Bioorthogonal antigens as tool for investigation of antigen processing and presentation

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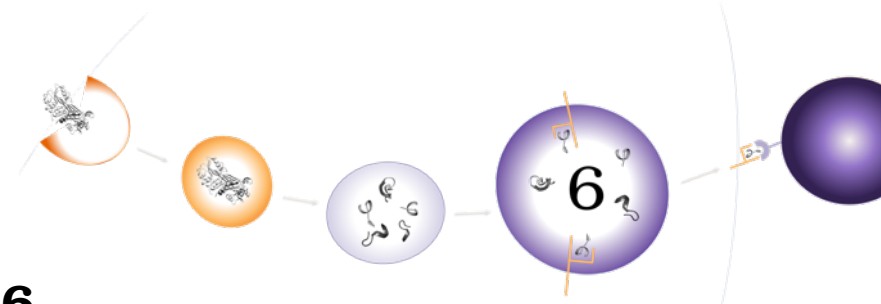


Chapter 6

Summary and future perspectives

6.1 Summary

This thesis illustrates research on the use of bioorthogonal protein as antigens in immunological studies. Bioorthogonal proteins are defined as proteins in which one of the canonical amino acids is replaced by a non-canonical one, the side-chain of which carries a bioorthogonal functionality - a functionality that is inert in physiological samples and that can be made to react effectively and selectively to bring added functionality at a desired time during the course of a biological experiment.

In the context of the research described in this thesis, bioorthogonal proteins are created and utilized in which methionine residues in the protein backbone are replaced by Aha or Hpg-biological Met isosteres that are small (thus are not likely to perturb protein functioning much), are physiologically largely inert, yet are reactive. Aha and Hpg can be used for the copper-catalysed Huisgen cycloaddition reaction, or - in case of Aha- in the Staudinger and strain-promoted azide-alkyne cycloaddition reactions as well, and can be readily introduced in recombinant proteins by utilizing methionine auxotroph bacterial expression strains.

The main focus of this thesis research has been to establish procedures that make use of bioorthogonal proteins to study proteolysis events that take place in dendritic cells during antigen presentation. Factors that partake in antigen processing, such as dynamics, hierarchy of proteolytic events (which proteases are involved and in which order), and in which subcellular compartments are currently very hard to study. Simply incubating APCs with antigenic peptides equipped with reporter molecules fails to give a good picture of what happens in natural antigen processing because antigen trafficking and proteolysis are intrinsically linked. The rate of proteolysis is highly dependent on the precise sequence and structure of the protein, which hampers or even obviates the use of engineered/modified antigenic proteins bearing (large, often hydrophobic) fluorophores as reporter entities (this thesis Chapter 2). A final complication comprises the harsh chemical conditions found within the antigen presenting pathway of the dendritic cell. On its way to being MHC-surface loaded, the peptide will encounter oxidative as well as reducing conditions, besides the acidic environment as found within lysosomes

- conditions that fluorophores may not withstand (and incidentally also precludes the use of alternative bioorthogonal tags such as trans-cyclooctenes). For these reasons, antigenic proteins containing either Aha or propargylglycine (Pg) as bioorthogonal antigens are put forward here for a variety of studies in the context of antigen processing and presentation. The research described in the preceding consecutive chapters of this thesis covers both the generation of such bioorthogonal antigens and the study of their fate in a number of *in vitro* and *in situ* proteolysis studies.

Chapter 2 introduces antigen processing and presentation as a central pathway in adaptive immunity in vertebrates, the techniques that are available to study processes involved in these, and bioorthogonal chemistry, its scope and limitations and its potential to aid in the study of antigen processing and presentation, the latter as indicated in Chapter 1 in which this thesis work is introduced. **Chapter 3** describes the expression of bioorthogonal variants of the model antigen, ovalbumin in a methionine auxotrophic *E. coli* strain. Replacing Met with Aha or Hpg during the expression yielded the antigen carrying 17 click-reactive groups per protein. These proteins were then assessed in their ability to activate cognate T cells after processing by DCs, proving to be near identical in their antigenic properties to the wildtype of the antigen. In addition, it was shown that the degradation of the antigen could be followed by SDS-PAGE, and the fate of the antigen tracked by confocal microscopy. These data combined suggest bioorthogonal antigens to be suitable reagents for studying antigen processing.

Chapter 4 describes the detailed study of the proteolysis of the bioorthogonal antigenic proteins that were prepared in Chapter 3. By assessing the cleavage rates by different recombinant proteases *in vitro*, the effects of the bioorthogonal modification on this degradation can be studied in detail. These experiments show that this fine specificity changes <20 % upon introduction of the click handles. It was also analysed whether the post-translational modification carbamylation and citrullination of the proteins did lead to altered processing. This was indeed observed: carbamylation in particular led to significant reductions in the rates of proteolysis for various endo-lysosomal cathepsins. Interestingly, this was also true for the auto-antigen vinculin of which post-translational citrullination and carbamylation have been implicated in the pathogenesis of rheumatoid arthritis. Thus, led to the research described in **Chapter 5**, in which the altered antigenicity of these proteins is described, confirming that bioorthogonal antigens were useful reagents for studying the processing of these modified proteins.

6.2 Future perspectives

Whereas the research as described in Chapter 3 - Chapter 5 on bioorthogonal antigenic proteins and their use in the study of antigen processing and presentation yielded interesting results, there are limitations associated with the approach. One of these is the choice of the bioorthogonal groups and reactions. The copper-catalysed Huisgen cycloaddition is advantageous in that the reagents are stable, and the reaction proceeds with low background reactivity. However, the reaction does not allow for the live cell imaging of the above processes, nor does it allow for the tracking of only the epitope. The development of bioorthogonal antigenic proteins that would enable the above two research objectives are future directions that would broaden the scope of the approach. One way to achieve this would be to use the azide-containing bioorthogonal antigens

and expose these to bioorthogonal reactions that can be performed in living cells, such as the strain-promoted azide-alkyne cycloaddition, or the Staudinger ligation. However, neither of these reactions are ideal, as the times need to require detectable conversions (> 1 h) do not allow for biologically meaningful data to be obtained. In other words: too much biology would have happened during the ligation step leading to a 'blurring' of the biological observation. Additionally, the reagents used for these reactions suffer from extensive side reactions, particularly under the redox conditions found in the antigen processing pathway. Thus, to achieve *in vivo* epitope/antigen tracking, both faster and more selective live cell compatible bioorthogonal reactions are needed. Although such ideal reactions do not yet exist, one that comes close - at least in terms of speed and live cell compatibility - are inverse Electron-Demand Diels-Alder (iEDDA) reactions: reactions between an electron poor diene and an electron rich (often strained) dienophile. Rates of up to 10^4 $M^{-1}s^{-1}$ have been reported for these reactions[303], which indicates that labelling under physiological conditions (ambient temperature and pressure, neutral pH, aqueous conditions) can possibly be achieved within a few minutes.

On the downside, and in contrast to the introduction of Aha and Hpg described in Chapter 2 - Chapter 5, non-canonical amino acids that can partake in an iEDDA process (that is, a tetrazine or strained alkyne containing amino acid) can only be used by amber codon suppression. This methodology, although much more flexible in terms of the nature of the non-canonical amino acid that can be introduced through adaptation of ribosomal protein synthesis, is complex in execution and normally returns small amounts of protein.

An easier way of using near isosteric iEDDA-reactive amino acids would be to incorporate them into polypeptide antigens using chemical approaches, thereby allowing control over the number and position of these click handles. Native chemical ligation can be used to produce proteins in this manner, but the synthesis of the proteins are complex, and not all sequences are amenable to ligation strategies or the subsequent refolding that is required to produce a native protein[304,305]. In terms of size, linearly synthesized (long) peptides (SLP) offer an interesting alternative. Not only can the readily be made to a size of approximately 40 amino acids. They are also currently being pursued in cancer vaccination strategies[52,60,65,66,306,307]. The synthesis of isosteric iEDDA-reactive amino acids, compatible with Fmoc-solid phase peptide synthesis would therefore be of great use.

In a first step towards this goal, it was assessed whether synthetic peptide antigens could be of use within the chemical constraints of this thesis (that is, whether they could be used in combination with the ccHc-reaction). For this purpose, some preliminary experiments were performed, in which a series of synthetic 'long'-peptides (LP) were designed based on two model epitopes: the Herpes Simplex Virus (HSV) glycoprotein (GP) B-derived T cell epitope SSIEFARL (HSV $GpB_{497-505}$, 8-mer), and the chicken ovalbumin epitope SIINFEKL ($OVA_{256-264}$, 8-mer). Analogues of these peptides were made carrying different N- and/or C-terminal extensions, and propargylglycine at position P4, P7 or P8 of the epitope (Table 6.1).

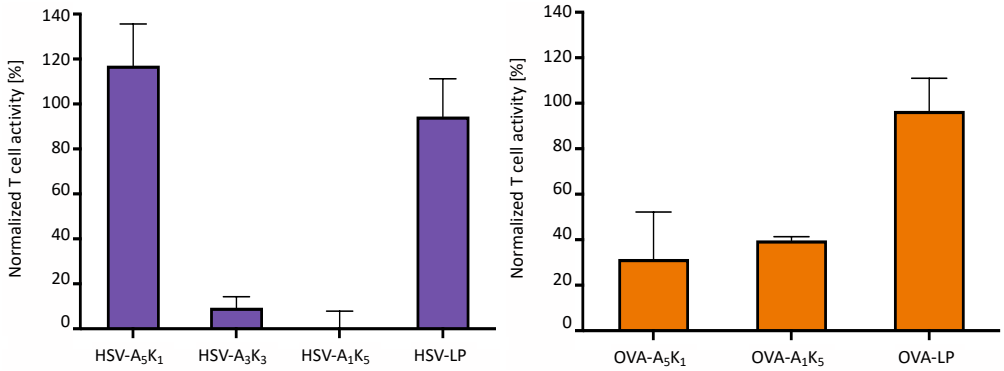
One of the variables that was chosen was to increase the number of positive charges in the C-terminal flanking region of the peptide. This was postulated to lead to an increased retention of these peptides in acidic organelles. All peptides were synthesized by solid-phase peptide synthesis, purified by HPLC. All peptides were found to be greater than 90 % pure. The structure of the SLPs was assessed by CD and showed structural changes upon increases in lysine content: HSV-A_5K_1-Wt peptide showed

Table 6.1: Overview of synthezised herpes simplex virus - and ovalbumin -derived peptides. The epitopes are indicated in bold.

Peptide				Sequence		
Abbreviation	Full name	Source	Length	N-term	Epitope	C-term
HSV-A_5K_1	HSV $Gp_{488-505}$-A_5K_1	HSV GpB	24-mer	NASVERIKTT	**SSIEFARL**	AAAAAK
HSV-A_3K_3	HSV $Gp_{488-505}$-A_3K_3	HSV GpB	24-mer	NASVERIKTT	**SSIEFARL**	AAAKKK
HSV-A_1K_5	HSV $Gp_{488-505}$-A_1K_5	HSV GpB	24-mer	NASVERIKTT	**SSIEFARL**	AKKKKK
HSV-LP	HSV $Gp_{488-500}$	HSV GpB	18-mer	NASVERIKTT	**SSIEFARL**	
HSV-A_5K_1-Pg_3	HSV $Gp_{488-505}$-A_5K_1	HSV GpB	24-mer	NASVERIKTT	**SS**Pg**EFARL**	AAAAAK
HSV-A_5K_1-Pg_4	HSV $Gp_{488-505}$-A_5K_1	HSV GpB	24-mer	NASVERIKTT	**SSI**Pg**FARL**	AAAAAK
HSV-A_5K_1-Pg_7	HSV $Gp_{488-505}$-A_5K_1	HSV GpB	24-mer	NASVERIKTT	**SSIEFA**Pg**L**	AAAAAK
HSV-A_5K_1-Pg_8	HSV $Gp_{488-505}$-A_5K_1	HSV GpB	24-mer	NASVERIKTT	**SSIEFAR**Pg	AAAAAK
OVA-A_5K_1	$OVA_{247-264}$-A_5K_1	OVA	24-mer	DEVSGLEQLE	**SIINFEKL**	AAAAAK
OVA-A_3K_3 $^+$	$OVA_{247-264}$-A_3K_3	OVA	24-mer	DEVSGLEQLE	**SIINFEKL**	AAAKKK
OVA-A_1K_5	$OVA_{247-264}$-A_1K_5	OVA	24-mer	DEVSGLEQLE	**SIINFEKL**	AKKKKK
OVA-LP	$OVA_{247-259}$	OVA	18-mer	DEVSGLEQLE	**SIINFEKL**	

HSV= Herpes Simplex Virus, GpB = glycoprotein B, LP = long peptide. OVA= Ovalbumin (*G. gallus*), $^+$peptides could not be synthesized.

mainly β-sheet behaviour (data available upon request), whereas the a-helical content dominated for HSV-A_3K_3-Wt, HSV-A_1K_5-Wt showed no secondary structure motifs. The obtained peptides were tested for their immunogenicity in a T cell using the D1 cell line as APC. The peptides and DCs were co-incubated for 3 h prior to washing and the addition of either a SSIEFARL-specific T cell hybridoma (LacZ-inducible T cell hybridoma HSV2.3.2E2)[148], or the SIINFEKL-specific B3Z T cell hybridoma (Figure 6.1).



(a) HSV-peptides **(b)** OVA-peptides

Figure 6.1: T cell assay with synthetic long peptides. Activation of (a) HSV-specific or (b) OVA-specific T cells after pulse with 10 μM final concentration of each indicated peptide. Measurement of activation by determination of IL-2 production by T cells after peptide uptake. n=3 independent with at least two experimental (N=2) replicates.

For the various HSV-peptides, the highest responses were observed for HSV-A_5K_1-Wt and the reference peptide HSV-LP. The HSV-A_3K_3-Wt peptide showed only $^1/_{10}$th

of the response at this concentration and HSV-A_1K_5-Wt failed to activate the T cells above the limit of detection. These data indicate that these model peptides are suitable reagents to assess bioorthogonal long peptides. The reason for the variations between the different C-terminal variations of the peptides remains to be investigated.

In order to study whether bioorthogonal variants of these peptide antigens could be used to track the fate of the epitope within dendritic cells, isosteric peptides carrying Pg at positions 3, 4, 7 and 8 in the epitope were made (Table 6.1). Interestingly, secondary structure determination of the P_3-modified peptides (see experimental details), showed that the substitutions at position 3 caused a major change in structure: all peptides displayed random coil structures after incorporation of the bioorthogonal groups. Preliminary microscopy experiments of these peptides showed extensive aggregation in cell culture media (data available upon request), leading to a rethink of the use of such click-enabled peptides as model antigens. However, the combination of such bioorthogonal peptide synthesis (particularly carrying PTMs at specific sites and live-cell compatible click handles), with the protein synthesis methodologies using e.g., native chemical ligation or expressed protein ligation strategies remain a tantalizing future prospect for this work.

6.3 Materials and methods

6.3.1 Materials

Chemicals

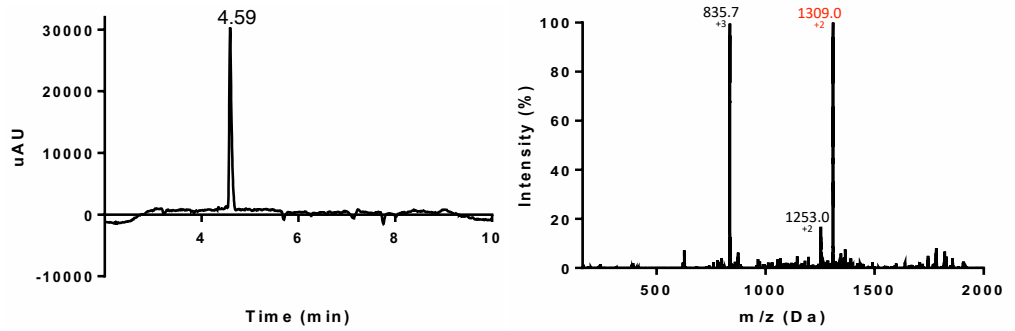
AlexaFluor488-azide (catalogue number: A10266) and all other fluorophores were purchased from Thermo Fisher Scientific. For solid phase synthesis, amino acids were purchased from Novabiochem (Fmoc-Glu(OtBu)-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Val-OH and Fmoc-Propargylglycin-OH (Pg)) and from Sigma Aldrich (Fmoc-Ala-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Ile-OH, Fmoc-Lys(Boc)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Arg(Bbf)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH) and resins were purchased from Rapp Polymere GmbH, Germany.

6.3.2 Methods

Peptide synthesis

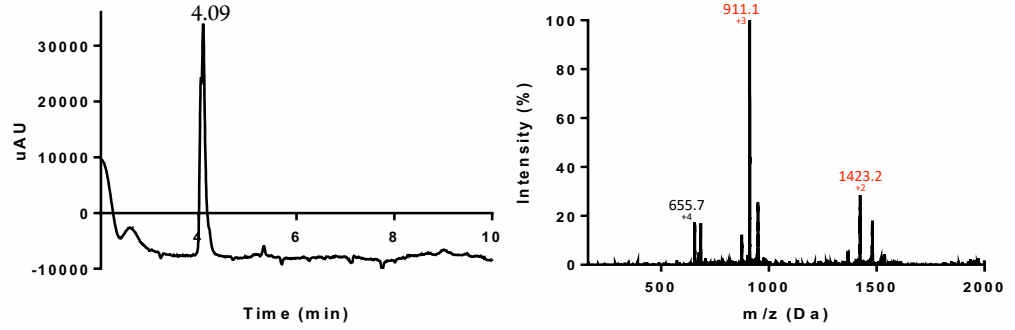
Peptides were synthesized using standard 9-fluorenylmethyloxycarbonyl (Fmoc) Solid Support Chemistry and purified using High Performances Liquid Chromatography (Prep column Gemini C18 110 Å 150 x 21.20 mm pore size, 5 μm particle size) using 15 to 45 % gradient (A: 0.1 % TFA in MilliQ H_2O, B: ACN). LC-MS measurements were done on an API 3000 Alltech 3300 with a Grace Vydac 214TP 4,6 mm x 50 mm C4 column.

Manual synthesis: Peptides were synthesized manually (or in a synthesizer) using solid phase peptide synthesis (SPPS). An adapted version of the protocol described by Amblard *et al.* (2006)[308] was followed. Three different 9-fluorenylmethyloxycarbonyl chloride (Fmoc) protected resins were used for the synthesis of the linker-peptides; Tentagel SRAM, Tentagel RRAM and Tentagel SPGH Lys (Boc) Fmoc (Rapp Polymere GmbH, Germany).



(a) LC-MS HSV-A_5K_1-Wt **(b)** Ion spectrum HSV-A_5K_1-Wt

Figure 6.2: Characterization of peptide HSV-A_5K_1. (a) analytical C18 RP-HPLC chromatogram of purified HSV-A_5K_1 peptide, linear gradient 10-90 % ACN. (b) corresponding mass spectrum of HSV-A_5K_1 peptide at t=4.59 min, expected mass: 2504.84 g/mol, observed mass: 2504.1 Da, 835.7 [M+3H], 1253.0 [M+2H], 1309.0 [M+2]+TFA.



(a) LC-MS HSV-A_3K_3-Wt **(b)** Ion spectrum HSV-A_3K_3-Wt

Figure 6.3: Characterization of peptide HSV-A_3K_3. (a) analytical C18 RP-HPLC chromatogram of purified HSV-A_3K_3 peptide, linear gradient 10-90 % ACN. (b) corresponding mass spectrum of HSV-A_3K_3 peptide at t= 4.09 min, expected mass: 2619.03 g/mol, observed mass: 2618.8 Da, 655.7 [M+4H], 911.1 [M+3]+TFA, 1423.2 [M+2]+2TFA.

For the manual synthesis following steps were performed. The temporary Fmoc protecting group was removed by reacting with 20 % piperidine (Biosolve, Netherlands) in dimethylformamide (DMF, Biosolve, Netherlands) for 3 min and then for 7 min[309]. After washing with DMF 5 equivalents of amino acids, preactivated in 4.9 eq O-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU, Biosolve, Netherlands) 0.5 M in DMF and 10 eq N,N-Diisopropylethylamine (DIPEA, Carl Roth GmbH & Co. KG., Germany), are added to the resin for at least 30 min followed by washing with DMF. To minimize by-product formation a capping step was added to the first six couplings[308]. The resin was incubated twice for 10 min with pyridine/acetic anhydride (9:1, v/v, Biosolve, Netherlands). For the coupling of the first amino acid, all reagents were used as double equivalents with respect to normal coupling conditions[308].

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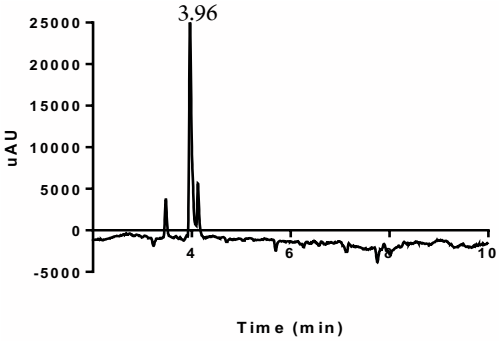
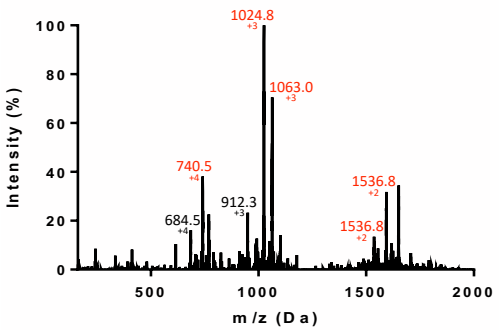
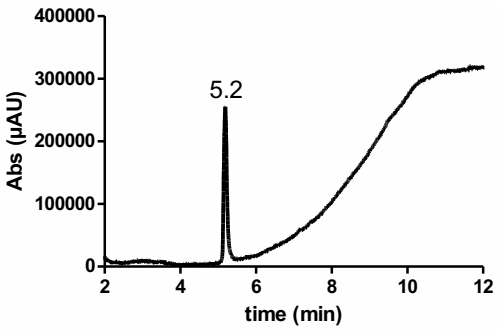


(a) LC-MS HSV-A_1K_5-Wt

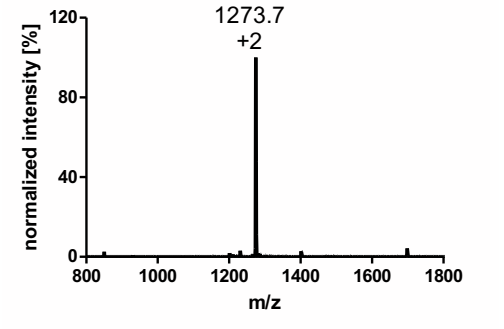


(b) Ion spectrum HSV-A_1K_5-Wt

Figure 6.4: Characterization of peptide HSV-A_1K_5. (a) analytical C18 RP-HPLC chromatogram of purified HSV-A_1K_5 peptide, linear gradient 10-90 % ACN. (b) corresponding mass spectrum of HSV-A_1K_5 peptide at t= 3.96 min, expected mass: 2733.22 g/mol, observed mass: 2734.0 Da, 684.5 [M+4H], 740.5 [M+4]+2TFA, 912.3 [M+3], 871.4 [M+3H], 909.3 [M+3]+TFA, 1363.2 [M+2]+TFA, 1420.5 [M+2]+2TFA,1063.0 [M+3]+4TFA, 1536.8 [M+2]+3TFA, 1593.6 [M+2]+4TFA, 1650.5 [M+2]+5TFA.



(a) LC-MS OVA-A_5K_1-Wt



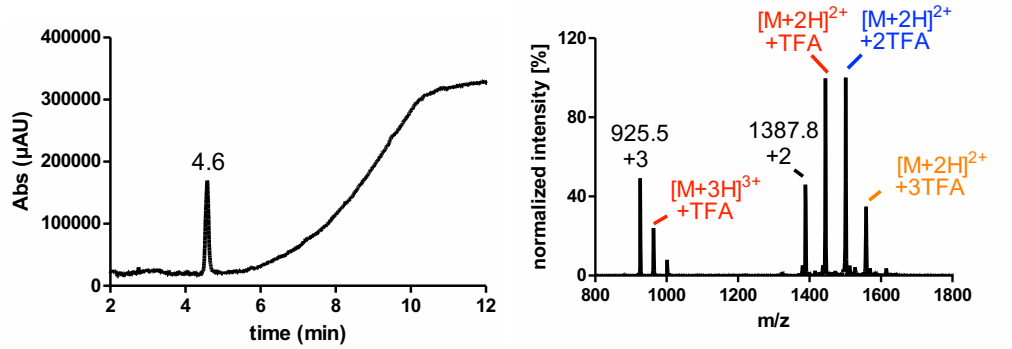
(b) Ion spectrum OVA-A_5K_1-Wt

Figure 6.5: Characterization of peptide OVA-A_5K_1. (a) analytical C18 RP-HPLC chromatogram of purified OVA-A_5K_1 peptide, linear gradient 10-90 % ACN. (b) corresponding mass spectrum of OVA-A_5K_1 peptide at t= 5.2 min, expected mass: 2545.84 g/mol, observed mass: 2545.0 g/mol, 1273.7 [M+2H].

Netherlands) was used. Washing of the resin was done with DMF. Amino acids were activated with 0.5 M HBTU. Amino acid coupling was performed for 1 h. Fmoc deprotection was done with 20 % piperidine in DMF. The deprotection and coupling efficiency across the couplings was followed by UV absorbance at 300 nm[310]. Capping after deprotection was performed using pyridine/acetic anhydride (9:1 v/v) mix.

Peptide cleavage

Cleavage from the resin was performed as follows: A mixture of trifluoroacetic acid (TFA) :water:triisopropyl silane (TIPS) (95:2.5:2.5) was added to the resin beads for at least



(a) LC-MS OVA-A_1K_5-Wt

(b) Ion spectrum OVA-A_1K_5-Wt

Figure 6.6: Characterization of peptide OVA-A_5K_1. (a) analytical C18 RP-HPLC chromatogram of purified OVA-A_5K_1 peptide, linear gradient 10-90 % ACN. (b) corresponding mass spectrum of OVA-A_5K_1 peptide at t= 4.6 min, expected mass: 2774.22 g/mol, observed mass: 2773.5 g/mol, 925.5 [M+3H], 963.13 [M+3H]+TFA, 1387.8 [M+2H], 1443.8 [M+2H]+TFA, 1500.5 [M+2H]+2TFA, 1557.8 [M+2H]+3TFA.

1.5 h. Then, the cleavage mixture was diluted in ddH_2O and a mixture of Buffer M (TBA/water/TFA, 1:1:1) to increase solubility or by treating it with diethyl ether (Sigma Aldrich, Germany), washing the precipitate twice with diethyl ether and dissolving the pellet in Buffer M. For LC-MS analysis, 20 μL of samples were injected and ran on a 10 % to 90 % B10 solvent B linear gradient using a Finnigan LCQ advantage max with a Gemini 3 μm C18 110 Å column, 150 x 21.20 mm pore size, 5 μm particle size. Absorbance was measured with the Surveyor PDA plus detector from 200 to 600 nm. The solvent system was; A: water, B: acetonitrile (ACN), C: 1 % TFA in water and D: MeOH.

Peptide purification

Peptides were purified via preparative high performances liquid chromatography (Preparative column Gemini C18 110 Å 150 x 21.20 mm pore size, 5 μm particle size) using a linear gradient of 15 to 35 % Solvent B (Solvent A: MilliQ-H_2O + 0.1 % TFA, Solvent B: ACN)

Peptide analysis

Peptides were characterized using electrospray ionization mass spectrometry (ESI-MS) on a Thermo Finnigan LCQ Advantage Max LC-MS instrument with a Surveyor PDA plus UV detector or an Agilent 6120 Quadrupole LC-MS instrument with an Agilent 1260 Infinity high-performance liquid chromatography (HPLC) system. On both systems, a Gemini-NX C18 column (Phenomenex, 3 μm, 110 Å, 50 mm x 4.6 mm) was used. Peptides were analysed using a gradient from 10 to 90 % or from 10 to 50 % of B over 10-20 min (eluent A, H_2O; eluent B, acetonitrile; eluent C, 1 % TFA in H_2O; eluent D, methanol). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all of the observed protonation states of the peptides using the Xcalibur

Qual Browser or LC-MS_6120B software.

Circular dichroism

Protein sample was dialysed into 20 mM Tris-HCl and concentrated to minimum 0.1 mg/ml. The secondary structure of protein variants has been characterized via CD spectroscopy. Far UV-CD spectra were recorded using a Jasco J815 CD spectrometer equipped with a Jasco PTC 123 Peltier temperature controller (Easton, MD) between 190 to 260 nm. The absorbance $(\Delta\varepsilon)^{215}$ was measured for every sample. Samples were loaded in a 1 mm path length quartz cuvette at 1 nm resolution. A minimum of five spectra with an acquisition time of 70 seconds for each scan in a 1 mm quartz cuvette at 1 nm resolution were acquired at rt and were averaged. For processing of raw data, GraphPad Prism 7.0 was used. The spectra are given in molar ellipticity $(\vartheta)^{215}$.

Beta-galactosidase antigen presentation assay

Used cells were cultured as described. The assay was performed in 96-well flat bottom microtiter plate. 0.05 x 10^6 D1 cells were seeded per well in 50 μL D1 Medium. D1 cells were allowed to adhere at 37 °C for 1 h. Peptides dissolved in DMSO were diluted in D1 medium and added to the respective wells at the required concentration and time before incubation at 37 °C. After incubation, medium and excess peptide was removed gently. 0.05 x 10^6 OVA-specific B3Z T cells or HSV-specific HSV I T cells were checked for viability and added in a volume of 100 μL medium at 37 °C for approximately 17 h. Subsequently, the cells were spun down at 1500 rpm for 3 min and supernatant was collected and saved. In order to measure β-galactose activity as an indication of T cell activation, 100 μL of CPRG lysis buffer (6 mg of CPRG (Chlorophenolred-β-D-galactopyranoside), 9.6 ml PBS, 90 μL $MgCl_2$ 1 M, 125 μL NP-40 and 71 μL β-mercaptoethanol) was added to each well and the plate was re-incubated at 37 °C. The resulting colour change of the converted substrate was then quantified measuring the absorbance at 570 nm and is a direct measure for T cell activation.

Cell culture

Dendritic cell line D1

Cells were cultured as earlier described in Chapter 3.4.2.

T cell line-hybridoma

Cells were cultured as earlier described in Chapter 3.4.2.