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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
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Article

Backbone Modifications of HLA-A2-Restricted Antigens Induce Diverse Binding and T Cell Activation Outcomes

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Cite This: J. Am. Chem. Soc. 2021, 143, 6470−6481
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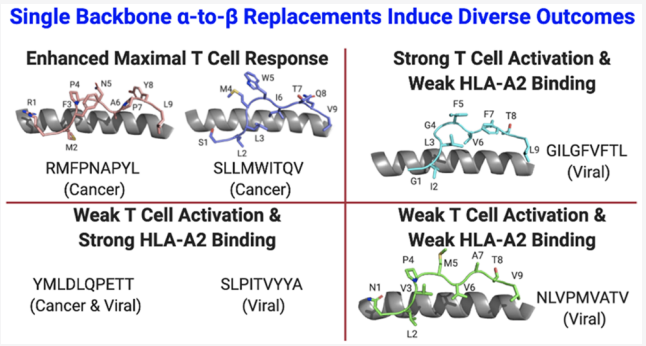
ABSTRACT: $CD8^+$ T cells express T cell receptors (TCRs) that recognize short peptide antigens in the context of major histocompatibility class I (MHC I) molecules. This recognition process produces an array of cytokine-mediated signals that help to govern immunological responses. Design of biostable MHC I peptide vaccines containing unnatural subunits is desirable, and synthetic antigens in which a native α-amino acid residue is replaced by a homologous β-amino acid residue (native side chain but extended backbone) might be useful in this regard. We have evaluated the impact of α-to-β backbone modification at a single site on T cell-mediated recognition of six clinically important viral and tumor-associated antigens bound to an MHC I. Effects of this modification on MHC I affinity and T cell activation were measured. Many of these modifications diminish or prevent T cell response. However, a number of α/β-peptide antigens were found to mimic the activity of natural antigens or to enhance maximal T cell response, as measured by interferon-γ release. Results from this broad exploratory study advance our understanding of immunological responses to antigens bearing unnatural modifications and suggest that α/β-peptides could be a source of potent and proteolytically stable variants of native antigens.



INTRODUCTION

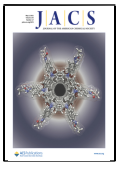
T cell receptors (TCRs) found on $CD8^+$ T cells orchestrate immune responses via their recognition of short peptide fragments (8−14 residues) bound in major histocompatibility class I (MHC I) molecules.[1] Peptide-MHC I (pMHC I) complex recognition[2] triggers T cell activation and leads to elimination of abnormal and infected cells. Although the TCR set is large ($\sim 2.5 \times 10^7$ unique receptors),[3,4] the pool of pMHC I complexes is far larger ($\sim 1.7 \times 10^{18}$).[5] A single TCR can bind to complexes formed by as many as 10^6 different peptides presented by the cognate MHC I.[6] This intrinsic diversity in TCR-pMHC I recognition has inspired a search for altered peptide ligands (APLs) capable of priming T cells that are effective at recognizing a natural viral or tumor-associated antigen.

To be useful, an APL must bind to the intended MHC I, and the resulting pMHC I complex must engage TCRs. Thus, when bound to the MHC I, the APL must at least partially mimic a natural peptide antigen. If an APL is to evoke T cell responses in a broad human population, then the APL-MHC I complex should be recognized by TCRs that are "public", i.e., that occur in all individuals who express that MHC I.[7] This strategy of targeting public TCRs is designated TCR-optimized peptide skewing of the repertoire of T cells (TOPSORT)[8] and has recently been used to enhance T cell responses to an antigen associated with melanoma.[9] Enhancement of T cell responses of public TCRs with APLs is particularly desirable against antigens that are intrinsically tolerogenic, as is the case with many tumor-associated antigens such as Melan-A;[10−12] well-designed APLs can be used to break tolerance to self-antigens.

Most APL candidates have been derived entirely from L-α-amino acids, as are the natural antigens that bind to the targeted MHC I.[8,13] This type of peptide, however, is rapidly degraded by proteases. Low stability *in vivo* of conventional peptides has motivated a few studies of APLs that contain other types of subunits, such as those derived from D-α-amino acids,[14] *N*-alkyl-glycines,[15] or β-amino acids.[16−19] Because the number of such studies is limited, and the MHC I and TCR ranges are so vast, it is difficult to reach any general conclusions about potential MHC I ligands containing these unnatural subunits from the available examples.

The work described here is motivated by our desire to understand the range of possible outcomes when a well-studied conventional MHC I antigen is altered by replacing a single L-α-amino acid residue with a β-amino acid residue. The

Received: January 6, 2021
Published: April 21, 2021



https://doi.org/10.1021/jacs.1c00016
J. Am. Chem. Soc. 2021, 143, 6470−6481

replacements we implement are "homologous" because the natural side chain is retained; structural change is limited to the peptide backbone (one extra CH_2 unit within one residue). One merit of the α-to-β replacement strategy is that a single β residue can inhibit enzymatic cleavage of nearby peptide bonds, even if they occur between α residues.[20−26] We wondered whether the resulting subtle change in peptide structure might modify the nature of the T cell response, at least in some cases. Possible problems with the α-to-β replacement strategy are that the structural alteration could diminish or even abolish binding to the MHC I, or the resulting α/β-peptide+MHC I complex (α/β-pMHC I) could be unrecognizable by the TCR.

We were motivated to undertake this study by previous reports on α-to-β replacement in other types of peptides that convey information by interacting with partner proteins. Our original interest in this backbone-modification strategy arose from the prospect that properly designed α/β-peptides might retain the "message" embedded in the all-α sequence but resist proteolysis because of the unnatural subunits. Unexpectedly, we found that α-to-β replacement could, in some cases, alter the nature of the message, relative to the original α-peptide. Examples included discovery of partner-selective α/β-peptides derived from a promiscuous α-peptide[27−29] and pathway-selective analogues of peptide hormones ("biased agonists").[23,30] Studies involving backbone modification via α-to-β replacement in derivatives of neuropeptide Y family members,[31−33] angiotensins,[21,34] and phosphopeptides that bind to 14−3−3 proteins[35] provide further evidence that replacing one or a few α residues in a polypeptide with β homologues can generate novel derivatives with interesting properties.[36,37] These precedents led us to wonder whether antigen analogues containing an α-to-β replacement might form MHC I complexes that retain the signaling properties of the native pMHC I, as manifested via engagement of TCRs, or perhaps display altered signaling properties.

Two studies have previously examined the impact of single-site α-to-β replacement in human MHC I antigens. In one such study, α/β-peptides derived from HLA-A*0201-restricted (HLA-A2 hereafter) Melan-A antigen ALGIGILTV were evaluated.[16] (In humans, MHC molecules are encoded within the human leukocyte antigen (HLA) locus, and the abbreviation HLA is often used in place of MHC.) Most of the ALGIGILTV α/β-peptides exhibited moderate to strong binding to HLA-A2. In addition, three α/β-peptides induced lysis of HLA-A2-bearing cells by two different T cell clones. A different study surveyed RRFFPYYV α/β-peptides presented by the MHC I HLA-B*2705.[17] These α/β-peptides were generally 10^2- to 10^5-fold less effectively recognized by cytotoxic $CD8^+$ T cells relative to the α-peptide.

Two additional reports evaluated antigens restricted to the murine MHC I H2−K^b (SIINFEKL)[18] or H2-D^b (KAVYNFATM).[19] All SIINFEKL α/β-peptides activated two distinct TCR clones, and two of these α/β-peptides were cocrystallized with H2−K^b to reveal the basis of their participation in the MHC-peptide-TCR complex. We evaluated KAVYNFATM-derived α/β-peptides containing two or three evenly spaced $\alpha \rightarrow \beta$ replacements using $\alpha\alpha\alpha\beta$ and $\alpha\alpha\beta\alpha\alpha\alpha\beta$ backbone repeat patterns. Previously, we had shown that these backbone repeat patterns confer substantial proteolytic resistance.[24,25] The KAVYNFATM α/β-peptides containing multiple $\alpha \rightarrow \beta$ replacements bound only weakly to MHC I H2-D^b, and these α/β-peptides were ineffective at activating T cells.

The new studies were aimed at generating a more comprehensive understanding of the possible outcomes from replacing a single α residue with a β homologue in terms of recognition by components of the human immune system. Previous observations that a single $\alpha \rightarrow \beta$ replacement in a peptide hormone could alter the "molecular message" conveyed via a cognate G protein-coupled receptor (GPCR)[30] raised the possibility that an analogous alteration could occur in signaling mediated by a pMHC I complex. In the former case, the signal is produced when the peptide hormone induces conformational rearrangements within the GPCR that are sensed by cytosolic partners (including G proteins and arrestins).[38] We wondered whether subtle changes in the pMHC I complex that result from $\alpha \rightarrow \beta$ replacement, i.e., the addition of a single methylene to the antigen backbone, could alter the message conveyed to TCRs. Previous work has shown that changes involving a single side-chain methylene group (e.g., Asp vs Glu) in an all-α antigen can alter the functional outcome of pMHC I-TCR engagement.[39,40]

We have systematically introduced single α-to-β replacements in six clinically important HLA-A2-restricted epitopes and determined the effects of these replacements on the ability of α/β-peptides to bind to HLA-A2. In addition, we have evaluated the abilities of the α/β-pMHC I complexes to activate patient-derived antigen-specific $CD8^+$ T cells. We find that different antigens display distinct responses to α-to-β replacements, in terms of both MHC I affinity and T cell activation, despite the fact that all antigens are presented in the same MHC I context. To our surprise, we discovered several α/β-analogues that elicit enhanced maximal T cell response in comparison to their natural α-peptide counterpart, as measured by release of interferon-γ (IFN-γ).

This study significantly expands our understanding of potential outcomes from homologous α-to-β replacements among MHC I peptide antigens by showing that this type of modification can generate distinctive and potentially useful immunological signaling properties. It was easy to discover these novel antigens because solid-phase synthesis of small "β-scan" libraries for a given antigen is straightforward. Our findings suggest that evaluating the α-to-β replacement series for other MHC I antigens might provide tools to probe immunological responses. In the long term, such efforts could lead to proteolysis-resistant HLA-A2 peptide vaccine candidates.

RESULTS

Synthesis and Evaluation of HLA-A2 α/β-Peptides. We replaced α residues with homologous β residues in which the native side chain is maintained but an additional carbon (CH_2 unit) is inserted into the backbone (Figure 1). The most straightforward approach is to use β^3 residues with absolute configuration corresponding to that of L-α residues and bearing the side chain on the carbon adjacent to nitrogen. L-α residues have *S* absolute configuration, but the absolute configuration of the homologous β^3 residues varies between *S*, in most cases, and *R*, for the β^3 homologues of Ser, Thr, Val, and Ile. We use the designation *S** for this stereochemical family of β^3 residues. All past studies of α/β-peptide analogues of MHC I antigens involved β^3 residues with *S** configuration.[16−19] Many Fmoc-protected (*S**)-β^3-amino acids are commercially available, and they are readily incorporated via solid-phase synthesis. Therefore, our studies included many α-to-β^3 replacements

https://doi.org/10.1021/jacs.1c00016
J. Am. Chem. Soc. 2021, 143, 6470−6481

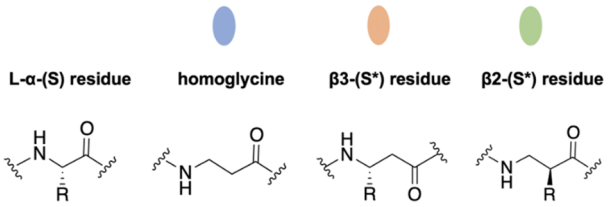


Figure 1. Structures of amino acids used in this study. Colored ovals indicate generic structures of β-amino acids and homoglycine (also called β-alanine). The colored ovals are used to identify sites at which α-to-β or α-to-homoglycine replacements have been introduced. [See text for explanation of the designation S*.]

for each antigen. β^2 homologues of α residues bear the native side chain on the carbon adjacent to the carbonyl. Relatively few protected β^2-amino acids are commercially available, and we therefore examined only a few α-to-β^2 replacements. Consequences of β^2 residue substitutions have previously been examined for only an MHC II antigen.[41] We use the designation S* for β^2 residues with absolute configuration corresponding to that of L-α residues.

We introduced single α-to-β replacements into six different HLA-A2 peptide epitopes derived from viral or tumor-associated antigens: RMFPNAPYL (WT-1), SLLMWITQV (NY-ESO-1), GILGFVFTL (M1), YMLDLQPETT (HPV E7), NLVPMVATV (CMV pp65), and SLPITVYYA (glycoprotein D1). HLA-A2 is one of the most abundant alleles in humans, and previous research has yielded a wealth of knowledge on diverse peptides bound to HLA-A2.[42] High-resolution structures are available for pMHC I or pMHC I-TCR involving four of the epitopes: RMFPNAPYL, SLLMWITQV, GILGFVFTL, and NLVPMVATV.[43−46] These data offer a framework for structure-based interpretation of our findings.

Cell-based experiments were undertaken to characterize binding and T cell activation behavior of the α/β-peptides. The 174 x CEM.T2 cell line (referred to as T2 cells below) was used for antigen presentation. The use of a single cell line for experiments evaluating binding or T cell activation permitted direct correlation of these two critical parameters. T2 cells express HLA-A2 but lack the transporter associated with antigen processing. Therefore, relatively few HLA-A2 molecules are present on the surface of a T2 cell in the absence of exogenously added peptide ligands.[47−49] Introduction of an HLA-A2-restricted peptide induces accumulation of HLA-A2 on the surface of T2 cells.[9,14,50]

An exogenous peptide that binds tighter to HLA-A2 will generate greater surface HLA-A2 levels on T2 cells.[50,51] We quantified HLA-A2 level changes using flow cytometry by staining T2 cells with a fluorescently labeled anti-HLA-A2 antibody. The maximum HLA-A2 surface level measured for each natural antigen was used to define "100% response," and the values for α/β-peptides were normalized against the natural antigen. Quantitation of peptide binding was independently assessed *in vitro* using purified HLA-A2 complexes. In these measurements, the affinity of the peptide was determined based on its ability to inhibit binding of a high-affinity radiolabeled peptide. The data from the *in vitro* assay are presented as IC_{50} values (i.e., concentration of a peptide required to produce a half-maximal inhibitory effect).

To quantify T cell activation, we measured levels of IFN-γ secreted by $CD8^+$ T cells upon pMHC I-TCR engagement (Figure 2). $CD8^+$ T cells secrete various cytokines upon

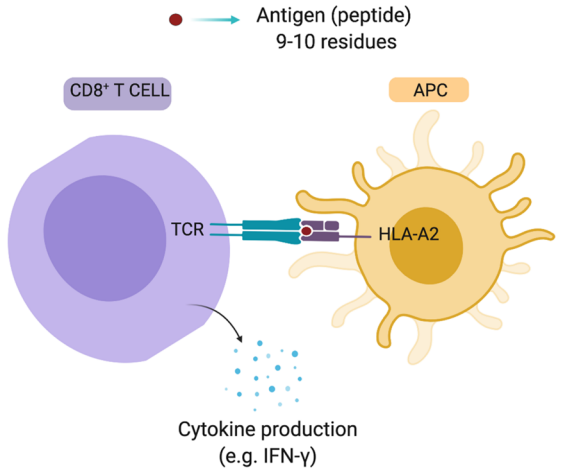


Figure 2. Cartoon illustration of HLA-A2 molecule, found on an antigen-presenting cell (APC), presenting a short peptide to the T cell receptor (TCR) expressed on a $CD8^+$ T cell. The formation of this ternary complex within the immune synapse leads to the secretion of IFN-γ and other cytokines by $CD8^+$ T cells.

activation, but the levels of IFN-γ are often higher than those of other cytokines.[52,53] Thus, IFN-γ levels offer a wider dynamic range and more accurate quantification relative to levels of other cytokines. IFN-γ levels were measured using ELISA. To compare activities among peptides, we used both EC_{50} (i.e., effective concentration of a peptide that produces a half-maximal T cell response) and maximal response (i.e., the maximum T cell response a peptide can produce). Recorded EC_{50} and maximal response values for α/β-peptides were normalized against the values for the natural antigen.

The patient-derived antigen-specific $CD8^+$ T cells used here are presumed to be polyclonal, which means that more than one TCR is represented among these cells. Different TCRs can recognize a pMHC complex in different ways. Relative to monoclonal T cells, polyclonal T cells are better mimics of a T cell response *in vivo* because each person is likely to have more than one type of T cell bearing a TCR that can recognize a single pMHC I.[54] This consideration motivated our decision to use polyclonal T cells in this study.

WT-1 and NY-ESO-1 Antigens: α-to-β Replacement Can Lead to Enhanced Maximal T Cell Responses. We first examined the activity of α/β-peptides based on the HLA-A2-restricted peptide RMFPNAPYL (RMF hereafter) derived from the WT-1 antigen. The RMF peptide is a promising lead for development of vaccines against hematopoietic and solid tumors.[55] A crystal structure of the RMF+HLA-A2 complex reveals an extended peptide conformation with a "bulging" region comprising positions 4 (p4) and p5 (Figure S1A).[43] The peptides in pMHC I complexes generally do not exhibit an entirely extended conformation. Instead, the middle region is forced to bulge away from the MHC because the N- and C-terminal segments cannot extend out of the MHC I binding groove.[56] This bulging region is almost always the main peptide segment recognized by the TCR. In the absence of a structure for a ternary RMF+HLA-A2+TCR complex, we assume that residues at p4 and p5 make primary contacts with TCRs in RMF-specific polyclonal $CD8^+$ T cells.

In comparison to the natural epitope, peptide **1A**, RMF α/β-peptides **1C**, **1D** and **1F** elicited 40−70% greater maximal T cell response at the highest measured concentration (10 μM) (Figure 3). **1C** and **1D** were 100-fold less potent (i.e.,

https://doi.org/10.1021/jacs.1c00016
J. Am. Chem. Soc. 2021, 143, 6470−6481

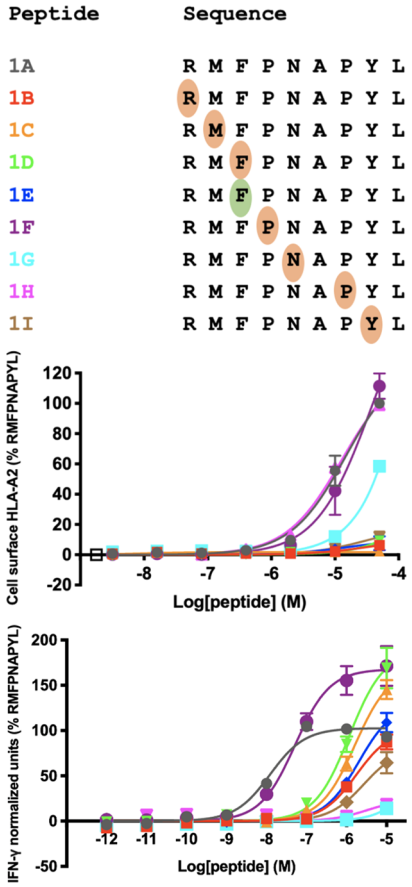


Figure 3. (Upper graph) Sequences of WT-1 α/β-peptides using the color-coding scheme for β residues from Figure 1. (Middle graph) Binding data points are the mean of ≥2 independent experiments. Error bars represent SEM. Normalized 100% represents the mean of the natural antigen **1A** at the highest concentration (50 μM). (Lower graph) T cell activation data points are the mean of ≥3 independent experiments. Error bars represent SEM. Data were normalized against the natural antigen **1A** and fit to three-parameter sigmoidal curves.

rightward shift in dose−response curves) than **1A**. However, α/β-peptide **1F**, which contains β^3-hPro at p4, displayed potency comparable to that of **1A**. It is intriguing that the T cells tolerated α-to-β substitution at a hypothetical TCR contact residue (Figure S1A). These observations show that a single α-to-β substitution in a known MHC I antigen can enhance T cell response without compromising potency.

To understand the basis for increased maximal T cell responses displayed by some RMF α/β-peptides, we asked whether these α/β-peptides exhibit a higher affinity for HLA-A2 relative to RMF itself. Using T2 cells, we observed no difference in the ability of native antigen **1A** and α/β-peptide **1F** to stabilize HLA-A2 on the cell surface (Figure 3). Thus, peptides **1A** and **1F** appear to exhibit similar affinity. In contrast, the other two analogues (**1C** and **1D**) displayed little to no binding in this assay. The T2 cell assay is relatively insensitive, losing efficacy at low peptide concentrations (<0.1−1 μM). We therefore turned to a complementary *in vitro* HLA-A2 binding assay, which indicated that **1C** and **1D** bound HLA-A2 with ∼130-fold lower affinity than **1A** (Table S1). The α/β-analogue **1F** matched the affinity of the natural antigen, which was consistent with results of the T2 cell assay.

The behavior of **1C** and **1D** indicates that α/β-peptide antigens can strongly activate T cells despite relatively weak HLA-A2 binding. Collectively, data obtained with the RMF α/β-peptides suggest that enhancement of maximal T cell response resulting from backbone modification at some sites arises from a change in the way the TCR engages with the pMHC I complex, relative to the native antigen pMHC I complex, rather than from altered peptide-MHC I affinity.

Paralleling behavior among RMF α/β-peptides, some α/β-analogues derived from the HLA-A2 epitope specific to NY-ESO-1 antigen were found to induce greater maximal T cell responses relative to the natural antigen (Figure 4). These

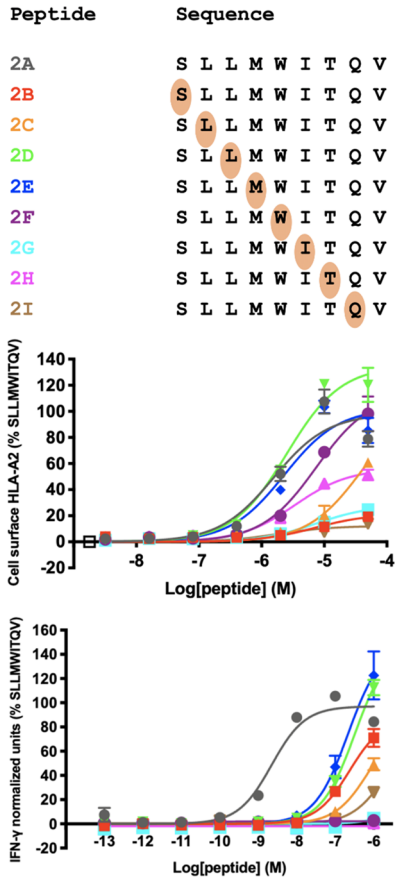


Figure 4. (Upper graph) Sequences of SLL α/β-peptides using the color-coding scheme for β residues from Figure 1. (Middle graph) Binding data points are the mean of ≥2 independent experiments. Error bars represent SEM. Data were normalized against the natural antigen **2A** and fit to three-parameter sigmoidal curves. (Lower graph) T cell activation data points are the mean of ≥3 independent experiments. Error bars represent SEM. Data were normalized against the natural antigen **2A** and fit to three-parameter sigmoidal curves.

observations are significant because they show that this intriguing outcome is not limited to a single antigen. However, as shown by results obtained with other antigens, described below, the discovery of analogues that cause enhanced maximal T cell response is not a universal result from β-scan analysis of an MHC I antigen.

NY-ESO-1 is not expressed in normal tissues but instead aberrantly overexpressed in many tumors, thus making this antigen an attractive target for therapeutic efforts.[57] The HLA-A2-restricted epitope SLLMWITQC has been previously targeted with TCRs in gene therapy trials, and this antigen triggered favorable responses in patients with melanoma and sarcoma.[58,59] For our assays, we synthesized α/β-analogues

https://doi.org/10.1021/jacs.1c00016
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based on a more chemically stable heteroclitic variant SLLMWITQV (SLL hereafter) containing a canonical valine residue at p9.[60]

Relative to their all-α counterpart **2A**, SLL α/β-peptides **2D** and **2E** caused increased maximal T cell response by 13 and 23% at the highest tested peptide concentration (1 μM) (Figure 4). We note that all evaluated SLL α/β-analogues were significantly less potent than **2A**, and none of them reached a maximal response over the concentration range evaluated. (Therefore, the top of the curve for **2D** and **2E**, which defines the maximal T cell response, might be even higher at higher concentrations.) In **2E**, $\alpha \rightarrow \beta$ replacement occurs at position p4, which is expected to engage the TCR (Figure S1B).[45] The result is noteworthy because despite a 65-fold reduction in potency, we find that a significantly larger maximal T cell response can be achieved by adding a single CH_2 unit to the backbone of a peptide antigen.

The affinities of the SLL α/β-peptide series for HLA-A2 were assessed based on their ability to stabilize HLA-A2 on the T2 cell surface (Figure 4). Three α/β-analogues (**2D**, **2E**, and **2F**) stabilized HLA-A2 as effectively as all-α antigen **2A**. These cell-based experimental results were consistent with results of *in vitro* HLA-A2 binding measurements (Table S2): **2D** and **2E** matched the IC_{50} of the all-α antigen, while **2F** displayed 10-fold lower affinity.

The results obtained with the "β-scan" series derived from the SLL sequence reveal an important correlation with those from the comparable set of RMF analogues, in that, both studies identified α/β antigens that can elicit a greater maximal T cell response relative to the all-α antigen. However, this comparison also reveals significant differences in outcome. For example, each series identified at least one α/β analogue that bound to HLA-A2 with affinity comparable to that of the all-α antigen (**1F** for RMF; **2D**, **2E**, and **2F** for SLL). However, α/β-peptide **1F** matched RMF itself in potency for T cell activation (EC_{50} in Figure 3), while **2D** and **2E** were considerably less potent than SLL itself (Figure 4). Moreover, the SLL series contained an α/β-peptide, **2F**, that binds to HLA-A2 with reasonably high affinity but does not elicit any T cell response; this behavior was not observed among RMF analogues. These distinct patterns of behavior presumably arise from differences in the way that cognate TCRs engage the peptide-MHC I complexes formed by RMF and α/β analogues relative to complexes formed by SLL and α/β analogues.

Why do α/β-peptides **2D**, **2E**, and **2F** bind HLA-A2 with affinity comparable to or approaching that of the all-α antigen (**2A**) but display low potency in terms of T cell activation, or cause no T cell activation at all? In considering this question, we note that the sites of $\alpha \rightarrow \beta$ replacement in **2D**, **2E**, and **2F** are p3, p4, and p5, respectively, which are expected to be involved in recognition of pMHC I by TCRs.[45] Thus, the peptide portions that are engaged directly by HLA-A2 are identical among these three α/β-peptides and the α-peptide, which could explain similar affinities for the MHC I. The extra backbone methylene group in each α/β antigen may weaken TCR engagement of pMHC I (**2D** and **2E**) or abolish TCR binding (**2F**). Alternatively, it is possible that TCRs engage the HLA-A2 complex of **2F** in a manner that does not cause T cell activation. Binding of different TCRs to pMHC I complexes can induce different conformations in the central portion of the SLL antigen peptide[60−62] or extended variants.[63] Perhaps the local conformation of the p3-p5 segment in **2D**-**2F** bound to HLA-A2 is not conducive to a productive interaction with TCRs, but TCR binding can induce a signaling-competent p3-p5 conformation for **2D** and **2E**.

Influenza Antigen: α-to-β Replacement Can Lead to Robust CD8$^+$ T Cell Activation Despite Weak Binding to HLA-A2. We analyzed a series of GILGFVFTL (GIL hereafter) α/β-peptides derived from the influenza A virus M1 protein. GIL is a highly immunogenic and conserved epitope,[64,65] and several recent reports evaluated this epitope for a potential peptide vaccine.[14,66,67] In contrast to most pHLA-A2 structures, GIL is considered to be a "featureless" peptide in its complex with HLA-A2 because there are no solvent-exposed side chains in the central region of GIL (p4-p6).[46,68−70] For example, the side chain of Phe at p5 points into the HLA-A2 binding groove as opposed to projecting into solvent (Figure S1C).

Binding measurements using T2 cells demonstrated that all GIL α/β-peptides exhibited inferior binding to HLA-A2 relative to GIL itself, as indicated by the ability to stabilize cell-surface HLA-A2 (Figure 5). α/β-Peptide **3H** caused the highest HLA-A2 stabilization (88% maximal response), while most other α/β analogues displayed 20−50% maximal

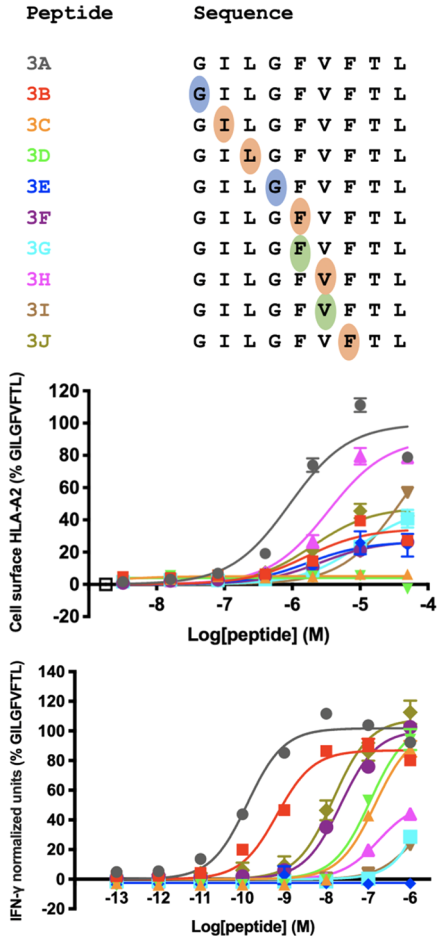

Figure 5. (Upper graph) Sequences of GIL α/β-peptides using the color-coding scheme for β residues and homoglycine from Figure 1. (Middle graph) Binding data points are the mean of ≥2 independent experiments. Error bars represent SEM. Data were normalized against the natural antigen **3A** and fit to three-parameter sigmoidal curves. (Lower graph) T cell activation data points are the mean of ≥3 independent experiments. Error bars represent SEM. Data were normalized against the **3A** and fit to three-parameter sigmoidal curves.

https://doi.org/10.1021/jacs.1c00016
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response at the highest peptide concentration. Complementary *in vitro* HLA-A2 binding measurements were consistent with the cell-based data, as all GIL α/β-analogues other than **3H** displayed a 10^2- to 10^4-fold higher IC_{50} relative to GIL (Table S3). We compared binding effects induced by a single $\alpha \rightarrow \beta^3$ vs $\alpha \rightarrow \beta^2$ replacement at two positions. β^3 and β^2 residues differ only in the location of the side chain within the residue (Figure 1). Peptide **3F**, containing a β^3-residue at p5, displayed higher HLA-A2 affinity than did peptide **3G**, containing a β^2 replacement (IC_{50} = 44 nM vs 180 nM; Table S3). A similar trend was observed at p6, where **3H** displayed a much higher HLA-A2 affinity than did **3I** (IC_{50} = 5.9 nM vs 350 nM).

Although all but one of the GIL α/β-peptides exhibited substantially weaker HLA-A2 binding relative to the natural antigen, we were surprised to observe that many of these α/β-peptides could induce substantial T cell responses. Five of the α/β-peptides (**3B**, **3C**, **3D**, **3F**, and **3J**) achieved maximal response levels comparable to that of the natural antigen (Figure 5), albeit at concentrations higher than required for the maximal response to GIL itself. The most active GIL α/β-analogue, **3B**, approached the potency and maximal T cell response exhibited by the natural antigen. The modest decrease in relative potency and T cell response observed with **3B** relative to **3A** might be linked in part to the weak HLA-A2 binding of **3B** (Figure 5; 48-fold higher IC_{50} for **3B** relative to **3A** in the *in vitro* binding assay; Table S3). Nevertheless, it seems remarkable that the maximum degree of binding in the cell-based assay (Figure 5) plateaus for **3B** well below the maximum level seen with native antigen **3A**, but **3B** approaches the maximum level of T cell activation seen with the natural antigen.

Although the pattern of responses to single-site α-to-β replacements observed among the GIL analogues differs from the patterns observed among the RMF and SLL analogues, the GIL-derived α/β series results strengthen the case that evaluating the β-scan series based on MHC I antigens is a productive discovery path. In particular, the intriguing behavior of analogue **3B** suggests that despite impaired MHC I affinity, the α-to-β replacement in this case may lead to highly effective signal transduction via cognate TCRs.

HPV E7 and Glycoprotein D1 Antigens: α-to-β Replacement Can Lead to Peptides that Bind Strongly to HLA-A2 but Activate T Cells Only Weakly. The HLA-A2-restricted epitope YMLDLQPETT (YML hereafter) derived from the HPV-16 E7 oncoprotein is of interest because vaccines based on E7 fragments could activate HPV-specific T lymphocytes against HPV-positive cancers.[71] There is no high-resolution structure of the HLA-A2-bound YML antigen.

T2 peptide binding experiments demonstrated that six out of nine YML α/β-analogues displayed analogous or enhanced binding to HLA-A2 when compared to natural antigen **4A** (Figure 6). Cell-based T2 binding measurements do not allow us to quantitate small differences in binding; thus, we turned to competition binding experiments involving purified HLA-A2 molecules. These *in vitro* measurements were consistent with results from the T2 cell assay (Table S4). Two α/β analogues, **4F** and **4G**, bound HLA-A2 with higher affinity (i.e., lower IC_{50}; by 3-fold and 2-fold, respectively) than did the natural antigen **4A**. The α/β-analogues **4E**, **4H**, **4I**, and **4J** bound HLA-A2 with similar or slightly lower affinity relative to the YML antigen itself. These observations contrast the binding trend among GIL-derived α/β-peptides (Figures 5), which all

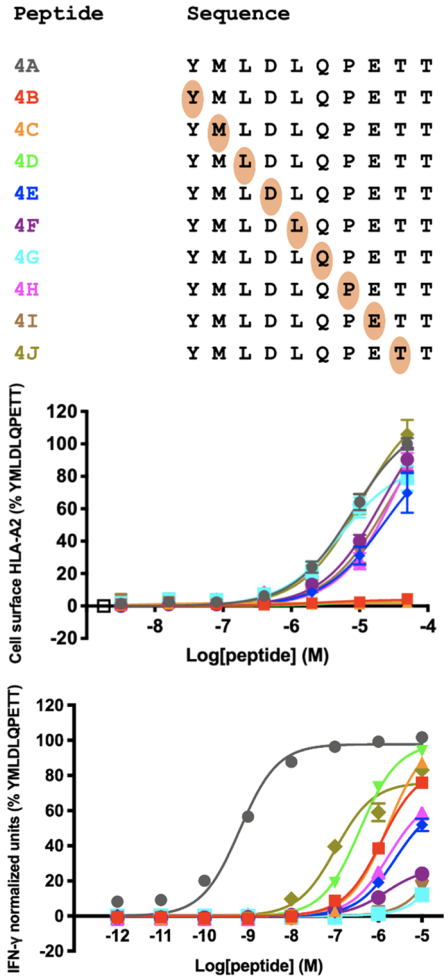

Figure 6. (Upper graph) Sequences of YML α/β-peptides using the color-coding scheme for β residues from Figure 1. (Middle graph) Binding data points are the mean of ≥2 independent experiments. Error bars represent SEM. Normalized 100% represents the mean of the natural antigen **4A** at the highest concentration (50 μM). (Lower graph) T cell activation data points are the mean of ≥3 independent experiments. Error bars represent SEM. Data were normalized against **4A** and fit to three-parameter sigmoidal curves.

displayed relatively weak HLA-A2 affinity. This difference in trends highlights the fact that a single MHC I can display different levels of tolerance of α-to-β substitutions depending on antigen sequence.

Given the relatively high MHC I affinities observed among α/β analogues of YML, we were surprised to find that most of these analogues were 10^3-fold less potent in T cell activation compared to the natural epitope (Figure 6). The most active α/β-peptide, **4J**, was 150-fold less potent than YML itself. Only one α/β-peptide, **4D**, matched the maximum level of T cell activation observed with YML.

We observed a similar outcome, strong HLA-A2 binding and weak T cell activation, for a set of α/β-peptides derived from the HSV-1 glycoprotein D1 antigen SLPITVYYA (SLP hereafter). In the SI, we provide the data for these SLL-derived α/β-peptides (Figure S4) and an interpretation of the data.

CMV pp65 Antigen: α-to-β Replacement Can Diminish Both HLA-A2 Binding and T Cell Activation. We analyzed α/β-peptides based on NLVPMVATV (NLV hereafter), an epitope derived from the pp65 protein of CMV. The

https://doi.org/10.1021/jacs.1c00016
J. Am. Chem. Soc. 2021, 143, 6470−6481

predominant $CD8^+$ T cell response to CMV infection in individuals carrying the widespread HLA-A2 allele is directed against the NLV epitope.[72] A crystal structure of the ternary pMHC I-TCR complex has been determined using the dominant public RA14 TCR from an immunocompromised patient.[44] The NLV peptide displays a canonical binding mode to HLA-A2. Side chains of Leu at p2, Val at p6 and Leu at p9 facilitate binding to HLA-A2. Solvent-exposed side chains are found at p4, p5 and p8. Out of these three sites, p5 and p8 are critical for RA14 TCR recognition (Figure S1D).

All NLV α/β-analogues displayed weak HLA-A2 binding and diminished ability to activate T cells relative to NLV itself. In the SI, we provide the data for these NLV-derived α/β-peptides (Figure S5) and an interpretation of the data.

DISCUSSION

The survey of HLA-A2-restricted antigens reported here was motivated by previous unexpected observations that α-to-β replacement could alter the informational properties of polypeptides. For example, the BIM BH3 domain binds promiscuously to proteins in the Bcl-2 family, such as Bcl-x_L and Mcl-1, but α/β-peptide analogues of a BIM BH3 18-mer that display high selectivity for either Bcl-x_L or Mcl-1 have been discovered.[27,28] Another example involves the N-terminal fragment of parathyroid hormone PTH(1−34), which is the active ingredient in the osteoporosis drug teriparatide. This peptide activates two natural GPCRs, the parathyroid hormone receptor 1 (PTHR1) and PTHR2. α-to-β replacement at positions 1 and 7 of PTH(1−34) generated a highly PTHR1-selective agonist, while α-to-β replacement at positions 2 and 6 generated a highly PTHR2-selective agonist.[29]

PTHR1 activation by agonist binding at the extracellular side stimulates diverse intracellular outcomes mediated by engagement of distinct cytosolic proteins, including heterotrimeric G proteins and β-arrestins.[38] Derivatives of PTH(1−34) containing a single α-to-β replacement function as "biased agonists" of the PTHR1, activating G-protein-mediated pathways in preference to recruiting β-arrestins.[30] The ability of a single α-to-β replacement to alter the signal transduced by an agonist-receptor complex led us to wonder whether a single α-to-β replacement within an antigen peptide could alter the signal transduced by a pMHC I complex via interaction with a T cell receptor; the results provided here begin to address this question.

Collectively, our findings show that implementation of a systematic α-to-β replacement strategy ("β scan") to modify six well-studied MHC I peptide epitopes can lead to discovery of novel antigens with diverse and, in some cases, potentially useful activity profiles. Because all six antigens we studied are displayed by HLA-A2, we have been able to show that the effect of α-to-β replacement on MHC I binding varies as a function of antigen sequence when the MHC I is held constant. In addition, we can conclude that the response of T cell receptors to α-to-β replacement in the context of pMHC I complexes varies as a function of epitope sequence when the MHC I is held constant.

We hypothesized that single α-to-β replacements near the peptide termini would be detrimental for MHC I binding, given that each peptide terminus contains an "anchor" residue at position 2 (p2) and at p9 or p10. To test this hypothesis, we evaluated α-to-β replacements at p1-p3 of each antigen; in all cases but one, peptide **2D**, which is substituted at p3 (Figure 4; Table S2), we observed a dramatic reduction in MHC I affinity compared to the natural epitope. Since TCRs typically do not make contacts near terminal antigen residues, it was unclear to us whether α-to-β replacements at p1-p3 would affect T cell activation. Our findings reveal that some antigen analogues with diminished MHC I binding because of α-to-β replacement near the N-terminus can nevertheless induce robust T cell activation (several peptides in Figure 5) or even enhanced maximal T cell response (peptides **1C** and **1D**; Figure 3).

We predicted that single α-to-β replacements in the central region of an antigen (p4-p6) would be tolerated in terms of MHC I binding. These central segments often protrude toward the TCR. With the exception of peptides **3E−3I** (Figure 5), α-to-β replacements at p4-p6 were tolerated well in terms of MHC I binding. Despite the strong MHC I binding, antigen analogues with α-to-β replacement at p4-p6 often prompted very weak responses by T cells.

In seven cases, distributed across several antigens, we were able to compare the impact of α-to-β^3 vs α-to-β^2 replacement on MHC I binding and T cell activation. α-to-β^3 replacements were consistently better accommodated than α-to-β^2 replacements in terms of MHC I affinity and in terms of productive pMHC I recognition by T cells, among the comparisons we made. However, we note that Cheloha et al. compared α-to-β^3 vs α-to-β^2 replacements at eight positions within an MHC II antigen and observed a more varied response to this subtle alteration, with some instances of β^2 residue superiority.[41] Among PTH(1−34) analogues, α-to-β^2 replacement was superior to α-to-β^3 replacement at several positions in terms of PTHR1 activation.[29]

The outcomes that resulted from α-to-β replacement in HLA-A2 peptides can be grouped into four categories, which we designate A−D. Outcome A, found for RMF and SLL, was the discovery of α/β-peptides that caused an enhanced maximal T cell response, as indicated by release of IFN-γ, relative to the all-α epitope. This behavior was observed for α/β-peptides with high affinity for HLA-A2, and for α/β-peptides that bound relatively weakly to HLA-A2, which indicates that the enhanced maximal T cell response does not require that an α/β antigen display high affinity for the MHC I. Outcome B, found for GIL, was the discovery of α/β analogues that bound HLA-A2 molecule weakly but nevertheless led to potent T cell activation. Outcome C, found for YML and SLP, was the discovery of α/β-peptides that exhibited strong HLA-A2 binding but were ineffective at T cell activation. Outcome D, found for NLV, was that all α/β-peptides bound only weakly to HLA-A2 and activated T cells only weakly. It should be noted, however, that among the antigens that led to outcomes A−C, most α-to-β replacements caused weak MHC I binding and weak T cell activation.

To the extent that outcomes A−C can be considered interesting, our survey suggests that conducting an α-to-β^3 scan of MHC I antigens may prove generally to be productive in the context of elucidating and manipulating T cell activation involving those antigens. The small set of peptides necessary for such a scan can be readily prepared from commercially available precursors via conventional solid-phase synthesis.

Activation of a $CD8^+$ T cell depends on recognition of the pMHC I by the T cell receptor along with subsequent events at the T cell surface and within the T cell, including formation of the immunological synapse between the antigen-presenting cell and the T cell, phosphorylation of intracellular immunoreceptor-tyrosine-based activation motifs in the

https://doi.org/10.1021/jacs.1c00016
J. Am. Chem. Soc. 2021, 143, 6470−6481

CD3ζ subunits of the TCR,[73] and recruitment of various kinases, phosphatases and signaling molecules to the intracellular portions of the TCR. Our focus on interferon-γ release as the indicator of T cell activation does not allow us to distinguish among possible effects of α-to-β substitution in the MHC I-displayed peptide on different aspects of T cell activation. However, this work has identified a small set of α/β-peptide antigens that would be most interesting to evaluate in terms of their impact on T cell activation. For example, RMF analogue **1F** and SLL analogues **2D** and **2E**, all examples of outcome A above, would be good candidates for efforts to understand how the maximal T cell response could be increased relative to the all-α antigen. Whether the strength of TCR binding to pMHC I or the kinetics of TCR-pMHC I association (or both factors) control T cell activation remains a topic of debate,[2,74−76] and it would be interesting to know how α-to-β modifications in selected RMF and SLL peptides affect the thermodynamic and kinetic profiles of TCR-pMHC I interactions.

More than half of the GIL α/β-peptides initiated robust T cell activation despite binding only weakly to HLA-A2 (outcome B; Figure 5; Table S3). The most striking example of this outcome is GIL α/β-analogue **3B**. In comparison to the natural epitope, **3B** bound HLA-A2 48-fold less strongly, and yet this α/β-peptide was indistinguishable from the natural antigen in terms of T cell response potency and maximal response. Previous studies found that a single pMHC complex on the surface of an antigen-presenting cell is sufficient to elicit a detectable T cell response, and 3 to 25 pMHC complexes are necessary to achieve a maximal response.[77−79] It is possible that TCRs responsive to the GIL antigen require only a small number of pMHC I complexes for strong T cell activation. In this case, even a weak MHC I ligand might be able to promote full T cell response. This hypothesis could be explored with an α/β-peptide such as **3B**.

Biochemical and cell-based peptide binding experiments revealed that most of the YML and SLP α/β-peptides were bound to HLA-A2 with affinities comparable to that of the natural antigen (Figures 6 and S4 and Tables S4,S5); none of the other systems studied here exhibited this behavior. Despite their high affinity for HLA-A2, none of the YML or SLP α/β-peptides activated T cells effectively. It is possible that the conformation in which these α/β-peptides bind does not generate a pMHC I complex that is recognized by TCRs, or that TCR association with such complexes does not lead to T cell activation. It would be interesting to determine whether these peptides could antagonize T cell activation mediated by the all-α antigen.

Related observations were made with SLL α/β-peptides **2D** and **2E**, which both bound HLA-A2 more tightly than the all-α antigen. However, both **2D** and **2E** displayed reduced potency relative to the all-α antigen for T cell activation. In addition, these α/β antigens exhibited maximal responses that were greater than that of the all-α peptide (Figure 4). Collectively, these data demonstrate that strong MHC I binding by an α/β-peptide does not guarantee a potent T cell response.

Peptides with low MHC affinity are often found to elicit a weak T cell response, as observed for many α/β antigens in our study, including all derived from NLV (Figure S5).[14] Under our experimental conditions, weaker HLA-A2 affinity by a peptide results in fewer pHLA-A2 complexes on the surface of T2 cells that can be accessed by T cells, which leads to a less effective T cell response. It is difficult to determine experimentally the level of pMHC affinity necessary to elicit an effective T cell response. This hypothetical "effective affinity" may vary among different epitopes, which could explain why some α/β antigens we discovered elicited robust T cell response despite weak MHC binding.

Our findings may suggest useful strategies for development of HLA-A2-targeting peptide vaccines. GIL α/β-peptide **3B**, for example, is a potent antigen, and the β-hGly substitution at p1 might protect the antigen against degradation by aminopeptidases. A different type of benefit might arise from an example such as RMF α/β-peptide **1F**, which is comparable to the natural antigen in MHC I affinity but elicits a larger maximal T cell response (Figure 3). Our immune system often tolerates tumor-associated antigens, such as the RMF epitope, since they are self-antigens. Because the β^3-hPro substitution in **1F** occurs at p4, which is presumed to be a TCR-contacting residue, this α/β-peptide might be recognized as nonself by RMF-responsive TCRs.[43] In this case, α/β-peptide **1F** might prime RMF-responsive TCRs and help break their self-tolerance to the natural all-α RMF antigen.

Although three previous studies examined single α-to-β replacements in MHC I antigens, in each prior case, only one antigen was evaluated, and a different MHC I was used in each case.[16−18] Thus, in comparison to these three precedents, our study substantially expands understanding of the characteristics that can result from single-site α-to-β replacement in MHC I antigens. Our demonstration that α-to-β replacements can induce differential effects on MHC I binding and T cell activation for different antigens would not have been possible if we had not conducted parallel "β scan" studies based on multiple antigens. We identified several α/β-peptide antigens that displayed enhanced maximal T cell response relative to their all-α counterpart. This outcome was not described in previous reports that focused on a single antigen,[16−18] and this behavior was not universal among the six antigens we evaluated. Thus, this finding depended on the breadth of our survey. It is noteworthy that enhanced maximal T cell response was observed for peptides containing single α-to-β replacements within the antigen region known to be critical for either MHC I binding or TCR engagement (p2-p4) (Figure 3,4). This trend can focus future efforts that aim to identify altered peptide antigens that evoke strong T cell response.

There is a clear need to enhance proteolytic stability and biological efficacy of MHC peptide-based T cell vaccines. Most previous attempts to address these needs sought to modify sequences at MHC or TCR contact sites by modifying side chains, i.e., by replacing native residues with other L-α-amino acid residues.[8,13] One undesired potential outcome from this approach is that the resulting peptides might prime T cells that recognize the altered peptide ligand (APL) but are ineffective at targeting the natural antigen found on abnormal or infected cells.[80−82] In contrast, peptides containing replacement subunits that retain the natural side chain (e.g., D-α-amino acid residues, peptoid residues, or β-amino acid residues) may avoid this outcome because they retain the complement of side chains displayed by the natural antigen. Furthermore, peptides containing these unnatural subunits can offer advantages in terms of immunogenicity, proteolytic stability, and bioavailability.[14−19,83,84]

Overall, this evaluation of singly substituted α/β-peptides derived from six well-studied HLA-A2-restricted peptides shows that single-site α-to-β modifications can cause a variety of effects on peptide binding to the MHC I and on T cell

https://doi.org/10.1021/jacs.1c00016
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activation by the pMHC I complex. Some of the α/β-peptide antigens described here might represent starting points for design of proteolytically stable HLA-A2 peptide vaccines capable of expanding T cells that display public and disease-specific TCRs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c00016.

Peptide synthesis procedures, MALDI-TOF and UPLC data for all peptides, additional figures, as well as protocols for cell culture maintenance, peptide-HLA-A2 binding, and T cell activation assays (PDF)

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Notes

The authors declare the following competing financial interest(s): S.H.G. is a co-founder of Longevity Biotech, Inc., which is pursuing biomedical applications of alpha/beta-peptides.

■ ACKNOWLEDGMENTS

This research was supported in part by the National Institutes of Health (R01-GM056414).

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