Non-Enzymatic Structural Modifications Reshape Peptide Presentation and Antigen Recognition

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ABSTRACT

For cytotoxic T-cells to identify and eliminate infected and transformed cells, the adaptive immune system requires the presentation of antigenic peptides by major histocompatibility complex class I (MHC-I) molecules. These peptides, typically derived from intracellular proteins, undergo a rigorous selection process to ensure self-tolerance. However, post-translational modifications (PTMs) and non-enzymatic chemical modifications can alter peptide structure and chemistry, potentially affecting immune recognition. In this study, we investigated the impact of non-enzymatic PTMs on antigen presentation and T cell recognition. Using the well-characterized model epitope SIINFEKL, we synthesized peptide variants incorporating common non-enzymatic PTMs and assessed their effects on MHC-I binding interactions and T cell recognition. We demonstrated that non-enzymatic PTMs markedly alter MHC-I affinity of peptides in a cancer-associated immunopeptidome. Additionally, we developed a novel enrichment strategy using an alkyne-modified probe to identify sites of non-enzymatic acylation prone to MHC-I display. These findings underscore the significance of non-enzymatic PTMs in altering the immunopeptidome and modulating immune responses.

INTRODUCTION

The adaptive immune system provides a specialized and formidable defense system against infected and transformed cells through the ability of immune cells to recognize and respond to specific antigens, which are often presented as peptides on the surface of host cells. Major histocompatibility complex (MHC) molecules, and the peptides they present, are pivotal to orchestrating this detection system.[1] MHC molecules are divided into two main classes: class I and class II.[2] While both classes are critical for immune function, MHC class I (MHC-I) molecules are the primary vehicle in presenting peptides to cytotoxic T cells.[3]

MHC-I molecules are expressed on the surface of nearly all nucleated cells and present a diverse array of peptides derived from protease digestion of intracellular proteins.[4] Once displayed on the surface of the host cells, this peptide-MHC complex (pMHC) engages with cytotoxic T cells, a subset of T lymphocytes specialized in identifying and eliminating peptides that are not recognized as “self”.[5] This designation of self vs non-self is paramount to the proper function of the adaptive immune system. The circulating T cell repertoire is shaped by a negative selection process in the thymus, where T cell receptors (TCRs) that bind self-peptides are eliminated from future propagation.[6] As a result, the remaining T cells in circulation can potentially recognize peptides that were absent from the negative selection process.[7]

Traditionally, non-self peptides are derived from pathogens (e.g., bacteria, fungi, and viruses) or transformed cells from genetic lesions that yield new protein sequences. However, non-self-peptides can be more broadly defined as any peptide sequence that has an altered chemical composition relative to endogenous expression.[8] Even a single amino acid alteration can drastically affect the TCR recognition of a peptide, potentially altering the activity of the immune response.[9] Therefore, understanding the impact of amino acid modifications on MHC-I-presented peptides is necessary for elucidating how the immune system responds to infections, cancers, and in autoimmunity.

It was recently revealed that most PTMs can escape negative selection due to their low abundance throughout the lifetime of the thymus, suggesting that PTM modified peptides may play a role in immune-related diseases.[12] PTMs, such as phosphorylation, glycosylation, and ubiquitination, can alter the structure and function of proteins, including those that are later processed into peptides for presentation.[13] One of the ways these modifications can affect the immune response is by altering peptide binding affinity to MHC-I or its recognition by TCRs.[14] Recently, we systematically assessed the impact of enzymatic PTMs by synthesizing modified peptides and assessing their MHC-I binding and T cell recognition relative to unmodified counterparts and found that they can play a significant role in altering these interactions.[15]

A less appreciated form of PTMs arises from non-enzymatic chemical entities. While some of these modifications are native and regulated (e.g., certain forms of cysteine

oxidation which occur during oxidative bursts), many non-enzymatic alterations are poorly controlled and, therefore, can generate a pool of non-self peptides.[18] Notably, whereas enzymatic PTMs are often reversible through specific “eraser” enzymes, non-enzymatic PTMs have not been reported to be natively reversed.[19] In most cases, non-enzymatic PTMs result from electrophilic chemical species reacting with nucleophilic amino acids (e.g., cysteine, arginine, lysine, histidine, tyrosine, and, to a lesser extent, aspartic acid and glutamic acid). The sources of these electrophiles are diverse, with well-characterized examples naturally formed in healthy cells including reactive oxygen species (ROS), reactive nitrogen species (RNS), oxidative byproducts of glycolysis, and non-native short-chain fatty acids that are activated to acetyl-co-enzyme A adducts.[20]

The consequences of amino acid modifications on peptide presentation are multifaceted. First, they can affect the stability of the peptide-MHC (pMHC) complex. The binding affinity between a peptide and MHC-I is a critical determinant of whether a peptide will be effectively presented to T cells. Amino acid modifications can either enhance or disrupt this binding, thereby influencing the stability of the complex and the likelihood of T cell recognition.[22, 23] Second, these modifications can directly impact the interaction between the presented peptide and the TCRs of cytotoxic T cells. Because TCRs are finely tuned to recognize specific pMHC combinations, any alteration in the peptide sequence due to amino acid modifications can potentially disrupt recognition and compromise immune response against the infected or transformed cell.[24] Furthermore, the presence of modified peptides can lead to the activation of autoimmune responses, where the immune system mistakenly targets healthy, self-cells.[25] Collectively, these non-enzymatic

modifications generate a chemically distinct pool of peptides that can escape negative selection, potentially altering immune recognition. In particular, these modified peptides may inadvertently trigger autoimmune pathologies, causing the immune system to target healthy cells.[21]

To investigate the role non-enzymatic PTMs play in antigen presentation through MHC-I, we determined the effects of non-enzymatic PTMs on the pMHC-I complex stability and T cell recognition. We also explored the effects that non-enzymatic PTMs have on peptides presented in the context of cancer cells. Finally, we developed a novel enrichment strategy to identify MHC-I associated peptides bearing non-enzymatic modifications to improve detection of chemically modified peptides presented on MHC-I molecules.

RESULTS AND DISCUSSION

To investigate the effects of non-enzymatic PTMs on peptide affinity for MHC, we used the model epitope SIINFEKL (**ovaWT**) derived from the protein ovalbumin (**Figure 1A**). SIINFEKL is extensively utilized in antigen presentation studies due to its well-defined interactions with H-2K^b and SIINFEKL-specific TCRs, providing a robust system to evaluate how non-enzymatic PTMs modulate antigen presentation (**Figure 1B**).[26] We first focused on the lysine residue of **ovaWT**, as lysine is among the most frequently modified amino acids. A primary source of non-enzymatic PTMs is oxidative stress, which can

induce lysine modifications, including side-chain formylation and the conversion of the γ-amine to aminoadipic acid or homocitrulline.[27] Another prominent class of lysine PTMs arises from reactions with reactive sugar metabolites, forming stable covalent adducts.[28] In particular, methylglyoxal, a byproduct of glycolysis, can promote the accumulation of *S*-D-lactoylglutathione and result in the non-enzymatically driven D-lactylation.[29] To examine these modifications, we synthesized **ovaWT** variants incorporating a formylated lysine side chain (**ovaK7$_{formyl}$**), aminoadipic acid (**ovaK7$_{aad}$**), homocitrulline (**ovaK7$_{hcit}$**), and a lactic acid adduct (**ovaK7$_{lac}$**).

Next, we considered asparagine residues, which have been observed to be presented on MHC-I following deamidation.[30] Deamidation often occurs through the formation of a succinimide intermediate, resulting in the conversion of asparagine to aspartate or the formation of an isoaspartate linkage in the peptide backbone.[31] Notably, deamidation can also occur enzymatically, as *N*-glycosidase enzymes remove glycosylated asparagine residues, thereby contributing to the presentation of deamidated peptides on MHC molecules.[32] To assess the impact of deamidation on **ovaWT**, we synthesized peptide variants incorporating an aspartate substituent (**ovaN4D**) or an isoaspartate bond (**ovaN4D$_{iso}$**) at the asparagine position (**Figure 1A**).

TCRs have been shown to have promiscuous binding to peptides displayed on MHC-I and can activate against peptides with similar sequences in a phenomenon known as cross-reactivity. Therefore, we examined the potential for non-enzymatic PTMs to

structurally and functionally mimic canonical amino acid residues, potentially eliciting cross-reactive immune responses. An illustrative example is the irreversible oxidation of cysteine to sulfonic acid which can structurally mimic acidic residues like glutamic acid.[33] To probe this, we synthesized a sulfonic acid-modified peptide variant (**ovaE6C$_{SO3}$**) to model where such a modification could mimic the glutamic acid residue within SIINFEKL (**Figure 1A**).

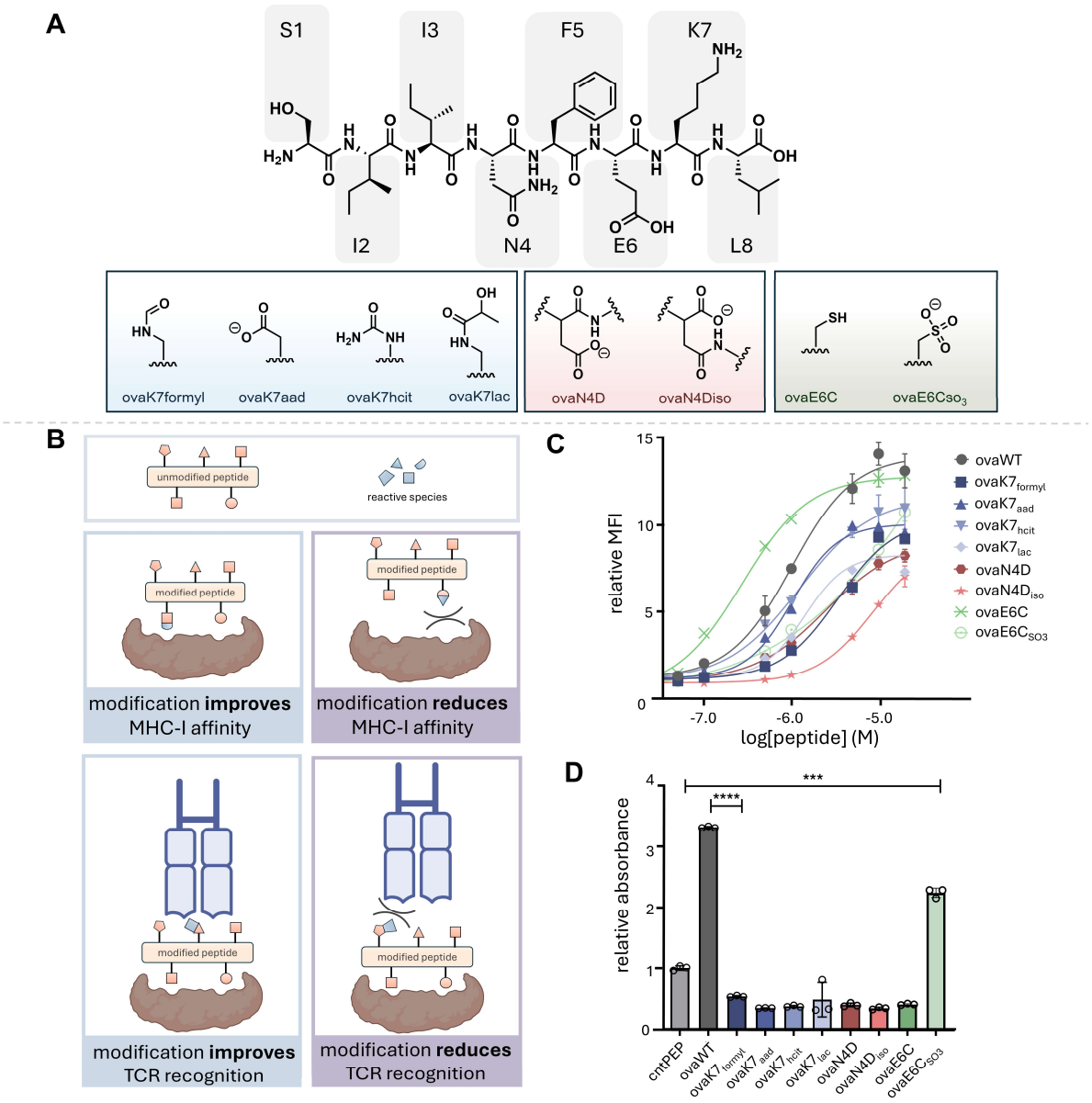

Figure 1. (A) Chemical structure of **ovaWT** and the respective modifications. **(B)** Schematical representation of how reactive chemical species might alter antigen presentation through MHC-I. **(C)** Dose-response curve from the RMA-S stabilization assay. RMA-S cells were incubated with the indicated concentration of peptide and analyzed via flow cytometry for H-2K^b expression. **(D)** B3Z T cell activation assay. RMA-S cells were incubated with 5 μM of indicated peptide for 1 h at 26 ºC. RMA-S cells

were subsequently co-incubated with B3Z T cells for 6 h. β-galactosidase expression was then measured via the conversion of the colorimetric reagent chlorophenol red-β-D-galactopyranoside (CPRG) on a plate reader at 570 nm. Adjusted p-values were determined by a one-way ANOVA with multiple comparisons (*** $p < 0.001$, **** $p < 0.0001$, ns = not significant).

Impact of Modifications on MHC-I Affinity and T Cell Activation

To investigate how non-enzymatic PTMs influence peptide affinity for MHC molecules, we employed RMA-S cells. These cells are deficient in the transporter associated with antigen processing (TAP), resulting in reduced surface expression of their MHC-I haplotype H-2K^b.[34] However, incubation with high-affinity peptides stabilizes H-2K^b molecules, restoring their surface expression. Previous studies have established a strong correlation between the abundance of surface MHC complexes and peptide affinity for H-2K^b.[35]

RMA-S cells were incubated with indicated peptide concentrations to assess relative changes in H-2K^b binding affinities. Lysine modifications at position 7 (**ovaK7$_{formyl}$**, **ovaK7$_{aad}$**, **ovaK7$_{hcit}$**, and **ovaK7$_{lac}$**) showed minimal impact on MHC-I affinity (**Figure 1C**). This outcome was anticipated, as position 7 in **ovaWT** is a solvent-exposed site with limited interactions with the H-2K^b molecule. Although such residues are less critical for MHC binding, they are more likely to influence TCR recognition.[36] In contrast, both

asparagine modifications (**ovaN4D** and **ovaN4D$_{iso}$**) demonstrated a marked decrease in pMHC surface expression on RMA-S cells. The changes in MHC-I affinity for **ovaN4D$_{iso}$** are consistent with previous reports that backbone modifications can alter pMHC stability.[37] We next assessed the impact of converting the glutamic acid residue in **ovaWT** to a sulfonic acid on H2-K^b binding affinity. Here, **ovaE6C$_{SO3}$** also showed a decrease in MHC-I surface display on RMA-S cells which may be due to the increased steric bulk of the sulfate group relative to the side chain of glutamic acid (**Figure 1C**). Overall, these results indicate that non-enzymatic PTMs can induce significant changes in the binding affinity of **ovaWT** to H-2K^b.

Next, we evaluated how non-enzymatic PTMs affect T cell recognition of their target ligand. RMA-S cells were incubated with 5 μM peptide and co-cultured with B3Z T cells, which express a TCR specific for **ovaWT** presented by H-2K^b and contain a β-galactosidase reporter gene under the control of an IL-2 inducible promoter. Upon recognition of its cognate ligand, B3Z activation induces β-galactosidase expression, which can be quantified on a plate reader via the enzymatic conversion of chlorophenol red β-D galactopyranoside (CPRG) to chlorophenol red.[38]

As previously mentioned, position 7 of **ovaWT** is a solvent-exposed residue that plays a key role in TCR interactions. Despite its minimal effects on H-2K^b affinity, modifications at position 7 (**ovaK7$_{formyl}$**, **ovaK7$_{aad}$**, **ovaK7$_{hcit}$**, and **ovaK7$_{lac}$**) eliminated T cell activation (**Figure 1D**). This likely reflects the loss of the lysine side chain's positive charge, as

previous studies have shown that charge alterations at TCR-contact residues can eliminate TCR recognition.[15, 39] Similarly, both asparagine modifications (**ovaN4D and ovaN4D$_{iso}$**) abolished T cell activation, further supporting the critical role of charge and side-chain chemistry in TCR engagement (**Figure 1D**).

Interestingly, the sulfonic acid-modified variant **ovaE6C$_{SO3}$** restored T cell activation relative to the inactive **ovaE6C** (**Figure 1D**). We attribute this response to the negatively charged sulfonic acid moiety mimicking the native glutamate residue. This finding suggests that certain non-enzymatic PTMs can functionally resemble canonical amino acids, potentially contributing to T cell cross-reactivity. Overall, these results demonstrate that non-enzymatic PTMs can influence both pMHC stability and T cell recognition, highlighting their potential to modulate antigen presentation and immune responses.

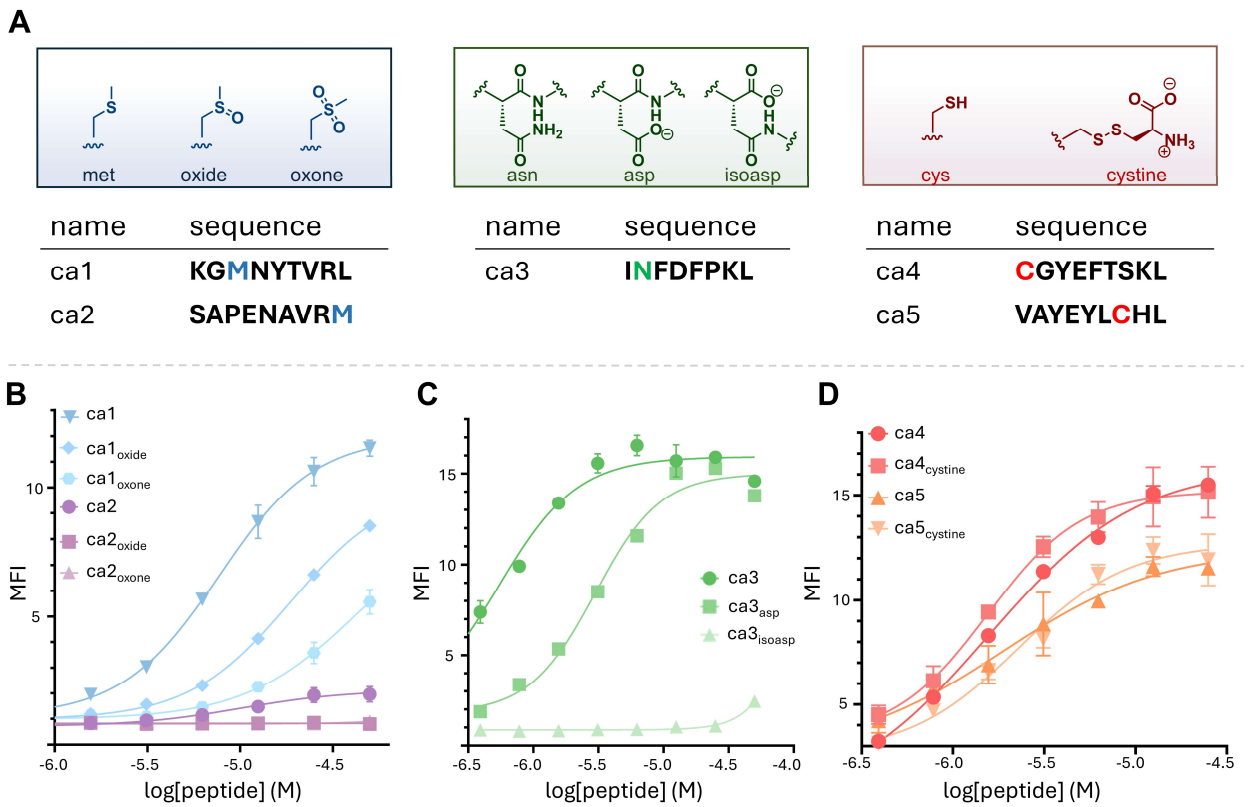

Figure 2. (A) Chemical structure of the unmodified and modified cancer-associated peptides. Dose-response analysis of the RMA-S stabilization assay for methionine oxidation **(B)**, asparagine deamidation **(C)**, or cysteinylation **(D)**. RMA-S cells were incubated with the indicated concentration of peptide and analyzed via flow cytometry for H-2K^b expression.

Non-Enzymatic PTMs on Cancer-Associated Peptides Influence MHC-I Binding Affinity

To examine the role of non-enzymatic PTMs in the presentation of cancer-associated peptides, we sought to synthesize modified peptides previously identified as being displayed on cancer cells. Kacen et al. developed a mass spectrometry-based platform that helped identify that non-enzymatic modifications constituted the majority of modified MHC-I-associated peptides and play a role in altering the cancer immunopeptidome.[22] The primary non-enzymatic modifications identified in their study included oxidation, deamidation, and cysteinylation. However, mass spectrometry alone cannot fully resolve the functional impact of these modifications on pMHC binding. To address this, we synthesized both unmodified and modified variants of peptides reported to be presented in cancer cells.[22]

We aimed to observe how modifications at various positions in the peptide may influence peptide binding to H-2K^b by using the RMA-S stabilization assay. Accordingly, we synthesized peptides bearing the aforementioned modifications at multiple positions along the peptide chain (**Figure 2A**). For cancer-associated peptides 1 (KGMNYTVRL, **ca1**) and cancer-associated peptide 2 (SAPENAVRM, **ca2**), methionine oxidation at positions 3 or 9, respectively, disrupted display on H-2K^b (**Figure 2B**). Increasing the degree of methionine oxidation from **ca1$_{oxide}$** to **ca1$_{oxone}$** also demonstrated a greater decrease in display on RMA-S cells. Additionally, deamidation at position 2 of **ca3**

(INFDFPKL) to make **ca3$_{isoasp}$** caused a >100-fold decrease in peptide display on RMA-S cells, further suggesting that backbone modifications may have a substantial negative impact on H-2K^b affinity (**Figure 2C**). In contrast, cysteinylation at positions 1 of **ca4** (CGYEFTSKL) to make **ca4$_{cystine}$** or position 7 of **ca5** (VAYEYLCHL) to make **ca5$_{cystine}$** resulted in minimal changes to pMHC-I affinity (**Figure 2D**). These results are also consistent with prior reports showing that cysteinylated peptides can be presented as immunodominant forms in viral infections and as HY antigens.[40,41] Overall, these results demonstrate that non-enzymatic PTMs can modulate MHC-I display in endogenously presented peptides, and it potentially represent an immune evasion mechanisms by reducing the display of potentially immunogenic epitopes on the surface of cancer cells.

Identification of Peptide Acylation Sites Displayed on MHC-I

We next aimed to develop a chemical enrichment strategy to identify non-enzymatically modified peptides presented on MHC-I molecules. Identifying modified peptides remains a considerable analytical challenge due to their typically low stoichiometric abundance within complex cellular environments, the structural diversity and unpredictability of PTMs, the complex fragmentation patterns, and the modification-induced effects on the ionization efficiency.[42 43 44] To overcome these limitations, we reasoned that a targeted pull-down approach would allow for selective enrichment and identification of modified species.

To achieve selective capture of a specific non-enzymatic PTM, we utilized a thioester-based alkyne chemical probe (**1**) developed by the Meier group, which was previously shown to label sites of non-enzymatic acylation.[45] Probe **1** reacts covalently with nucleophilic residues at sites of non-enzymatic acylation and contains an alkyne handle to enable bioorthogonal conjugation and enrichment (**Figure 3A**). We postulated that labeling with **1** would allow specific tagging and isolation of modified peptides.

First, we confirmed cellular incorporation of **1** by treating MDA-MB-231 cells with increasing concentrations of **1**. Cells were subsequently reacted with FAM-N_3 through a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction and analyzed by flow cytometry, revealing a concentration-dependent increase in fluorescence and confirming successful probe labeling (**Figure 3B**). Consistent with these results, fluorescent SDS-PAGE analysis of lysates from cells treated with **1** demonstrated robust incorporation of **1** into protein targets (**Figure 3C**).

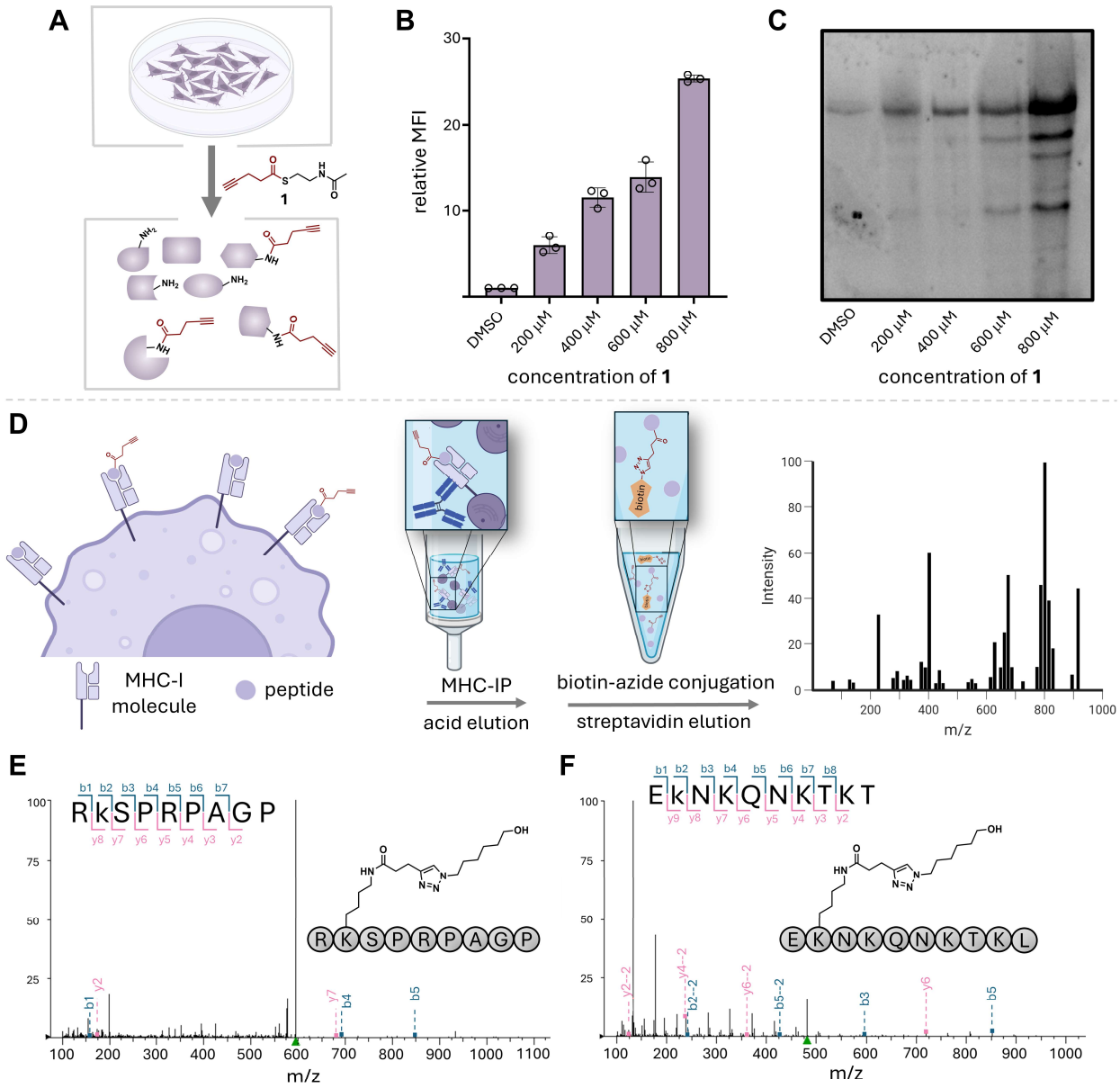

Figure 3 (A) Schematic representation of labeling sites of non-enzymatic modifications by probe **1**. **(B)** Flow cytometry analysis of MDA-MB-231 cells treated with the indicated concentration of **1** followed by a reaction FAM-N_3 in the presence of $CuSO_4$, ascorbic acid, and THPTA. **(C)** In-gel fluorescence analysis of **1**-treated MDA-MB-231 cells after labeling with FAM-N_3 in the presence of $CuSO_4$, ascorbic acid, and THPTA and resolved by SDS-PAGE **(D)** Schematic of the chemical enrichment workflow using **1**.

Peptides from the immunopeptidome of **1**-treated cells were reacted with DADPS-biotin azide and enriched on streptavidin beads prior to LC-MS/MS analysis. Representative LC-MS/MS spectra of modified peptides RKSPRPAGP **(E)** and EKNKQNKTKL **(F)**. Detected b+ ion fragments are shown in blue, and y+ ion fragments are shown in pink.

After validating efficient cellular and proteomic labeling, we applied this approach to isolate modified MHC-I peptides (**Figure 3D**). Approximately 2 x 10^8 MDA-MB-231 cells were treated with **1** for 6 h, followed by isolation of MHC-I-associated peptides by immunoaffinity purification.[46,47] The enriched peptide fractions were conjugated to an acid-cleavable dialkoxydiphenylsilane (DADPS)-biotin azide linker via CuAAC reaction, enabling selective capture of probe-modified peptides on streptavidin-functionalized beads. Bound peptides were then released under acidic conditions and identified by LC-MS/MS.

Using this workflow, we identified two MHC-I associated peptides bearing the modification introduced by **1** (**Figure 3E-F**). Notably, both peptides originated from nuclear proteins implicated in DNA damage and repair, suggesting that nuclear repair enzymes may be particularly susceptible to non-enzymatic acylation or more abundantly displayed on MHC-I. These results also demonstrate the feasibility of chemically tagging proteins with a bioorthogonal handle to enrich modified peptide targets from the immunopeptidome. More broadly, this approach establishes a chemical proteomics framework for the direct

identification of non-enzymatically modified peptides presented by MHC-I, which may play underappreciated roles in immune surveillance and tumor immunogenicity.

Conclusion

In this study, we investigated how non-enzymatic PTMs influence MHC-I antigen presentation and T cell recognition. Our results demonstrate that non-enzymatic modifications can markedly alter peptide-MHC-I binding affinity and modulate TCR recognition, underscoring the potential for non-enzymatic PTMs to reshape the immunopeptidome in ways that may either enhance or disrupt immune responses. Our analysis of cancer-associated peptides further revealed that non-enzymatic PTMs can affect MHC-I stability, suggesting a potential role in immune evasion or the generation of novel antigenic targets.

To facilitate the identification of these modified antigens, we developed a chemical enrichment strategy to directly identify non-enzymatically acylated peptides displayed on MHC-I molecules. We demonstrated selective tagging, enrichment, and LC-MS/MS identification of modified peptides by using a chemical probe with a bioorthogonal handle. This approach establishes a foundation for mapping modified peptide antigens that are otherwise difficult to detect due to their low abundance and chemical diversity.

Characterizing such modified MHC-I-associated peptides is critical, as they may escape thymic negative selection and elicit immune responses against self or tumor-associated antigens. One physiologically relevant source of non-enzymatic acylation arises from short-chain fatty acids (SCFAs), metabolites produced by the gut microbiota through fermentation of dietary fibers. SCFAs can form acyl-CoA intermediates that modify lysine residues and have been detected at millimolar concentrations in the intestinal lumen, where they induce widespread protein acylation.[48] Given emerging links between SCFA metabolism and inflammatory conditions such as irritable bowel syndrome (IBS),[49] investigating SCFA-induced modifications on MHC-I peptides could reveal new connections between metabolic dysregulation, antigen presentation, and immune-mediated diseases. Together, these findings and methodological advances highlight the underexplored role of non-enzymatic PTMs in shaping the antigenic landscape and provide a framework for systematically uncovering modified peptide antigens relevant to cancer and autoimmunity.

SUPPORTING INFORMATION

- Additional figures, tables, and materials/methods are included in the Supporting Information File (PDF)

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

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