential of this approach using a synthetic peptide vaccine targeting both CD4⁺ and CD8⁺ T cells against a clinically-relevant tumor antigen, Trp2. Our findings represent a significant improvement to class I-restricted tumor-derived peptide vaccines and highlight an increased potential for their clinical translation.

We believe that ahCD27 enhances vaccine immunogenicity by coordinating CD4⁺ and CD8⁺ T cell responses, thereby increasing the helper function of vaccine-specific CD4⁺ T cells and the resultant antitumor effector function of tumor-specific CD8⁺ T cells. Indeed, while neither adjuvant ahCD27 nor a linked class II epitope alone is sufficient to promote antitumor efficacy, their combination has a substantial therapeutic effect. These data indicate that a synergistic relationship exists between CD27 stimulation and CD4⁺ T cell help. CD27 signaling has been previously shown to drive T_H1 polarization in mouse³³ and human³⁴ CD4⁺ T cells by upregulating IL-12R β 2 and T-bet expression and was also found to enhance

their helper activity towards CD8⁺ T cells.³⁵ Additionally, we surmise that CD4⁺ T cell help may render CD8⁺ T cells more sensitive to CD27 signaling, thus amplifying the effect of ahCD27 on the CD8⁺ T cell response compared to the effect that ahCD27 would have in the absence of CD4⁺ T cell help. We believe that these characteristics of CD27 biology make ahCD27 uniquely poised as a promising vaccine adjuvant for enhancing the response to peptide vaccines containing linked class I/II epitopes.

It is also worth noting that CD27 is expressed by other cell types, including natural killer (NK) cells, regulatory T cells (Tregs), medullary thymocytes, and a subset of peripheral blood B cells.³⁶ It is possible that potential off target effects of ahCD27 could result in the activation of these cell subsets and engender bystander functionality. The activation of NK or B cells could contribute to inflammatory immunopathology, while the stimulation of CD27 on Tregs could increase immunosuppression. Additionally, while ahCD27 only stimulates T cells with engaged T cell receptors, ahCD27 could result in the activation of CD4⁺ and CD8⁺ T cells specific for available self-antigens. Stimulation of T cells specific for self-antigen could engender autoimmune reactivity and pathology. However, the ahCD27 monoclonal antibody employed in our studies is currently being evaluated in clinical studies as a single agent (Varlilumab[®]) and in combination therapy with checkpoint inhibitors in patients with solid tumors (NCT02335918). These studies have shown that Varlilumab[®] is safe and well tolerated, with no severe adverse events, systemic immunosuppression, or autoimmune reactivity reported in over 50 patients that have received this experimental therapy.³⁷ Therefore, while off-target effects are always a possibility for immunomodulatory antibodies, the ahCD27 monoclonal antibody Varlilumab[®] has shown an extremely safe profile, making it a suitable agent to use as a vaccine adjuvant.

There are a variety of clinically-available peptide vaccines comprised of a tumor-derived class I epitope linked to a universal helper epitope or tumor-derived class II epitope,^{13,38,39} which would make for promising vaccine candidates for combination with adjuvant ahCD27. In addition to peptide vaccines targeting high-frequency tumor mutations, we believe that adjuvant ahCD27 may enhance the clinical benefit of peptide vaccines derived from class I-restricted tumor neo-antigens, especially when linked to immunogenic class II epitopes. The therapeutic promise of such neo-epitope peptide vaccines has recently been demonstrated in the setting of advanced melanoma, where the administration of long peptides derived from melanoma neo-antigens has resulted in effective antitumor T cell responses.^{40,41} To broaden the scope of this approach, we envision that putatively any class I tumor neo-antigen could be linked to a helper epitope (e.g., P30) and administered alongside adjuvant ahCD27 to enhance its immunogenicity and efficacy, giving rise to a customizable vaccine/adjuvant treatment modality.

ahCD27 was previously shown to promote effective tumor immune responses as a single agent in mice bearing immunogenic tumors.²³ Herein, we demonstrate that this antibody can

also be employed as a vaccine adjuvant with a preferential effect on linked class I/II peptide vaccines to engender antitumor efficacy against poorly immunogenic intracranial tumor. However, it is worth noting that while ahCD27 dramatically elevated vaccine immunogenicity, that there was variability in these responses despite age- and gender-matched genetically identical recipients. While vaccine variability in humans is often attributed to differing genetic backgrounds, we attribute vaccine variability in mice to the technical aspects of vaccine emulsification and intradermal administration. These results within genetically identical murine models underscore the importance of establishing standard operating procedures to achieve the most consistent results possible in human clinical trials as well. We believe our treatment strategy of combinatorial ahCD27 and peptide vaccination has high translational relevance, as both a monotherapy for potentiating weak endogenous tumor immune responses and as a vaccine adjuvant for potentiating the immunogenicity of class I-restricted tumor-derived antigens. Importantly, the addition of a class II epitope alone was not sufficient to promote efficacy of a class I-restricted peptide vaccine in the absence of adjuvant ahCD27, indicating the requirement for both CD27 stimulation and CD4⁺ T cell help in promoting the efficacy of class I-restricted peptides.

However, despite the improved survival observed in vaccinated mice treated with adjuvant ahCD27, our therapy was not curative. Several mechanisms could be responsible for limiting the therapeutic efficacy of our treatment platform, such as the differences in tumor infiltration of CD4⁺ and CD8⁺ T cells, tumor antigen loss, decreased or lost CD27 expression on T cells, expression of pro-apoptotic molecules, the expression of checkpoint inhibition molecules, and the activity of immune-suppressive regulatory T cells (Tregs). Recent studies have demonstrated that CD27 engagement can lead to Fas expression and T cell apoptosis, while other studies have seen an increased in the expression of the immune checkpoint PD-1.^{42,43} In support of these observations, our supplemental [Figure 4](#) demonstrates that administration of ahCD27 with vaccination increases the expression of PD-1 on CD8⁺ T cells. Importantly, a recent study showed that PD-1 blockade can augment the efficacy of ahCD27 administration in the absence of vaccination through increasing T cell cytotoxicity.^{44,45} Therefore, despite the expansion we observed in antigen-specific T cells after vaccination and ahCD27, the antitumor efficacy of these T cells could potentially be improved by the incorporation of a checkpoint inhibitor. In addition to effector T cell dysfunction from PD-1 expression, an increase in suppressive regulatory T cell (Treg) levels and/or activity could also limit the therapeutic efficacy we observed. CD27 has been shown to be expressed at high levels on Tregs and to increase their suppressive function.^{46,47} However, recent studies in both the pre-clinical and clinical settings have demonstrated that ahCD27 administration does not potentiate Treg function but instead results in Treg depletion and a lack of suppressive function.^{37,42} Therefore, we believe an increase in Treg suppression is unlikely to be impairing efficacy in our model. Finally, tumors can still overcome potent immune responses through tumor editing, a scenario in which antigen-loss variants arise as a result of antigen-specific immune pressure. Overcoming tumor-editing may well depend upon increasing tumor antigen

presentation; this could be achieved by tumoricidal chemotherapy to promote the shedding of novel tumor antigens and their subsequent presentation to T cells by professional antigen-presenting cells. Importantly, ahCD27 could function synergistically in this context through the expansion of T cells recognizing linked class I and class II tumor epitopes from newly presented tumor antigen.⁴⁸ Furthermore, our therapeutic platform lends itself to the use of multivalent vaccination containing multiple known tumor-specific or tumor-associated class I epitopes linked to universal class II epitopes in the context of ahCD27 administration. Targeting multiple tumor antigens could potentially impair the generation of antigen loss variants and further increase the efficacy of our vaccine approach.

We believe that our study serves as a proof-of-principle demonstration of the adjuvant potential of exogenous CD27 stimulation. As such, our findings warrant further clinical investigation into ahCD27 as an adjuvant for peptide vaccines consisting of a class I-restricted tumor antigen linked with a class II helper epitope for instilling effective tumor-specific T cell immunity.

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Author contributions

JHS, LSP, and KAR jointly conceived and implemented this study; KAR, PKN, and AS performed *ex vivo* immune monitoring; KAR and CMS performed animal vaccine and tumor studies; KAR, LSP, EAR, LH, TK, KLC and JHS performed data analysis and interpretation; KAR, LSP, KLC and JHS wrote the manuscript. All authors have provided final approval of this manuscript.

Disclosure of Potential Conflicts of Interests

TK and LH are both shareholders and employees of Celldex Therapeutics, Inc.

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