Antigens Hot Paper

Deutsche Ausgabe: DOI: 10.1002/ange.201500301
Internationale Ausgabe: DOI: 10.1002/anie.201500301

Bioorthogonal Deprotection on the Dendritic Cell Surface for Chemical Control of Antigen Cross-Presentation**

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Abstract: *The activation of $CD8^+$ T-cells requires the uptake of exogenous polypeptide antigens and proteolytic processing of these antigens to octamer or nonamer peptides, which are loaded on MHC-I complexes and presented to the T-cell. By using an azide as a bioorthogonal protecting group rather than as a ligation handle, masked antigens were generated—antigens that are not recognized by their cognate T-cell unless they are deprotected on the cell using a Staudinger reduction.*

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.[5,6] 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 (Figure 1 A).[7] This process is essential for both self-tolerance and priming of $CD8^+$ T-cells against virus-infected and malignantly transformed self-cells[8] and is therefore of pivotal importance, for example, in cancer immunotherapy.[9] 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.[10] Our aim was to develop a new method for studying this process that would give us chemical control over the final activation step while causing only minimal structural alteration of the epitope.[11]

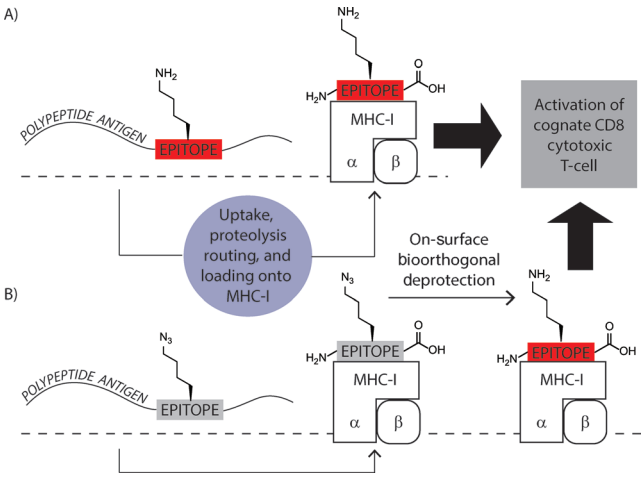

Figure 1. A) Cross-presentation of a polypeptide antigen. B) Processing and presentation of azide-protected latent epitopes.

Organic azides are the most extensively used bioorthogonal group.[12] They have been incorporated into glycoproteins,[13] polypeptides,[14] and lipids[15] in bacteria,[16] eukaryotes,[17] and metazoans.[18] Azides are readily incorporated by hijacking the cell's biosynthetic machinery[19] with minimal structural perturbation to the biomolecule and minimal cytotoxicity. Three different bioorthogonal reactions exist for ligating this handle: Staudinger–Bertozzi ligation,[13] copper-catalyzed [3+2] Huisgen cycloaddition (ccHc),[20] and strain-promoted [3+2] cycloaddition (SPC) reactions.[21] Owing to their versatility, stability, and ease of use, azides have become the functional group of choice for in vivo bioorthogonal chemistry.[22] However, one aspect of the azide that has been relatively underexplored to date is its function as a bioorthogonal protecting group for amines.

We envisaged an approach whereby we could use the azide 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 unmasking would liberate the native epitope and thus activate the T-cell (Figure 1 B). This approach offers advantages over existing methods that employ photocaged epitopes[23,24] as very low conversions into the native antigen are observed in this approach.[23a] Furthermore, photocaged epitopes have not been shown to be

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[**] G.P.P.G., F.A.O., and D.V.F. received funding from the Netherlands Organization for Scientific Research. S.I.v.K. was supported by a Veni grant from the Netherlands Organization for Scientific Research (700.59.402) and an ERC Starting Grant (639005). T.J.R. is a member of the Viral Pathogenesis Laboratory (Barney Graham/NIAID/VRC) and is supported by intramural NIAID funding.

Supporting information for this article, including the conditions for peptide synthesis and characterization and the immune assays, is available on the WWW under http://dx.doi.org/10.1002/anie.201500301.

compatible with intracellular processing and routing. We herein report 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.

We chose the H2-K^b-restricted immunodominant epitope from chicken egg white ovalbumin $OVA_{257-264}$ (OT-I, SIINFEKL; Figure 2A) as our starting epitope for modification. This extensively studied epitope has three residues that mediate the interaction with its cognate TCR:[25] P3, P6, and P7. Three further residues ensure MHC-I anchoring:[26] P2, P5, and P8. We envisaged 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.[26a]

We synthesized the OT-I epitope peptide and a variant peptide bearing a Lys to ANL substitution (OT-Az; Figure 2B) and assessed the recognition of this epitope by an OT-I-specific T-cell. We exploited the fact that high-affinity epitopes can be loaded onto receptive MHC-I complexes on the surface of APCs by simple co-incubation.[27] We studied the antigenicity of the OT-I and OT-Az peptides using the immortal LacZ-containing reporter T-cell line, B3Z.[28] This T-cell line allows the quantitation of T-cell activation through monitoring of the β-galactosidase-mediated conversion of a fluorogenic substrate.[29] H2-K^b-positive bone-marrow-derived dendritic cells (BM-DCs) were used as the APCs.[30] After peptide loading and overnight incubation with B3Z, we observed no T-cell activation by OT-Az at concentrations as high as 10 μM (Figure 2C). 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.

To assess the potential of the azide moiety as a bioorthogonal protecting group, we explored the Staudinger reduction—the aqueous reduction of azides by trivalent phosphorus species—as a possible bioorthogonal deprotection reaction.[31] 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.[13] We screened a series of phosphorus reagents for their ability to reduce azides (Supporting Information, Figure 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

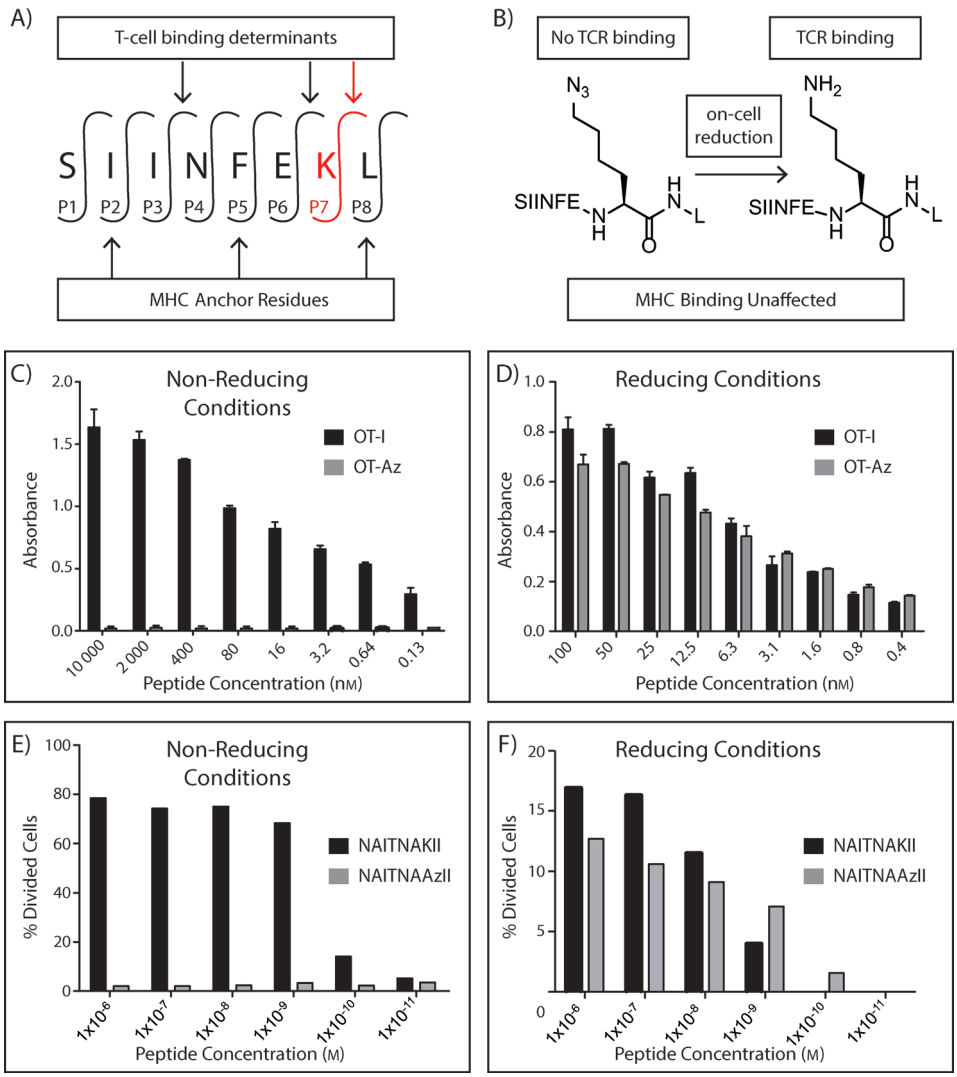

Figure 2. Chemical unmasking of azido epitopes restores T-cell activation. A) Certain residues are key for anchoring to MHC-I and others are key T-cell recognition determinants. B) Lysine at P7 was chosen as the target residue for masking: Converting the cognate epitope OT-I into the azido analogue OT-Az 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 $D^bM_{187-195}$-specific transgenic CD8$^+$ T-cells by NAITNAKII or NAITNAAzII follows a similar trend: Azido epitopes are not recognized. F) After reduction as above, the recognition of NAITNAAzII is restored.

observed by NMR spectroscopy (Figure S2; OT-OH). 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.

We next focused on the on-cell TCEP-mediated unmasking of the OT-Az epitope on BM-DCs. We found that 100 mM TCEP resulted in optimal on-surface deprotection (Figure S3A). Unmasking appeared to be complete within a reaction time of 30 minutes (Figure S3B). Under these conditions (100 mM TCEP, 30 min), the T-cell reactivity on the cell surface of BM-DCs 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 BM-DCs 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^{b}M$_{187\text{-}195}$ epitope from respiratory syncytial virus (RSV).[33] RSV is the main causative agent of respiratory failure in infants, and the role of CD8-mediated T-cell immunity remains somewhat controversial. D^{b}M$_{187\text{-}195}$ is a dominant epitope in C57BL/6 mice[32] and a highly functional subdominant epitope in CB6F1 mice.[33] The D^b-binding NAITNAKII nonamer is critically dependent on Lys-193 for T-cell recognition,[34] and we postulated that masking this residue would similarly ablate T-cell recognition. 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^{b}M$_{187\text{-}195}$ 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, our 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, we synthesized long peptides containing either the OT-I or OT-Az epitopes (LP-I and LP-Az; Figure 3A). We added one or another of these long peptides to BM-DCs 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 3B). 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 3C). A marked reduction in rescue was observed (>50% rescue) at higher peptide concentrations, which could in part be explained as before,

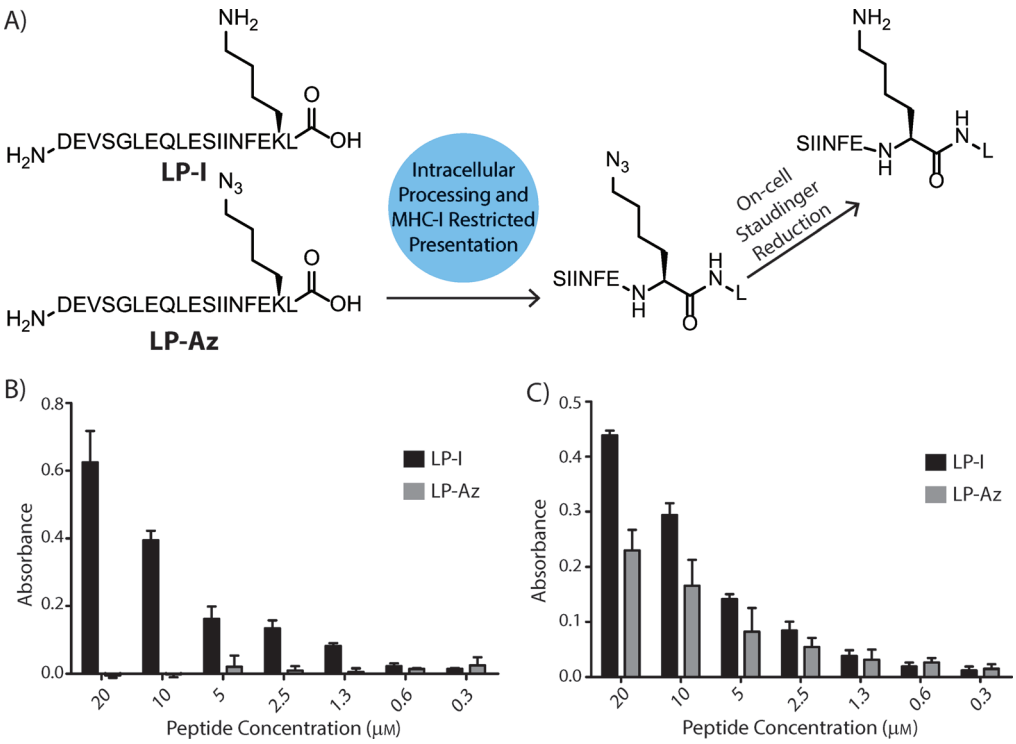

Figure 3. Presentation of long peptides to B3Z-hybridoma. A) Design of long peptides for studying the suitability of azido epitopes as latent antigens for intracellular routing. B) Intracellular routing of LP-Az results in no activation of the OT-I cognate B3Z T-cell clone. C) Reduction with TCEP three hours after the initial peptide addition resulted in partial recovery of T-cell activation.

and in part be due to minor differences in processing efficiency resulting from the amine-to-azide modification.

In conclusion, our results demonstrate that organic azides are not only valuable bioorthogonal ligation handles, but are equally applicable to bioorthogonal protection. We have exploited this phenomenon 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.[23] 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,[35] although—like the photouncaging reaction—it employs a bulky protecting group, which may preclude normal intracellular routing and proteolytic processing.

The main limitation of our current 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.

Keywords: antigens · azides · bioorthogonal chemistry · cell recognition · Staudinger reduction

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 5628–5631
Angew. Chem. **2015**, *127*, 5720–5723

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Received: January 13, 2015
Published online: March 17, 2015

