Molecular approaches to identify cancer T cell antigens and improve immunogenicity
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Chapter 6

The Optimization Of Bioorthogonal Epitope Ligation Within MHC-I Complexes

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Abstract

Antigen recognition followed by the activation of cytotoxic T-cells (CTLs) is a key step in adaptive immunity, resulting in clearance of viruses and cancers. The repertoire of peptides that have the ability to bind to the major histocompatibility type-I (MHC-I) is enormous, but the available approaches to study the diversity of the peptide repertoire on a cell are limited. Here we explore the use of bioorthogonal chemistry to quantify specific peptide-MHC-I complexes (pMHC-I) on cells. We show that modifying epitope peptides with bioorthogonal groups in surface accessible positions allows wild-type-like MHC-I binding and bioorthogonal ligation using fluorogenic chromophores in combination with a Cu(I)-catalyzed Huisgen cycloaddition reaction. We expect that this approach will make a powerful addition to the antigen presentation toolkit as for the first time it allows quantification of antigenic peptides for which no detection tools exist.
Introduction

The interaction between peptides in major histocompatibility type-I complexes (pMHC-I) and T-cell receptors (TCRs) is key in both the activation of cytotoxic T-cells (CTLs) and their killing capacity. CTLs respond to very small numbers of pMHC-Is on their target cells and as a result undergo alteration of phosphorylation of their T-cell receptor complex, polarization and secretion of lytic granules at the contact site with the target cell. This recognition and killing is essential for the clearance of viruses and cancers. As a result, the diversity of both the MHC-I and peptide repertoires are very high in the population. In humans, over 2000 different MHC-alleles are known.

The repertoire of self and foreign peptides that have the ability to bind to MHC-I is enormous, due to the promiscuous binding mode of the receptor complex. The peptide-binding region of MHC-I consists of a beta-sheet ‘floor’ on which two alpha-helices define a closed peptide binding groove and most polymorphic positions line this binding groove. These positions define binding pockets (of which usually two dominate) which lend specific MHC molecules their specificity for anchor residues of antigenic peptide. For example, the mouse MHC-I molecule H2-Kb binds peptides through a deep hydrophobic primary anchor pocket selective for aromatic residues at position 5 in a peptide and a second hydrophobic pocket specific for alkyl side chains at position P8. The sidechains of P4, P6, and P7 contribute minimally to the binding to MHC-I and display the highest tolerance to amino acid variability.

The study of the nature and diversity of peptides presented by a specific cell is complicated. Reagents are mostly available for the specific study of known peptides, such as T-cells specific for particular peptide-MHC-I (pMHC-I) and recombinant T-cell receptors (TCRs). Alternatively, TCR-like antibodies specific for a given pMHC-I complex can be used. Alternatively, the whole MHC-bound peptidome can be analyzed by sequencing or mass spectrometry. Recently the use of prediction software has gained interest. This can for instance be used in combination with whole-tumor sequencing to predict the presentation of tumor-specific neoepitopes for exploitation in cancer therapy.

Our aim was to explore whether bioorthogonal chemistry could serve as a tool to quantify specific pMHC-I complexes on cells. We envisaged that bioorthogonal peptides would allow the quantification of its surface concentration in MHC-I complexes by specifically incorporating bioorthogonal functional groups in tolerated positions in epitopes, followed by an on-cell ligation reaction. This would mean that the actual epitope could be quantified independent of T-cell-based reagents, which would greatly facilitate the study of pMHC-Is for which TCR-based reagents are not available. Unlike larger detectable groups, bioorthogonal groups are small enough not to affect loading and their in vivo stability makes them ideally suited to the study of pMHC-I complexes on surface of the cell. Quantification of the bioorthogonal antigens can be achieved by ligation of a complementary fluorophore to the bioorthogonal amino acid side chain at the end of the experiment. We realized this would be challenging, due to the low numbers of specific pMHC-Is available on the cell surface. Typically, 105-106 MHC-I molecules are present per cell, which would require sensitive and selective ligation of these peptides. Furthermore, these ligations have to be done on peptides within MHC-I complexes, adding to the experimental stringency.

Here, we present our optimization of the bioorthogonal quantification of peptides loaded on MHC-I complexes. We establish binding and ligation parameters that allow the quantification of exogenous bioorthogonal epitopes within a pMHC-I complex. We optimize and assess the effect of bioorthogonal modification of epitopes on MHC-I binding, TCR recognition, and bioorthogonal reaction efficiency in various possible ligation reactions to facilitate further translation to the quantification and imaging of the antigen presentation pathway.
**Figure 1. Bioorthogonal antigens.** (A) Crystal structure of epitopes within MHC-I complexes allows the prediction of solvent accessible residues available for bioorthogonal modification. Here HSV-Gp498-505 in H2-Kb is shown with accessible positions 4 and 7 in magenta. (B) H2-Kb rescue by bioorthogonal analogues of SIINFEKL: Peptides are exogenously loaded on RMA-S with 1 μM of the indicated bioorthogonal rescue peptides at 37 °C for 4 h to allow affinity-dependent stabilization of MHC-I. Positions 1, 4, 6, and 7 can be modified with both azides and alkynes without affecting MHC-I binding affinity, and additionally positions 2, 3, and 8 also tolerate azide substitutions. Data obtained from experiments were normalized to the corresponding control of each sample, where the control values were set to equal 1 to account for batch-to-batch variation of MHC expression on RMA-S cells. All error bars represent the SD of the mean from three independent experiments. (C) MHC-I stabilization on RMA-S cells of the nonligatable and ligatable variants of HSV-Gp498-505 modified with Pg at the indicated positions: binding of these epitopes is similar to that of the wildtype epitope.
Results and discussion

In order to be applicable to the study of pMHC-I biology, bioorthogonal modifications must not negate binding to MHC-I. Once bound to MHC-I, the steric hindrance should be sufficiently small to allow on-surface ligation of the bioorthogonal group to allow the quantification of the pMHC-I preferably below MHC-I saturation levels.

Bioorthogonal modification and pMHC-I stability

We first determined whether bioorthogonal chemical functionalities could be incorporated into minimal MHC-I epitopes without affecting binding to MHC-I. We focused our efforts on the two smallest available bioorthogonal groups with the lowest sidereactivity: the alkyne and azide. Both minimally impact structure due to their small size and bioorthogonality and are readily incorporated into peptides and proteins in the side-chain modified amino acids azidohomoalanine (Aha) and propargylglycine (Pg). Furthermore, they have exceptional stability profiles as only very few biological sequestration reactions of alkynes and azides have been reported.

To test MHC-I binding and to establish the constraints of performing bioorthogonal ligations within the MHC-I complex, we generated a library of 16 bioorthogonal analogues of the major epitope peptide spanning residues 257-264 of the model antigen ovalbumin (OVA; SIINFEKL, Table S2) modified with either Aha or Pg at each of the positions within the epitope (P1-P8). We assessed binding of the 16 bioorthogonal peptides to the MHC-I molecule H2-Kb and compared it against the affinity of the parent epitope, SIINFEKL.

Binding was assessed using the TAP-deficient RMA-S cell line, which expresses a large fraction of its MHC-I molecules with low affinity peptides, when incubated at 26°C. These low affinity peptides rapidly dissociate when the temperature is raised to 37°C, resulting in the internalization and degradation of the empty MHC-I complexes. These can, however, be stabilized by coincubation with a high affinity peptide. Quantification of MHC-I at the cell surface with an anti MHC-I-antibody after incubation at 37°C for 4 h thus provides an indirect quantification of MHC-I binding affinity of a particular peptide.

This assay revealed that Aha-substitutions were tolerated in terms of H2-Kb binding at all positions, except the primary anchor residue Phe-5 (Figure 1B). Even modifications of the secondary anchor residues (P2, P3, or P8) were well-tolerated. Pg substitutions in SIINFEKL were tolerated less broadly, but still non-anchor positions could be substituted without loss of affinity (Figure 1B). As for our previously reported azido-antigen, none of the modified antigens are recognized by the SIINFEKL cognate T-cell clone B3Z (Figure S3A), with the exception of Pg-8. Binding of the TCR-like antibody that is specific for the peptide SIINFEKL in complex with H2-Kb (antibody 25-D1.16) was in alignment with the known contact sites from the crystal structure of the complex: modifications of P1-P4 (known not to interact with the antibody) were tolerated, whereas modifications of the antibody contact positions P5-P8 abolished 25-D1.16-binding (Figure S3B). This discrepancy between B3Z and 25-D1.16 binding highlights the difference in fine specificity between antibody and TCR.

Optimizing on-surface ligation chemistry

Having shown that positions 1, 4, 6 and 7 can be substituted to Aha and Pg without loss of affinity for H2-Kb, we next analyzed whether the bioorthogonal epitopes could be ligated when bound to MHC-I. We tested the suitability of the available azide- and alkyne-reactive bioorthogonal ligation reaction chemistries by comparing the reactivity of unmodified SI-
InFEKL and the epitopes modified with either azides or alkynes at P7. First RMA-S cells were pulsed with the different peptides, then ligated to complementary bioorthogonal fluorophores under various conditions and analyzed by flow cytometry for the increase in mean fluorescence intensity upon ligation. In these experiments, none of the attempted modifications of azide-containing epitopes, such as the strain-promoted azide-alkyne cycloaddition reactions (SPAAC; Figure S4), Staudinger ligations and copper(I)-catalyzed Huisgen cycloaddition-reactions (ccHc; Figure S4) gave statistically significant signal-to-noise ratios.

Only when the recently reported fluorogenic azide-reactive CalFluor-488, of which the fluorescence quantum yield increases 250-fold upon ccHc-ligation37 (3, Figure 2A) was combined with a labeling protocol in which the cells were first fixed mildly (0.5% paraformal-
dehydine in PBS) and extensively blocked with 1% BSA and 1% fish gelatin before and after the reaction, were robust and significant signal-to-noise ratios obtained (Figure 2B/C). This implies that our approach is limited to the quantification of alkynyl epitopes as Calfluors are exclusively alkyn reactive.

The reaction showed a strong dependence on solvent accessibility of the amino acid side chain in the crystal structure: OVA\textsubscript{257-264}\textsuperscript{Pg-8} (of which the Pg-sidechain likely resides in a hydrophobic pocket, Figure 3A) showed no labeling even at very high peptide concentrations (up to 20 µM). This is in contrast to OVA\textsubscript{257-264}\textsuperscript{Pg-7}, which carries the bioorthogonal side chain in a solvent exposed area. This epitope showed concentration dependent ligation efficiency (Figure 2B). This supports the hypothesis that the bioorthogonal peptides labeled are indeed bound to H2-K\textsuperscript{b}. The reaction also shows an increase in reactivity with an increase in pH (Figure 2C), likely due to the reported increased stability of CalFluor at higher pH-values.\textsuperscript{37}

To assess whether fixation conditions used for the ccHc permeabilized the cells, resulting in labeling of intracellular pMHC complexes undergoing recycling\textsuperscript{38}, we also attempted the modification reaction on unfixed live cells. Using short reaction times, we showed similar labeling for live cell and fixed cell labeling, suggesting that the contribution of intracellular labeling of recycling pMHC complexes does not contribute to the overall signal observed in these experiments (Figure S5).

With these optimized ligation conditions in hand, we performed a positional scan of ccHc-reactivity of all positions in OVA\textsubscript{257-264}.
on RMA-S, again quantified by flow cytometry (Figure 3B). These experiments showed a strong correlation of reactivity with solvent-accessibility (as estimated from the reported crystal structure33; Figure 3): solvent-accessible39 positions P4 and P7 showed significant signal-to-noise ratios, whereas P1, P2, P3, P5 and P8 showed no signal over background.

Application to other H2-Kb-binding epitopes

We then determined whether our strategy was restricted only to SIINFEKL variants, or whether other H2-Kb-binding epitopes could also be ligated. We focused on epitopes for which crystal structures are known and found that nucleoprotein52-59 epitope from the vesicular stomatitis virus (RGVYVQGL)7,40 also showed significant labeling when modified at the solvent-accessible residues P4 and P7 (Figure 3C). A second disease-relevant viral epitope, Herpes simplex virus (HSV) glycoprotein B4498-505 (HSV-Gp498-505)41 also showed reactivity in line with the crystal structure: solvent accessible P4 and P7 could be ligated, and solvent inaccessible P2 could not (Figure 4A,D). MHC affinity of these peptides was identical to the w.t.-HSV-epitope and to SIINFEKL (Figure 1C).

We were also intrigued whether the approach could be used to determine MHC-loading of epitopes for which no TCR or antibody reagents exist. We therefore looked at other reported H2-Kb binding peptides from OVA, against which no T-cell responses are observed during immune responses, despite a reported affinity39 for H2-Kb haplotype MHC-I42, namely OVA55-62. This peptide too showed cChc-reactivity for the predicted solvent accessible modification Pg-7 and not for Pg-2 (Figure 3C).

Due to its robust MHC-I binding, relevance in the immune response against HSV, and robust bioorthogonal labeling, we further explored the Pg-7-modified variant of HSV-GpB498-505 epitope by performing a serial dilution of HSV-GpB498-505-P7 on RMA-S (Figure 4B). Here we found that a detectable signal over background was still obtained after incubation with 19 nM peptide, which is similar to the sensitivity of detection that can be obtained with a TCR-like antibody17 highlighting the power of this research tool for the study of antigen presentation.

Reactivity of MHC-bound epitopes

To determine what percentage saturation of the 10^5 MHC-Is44 could be detected, we performed a competition experiment: bioorthogonal peptides were co-incubated with increasing concentrations of unlabeled control peptides (Figure 4C). Bioorthogonal ligation yields indeed showed an inverse correlation with concentration of the unlabeled competition peptide. However, even at a concentration of competing peptide of 32 µM, a detectable signal could be observed. Presuming equal affinity of the two peptides for MHC-I, this means that the peptide could be detected at <10% saturation level within the H2-Kb peptide binding groove, suggesting this approach could be used to image 10^4 molecules per cell.

In conclusion, the here presented data suggest that bioorthogonal epitopes can bind and be quantified within MHC-complexes at physiologically relevant concentrations and may serve
as a useful tool for studying pMHC-I biology on the surface of cells. The requirement is that nonanchor residues in solvent-accessible positions are modified, which results in peptides capable of both binding MHC-I and of being ligated using a fluorogenic ccHc-variant.

We expect that our approach can make a powerful addition to the antigen presentation toolkit as for the first time it allows facile quantification of antigenic peptides for which no T-cell (or other) reagents are available, such as the H2-Kb-binding peptide OVA55-62 reported herein. The broad scope of bioorthogonal chemistry45 and the breadth of tools available to incorporate minimal bioorthogonal functionalities into peptides, proteins46 from both prokaryotic expression systems47, as well as eukaryotic ones48,49, and whole cells27 including various pathogens50 suggests that this approach could potentially extend to the study the rates at which antigen presenting cells process exogenous antigens for the activation of CTLs – so-called antigen cross-priming. The stability of bioorthogonal groups would be very beneficial here, as antigens encounter some of the harshest conditions known in the human body in...
the endolysosomes of antigen presenting cells, with both strongly oxidizing and reducing conditions found during cross-presentation. We have previously shown azides to be stable to these conditions and are currently pursuing the stability of other bioorthogonal groups in the endolysosomal environment to image the entire cross-presentation routing start-to-finish.

Method and materials

General Reagents

Alexa Fluor 488 Azide (catalogue number: A10266) and all other fluorophores were purchased from Thermo Fisher Scientific. Azidohomoalanine and Propargylglycine-Fmoc were purchased from Anaspec. Tris(3-hydroxypropyl-triazolylmethylamine) (THPTA) was purchased from Sigma-Aldrich, as were all other reagents at the highest available grade. Mouse Anti-Mouse H2-Kb (B8-24-3 clone) was made in-house. 25-D1.16-APC conjugated was purchased from eBioscience (Cat. #: 12-5743-81) (APC conjugated in-house). Secondary antibody (Goat anti-Mouse IgG conjugated to Alexa Fluor-647 (catalogue number: A-21235) was purchased from Thermo Fisher Scientific. All solvents were purchased from Biosolve Ltd. Phosphate buffered saline (PBS) is 5 mM KH2PO4, 15 mM Na2HPO4, 150 mM NaCl, pH 7.4.

NMR spectra (1H and 13C) were measured on a Bruker AV-400MHz spectrometer at ambient temperature at the Leiden Institute of Chemistry NMR Facility. Chemical shifts are recorded in ppm. Residual solvent peaks were used as an internal standard.

Peptide Synthesis

All peptides were synthesized using standard Fmoc Solid Support Chemistry and purified using High Performances Liquid Chromatography (Prep column Gemini C18 110A 150x21.20 5μm) using 15 to 45 % gradient (A: 0.1% TFA in MilliQ H2O, 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 and analyzed by electrospray LC-MS analysis on a PE SCIEX: API 3000 LC/MS/MS system using a Gemini 3u C18 110A analytical column (5μ particle size, flow: 1.0 ml/min), on which the absorbance was also measured at 214 and 254 nm. Solvent system for LC-MS: A: 100% water, B: 100% acetonitrile, C: 1% TFA (aq).

Calfluor-488 Synthesis

Calfluor-488 was synthesized as described previously. Analytical data: M/z found: 836.3 [M=H]+.

RMA-S MHC I-binding and stability assays

RMA-S assays were essentially performed as described previously. Briefly, RMA-S cells were grown and passaged at 37 °C, 5% CO2 in RPMI-1440 augmented with 10% FCS and antibiotics. Prior to the experiment, the incubation temperature was lowered to 26 °C for 48 hours (106 cells/mL) to ensure metastable MHC-I surface expression. For affinity tests, cells were incubated with rescue peptides in serum free medium at the indicated concentrations for 4 hours and washed in protein blocking agent (PBA: 5% BSA in PBS + 0.1% w/v NaN3). For MHC I-peptide complex stability assays, 26°C RMA-S cells were pulsed for 1 hour with respective peptides at the indicated concentrations, and washed thoroughly in ice-cold serum free medium, after which they were placed back at 37 °C and chased for the indicated time. After each timepoint, cells were fixated in 4% fixation buffer (Cat #420801, Biolegend) for 30 minutes. After this time, the cells were washed with PBA. Both assays were subse-
quently stained with anti-Mouse-H-2Kb (400 ng/mL; >60 μL/well) in PBA for 30 minutes on ice, prior to washing with PBA twice. Secondary antibody (Goat anti-Mouse IgG conjugated to AlexaFluor-647; 5 μg/mL) was added and the cells were again incubated on ice for 30 minutes prior to washing twice with PBA before analysis. Analysis was performed on a BD Accuri™ C6 Plus Flow Cytometer. All flow cytometry data was analyzed using FlowJo v10.1 (Miltenyi Biosciences).

25-D1.16-binding of bioorthogonal epitopes

RMA-S cells were incubated with a serial dilution of peptides as above. After the 4 hour loading period and blocking, cells were incubated with 25-D1.16-APC conjugated antibody (1.3 μg/mL; >60 μL/well, conjugated in-house) in PBA for 30 minutes on ice, prior to washing with PBA twice. Cells were analyzed by flow cytometry as described above. Fluorescence intensity in the APC-channel were plotted against peptide concentration at 1μM was used for Figure S3B.

Bioorthogonal Modification reactions on RMA-S Cells

RMA-S cells were grown and as described above for the binding assay and plated in 96-well v-bottom microtiter plate (400,000 cells/well) in serum free medium and incubated for ~1h at 37°C with respective peptides at the indicated concentrations. After the incubation the cells were washed twice in PBS and subsequently fixed for 1 hour at RT in 0.5% PFA in PBS (Cat #420801, Biolegend; diluted 1:8) and washed twice more with PBS. Cells were then exposed to the bioorthogonal labeling mixture (1 mM CuSO₄, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM aminoguanidine, 100 mM HEPES, pH 8.4, CalFluor-488 10 μM). After 45 minutes at RT, the reaction mixture was aspirated and the cells were blocked with 1% BSA and 1% fish gelatin before being washed twice with PBS prior to analysis by flow cytometry. Assays were set up in triplicate, unless otherwise indicated. The statistical significance of the indicated differences was analyzed by the two-tailed student’s t-test with the significance specified using p values with *p≤0.05, **p≤0.01, ***p≤0.001, and ****p≤0.0001. All error bars correspond to SD of the mean.

Reactivity of bioorthogonal SIINFEKL peptides in a B3Z T cell assay

RMA-S cells were grown as described above and plated in 96-well tissue-culture treated microtiter plate (50,000 cells/well) and incubated for 1h at 37 °C with SIINFEKL modified peptides at the indicated concentrations, followed by a wash with complete IMDM. After the wash, the T cell hybridoma B3Z cells (50,000 cells/well) were added. The RMA-S 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.54

Competition assay with unlabeled peptide

RMA-S cells were grown and treated as described above for the bindings assay, prior to loading. Incubation of RMA-S cells with HSV-Gp₄₉₈-₅₀₅-Pg7 epitopes was performed for 4 hours at 4 μM, in presence of increasing amounts of w.t. HSV-Gp₄₉₈-₅₀₅ at the indicated concentrations. The amount of peptide in all samples was equalized to 36 μM by adding the lacking amount of adenoviral H-2D⁴ binding epitope of the human Adenovirus 5 E1a protein Ad10₂₃₄-₂₄₃.55 Bioorthogonal ligation was subsequently performed as described above.
References


Supplementary figures

![Figure S1. Structures of Aha (1), Pg (2)](image)

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Table S2. Overview of all modified epitope peptides used in this study and corresponding nomenclature.
Figure S3. (A) Reactivity of biorthogonal antigens with the SIINFEKL-specific T-cell clone B3Z T-cell assay of Pg-modified SIINFEKL. Peptides were serially diluted 1:5 from 10 μM and incubated with B3Z using standard conditions12. Pg-modified SIINFEKL on the anchor position P8 shows a T cell response, but no other position does. (B) Binding of complex-specific antibody 25-D1.16 to H-2Kb-bound SIINFEKL analogues on RMA-S cells.
**Figure S4.** Normalized mean fluorescence intensity (MFI) in FL-4 channel (647 nm) of the strain-promoted azide-alkyne cycloaddition (SPAAC) with Alexa Fluor 647 DIBO Alkyne (DIBO-647) and Copper(I)-Catalyzed Azide-Alkyne Huisgen Cycloaddition (ccHc) with Alexa Fluor-647-alkyne. Both reactions were performed with OVA257-264-Aha-7 and no peptide as control. Data obtained from experiments were normalized to the corresponding control of each samples, where the control equals 1. All error bars represent the SD of the mean from at least 2 independent experiments.

**Figure S5.** Comparison of ccHc conditions on cells fixed with 0.5% PFA to ccHc reaction performed on live cells (for these reactions the reaction time was reduced to