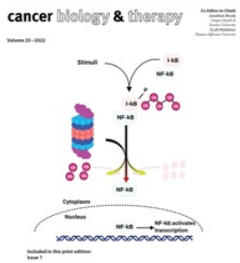

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β-amino acid substitution in the SIINFEKL antigen alters immunological recognition

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ABSTRACT

Background: Peptide vaccines offer a direct way to initiate an immunogenic response to a defined antigen epitope. However, peptide vaccines are unstable *in vivo*, subject to rapid enzymatic proteolysis. Replacement of an α-amino acid residue with a homologous β-amino acid residue (native side chain, but backbone extended by a single CH_2 unit) impairs proteolysis at nearby amide bonds. Therefore, antigen analogues containing α-to-β replacements have been examined for functional mimicry of native all-α antigens. Another group previously took this approach in the ovalbumin (OVA) antigen model by evaluating single α-to-β analogues of the murine major histocompatibility complex (MHC) I-restricted peptide SIINFEKL.

Methods: We re-examined this set of α/β SIINFEKL antigens. We tested the susceptibility to proteolysis in mouse serum and their ability to activate OVA-antigen-specific CD8 T cells *in vitro*. Additionally, we tested the α/β antigens *in vivo* for their ability to induce an antigen-specific immunogenic response in naïve mice and in OVA-expressing tumor-bearing mice.

Results: The α/β antigens were comparable to the native antigen in their susceptibility to proteolysis in serum. Each α/β antigen was capable of activating antigen-specific CD8 T cells *in vitro*. However, antigen-specific CD8 T cells induced against α/β antigens *in vivo* were not cross-reactive to the native antigen. Moreover, immunization with α/β analogues did not elicit anti-tumor effects in tumor-bearing mice.

Conclusions: We conclude that even though α/β analogues of the SIINFEKL antigen can elicit a T cell-based response, this class of backbone-modified peptides is not promising from the perspective of antitumor vaccine development.

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Peptide vaccines; β-amino acid substitutions; proteolysis-resistant peptides; MHC I; CD8 T cells

Introduction

T cells are a major component of natural cancer detection and surveillance. The recognition of, and immunological response to, cancer cells depend on the recognition by T cell receptors (TCRs) of tumor-associated antigens (TAAs) as presented by major histocompatibility complexes (MHCs) on the cancer cell surface.[1] The natural anti-tumor function of T cells has led to the development of vaccines as treatments for cancer; immunization with such vaccines is intended to expand and activate tumor-reactive T cells.[2] For cytolytic CD8 T cells, the target antigens are short peptide epitopes (typically 8–10 residues) derived from tumor-expressed proteins that are presented by MHC class I molecules.[1] While vector immunization strategies using virus, bacteria, plasmid DNA or mRNA encoding a target antigen are being explored, the simplest means of immunization might involve direct delivery of the peptide epitope recognized by CD8 T cells.[3] This approach has been investigated using epitopes presented by a highly prevalent human leukocyte antigen-A2 (HLA-A2) targeting WT1, TARP or HER-2/neu for tumors expressing these antigens, and HLA-A24-restricted epitopes targeting MAGE-A3, NY-ESO-1, hTERT or MART-1 among many other proteins.[4,5]

The E75 peptide, an HLA-A2-specific peptide derived from the HER-2/neu protein, is an example of a peptide that has been evaluated in multiple clinical trials, including those targeting breast and prostate cancers.[6] Mittendorf et al. vaccinated high-risk breast cancer patients with E75 peptide vaccine and GM-CSF in the adjuvant setting. They reported minimal toxicity and a 5-y disease free survival (DFS) of ~95% with only one recurrence in comparison to DFS of ~80% in the control group, demonstrating clinical efficacy.[7] In another phase II study, NeuVax (E75 peptide vaccine + GM-CSF) was evaluated in combination with Herceptin (HER2/neu receptor inhibitor) for treatment of breast cancer patients with HER1- and HER2-expressing tumors.[8] The study concluded that the combination treatment was safe but did not report any significant improvement in outcome for patients with HER2-low-expressing breast cancer. However, significant clinical benefit was reported in patients with triple-negative breast cancer.[8]

A growing interest has emerged in vaccines based on neoantigens, which are novel peptide sequences that arise from tumor-specific mutations and are not found in normal tissues. These neoantigens are highly immunogenic due to

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their tumor-specific nature, making them ideal targets for personalized cancer vaccines.[9] Clinical trials have demonstrated the feasibility of neoantigen-based vaccines, particularly in melanoma and other solid tumors, where they have shown promise in eliciting robust T cell responses and clinical benefit.[10] Many groups are utilizing prediction algorithms to identify mutation-associated neoantigens (MANA) that can be developed as vaccine candidates.[11] The ability to quickly and inexpensively manufacture peptides corresponding to unique mutation-associated epitopes has led to renewed interest in the development of peptide vaccines.

Unfortunately, most vaccines that contain only short peptides that function as CD8 T cell epitopes (i.e., lacking a carrier protein and/or an adjuvant) have not been highly immunogenic. The achievements summarized above have resulted from more complex approaches involving adjuvants and/or delivery of dendritic cells that had been pre-loaded with an antigen peptide. For example, favorable clinical results were obtained when the WT1 peptide vaccine was combined with Montanide ISA-51 and GM-CSF adjuvants.[12,13] Alternatively, the WT1 and MAGE-A3 peptides have been loaded onto dendritic cells to improve vaccine efficacy.[14,15] Other investigations have used longer peptides containing multiple CD8 epitopes and/or MHC class II epitopes (which, typically consist of 12–25 residues) in an effort to increase their immunogenicity.[15–18]

Many groups have described modifications to the amino acid sequence of a peptide antigen that increase the immune response elicited; these modified agents are referred to as heteroclitic peptides.[19] In these cases, the α-amino acid residue backbone is retained, but at least one side chain is altered relative to the native antigen. Modifications to MHC class I-binding anchor residues, for example, can enhance affinity for MHC class I and increase the number of CD8 T cells elicited with vaccination.[20] We have reported that immunization with epitopes altered for increased affinity to the MHC resulted in greater numbers of antigen-specific CD8 T cells, but these T cells displayed increased expression of programmed cell death protein 1 (PD-1) and manifested greatly decreased anti-tumor efficacies relative to T cells elicited with the native antigen.[21] Other groups have investigated peptide vaccines with amino acid substitutions among antigen residues that contact the TCR to increase the recognition or activation of antigen-specific CD8 T cells.[22] Some of these efforts have generated favorable outcomes, but others have shown that even subtle changes to the antigen sequence can abrogate recognition of the native antigen following immunization with the altered peptides.[23–26]

Short peptides are susceptible to rapid proteolysis by proteases at the site of immunization,[27] and this feature constitutes an inherent liability of peptide-based vaccine strategies. Efforts to circumvent this problem have included the use of oil-based adjuvants or loading of peptides directly onto dendritic cells, which are then delivered *in vivo*. Alteration of the peptide backbone represents another approach to minimizing proteolytic susceptibility. Replacing α-amino acid residues with β-amino acid residues, for example, can decrease susceptibility to proteolysis.[28] This approach has been pursued in several systems.[29–33] Webb et al. evaluated this strategy in the well-established ovalbumin antigen model using the dominant murine H-2k^b (MHC I) epitope SIINFEKL.[31] Some variants of SIINFEKL containing single α-to-β substitutions were reported to display modestly enhanced stability in mouse serum relative to SIINFEKL and were shown to bind H-2k^b with an affinity similar to that of SIINFEKL.[31] This group demonstrated that immunization with certain SIINFEKL α/β antigens resulted in the generation of cross-reactive cytotoxic CD8 T cells in C57BL/6 mice. However, the possibility that vaccination with any of these α/β antigens could produce an anti-tumor effect on tumors expressing the ovalbumin antigen was not explored.

Our study is based on the SIINFEKL analogues containing single α-to-β substitutions previously examined by Webb et al.[31] These analogues retain the natural side chains, in the natural order, but their backbone is altered via introduction of an "extra" CH_2 unit between the alpha carbon and the carbonyl carbon (β^3 amino acid substitution) at one site. We were drawn to backbone-modified antigens because they represent an alternative design approach relative to heteroclitic peptides, in which the backbone is preserved but at least one side chain is altered. We tested the α/β analogues of SIINFEKL *in vitro* for their susceptibility to proteolysis in mouse serum, specificity, and ability to activate antigen-specific CD8 T cells, and a subset of these α/β analogues was then evaluated *in vivo* for their ability to generate an immunogenic response and anti-tumor function.

Materials and methods

Cell lines and mouse models

E.G7-OVA (derivative of EL4, with constitutive expression of ovalbumin) cell line was purchased from ATCC (Manassas, VA, Cat. # CRL-2113) and maintained according to ATCC recommended guidelines.

C57BL/6 mice (stock no. 000664) and OT1 mice (stock no. 003831) were obtained from the Jackson laboratory (Bar Harbor, ME) and were housed and monitored by the Wisconsin Institute of Medical Research vivarium facility. All mice were maintained under aseptic conditions and all experiments were conducted under a protocol (M005690) approved by the University of Wisconsin Institutional Animal Care and Use Committee (IACUC). The studies with mice adhered to Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Reagents

RPMI-1640 (Cat.# 10–040-cv), penicillin/streptomycin solution (Cat.# 15140122) was purchased from Thermo Fisher Scientific (Waltham, MA). BenchMark FBS (Cat.# 100–106 500 ml) was purchased from Gemini Bio (Sacramento, CA). TLR3 agonist (Poly I:C) (Cat.# vac-pic) and TLR9 agonist, CpG (Cat.# trlr-2395) were purchased from InvivoGen (San Diego, CA). TMB-substrate (Cat.# 50-76-00) was purchased from Sera Care Life Sciences (Milford, MA). BCIP (5-Bromo-4-chloro-3-indolyl phosphate) (Cat.# BP1610–500) was purchased from VWR (Radnor, PA), NBT (Nitro Blue Tetrazolium) (Cat. # N66000–1.0) was purchased from Research Products

Table 1. Affinity of SIINFEKL and α/β analogues for H-2K^b.

Peptide	H-2K^b binding (IC_{50} ± SD; nM)	Relative binding
Native	1.2 ± 1.3	1
β3-Ser1	48 ± 1.9	41
β3-Ile2	1428 ± 1.4	1224
β3-Ile3	38 ± 1.5	33
β3-Asn4	2.3 ± 1.2	2
β3-Phe5	14 ± 1.1	12
β3-Glu6	11 ± 1.2	9.4
β3-Lys7	2.3 ± 1.3	2

Data represents 6 independent experiments for SIINFEKL and 3 independent experiments for each α/β analogue.

(RPI) (Mount Prospect, IL), Brillian Stain Buffer (Cat.# 566349) was purchased from BD Biosciences (Franklin Lakes, NJ), complete (Cat.# F5881) and incomplete (Cat.# F5506) Freund's adjuvant were purchased from Sigma-Aldrich (St. Louis, MO). Normal Mouse Serum (NMS) (SIAL-NS03L-1 ML) from Sigma Aldrich. Trichloroacetic acid (TCA) (T036925G) was from Fisher Scientific. Dulbecco's Phosphate Buffered Saline (DPBS) (14190–136) was from Gibco. Glibenclamide (QA-1040) was obtained from Combi-Blocks. Antibodies used are described in Supplemental Table 1.

Peptides

SIINFEKL, the H-2k^b-restricted epitope from chicken ovalbumin, was obtained commercially (LifeTein, LLC., Hillsborough, NJ), and the purity and identity were confirmed by mass spectrometry and HPLC. Native or analogues of SIINFEKL antigen, each containing a single α-to-β replacement was prepared as described in the supplemental methods. Mass spectrometry characterization and UPLC chromatogram of each analogue are shown in Supplemental Figures S1-S14, and summarized in Supplemental Table S2.

Proteolysis in mouse serum

Detailed protocol is described in the supplemental methods. Briefly, stock solutions of Normal Mouse Serum (NMS) were prepared using sterile Nanopure water. For each proteolysis reaction, each peptide was diluted to 0.5 mm with DPBS. A 20 µL aliquot of peptide solution was added to 180 µL of pre-warmed NMS, and the solution was incubated at 37°C. At each timepoint, a 20 µL aliquot of the reaction solution was removed and mixed with 20 µL of the quenching solution. For each reaction, a "0 min" control was prepared. After quenching, the aliquots were allowed to incubate at 4°C for 40 min. The aliquots were then centrifuged, 20 µL of the supernatant was mixed with 13.5 µL of 1 M NaOH, and this was followed by a second round of centrifugation. A 10 µL aliquot of each quenched and neutralized reaction solution was injected onto a Waters Acquity H-Class UPLC. The relative amount of peptide was quantified by integration of the SII/analogue peak at 220 nm and normalization to the glibenclamide internal standard. For each sample, the % peptide remaining was calculated by dividing the normalized peak area by the normalized peak area from the "0 min" control. Half-life values were determined by fitting normalized data to a one-phase decay model in Origin 2020.

H-2K^b affinity measurements

Quantitative binding affinity measurements for the mouse class I MHC molecule H-2K^b were obtained using classical competition assays based on the inhibition of binding of a high affinity radiolabeled peptide to purified K^b molecules. The assays were performed as detailed elsewhere[34] and in supplemental information.

In vitro *activation of OT-1*

Spleens were collected from OT-1 mice and made into single-cell suspensions. A 0.5×10^6 cells per well were plated in a 96-well plate and stimulated with either native or β-substituted peptide at variable concentrations ranging from 1 µg/ml to 100 ag/ml. PBS was used as a negative control (untreated) and CD3-CD28 beads were used as a positive control for stimulation. After 24 h, cells were washed twice with PBS and then stained for analysis by flow cytometry;[35] the media supernatant was collected for quantification of secreted IFN-γ by ELISA.[36] Reagents used are listed in supplemental Table 1.

In vivo *immunization studies*

C57BL/6 naïve male mice (8–10 weeks old) were immunized with either native or β-substituted peptide. A 100 µg of peptide was resuspended in PBS and delivered subcutaneously twice, one week apart. One week after the second immunization, spleens were collected, processed into a single-cell suspension and then tested by flow cytometry for tetramer-specific CD8 T cells. PBS was used as a negative control and 100 µg of pTVG4-sOVA[37] plasmid DNA encoding ovalbumin, delivered intradermally with the same weekly schedule, was used as a positive control. For studies using Freund's adjuvant, the first immunization was with complete Freund's adjuvant and the second immunization was with incomplete Freund's adjuvant mixed 1:1 with peptide in PBS. For studies testing cross-reactivity of antigen-specific CD8 T cells, the mice were immunized with either SIINFEKL or an α/β analogue, co-delivered subcutaneously with TLR3 (Poly I:C – 100 µg) and TLR9 (CpG-ODN 1826–50 µg) agonists. Splenocytes from these mice were then stimulated *in vitro* with either native SIINFEKL peptide or with the specific β-substituted

peptide that was used to immunize the mice. After 48 hours, the cells were analyzed using ELISpot to quantify IFN-γ secreting cells.[38] For the ELISpot studies, media alone was used as negative control and CD3-CD28 beads were used as positive controls. Reagents used for ELISpot are listed in supplemental Table S1.

In vivo *tumor studies*

C57BL/6 male mice were implanted with 1×10^6 E.G7-OVA cells subcutaneously. Nine days post implantation 2×10^6 OT-1 splenocytes were adoptively transferred intraperitoneally. The following day, mice were randomly assigned to treatment groups and immunized with 100 µg of either native or different β-substituted peptide subcutaneously. TLR3 agonist (100 µg PolyI:C) and TLR9 agonists (50 µg CpG) were mixed with peptide for some experiments. Tumor growth was monitored and measured using calipers to calculate tumor volumes using the formula $(\pi/6) \times (\text{long axis}) \times (\text{short axis})^2$. Mice were euthanized once the tumor volume reached 2 cm^3. For analysis of tumor infiltrating lymphocytes, tumors were collected on day 16 post-implantation and processed to form single-cell suspensions as previously described.[39] The cells were then stained for analysis by flow cytometry as described above.

Identification of SIINFEKL bound to H-2K^b

Dendritic cells were harvested from the spleens of C57BL/6 mice bearing Flt3L-expressing tumors, as previously reported.[37] A 5×10^6 cells per well were plated in a 96-well plate. The cells were then incubated with 1 µg/ml of native or β-substituted peptide for 4 h. Cells were then washed with PBS twice and then stained with an antibody that recognized SIINFEKL bound to H-2K^b (clone 25-D1.16) from BioLegend (San Diego, CA). The cells were then analyzed by flow cytometry.

Statistical analysis

Tumor growth data were analyzed by fitting a linear mixed-effects model with Geisser-Greenhouse correction and used to compare group means among treatment groups. Survival analysis was conducted using a Mantel-Cox log-rank test. All data presented are representative of at least three or more replicates for each experiment or assay. In most studies, a group size of five animals per group was used, sufficient to detect large effect differences of at least 2.0 in outcome measures between groups at a two-sided 0.05 significance level with 80% power. Data are expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to calculate statistical significance for all data presented that had more than two groups for comparison. Two-way ANOVA was used for experiments that had only two experimental groups. $p < .05$ was considered statistically significant. All analysis was performed using either GraphPad Prism V.10.0.0 or Origin 2020.

Results

SIINFEKL analogues containing a single α-to-β modification bind to the MHC-I and activate OT-1 T cells in vitro *but do not resist proteolysis*

To study the ability of α/β antigens to activate CD8 T cells, we focused on OT-1 mice, which are genetically modified to express TCRs restricted for identification of the peptide corresponding to ovalbumin residues 257–264 (SIINFEKL). We first asked whether SIINFEKL analogues containing a single α-to-β substitution would retain their binding capacity to the murine MHC-I H-2K^b with a competition assay that requires displacement of a radiolabeled peptide that binds tightly to H-2K^b (Table 1). Affinities varied depending on the site of backbone modification. Substitution at Asn4 or Lys7 had little effect on affinity, as indicated by IC_{50} values within 2-fold of the value for SIINFEKL. The side chains of Asn4 and Lys7 point away from the H-2K^b pocket; upon formation of the TCR-antigen-MHC complex, these side chains are oriented toward the TCR.[40] Substitution at Ser1, Ile3, Phe5 or Glu6 caused ~9- to 40-fold increases in IC_{50} relative to the native peptide, indicating significant decreases in MHC-I affinity for these α/β antigens. The side chains of Ser1, Ile3 and Phe5 project into the peptide-binding groove of the MHC-I. Modification at Ile2 caused a profound reduction in affinity for H-2K^b (>1000-fold increase in IC_{50}); Ile2 is a critical binding anchor for SIINFEKL, and the side chain makes extensive contacts with the MHC I surface.[40]

We analyzed susceptibility of the α/β analogues to proteolysis in mouse serum. Our data revealed that none of the single α-to-β modifications improved stability relative to the native peptide (Figure S15). The calculated half-life values for SIINFEKL and the α/β analogues were indistinguishable from one another.

To determine whether α/β analogues of SIINFEKL could activate SIINFEKL-specific CD8 T cells, OT-1 splenocytes were stimulated with native antigen or one of the α/β analogues for 24 h *in vitro* and then assessed by flow cytometry. The activation and effector functions were measured by monitoring the expression of 4-1BB, IFN-γ, TNF-α and PD-1 (Figure 1a–d) on CD8 T cells. Other cytokines evaluated included IL-2, IL-4, IL-10 and IL-17a (Figure 1 e-h). Among the α/β antigens, the peptide modified at Phe5 was the most potent for activation of OT-1 CD8 T cells *in vitro* (Table 2). For most of the markers evaluated, EC_{50} for the analogue containing β^3-hPhe at position 5 was within 2-fold of the value for SIINFEKL itself. PD-1 was a notable outlier, however, with EC_{50} nearly 100-fold higher for this particular α/β antigen relative to the native antigen. The other α/β antigens were substantially less potent in T cell activation, with EC_{50} values for most or all markers >10-fold higher relative to SIINFEKL.

For some markers, all α/β analogues seemed to reach a maximum in terms of percentage of T cells that was comparable to the maximum reached by SIINFEKL. The clearest example of this behavior was 4-1BB. For other markers, there was considerable spread among the maximum values; this trend was most evident for PD-1. We reasoned that these observations may reflect the prolonged

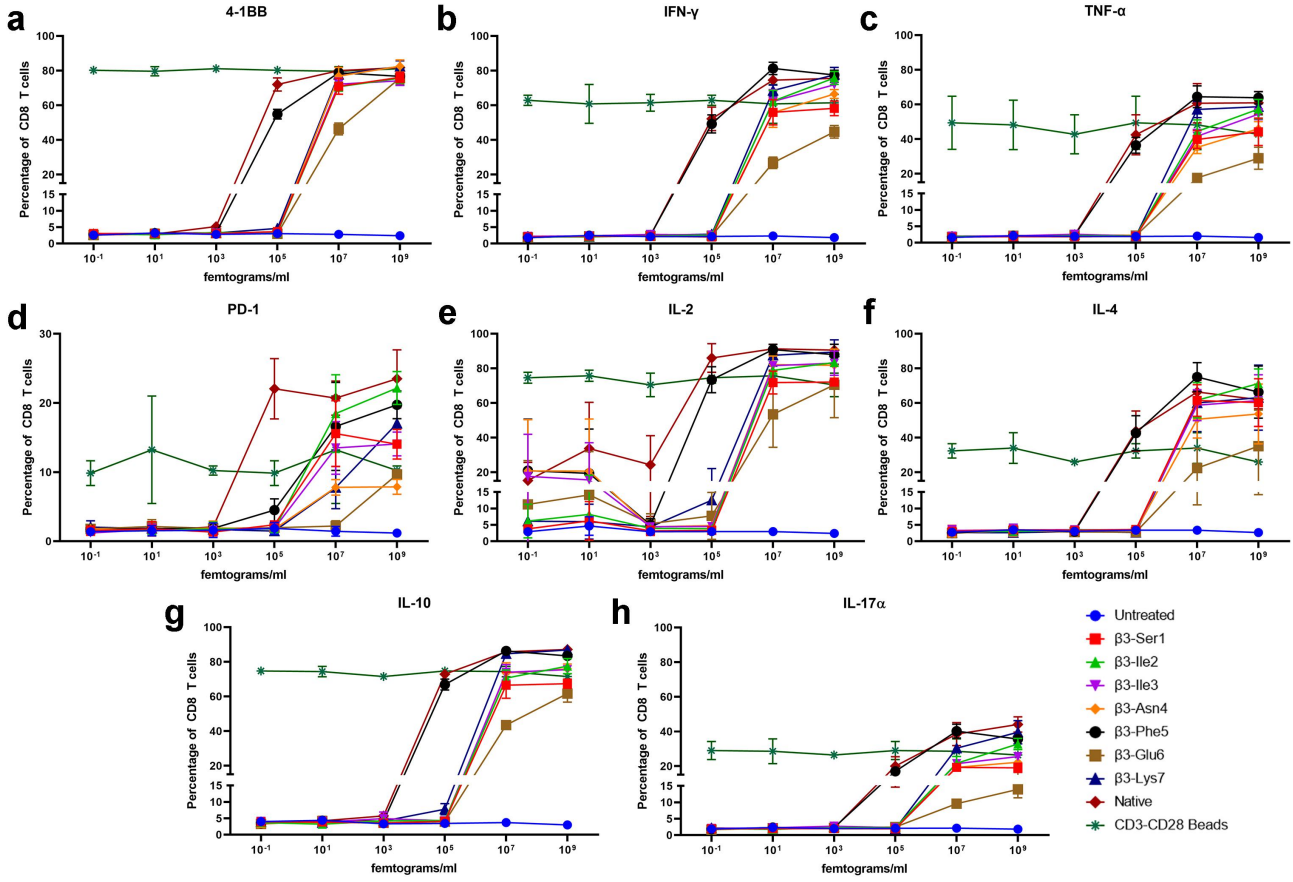

Figure 1. α/β-antigens activated OT-1 T cells *in vitro*. OT-1 splenocytes (from three OT-1 mice) were plated in a 96-well plate at 0.5×10^6 cells per well and then treated with varying concentrations (1 µg/ml – 11 ag/ml) of each peptide. CD3-CD28 beads were used as positive control. Cells were incubated overnight and then washed twice with PBS before staining for flow cytometry. For intracellular staining, GolgiStop was added to the cells for 6 h and then the cells were fixed. Gating was performed to identify live CD4- CD8+ single cells. These cells were then analyzed for expression of 4-1BB (panel A), IFN-g (panel B), TNF-α (panel C), PD-1 (panel D), IL-2 (panel E), IL-4 (panel F), IL-10 (panel G), and IL-17α (panel H). These are represented in graphs as percentage positive of each analyte among total CD8 T cells. This experiment is representative of three independent experiments, each with three biological replicates.

Table 2. EC_{50} (nM) of each β-modified peptide analog and native peptide for expression of activation markers and cytokines in OT-1 CD8 T cells.

	4-1BB	IFN-γ	PD-1	TNF-α	IL-2	IL-4	IL-10	IL-17a
Native	0.01	0.05	0.01	0.05	0.01	0.05	0.02	0.12
β3-Ser1	1.31	1.19	0.65	1.55	1.09	0.96	1.07	0.99
β3-Ile2	1.39	2.45	2.40	3.211	1.48	1.97	1.54	5.88
β3-Ile3	1.21	1.94	1.16	3.43	1.57	1.30	1.21	2.36
β3-Asn4	1.41	2.25	1.15	3.40	1.58	1.36	1.22	2.00
β3-Phe5	0.04	0.06	0.90	0.08	0.03	0.07	0.03	0.14
β3-Glu6	7.01	7.58	Incalculable	7.58	4.15	6.69	4.76	6.00
β3-Lys7	1.29	1.75	16.23	1.23	0.84	1.33	1.03	3.47

Data is from one experiment with each group tested in triplicates and is representative of three such independent experiments. The EC50 values reported were derived from dose-responses found in Figure 1 and were calculated using Prism v10.

activation of cells *in vitro*. Hence, in a separate study, we stimulated OT-1 splenocytes with SIINFEKL or an α/β analogue at a single, high concentration (1 µg/ml), washed away excess peptide after 4 h, and then measured activation after 24 h as above (Figure S16). The T cell activation pattern observed among the α/β antigens in this assay differed from the pattern observed at 1 µg/ml in the studies summarized in Figure 1. These observations suggested that some analogues were less efficient (e.g., in terms of association with or dissociation from the H-$2K^b$ or TCR or both) relative to SIINFEKL in activating OT-1 T cells without prolonged exposure.

Direct immunization with SIINFEKL or an α/β analogue did not result in generation of antigen-specific CD8 T cells

To evaluate the α/β antigens *in vivo*, we immunized naïve C57BL/6 mice with PBS or 100 µg of SIINFEKL or an α/β analogue subcutaneously twice, 1 week apart. Splenocytes from immunized mice were evaluated by tetramer staining for SIINFEKL-specific CD8 T cells (Figure 2). As shown in Figure 2a,c, not a single peptide, among the native or α/β antigens, was able to induce an antigen-specific CD8 T cell response. Similar results were obtained when immunization was performed with Freund's adjuvant (data not shown). In contrast, intradermal immunization of naïve C57BL/6 mice

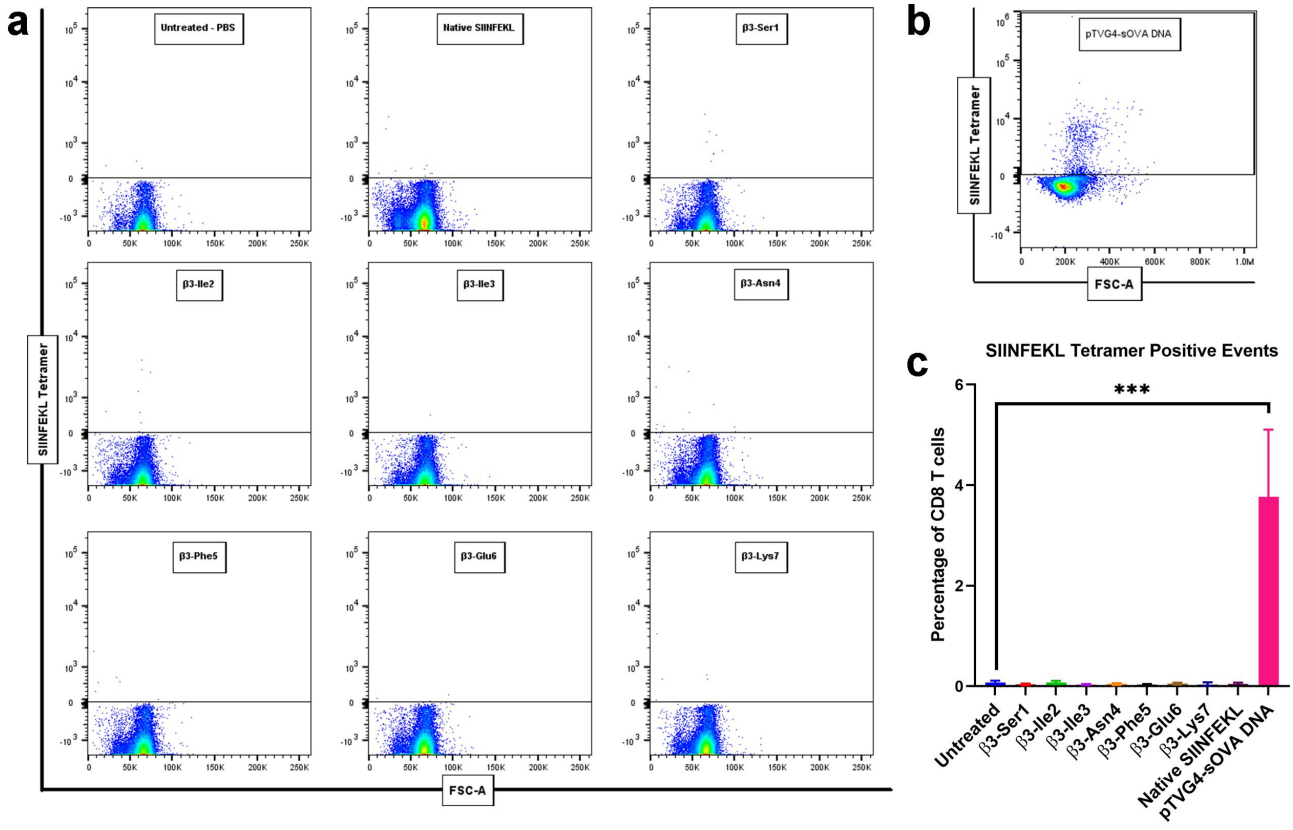

Figure 2. Direct immunization with native or β-modified SIINFEKL peptides did not result in generation of antigen-specific CD8 T cells. Naïve C57BL/6 mice ($n = 4$ per group, 39 mice total) were immunized twice with native or modified peptides (100 μg) subcutaneously, PBS was used as a negative control immunization and pTVG4-sOVA plasmid DNA (100 μg) was administered intradermally as a positive control. The immunizations were 1 week apart, and the mice were euthanized 1 week after the second immunization. Spleens were collected and processed for analysis by flow cytometry. (a–b). Gating was performed to identify live CD3+ CD8+ SIINFEKL tetramer+ single cells. The dot plots show the tetramer positive events from one of the representative mice from each group, and a bar plot for all the groups with mean and standard deviation is shown in (c). This experiment is representative of three studies, each with $n \geq 4$ mice per group.

with plasmid DNA encoding ovalbumin elicited a significant expansion of tetramer-specific CD8 T cells (Figures 2b,c). Overall, these results demonstrated that even though SIINFEKL and the α/β analogues were able to activate CD8 T cells *in vitro*, these peptides were unable to induce an antigen-specific CD8 T cell response *in vivo*, at least with the immunization protocol we employed.

Immunization with SIINFEKL or an α/β analogue did not result in tumor suppression or improved survival

Since we found that SIINFEKL-specific CD8 T cells could be activated by the α/β antigens *in vitro* (most notably by the analogue substituted at Phe5), but direct *in vivo* immunization did not result in generation of antigen-specific CD8 T cells at detectable levels, we asked whether selected α/β antigens could activate the anti-tumor effector function of SIINFEKL-specific CD8 T cells adoptively transferred to tumor-bearing mice. Specifically, OT-1 splenocytes were adoptively transferred into E.G7-OVA tumor-bearing mice, which were then immunized with SIINFEKL or an α/β analogue (Figure 3a). The α/β antigens in this experiment were chosen based on *in vitro* data showing low PD-1 expression with strong expression of activation-related markers including 4-1BB and IFN-γ (Figure 1 and Table 2). These criteria directed our attention to analogues with an α-to-β replacement at Asn4, Phe5 or Glu6. Unfortunately, neither the native antigen nor any among these three α/β antigens induced tumor growth suppression (Figure 3b) or improvement in overall survival of mice (Figure 3c). These data imply that immunization alone with SIINFEKL or with an α/β analogue was not sufficient to activate the anti-tumor function of adoptively transferred antigen-specific CD8 T cells *in vivo*.

Immunization with native peptides but not with α/β analogues along with TLR3 and TLR9 agonists improved survival in tumor bearing mice

We have previously shown that peptide immunization delivered with concurrent TLR3 and TLR9 activation can improve the effector function of vaccine-activated antigen-specific CD8 T cells.[41] Since peptide immunization alone did not activate the anti-tumor function of antigen-specific T cells *in vivo*, we vaccinated mice with native or selected α/β antigens along with TLR3 and TLR9 agonists (Figure 4a). As shown in Figure 4, treatment of E.G7-OVA tumor-bearing mice by adoptive transfer of OT-1 T cells, and then immunization with the native antigen SIINFEKL in combination with a TLR3 agonist (poly I:C) and a TLR9 agonist (CpG), resulted in significant suppression of tumor growth (Figure 4b) and significant increase in overall survival (Figure 4c). However, treatment with any of the three α/β antigens did not result in any

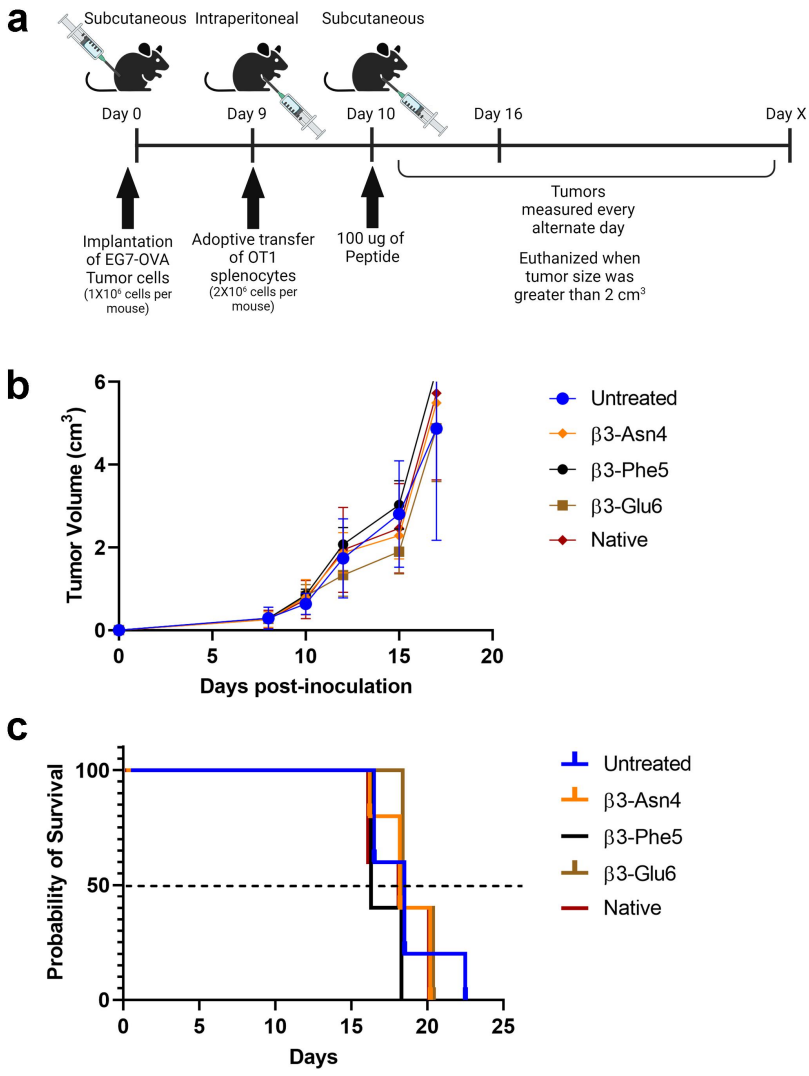

Figure 3. Immunization with native or β-modified SIINFEKL peptides did not result in tumor suppression or improved survival. C57BL/6 mice ($n = 5$ per group, 25 mice total) were implanted with E.G7-OVA tumor cells (1×10^6 cells per mouse) subcutaneously. Nine days post implantation, 2×10^6 OT-I splenocytes (from one OT-I mouse) were adoptively transferred intraperitoneally. The following day, mice were immunized with either PBS (untreated) or 100 µg of native or β-modified peptide (diluted in PBS) via subcutaneous injections. (a). Schematic of the study design represented on a timeline. (b). Tumor volumes were measured every other day and plotted. Mice were euthanized when the tumor size reached >2 cm^3. (c). Survival curve was plotted.

suppression of tumor growth (Figure 4b) or improved survival (Figure 4c). TLR3 and TLR9 agonist treatment alone similarly did not result in any suppression of tumor growth.

In a separate study, tumors from mice treated with SIINFEKL or with an α/β analogue and TLR agonists were collected at day 16 for analysis of tumor-infiltrating lymphocytes. As shown in Figure 4d, we detected antigen-specific CD8 T cells in the tumors of mice that were treated with SIINFEKL in combination with the TLR3 and TLR9 agonists. In contrast, treatment with any of the α/β antigens did not result in infiltration of antigen-specific CD8 T cells.

α/β antigens were immunogenic but resulted in antigen-specific CD8 T cells that did not cross-react with the native epitope

Because the α/β antigens derived from SIINFEKL could activate OT-1 T cells *in vitro* but could not activate anti-tumor effector function of these CD8 T cells *in vivo*, we evaluated the cross-reactivity of the α/β antigens to the native epitope. First, dendritic cells, as professional antigen-presenting cells, were incubated with the native peptide or an α/β analogue for 4 h, and then excess peptide was washed away. These cells were then stained with an antibody that specifically detects SIINFEKL bound to H-$2K^b$. Only the native peptide and the analogues modified at Ser1, Ile3 or Asn4 were detectable by this antibody (Figure 5a). Curiously, while all α/β antigens bound to some degree to H-$2K^b$ (Table 2), these modified antigens were not all specifically detected by the antibody (Figure 5a). In particular, the Phe5-modified antigen, which displayed native-like affinity for H-$2K^b$ (Table 2), and was most potent for activating OT-1 T cells (Figure 1), was not recognized by this antibody (Figure 5a). Thus, the recognition properties of the MHC-I+peptide complex containing the Phe5-modified α/β antigen differed significantly from the recognition properties of the MHC-I+SIINFEKL complex.

In a complementary study, C57BL/6 naïve mice were immunized twice, 1 week apart, with each of the α/β antigens or with SIINFEKL, with concurrent administration of TLR3 and TLR9

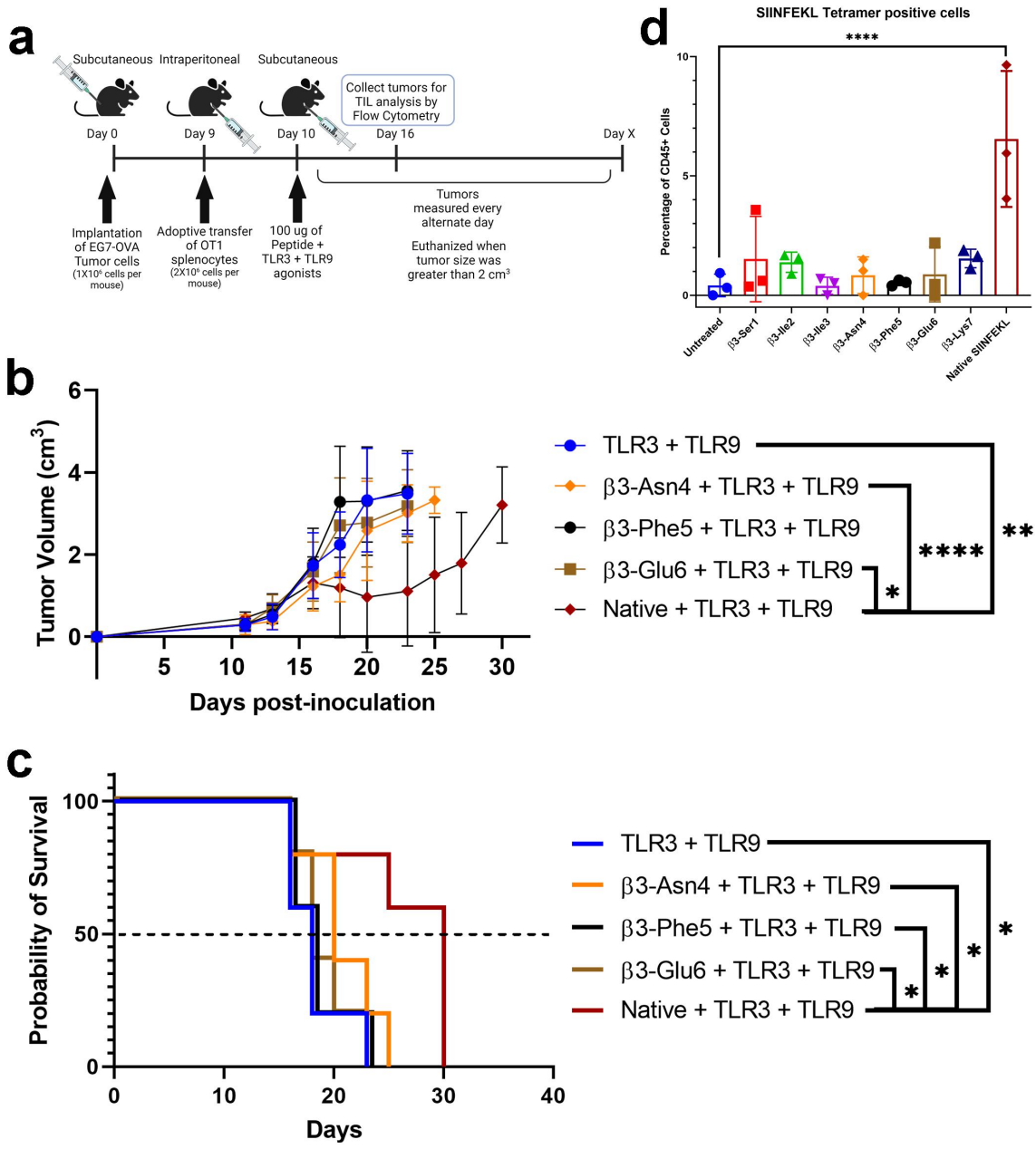

Figure 4. Immunization with native peptides, but not with β-modified peptides, along with TLR3 and TLR9 agonists improved survival in tumor-bearing mice. C57BL/6 mice ($n = 5$ mice per group, 25 mice total) were implanted with E.G7-OVA cells (1×10^6 cells per mouse) subcutaneously. Nine days post implantation 2×10^6 OT-I splenocytes (from one OT-I mouse) were adoptively transferred intraperitoneally. The following day, mice were immunized with either TLR3 (100 µg) and TLR9 (50 µg) agonists only or with native or β-modified peptide (100 µg) combined with TLR3 and TLR9 agonists subcutaneously. (a). Schematic of the study design is represented on a timeline. (b). Tumor volumes were measured every other day and plotted. Statistical analysis performed using two-way ANOVA (mixed-effects model) for measurements from day 20. (c). Survival curve was plotted, and statistical analysis was performed using Log-rank (Mantel-Cox) test. Mice were euthanized when the tumor size reached >2 cm^3. The experiment was repeated twice, and each group consisted of five mice. Tumors were collected on day 16 (additional 25 mice) and processed into single-cell suspension to be analyzed by flow cytometry. (d).Percentage of tumor-infiltrating tetramer positive CD8 T cells were plotted as a bar graph, statistical analysis was performed using one-way ANOVA. *$p < .05$, **$p < .01$, and ****$p < .0001$.

agonists, consistent with the protocol described in Figure 4. Splenocytes were evaluated for SIINFEKL-specific tetramer staining. As shown in Figure 5b, immunization with the native antigen SIINFEKL (with adjuvants) elicited tetramer-specific CD8 T cells, but none of the α/β analogues elicited tetramer-specific CD8 T cells. These splenocytes were also tested *in vitro* for antigen-specific IFN-γ secretion by ELISpot using either the immunizing peptide or the native SIINFEKL peptide as an antigen. We found that several of the modified peptides were immunogenic (notably, the α/β antigens with substitution at Ile3 or Lys7) and able to elicit an immune response to the immunizing peptide; however, there was no cross-reactivity to the native SIINFEKL epitope (Figure 5c and Figure S17). Overall, these data suggest that while some α/β analogues of SIINFEKL could activate antigen-specific CD8 T cells *in vitro*, when these α/β analogues were delivered directly as vaccines, they elicited CD8 T cells that were uniquely reactive to the modified epitope but did not cross-react with the native antigen. Thus, these α/β antigens were not competent to serve as vaccines to improve anti-tumor T cell immunity.

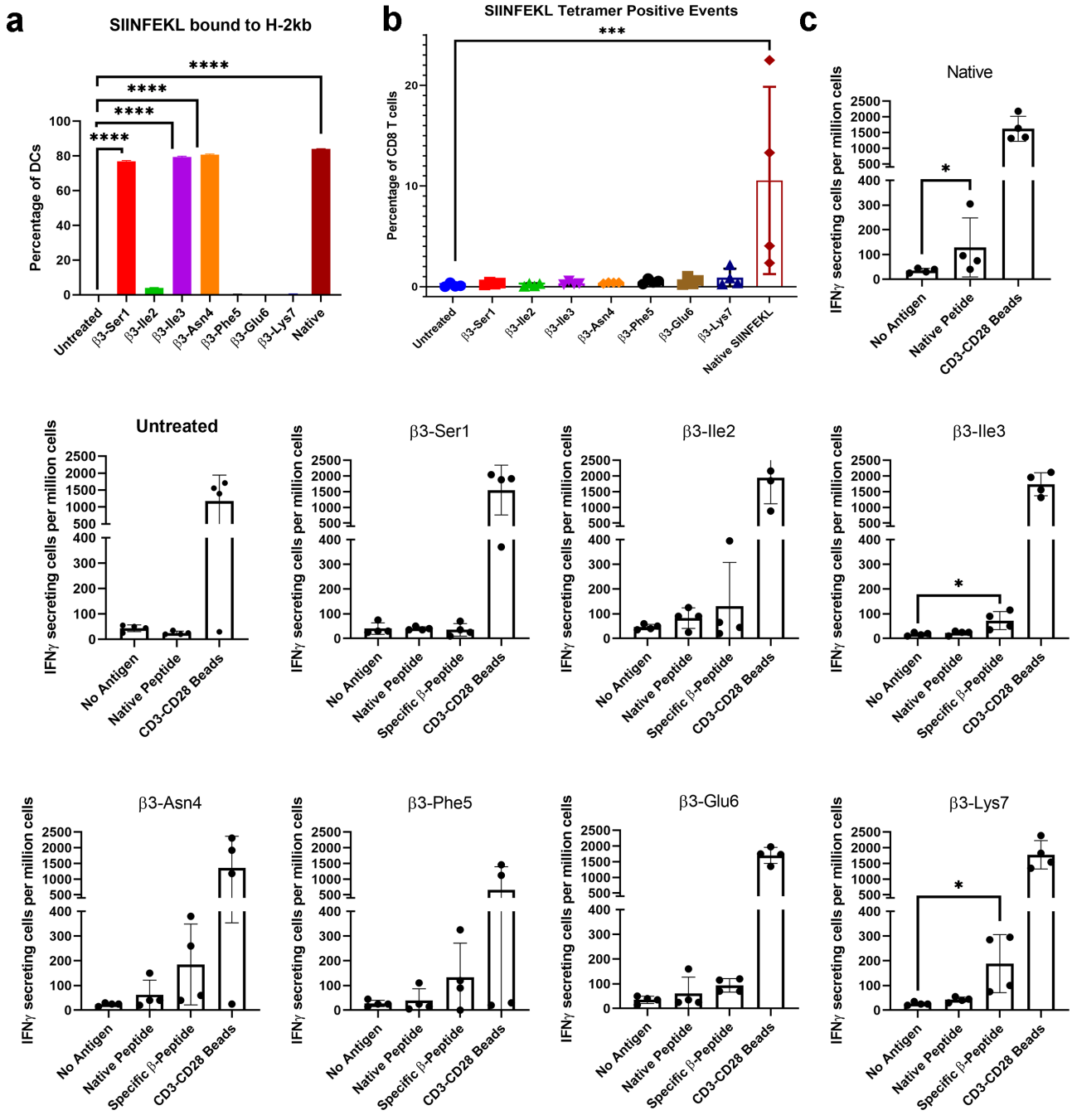

Figure 5. β-modified peptides are immunogenic but result in antigen-specific CD8 T cells do not cross react with the native epitope. Dendritic cells were isolated from C57BL/6 splenocytes using negative selection and incubated with 1 μg/ml of native or β-modified peptides for 4 h. DCs were then washed and stained for analysis by flow cytometry. (a). Percentage of DCs that are stained for SIINFEKL bound to H2-k^b antibody. (b). Naïve C57BL/6 mice ($n = 4$ per group, 36 mice total) were immunized twice with either TLR3 (100 μg) and TLR9 (50 μg) agonists only (untreated) or with native or β-modified peptide (100 μg) combined with TLR3 and TLR9 agonist subcutaneously. The immunizations were 1 week apart, and the mice were euthanized 1 week after the second immunization. Spleens were collected and processed for analysis by flow cytometry. Gating was performed to identify live CD3+ CD8+ SIINFEKL tetramer+ single cells. (c). ELISpot plate was coated with anti-mouse IFN-γ antibody and 0.2×10^6 splenocytes from each mouse spleen were plated. Cells were stimulated for 48 h with either media alone (no antigen), native peptide (2 μg/ml) or the β-specific peptide (2 μg/ml) that was used to immunize the mice. IFN-γ-secreting cells were counted, with the data for each mouse presented as bar graphs. Statistical analysis was performed using one-way ANOVA, *$p < .05$, ***$p < .001$ and ****$p < .0001$.

Discussion

Peptide vaccines offer a precise and safe approach to immunotherapy by targeting specific antigens, making these vaccines effective against cancer and infectious diseases. The development of MANA has reignited interest in anticancer vaccines, as MANA-based vaccines potentially enable personalized cancer treatments by exploiting unique tumor-specific epitopes. Despite their promise, however, peptide vaccines face notable challenges because of their low stability *in vivo*. One approach to enhance stability is the modification of peptide structure to resist proteolysis. Previous studies have explored unnatural antigen analogues in which a single α-amino acid residue is replaced with a homologous β-amino acid residue (natural side chain preserved, but the backbone is extended),

a modification that can protect nearby backbone amide bonds from enzymatic degradation. Encouraging behavior of α/β antigens *in vitro* has been reported.[29–33] However, the performance of α/β antigens as anti-tumor vaccines has not been previously evaluated.

Our proteolysis study indicated that single α-to-β replacements did not improve the stability of backbone-modified analogues in murine plasma relative to the well-studied mouse antigen SIINFEKL. This result is not surprising, because the protective effect of an α-to-β substitution is limited to nearby amide groups, and one α-to-β modification in an 8-mer peptide is unlikely to inhibit proteolysis across the entire backbone.[42] Webb et al. previously compared the effects of murine plasma on SIINFEKL and the analogues containing a single α-to-β in terms of remaining peptide after 2 h; this analysis showed that all peptides were rapidly degraded but suggested a modestly enhanced stability for some of the analogues.[42] Regarding the slightly different conclusions between the previous study and our own, we note that there was considerable variation among our measurements, while Webb et al. reported results of a single measurement. In addition, it is possible that the murine plasma samples had different protease profiles. Multiple α-to-β substitutions would likely provide significant resistance to proteolysis.[33] However, we have previously observed that two or three α-to-β replacements in an MHC I antigen peptide can greatly diminish MHC I binding and abrogate TCR recognition.[43] A subsequent study[33] and data provided here show that even a single α-to-β replacement can disrupt antigen binding or TCR recognition. Therefore, we did not pursue multiple substitutions in this study.

Our data suggest that single α-to-β replacements at a few positions in SIINFEKL can be tolerated without profound loss of affinity for the MHC-I H-2K^b. However, at other positions, α-to-β replacement causes moderate-to-severe loss of MHC-I affinity. A previous study suggested a greater tolerance of α-to-β replacement.[31] This difference in outcomes was likely related to differences in binding assays: we used a competition-based biochemical assay, while the previous study used the RMA-S cell-based MHC stabilization assay.

Despite the differences in affinity for H-2K^b indicated by our studies, each α/β analogue of SIINFEKL was able to activate OT-1 T cells *in vitro*. Some of this may be explained by the simple *in vitro* system in which all CD8 T cells expressed the same TCR; it is conceivable that peptides with low affinity for the MHC could still have sufficient affinity for the TCR to activate these T cells. However, the potencies of activation varied depending on the site of α-to-β replacement, and, to a lesser extent, on the marker monitored. The α/β antigen with substitution at Phe5 was the most potent among these analogues, approaching the efficacy of SIINFEKL itself for most markers. No differences were observed in T cell activation when comparing different α/β antigens at high peptide concentration (1 μg/ml), which could be a result of a large excess of the antigen in the culture medium. However, when we washed away the excess peptide after 4 h and analyzed T cell activation at 24 h, there were clear peptide-based differences in the activation profiles of CD8 T cells. Affinity for H-2K^b and potency for T cell activation were not correlated. For example, the antigen with α-to-β replacement at Ile2 bound the MHC-I >1000-fold less strongly than SIINFEKL, while the antigen with α-to-β replacement at Glu6 bound only ~10-fold less strongly; however, the antigen with modification at Ile2 was considerably more potent for all markers of activation than the antigen with modification at Glu6.

SIINFEKL and its α/β analogues failed to elicit an immunogenic response following the delivery of peptide alone *in vivo*. However, in the presence of TLR3 and TLR9 agonists, some α/β analogues generated an antigen-specific CD8 T cell response. This finding underscores the necessity of antigen-presenting cell (APC) activation via TLRs for effective peptide presentation and T cell activation *in vivo*. These results corroborate the findings of Jeon et al., who demonstrated that TLR-mediated APC activation is crucial for peptide-based immunization.[41] In a study with the E.G7-OVA tumor model, we found no response to peptide immunization alone, reinforcing the need for adjuvants to enhance peptide vaccine efficacy. When peptide immunization was augmented with TLR adjuvants following adoptive transfer of OT-1 T cells, an anti-tumor effect was observed in mice immunized with SIINFEKL but not in mice immunized with any of the α/β analogues. The absence of OT-1 tumor-infiltrating lymphocytes (TILs) following immunization with the α/β antigens suggested potential problems with peptide-induced T cell activation or T cell trafficking *in vivo*. However, direct α/β antigen immunization studies using TLR agonists as adjuvants resulted in CD8 T cells with altered specificity that did not recognize the native antigen.

To our knowledge, this is the first study to ask whether immunization with an antigen containing an α-to-β modification can elicit anti-tumor effects. Previous reports have tested the immunogenicity of these modified peptides and cross reactivity of the resulting T cells with the native antigen.[31] Webb et al. showed that immunization with the SIINFEKL analogue containing α-to-β replacement for Phe5 (with adjuvants) resulted in an immunogenic response *in vivo*. The resulting T cells were found to be cross reactive to the native epitope. In our studies, the α/β antigen with substitution for Phe5 was the only analogue that activated T cells similarly to the native peptide *in vitro*, and immunization with this α/β antigen in conjunction with TLR3 and TLR9 agonists resulted in the generation of antigen-specific T cells *in vivo*. However, we did not observe cross-reactivity of these T cells with the native epitope or any anti-tumor effect in mice immunized with this α/β antigen. It is not clear why our study indicated a lack of T cell cross-reactivity, SIINFEKL vs. the analogue with α-to-β replacement at position 5, when Webb et al. previously observed such cross-reactivity, albeit with a different experimental design. It is possible that the prolonged *in vitro* exposure to the antigen, with lymphocytes loaded with SIINFEKL antigen, used by Webb et al. to expand and detect antigen-specific CD8 T cells, altered the phenotype of cells elicited directly by vaccination and sensitized these cells toward the α/β antigen. This hypothesis would account for the difference in outcome between our study and that of Webb et al. Alternatively, there may be a small number of SIINFEKL-specific clonal cells elicited by immunization with the analogue containing α-to-β replacement at position 5 that could undergo expansion under continuous stimulation over 13 d,

but not be detected by ELISPOT without this *in vitro* stimulation. We conducted tetramer staining on the day of spleen collection as a direct readout for the presence of SIINFEKL-specific CD8 T cells; a small number of these cells present at that time might have been below our limit of detection. These differences in experimental setup may explain the disparities between our results and those of Webb and colleagues.

We found that immunization with α/β antigens modified at Ile3, Asn4, Phe5 or Lys7 resulted in CD8 T cells specific to the α/β antigen, with no cross-reactivity to the native antigen SIINFEKL. This lack of TCR cross-reactivity may reflect differences in antigen presentation by the MHC-I that are caused by the backbone extension in the α/β antigens relative to the all-α antigen SIINFEKL. Comparable effects have been previously described with heteroclitic peptides, which contain side-chain modifications that can influence TCR recognition.[44] The inability of the antibody directed against H-2K^b-bound SIINFEKL to recognize α/β antigens loaded onto APCs may indicate that antibody binding is hindered by backbone modification, which would align with observations of Johnson et al.[44] on the effects of side-chain changes. We do not know if our results using these modified peptides as vaccine antigens are generalizable to all MHC-I peptide epitopes; it is conceivable that other MHC:peptide:TCR complexes are more tolerant of changes to the peptide backbone.

Interestingly, we observed that the α/β antigens activated OT-1 CD8 T cells *in vitro*, whereas CD8 T cells obtained from α/β antigen immunized mice were not activated by the native antigen. This discrepancy is likely due to the fact that all CD8 T cells in the *in vitro* assay expressed the same TCR, and these T cells could be activated by peptides despite modest changes in their MHC or TCR affinity. However, following immunization *in vivo*, in which the T-cell repertoire is vastly larger, we expect that immunization with these peptides led to activation of CD8 T cells expressing different TCR that had no cross-reactivity for the native SIINFEKL epitope, similar to what has been observed using peptides with altered amino acid side chains.[44]

We were not able to identify any pattern in the effects of α-to-β replacement on immunogenicity. Ile2 and Phe5 are anchor residues for SIINFEKL,[40] i.e., the side chains of these residues interact extensively with the MHC I surface. Backbone modification at these two positions led to different outcomes: α-to-β replacement at Ile2 reduced MHC-I affinity, while replacement at Phe5 resulted in native-like affinity. These differences were reflected in the immunization experiment, where the peptide with α-to-β substitution at Ile2 failed to induce an immunogenic response, but an immunogenic response was generated by the peptide with α-to-β at Phe5. Glu6 and Lys7 are TCR contact points for SIINFEKL,[45] and α-to-β replacement at these positions led to opposing outcomes. The antigen modified at Glu6 was least effective in activating T cells *in vitro* and failed to induce an antigen-specific response *in vivo*. In contrast, the antigen modified at Lys7 activated T cells similarly to other modified antigens and resulted in antigen-specific CD8 T cells after immunization.

Overall, this study suggests that modifying the SIINFEKL antigen with a single α-to-β replacement exerted variable impact on binding to a cognate MHC-I, depending on the substitution site. Most of the resulting α/β antigens could activate T cells *in vitro*, as monitored with a variety of markers. Potency of activation varied with the site of substitution; α-to-β replacement at Phe5 led to the highest expression for most markers. Vaccination with these antigen analogues, in the presence of TLR3 and TLR9 agonists, could elicit responsive T cells, but these T cells did not cross-react with the native antigen. These findings suggest that the α-to-β replacement strategy may not be productive for anti-cancer vaccine development, although it is possible that more favorable outcomes would be observed with other tumor-associated MHC-I antigens.

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Author contributions

CRediT: **Ichwaku Rastogi:** Conceptualization, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing; **John A. Mannone:** Formal analysis, Investigation, Methodology, Writing: review and editing; **Ruslan Gibadullin:** Conceptualization, Formal analysis, Writing – review & editing; **Jena E. Moseman:** Investigation, Writing – review & editing; **John Sidney:** Formal analysis, Investigation, Writing – review & editing; **Alessandro Sette:** Investigation, Writing – review & editing; **Douglas G. McNeel:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing; **Samuel H. Gellman:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Disclosure statement

IR, JAM, RG, JEM, JS, and DGM have no relevant potential conflicts of interest. AS is a consultant for Alcimed, Gritstone, Darwin Health, EmerVax, Gilead Sciences, Guggenheim Securities, Link University, RiverVest Venture Partners, and Arcturus. LJI has filed for patent protection for various aspects of T cell epitope and vaccine design work. SHG is a co-founder of Longevity Biotech, Inc., which is pursuing biomedical applications of α/β peptides.

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Authors' contributions

IR wrote the manuscript, designed and performed immunology-based experiments, and carried out data analysis. JEM performed experiments. RG and JAM generated α/β-modified peptides and performed proteolysis experiments and its analysis. JS performed H2-k^b binding affinity experiment and its analysis. DGM, SHG and AS oversaw the experimental design. DGM and SHG are responsible for the overall content as guarantors. All authors contributed to the writing and approval of the final manuscript.

Data availability statement

The data generated and/or analyzed during this study are available from the corresponding author on reasonable request.

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