Bioorthogonal Antigens

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Bioorthogonal deprotection on the dendritic cell surface allows chemical control of antigen cross-presentation

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5.1 Introduction

In the process of antigen cross-presentation[1], long polypeptides are taken up by phago-[2], endo-[3], or macropinocytosis[4] and proteolytically degraded inside the cell to octamer or nonamer peptides by a host of different proteases[5]. During processing, the polypeptides pass through a series of organelles[1] to end up loaded on major histocompatibility complex class I (MHC-I) receptors for immune surveillance by $CD8^+$ T cells[6] (Figure 1A). This process is essential for both self-tolerance and priming of $CD8^+$ T cells against virus-infected and malignantly transformed self-cells[7] and is therefore of pivotal importance, for example, in cancer immunotherapy[8]. The biochemistry of antigen cross-presentation is complex[1]: different organelles, channels, and chaperones have been implicated in the routing of the antigen, and many proteases are involved in the proteolytic liberation of the epitope peptides during this routing[9] (see chapter 2). This chapter concerns the development of a new method for studying this process that would allow chemical control over the final activation step while causing only minimal structural alteration of the epitope[10]. Organic azides are the most extensively used bioorthogonal group[11]. They have been incorporated into glycoproteins[12], polypeptides[13], and lipids[14] in bacteria[15], eukaryotes[16], and metazoans[17]. Azides are readily incorporated by hijacking the

cell's biosynthetic machinery[18] with minimal structural perturbation to the biomolecule and minimal cytotoxicity. Three different bioorthogonal reactions exist for ligating this handle: Staudinger–Bertozzi ligation[12], copper-catalyzed [3+2] Huisgen cycloaddition (ccHc)[19], and strain-promoted [3+2] cycloaddition (SPC) reactions[20]. Owing to their versatility, stability, and ease of use, azides have become the functional group of choice for *in vivo* bioorthogonal chemistry[21]. However, one aspect of the azide that has been relatively underexplored to date is its function as a bioorthogonal protecting group for amines. Here a different use of the azide is described: not for ligation, but instead as a bioorthogonal protecting group to mask the amine groups in a $CD8^+$ T cell epitope and render it unrecognized by its cognate T cell. Combining this "latent epitope" with on-surface deprotection chemistry would liberate the native epitope and thus activate the T cell (Figure 1B). This approach offers advantages over existing methods that employ photocaged epitopes[22] as very low conversions into the native antigen are observed in this approach[22b]. Furthermore, photocaged epitopes have not been shown to be compatible with intracellular processing and routing. This study herein reports that masked epitopes bearing organic azides are 1) cross-presented by antigen-presenting cells (APCs) with near-equal efficiency compared to their native counterparts, and 2) are unmasked with high efficiency by a Staudinger reduction to yield a fully operational MHC-I/peptide epitope complex.

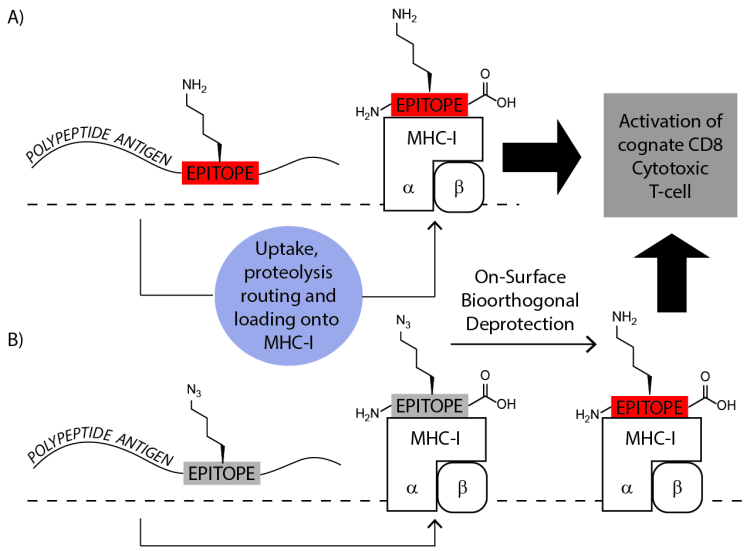


Figure 1. *A) Cross-presentation of a polypeptide antigen. During this process the polypeptide is taken up and routed to the MHC-I loading complex. Meanwhile the polypeptide is degraded to liberate an 8-9-mer epitope peptide that is loaded. B) Processing and presentation of azide-protected latent epitopes. Azido-antigens are processed and presented on MHC-I as normal, but not recognized by the epitope-specific cognate T cell clone. Only after an on-cell deprotection is the native epitope liberated and the T cell activated.*

5.2 Results and discussion

The H2-K^b-restricted immunodominant epitope from chicken egg white ovalbumin $OVA_{257-264}$ (OT-I, SIINFEKL; Figure 2A) was chosen as a starting epitope for modification.

This extensively studied epitope has three residues that mediate the interaction with its cognate TCR[23]: P4, P6, and P7. Four other residues ensure MHC-I anchoring[24]: P2, P3, P5, and P8. It can be thus anticipated that a chemical mutation of Lys_{263} (P7) to an azidonorleucine (ANL; Figure 2B) would strongly reduce T cell recognition while minimally affecting MHC-I binding. Mutation of this residue to alanine had a minor effect on peptide/MHC-I stability, but reduced T cell recognition of cognate clones by a factor of 100–1000[24]. The OT-I epitope peptide and a variant peptide bearing a Lys to ANL substitution (OT-Az; Figure 2B) was synthesized and the recognition of this epitope by an OT-I- specific T cell was assessed. Further the fact that high-affinity epitopes can be loaded onto receptive MHC-I complexes on the surface of APCs by simple co-incubation was exploited[25] and the antigenicity of the OT-I and OT-Az peptides using the immortal LacZ-containing reporter T cell line, B3Z[26] was measured. This T cell line allows the quantitation of T cell activation through monitoring of the β-galactosidase- mediated conversion of a fluorogenic substrate[27]. H2-K^b-positive bone-marrow- derived dendritic cells (BMDCs) were used as the APCs[28]. After peptide loading and overnight incubation with B3Z, no T cell activation by OT-Az at concentrations as high as 10 µM (Figure 2C) was observed. This represents a reduction in T cell activation by more than five orders of magnitude, which underscores the key role of the lysine *ε*-amino group for OT-I recognition by the T cell.

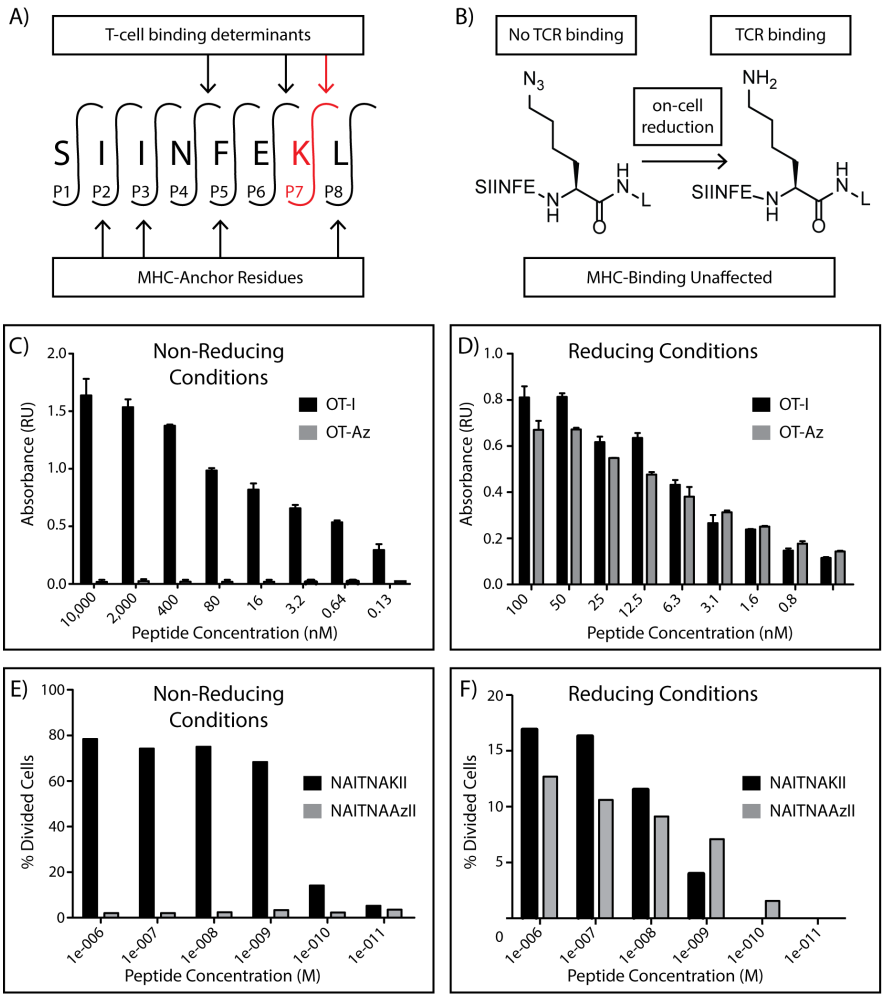


Figure 2. *Chemical unmasking of azido epitopes restored T cell activation. A) Certain residues are key for anchoring to MHC-I and others are key T cell recognition determinants. B) Lysine at P7 was chosen as the target residue for masking: converting the cognate epitope OT-I into the azido analogue OT-Az was postulated to prevent T cell recognition while minimally affecting MHC-I binding. C) OT-Az is indeed not recognized by B3Z T cells. D) Upon reduction with TCEP (100 mM), OT-Az is converted into an epitope that is recognized by B3Z. E) The activation of $D^bM_{187\text{-}195}$-specific transgenic $CD8^+$ T cells by NAITNAKII or NAITNAAzII follows a similar trend: Azido epitopes were not recognized. F) After reduction as above, the recognition of NAITNAAzII was restored.*

To assess the potential of the azide moiety as a bioorthogonal protecting group, the Staudinger reduction—the aqueous reduction of azides by trivalent phosphorus species was explored as a possible bioorthogonal deprotection reaction[29]. The biocompatibility of this reaction was established by the group of Bertozzi, who showed that tris(2-carboxyethyl) phosphine hydrochloride (TCEP) partially reduces azido groups on mammalian cell surfaces[12]. A series of phosphorus reagents were screened for their ability to reduce azides (Supporting Table 1 (S1)). Interestingly, when the phosphine-mediated reduction of the azide with TCEP was monitored

(Figure S1A), the azide was observed to disappear almost completely within the first 20 minutes. Alongside the formation of the expected OT-I epitope, the conversion of the azide into the primary alcohol was also observed by NMR spectroscopy. Its formation may be due to nucleophilic substitution of the intermediate iminophosphorane group by water. The formation of a small amount of alkene, as detected by LC-MS, is consistent with the idea that the iminophosphorane can also serve as a leaving group. Treatment of OT-Az with bulkier and less nucleophilic triphenylphosphine-3,3',3''-trisulfonic acid proceeded sluggishly (Figure S1B) and did not yield any OT-I; instead, an approximately 2:1 mixture of OT-OH and the alkene was formed. In future, the study of more reactive water-soluble phosphines, such as those containing alkyl sulfonates, PEGylated variants as well as other azide reducing agents might be considered. Finally the on-cell TCEP-mediated unmasking of the OT-Az epitope on BMDCs was performed. It has been found that 100 mM TCEP resulted in optimal on-surface deprotection (Figure 3A). Unmasking appeared to be complete within a reaction time of 15 minutes (Figure 3B).

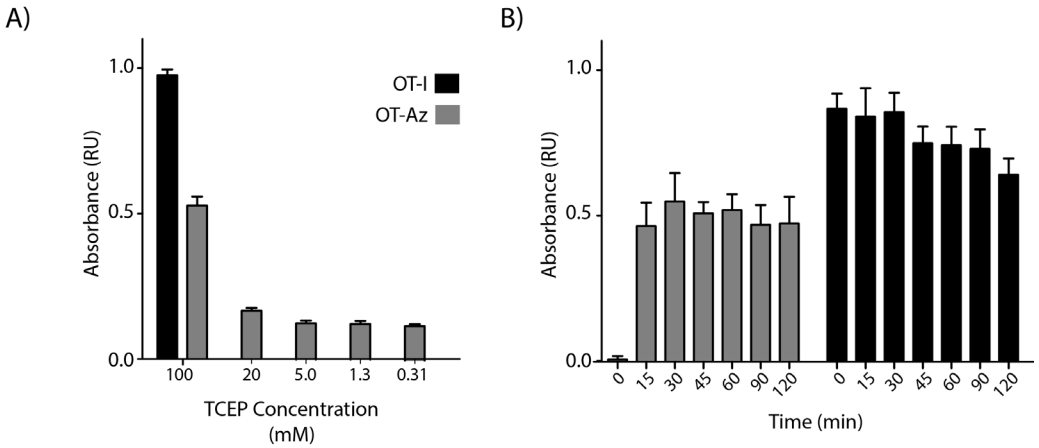


Figure 3. *A) B3Z Tcells response to OT-I (10 nM) and OT-Az (10 nM) in presence of the reducing agent TCEP for 30 minutes at the indicated concentrations. B) reduction of OT-Az and OT-I (10 nM) with TCEP (100 mM) for indicated time periods. Reduction was complete within 15 minutes.*

Under these conditions (100 mM TCEP, 30 min), the T cell reactivity on the cell surface of BMDCs was fully rescued at dose-limiting peptide concentrations. At high concentrations, partial rescue was observed (>80%, Figure 2D), which could be due to inefficient conversion at these concentrations or competition of the aforementioned side reactions, which leads to unrecognized side products. Effects that are due to toxicity were ruled out (Table S1) as pH-adjusted TCEP was found to be non-toxic to BMDCs at the concentrations and reaction times required for on-surface unmasking (viability >98%; Table S1). To exclude artifacts stemming from the specific epitope

(OT-I) and the specific MHC-I haplotype (H2-K^b), a second epitope and MHC-I haplotype was also tested: the $D^bM_{187\text{-}195}$ epitope from respiratory syncytial virus (RSV)[30]. RSV is the main causative agent of respiratory failure in infants, and the role of $CD8^-$ mediated T cell immunity remains somewhat controversial. $D^bM_{187\text{-}195}$ is a dominant epitope in C57BL/6 mice[31] and a highly functional subdominant epitope in CB6F1 mice[30]. The D^b-binding NAITNAKII nonamer is critically dependent on Lys_{193} for T cell recognition[32], thus masking of this residue would presumably ablate T cell recognition similarly to the OT-I epitope. Residue 193 was therefore subjected to a chemical mutation from Lys to ANL (NAITNAAzII). Masking successfully prevented recognition of the ANL-variant peptide by T cell receptor transgenic $CD8^+$ T cells specific for the $D^bM_{187\text{-}195}$ epitope, even at high peptide concentrations (up to 1 µM tested, Figure 2E). Upon addition of TCEP, T cell recognition was restored to a similar extent as for OT-Az/OT-I (Figure 2F). These results indicate that the azide group can indeed be used to generate masked epitopes and that the unmasking reaction can be chemically controlled and proceeds with good yields. However, the pivotal aim was to develop a reagent that could be used to unmask antigens after intracellular processing, to allow the separation of intracellular cross-presentation kinetics and on-cell pMHC dynamics. To study whether this approach was compatible with the biochemistry that an antigen encounters during cross-presentation, long peptides containing either the OT-I or OT-Az epitopes (LP-I and LP-Az; Figure 4A) were synthesized. Subsequently, these long peptides were added to BMDCs and after 3 hours, they were subjected to a reduction with TCEP. The cells were washed prior to addition of B3Z T cells for immune surveillance. No intracellular reduction of the azide to the corresponding amine was observed during cross-presentation (Figure 4B). When TCEP was added after the addition of one of the peptides, full T cell reactivity against OT-I could be recovered at low peptide concentrations (Figure 4C). A marked reduction in rescue was observed (>50% rescue) at higher peptide concentrations, which could in part be explained as before, and in part be due to minor differences in processing efficiency resulting from the amine-to-azide modification.

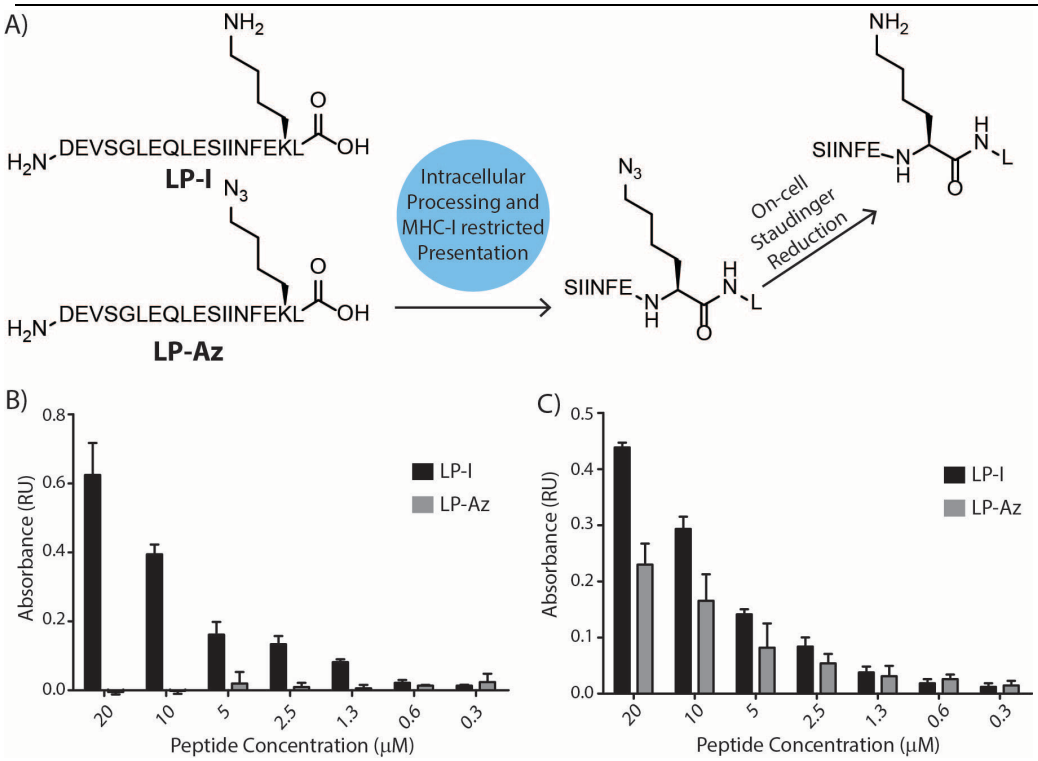


Figure 4. *Presentation of long peptides to B3Z-hybridoma. A) Design of long peptides for studying the suitability of azido epitopes as latent antigens for intracellular routing. B) Intracellular routing of LP-Az resulted in no activation of the OT-I cognate B3Z T cell clone. C) Reduction with TCEP three hours after the initial peptide addition resulted in partial recovery of T cell activation.*

In summary, the results have demonstrated that organic azides are not only valuable bioorthogonal ligation handles, but are equally applicable to bioorthogonal protection. This phenomenon was exploited to produce latent epitopes that enabled the controlled activation of epitopes on the surface of APCs after uptake, intracellular routing, and proteolysis for the first time. Azide-masked epitopes represent a powerful new approach for the study of antigen cross-presentation. They are mutually orthogonal to photocaged epitopes[22b, 33]. Applying this approach to whole protein antigens would also offer an interesting comparison of the presentation kinetics of these different antigen classes. The chemical unmasking of a bioorthogonal group using a Diels–Alder reaction on a whole protein can be envisaged to be of use to this approach[34], although—like the photouncaging reaction— it employs a bulky protecting group, which may preclude normal intracellular routing and proteolytic processing. The main limitation of this approach is that it is currently limited to epitopes with lysine at key positions for T cell recognition. The application of other bioorthogonal reactions to mask other natural epitopes would broaden the scope of this approach and offer even further additions to the immunologist's toolkit, as it

allows the separation of early and late-appearing antigens for the first time, which would allow the determination of the contribution of such populations to the overall immune response.

Despite the above application of this reagent for studying for example early appearing antigen, the importance laid in the interest in the study of later-appearing peptide-MHC-I complexes. Therefore a reaction that could permanently block the latent epitopes early in the immune response was sought. For this, other azide chemistry, namely the biocompatible strain-promoted alkyne-azide [3+2] cycloaddition reactions (SPAAC)[20] was applied. These reactions (Figure 5A) can selectively form a new triazolyl-species in a bioorthogonal fashion at the cell surface[21]. The potential of the SPAAC-reaction was studied by incubating OT-Az-pulsed BMDCs with bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN-NH_2)[35], as this strained alkyne has the most favorable properties with respect to aspecific binding[27]. First, the BMDCs were OT-Az pulsed for 1 hour followed by reaction with BCN-NH_2. After subsequent reduction with TCEP, it was found that BCN-NH_2 prevented uncloaking by TCEP (Figure 5B). Antigen presentation of OT-I peptide-pulsed BM-DCs treated under these same conditions was unaffected. Next this sequence was applied to the study of long peptide antigen processing. BMDCs were pulsed for 3 hours with LP-1 or LP-Az, followed by a blocking with BCN-NH_2 at the end of this 3 hour pulse period followed by a reduction (Figure 5C). This sequence showed that presentation of OT-Az could be blocked by this reagent without affecting routing and presentation of LP-Az.

To test whether this reaction could be used to isolate the contribution of late appearing antigen during the immune response against the OVA-long peptide, the BMDCs were first pulsed for 3 hours (no immune responses were observed with shorter pulses of LP-I) followed by the blocking step with BCN-NH_2. Reductions were then performed at different time points after this initial blocking step (Figure 5D). Strikingly, no new antigen appeared after the initial 3 hour block, suggesting a rapid burst in processing kinetics.

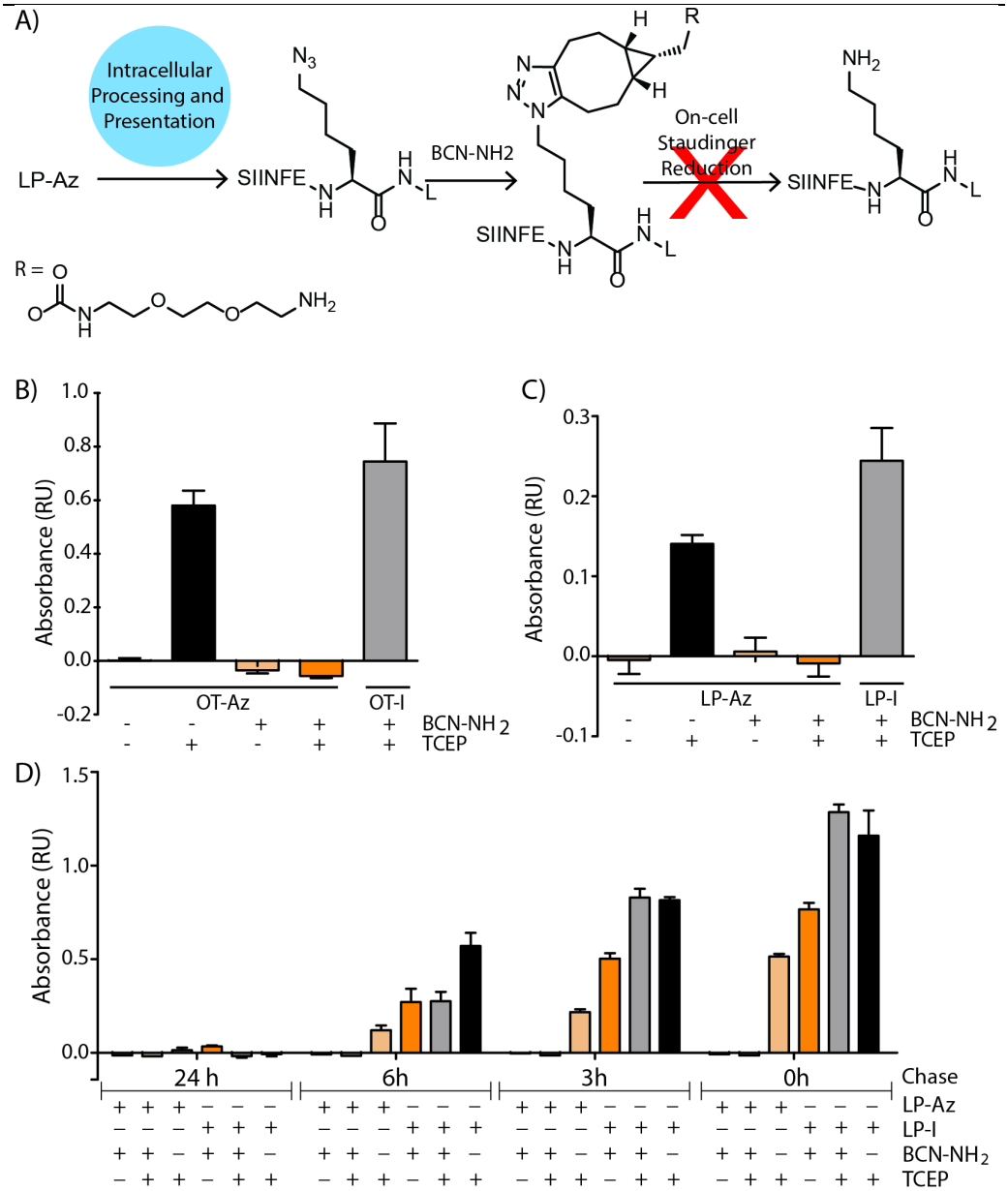


Figure 5. *Latent epitopes allow temporal separation of antigen populations. A) A strain-promoted alkyne-azide [3+2] cycloaddition reactions (SPAAC) strategy. These reactions can selectively form a new triazolyl-species in a bioorthogonal fashion at the cell surface by incubating OT-Az-pulsed BMDCs with bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN-NH$_2$). B) BCN-NH$_2$ prevented uncloaking of OT-Az by TCEP. C) BCN-NH$_2$ prevented uncloaking of LP-Az by TCEP. D) Reductions performed at different time points after this initial blocking step. No new antigen appeared after the initial 3 hour block, suggesting a rapid burst in processing kinetics.*

5.3 Conclusion

These results demonstrate that azide-modified epitopes are a powerful new class of reagents for the study of antigen cross-presentation. Not only can they be uncloaked in robust yields on the surface of cells to reveal lysines, they can also be used to visualize the presence of peptide MHC-I on the cell surface. In the future, it would be exciting to use these peptides with the previously reported photocaged epitopes[22a], if an increase in on-cell uncaging yields can be achieved for this approach[22b]. Also, the incorporation of these handles into protein antigens, or the combination with the recently reported chemical uncloaking of a bioorthogonal handle that was incorporated into a whole protein[36] would allow further expansion of this approach.

5.4 Supporting table and figure

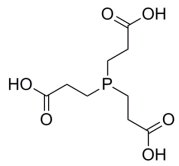
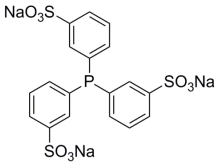
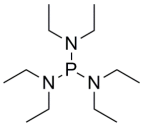
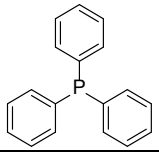
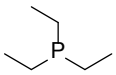
Name	Structure	Solubility in PBS			Incubation Time (h)	Percentage viable Cells			
Tris-(2-carboxyethyl) phosphine (**TCEP**)		500 mM	100 mM	10 mM		100 mM	10 mM	1 mM	0 mM
		Yes	Yes	Yes	0.5	98	94	96	94
					1.0	94	93	94	98
					1.5	57	89	95	99
					2.0	1	89	93	97
Triphenyl-phosphine-3,3',3''-trisulfonic acid (**TPPTS**)		500 mM	100 mM	10 mM		100 mM	10 mM	1 mM	
		Yes	Yes	Yes	0.5	98	91	99	
					1.0	98	99	98	
					1.5	87	99	99	
					2.0	0	99	99	
Sodium hypo-phosphite (**SHP**)		500 mM	100 mM	10 mM		100 mM	10 mM	1 mM	
		Yes	Yes	Yes	0.5	97	99	93	
					1.0	100	98	99	
					1.5	97	99	99	
					2.0	97	99	99	
Insoluble phosphine also tested									
Tris-(diethyl amino)-phosphine (**TDAP**)		Triphenyl Phosphine (**TPP**)				Triethyl Phosphine (**TEP**)			

Table S1. *Overview of all phosphines tested in this study.*

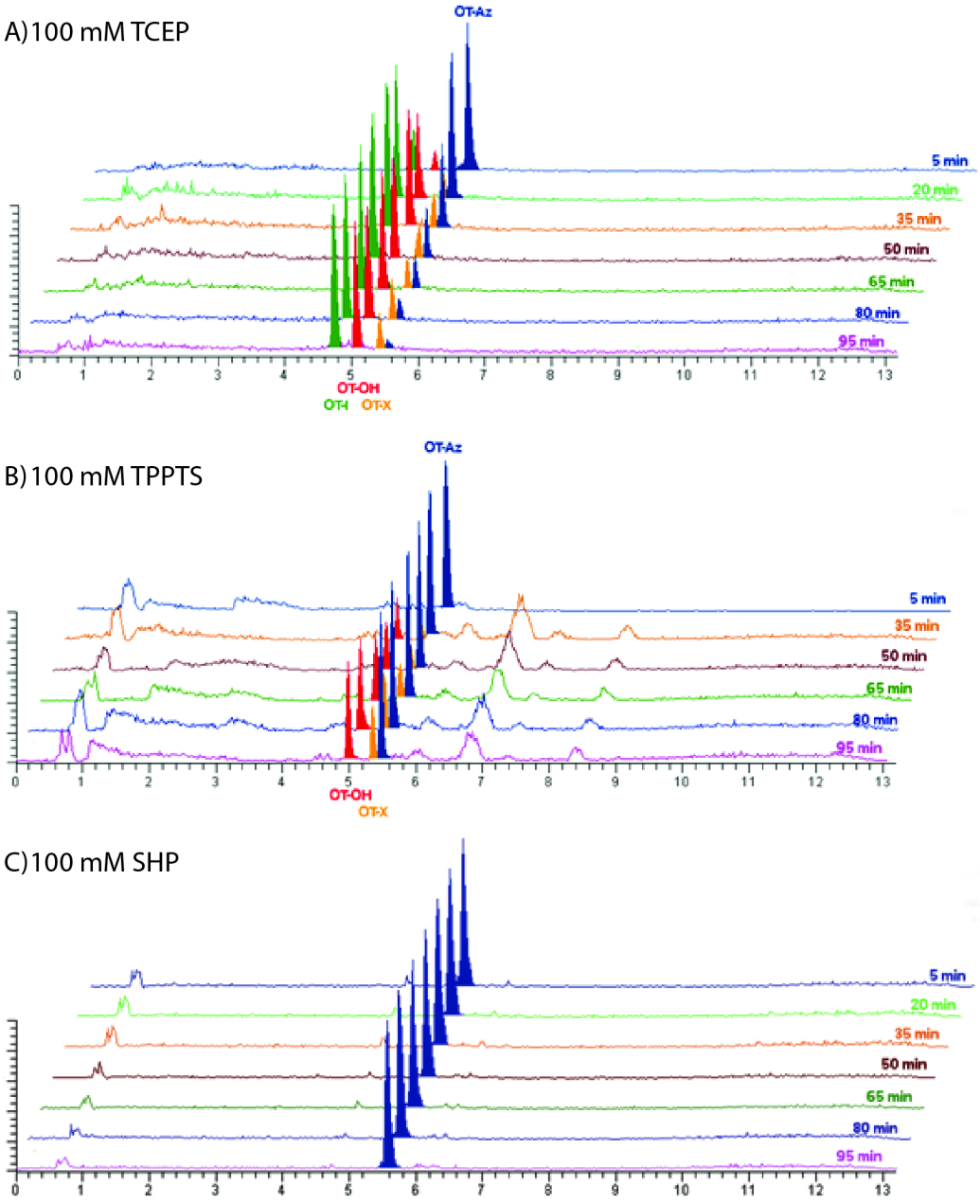


Figure S1. *0.1mM OT-Az solution (in PBS) was reduced with 100 mM of phosphines. The reaction was performed at room temperature and monitored every 15 minutes using LCMS. A) Reduction with 100 mM TCEP. B) Reduction with 100 mM TTPTS. C) Reduction with SHP. LCMS indicated the formation of 2 major side products: OT-OH – the ε-hydroxylysine - and OT-X – the δ-ε-alkene, the result of elimination via an E2 mechanism.*

5.5 Experimental section

Reagents:
Solvents used for column chromatography were of technical grade from Sigma Aldrich and used directly. Chemicals tris(2-carboxyethyl)phosphine, triphenyl-phosphine-3,3',3''-trisulfonic acid, sodium hypophosphite, tris-(diethylamino)-phosphine, streptomycin, chlorophenol red-β-D-galactopyranoside, IMDM-medium were purchased from Sigma Aldrich and used without further purifications. OT-I was purchased from Invivogen. Endotoxin-free PBS was Gibco-brand purchased from Life Technologies. GM-CSF was purchased from ImmunoTools and hygromycin B from AG technologies.

HPLC kinetics:
In 700µL of PBS was added 100µL of 1mM SIINFEAzL solution in PBS. Then 200µL of 0.5M solution of desired phosphine and the reaction was performed at room temperature without stirring. Monitoring the reaction was done using LCMS every 15 min. For LC-MS analysis a JASCO HPLC-system (detection simultaneously at 214 and 254 nm) equipped with an analytical C18 column (4.6 mmD × 50 mmL, 3µ particle size) in combination with buffers A: H2O, B: MeCN and C: 0.5% aq. TFA and coupled to a PE/SCIEX API 165 single quadruple mass spectrometer (Perkin-Elmer) was used.

Bone marrow derived dendritic cells:
BMDCs were generated from B57BL/6 mice bone marrow essentially as described[37] with some modifications. Briefly, bone marrow was flushed from femurs and tibia and cells were cultured in IMDM supplemented with 8% heat-inactivated fetal calf serum, 2mM glutamax, 20µM 2-Mercaptoethanol, penicillin 100 l.U./mL and streptomycin 50µg/mL in the presence of 20ng/mL GM-CSF. Medium was replaced on day 3 and 7 of culture and the cells were generally used between days 10 and 13.
B3Z-hybridoma culturing: The $OVA_{257-264}$-specific, H-$2K^b$-restricted CTL hybridoma, B3Z[26] was cultured in IMDM medium supplemented with 10% FCS, 2mM glutamax, 0.25mM 2-Mercaptoethanol, penicillin 100 l.U./mL and streptomycin 50µg/mL in the presence of hygromycin B (500µg/mL)[38].

Antigen presentation assays:
BMDCs were plated in 96-well tissue-culture treated microtiterplates ($5x10^4$ cells/well) for 1h and allowed to adhere at 37 °C for 1 h prior the addition of peptides at the indicated concentrations. BMDCs were incubated with the peptides for the indicated times (usually 1h for minimal epitopes and 3h for SLPs), followed by a wash with complete IMDM. Peptide-pulsed BMDCs were then treated with 100 mM TCEP in 1% fetal calf serum for 1h at 37°C. After removal of the reduction medium, the cells were washed with complete IMDM and resuspended in 100 µL/well cIMDM before the addition of the T cell hybridoma B3Z cells ($5x10^4$ cells/well). The BMDCs and T cells were co-cultured for 17 h at 37°C. Stimulation of the B3Z hybridoma was

measured by a colorimetric assay using CPRG (chlorophenol red-β-D-galactopyranoside) as a substrate as described[39].

Mixed splenocyte T cell activation assay:
To assess the ability of T cells to respond to the natural Db-binding NAITNAKII peptide of RSV or the chemically-modified NAITNAAzII peptide, splenocytes were isolated from CB6F1 (Db-bearing) mice using Fico/Lite-LM. Following isolation, splenocytes were incubated with the indicated concentrations of the natural or modified peptide for one hour at 37°C in R-10 (RPMI supplemented with 10% fetal bovine serum, 2mM L-Glutamine, 1mM sodium pyruvate, non-essential amino acids, 25mM HEPES, 5x10-5M β-mercaptoethanol and pen/strep antibiotics) prior to washing and incubating in either PBS with 1% serum (control), or 100mM TCEP in PBS/1% serum. After an additional hour at 37°C, the splenocytes were washed with media, and cocultured with CFSE-labeled $D^bM_{187\text{-}195}$-specific transgenic $CD8^+$ T cells isolated using an untouched $CD8\alpha^+$ T cell isolation kit (MiltenyiBiotec) as previously described[40]. Following three days of culture, samples were stained for $CD8^+$, $CD3^+$, and viability as previously described[40], and the percent of the labeled transgenic $CD8^+$ T cell population that divided was determined using the proliferation module of FlowJo 9.7.4.

Cell viability assay:
BMDCs were plated into 24-well tissue-culture treated flat bottom transparent plate ($3x10^5$ cells/well) and allowed to adhere for 1 hour at 37°C. The cells were incubated with different phosphines at the indicated concentration and time (½ hour, 1h, 1½ hour and 2h) in 1% fetal calf serum in PBS at 37°C. After removal of the phosphine solutions, the cells were washed with cIMDM and incubated for 2h at 37°C before the addition of propidium iodide (2µg/mL) and Hoechst 33258 (2µg/mL) and incubation for 15min at RT. The cells were imaged on Olympus IX81 using 4x objective. 10 images were collected per condition and counted for Hoechst 33258 and propidium iodide analyzed with LASAF software and live-dead cell counting was performed automatically using the particle counting functionality in the ImageJ analysis software.

Peptide synthesis:
Peptides were synthesized using standard Fmoc Solid Support Chemistry and purified using high performance liquid chromatography (Prep column Gemini C18 110A 150x21.20 5µm) using 15 to 45 % gradient (A: 0.1% TFA in MilliQH2O, B: ACN). LC-MS measurements were done on an API 3000 Alltech 3300 with a Grace Vydac 214TP 4,6 mm x 50 mm C4 column.

5.6 References

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