# Bioorthogonal deprotection strategy to study T-cell activation and cross-presentation

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# 3

# A Bioorthogonal Deprotection Strategy to Study Uptake, Processing and Cross-Presentation of a Long Peptide Antigen

## 3.1 Introduction

Antigen presentation is a vital part of the immune system. It allows for the distinction between native and foreign cells and for surveillance of the internal condition of the cell.[1] Intracellular peptides are presented in major histocompatibility complex (MHC) class I and extracellular peptides in MHC class II.[1] $CD8^+$ T-cells recognize epitopes presented by MHC class I and $CD4^+$ T-cells recognize epitopes presented by MHC class II. MHC class I complexes are found on almost all cell types whereas MHC class II complexes are found exclusively on antigen presenting cells (APCs).[2] Cross-presentation of peptides derived from extracellular material, such as apoptotic/necrotic material[3], soluble matter[4], or tumor exosomes[5] which are taken up by endo-, phago-, or micropinocytosis[6], allows for the presentation of these peptides on MHC-I of APCs. Through cross-presentation APCs are capable of priming/pre-activating $CD8^+$ T-cells (cytotoxic killer T-cells).[7] These activated T-cells can, after priming in a lymph, re-enter the circulation where they are now capable of killing any (tumor) cell presenting the peptide they were activated with.[8]

The exact mechanism of cross-presentation has not been unraveled yet. So far two major pathways have been described: the cytosolic pathway and the vacuolar pathway (Figure 1).[7,9] In the cytosolic pathway the endogenous proteins are degraded into small peptide sequences by proteasomes. They are

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then transported to the endoplasmic reticulum (ER) by the transporter associated with antigen processing 1 (TAP1). In the ER the peptides are loaded on newly synthesized MHC-I molecules by TAP2. Trimming of these epitopes is done by ER-associated aminopeptidase 1 (ERAP1) and endosomal insulin-responsive aminopeptidase (IRAP). Finally, the MHC-I-epitope complex will be transported via the Golgi apparatus to the cell surface.[10,11]

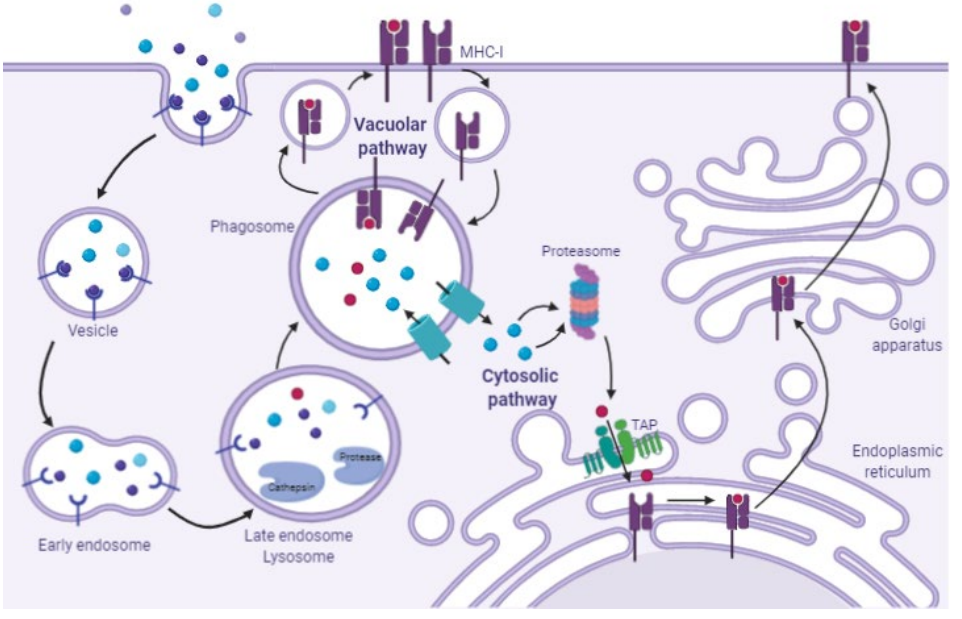


**Figure 1 | Proposed cross-presentation processing pathways.** Extracellular material is taken up going from vessicle, to ealy endeosome, to late endosome and ending up in the phagosome. From here extracellular proteins can either be degraded in phago/lysosome and loaded onto MHC-I (originating from ER or cell surface) = vacuolar pathway, or proteins escape from the phagosome by transporters ending up in the cytosol where they are degraded by the protesome. After degration the fragments are transported into the ER by TAP where they are loaded on newly synthesized MHC-I. The loaded MHC-I complexes are transported to the cell membrane via the Golgi apparatus.

When the protein enters the vacuolar pathway, the proteins are degraded into peptides within an endo-lysosomal-like compartment and loaded onto MHC-I inside this compartment.[12] It is not clear where these MHCs originate from.[7,9] Currently there are two views: the first being that the MHC-I for the vacuolar pathway comes from the ER, and the second that the MHC-I originate from the cell surface and are thus re-used.[7,9] It is not clear which pathway is at work *in*

*vivo,* and whether both pathways are employed equally by dendritic cell subsets.

Better understanding of antigen cross-presentation is of increasing therapeutic importance: the recent spate in anti-viral and anti-tumor therapeutic vaccines utilize cross-presentation for CTL-activation in established disease models.[13] Of particular interest in this field are the long peptide-based synthetic vaccines that provide a potentially pure, safe to develop and customizable vaccine.[14] For example, in 2017 the first clinical trial of patient-specific synthetic long peptide (SLP) vaccine specific for a highly mutated form of melanoma cancer, resulted in all 6 participants showing a durable response. Originally the mutations of 10 patients were characterized, unfortunately for four patients the level of mutations in their melanoma was not high enough to create an effective vaccine.[15,16]

Many fundamental aspects of the cross-presentation of these vaccines remain unknown, and this can be highlighted by the fact that in the above-mentioned clinical trial only 12% of the peptides induced CTL-responses, despite the vaccine candidates being fully designed for CTL-activation. A more in-depth understanding of the immune system is required to optimize vaccination protocols and antigen peptide selection.[14]

One aspect that has been largely overlooked in the study of SLP cross-presentation has been the kinetics of the process(es). There are currently no tools that allow the facile study of how fast an antigen is processed in immune cells *in vivo* leading to cross-presentation. Nor is it known whether variations in the presentation kinetics positively, or negatively, impact SLP-vaccine efficacy.

It was envisaged that the bioorthogonal deprotection strategy described in Chapter 2 could be used to gain understanding of antigen processing and presentation speeds of SLPs. For this a peptide that could not be loaded exogenously had to be designed with which T-cell activation could be controlled. Such a peptide – in combination with appropriate localized deprotection mechanisms – could provide a new approach to study processing and presentation kinetics. It could even be envisaged that local subcellular unmasking of epitopes could be used to truly confirm the direct participation of an organelle in processing.

In this Chapter, the exploration of the IEDDA-pyridazine deprotection – as described for the minimal epitope characterization in Chapters 2 and 4 – to study SLP-processing is described. The goal is to acquire more understanding of surface appearance, intracellular storage and what cellular routing these SLPs take through control over the availability of recognizable peptide. This knowledge can aid in the rational design of SLP vaccines for tumor destruction.

### 3.2 Results and Discussion

In order to determine whether 2-TCO-modified peptides can be used to study antigen cross-presentation rates, the N-terminally extended 18-mer containing the SIINFEKL sequence, mbTCO-$OVA_{247-264}$, was chosen as the model antigen. This 18-mer oligopeptide has previously been shown to lead to robust cross-presentation[17] and only contains a single lysine (in the epitope), facilitating its synthesis using the same protocols used for the preparation of protected SIINFEKL.[18] The mbTCO and wt-variants of $OVA_{247-264}$, were therefore synthesized for use in the studies described in this Chapter (Figure 2).

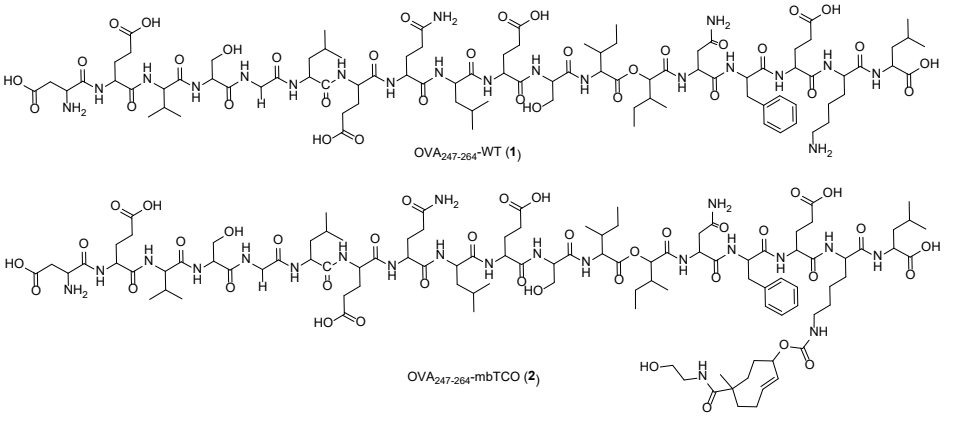


**Figure 2 | wild type (WT-) and caged (mbTCO-) variants of $OVA_{247-264}$ used in the research described in this Chapter.**

It was first assessed whether the mbTCO-protection of the peptide indeed prevented T-cell recognition after processing. Therefore, D1 dendritic cells were incubated with a concentration-range of the caged (**2**) and wild type (**1**) 18mer for 3 hours, followed by the addition of SIINFEKL-specific B3Z T-cells for 18h. It was observed (Figure 3A), that mbTCO-modified $OVA_{247-264}$ (**2**) did not give a T-cell response at any of the concentrations tested. Addition of tetrazine **Tz1** (described in Chapter 2) prior to addition of the T-cells resulted in

restoration of +/- 54% of the B3Z-responses. Longer incubation times of the D1s with the peptide (10µM, 1, 2, 3, 5- or 24-hours pulse time) showed that longer pulse times, resulted in the relative T-cell activation going up to 95% after a 24h pulse. These data led to the hypothesis that mbTCO-$OVA_{247-264}$ was taken up and/or processed more slowly than the unmodified SLP. This difference could be magnified, however, by the fact that any antigen appearing after the addition of the tetrazine (i.e. at the point of B3Z addition) remained protected (i.e. not contributing to B3Z activation), whereas any wt-SLP appearing after this time could contribute to B3Z-activation.

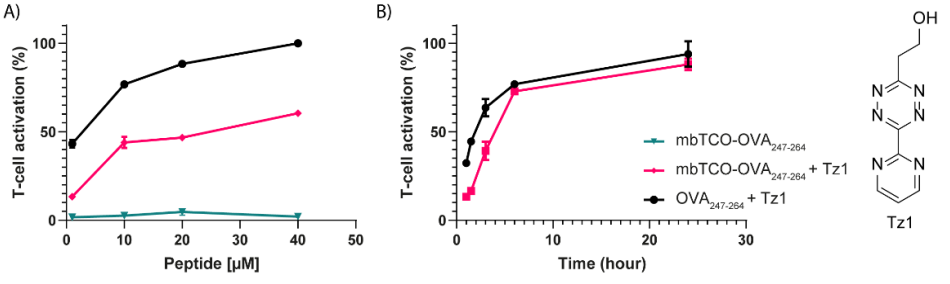


**Figure 3 | T-cell activation assay for n-terminal extended mbTCO-SIINFEKL peptide: mbTCO-$OVA_{247-264}$.** a) Percentage of T-cell activation of B3Z stimulated by D1 cells loaded with different concentrations of long peptide incubated for 3 hours. After 3 hours the cells were treated with 50 µM Tz1 for 30 minutes. b) D1 cells were incubated with 10 µM of long peptide for different length of time: 1, 2, 3, 5 and 24 hours. After incubation times cells were treated with 50 µM Tz1 for 30 minutes.

### 3.2.1 Processing: pathway inhibitors

To assess whether the wild type and caged $OVA_{247-264}$ are processed by the 'vacuolar' and/or 'cytosolic' route, D1s were treated with commonly used commercially available inhibitors for the study of these routes (Figure 4). 7 inhibitors were selected, as these are commonly used in processing pathway studies, which can be divided in two groups: inhibiting part of the vacuolar or cytosolic pathway. E64 is an irreversible cysteine protease inhibitor capable of inhibiting cathepsin X, H, L, B and S[20], proteases that break down proteins to peptides in endosomes/lysosome. Pepstatin A inhibits lysosomal proteases such as cathepsin D and E;[24] bafilomycin A1, inhibits the acidification of the lysosome and inhibits phagosome lysosome fusion; [21] chloroquine inhibits the lysosome by blocking lysosomal acidification, which results in swelling of the lysosomes *in vitro*.[22] Chloroquine, in a different study, has been shown to

increase in cross-presentation,[25] making this not an ideal inhibitor for pathway classification, though establishing its effect on fate of the caged peptide would be of interest.

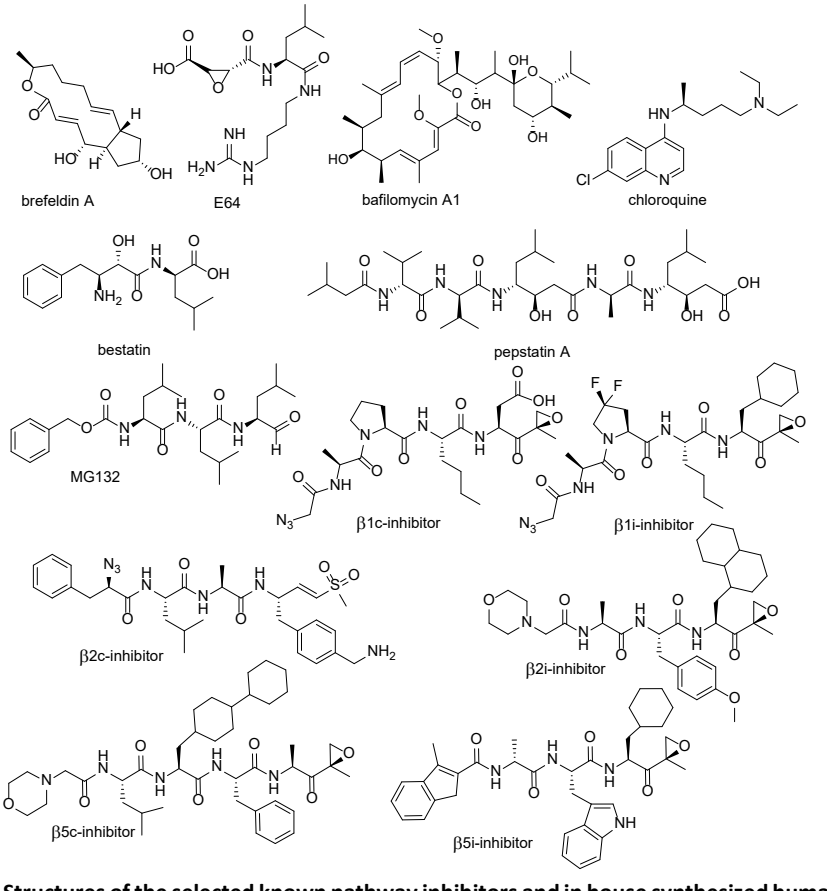


**Figure 4 | Structures of the selected known pathway inhibitors and in house synthesized human proteasome inhibitors.**

Brefeldin A blocks protein transport from the Golgi apparatus thereby withholding peptide-MHC I complexes from reaching the cell surface.[19] Bestatin inhibits cytosolic and ER aminopeptidases involved in trimming peptides at the N-terminus to yield MHCI epitopes.[23]MG132 finally is a broad-spectrum inhibitor of proteasomes, which are the starting points for the generation of MHCI epitopes from cytosolic and nuclear proteins.[1] Next to these known inhibitors, in-house synthesized inhibitors of specific human proteasome

subunits (β1, β2 or β5-specific)[34] were also used. There is a significant genomic overlap between human and mouse proteasome,[26] however, the specificity of these inhibitors in mice has yet to be determined at this stage.

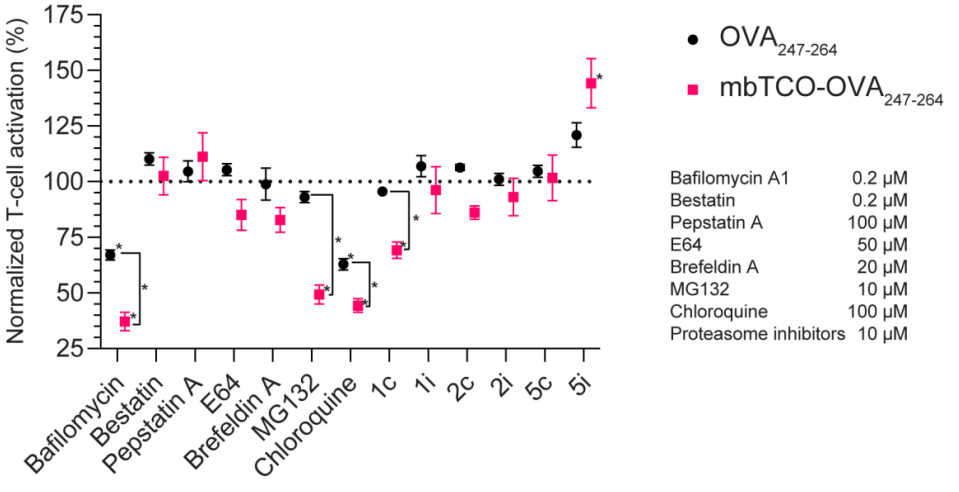


**Figure 5 | T-cell activation assay of D1 cells treated with inhibitors associated with the cytosolic or vacuolar processing pathway and the proteasome subunits of D1 cells.** a) Several commercially available inhibitors and proteasome inhibitors were given for 3 hours together with 10 µM peptide to D1 cells. Next the cells were treated with 50 µM **Tz1** for 60 minutes. After tetrazine treatment the cells were washed and left overnight with B3z T-cells. Inhibition percentage given for each compound is compared to no inhibitor for each peptide individually. Error bars represent the standard error of the mean (N=2, n=3). * Indicates significant difference of $p < 0.05$ compared to no inhibitor or between caged wild type epitope as indicated with black lines. Significance was determined with multiple t-test, corrected for comparison using the Holm-Sidak method.

Co-incubation of the inhibitors and peptides for 3 hours, prior to the addition of tetrazine **Tz1**, for 1 hour (Figure 5), showed a differential effect of the inhibitors on the caged SLP **2** and the wt-variant **1**. T-cell response in Figure 6 is normalized towards the maximal response for each individual peptide (**1** or **2**). In general, the presentation of the wild type epitope is far less affected by the inhibitors than the caged variant. Proteasome inhibition has no influence on the presentation of the wild type epitope, but β1c-inhibition negatively impacts the cross-presentation of **2**. β5i-inhibition on the other hand enhances the cross-presentation of **2**. Both bafilomycin and chloroquine inhibit presentation of wild

type and caged epitopes significantly ($p<0.05$, multiple t-test) compared to no inhibitor. For these inhibitors, T-cell activation by the caged epitope was inhibited significantly more ($p<0.05$, multiple t-test) compared to wildtype, suggesting that the lysosome plays a rate-limiting role in the processing and presentation of both antigens. To look further into the effects of these inhibitors, a serial dilution experiment of four of these inhibitors (β1c-inhibitor, β5i-inhibitor, bafilomycin A1 and chloroquine) was performed (Figure 7).

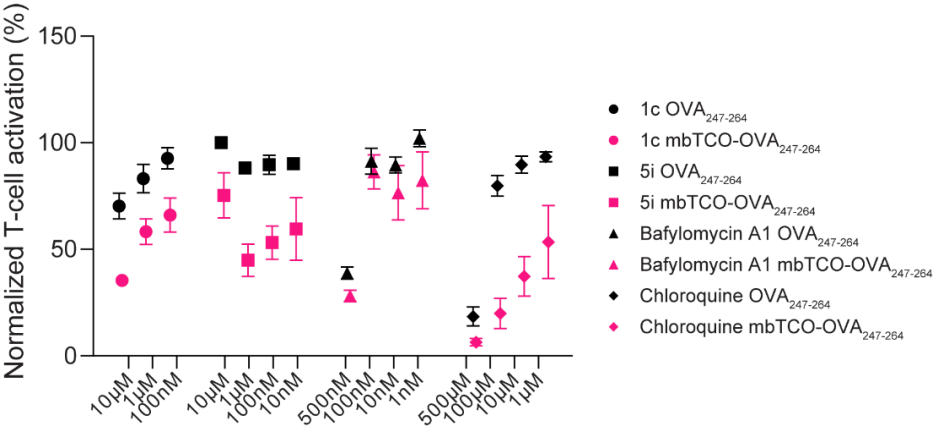


**Figure 6 | T-cell activation assay of D1 cells treated with different concentration of pathway inhibitors.** D1 cells were incubated with 10 µM of $OVA_{247-264}$ or mbTCO- $OVA_{247-264}$ and the indicated concentration of inhibitor for 3 hours. After washing the cells were treated for 30 minutes with 50 µM tetrazine Tz1. The T-cell activity was measured the following day with the CPRG assay. The signal obtained for T-cell activation was normalized for each peptide towards DMSO added instead of inhibitor (=100%). The error bars represent the standard deviation (N=2, n=3).

All inhibitors show a concentration dependent effect on antigen presentation (Figure 6). Surprisingly, the difference between wild type and caged epitope are less pronounced for the highest concentration of bafilomycin A1 and chloroquine. The difference in concentration between Figure 6 and Figure 5 for these compounds (bafilomycin A1: 500 nM vs 200 nM; chloroquine 500 µM vs 100 µM), can explain the decrease of T-cell activation for the wild type epitope. This is confirmed by the loss of effect of chloroquine on wild type epitope presentation at lower concentrations. Noteworthy is inhibitor 5i which inhibits subunit β5i of the immunoproteasome. This inhibitor gave in previous experiments an increase in T-cell activation, thus probably an increase in

antigen presentation. Now this effect is no longer seen, though at the highest concentration of 10 μM a higher T-cell response is observed than for the lowest concentration of 10 nM. This increase in antigen presentation only occurs at a concentration of 10 μM. The question arises whether this effect increases with even higher concentrations of β5i inhibitor, though it should be kept in mind that adding too much compound can give side effects which have nothing to do with the actual effect of proteasome inhibition. From the pathway inhibitor data, it was concluded that proteasomes are not required for wild type epitope processing/presentation. The caged epitope seems to be processed differently than the wild type epitope as proteasome inhibition decreases T-cell activation. It might be that both routes are taken for the processing of the caged epitope, as proteasome inhibition does have an effect on T-cell activation.

A disadvantage of these experiments is that tetrazine **Tz1** used for decaging is cell permeable. As also the cells are not fixed, the chase time (time after excess peptide and tetrazine are removed) is 18 hours with T-cells present. This is a lot of time in which it is hard to distinguish the exact effect of the different compounds. Therefore, a more precise system is required. Initially the use of fixatives to look into different chase times was assessed.

### 3.2.2 Pulse and Chase

To assess whether the continued processing of the wt-$OVA_{247-264}$ (**1**) during the incubation with B3Z influenced the outcome of the experiments, and to determine whether intracellular uncaging of mbTCO-$OVA_{247-264}$ by tetrazine **Tz1** obfuscated the above experiments, the following pulse-chase experiment was designed: D1 cells were treated with peptide for 2 hours (followed by washing of the non-internalized SLP). The cells were then chased for different times (to allow processing and presentation of the antigen). To then exclude any effects of processing and appearance after the addition of tetrazine and B3Z, the D1 cells were fixed after the 1h tetrazine pulse (Figure 7).

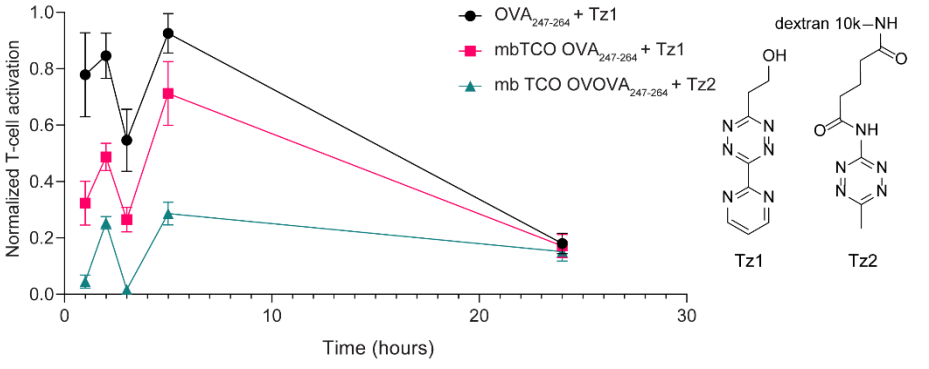


**Figure 7 | Pulse and chase experiment of $OVA_{247-264}$ compared to its caged variant.** Long caged peptide is given to D1 cells for the indicated times after which the cells were treated with 50 µM Tz1/Tz2 for 60 minutes. After uncaging the cells were fixed and B3Z T-cells were co incubated with the fixed cells O/N. Error bars represent standard deviation, (N=2, n=3).

These data show that the presentation kinetics of **1** and **2** are similar. Interestingly, a drop in T-cell activation at the 3-hour chase point suggesting that presented peptide is taken away from the cell membrane for recycling/degradation.

The use of the fully extracellularly retained dextran-tetrazine conjugate **Tz2** resulted in a much lower T-cell response at the first 4 time points, with all activation levels converging at the 24-hour time point. In Chapter 2 (Figure 6) **Tz2** showed only ±50% T-cell activity whereas **Tz1** showed ±80% T-cell activity compared to SIINFEKL. These results suggest that caged peptide appears at the surface 2 hours after the initial pulse, and remains there at a constant level (with the exception of the unexplained dip in activation observed at t = 4h). The unmodified $OVA_{247-264}$ shows a higher appearance at early time points after the chase, with a loss of T-cell activation capacity over time until 24h. The exact biological significance is yet to be determined, but one hypothesis that emerges from these observations is that mbTCO-modified antigen was actually excluded from certain parts of the cross-presentation pathways compared to the unmodified antigen, perhaps due to the steric bulk of the 2-TCO-moeity.

### 3.2.3 Tetrazine Cell Penetrance

To determine the rate of cellular penetrance of the small-molecule tetrazines, a second family of tetrazines was used, namely those capable of only ligating to the 2-TCO without resulting in elimination of the allylic substituent.[27] Tetrazine

**Tz3** described also in Chapter 2 (synthesized by Mark de Geus). After incubation of 3 hours with the epitopes the cells were first treated between 5 and 20 minutes with non-eliminating tetrazine **Tz3** (10 µM) to assess how long it would take before the entire pool of mb-TCO-OVA$_{247-264}$ was ligated. The cells were next treated with an eliminating tetrazine **Tz1** for 30 minutes (10 µM) followed by addition of B3Z T-cells to assess how much of the intracellular pool had been blocked by **Tz3** (Figure 8). After 20 minutes complete blockage of the mbTCO was already observed, suggesting that the blocking tetrazine **Tz3** has completely ligated the entire intra- and extracellular peptide pool at this timepoint. The fact that full T-cell activity could be achieved within 30 minutes of incubating with the tetrazine **Tz1** suggests that this tetrazine too must be considered as fully cell permeable, lending further importance to the use of the extracellularly retained tetrazine **Tz2**, and others[28] reported in literature.

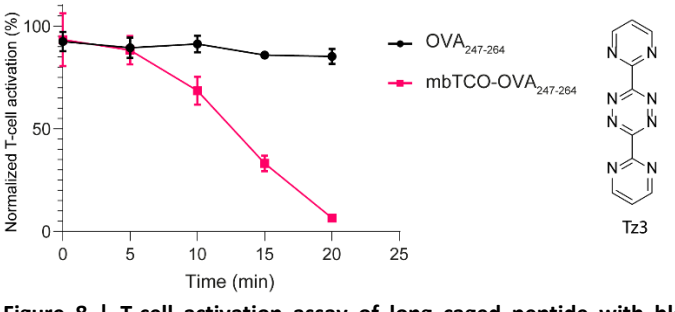


**Figure 8 | T-cell activation assay of long caged peptide with blocking tetrazine.** D1 cells, preloaded with long peptide **1**/ **2** for 3 hours, were first treated with 10 µM **Tz3** for indicated times. After blocking the remaining available handles were decaged with **Tz1** 10 µM for 30 minutes. Signal is normalized to the highest OVA$_{247-264}$ level obtained. Error bars represent the standard deviation (N=2, n=3).

### 3.2.4 Tetrazine coated beads

To further assess the hypothesis that lysosomal routing is a critical component of the cross-presentation pathway of mbTCO-OVA$_{247-264}$, another tool was envisaged: a tetrazine that would accumulate in the lysosome and therefore only deprotect antigen that passed through the lysosome. For this tetrazine **Tz4** (Figure 9) was conjugated to 100 nm-sized latex beads, which have been reported to accumulate in lysosomes of DCs[29] after 30 minutes incubation.[30] When mbTCO-OVA$_{247-264}$ is then incubated with these cells, it will only be

deprotected if the vesicles in which it is contained fuse with the bead-containing vesicles, thereby revealing the B3Z-activating T-cell epitope.

D1 cells were therefore pulsed with these beads for 30 minutes, after which time they could be detected inside the cells with fluorescence microscopy. To confirm that all beads were internalized, the cells were pulsed with mbTCO-$OVA_{257-264}$ minimal epitope peptide. As this short peptide (epitope) can bind to MHC-I present on the outside of a cell its activation would indicate the presence of beads on the outside of the cell. No T-cell activation (Figure 9; "beads in D1") was observed for these beads. As a control an excess of beads was given to cells which were already incubated with only the short peptide (Figure 8). The cells with beads could not induce T-cell activation and the cells which were given an excess of beads after peptide incubation do show that the tetrazine on the beads can decage the TCO. These beads need to be characterized further for future use with the caged 18mer to study trafficking of the 18mer peptide.

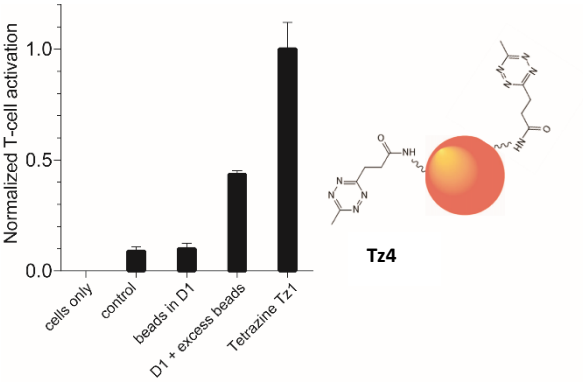


**Figure 9 | T-cell activation assay of D1 cells with tetrazine coated beads and mbTCO-SIINFEKL.** D1 cells were either treated with tetrazine coated beads for 30 minutes or not, after which 100 nM of mbTCO-SIINFEKL was given to the D1 cells for 60 minutes. D1 cells without beads were treated with 50 µM tetrazine **Tz1** or an excess of tetrazine coated beads for 30 minutes.

### 3.3 Conclusion

In this Chapter the *in vivo* compatibility of uncaging SLPs was evaluated. The TCO-cage seems to be compatible with processing of the 18mer variant of SIINFEKL. The caged long peptide requires more time (6 hours) to reach similar presentation levels. The caged long peptide is processed differently compared to wild type long peptide, which explains this delay in SIINFEKL levels presented on the cell surface. This difference in processing was confirmed by the influence

of known pathway inhibitors on the T-cell activation of wild type long peptide (**1**) compared to caged long peptide (**2**). The proteasome, which is known to cleave a c-terminally extended version of the SIINFEKL epitope,[31] is not involved in processing of $OVA_{247-264}$, as expected. However, the proteasome seems to be involved in the processing of the caged epitope. Inhibition of the proteasome inhibits up to 60% of the maximal T-cell activation obtained for caged long peptide without inhibitor. It can be concluded that the caged epitope is processed by both vacuolar and cytosolic route. This is further confirmed by the significant effect of inhibition on T-cell activation by chloroquine and bafilomycin A1, both influencing the lysosomal degradation. This difference in processing, though not initially expected, offers great opportunities to evaluate the effect of small changes in epitopes to processing, possibly aiding the design of more effective SLPs.

## 3.4 Experimental section

***General reagents***

Phosphate buffered saline (PBS) is 5 mM KH2PO4, 15 mM Na2HPO4, 150 mM NaCl, pH 7.4. CPRG (chlorophenol red-β-D-galactopyranoside) was purchased from Calbiochem (cat: 220588-250MG). Glutaraldehyde 25% in $H_2O$ (111-30-8) was purchased from Sigma-Aldrich. All media, streptomycin, penicillin, L-glutamine and FCS (fetal calf serum) were purchased from Sigma Aldrich.

***Synthesis of peptides and tetrazines***

Peptides were synthesized using standard Fmoc solid phase peptide synthesis protocols. TCO modifications and peptides were made by Mark de Geus, and detailed synthesis can be found in his thesis. Tetrazines **Tz1** and **Tz2** were made by Mark de Geus and tetrazine-dextran (**Tz3**) was kindly provided by Marc Robbilard from Tagworks. **Tz4** was made by Michel van de Graaff, and detailed synthesis can be found in his thesis. All compounds were HPLC purified before testing in biological systems.

***Cell culture***

The $OVA_{257-264}$-specific, H-2Kb-restricted CTL hybridoma, B3Z[32] was cultured in IMDM supplemented with 10% heat inactivated fetal calf serum (FCS), penicillin 100 IU/ml, streptomycin 50 µg/ml, 2 mM L-glutamine and 0,25 mM 2-mercaptoethanol. RMA-s cells were cultured in DMEM high glucose supplemented with 10% heat inactivated FCS, penicillin 100 IU/ml, streptomycin 50 µg/ml and 2 mM glutamax. D1[33] cells were cultured in IMDM containing 10% heat-inactivated FCS, 100 IU/ml penicillin, 50 µg/ml streptomycin, 2 mM L-glutamine, and 50 µM 2-mercaptoethanol (complete IMDM).

This medium was supplemented with 30% fibroblast supernatant (SN) from NIH/3T3 cells (collected from confluent cultures and filtered) containing 10-20 ng/ml mouse rGM-CSF.[33] The complete medium for D1 cells is referred to as R1 medium. Culture conditions were 5% $CO_2$ at 37°C.

***T-cell activity assay in vitro SIINFEKL***

DCs were plated in 96-well tissue-culture treated microtiter plates (50,000 cells/well) and allowed to adhere at 37°C for 1 h. Peptide at indicated concentration was added. DCs were incubated with short peptides for 1 h and long peptides for at least 3 hours, followed by a wash with complete Medium. Peptide-pulsed DCs were then treated with indicated concentration of tetrazine in complete Medium for 60 minutes unless indicated differently. After removal of the reduction medium, the cells were washed with complete Medium. If cells were fixed, cells were treated with 0.2% Glutaraldehyde in PBS for 60 seconds on ice and immediately quenched with 200 µl of complete medium. Medium was removed and T-cell hybridoma B3Z cells (50,000 cells/well) were added in full IMDM medium. The DCs and T-Cells were co-cultured for 17 hours at 37°C. B3Z activity was measured by a colorimetric assay using CPRG (chlorophenol red-β-D-galactopyranoside) as a substrate. To every well 100 µl of lysis buffer (9,6 ml PBS; 90 µl $MgCl_2$ 1M; 125 µl IGEPAL; 71 µl 2-mercaptoethanol; ±6 mg CPRG) was added followed by short shaking of the plate and 30 minutes incubation at 37°C in the dark. B3Z has the LacZ reporter gene, which produces the β-galactosidase enzyme, incorporated behind the IL-2 promoter. Upon activation of the T-cell the IL-2 promoter will become activated thus producing IL-2 and β-galactosidase. The levels of expression of β-galactosidase correlate directly with the IL-2 levels and can be measured by the catalytic hydrolysis of the CPRG substrate from yellow to a dark red product, which can be measured at the optical density of 570 nm (OD570 ClarioStar plate reader).

## 3.5 References

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