# Endogenous and Exogenous Electrophilic Modifications Reshape Antigen Presentation and Recognition

Sarah E. Newkirk[1#], Joey J. Kelly[1#], Sameek Singh[2], Karl Ocius[1], Carson Starnes[1], Tian Zhang[2], and Marcos M. Pires[1,3*]

[1] Department of Chemistry, University of Virginia, Charlottesville, VA 22904, United States

[2] Department of Biochemistry and Molecular Genetics, School of Medicine, University of Virginia, Charlottesville, VA 22904, United States

[3] Department of Microbiology, Immunology, and Cancer, University of Virginia, Charlottesville, VA 22904, United States

* corresponding author: mpires@virginia.edu

[#] *S.E.N. and J.J.K. contributed equally to this work*

**ABSTRACT**

Cytotoxic T lymphocytes recognize infected or transformed cells through peptide antigens presented by major histocompatibility complex class I (MHC-I) molecules. Although antigen recognition is typically defined by peptide sequence, chemical modifications to amino acid sidechains can generate structurally distinct epitopes that alter immune recognition. Here, we investigated how endogenous and exogenous electrophiles can install non-enzymatic post-translational modifications (PTMs), thereby influencing antigen presentation and T cell activation. Notably, these structural modifications are typically irreversible and can persist through protein processing and subsequent peptide presentation. We comprehensively mapped out these potential modifications and found that peptide variants bearing non-enzymatic PTMs altered MHC-I stability and disrupted T cell recognition, particularly when modifications occurred at TCR-contact residues. To identify such species on MHC-I of cells, we developed a chemical enrichment strategy using an alkyne-tagged probe to capture non-enzymatically acylated peptides associated with MHC-I. Finally, we show that electrophilic environmental chemicals and dietary isothiocyanates (ITCs) can covalently modify antigenic peptides and abolish T cell activation despite preserved MHC-I binding. Together, these findings demonstrate that endogenous and exogenous chemical modifications can reshape the immunopeptidome and generate chemically distinct peptide antigens that alter adaptive immune recognition.

## INTRODUCTION

The adaptive immune system defends against infected and transformed cells by recognizing specific antigens, often presented as peptides on host cell surfaces. Major histocompatibility complex (MHC) molecules are central to this process, presenting peptides that guide immune detection.[1, 2] MHC molecules are divided into class I and class II, with MHC class I (MHC-I) primarily presenting peptides to cytotoxic T cells.[3,4] MHC-I molecules are expressed on the surface of nearly all nucleated cells and display peptides derived from intracellular peptide pools.[5] Peptide-MHC complexes (pMHC) on cell surfaces engage cytotoxic T cells, which eliminate cells presenting peptides not recognized as “self”.[6] The differentiation between self and non-self underpins adaptive immunity, positioning neoantigens as valuable targets for both personalized cancer immunotherapy and vaccination strategies.

The foundation for this precise discrimination is established early in immune development. 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.[7] Importantly, the stringency of this selection declines over time due to thymic involution. This temporal limitation in central tolerance means that late-emerging or structurally modified peptides can act as blind spots in the immune system's 'self' registry, leaving the remaining T cells fully capable of recognizing them as foreign.[8] Indeed, such aberrant modifications to presented peptides are well-established drivers of

autoimmune pathologies. Yet, to the best of our knowledge, there have been few comprehensive efforts to assess the types of molecules capable of driving these reactions, or to evaluate their subsequent impact on MHC binding and the alteration of T cell engagement.

A major driver of these structural alterations is the covalent modification of proteins by reactive electrophiles, arising from both endogenous biochemical processes and exogenous chemical exposures. Structural modification to amino acids, also known as post-translational modifications (PTMs), broadly fall into two categories: those regulated by enzymatic machinery and those induced non-enzymatically by reactive chemical species. Canonical enzymatic PTMs, such as phosphorylation, glycosylation, and ubiquitination, are well-established modulators of protein structure and function.[9, 10] These controlled modifications can influence the immune response by affecting peptide binding affinity to MHC-I or recognition by TCRs.[11-15] Building on this, our lab has systematically assessed the impact of enzymatic PTMs on MHC-I binding and T cell recognition, demonstrating that they substantially modulate these interactions.[16] A key feature of enzymatic PTMs is that they are typically reversible and can be removed by specific 'eraser' enzymes (**Fig. 1a**).[17] In sharp contrast, non-enzymatic modifications (particularly those driven by highly reactive exogenous molecules) are largely irreversible, as the host lacks the evolved enzymatic machinery to remove them. Consequently, these stable modifications should persist in peptides presented on surface-bound pMHCs.[18-22] Critically, protein modifications by electrophiles after age-related thymic involution poses

a particularly high risk, as the immune system lacks prior tolerization to these altered structures and can readily perceive the modified peptides as non-self neoantigens.

The sources of electrophiles that can install PTMs are diverse, encompassing both endogenous metabolites and exogenous chemical species (**Fig. 1b**). Endogenous electrophiles can originate from reactive oxygen species (ROS; ex: hydrogen peroxide), reactive nitrogen species (RNS; ex: peroxynitrite), and reactive carbon species (RCS; ex: methylglyoxal). ROS are known to modify sulfur containing amino acids such as cysteine and methionine. Oxidation of methionine has been associated with the creation of structurally modified peptides that can bypass early central tolerance, acting as neoantigens that provoke unexpected T cell activation.[23] Similarly, reactive nitrogen species (RNS) are known to modify aromatic amino acids, most notably through the nitration of tyrosine. Tyrosine nitration has been associated with the creation of structurally modified neoepitopes, such as the nitration of tyrosine residues within viral peptides (e.g., LCMV gp33), which alters the molecular surface of the MHC class I complex and dramatically shifts CD8+ T cell recognition.[24] Finally, reactive carbon species (RCS), particularly glycolytic byproducts like methylglyoxal (MGO), readily modify basic amino acids such as arginine and lysine to form irreversible advanced glycation end-products (AGEs). This carbon-driven modification has been associated with a mounting burden of structurally altered neoantigens. Because these irreversible AGEs accumulate continuously on long-lived proteins, they present a unique, chemically diverse challenge to the restricted T cell repertoire and waning central tolerance associated with age-related thymic involution.[25] Additionally, short-chain fatty acids (SCFAs) are

produced by the gut microbiota through the fermentation of dietary fibers.[26] Reaching millimolar concentrations in the intestinal lumen, SCFAs can be activated into reactive acyl-coenzyme A intermediates in human cells, which subsequently induce widespread protein acylation by modifying lysine residues.[27, 28] Given the widespread non-enzymatic modification of lysines by SCFAs across the proteome, coupled with emerging links between SCFA metabolism and inflammatory conditions such as irritable bowel syndrome (IBS),[29] investigating SCFA-altered peptides on MHC-I could uncover novel mechanistic links between metabolic dysregulation, antigen presentation, and immune-mediated diseases.

Beyond endogenous metabolic byproducts, an array of entirely exogenous electrophiles spanning environmental pesticides,[30-33] pharmaceuticals,[34-36] and dietary metabolites,[37-39] exert a comparable chemical burden, covalently modifying host proteins through similar non-enzymatic mechanisms (**Fig. 1c**). By their chemical nature, the majority of environmental pesticides are highly reactive electrophiles. Their reactivity often rivals that of classic anticancer drugs, which can sometimes rely on poorly discriminate covalent protein modification to destroy tumor cells; consequently, chronic pesticide exposure drives similar non-enzymatic modifications on host proteins. An analogous phenomenon occurs with many clinically approved pharmaceuticals (e.g., β-lactam drugs and many next generation cancer drugs directed at hyperactive cysteines). While these agents are specifically engineered for therapeutic benefit, their mechanisms of action, or their subsequent metabolic processing, can sometimes rely on inherent electrophilic properties. Consequently, alongside engaging their intended clinical targets, these drugs

can inadvertently drive off-target, non-enzymatic modifications on bystander proteins, contributing to the pool of chemically altered peptides available for MHC-I presentation. Indeed, this mechanism has recently been exploited in clinically targeted therapies, where the covalent KRAS(G12C) inhibitor ARS1620 was shown to act as a hapten, generating uniquely alkylated peptide neoepitopes on MHC-I that can be directly targeted by cytotoxic T cell immunotherapies.[40] Crucially, the significance of this study extends beyond the discovery of a novel neoepitope; it exposes a profound limitation in how the field currently validates non-enzymatic PTMs. To date, researchers have been unable to physically isolate and sequence this specific haptenated peptide directly from the cancer cells using standard analytical techniques. Yet, its existence is indisputably proven by the biological readout: these modified complexes successfully guide targeted T cell cytotoxicity, an approach currently advancing toward clinical evaluation. This powerful dichotomy challenges the field's heavy reliance on the physical detection of modified peptides to prove their role in driving disease phenotypes. As this clinical candidate demonstrates, non-enzymatic modifications can exert profound immunological modulation even when their abundance falls completely below the detection threshold of standard proteomic technologies.

β-lactam antibiotics represent another example of exogenous electrophiles, covalently modifying protein targets through acylation.[41] For instance, irreversible modification by flucloxacillin has been shown to generate flucloxacillin-modified peptides presented on MHC-I.[36] Beyond pharmaceuticals, natural environmental exposures also drive protein adduct formation. A prominent example is urushiol, the primary immunogen of poison ivy.

While unmodified urushiol can be presented on CD1a, a transmembrane glycoprotein that presents lipid and glycolipid antigens to T cells,[42] it can also covalently modify self-proteins in the skin to produce neoantigens and trigger T cell-mediated allergic responses.[43-45] Collectively, these examples highlight that immune responses to exogenous electrophiles, particularly hose driving allergic reactions, may not involve only traditional immunoglobulin E (IgE)-based responses, but also MHC- and T cell-mediated recognition of covalently modified non-self-antigens.

Non-enzymatic modifications can influence peptide presentation in multiple ways. First, they can affect the stability of the peptide-MHC (pMHC) complex, as binding affinity is a critical determinant of whether a peptide is effectively presented to T cells. Modifications can either enhance or disrupt this binding, altering complex stability and the likelihood of T cell recognition.[46, 47] Second, modifications can directly impact interactions with TCRs of cytotoxic T cells, which are finely tuned to recognize specific pMHC combinations. Even subtle changes in peptide sequence can disrupt TCR recognition, compromising immune response against infected or transformed cells[48, 49 50, 51] Collectively, non-enzymatic and exogenous chemical modifications generate a chemically distinct pool of peptides that may escape negative selection and potentially trigger autoimmune pathologies. Despite their ubiquity, the immunological consequences of exogenous chemical modifications on antigen presentation remain poorly understood. These modifications may occur at multiple stages: they may alter intracellular proteins prior to processing, or modify peptides in the cytosol after processing but before presentation, thereby generating chemically altered peptides (**Fig. 1d**). Alternatively, modifications may act directly on

peptides already displayed on MHC-I molecules at the cell surface. Regardless of the precise spatiotemporal pathway of adduct formation, the overarching consequence is the generation of a chemically distinct antigenic landscape.

In this study, we investigate how non-enzymatic modifications (driven by both endogenous and exogenous electrophiles) influence MHC-I antigen presentation. To enable the direct interrogation of these events, we developed a novel enrichment strategy to detect MHC-I-associated peptides bearing non-enzymatic adducts. Using this platform, we examine how these chemically altered peptides are presented and recognized in cellular and cancer-relevant contexts. We further apply this framework to assess how exposure to exogenous electrophiles, including environmental herbicides and dietary isothiocyanates (ITCs), alters proteome-wide reactivity, pMHC-I stability, and T cell engagement. Ultimately, this work expands the immunological definition of 'non-self' to encompass exogenously and non-enzymatically modified peptides, providing a mechanistic basis for how chemical exposures shape immune recognition.

## RESULTS AND DISCUSSION

### Impact of Non-Enzymatic Modifications on pMHC-I Stability and T Cell Activation

To investigate the effects of non-enzymatic PTMs on peptide affinity for MHC, we used the model epitope SIINFEKL (**ovaWT**) derived from ovalbumin (**Fig. 2a**). 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 chemical modifications modulate antigen presentation.[52] To this end, recent work from our lab systematically evaluated various peptidomimetics of SIINFEKL on pMHC stability, TCR recognition, serum stability, and cellular permeability.[53] In this work, we first assessed pMHC-I binding using RMA-S cells, which lack functional transporters associated with antigen processing (TAP).[54, 55] In TAP-deficient cells, impaired endogenous peptide loading leaves MHC-I molecules largely empty or occupied by low-affinity peptides, resulting in a marked reduction of surface H-2K$^b$ expression. However, reducing the ambient temperature promotes the transient surface accumulation of these unstable MHC-I molecules. Subsequent incubation with high-affinity exogenous peptides thermodynamically stabilizes the H-2K$^b$ complexes, enabling their persistent surface display even upon return to physiological temperatures. Previous studies have established a strong correlation between the abundance of surface MHC complexes and peptide affinity for H-2K$^b$.[56-58] To establish a baseline for these interactions, we employed the peptide SNFVSAGI (**cntPEP**) as a negative control, given its well-documented inability to bind H-2K$^b$.[59]

We first focused on lysine (position 7 in **ovaWT**), one of the most frequently modified amino acids in the proteome likely due to its nucleophilic nature. To investigate how oxidative stress can induce lysine modifications, we synthesized targeted **ovaWT** variants

incorporating formylated lysine (**ovaK7$_{formyl}$**), aminoadipic acid (**ovaK7$_{aad}$**), and homocitrulline (**ovaK7$_{hcit}$**) (**Fig. 2a**).[60, 61] In all, we found that these diverse set of oxidative mediated modification of the amino sidechain of lysine in this specific position had minimal impact on pMHC stability, consistent with this residue being solvent-exposed and having limited interactions with the H-2K$^b$ molecule (**Fig. 2b**).[62] Another prominent class of lysine PTMs arises from reactions with reactive sugar metabolites, forming stable covalent adducts.[63, 64] In particular, methylglyoxal, a byproduct of glycolysis, can promote the accumulation of *S*-D-lactoylglutathione and result in the non-enzymatically driven D-lactylation.[65] We synthesized lactic acid-modified variant (**ovaK7$_{lac}$**) and found that, again, this specific site was able to tolerate the modification with minimal impact on pMHC stability. While this specific site has minimal engagement with the MHC molecule, it is expected that given its position lysine modifications are more likely to influence TCR recognition.[66]

Next, we examined asparagine (position 4), which can undergo deamidation through formation of a succinimide intermediate, yielding aspartate or isoaspartate linkages in the peptide backbone (**Fig. 2a**).[67] Notably, these modifications have been observed to be presented on MHC-I following deamidation,[68] which can also occur enzymatically by the *N*-glycosidase-mediated removal of glycosylated asparagine.[69] In contrast to the lysine modifications, peptide variants incorporating aspartate (**ovaN4D**) or isoaspartate (**ovaN4D$_{iso}$**) at this position showed a marked decrease in pMHC surface expression, consistent with previous reports that that backbone modifications can alter pMHC stability (**Fig. 2b**).[70]

We also investigated whether non-enzymatic PTMs can mimic canonical residues to induce cross-reactive responses, given the promiscuity that TCRs have been shown to display towards MHC-I-presented peptides. An illustrative example is the irreversible oxidation of cysteine to sulfonic acid, which can structurally resemble acidic residues.[71] To test this, we synthesized **ovaE6C$_{SO3}$**, a sulfonic acid-modified peptide, to model where such a modification could mimic the glutamic acid residue at position 6 within SIINFEKL (**Fig. 2a**). This variant also showed reduced pMHC stability, likely due to the increased steric bulk of the sulfate group relative to the side chain of glutamic acid (**Fig. 2b**). Overall, these results indicate that non-enzymatic PTMs can induce significant changes in the binding affinity of **ovaWT** to H-2K$^b$.

We then aimed to evaluate how non-enzymatic PTMs affect T cell recognition of their target ligand. To do so, RMA-S cells were incubated with 5 μM peptide and co-cultured with B3Z T cells, which express a SIINFEKL-specific TCR and a β-galactosidase reporter under an IL-2 inducible promoter. Recognition of the cognate ligand triggers enzymatic conversion of chlorophenol red β-D galactopyranoside (CPRG) to chlorophenol red, producing a colorimetric change that can be monitored spectrophotometrically, with the intensity of the signal reflecting functional T cell activation.[72] Consistent with pMHC stability data, modifications at lysine 7 (**ovaK7$_{formyl}$**, **ovaK7$_{aad}$**, **ovaK7$_{hcit}$**, and **ovaK7$_{lac}$**) eliminated T cell activation (**Fig. 2c**). This likely reflects the loss of the positive charge, as previous studies have demonstrated that charge alterations at TCR-contact residues can

abolish T cell activation.[16, 73] Similarly, both asparagine modifications (**ovaN4D and ovaN4D$_{iso}$**) eliminated T cell activation, further highlighting the importance of side-chain chemistry in TCR engagement. Interestingly, the sulfonic acid-modified variant **ovaE6C$_{SO3}$** restored T cell activation relative to the inactive **ovaE6C**. We attribute this response to the functional mimicry of the negatively charged sulfonic acid moiety for the native glutamate residue. This finding suggests that certain non-enzymatic PTMs can 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.

**Non-Enzymatic PTMs on Cancer-Associated Peptides Influence pMHC-I Stability**

To investigate the role of non-enzymatic PTMs in the presentation of cancer-associated peptides, we synthesized modified variants of peptides previously identified as being displayed on cancer cells. Kacen et al. used a mass spectrometry-based platform to show that non-enzymatic modifications constitute the majority of modified MHC-I-associated peptides and play a significant role in shaping the cancer immunopeptidome.[46] 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 reported cancer-associated peptides[46] (**Fig. 3a**) and assessed their H-2K$^b$ binding using the RMA-S stabilization assay (**Fig. 3b**). Modifications were introduced at multiple positions along the peptide chain to probe their effects on pMHC display. For cancer-associated peptide 1 (KGMNYTVRL, **ca1**) and cancer-associated peptide 2 (SAPENAVRM, **ca2**), methionine oxidation at positions 3 and 9, respectively, disrupted display on H-2K$^b$. Increasing the degree of methionine oxidation (from **ca1$_{oxide}$** to **ca1$_{oxone}$**) further reduced pMHC surface display on RMA-S cells. In cancer-associated peptide 3 (INFDFPKL, **ca3**), deamidation at position 2 (**ca3$_{isoasp}$**) caused a >100-fold decrease in peptide display, highlighting the substantial impact of backbone modifications H-2K$^b$ affinity. In contrast, with cancer-associated peptide 4 (CGYEFTSKL, **ca4**) and cancer-associated peptide 5 (VAYEYLCHL, **ca5**), cysteinylation at positions 1 (**ca4$_{cystine}$**) and position 7 (**ca5$_{cystine}$**), respectively, had minimal effect on pMHC stability. HPLC analysis of these peptides incubated in cell culture medium confirmed that cystine reduction did not occur over the course of the assay **(Fig. S1** and **S2**). These findings are also consistent with prior reports showing that cysteinylated peptides can be presented as immunodominant forms in viral infections and as histocompatibility-Y (H-Y) antigens.[74,75] Collectively, these results demonstrate that non-enzymatic PTMs can modulate MHC-I display of endogenously presented peptides. In particular, modifications that reduce pMHC-I surface expression may represent a potential immune evasion mechanism by decreasing the display of immunogenic epitopes on cancer cells.

**Chemical Enrichment of Non-Enzymatically Modified MHC-I Peptides**

We next sought to develop a chemical enrichment strategy to identify non-enzymatically modified peptides presented on MHC-I molecules. Detecting such peptides remains challenging due to their low stoichiometric abundance within complex cellular environments, the structural diversity and unpredictability of PTMs, complex fragmentation patterns, and modification-induced effects on the ionization efficiency.[76] [77] [78] To overcome these limitations, we reasoned that a targeted pull-down approach could selectively enrich and identify modified species.

In order to capture a specific non-enzymatic PTM, we employed a thioester-based alkyne chemical probe (**1**) developed by the Meier group, previously shown to label sites of non-enzymatic acylation.[79] Probe **1** covalently reacts with nucleophilic residues at acylation sites and contains an alkyne handle for bioorthogonal conjugation and enrichment (**Fig. 4a**). We hypothesized that labeling with **1** would allow selective tagging and isolation of modified MHC-I-presented peptides. We first confirmed cellular incorporation of **1** by treating MDA-MB-231 cells with increasing concentrations of the probe. Cells were subsequently reacted with fluorescein azide via a copper-catalyzed azide-alkyne cycloaddition (CuAAC) and analyzed by flow cytometry, revealing a concentration-dependent increase in fluorescence (**Fig. 4b**). Fluorescent SDS-PAGE analysis of lysates further confirmed robust incorporation of **1** into protein targets (**Fig. 4c**).

After validating efficient cellular and proteomic labeling, we applied this strategy to isolate modified MHC-I peptides (**Fig. 4d**). Approximately 2 x $10^8$ MDA-MB-231 cells were treated overnight with **1**, followed by immunoaffinity purification of MHC-I-associated peptides.[80,81] Enriched peptides were conjugated to an acid-cleavable dialkoxydiphenylsilane (DADPS)-biotin azide linker via CuAAC, enabling selective capture on streptavidin-functionalized beads. Bound peptides were then released under acidic conditions and analyzed by LC-MS/MS. Using this workflow, we identified two MHC-I associated peptides bearing the modification introduced by **1** (**Fig. 4e** and **4f)**. 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 frequently presented 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.

## Impact of Electrophilic Environmental Chemicals and Pharmaceuticals on MHC-I Binding and T Cell Activation

Building on our findings that non-enzymatic PTMs can modulate immune responses and reshape the immunopeptidome, we next sought to determine whether additional classes

of exogenous chemical modifications might exert similar effects. Electrophilic pesticides, herbicides, and fungicides represent a particularly understudied yet potentially important group of agents in this context.[82] Owing to their inherent chemical reactivity, these compounds can directly and covalently modify nucleophilic amino acid side chains in proteins,[30-33] a process known as haptenation.[83] The Cravatt laboratory has demonstrated through activity-based protein profiling (ABPP) that the nucleophilicity of specific amino acids depends on their local microenvironment, identifying certain residues as "hyper-reactive" hotspots.[84] Considering this, modifications at these hyper-reactive sites likely have the potential to trigger adaptive immune responses and may contribute to chronic low-grade inflammation or autoimmunity if the modified peptides are processed and presented via MHC molecules.

Previous work from the Nomura laboratory has demonstrated, using reactivity-based chemoproteomic approaches, that a broad range of environmental chemicals are capable of covalently modifying complex proteomes.[31] To recapitulate and extend these observations, we employed a similar competitive gel-based fluorescence assay to assess the proteome-wide reactivity of cysteine- and lysine-reactive herbicides, pesticides, and fungicides. The objective of this analysis was to identify electrophilic environmental chemicals within our panel that exhibit the greatest potential to covalently modify protein antigens and, therefore, may be most likely to elicit immune responses. These results were used to prioritize candidates for subsequent immunological studies.

Briefly, whole-cell lysates from MDA-MB-231 cells were treated with a panel of electrophilic environmental chemicals (**Fig. 5a**), allowing for covalent modification of nucleophilic residues across the proteome. Lysates were subsequently incubated with either the cysteine-reactive iodoacetamide alkyne (IA-alkyne) probe or lysine-reactive NHS-ester alkyne probe (4-pentynoic acid succinimidyl ester)[85] to label unoccupied nucleophilic sites. Following probe labeling, proteins were conjugated to the fluorophore FAM-$N_3$ via copper-catalyzed azide-alkyne cycloaddition (CuAAC) and resolved by SDS-PAGE for in-gel fluorescence analysis. Consistent with the competitive nature of the assay, a reduction in fluorescent signal indicated increased proteome-wide reactivity of the corresponding electrophilic chemical. To complement the gel-based measurements and assess reactivity in a cellular context, we also performed live-cell competitive flow cytometry-based assays. In this parallel workflow, cells were treated with the same panel of electrophilic herbicides, followed by competition with either IA-alkyne or NHS-ester alkyne to probe cysteine and lysine reactivity, respectively. Cells then underwent a CuAAC reaction with coumarin azide and fluorescence was quantified by flow cytometry.

Consistent with the gel-based analyses (**Fig. S4**), lysine-directed labeling exhibited minimal signal reduction across most herbicides (**Fig. S5**). This may reflect differential selectivity of the NHS-ester alkyne probe, which can react with nucleophiles beyond lysine residues such as threonine and serine, potentially obscuring decreases in labeling caused by prior electrophile engagement.[86] As a result, samples may appear similarly reactive to probe-only controls. Although glyphosate produced a modest decrease in fluorescent signal in the gel-based assay (**Fig. S4**), flow cytometry identified chlorothalonil

as the most lysine-reactive compound in the panel (**Fig. S5**). These discrepancies likely reflect differences in cellular permeability, as gel-based labeling in lysates bypasses membrane barriers that may limit intracellular access and apparent reactivity in live-cell assays. In contrast, cysteine-directed labeling produced more pronounced effects and closely mirrored the gel-based results, with a strong reduction in signal observed for the positive control iodoacetamide and a particularly robust decrease following chlorothalonil treatment (**Fig. S6** and **S7**). Taken together, these findings indicate that chlorothalonil displays the highest proteome-wide reactivity among the herbicides tested, particularly toward cysteine residues. Based on its consistent performance across both assay formats, chlorothalonil was selected for subsequent immunological studies.

We also examined acetylsalicylic acid (ASA), or aspirin, which has been reported to covalently modify mostly serine residues, but also other amino acid side chains like lysine, via acetylation.[87] Under our experimental conditions, we did not observe substantial reductions in lysine-directed probe labeling by either gel-based or flow cytometric analysis (**Fig. S8** and **S9**). This may again reflect limited selectivity of the NHS-ester alkyne probe, whose reactivity with multiple nucleophiles could obscure changes in lysine-specific modulation. Additionally, ASA-mediated lysine acetylation may occur more slowly than many electrophile-thiol reactions, often over several hours.[88] Additionally, others have found that even after high doses of aspirin, lysine acetylation site occupancies remain low unless cellular deacetylases are inhibited.[89] Consequently, our one-hour incubation, chosen to match conditions for other reactive species, may have been insufficient to capture robust lysine engagement. Notably, we observed a slight fluorescence reduction

in the cysteine-directed gel-based assay (**Fig. S10**), potentially reflecting low-level thiol acylation by ASA,[90, 91] although this effect was substantially weaker than for the most reactive electrophiles in our panel.

Guided by these proteome-wide reactivity profiles, we synthesized variants of the model antigen SIINFEKL in which the lysine residue was covalently modified with selected electrophilic chemicals or pharmaceuticals (**Fig. 5b**). These modified peptides allowed us to examine how site-specific exogenous covalent modifications affect T cell recognition. Specifically, we generated a chlorothalonil-modified SIINFEKL peptide (**ovaCTN**), corresponding to the most reactive fungicide identified in our panel, to model haptenation of a lysine residue within a protein or peptide in cellular context. For aspirin, the resulting modification is chemically analogous to lysine acetylation, a PTM we demonstrated in previous work to modulate T cell activation.[20] Accordingly, we included this ASA-modified SIINFEKL variant (**ovaASA**) containing an acetylated lysine residue.

Using the RMA-S stabilization assay, we evaluated the impact of these hapten modifications on peptide-MHC-I (pMHC-I) stability as a proxy for relative MHC binding affinity (**Fig. 5c**). Compared to unmodified SIINFEKL (**ovaWT**), both **ovaCTN** and **ovaASA** exhibited comparable pMHC stability, consistent with our prior observations that lysine-directed modifications often minimally impact MHC-I binding, despite their potential to alter T cell recognition. We then assessed antigen-specific T cell activation using the B3Z reporter assay. Both **ovaCTN** and **ovaASA** failed to elicit detectable T cell activation,

indicating a complete loss of productive TCR engagement. This demonstrates that covalent modification of a single lysine residue can render peptides immunologically distinct despite preserved MHC-I binding.

While the use of a pre-conjugated SIINFEKL variant allows for precise control over peptide composition, the TAP-deficient RMA-S cell line does not fully capture the physiological context in which antigens are generated endogenously. To determine whether the observed effects extend to intact antigen processing pathways, we adapted the B3Z assay for co-incubation with B16-OVA cells, a murine melanoma cell line engineered to express full-length ovalbumin. In this system, ovalbumin undergoes intracellular processing to generate the SIINFEKL epitope, which is subsequently presented on H-2K$^b$ molecules and recognized by the SIINFEKL-specific B3Z TCR. This approach allows us to assess whether electrophilic compounds disrupt T cell engagement in a setting where modifications could occur either on peptides or proteins prior to MHC-I presentation, or directly on peptides already bound to MHC-I at the cell surface. Treatment of B16-OVA cells with chlorothalonil reduced T cell activation to background levels, whereas ASA treatment did not, likely reflecting the slower kinetics of lysine acetylation under our one-hour incubation (**Fig. 5d**). Together, these results demonstrate the ability of a particularly reactive electrophilic fungicide to disrupt T cell engagement in a physiologically relevant antigen presentation context.

**Impact of Dietary Isothiocyanates on MHC-I Binding and T Cell Activation**

In our comprehensive examination of exogenous electrophilic species, we next investigated isothiocyanates (ITCs) as a distinct class of reactive small molecules capable of haptenating proteins or peptides, thereby modulating immune recognition (**Fig. 5e**). ITCs, including benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), allyl isothiocyanate (AITC), and sulforaphane (SFN), are abundant in dietary sources such as cruciferous vegetables and are well documented to covalently modify proteins through reaction with nucleophilic residues. These compounds also exhibit established anti-cancer activity in both *in vitro* and *in vivo* models,[92, 93] and have demonstrated potential in fighting oxidative stress and inflammation in various organ systems.[94] Quantitative chemical proteomic studies have identified numerous protein targets of ITCs, underscoring their capacity for broad proteome engagement.[38, 95-97]

Using the same gel-based and flow cytometry competitive assays, we evaluated the proteome-wide reactivity of representative ITCs, with a particular focus on PEITC, AITC, and SFN. Across both assay formats, PEITC and AITC showed robust competition with cysteine-reactive probes, consistent with their known electrophilic reactivity toward thiols (**Figs. S10** and **S11**). In contrast, SFN displayed comparatively weaker competition under the conditions tested. Lysine-modified SIINFEKL variants were synthesized for PEITC (**ovaPEITC**) and AITC (**ovaAITC**) and tested for their pMHC stability and T cell engagement (**Fig. 5f**). As observed for the chlorothalonil and aspirin variants, **ovaPEITC** and **ovaAITC** retained binding comparable to **ovaWT** but showed markedly reduced B3Z

T cell activation (**Fig. 5g**), indicating that covalent lysine modification by dietary ITCs also renders peptides immunologically distinct.

In the B16-OVA system, treatment with PEITC or AITC reduced T cell engagement of endogenously processed SIINFEKL, whereas SFN did not (**Fig. 5h**), aligning with their proteome-wide reactivity results. One possible explanation is that sulforaphane's electrophilicity is functionally biased towards thiol-containing residues, such as cysteines, and does not necessarily translate to efficient or stable modification of lysine ε-amines within antigenic peptides. In addition, sulforaphane's increased polarity and steric bulk relative to PEITC and AITC may limit its ability to access or productively orient toward the lysine residue within the conformationally constrained environment of the pMHC complex. It is also conceivable that sulforaphane-lysine adducts are comparatively less stable or more reversible, such that transient modification is insufficient to perturb TCR recognition under these conditions. These possibilities suggest that while dietary ITCs represent a class of electrophilic compounds that have the potential to modulate immune response, electrophile reactivity alone is not predictive of antigen-modifying potential and that residue selectivity, adduct stability, and molecular context are likely critical determinants of whether covalent modification translates into altered immune recognition.

**Surface-Bound MHC-I Peptides are Susceptible to Direct Chemical Modification**

Notably, because antigens in B16-OVA cells are generated intracellularly, the previous experiments do not distinguish between modifications that occur during antigen processing and presentation, and those that might occur directly on peptides already bound to MHC-I at the cell surface. As such, either (or both) mechanisms may contribute to the observed loss of T cell recognition. If modifications occur intracellularly, the accumulation of electrophilic species is critical, as the compound must traverse the cell membrane to reach its target. In contrast, surface-bound modifications could induce immunogenic changes, even if the electrophilic species has limited cellular permeability.

To specifically probe the latter mechanism, we exploited the unique TAP deficiency of RMA-S cells, permitting them to exclusively present exogenously loaded peptides (**Fig. 6a**). In this system, RMA-S cells were first loaded with unmodified SIINFEKL (**ovaWT**) to generate stable pMHC-I complexes on the cell surface. After extensive washing to remove any free peptide, the cells were treated with electrophilic compounds for 1.5 h. Under these conditions, any chemical can only occur on peptides already bound to H-2K$^b$ molecules at the cell surface, eliminating the possibility of intracellular processing or new peptide presentation contributing to the observed effects. The **ovaWT**-preloaded RMA-S cells were then co-incubated with B3Z T cells to assess TCR engagement. This approach therefore enables the unambiguous determination of whether electrophilic compounds can directly alter surface-bound pMHC complexes and disrupt T cell recognition, independently of any effects on endogenous protein processing or antigen generation. Treatment of SIINFEKL-loaded RMA-S cells with chlorothalonil, PEITC, or AITC reduced T cell activation to background levels, whereas SFN had no effect (**Fig. 6b**). These

findings indicate that select electrophilic compounds can directly modify peptides already presented on MHC-I, thereby disrupting TCR recognition independently of intracellular antigen processing.

## CONCLUSION

In this study, we demonstrate that non-enzymatic PTMs and exogenous chemical modifications can substantially reshape MHC-I antigen presentation and T cell recognition. Using the model SIINFEKL epitope, we show that chemical modifications can alter pMHC-I stability and, in many cases, abolish T cell recognition even when MHC binding is preserved. Extending these findings to cancer-associated peptides suggests a potential role for such modifications in immune evasion or in generating novel antigenic targets. These results underscore how even single chemical modifications at specific residues can create immunologically distinct epitopes. We further establish a chemical enrichment strategy that enables the identification of non-enzymatically acylated MHC-I-associated peptides. This approach provides a framework for systematically mapping chemically modified immunopeptidomes, which are otherwise challenging to detect due to low abundance and chemical diversity. Such methodologies are critical for uncovering modified peptide antigens relevant to cancer and autoimmune disease. Importantly, our findings also reveal that electrophilic environmental chemicals and dietary metabolites can directly modify proteins and MHC-I-presented peptides at the cell surface. These

results suggest that both environmental exposure and metabolic state can dynamically influence immune recognition by generating chemically modified antigens.

The observation that chemically diverse molecules—those broadly described as “harmful”, such as environmental chemicals, and those considered “beneficial”, like ITCs in cruciferous vegetables—can product similar net effect of reducing T cell activation, warrants further consideration. From an immunological perspective, ITC-mediated protein modification may represent a mechanistically distinct scenario from electrophilic herbicide exposure (**Fig. 6c**). Whereas environmental toxicants may introduce novel chemical modifications that disrupt immune tolerance, ITCs could hypothetically act in a protective manner by modifying nucleophilic residues during immune education. If such modifications occur during thymic selection, T cells may be tolerized to ITC-modified epitopes, potentially reducing reactivity toward these antigens later in life. Conversely, subsequent loss of ITC exposure could result in the presentation of unmodified peptides that differ from those encountered during T cell training, generating a mismatch in immune recognition. Alternatively, the absence of ITC-mediated modification could leave reactive nucleophilic residues unprotected, rendering them more susceptible to modification by “harmful” electrophiles such as environmental toxicants. While entirely speculative, this working model illustrates how chemically distinct classes of reactive small molecules may differentially shape immune tolerance and responsiveness through their effects on antigen modification.

Taken together, these findings and methodological advances highlight the underexplored role of endogenous and exogenous chemical modifications in shaping the antigenic landscape and provide a framework for systematically uncovering modified peptide antigens relevant to cancer and autoimmunity. They also highlight how chemically distinct classes of reactive small molecules may differentially shape immune surveillance by altering antigen identity at the peptide level. Collectively, this work expands the concept of antigenic diversity to include chemically modified epitopes, representing a potentially important class of non-mutational neoantigens in cancer, inflammation, and autoimmune disease.

## ACKNOWLEDGEMENTS

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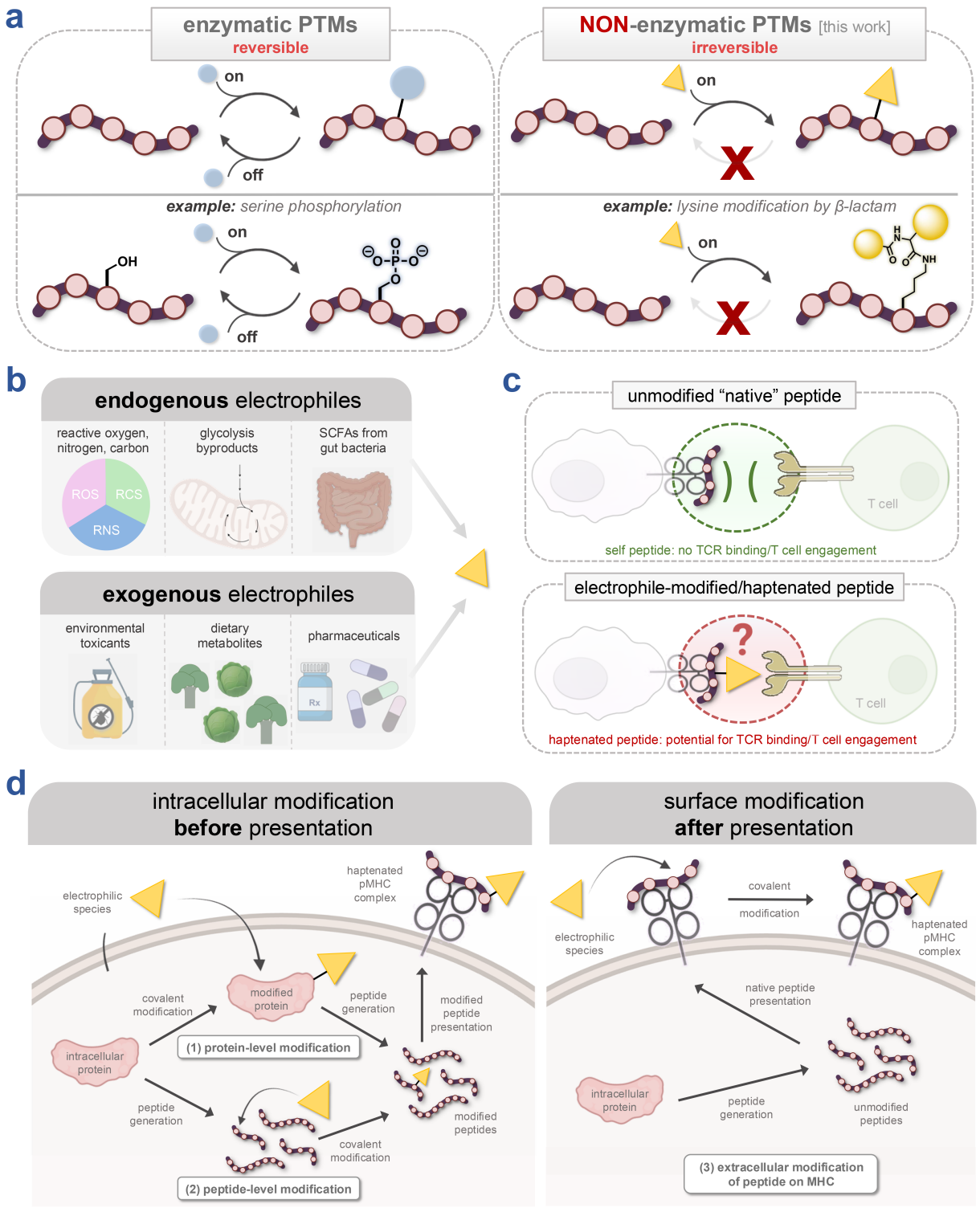
## SUPPORTING INFORMATION

Additional figures, tables, and materials/methods are included in the supporting information file.

## COMPETING INTERESTS

The authors declare no competing interests.

**Figure 1**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reversible
on
off
example: serine phosphorylation
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irreversible
example: lysine modification by β-lactam
b
endogenous electrophiles
reactive oxygen, nitrogen, carbon
ROS
RCS
RNS
glycolysis byproducts
SCFAs from gut bacteria
exogenous electrophiles
environmental toxicants
dietary metabolites
pharmaceuticals
Rx
c
unmodified "native" peptide
T cell
self peptide: no TCR binding/T cell engagement
electrophile-modified/haptenated peptide
haptenated peptide: potential for TCR binding/T cell engagement
d
intracellular modification before presentation
electrophilic species
haptenated pMHC complex
covalent modification
modified protein
peptide generation
modified peptide presentation
intracellular protein
(1) protein-level modification
modified peptides
(2) peptide-level modification
surface modification after presentation
covalent modification
native peptide presentation
unmodified peptides
(3) extracellular modification of peptide on MHC

**Figure 1. (a)** Representation of the reversibility of most canonical enzymatic PTMs (left) relative to the typically irreversible non-enzymatic PTMs (right). **(b)** Schematic illustrating the sources of endogenous and exogenous electrophiles capable of modifying nucleophilic amino acid residues. **(c)** Representation of how electrophilic chemical species may alter immune response through modification of an MHC-I presented peptide. **(d)** Schematic depiction of potential pathways of electrophilic modification, including intracellular modification of proteins or peptides prior to MHC-I presentation, and modification of MHC-I-presented peptides on the cell surface.

**Figure 2**

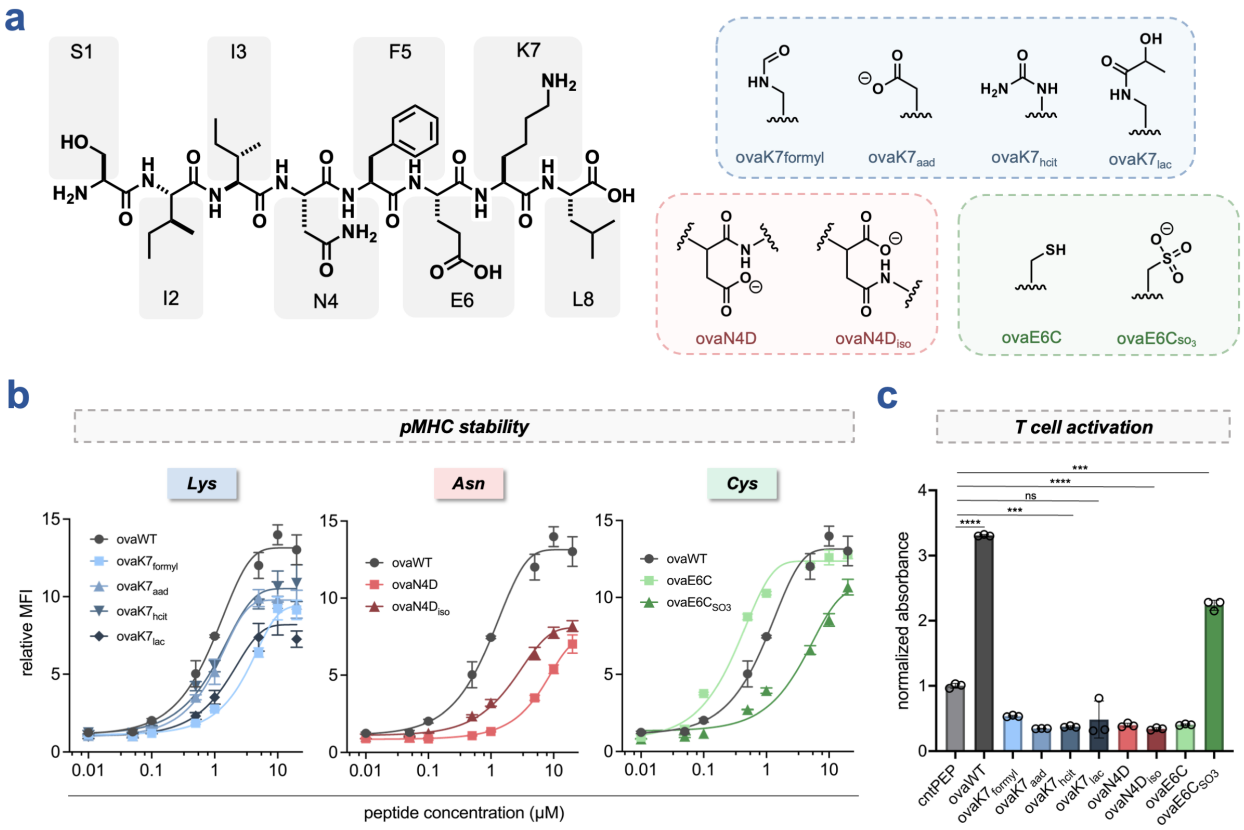



**Figure 2. (a)** Chemical structure of **ovaWT** and associated non-enzymatic PTMs to lysine (blue), asparagine (red), and cysteine (green). **(b)** Dose-response curves from flow cytometry analysis of the RMA-S stabilization assay for non-enzymatic PTM SIINFEKL variants. RMA-S cells were incubated with the indicated concentration of peptides and analyzed via flow cytometry for H-2K$^b$ expression by APC anti-mouse H-2K$^b$ antibody. MFI is the mean fluorescence intensity of the level of fluorescence relative to the negative control peptide (**cntPEP**). Data are represented as mean ± SD ($n$ = 3), and Boltzmann sigmoidal curves were fitted to the data using GraphPad Prism. **(c)** B3Z T cell activation assay. RMA-S cells were incubated with 5 μM of indicated peptides for 1 h at 26°C. RMA-S cells were subsequently co-incubated with B3Z T cells for 6 h at 37°C. 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. The data presented has been normalized to the absorbance of the negative control peptide (**cntPEP**). Data are represented as mean ± SD ($n$ = 3). $P$-values were determined by a two-tailed $t$-test (ns = not significant, *** $p < 0.001$, **** $p < 0.0001$).

**Figure 3**

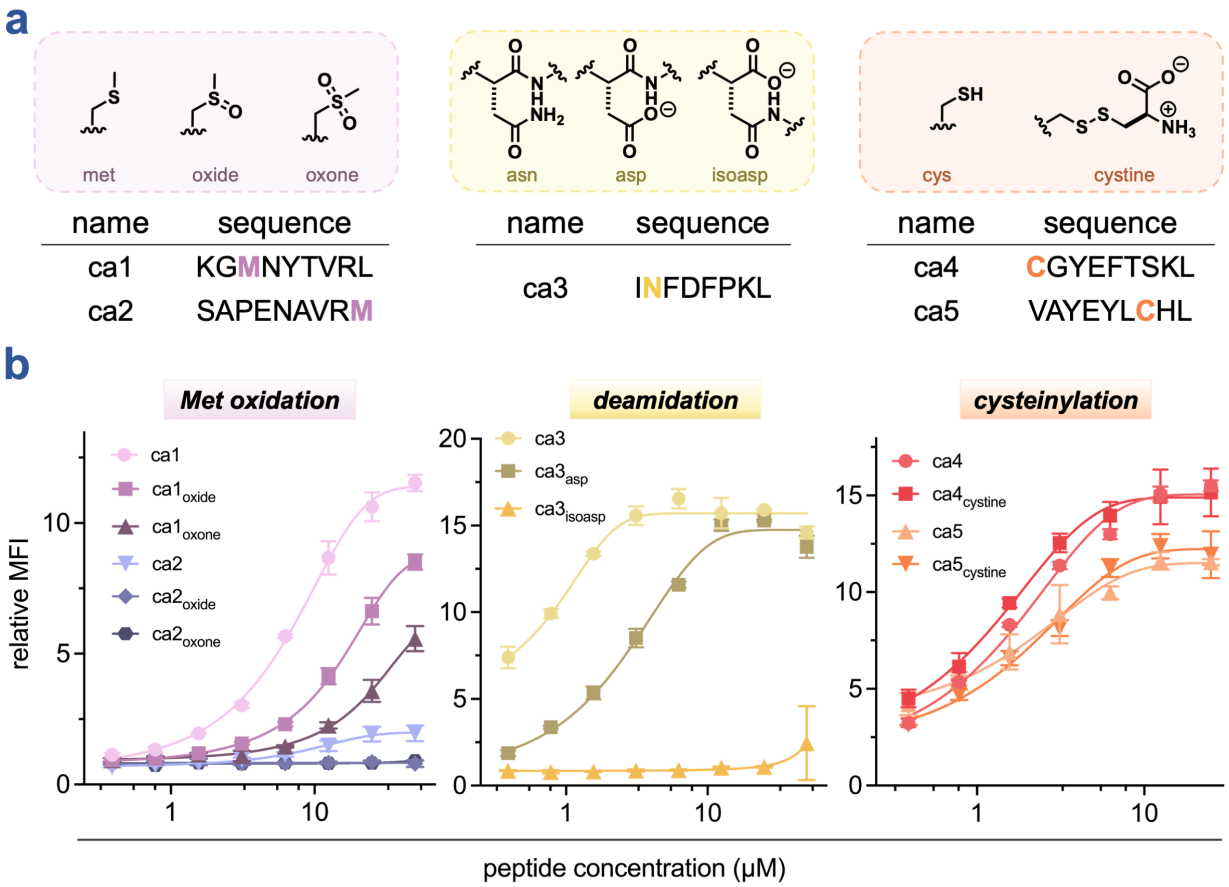



**Figure 3. (a)** Sequences of cancer-associated peptides and respective non-enzymatic PTMs of methionine oxidation (pink), deamidation (yellow), and cysteinylation (orange). **(b)** Dose-response curves from flow cytometry analysis of the RMA-S stabilization assay for non-enzymatic PTM cancer-associated peptides. RMA-S cells were incubated with the indicated concentration of peptides and analyzed via flow cytometry for H-2K$^b$ expression by APC anti-mouse H-2K$^b$ antibody. MFI is the mean fluorescence intensity of the level of fluorescence relative to the negative control peptide (**cntPEP**). Data are represented as mean ± SD ($n$ = 3), and Boltzmann sigmoidal curves were fitted to the data using GraphPad Prism.

**Figure 4**

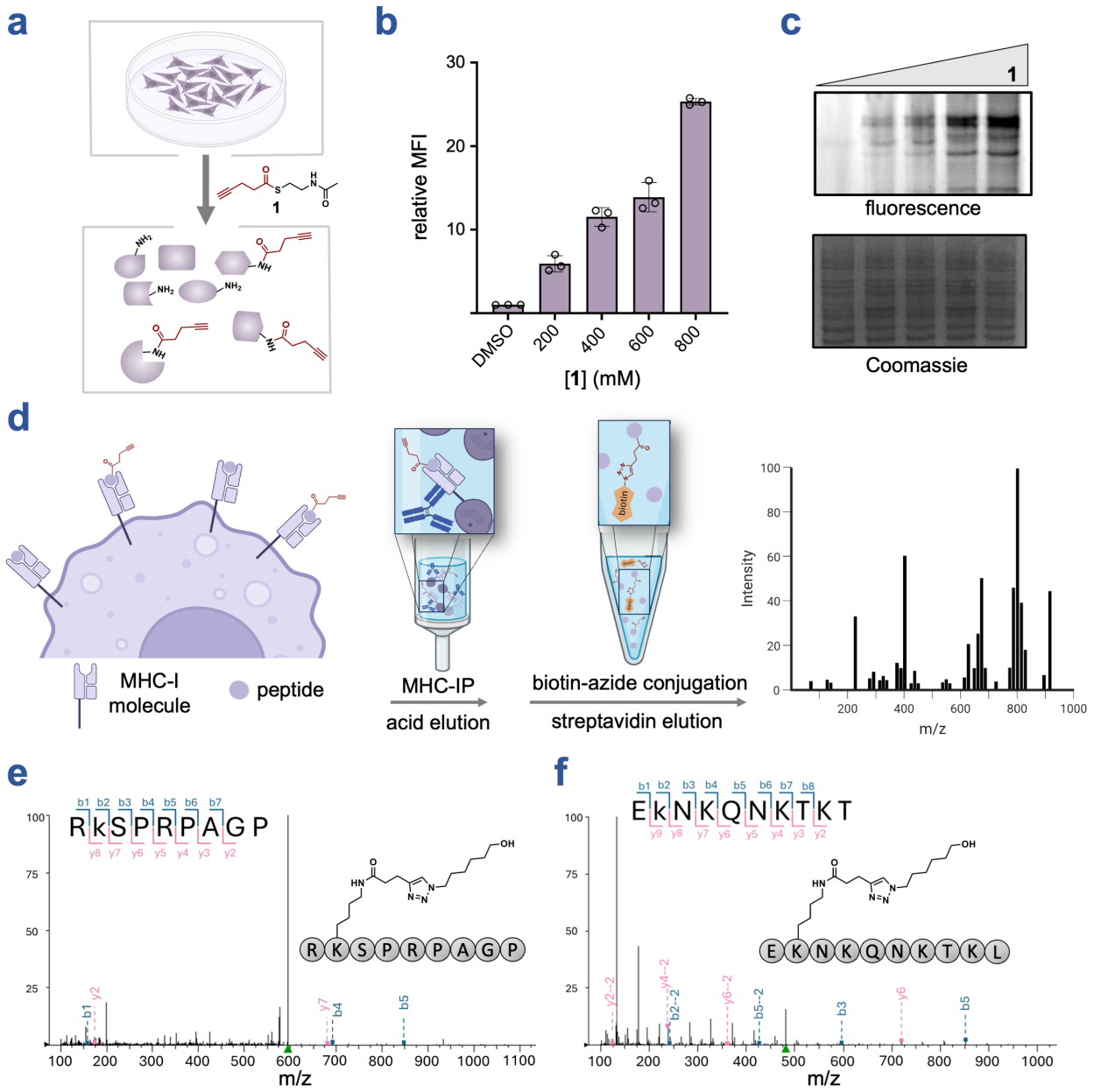
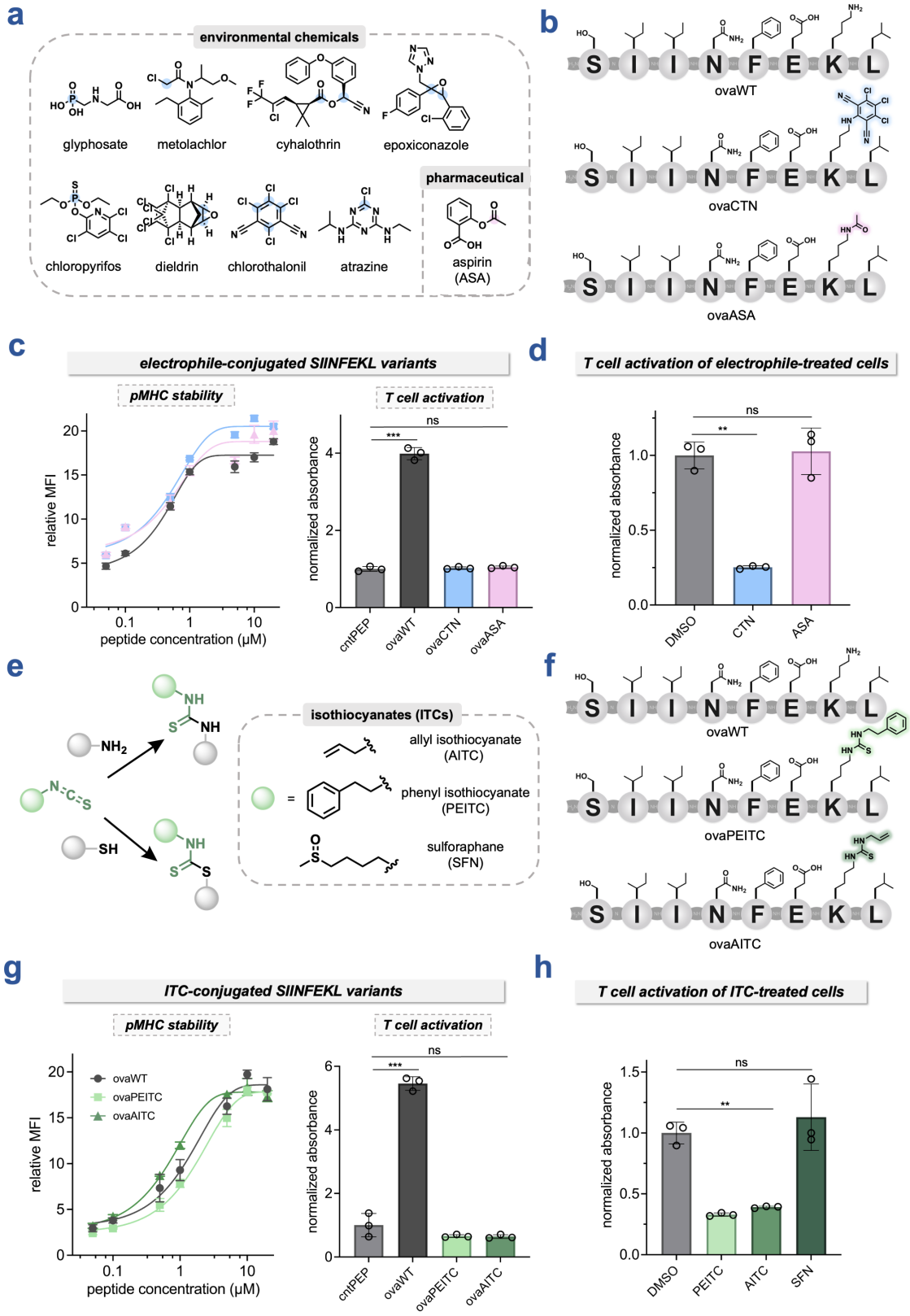



**Figure 4. (a)** Schematic representation of labeling sites of non-enzymatic modifications by probe **1**. **(b)** Flow cytometry analysis of MDA-MB-231 cells treated with the indicated concentration of **1** followed by a reaction with FAM-$N_3$ in the presence of $CuSO_4$, ascorbic acid, and THPTA. MFI is the mean fluorescence intensity of the level of fluorescence relative to the DMSO control. Data are represented as mean ± SD ($n$ = 3) **(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 **(e)** RKSPRPAGP and **(f)** EKNKQNKTKL. Detected b+ ion fragments are shown in blue, and y+ ion fragments are shown in pink.

## Figure 5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environmental chemicals
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chlorothalonil
atrazine
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b
ovaWT
ovaCTN
ovaASA
c
electrophile-conjugated SIINFEKL variants
pMHC stability
T cell activation
relative MFI
peptide concentration (μM)
normalized absorbance
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ovaWT
ovaCTN
ovaASA
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T cell activation of electrophile-treated cells
normalized absorbance
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CTN
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e
isothiocyanates (ITCs)
allyl isothiocyanate (AITC)
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ovaAITC
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PEITC
AITC
SFN

**Figure 5. (a)** Chemical structures of exogenous reactive species, with electrophilic centers are highlighted. **(b)** Chemical structures of **ovaWT**, **ovaCTN**, and **ovaASA**. **(c)** Dose-response curves from flow cytometry analysis of the RMA-S stabilization assay, and T cell activation from B3Z assay for chlorothalonil-modified (**ovaCTN**) and aspirin-modified (**ovaASA**) SIINFEKL variants. For the RMA-S assay, RMA-S cells were incubated with the indicated concentration of peptides and analyzed via flow cytometry for H-2K$^b$ expression by APC anti-mouse H-2K$^b$ antibody. MFI is the mean fluorescence intensity of the level of fluorescence relative to the negative control peptide (**cntPEP**). Boltzmann sigmoidal curves were fitted to the data using GraphPad Prism. For the B3Z T cell activation assay, RMA-S cells were incubated with 5 μM of indicated peptides for 1 h at 26°C. RMA-S cells were subsequently co-incubated with B3Z T cells for 6 h at 37°C. 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. Data for both assays were normalized to the negative control peptide (**cntPEP**) and are represented as mean ± SD ($n$ = 3). $P$-values were determined by a two-tailed $t$-test (ns = not significant, *** $p < 0.001$). **(c)** B3Z T cell activation assay with electrophile-treated B16-OVA cells. IFN-γ-pre-treated B16-OVA cells were incubated with 100 μM of CTN or 1 mM of ASA for 1.5 h, washed, then 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. The data presented has been normalized to the absorbance of the DMSO control. Data are represented as mean ± SD ($n$ = 3). $P$-values were determined by a two-tailed $t$-test (ns = not significant, ** $p < 0.01$). **(e)** Schematic depicting reactivity of isothiocyanate

(ITC) with amines and thiols, and chemical structures of example ITCs. **(f)** Chemical structures of **ovaWT**, **ovaPEITC**, and **ovaAITC**. **(g)** Dose-response curves from flow cytometry analysis of the RMA-S stabilization assay, and T cell activation from B3Z assay for ITC-modified SIINFEKL variants. For the RMA-S assay, RMA-S cells were incubated with the indicated concentration of peptides and analyzed via flow cytometry for H-2K$^b$ expression by APC anti-mouse H-2K$^b$ antibody. MFI is the mean fluorescence intensity of the level of fluorescence relative to the negative control peptide (**cntPEP**). Boltzmann sigmoidal curves were fitted to the data using GraphPad Prism. For the B3Z T cell activation assay, RMA-S cells were incubated with 5 μM of indicated peptides for 1 h at 26°C. RMA-S cells were subsequently co-incubated with B3Z T cells for 6 h at 37°C. 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. Data for both assays were normalized to the negative control peptide (**cntPEP**) and are represented as mean ± SD ($n$ = 3). $P$-values were determined by a two-tailed $t$-test (ns = not significant, *** $p < 0.001$). **(h)** B3Z T cell activation assay with ITC-treated B16-OVA cells. IFN-γ-pre-treated B16-OVA cells were incubated with 100 μM of ITCs for 1.5 h, washed, then 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. The data presented has been normalized to the absorbance of the DMSO control. Data are represented as mean ± SD ($n$ = 3). $P$-values were determined by a two-tailed $t$-test (ns = not significant, ** $p < 0.01$).

**Figure 6**

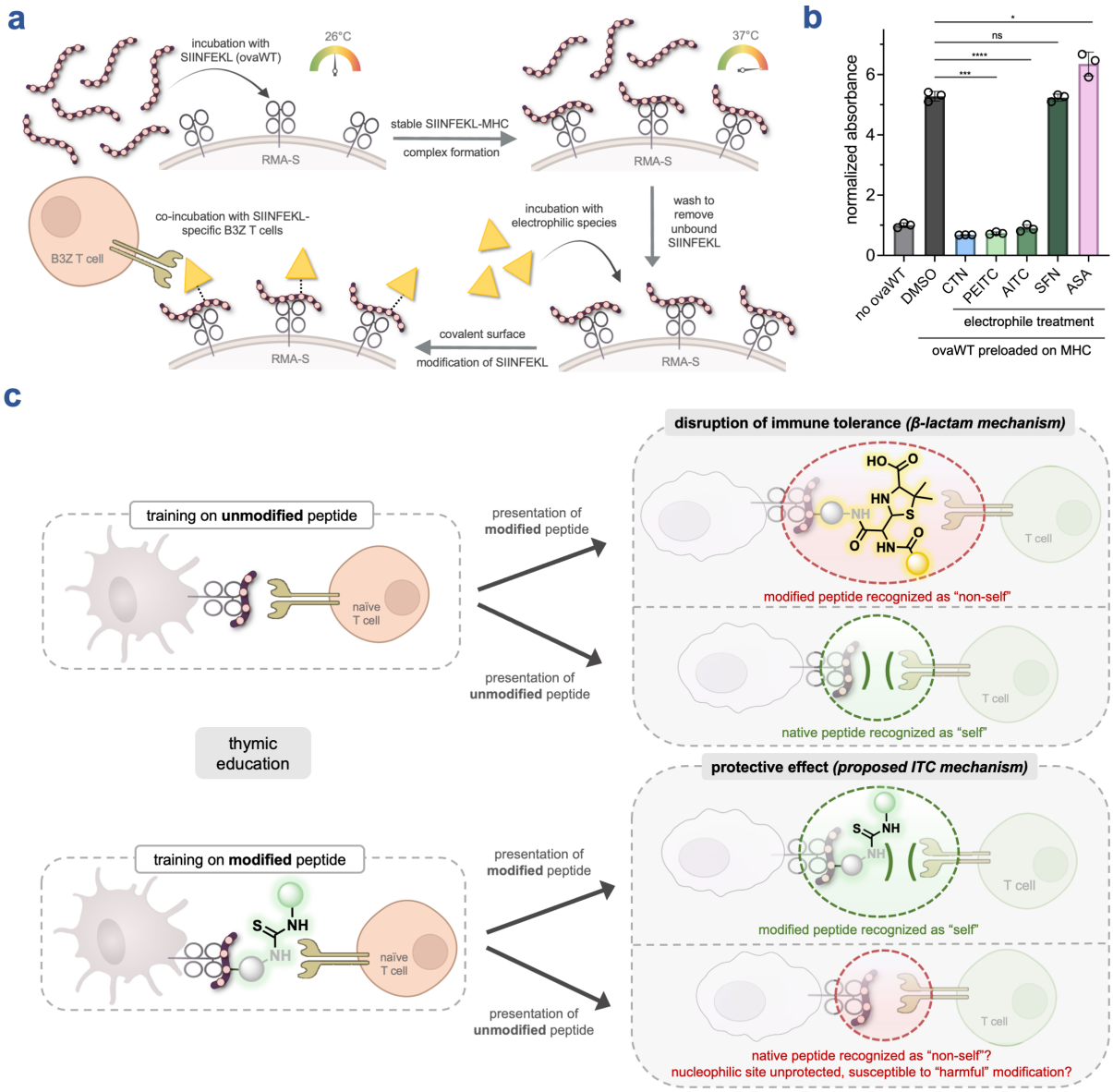


**Figure 6. (a)** Schematic illustrating the B3Z T cell activation assay using RMA-S cells with SIINFEKL pre-loaded on MHC to isolate the impact of exclusively surface modification of MHC-I-presented peptides by electrophilic species. **(b)** B3Z T cell activation assay with electrophile-treated, SIINFEKL-pre-loaded RMA-S cells. RMA-S cells were incubated with 5 μM **ovaWT** for 1 h at 26°C in order to occupy empty MHC on the surface, then washed thoroughly to remove unbound **ovaWT** from the culture media. RMA-S cells were

then treated with 100 μM of electrophilic compounds (except for ASA, which was 1 mM) for 1.5 h, washed, then co-incubated with B3Z T cells for 6 h at 37°C. 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. The data presented has been normalized to the absorbance of the DMSO control. Data are represented as mean ± SD ($n$ = 3). $P$-values were determined by a two-tailed $t$-test (ns = not significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$). **(c)** Proposed model illustrating how "harmful" and "beneficial" electrophilic species may differentially shape immune recognition. In the conventional scenario, T cells are educated during thymic selection to recognize native, unmodified epitopes. Subsequent modification of these epitopes by "harmful" electrophiles, such as environmental toxicants or β-lactam antibiotics, may disrupt immune tolerance, as T cells were originally trained to recognize the unmodified form of the antigen. We propose a hypothetical second scenario, whereby exposure to "beneficial" electrophilic molecules, such as dietary ITCs, can occur during thymic education and may lead to T cell training against a chemically modified epitope. In this model, the modification itself may exert a protective effect by shaping immune tolerance toward the modified antigen. Loss of this modification later in life could therefore generate an antigenic mismatch, in which the unmodified peptide differs from the epitope encountered during T cell education and may be less efficiently recognized.

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