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Chemical Control over T-cell Activation In Vivo using Deprotection of Trans-cyclooctene Modified Epitopes

2.1 Introduction
Cytotoxic T-cell activation is a crucial step in the body’s defensive mechanism against viruses and tumors. These cells are both activated and secrete cytokines to kill their target cell upon recognition of short (8-10 amino acid) peptides presented on MHC-I complexes to their T-cell receptors (TCRs). However, the correlation between this recognition and T-cell activation remains poorly understood. The kinetic proofreading model, which is supported by computational studies, dictates that a series of signaling events must occur over time to initiate a critical activation event before the TCR-pMHC interaction is broken. Experimental evidence has shown that fast dissociating antigens can never reach the same level of response that long residence time antigens accomplish. This leads to the conclusion that activity of an antigen, namely, the T-cell response that is created, is determined by both potency and duration. Further supporting the theory that a series of signaling events are required for activation is that MHC-I-TCR interaction with CD8 enhances peptide sensitivity one million-fold.

Studying T-cell activation using exogenously loaded epitopes (“short peptide”) on MHC-I on APCs is common, but suffers from disadvantages such as asynchronous T-cell activation and low levels of activation. To allow the in vivo correlation of activation parameters to functional killer T-cells in tumor models,
more precise insights into the mode of activation of CTLs are needed. Studies as done by Mempel et al. and Lodygin et al. show with intravital imaging after vaccination with peptide-loaded APC - that T-cell activation in vivo does not occur via the prolonged contact leading to a synapse formation, but rather via multiple transient contacts in the first 8 hours after entering the lymph node that lead to the second stage of activation and the eventual full T-cell activation and proliferation.\textsuperscript{7,8} The lack of a inducible synchronized starting point for the pMHC-I-TCR interactions complicates the study of early T-cell activation. Therefore, new tools with control over timed activation are needed to study these early events.

Protecting group manipulations in chemical biology has gained success over recent years using either photo- or chemoactivation. Photo-cleavable agonists, such as 6-nitroveratryloxycarbonyl (NVOC), ortho-nitrobenzyl, substituted azobenzenes and 4,5-dimethoxy-2-nitrobenzyl (DMNB) (Figure 1), have been used as uncaging mechanism with good results in vitro.\textsuperscript{9–11} However when considering the applicability in vivo, photo-uncaging is limited by the poor penetration of infra-red light in deep tissue and animals.\textsuperscript{12}

![Chemical structures of protecting groups for photo/chemo activation.](image)

**Figure 1** | **Chemical structures of protecting groups for photo/chemo activation.** The top four structures are photo-cleavable protecting groups/agonists.\textsuperscript{8–10} The bottom three structures are chemical protection groups.\textsuperscript{13–15}

Chemical deprotection strategies come in many flavors, such as Staudinger reduction\textsuperscript{13}, inverse-electron demand Diels Alder click and release\textsuperscript{14} and
mesoionic click and release (Figure 1). These protecting group strategies might be translatable to achieve temporal control in vitro over T-cell activation. The activation of T-cells by deprotection of a peptide in MHC has been reported with the use of Staudinger reduction. However, the Staudinger reaction for in vivo purposes is too slow (60 minutes to reduce an azide to amine on cells) and phosphines are sensitive to oxidation.

This Chapter reports on a new approach: the in vivo compatible deprotection of T-cell epitopes using the inverse electron demand Diels-Alder reaction (IEDDA) reaction. This reaction between a tetrazine and a trans-cyclooctene was initially reported as an ultra-fast bioorthogonal ligation reaction by the Fox group. More recently this reaction was reported to also be a very suitable in vivo deprotection reaction where a ligation step is followed by an elimination step resulting for example in local toxin release as published by Versteegen et al.

Briefly, IEDDA pyridazine elimination is based on a “click-to-release” reaction between a trans-cyclooctene (TCO) and a tetrazine (Figure 2). The TCO used in these experiments is functionalized with an ethanolamine group (mbTCO) to improve solubility and cellular uptake. The mbTCO reacts rapidly with the tetrazine to form 4, 5-dihydropyridazine by means of cycloaddition. The lone electron pair of the NH group eliminates CO2 and the mbTCO through an electron cascade, leaving a 1,4-dihydropyridazine which can rearrange to an aromatic pyridazine.

Figure 2 | Mechanism of the inverse-electron-demand Diels Alder “click-to-release” reaction between mbTCO and tetrazine. The ethanolamine functionalized trans-cyclooctene and tetrazine undergo ligation via [4+2] cycloaddition, yielding 4,5-dihydropyridazine, followed by tautomerization to the 1,4-dihydropyridazine which can rearrange to an aromatic pyridazine, resulting in the elimination of a carbamate-linked biomolecule.
The IEDDA uncaging strategy was employed as an IEDDA pyridazine elimination reaction between a silent trans-cyclooctene modified epitope and a tetrazine, which liberated the amine of a crucial lysine upon which T-cell-pMHC recognition is restored. The uncaging showed promising yields and speed \textit{in vitro}. Further optimization led to \textit{in vivo} controlled local T cell activation being observed even in the first 3 hours upon uncaging.

2.2 Results and Discussion

Figure 3 | Schematic overview of uncaging mechanism of caged SIINFEKL using the inverse electron-demand Diels-Alder reaction (IEDDA). IEDDA pyridazine elimination between a silent trans-cyclooctene-modified epitope and a tetrazine liberates antigenicity of the peptide. After initial cycloaddition, tautomerization and elimination results in the free lysine ε-amine upon which a T-cell can recognize the epitope again and become activated; SIINFEK[CCO]L (4), SIINFEK[TCO]L (5) and SIINFEK[mbTCO]L (7).

As model epitope OVA\textsubscript{257-264} (OT-I, SIINFEKL) was selected. This well studied epitope has a lysine crucial for T-cell recognition, but which is not involved in MHC-I binding.\textsuperscript{13} Three caged epitopes were synthesized, the cis- and trans-cyclooct-2-en-1-yl carbamate derivatives, SIINFEK[CCO]L (4) and SIINFEK[TCO]L (5) respectively, and a more soluble variant of the TCO, SIINFEK[mbTCO]L (7) (Figure 3) where the cage was introduced at Lys-7, which should not interfere with the binding to MHC. Positions 2, 5 and 8 of SIINFEKL are the main anchoring positions effecting binding in the MHC-I binding groove,\textsuperscript{21} while positions 3, 6 and 7 are crucial for T-cells recognition of the epitope.\textsuperscript{22} To confirm that the
caged peptides can bind similar to the wild type epitope a binding affinity assay on the tap deficient cell line RMA-S²³ was performed (Figure 4a).

**Figure 4 | Optimization of *in vitro* deprotection of the trans-cyclooctene protected epitope SIINFEKL and TCO stability.**

**a)** Binding affinity (MFI) of the caged epitopes, compared to SIINFEKL, to cell surface H2-Kb at low temperatures of the TAP deficient cell-line RMA-S. Analysis was performed with anti H2-Kb and H2-Kb-specific antibody (25-D1-APC). SIINFEK[mbTCO]L (7, purple), CCO (4, green) and TCO (5, blue), SIINFEKL (red); **b/c)** Deprotection of caged peptides (4, 5, 7) using DC2.4 cells as APCs and B3Z cells as T-cells. T-cell activation was compared to wild-type response (SIINFEKL; red) by measuring absorption (AU) of beta-galactosidase-directed CPRG hydrolysis. All experiments have been done twice in triplicate, error bars represent the standard error of the mean; **b)** Deprotection of CCO (4, green), TCO (5, blue) and mbTCO (7, purple) in the presence of 50 µM 3,6-dimethyltetrazine (8) for 30 minutes and indicated peptide concentrations; **c)** Deprotection of 100 nM mbTCO (7) after incubation with 50 µM 8 for the indicated times. After 1 minute incubation, a significant (p = 0.04) T-cell response could already be detected. **d/e)** stability of TCO constructs in complete medium (d) and serum (e). The TCO constructs were incubated with a Bodipy-tetrazine. The Bodipy fluorescent signal measured as a measure for the amount of intact TCO. 5 was not soluble under these conditions and 7 was stable up to at least 4 hours.
Without Tap no endogenous peptides can be loaded on MHC-I which results in thermolabile MHC-I complexes which are recycled and stored in the ER-Golgi system.\(^{24}\) When these cells are incubated at 26°C the empty MHC-I complexes become stable by binding low affinity peptides and appear on the cell surface.\(^{24}\) After low temperature incubation the cells were put back at 37°C, and now the low affinity peptides dissociate easily and the empty MHC-I molecules can bind newly provided peptides. When these peptides are strong binding epitopes, these MHC-I molecules stay stable on the cell surface presenting that epitope. By FACS analysis after MHC-I antibody staining it can be determined whether MHC-I is present on the cell surface and thus has a bound epitope. Figure 4a shows no significant difference in binding between the caged epitopes and the wild type epitope, confirming that the cage has no influence on MHC-I binding. As well, the SIINFEKL-H2K\(b\) specific antibody (25-D1) was incubated with these peptide-loaded RMA-s cells showing that the cage hinders the epitope-MHC-I binding site of the antibody (Figure 4a).

Next it was determined to what extent and how fast the caged peptides could be deprotected \textit{in vitro}. Caged epitopes 4, 5, and 7 were loaded on dendritic cells (DC2.4 cells\(^{25}\)) and incubated with 50 \(\mu\)M of 3,6-dimethyl-tetrazine (8) for 30 minutes (Figure 4b). The B3Z T-cell response was measured as beta-galactosidase-directed CPRG (chlorophenol red-\(\beta\)-D-galactopyranoside) hydrolysis, which is in direct correlation with IL-2 promotor activity, due to its inclusion under the NFAT-promotor in the B3Z T-cell line.\(^{26}\) At the highest concentration of peptide no T-cell response was observed for the tetrazine-unreactive peptide 4. However, tetrazine-reactive peptide 5 gave 42\% \pm 4.2\% of the response observed for the wild type epitope. The mbTCO-modified peptide gave 82\% \pm 4.4\% of the wildtype response at this time point. The response was also rapid: cells loaded with 100 nM of 7 yielded significant (\(p = 0.04\)) T-cell responses after 1 minute of uncaging with 50 \(\mu\)M 8, (Figure 4c). The stability of the TCO-moiety for peptides 5 and 7 in full medium and FCS was compared (Figure 4d/e), revealing poor solubility for 5 and stability up to 4 hours in FCS for 7. Due to a superior uncaging yield, ease of purification and enhanced solubility, all further assays only include epitope 7.
Chemical control over T-cell activation \textit{in vivo} using deprotection of trans-cyclooctene modified epitopes

Figure 5 | Uncaging of caged epitopes (4, 5, 7) on different cell lines. \(\textbf{a/ b/ c/ d)}\) Deprotection of caged peptides (4, 5, 7) using various APCs and B3Z cells as T-cells. Experiments were performed at indicated peptide concentrations using 50 µM 3,6-dimethyltetrazine (8) for 30 minutes. T-cell activation was compared to wild-type response (SIINFEKL; red) by measuring absorption (AU) of beta-galactosidase-directed CPRG hydrolysis. All experiments have been done twice in triplicate, error bars represent the standard error of the mean; \(\textbf{a)}\) D1 cell line; \(\textbf{b)}\) Bone marrow DCs frozen at day 7, thawed and used after 3 days; \(\textbf{c)}\) DC2.4 cells grown in RPMI medium; \(\textbf{d)}\) DC2.4 cells grown in IMDM medium; \(\textbf{e)}\) Recovery percentages (compared to the natural epitope) at 100 nM peptide concentration of the different cell lines.

The uncaging strategy was extrapolated to other antigen presenting cells (the D1 cell line\textsuperscript{27} and bone-marrow derived dendritic cells, BM-DCs\textsuperscript{28}). Both these cell types showed significant and comparable levels of deprotection of the caged epitope (7) compared to DC2.4 under the same conditions, >85% T-cell activation for D1 cells and >48% T-cell activation for BMDCs compared to SIINFEKL (Figure 5).
Figure 6 | *In-vitro* kinetics of uncaging of SIINFEK[mbTCO]L (7) using different tetrazines. a) Structures of the four different tetrazines; b/c) Deprotection of 100 nM 7 using DC2.4 cells as APCs and B3Z cells as T-cells. T-cell activation was compared to wild-type response (SIINFEKL; set at 1.0 normalized T-cell response) by measuring absorption (AU) of beta-galactosidase-directed CPRG hydrolysis. All experiments have been done twice in triplicate, error bars represent the standard deviation; b) Uncaging of (7) with tetrazines 8-12 for 30 minutes at the indicated concentrations. c) Deprotection reaction of (7) with tetrazines 8-12 at 10 µM of tetrazine at increasing incubation times. Tetrazine 9 blocks T-cell activation and tetrazine 10 and 11 show improved uncaging speed compared to tetrazine 8. Tetrazine 12 shows reduced uncaging speed and increases linear. Relative T-cell response is normalized between SIINFEKL 100 nM response as 1.0 and no peptide background signal 0.0.

The speed of the uncaging of mbTCO-SIINFEKL (7) was investigated using the reported asymmetric tetrazines29 (Figure 6a), which were shown to have improved kinetics due to a combination of electron donating and withdrawing substituents on the tetrazine ring. 3,6-Dipyrimidinyl-tetrazine (9) (two EWGs) showed no detectable elimination, whereas 3-methyl-6-pyrimidinyl-tetrazine (10), and 3-hydroxyethyl-6-pyrimidinyl-tetrazine (11),29 indeed showed improved uncaging rates and efficacy (Figure 6b/c) compared to 8. At the first time point (1 minute incubation with tetrazine) the maximal T-cell activation was already reached, while for 8 maximal T cell activation is reached after 30 minutes of incubation. These results concur with the published data (Fan et al.)29 of very fast ligation for tetrazine 11 compared to 8. Additionally, the previously reported dextran-functionalized tetrazine (12), which has reduced yield and uncaging speed compared to 8 *in vitro*, though performs better *in vivo* due to reduced clearance,20,30 was tested in this *in vitro* system. Tetrazine 12 showed similar concentration dependent behavior as 8, but slower uncaging speed although linear in time. For further experiments only tetrazine 8, 11 and 12 were of interest. Even though tetrazine 8 has been reported to be non-toxic *in vivo* up to 140 mg/kg (1.25mmol/kg)31 in mice, the toxicity of tetrazines 8, 11 and 12 was determined on APCs. Negligible loss of cell viability was observed up to 10 µM of tetrazines (Figure 7). Only at 24 hours of 100 µM tetrazine was
a decrease in cell viability observed. Therefore it was concluded that tetrazines have negligible toxicity on APCs.

To assess whether the approach could be used for other key lysine residues as well as other MHC-I haplotypes, a second epitope was used in which T-cell recognition is dependent on a critical lysine, namely the D<sup>b</sup>M<sub>187-195</sub> peptide (NAITNAKII) from respiratory syncytial virus (RSV)<sup>32</sup>. This virus is the main causative agent of respiratory failure in infants and responsible for significant mortality in the very young (<2 years) and the elderly<sup>33</sup>. In C57BL/6 mice, M<sub>187-195</sub> is a dominant epitope<sup>34</sup>, and a highly functional subdominant epitope in CB6F1 mice<sup>35</sup>. The peptide (sequence NAITNAKII) is a nonamer that binds the MHC-I haplotype D<sup>b</sup> and the recognition by T-cells is critically dependent on Lys-193 recognition<sup>37</sup>, which has previously been shown to be amenable to caging<sup>13</sup>. Synthesis of an mbTCO-caged variant of this peptide (NAITNAK[mbTCO]II, <strong>13</strong>) followed by a mixed splenocyte assay showed the same level of control over T-cell activation as seen for SIINFEKL/OT-I (Figure 8), suggesting application to lysine-cognate TCRs in general.

T-cell hybridomas (e.g. B3Z) lack some key hallmarks of native T-cell activation, due to their immortalized nature. For instance, hybridoma cells are in a continually dividing state, which makes them unsuitable for studying the switch from quiescence to activation as this is marked by the switch from a non-proliferative to a highly proliferative state. Alterations in surface marker expression of these T-cells associated with this activation are also absent in these cell lines.<sup>26</sup> Naïve primary T-cells do allow the study of this activation
switch, as they show these properties upon activation. It needed to be determined whether the approach was compatible with primary CTLs. Primary CTLs were isolated from OT-I-mice, which has a homogeneous T-cell population selective for the SIINFEKL-epitope. CD62L and CD69 are the first markers that show changes in cell-surface expression levels upon T-cell activation in vitro. Changes in surface expression levels of these early markers, and the induction of proliferation, upon IEDDA-deprotection on primary naïve CTLs were quantified.

Figure 8 | Uncaging of NAITNAK[mbTCO]II (13). This caged epitope with a critical lysine can be uncaged just as efficiently as caged SIINFEKL. The uncaging efficiency is measured as percentage of divided T-cells after 3 days. a) Histograms of proliferation of T cells with a specific TCR for NAITNAKII after 3 days of incubation at peptide concentrations ranging from 1 µM. b) Percentage of divided T-cells after 3 days at different peptide concentrations. At 1 nM the caged peptide gave no background proliferation (data of 2 individual experiments represented with standard error of the mean).

Analysis of early activation markers showed similar kinetics of CD62L downregulation and CD69 upregulation upon pulsing with SIINFEKL or upon pre-loading with 7, followed by tetrazine-mediated uncaging with 11 (Figure 9a). Uncaged 7 shows a slight delay for both markers, however after 180 minutes a similar level of early markers is reached. These early markers demonstrate that the activation of T-cells using a caged epitope shows a similar profile of activation upon addition of tetrazine 11 compared to the natural epitope. The full activation of T-cells is a multi-step process. T-cells are
considered as fully activated when they proliferate and produce/excrete cytokines. To check whether these observed early changes in T-cells will lead to full activation the same experimental setup was repeated, 100 pM peptide and 10 µM 11, with addition of the pre-staining of the OT-I T-cells with CFSE and that these cells were incubated for 3 days after uncaging. Addition of 11 to 7-pulsed OT-I cells induced T-cell proliferation (Figure 9b) comparable to SIINFEKL with 11. However, the proliferation pattern of SIINFEKL was changed by addition of 11. These differences were assigned to earlier observed sensitivity of these cells; even slight changes in environment have an effect on activation/proliferation and in this setup tetrazine 11 is present at a concentration of 33 µM for 3 days.

Figure 9 | Primary T-cell (OT-I) proliferation and the early T-cell activation of SIINFEK[mbTCO]L (7) can be controlled by tetrazine elimination. a) Detection (MFI) of early activation markers upon deprotection of 7 (100 pM) with 11 (10 µM) using primary T-cells (OT-I). Early activation markers CD62L and CD69 were compared to wild-type response (SIINFEKL, 100 pM) by using fluorescent antibodies (CD62L-APC and CD69-PE, respectively). Data of 3 individual experiments with SD, normalized between highest signal obtained and zero fluorescence intensity; b) OT-I proliferation at day 3 after incubation with 100 pM of peptide (SIINFEKL or 7) and 10µM of tetrazine 11; representative figure of experiment performed twice.

As these OT-I primary T-cells are very sensitive the possible background activation of caged epitope and tetrazine only was investigated by looking at
proliferation profiles, Figure 10. The caged epitope 7 induced no background proliferation up to 100 pM of peptide and addition of only tetrazine 11 at 10 µM had no effect on proliferation. Further a dose dependency was observed for both SIINFEKL and uncaged peptide 7.

![Figure 10](image)

**Figure 10** | Primary OT-I T-cell proliferation *in vitro* of caged, uncaged and natural epitope. OT-I proliferation at day 3 after incubation with indicated peptide concentrations (1 nM to 0.1 pM; SIINFEKL or 7) and 10 µM of tetrazine 11 or PBS as control. The results show that 100 pM gives the optimal response without any background proliferation for the caged peptide (7). Tetrazine (11) gives no background proliferation of OT-I T-cells when given to the cells.

Encouraged by these *in vitro* results, the next challenge was to translate the chemical control over early activation events, CD62L shedding and CD69 upregulation, and T-cell proliferation from an *in vitro* to *in vivo* setting. For this, OT-I cells were adoptively transferred on day -1 i.v. in tail the base vein, allowing
distribution of the cells throughout the body,\textsuperscript{41} followed by tail base subcutaneously (s.c.) injection of 7 at day 0. 1 hour later tetrazine 11 was injected s.c. in the right flank above the right inguinal lymph node (iLN) and incubated for 1, 2 or 3 hours. Afterwards, mice were sacrificed and iLNs harvested. iLN Cells were stained and analyzed by FACS. At 3 hours, 70\% of CD8α\textsuperscript{+}-CD45.1\textsuperscript{+} T-cells were double positive (CD62L\textsuperscript{−} and CD69\textsuperscript{+}) in the right inguinal lymph node (iLN) compared to 17\% in the left (Figure 11). The mouse given SIINFEKL was also incubated for 3 hours with tetrazine 11. Both iLNs of this mouse showed similar expression levels of CD69 and CD62L on the OT-I T-cells and similar percentage of double positive T-cells, suggesting that peptide distributed equally to the right and left side of the mouse.

**Figure 11 | Early markers in vivo pilot experiment.** Each group contains 1 mouse. All mice were injected in the tail base area s.c. with 10 nmol peptide (SIINFEKL or caged epitope 7). Mice were injected with 100 nmol tetrazine 11 in the right flank s.c. on top of the right inguinal lymph node and left for different time points. The right and left inguinal lymph node were extracted after cervical dislocation. Activation of T-cells was determined by looking at downregulation of CD62L and upregulation of CD69 (using fluorescent antibodies CD62L-APC and CD69-PE for flow cytometry). The percentage of activated cells is given in the graph on the right as percentage of CD62L negative and CD69 positive cells of total OT-I population selected by anti-CD8α and anti-CD45.1. The results show that there is a difference between left and right and that the right lymph node activation of caged peptide 7 with tetrazine 11 almost reaches the SIINFEKL levels. No statistics could be performed.

Based on these initial results the experiment was repeated with 3 mice per group at 3 hours of tetrazine incubation (Figure 12a/b, dot plots can be found in Supporting Figures). Interestingly, even injecting only the caged peptide 7 already resulted in significant down- and upregulation of CD62L and CD69 respectively (p = 0.0004 and p < 0.0002), indicating the presence of an in vivo
mechanism for T-cell activation by antigens for which a TCR has low affinity. However, when tetrazine 11 was also injected, a significant difference was detected for CD62L compared to protected peptide 7 alone (p < 0.05). Dextran tetrazine 12 induced significant shifts for both markers. Furthermore, significant differences were observed between left and right iLN for both markers (CD62L p < 0.05 and CD69 p < 0.01). When assessing both markers in combination as a more robust method for selecting activated T-cells18, left over right differences became clearer. A similar percentage of double activated T-cells were observed in the right iLN (84.7% ± 6.9% of CD8α⁺-CD45.1⁺ T-cells; compared to 87.3% ± 1.3% for SIINFEKL in the right iLN), whereas only 43.1% ± 12% cells showed activation of both markers in the left iLN. The dextran-functionalized tetrazine 12 has been reported to have slower clearing properties20 and therefore the hypothesis is that this slower diffusion time explains the increased control over localized activation. These results show the regioselective potential of the approach.

To correlate these early activation events observed in vivo to full activation, the proliferation of OT-I T-cells was studied after 3 days. CFSE-labeled OT-I T-cells37 were adoptively transferred in recipient C57BL/6 mice on day -1. On day 0 the mice were either injected s.c. with mbTCO SIINFEKL (7) or SIINFEKL in the tail base area. 30 minutes later a second injection was given s.c. in the tail base area with either tetrazine 8 or PBS. After 3 days, the amount of T-cell proliferation was assessed by flow cytometry through CFSE-dilution (Figure 12d).42 Under these conditions, compound 7 induced very low levels of division of OT-I CTLs (and no proliferation) and upon injection with tetrazine 8, CTL proliferation was induced similar to SIINFEKL (3.1% ± 0.11% vs 4.4% ± 0.05 % divided OT-I of total lymphocytes; Figure 12c).
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Figure 12 | In vivo activation and proliferation of OT-I cells after local tail base injection of SIINFEKL[mbTCO]L (7) and subsequent injection of tetrazine 8, 11 or 12. a/b) CD62L and CD69 cell surface appearances were measured on OT-I T-cells using fluorescent antibodies: CD62L-APC and CD69-PE. When CD62L was decreased and CD69 was increased, the T-cells were qualified as activated. Mice were injected in the tail base area on both sides of the tail with a total amount of 10 nmol peptide (7 or SIINFEKL) and later injected with 100 nmol tetrazine or PBS only s.c. in the right flank right above the right iLN; a) Activation of right and left iLN represented as MFI of CD62L and CD69, respectively. 3 mice per experimental group and 2 for each control group. Dots represent individual lymph nodes; b) Bar chart representation of percentage of OT-I cells fully activated, positive for CD69 and negative for CD62L, with error bars as SD. There is a significant difference between 7 and 7 + 12 with \( p < 0.0002 \). There is even a significant difference between the left and right iLN for tetrazine dextran (12) of \( p < 0.01 \). P values were determined using an unpaired T test without assuming consistent SD; c/d) After adoptive transfer of CSFE labeled OT-I T-cells, mice were injected with 10 nmol 7 or SIINFEKL in the tail base area followed by an injection of 1 \( \mu \)mol 8 or PBS control after 30 minutes; c) Percentage of divided OT-I T-cells of total lymphocytes (data of 2 mice per group, represented with standard error of the mean); d) Histograms of the OT-I T-cell proliferation after 3 days.
2.3 Conclusion

The IEDDA-pyridazine elimination reaction can be used to exert chemical control over T-cell activation *in vitro* and *in vivo*. The technique complements other bio-orthogonal deprotection strategies *in vitro*, such as palladium-mediated reductions\(^{43,44}\) or Staudinger-based chemistry\(^{45}\), and adds to these techniques an *in vivo* applicability. Without the presence of a tetrazine, the lysine-caged epitopes show no T-cell receptor activation while MHC-I binding was not affected. Upon deprotection, T-cell receptor activation was restored. The lysine cage was implemented in two different epitopes, suggesting a generic application to lysine-sensitive TCRs.

*In vivo*, chemical deprotection of a caged peptide epitope could be achieved selectively three-hour post epitope injection. Using this decaging approach, local early activation of T-cells could be detected by quantifying cell surface expression of two early markers of T-cell activation, CD69 and CD62L, showing significant T-cell activation with tetrazine 12. Furthermore, *in vivo* results showed very similar T-cell proliferation potency upon decaging epitope 7 compared to the natural epitope, whereas the caged epitope showed no proliferation by itself. By combining this uncaging technique with injectable tetrazine-hydrogels\(^{46}\) or antibody-epitope conjugates\(^{20}\), the activation of T-cells could even be controlled more precisely in future experiments. Combining IEDDA-pyridazine uncaging with sensitive reporter systems, such as calcium biosensors\(^{47}\), will hopefully shed new lights on the dynamics of the activation of T-cells *in vivo*. This can provide new angles to the study of CTL-activation *in vivo*, analogous to that which has been achieved *in vitro* using photo-\(^{9,10,48}\) and chemo\(^{13}\)-deprotection.

Short peptides (epitopes) have been used as vaccines against cancer. However, they have several disadvantages such as low asynchronous T-cell activation and aspecific binding to MHC-I’s of healthy cells.\(^{6}\) The technique presented here might be useful to circumvent the asynchronous activation of T-cells, but also the aspecific binding when combined with localization tags such as antibodies, resulting in less side reactions.
2.4 Supporting figures

**Supporting Figure | Early markers in vivo experiment, raw data dot plots.** In vivo local activation of right inguinal lymphnode. 10 nmol of peptide, either SIINFEKL or SIINFEK[mbTCO]L (7), was injected in the tail base area at the same time or 1 hour previous to tetrazine injection, respectively. Tetrazine 11 or 12 was added; 100 nmol per mouse on top of the right inguinal lymph node s.c. On the x axis are the CD62L fluorescence and on the y axis the CD69 fluorescence (measured using the fluorescent antibodies CD62L-APC and CD69-PE, respectively). Cells shift from the right bottom corner, where they are unactivated, as for the top row with only tetrazine added, to the upper left corner (as is seen for the second row containing control peptide SIINFEKL). The bottom three rows have the caged epitope 7 without and with tetrazine 11 or 12, respectively. Shifts from right bottom corner to more upper left corner are visible for both groups containing tetrazine.
2.5 Experimental section

General reagents
Phosphate buffered saline (PBS) is 5 mM KH2PO4, 15 mM Na2HPO4, 150 mM NaCl, pH 7.4. Mouse Anti-Mouse H-2Kb (B8-24-3 clone). 25-D1.16-APC conjugated was purchased from eBioscience (Cat. #: 12-5743-81). Secondary antibody (Goat anti-Mouse IgG conjugated to Alexa Fluor-647 (catalogue number: A-21235) was purchased from Thermo Fisher Scientific. CPRG (chlorophenol red-β-D-galactopyranoside) was purchased from Calbiochem (cat: 220588-250MG). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Sigma Aldrich (CAS 298-93-1). CFSE was purchased from Molecular Probes. Bodipy-tetrazine (28) was purchased from lumiprobe.com. 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 Support chemistry. TCO modifications were made by Mark de Geus, detailed synthesis can be found in his thesis and as published. Tetrazines 8-11 were also made by Mark de Geus and tetrazine 12 was kindly provided by Marc Robbilard from Tagworks. All compounds were HPLC purified before testing in biological systems.

Stability of TCO constructs
Stability of TCO constructs were determined by incubation in the desired solvent (Full Medium or Fetal Calf Serum) at 37°C over a time period of 24 hours. 30 µl of a 200 µM solution of TCO-construct (4, 5, 7, 13 or 16) in DMSO was dissolved in 2970 µl solvent. The resulting 2 µM solution was incubated at 37°C. At time points 0, 1, 2, 3, 4 and 24 hours three times 100 µl was transferred to separate wells in a Greiner flat black 96-well plate and each was diluted with 100 µl of a freshly prepared solution of Bodipy-Tz 28 (10 µM), resulting in three 200 µl solutions (1 µM TCO-construct, 5 µM Bodipy-Tz 28). Solution fluorescence was measured using a Tecan Infinite M1000 Pro (λex = 491 nm, λem = 525 nm) for 60 minutes at 1 minute intervals and TCO stability was determined as a relative percentage to time point 0 (for each compound individually). Control samples containing 100 µl solvent (1% DMSO) were diluted with 100 µl Bodipy-Tz (28, 10 µM) and measured at all time points to establish a baseline.

Formula after baseline correction:
Time point x (t = 50 fluorescence) / Time point 0 (t = 50 fluorescence) * 100% = % TCO intact.

Cell culture
DC2.4 cells were cultured in RPMI supplemented with 10% heat inactivated fetal calf serum, penicillin 100 IU/ml, streptomycin 50 µg/ml, 25 mM Hepes pH 7, 2 mM L-
Chemical control over T-cell activation in vivo using deprotection of trans-cyclooctene modified epitopes

glutamine, 1mM pyruvate and 1x NEAA (non-essential amino acids). The OVA257-264-specific, H-2Kb-restricted CTL hybridoma, B3Z 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 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. The complete medium for D1 cells is referred to as R1 medium. Culture conditions were 5% CO₂ at 37°C.

**Cell culture of primary cells**
Immature dendritic cells were obtained from the bone marrow of C75BL/6 mice and were a gift from the Tumor Immunology Department (Leiden University Medical Center). The use of animals was approved by the ethics committee of Leiden University. Mice were euthanized by cervical dislocation; bone marrow of tibiae and femurs was flushed out and washed with PBS. Cells were grown in dendritic cell selection medium (IMDM containing granulocyte-macrophage colony stimulating factor (GM-CSF) (2:1 v/v) containing 8% FCS, penicillin/streptomycin (100 units/mL), glutamax (2 mM) and 2-mercaptoethanol (20 µM). Cells were selected for 10 days (37°C; 5% CO₂) and passaged every 2-3 days before use in the assays.

**Cell viability assay**
50,000 DCs per well were plated in a 96-well tissue-culture treated microtiter plate and allowed to adhere for 1 hour at 37°C. Different tetrazines (8, 11, 12) dissolved in complete media were added to the cells at 10 and 100 µM final concentration and incubated with the cells for 3 or 24 hours. Cell viability was checked for all samples after 24 hours. All media was removed from cells and 50µl of complete media was added to each well, as well as an only media control. 10 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (2.5mg/ml in PBS) was added to each well and incubated at 37°C for 45 minutes. Media was removed and 50 µl of isopropanol was added to each well. The absorbance at 565 nm was measured in a Tecan infinite M1000 after the plate was shaken for 3 minutes in the Tecan reader (orbital, 1mm amplitude). The optical density of the formed formazan is a measure of how alive (respiring) cells are. Results are normalized between untreated cells as 100% and only media as 0% live cells.

Alternatively, a live dead count was performed on D1 and BMDCs using trypan blue and a cell counter. For this type of measurement the adherent cells need to be detached and some cells are damaged in the process and can be accounted for as dead.
**T-cell activity assay in vitro SIINFEKL**

DCs were plated in 96-well tissue-culture treated microwell 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 the peptides for 1 h followed by a wash with complete Medium. Peptide-pulsed DCs were then treated with indicated concentration of 7Tetrazine in complete Medium for 30 minutes unless indicated differently. After removal of the reduction medium, the cells were washed with 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 MgCl₂ 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 Tecan infinite M1000 plate reader).

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

20,000 iCD103⁺-DC’s (in vitro developed CD103⁺ DC51) were seeded per well in a 96-well plate. NAITNAKII or NAITNAK(mbTCO)II (13) as added and incubated for 1 hour at 37°C. Cells were washed once with media. To half of the samples 50 µM 3,6-dimethyl-tetrazine (8) was added and incubated for 30 minutes at 37°C. Cells were washed twice with media. 100,000 CFSE-labeled G (beta chain-only transgenic) or E (full TCR transgenic) T-cells were added and were incubated for 3 days at 37°C. Cells were washed and stained for CD8 and viability. Samples were analyzed on a BD LSR-II flow cytometer. Data shown was gated on CD8⁺ live T cells.

**RMA-S MHC-I binding assay**

RMA-S cells have a decreased cell surface expression of MHC-I molecules at 37°C, but obtain metastable MHC-I surface expression when incubated at 26°C. The RMA-S cells were grown and passaged as described above. To ensure the metastable state of the MHC-I molecules on the cell surface, the cells were incubated for 48 hours at 26°C. The cells were seeded in a 96-well tissue treated microtiter plate (2x10⁵ per well) and then incubated with peptides (in medium) at the indicated concentrations for at least 3 hours. After the incubation period, the cells were washed twice in protein blocking agent (PBA: 5% BSA in PBS, 0.1% w/v NaN₃). Subsequently, the cells were stained with primary antibody Mouse Anti-Mouse-anti-H-2Kb (0.5 µg/mL; 25 µL) or Anti-mouse-anti OVA-Kb-PE (25-D1.16 0.2 µg/mL; 25 µL) in PBA for 30 minutes on ice. After the
incubation, the cells were washed twice with PBA and stained for 30 minutes on ice with secondary antibody Goat-anti-Mouse IgG conjugated to Alexa Fluor-488 (1 µg/mL; 25 µL). Cells stained with 25-D1 were left in PBA, no secondary staining. After washing twice with PBA, the cells were analyzed on a BD Accuri™ C6 Plus Flow Cytometer. Obtained data was analyzed using FlowJo version 10.2 (Miltenyi Biosciences).

**T-cell proliferation assay in vitro**

OT-I T-cells were extracted from OT-I/CD45.1 mice spleen and lymph nodes. To follow T-cell proliferation in vivo, OT-I/CD45.1 T-cells were labeled with the intracellular fluorescent dye CFSE (Molecular Probes). After a FACS analysis to assess the CFSE labeling efficiency, the OT-I cells were seeded in a 96-well microtiter plate (50,000 cells/well in 50 µL). Peptide and tetrazine (10 µM) were added at indicated concentrations and incubated for 1 hour at 37°C. 100 µL of complete media was added and cells were left for 3 days. After incubation cells were washed with PBA and incubated with anti-CD8α-APC antibody and anti-CD45.1-V450 antibody to gate for live CD8+/CD45.1+ T cells. Proliferation was assessed by flow cytometric analysis of the CFSE dilution on a BD LSR-II cytometer. Data was analyzed using the FlowJo software 10.2 (Miltenyi Biosciences).

**T-cell proliferation assay in vivo**

*Mice*

Female C57BL/6JRccHsd (B6; H-2b) mice were purchased from Envigo (The Netherlands). OT-I/CD45.1 mice, which have a transgenic Vα2Vβ5 TCR specific for the OVA257–264 epitope in the context of H2-Kb, were bred in the specific pathogen-free animal facility of the Leiden University Medical Center. The animal experiments have been reviewed and approved by the animal experimental committee of Leiden University.

*Day -1 adoptive transfer of CFSE-labeled OT-I cells*

OT-I T-cells were extracted from OT-I/CD45.1 mice spleen and lymph nodes. To follow T-cell proliferation in vivo, OT-I/CD45.1 T-cells were labeled with the intracellular fluorescent dye CFSE (Molecular Probes). After a FACS analysis to assess the CFSE labeling efficiency, C57BL/6 mice were injected i.v. with 1 x 10^6 CD8 positive OT-I cells in 200 µL PBS/ 0.1% BSA.

*Day 0 injections*

10 nmol of the control peptide SIINFEKL or SIINFEK[mbTCO]L (7) was injected i.d. in a total volume of 30 µL PBS in the tail base area of the mouse. After 30 minutes the mice were injected s.c. in the right and left flank in the vicinity of the inguinal lymph nodes with 1 µmol 3,6-dimethyl-tetrazine (8) in 200 µL PBS or as control 200 µL PBS.
Day 3 analysis
To analyze proliferation of the CD8+ T-cells derived from OT-I/CD45.1+ mice in vivo, both inguinal lymph nodes were collected from the recipient mice 3 days after the injections. Lymph cells were incubated with anti-CD8α antibody and anti-CD45.1 antibody to gate for live CD8+/CD45.1+ T cells. Proliferation was assessed by flow cytometric analysis of the CFSE dilution on a BD LSR-II cytometer. Division index and percentage dividing cells were calculated using the FlowJo software.

T-cell early activation markers in vitro
OT-I T-cells were extracted from OT-I/CD45.1 mice spleens and lymph nodes. After isolation with FACS sorting the cells were stored at 4°C overnight. The following day OT-I cells were seeded in a 96-well round bottom plate at 50,000 per well. The T cells were preincubated for 1 hour at 37°C with D1 cells and 10nM SIINFEKL(mbTCO)L (7), or D1 cells only, or 100pM SIINFEKL(mbTCO)L (7), or only OT-I cells. After pre incubation the plate was spun down for 3 minutes at 1500 rpm. The medium was removed for the samples with D1 cells. At indicated time points 3-hydroxyethyl-6-pyrimidinyl-tetrazine (11) was added at a final concentration of 10 µM. To the control samples, which so far contain no peptide, the control peptide SIINFEKL was added at the same concentration as the caged peptide (7) and at the same time given as tetrazine (11). After 3 hours of total incubation times the cells were spun down and washed. Cells were transferred to a 96 v-well plate and given 25µl of antibody mix containing CD8α-FITC 1:150, CD45.1-V450 1:500, CD69-PE 1:600 and CD62L-APC 1:800. Antibodies were diluted in PBA (PBS containing 0.5% BSA and 0.02% NaN3). After 30 minutes incubation the plate was washed twice with PBA. Cells were fixed for 20 minutes in 0.5% PFA in PBA and then diluted to 0.125% PFA in PBA and left over the weekend at 4°C. Samples were analysed by flow cytometric analysis of the 4 different colours on a BD LSR-II cytometer and calculations were done using FlowJo software.

T-cell early activation markers in vivo
Mice
Female C57BL/6JRccHsd (B6; H-2b) mice were purchased from Envigo (The Netherlands). OT-I/CD45.1 mice, which have a transgenic Vα2Vβ5 TCR specific for the OVA257–264 epitope in the context of H2-Kb, were bred in the specific pathogen-free animal facility of the Leiden University Medical Center. The animal experiments have been reviewed and approved by the animal experimental committee of Leiden University.

Day 0 adoptive transfer of OT-I T-cells
OT-I T-cells were extracted from OT-I/CD45.1 mice spleen and lymph nodes. C57BL/6 mice were injected i.v. with 1 x 10^6 CD8 positive OT-I cells in 200 µl PBS/ 0.1% BSA.
Day 1 injections and analysis
10 nmol of the control peptide SIINFEKL or SIINFEK[mbTCO]L (7) was injected s.c. in a total volume of 60 µl PBS in the tail base area of the mouse, 30µl on each side of tail. After 60 minutes the mice were injected s.c. in the right flank in the vicinity of the inguinal lymph nodes with 100 nmol 3-hydroxyethyl-6-pyrimidinyl-tetrazine (11) or tetrazine-dextran (12) in 100 µl PBS or as control 100 µl PBS. After 3 hours of incubation mice were euthanized by cervical dislocation and both inguinal lymph nodes were collected. Lymph cells were incubated with anti-CD8α-APC-R700 and anti-CD45.1-V450 antibody to gate for live CD8+/CD45.1+ T cells and cells were incubated with anti-CD62L-APC and anti-CD69-PE antibody for flow cytometric analysis of the early markers CD62L and CD69 on a BD LSR-II cytometer. Mean fluorescence intensity and percentage positive activated cells were calculated using the FlowJo software.

2.6 References
Chapter 2


Chemical control over T-cell activation in vivo using deprotection of trans-cyclooctene modified epitopes


