

Research Article

Antigen-Specific Bacterial Vaccine Combined with Anti-PD-L1 Rescues Dysfunctional Endogenous T Cells to Reject Long-Established CancerDavid C. Binder^{1,3}, Boris Engels^{2,3}, Ainhoa Arina^{2,3}, Ping Yu^{2,4}, James M. Schlauch⁷, Yang-Xin Fu^{2,3}, Theodore Karrison⁵, Byron Burnette⁶, Christian Idei^{2,3,8}, Ming Zhao⁹, Robert M. Hoffman^{9,10}, David H. Munn¹¹, Donald A. Rowley³, and Hans Schreiber^{1,2,3}**Abstract**

Immunogenic tumors grow progressively even when heavily infiltrated by CD8⁺ T cells. We investigated how to rescue CD8⁺ T-cell function in long-established immunogenic melanomas that contained a high percentage of endogenous PD-1⁺ tumor-specific CD8⁺ T cells that were dysfunctional. Treatment with α PD-L1- and α CTLA-4-blocking antibodies did not prevent tumors from progressing rapidly. We then tested exogenous tumor-specific antigen delivery into tumors using *Salmonella* Typhimurium A1-R (A1-R) to increase antigen levels and generate a proinflammatory tumor microenvironment. Antigen-producing A1-R rescued the endogenous tumor-specific CD8⁺ T-cell response: Proliferation was induced in the lymphoid organs and effector function was recovered in the tumor. Treatment with antigen-producing A1-R led to improved mouse survival and resulted in 32% rejection of long-established immunogenic melanomas. Following treatment with antigen-producing A1-R, the majority of tumor-specific CD8⁺ T cells still expressed a high level of PD-1 in the tumor. Combining antigen-producing A1-R with α PD-L1-blocking antibody enhanced the expansion of tumor-specific CD8⁺ T cells and resulted in 80% tumor rejection. Collectively, these data show a powerful new therapeutic approach to rescue dysfunctional endogenous tumor-specific CD8⁺ T cells and eradicate advanced immunogenic tumors. *Cancer Immunol Res*; 1(2); 123–33. ©2013 AACR.

Introduction

Tumors can escape immune control despite expressing tumor-specific antigens arising from mutations (1–3). Although immunogenic cancer cells can induce functional CD8⁺ T-cell responses, this is usually restricted to early stages of tumor growth (4–6). Immunity rapidly decays with tumor growth, concurrent with the establishment of a tumor microenvironment in which cancer cells are embedded in a suppressive tumor stroma (7). Human tumors are regularly infiltrated by dysfunctional endogenous PD-1-expressing CD8⁺ T cells (8–10). Proliferation and effector function of these CD8⁺

T cells are likely impaired because of engagement of PD-1 with PD-L1 expressed by cancer cells and/or antigen-presenting cells (APC; refs. 11, 12). Although clinical trials blocking PD-1/PD-L1 interactions with antagonistic antibodies have reported promising results (13, 14), new therapeutic approaches need to be developed to rescue antitumor T-cell responses for patients who do not respond to PD-1 blockade alone.

Therapeutic vaccination has been extensively tested as a second approach to generate or rescue endogenous tumor-reactive T cells. Because bacterial products can strongly activate APCs through Toll-like receptor (TLR) signaling, many vaccine studies have used bacterial products as adjuvants (15) or used live bacteria that express tumor antigen (16, 17). Nevertheless, therapeutic vaccination approaches have not reported eradication of long-established experimental tumors (18) or human tumors (for review see ref. 19). We define an experimental tumor as long-established if 2 or more weeks old and exceeding 100 mm³. It is only after 2 weeks that artifacts from cancer cell inoculation—significant necrosis, acute inflammation, and an initial functional T-cell response—finally resolve (4–6, 20).

The limited success of previous therapeutic vaccination approaches is likely due to vaccine-generated T cells becoming dysfunctional after infiltrating long-established suppressive tumors (21, 22). There have been extensive studies showing that intravenously-injected attenuated *Salmonella* Typhimurium strains preferentially accumulate in murine tumors (for

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (<http://cancerimmunolres.aacrjournals.org/>).

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review see ref. 23) and reduce immunosuppression in the tumor and tumor-draining lymph node (24, 25). However, previous studies using *S. Typhimurium*, either unmodified or genetically modified to deliver recombinant antitumor proteins or short hairpin RNA (shRNA) into tumors, have not eradicated long-established tumors in immunocompetent mice (23–26). We hypothesized that intravenous injection of antigen-producing *S. Typhimurium* could be used effectively to rescue T-cell dysfunction by (i) coupling antigen delivery and TLR stimulation to APCs and (ii) generating a proinflammatory tumor microenvironment. To test this hypothesis, we treated long-established B16 melanoma tumors that expressed the model tumor-specific antigen ovalbumin (OVA). This model provided the following advantages: (i) B16-OVA tumors resembled human tumors that are also infiltrated by dysfunctional endogenous PD-1⁺ CD8⁺ T cells (8–10), (ii) targeting the SIINFEKL (SIINF) epitope of OVA provided valuable immunologic tools to detect the SIINF epitope and track SIINF-specific CD8⁺ T cells, and (iii) the SIINF epitope has high affinity for H-2K^b (27), similar to a natural unmodified tumor-specific rejection epitope also presented by H-2K^b (28). Indeed, treating mice bearing long-established B16-OVA tumors with *S. Typhimurium* A1-R (A1-R)-producing SIINF rescued the endogenous dysfunctional tumor-specific CD8⁺ T-cell response, resulting in tumor eradication in about one third of the experimental mice. Anti-PD-L1 antibody has been shown to rescue dysfunctional T cells (13, 29), but when used alone, it was ineffective in treating B16-OVA tumors. However, anti-PD-L1 synergized with antigen-expressing A1-R leading to tumor rejection in a large majority of tumor-bearing mice.

Materials and Methods

Cloning of antigen constructs and verifying antigen expression

Antigen constructs were cloned into the pEGFP (Clontech) plasmid. We codon-optimized the OVA antigen construct (Invitrogen) encoding the first 104 amino acids of the *SopE* gene, the M45 epitope from the adenovirus E4-6/7 protein (30), and amino acids 248 to 357 of ovalbumin before inserting this antigen construct into the pEGFP backbone. Using standard cloning techniques, the SIINFEKL epitope of OVA was replaced by the irrelevant SNFVFAGI (31) epitope to make a control antigen construct. Expression plasmids were electroporated into A1-R bacteria. Antigen expression by A1-R was verified by Western blot analysis using an antibody against the M45 epitope (30) as described previously (16).

Mice, cell lines, and tumor experiments

C57BL/6 and C57BL/6 CD8^{-/-} (B6.129S2-CD8a^{tm1Mak/J}) mice were purchased from The Jackson Laboratory and maintained in a specific pathogen-free facility at the University of Chicago (Chicago, IL). Female mice were used at 8 to 14 weeks of age. All animal experimentation was carried out in accordance with the University of Chicago Institutional Animal Care and Use Committee (IACUC) protocols.

The B16-OVA M04 cell line, a gift from Mary Jo Turk (Dartmouth Medical School, Hanover, NH) received in 2009, has

been described previously (32). OVA expression was verified using the 25-D1.16 antibody that recognizes the SIINFEKL peptide bound to H-2K^b. The M04 line was regularly confirmed to be *Mycoplasma*-free by using the ATCC Universal Mycoplasma Detection Kit [American Type Culture Collection (ATCC)]. Cancer cells were trypsinized, washed 1× in PBS, and injected at a dose of 5 to 10 × 10⁶ s.c. on the backs of mice. B16-OVA tumor-take was more than 60% and all established tumors invariably progressed rapidly and killed the host (20 of 20 tumors from 8 independent experiments). Tumor size was measured along 3 orthogonal axes (*a*, *b*, and *c*). Tumor volume = $abc/2$. Mice were sacrificed once tumors exceeded 1.5 cm³.

The leucine-arginine auxotrophic *S. Typhimurium* A1-R strain (refs. 33, 34; AntiCancer, Inc.) was intravenously injected at a dose of approximately 2 × 10⁷ colony formation units in 200 μL 1× PBS per mouse. To study the effect of PD-L1 and CTLA-4 blockade, mice were treated with 150 μg of anti-PD-L1 (10F.9G2) and/or 100 μg of anti-CTLA-4 (UC10-4F10-11) intraperitoneally every third day. Anti-CTLA-4 was purchased from the Fitch Monoclonal Facility (University of Chicago) and anti-PD-L1 was purchased from BioXCell. Further experimental details are in the Supplementary Experimental Procedures.

Flow cytometry

A staining solution, referred to as SIINF-dX, containing SIINFEKL peptide-loaded K^b-DimerX [(K^b)₂-IgG], anti-mouse IgG1-phycoerythrin (PE) or -APC (BD Biosciences), and mouse immunoglobulin G1 (IgG1) isotype control was used to detect SIINF-specific CD8⁺ T cells. A staining solution, referred to as SIYR-dX, was loaded with the irrelevant SIYRYGL peptide and used as control. Details about other antibodies and flow-cytometric analyses are in the Supplementary Experimental Procedures.

Peptide restimulation *in vitro*

Single-cell suspensions from the tumor were used in a peptide restimulation assay. Cells were placed into a 96-well plate with each well containing 1 to 2 × 10⁶ viable cells in 200 μL RPMI with 20 μg/mL Brefeldin A, and 1 μg/mL SIINFEKL peptide (generously provided by S. Meredith, University of Chicago). Cells were restimulated with peptide for 5 hours at 37°C. Experimental details are provided in the Supplementary Experimental Procedures.

Statistical analysis

A Mann-Whitney test was used to conduct pairwise comparisons of tumor sizes between different groups during outgrowth. Both nonparametric Kruskal-Wallis and parametric analysis of variance (followed by Sidak's multiple comparisons procedure) were used for analysis of 3 or more groups. Fisher exact test was used to compare survival rates of mice at an indicated time or tumor rejection rates between different treatment groups. Wilcoxon-signed rank and paired *t* tests following parametric transformation of the data were used to compare the percentage of CD8⁺ T cells that were stained by SIINF-dX or SIYR-dX. The *P* values reported in this article were derived from parametric analysis. All results reported to be significant following parametric analysis were also significant

or at least marginally significant ($P < 0.06$) using the nonparametric tests.

Results

A1-R SIINF can deliver the SIINF EKL epitope into APCs for MHC class I presentation

S. Typhimurium oral vaccines depend on type III secretion to deliver heterologous antigen to APCs resulting in the generation of antigen-specific CD8⁺ T cells (16, 35). Similar to these studies, we constructed a fusion protein consisting of the SopE type III secretion/translocation domain (16), an M45 epitope tag (30), and a SIINF EKL (SIINF)-containing OVA domain (amino acids 248–357). As SIINF-negative controls, we used (i) a similar fusion protein in which the irrelevant SNFV FAGI (SNFV) epitope (31) replaced the SIINF epitope or (ii) the enhanced GFP (EGFP; diagrams in Fig. 1A).

A1-R SIINF and A1-R SNFV were generated by introducing expression plasmids encoding the corresponding fusion proteins into *S. Typhimurium* A1-R. A1-R EGFP has been described previously (33). Western blot analyses were used to verify the expression of the A1-R SIINF and A1-R SNFV fusion protein (Fig. 1B).

We evaluated whether A1-R SIINF could deliver the SIINF epitope into APCs for correct MHC class I processing and presentation to CD8⁺ T cells. J774 K^b-expressing macrophages were infected *in vitro* with A1-R SIINF or A1-R SNFV. The capacity of the infected macrophages to present the SIINF epitope to CD8⁺ T cells was determined by stimulation of B3Z, a CD8⁺ T-cell hybridoma specific for SIINF. A1-R SIINF- but not A1-R SNFV-infected macrophages stimulated B3Z to secrete IL-2 (Fig. 1C), showing that A1-R SIINF can deliver the SIINF epitope into APCs for MHC class I presentation.

B16-OVA tumors are heavily infiltrated by endogenous SIINF-specific CD8⁺ T cells

We evaluated the endogenous SIINF-specific CD8⁺ T-cell response to B16-OVA cancer cell inoculation. At 10 days post-B16-OVA inoculation, the peripheral blood from C57BL/6 mice contained a population of SIINF-specific CD8⁺ T cells (Fig. 2A). B16-OVA outgrowth in wild-type mice was delayed compared with both parental B16 in wild-type mice and B16-OVA in CD8^{-/-} mice (Fig. 2B), suggesting that SIINF-specific CD8⁺ T cells delayed the outgrowth of B16-OVA tumors.

Despite this initial SIINF-specific CD8⁺ T-cell response, B16-OVA tumors grew progressively and killed the host. As tumors reached 100 to 168 mm³, SIINF-specific CD8⁺ T cells reached a high percentage of total CD8⁺ T cells in the tumor (mean of 22%; ranging between 9% and 30%) but were at an undetectable or low percentage in the tumor-draining lymph node, spleen, and lungs (Fig. 2C).

A1-R SIINF preferentially colonizes established B16-OVA tumors

Previous studies showed that intravenously injected A1-R can preferentially colonize established tumors (33, 34). To determine if this occurs in our model, we intravenously injected A1-R SIINF into B16-OVA tumor-bearing mice. Eight

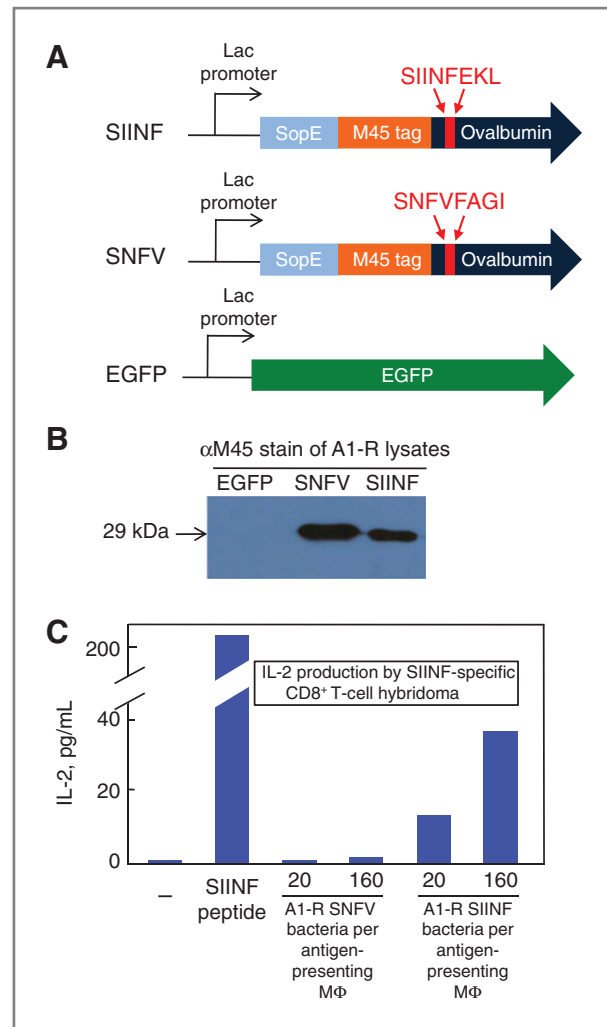


Figure 1. A1-R SIINF delivers the SIINF EKL epitope into APCs for MHC class I presentation. A, diagram of the SIINF, SNFV, and EGFP constructs used in this study. B, high-copy number plasmids encoding the respective SIINF and SNFV fusion protein constructs were introduced into the A1-R strain. Whole bacterial lysates were examined for fusion protein expression using the anti-M45 antibody. C, J774 K^b-expressing macrophages were infected with A1-R SIINF or A1-R SNFV. Infected macrophages were then incubated with the B3Z (SIINF EKL-specific CD8⁺ T cell) hybridoma for 24 hours. B3Z stimulation was evaluated by the amount of IL-2 secreted into the culture as determined by ELISA. Data are representative of 2 independent experiments.

days postinjection, the mean number of bacteria per gram of tissue was 10,000-fold greater in the tumor compared with the lungs and spleen, and 800-fold greater in the tumor compared with the liver (Supplementary Fig. S1A). A1-R SIINF colonization in tumors was confirmed by α -*Salmonella* immunohistochemistry (Supplementary Fig. S1B).

A1-R SIINF induces systemic SIINF-specific CD8⁺ T-cell proliferation in B16 tumor-bearing mice

We analyzed the endogenous SIINF-specific CD8⁺ T-cell response to intravenously injected A1-R SIINF in mice bearing established B16 parental (non-OVA) tumors. Nine days

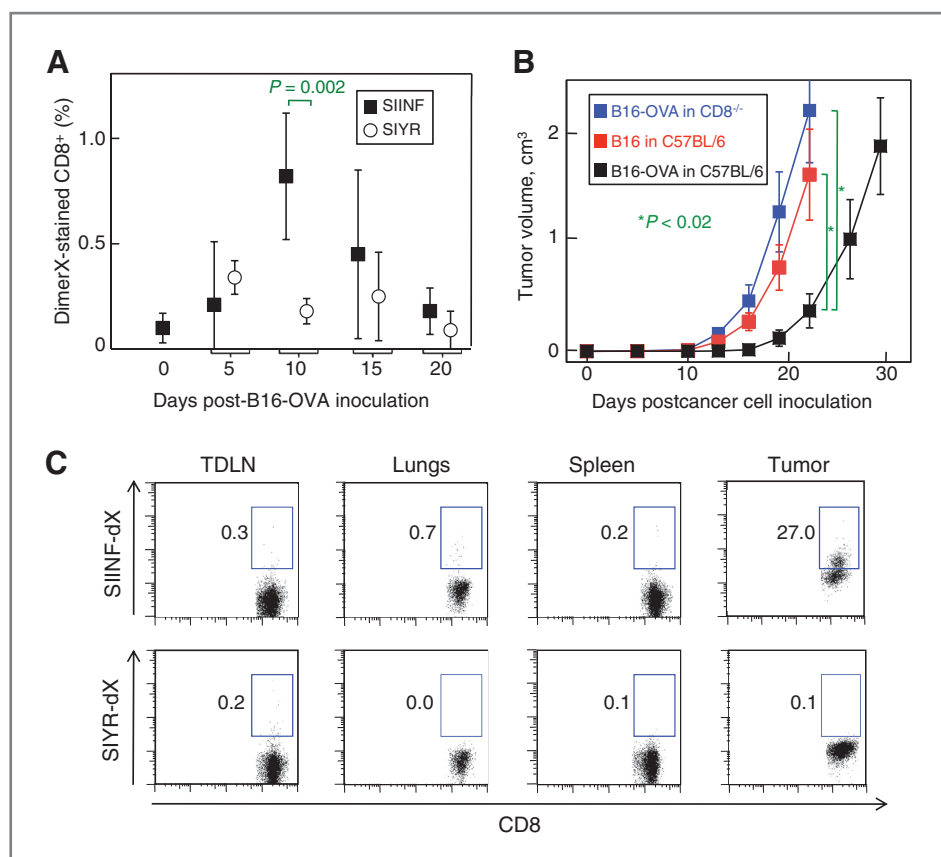


Figure 2. B16-OVA cancer cell inoculation induces priming of endogenous SIINF-specific CD8⁺ T cells that heavily infiltrate tumors. **A**, C57BL/6 mice were inoculated with B16-OVA cancer cells. At the indicated times, the peripheral blood leukocytes were stained with anti-CD8 and either SIINF/K^b-dimerX or control SIYR/K^b-dimerX. Data were pooled from 6 total mice with progressively-growing tumors compiled from 2 independent experiments. **B**, B16-OVA and B16 tumor growth was measured following injection of 5×10^6 cancer cells into C57BL/6 or C57BL/6 CD8^{-/-} mice. The mean tumor volume (\pm SD) was calculated for each time-point. Each group consisted of 4 mice with progressively-growing tumors; data are representative of 2 independent experiments. **C**, B16-OVA cancer cells were inoculated in a C57BL/6 mouse. Eighteen days later, the B16-OVA tumor reached 144 mm³. Single-cell suspensions were made from the tumor-draining lymph node (TDLN), lungs, spleen, and tumor. The suspensions were stained with anti-CD8 and either SIINF/K^b-dimerX (SIINF-dX) or control SIYR/K^b-dimerX (SIYR-dX). Data are representative of 3 total mice from 2 independent experiments.

postinjection, a high percentage of endogenous SIINF-specific CD8⁺ T cells were present in the tumor-draining lymph node, non-draining lymph nodes, spleen, and tumor. A 1-hour bromodeoxyuridine (BrdUrd) pulse showed that SIINF-specific CD8⁺ T cells were proliferating rapidly in all lymphoid organs and the tumor (Supplementary Fig. S2A). These data show that A1-R SIINF induces a systemic SIINF-specific CD8⁺ T-cell response.

A1-R SIINF rescues the dysfunctional SIINF-specific CD8⁺ T-cell response in the periphery and tumor of B16-OVA tumor-bearing mice

Mice bearing untreated B16-OVA tumors, established for at least 14 days and reaching 100 to 168 mm³, did not have detectable SIINF-specific CD8⁺ T cells in the lymph nodes, spleen, or blood (Figs. 2C and 3A). This group of mice was defined as "Day 0 untreated" as mice were treated throughout this study at this tumor size. Treating B16-OVA tumor-bearing mice with intravenously-injected A1-R SIINF induced systemic SIINF-specific CD8⁺ T-cell proliferation in the lymphoid

organs (Supplementary Fig. S2B) and generated a high percentage of SIINF-specific CD8⁺ T cells in the peripheral blood (Fig. 3A). To determine if this effect required A1-R to deliver SIINF, we used 2 independent SIINF-negative controls (A1-R SNFV and A1-R EGFP) collectively referred to as A1-R control. Mice treated with A1-R control did not have a high percentage of SIINF-specific CD8⁺ T cells in the peripheral blood (Fig. 3A), showing that A1-R is required to deliver SIINF to rescue the SIINF-specific CD8⁺ T-cell response in the periphery.

In mice bearing untreated B16-OVA tumors between 100 and 168 mm³, SIINF-specific CD8⁺ T cells reached a high percentage of total CD8⁺ T cells in the tumor (Figs. 2C and 3B). However, these SIINF-specific CD8⁺ T cells were dysfunctional as they produced little IFN- γ and TNF- α upon peptide restimulation (Fig. 3B). Treatment with A1-R SIINF or A1-R control did not significantly change the percentage of SIINF-specific CD8⁺ T cells in the tumor (Fig. 3B). However, treatment with A1-R SIINF, but not A1-R control, rescued the capacity of SIINF-specific CD8⁺ T cells to produce IFN- γ and TNF- α (Fig. 3B), indicating that the A1-R-produced SIINF

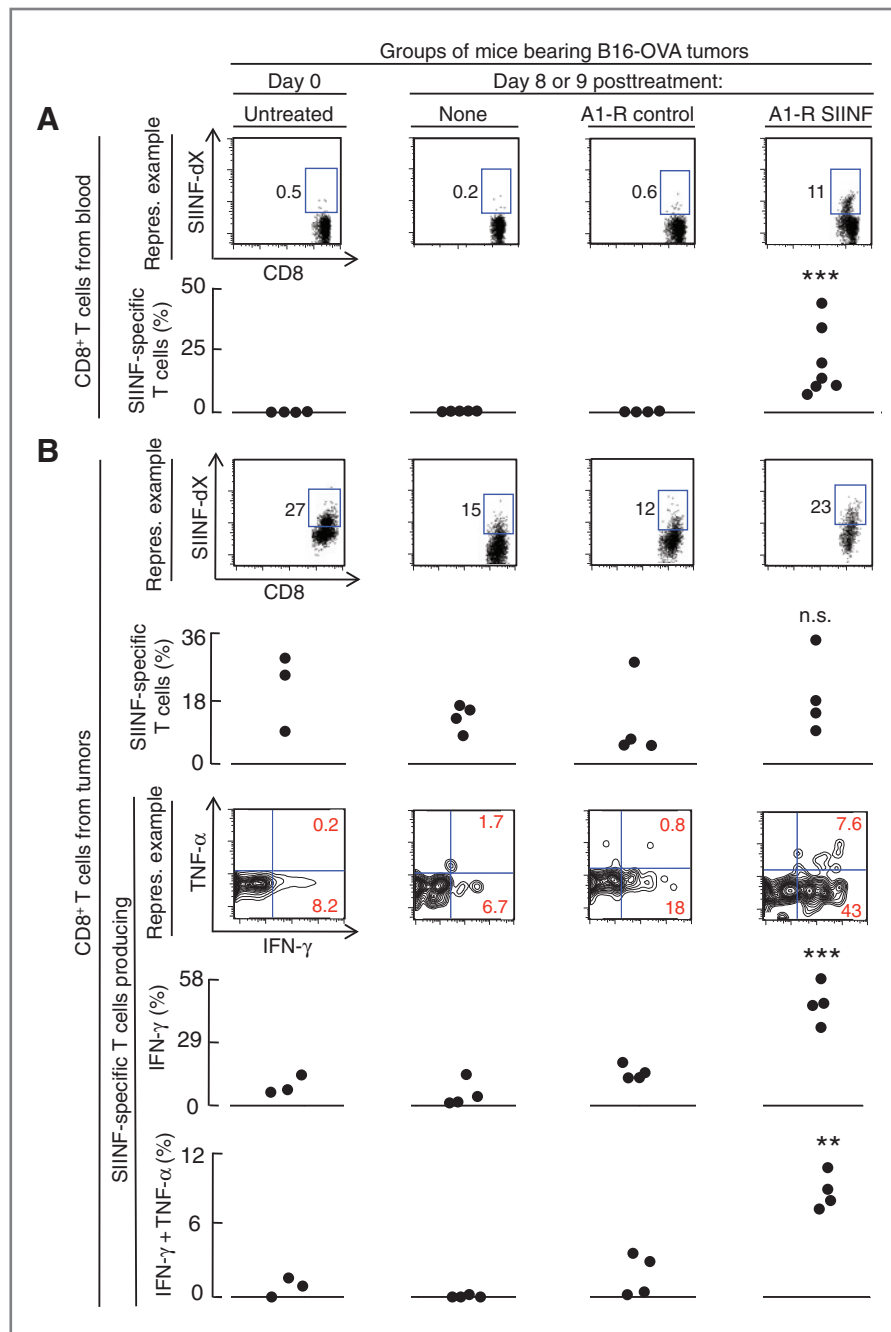


Figure 3. A1-R SIINF treatment rescues the SIINF-specific CD8⁺ T-cell response in the blood and tumor. C57BL/6 mice bearing B16-OVA tumors were analyzed from the following groups: untreated tumors at day 0 (defined when tumors reached 100–168 mm³); untreated tumors at day 8; A1-R control-treated tumors 8 to 9 days posttreatment; and A1-R SIINF-treated tumors 8 to 9 days posttreatment. **A**, the peripheral blood was stained with anti-CD8 and SIINF/K^b-dimerX. Top, representative stain from 1 mouse; bottom, contains pooled data with each mouse represented by a single dot. The A1-R control treatment group consisted of 2 mice treated with A1-R SNFV and 2 mice treated with A1-R EGFP. ***, $P < 0.001$ when comparing A1-R SIINF with each other group. **B**, top 2 rows analyzed the percentage of SIINF-specific CD8⁺ T cells from tumors. Single-cell suspensions from tumors were stained with anti-CD8 and either SIINF/K^b-dimerX or control SIYR/K^b-dimerX. Top, representative stain from 1 mouse per group. Bottom, contains pooled data from individual mice derived from at least 2 independent experiments per treatment group. The percentage of SIINF-specific CD8⁺ T cells represents the percentage of cells that stained positive with SIINF/K^b-dimerX subtracted by the background percentage of cells that stained positive with SIYR/K^b-dimerX. The difference between the A1-R SIINF group and each other group was nonsignificant (n.s.). The bottom 3 rows analyzed cytokine production by SIINF-specific CD8⁺ T cells from the tumor. The same tumor cell suspensions, as analyzed in the top 2 rows, were restimulated with SIINF peptide in the presence of Brefeldin A for 5 hours. Cells were stained with SIINF/K^b-dimerX or control SIYR/K^b-dimerX, anti-CD8, anti-IFN- γ , and anti-TNF- α . Top, a representative anti-IFN- γ and anti-TNF- α stain from gated SIINF/K^b-dimerX⁺ CD8⁺ double-positive cells. Bottom, pooled data. The percentage of IFN- γ ⁺ or IFN- γ ⁺ TNF- α ⁺ cells was defined as the percentage of SIINF-specific CD8⁺ T cells that stained positive with anti-IFN- γ and/or anti-TNF- α compared with the isotype controls. The A1-R control treatment group consisted of 4 mice treated with A1-R SNFV. ***, $P \leq 0.001$ when comparing A1-R SIINF with each other group. **, $P \leq 0.002$ when comparing A1-R OVA with each other group.

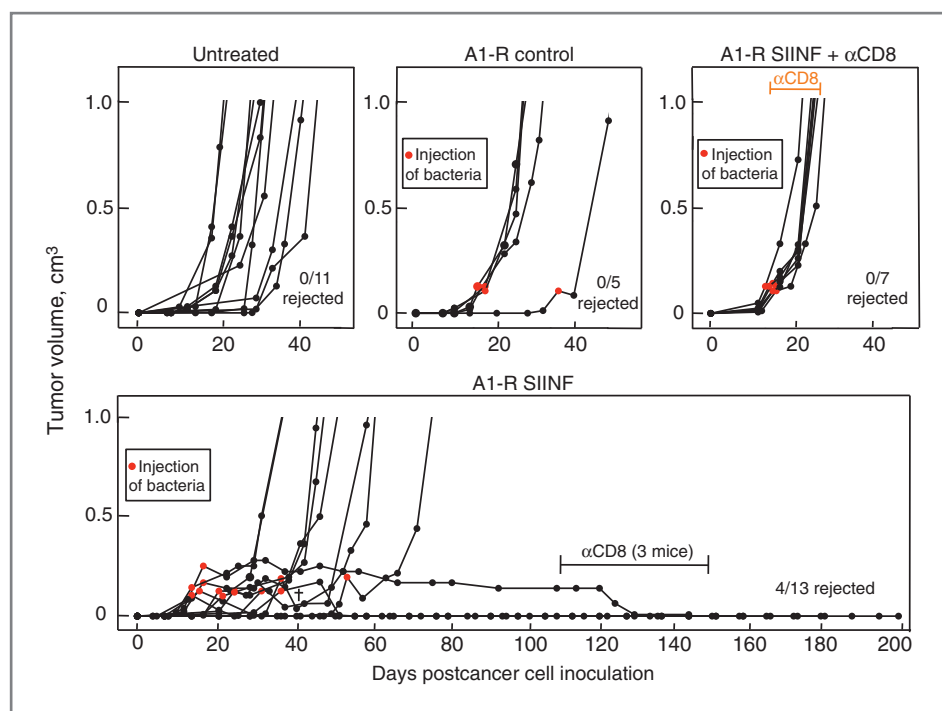


Figure 4. A1-R SIINF treatment results in a SIINF-specific CD8⁺ T cell-dependent antitumor effect on established B16-OVA tumors. C57BL/6 mice bearing established B16-OVA tumors were either left untreated, treated 1 time with A1-R control, treated 1 time with A1-R SIINF, or treated 1 time with A1-R SIINF followed by treatment with the α CD8 depletion antibody. Lines indicate individual mice. The 4 mice that rejected the tumor following A1-R SIINF treatment were held for at least 100 days posttreatment. To determine if the tumors were eradicated, 3 of these mice were injected with 3 doses of 200 μ g α CD8 at 3-day intervals. There was no tumor outgrowth for the 34 days following CD8⁺ T-cell depletion before the mice were sacrificed. The A1-R control treatment group consisted of 3 mice treated with A1-R EGFP and 2 mice treated with A1-R SNFV. †, a single mouse that died during the experiment.

rescued SIINF-specific CD8⁺ T-cell effector function in the tumor.

A1-R SIINF treatment improves mouse survival by a SIINF-specific CD8⁺ T cell-dependent mechanism

Mice bearing long-established B16-OVA tumors were treated with either a single injection of A1-R control, A1-R SIINF, or A1-R SIINF plus α CD8 depletion antibody (Fig. 4; Tables 1 and 2). At 25 days posttreatment with a single A1-R injection, 11 of 13 mice treated with A1-R SIINF were alive compared with 0 of 5 mice treated with A1-R control ($P = 0.002$) and 0 of 7 mice

treated with A1-R SIINF plus α CD8 ($P < 0.0005$). B16-OVA tumors were rejected in 4 of 13 mice treated with A1-R SIINF. We then tested if treating mice with weekly A1-R SIINF injections could maintain a stronger SIINF-specific CD8⁺ T-cell response leading to more consistent tumor rejection (Supplementary Fig. S3 and Table 1). At 25 days posttreatment, 7 of 9 mice treated with A1-R SIINF were alive compared with 1 of 10 mice treated with A1-R control ($P = 0.005$). Tumors were rejected in 3 of 9 mice treated with weekly A1-R SIINF. Therefore, A1-R SIINF treatment improves mouse survival by a SIINF-specific CD8⁺ T cell-dependent mechanism, and

Table 1. Compilation of experiments treating established B16-OVA tumors with A1-R control or A1-R SIINF

Treatment	B16 OVA tumor at treatment		Tumor rejection in experiment #												
	Tumor size, mm ³	Duration of growth, d	1	2	3	4	5	6	7	8	9	10	11	12	Total
None	97 ± 76	22 ± 8	0/3	0/2	—	0/2	0/1	0/1	0/2	—	—	—	—	—	0/11
A1-R control single injection	116 ± 11	21 ± 9	—	0/1 ^a	0/2 ^a	—	—	0/2 ^b	—	—	—	—	—	—	0/5
A1-R control weekly injection	104 ± 31	18 ± 4	—	—	—	—	0/4 ^a	0/2 ^b	0/1 ^b	0/2 ^b	0/1 ^b	—	—	—	0/10
A1-R SIINF single injection	147 ± 43	25 ± 11	1/2	0/2	0/2	—	1/1	—	—	1/1	—	0/1	1/1	0/3	4/13
A1-R SIINF weekly injection	143 ± 24	24 ± 9	—	—	0/1	2/2	0/1	0/1	0/1	1/2	0/1	—	—	—	3/9

^aA1-R EGFP.

^bA1-R SNFV.

Table 2. Compilation of experiments treating established B16-OVA tumors with A1-R SIINF and/or antibodies

Treatment	B16 OVA tumor at treatment		Tumor rejection in experiment #						
	Tumor size, mm ³	Duration of growth, d	1	2	3	4	5	6	Total
α CTLA-4 + α PD-L1	115 \pm 11	28 \pm 10	—	—	—	0/2	0/3	—	0/5
A1-R SIINF + α CD8	125 \pm 16	17 \pm 1	—	0/1	—	—	0/6	—	0/7
A1-R SIINF + α CTLA-4	119 \pm 10	25 \pm 5	—	—	—	—	0/3	0/1	0/4
A1-R SIINF + α PD-L1	122 \pm 9	20 \pm 1	—	—	—	—	2/3	2/2	4/5 ^a
A1-R SIINF + α CTLA-4 + α PD-L1	121 \pm 9	19 \pm 8	1/1	0/1	2/2	1/1	—	—	4/5 ^a

^a $P < 0.05$ compared with the α CTLA-4 + α PD-L1 group.

treatment with repeated doses does not provide additional therapeutic benefit.

We tested whether tumors were fully eradicated in 4 mice that rejected the tumor. Three of the mice were originally treated with a single injection of A1-R SIINF and 1 mouse was originally treated with weekly injections of A1-R SIINF. All 4 mice were held for at least 90 days posttreatment and then depleted of CD8⁺ T cells. The tumors did not relapse (Fig. 4 and Supplementary Fig. S3), strongly suggesting that A1-R SIINF treatment completely eradicated B16-OVA tumors.

Tumor resistance to A1-R SIINF-mediated rejection is not due to immune selection of antigen-loss variants

We investigated whether tumor relapse following A1-R SIINF treatment was due to outgrowth of antigen-loss variants. Cancer cells reisolated from six relapsed tumors retained the SIINF epitope as shown by staining with the K^b-SIINF-EKL antibody (data not shown).

The majority of intratumoral SIINF-specific CD8⁺ T cells still express PD-1 following A1-R SIINF treatment

Tumor relapse with retention of the SIINF epitope indicated that the SIINF-specific CD8⁺ T-cell response eventually failed to control tumor progression. Because CD8⁺ T cells can become dysfunctional through PD-1/PD-L1 interactions following chronic antigen exposure (11, 29, 36), we evaluated PD-1 expression before and after treatment with A1-R. As B16-OVA tumors became established at the 100 to 168 mm³ tumor size, more than 90% of intratumoral SIINF-specific CD8⁺ T cells expressed a high level of PD-1 (Supplementary Fig. S4). Treatment with A1-R control or A1-R SIINF did not reverse high PD-1 expression on the majority of SIINF-specific CD8⁺ T cells in the tumor. However, there was a nonsignificant trend suggesting that A1-R SIINF treatment may partially reduce the percentage of high PD-1-expressing SIINF-specific CD8⁺ T cells in the tumor (Supplementary Fig. S4). In the same A1-R SIINF-treated mice, SIINF-specific CD8⁺ T cells from the spleen did not express a high level of PD-1 (Supplementary Fig. S4). This showed that high PD-1 expression was specific to SIINF-specific CD8⁺ T cells from the tumor and suggested a

potential mechanism accounting for relapse following A1-R SIINF treatment.

Treatment with A1-R SIINF combined with α PD-L1 results in consistent tumor rejection

We tested whether the antitumor effects of A1-R SIINF could be enhanced by blocking the immunoinhibitory PD-1 and/or CTLA-4 pathway, which has also been implicated in suppressing T-cell responses to tumors (37). B16-OVA tumors responded minimally to treatment with a combination of α PD-L1 and α CTLA-4 (Fig. 5A and Table 2). Similarly, combining A1-R SIINF with α CTLA-4 was not effective because 0 of 4 tumors were rejected. However, combining A1-R SIINF with α PD-L1 or both α PD-L1 and α CTLA-4 was synergistic because 4 of 5 tumors were rejected in each group (Fig. 5A). Tumor rejection (4 of 5 mice) in each group was significant compared with the lack of rejection (0 of 5 mice) in the α CTLA-4 and α PD-L1 treatment group ($P < 0.05$; Table 2). Depleting CD8⁺ T cells in mice that rejected tumors did not result in relapse, strongly suggesting that treatment with A1-R SIINF combined with α PD-L1 resulted in complete tumor eradication (Fig. 5A).

To evaluate whether tumor rejection versus relapse was dependent on the magnitude of the SIINF-specific CD8⁺ T-cell response, we measured the percentage of SIINF-specific CD8⁺ T cells in the blood of mice following the different treatments. The peripheral blood was a reliable indicator of the therapeutic response to A1-R SIINF treatment, because mice that rejected B16-OVA tumors had a significantly higher percentage of SIINF-specific CD8⁺ T cells compared with mice that had tumor relapse (Fig. 5B). Consistent with this finding, mice rejecting tumors following treatment with A1-R SIINF combined with α PD-L1 had a similar percentage of SIINF-specific CD8⁺ T cells as mice rejecting tumors following treatment with A1-R SIINF alone (Fig. 5B). These data show that the SIINF-specific CD8⁺ T-cell response generated by A1-R SIINF can be enhanced by blocking PD-L1, most likely accounting for the consistent tumor eradication observed in these mice.

Discussion

To our knowledge, this is the first study reporting that therapeutic vaccination can rescue the dysfunctional endogenous tumor-specific CD8⁺ T-cell response leading to

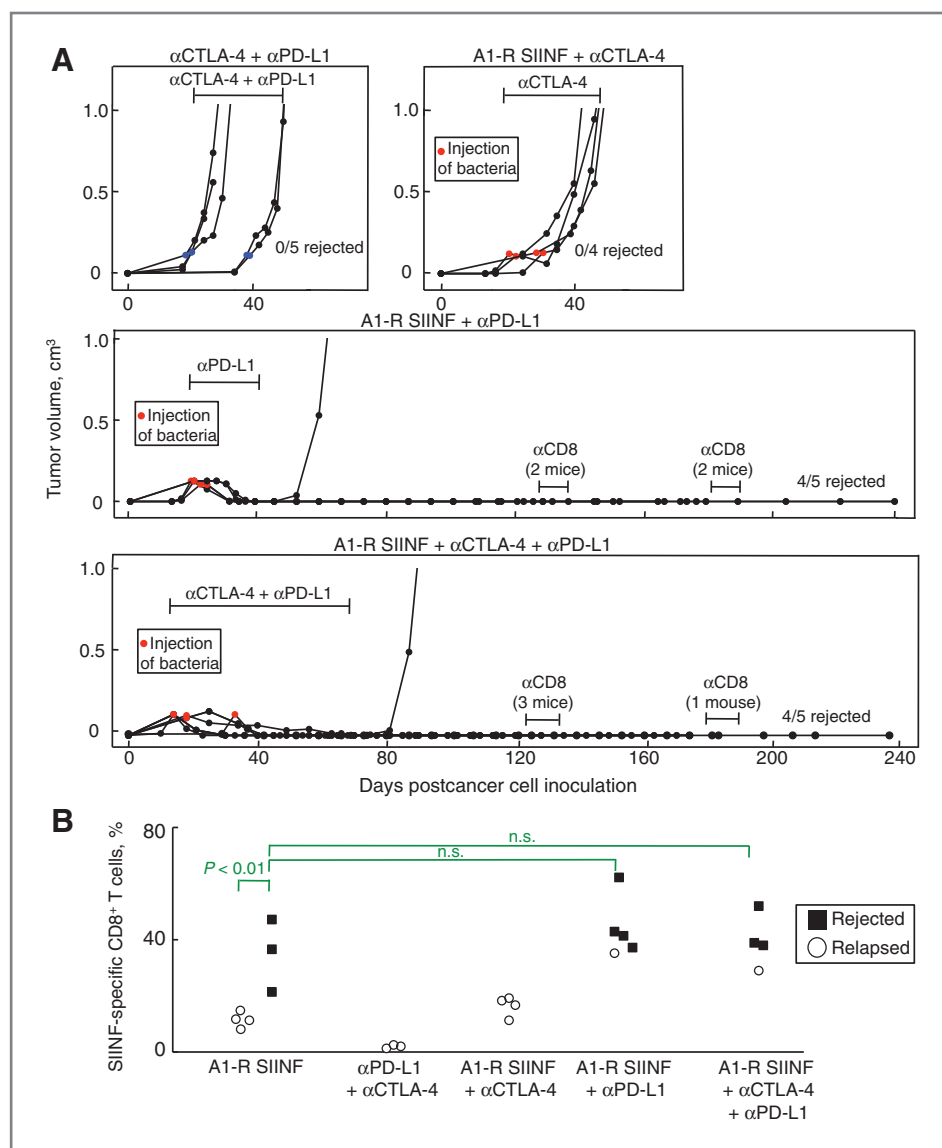


Figure 5. Treatment with A1-R SIINF combined with anti-PD-L1 results in enhanced SIINF-specific CD8⁺ T-cell expansion and consistent tumor rejection. **A**, C57BL/6 mice bearing established B16-OVA tumors were treated as indicated. A blue dot represents the initial time of treatment with α CTLA-4 and α PD-L1 for an individual mouse. A red dot represents treatment with A1-R SIINF for an individual mouse. In each treatment group, mice were treated with 100 μ g α CTLA-4 and/or 150 μ g α PD-L1 every third day until the tumor relapsed completely (>1.5 cm³) or was rejected. Mice that rejected tumors were held for at least 100 days posttreatment before administration of 3 doses of 200 μ g α CD8 at 3-day intervals. There was no tumor outgrowth for at least 40 days post-CD8⁺ T-cell depletion before the mice were sacrificed, showing that tumors were fully eradicated. **B**, at 8 or 9 days posttreatment, the peripheral blood from mice in the indicated treatment groups was stained with anti-CD8 and SIINF/K^b-dimerX. The percentage of SIINF-specific CD8⁺ T cells from mice that rejected B16-OVA versus relapsed following A1-R SIINF treatment was significantly different ($P < 0.01$); n.s., nonsignificant.

eradication of long-established tumors. Treating mice with our antigen-producing *S. Typhimurium* A1-R vaccine rescued the tumor-specific CD8⁺ T-cell response: proliferation was induced in the lymphoid organs and cytokine production was recovered in the tumor. Treatment with our vaccine resulted in improved mouse survival and rejection of established tumors in approximately one third of the mice. We discovered that mice rejecting tumors had a significantly higher percentage of tumor-specific CD8⁺ T cells in their blood compared with mice that relapsed following treatment, suggesting that the magnitude of the generated antigen-specific CD8⁺ T-cell response determined vaccine efficacy in our model. By combining our vaccine with α PD-L1, we enhanced the expansion of vaccine-generated CD8⁺ T cells and achieved consistent tumor rejection.

Our analysis focused on how to rescue tumor-specific CD8⁺ T cells which were specific to the SIINF epitope. By using 2 independent A1-R controls that did not produce SIINF, we

determined that A1-R must produce SIINF to rescue the SIINF-specific CD8⁺ T-cell response in the periphery and tumor. However, because A1-R SIINF expressed a large portion of OVA, it is possible that A1-R SIINF activated additional OVA-specific T cells (e.g., CD4⁺ T cells specific to the OVA₃₂₃₋₃₃₉ MHC class II epitope) that had direct antitumor effector function or contributed to rescuing SIINF-specific CD8⁺ T cells. The requirement for A1-R to deliver SIINF strongly suggests that APCs mediating T-cell rescue presented the SIINF epitope derived from A1-R rather than from B16 cancer cells. Because A1-R SIINF treatment induced systemic SIINF-specific CD8⁺ T-cell proliferation, this suggests that A1-R delivered the SIINF epitope to APCs localized both within and outside the tumor. This is probable as A1-R SIINF persisted at a low level in normal organs following intravenous injection. Because A1-R SIINF generated a systemic SIINF-specific CD8⁺ T-cell response, it is unknown whether tumor colonization by bacteria is required for tumor eradication. This is an important question to address

as clinical experience with intravenously injected *S. Typhimurium* VNP20009 showed that bacterial colonization of human tumors can be less consistent compared with murine experimental tumors (26, 38). Thus, if bacterial colonization of tumors is required for CD8⁺ T-cell rescue, this would support the continuation of studies investigating the mechanisms responsible for bacterial colonization of tumors and how colonization may be optimized in humans (39).

Rescuing CD8⁺ T-cell dysfunction remains a significant challenge in cancer (13, 14) and chronic viral infection (29). Patients with the strongest responses to α PD-1 have tumors expressing PD-L1 (14), which seems to be indicative of a preexisting T-cell response (12, 40). However, the objective response rate for this PD-L1⁺ patient subset is still only 36% (14), suggesting that blocking PD-1/PD-L1 may be insufficient to rescue T-cell dysfunction in many advanced tumors. In our model, long-established B16-OVA tumors were resistant to treatment with α PD-L1 and α CTLA-4 but were eradicated following treatment with A1-R SIINF combined with α PD-L1. Although the exact mechanism accounting for synergy between A1-R SIINF and α PD-L1 needs to be further investigated, our data suggest that synergy may have occurred in both the lymphoid organs and tumor. A1-R SIINF-mediated expansion of SIINF-specific CD8⁺ T cells was enhanced by blocking PD-L1, suggesting that PD-L1 expression by APCs inhibited T-cell expansion in the lymphoid organs. It is probable that A1-R treatment induced PD-L1 upregulation on APCs by inducing a strong inflammatory response (41) and/or through an LPS-mediated mechanism (40). In A1-R SIINF-treated tumors, it is likely that SIINF-specific CD8⁺ T cells were inhibited by PD-1/PD-L1 interactions as (i) the majority of SIINF-specific CD8⁺ T cells expressed PD-1 at a high level and (ii) SIINF-specific CD8⁺ T cells had rescued capacity to produce IFN- γ , a potent stimulator of PD-L1 expression on B16 (42) and human melanocytes (12) in addition to stromal cells (12).

This study used B16-OVA to determine how to rescue tumor-specific CD8⁺ T cells in long-established tumors. We targeted a model tumor-specific antigen as targeting tumor-associated self-antigens can lead to normal tissue damage and sometimes even patient death (43, 44). One might argue that the tumor eradication observed in this study was biased on the basis of the high affinity of SIINF-EKL for MHC class I. However, recent data from Robbins and colleagues strongly suggest that high-affinity peptides may be the only clinically relevant peptide targets in human cancers (45). Future studies will evaluate whether our vaccination approach is similarly effective when targeting natural tumor-specific epitopes on unmodified cell lines. If successful, translating our approach could be achieved using genomic technologies (45, 46) and peptide affinity measurements/algorithms (27, 45) to identify targetable high-affinity tumor-specific peptides for each individual patient.

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The ease in genetically modifying *S. Typhimurium* creates the opportunity to construct *S. Typhimurium* strains that could deliver the individual or a collection of unique tumor-specific antigens present on a patient's cancer cells, thereby rescuing the tumor-specific T-cell response unique to that patient. Targeting a collection of antigens may be required to prevent relapse of tumors as antigen-loss variants (47).

Our approach to cancer vaccine development could also be used to rescue or induce T cells that are isolated from the patient and used for adoptive T-cell therapy protocols. This could be especially beneficial for treating patients with larger tumor loads, as adoptive T-cell therapy has shown great potential for treating large established tumors in experimental and clinical studies (18, 48, 49). As isolating sufficient numbers of functional tumor-specific T cells from patients remains a challenge, treating patients with our antigen-expressing bacterial vaccine combined with α PD-L1 before T-cell isolation may significantly improve the quality of T cells that could be expanded *in vitro* and reinfused into patients. Therefore, the capacity to rescue a dysfunctional endogenous CD8⁺ T-cell response in tumor-bearers may also create new opportunities to target tumor-specific antigens by adoptive T-cell therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: D.C. Binder, A. Arina, P. Yu, D.A. Rowley
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.C. Binder, C. Idel
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.C. Binder, B. Engels, Y.-X. Fu, T. Karrison, C. Idel
Writing, review, and/or revision of the manuscript: D.C. Binder, B. Engels, A. Arina, P. Yu, J.M. Slauch, Y.-X. Fu, T. Karrison, B. Burnette, C. Idel, M. Zhao, R.M. Hoffman, D.H. Munn, D.A. Rowley, H. Schreiber
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D.C. Binder
Provided *Salmonella typhimurium* A1-R for the study: M. Zhao, R.M. Hoffman

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Cancer Immunology Research

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