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Bioorthogonal antigens as tool for investigation of antigen processing and presentation

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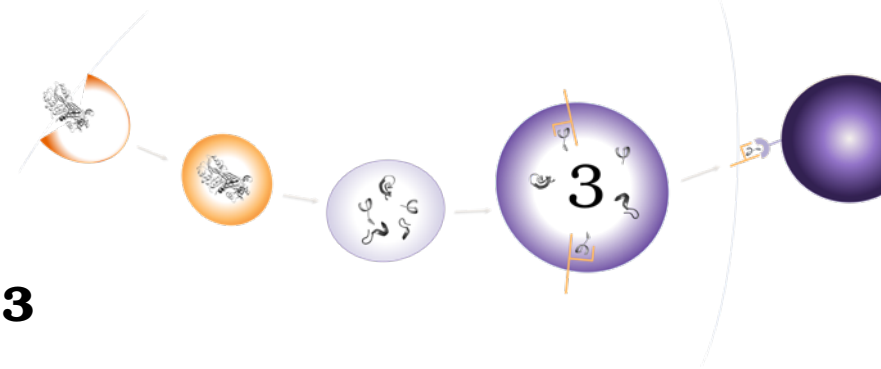


Chapter 3

Bioorthogonal antigens as model for antigen processing and presentation

3.1 Introduction

The study of the proteolysis underpinning antigen presentation is complex. Current strategies to follow the uptake and routing of a particular antigen within the cell rely on either genetic fusions of the antigen of interest with reporter proteins[209] and tracking of the reporter protein[121], or the mixing of a fluorophore modified variant of an antigen with an unmodified version[125,127] (refer to Section 2.4.3). Both approaches are based on the assumption that the detectable groups (reporter protein/fluorophore) only minimally affect the behaviour of the antigen. However, reporter modalities may significantly alter the size, shape, lipophilicity, pH(I) (isoelectric point (IEP)) and other properties of the antigenic protein (refer Section 2.3). For instance, native PAGE analysis showed that AlexaFluor647- ovalbumin and AlexaFluor488- ovalbumin, two fluorescent derivatives of the model antigen ovalbumin (Ova), differ in migration profile, also compared to native Ova (discussed in more detail later in this Chapter). In a same way, fluorophores may alter proteolytic processing that is part of the antigen presentation pathway, especially since a single point mutation within an antigen may alter or even block generation and presentation of a specific epitope. An additional problem is that fluorescent protein reporters can become invisible during processing due to proteolysis of the constructs, and that small molecule fluorophores can be rendered invisible due to oxidative quenching in the endo-lysosomal system (see Figure 3.1).

It was postulated that bioorthogonal chemistry could offer a solution in which the protein properties are minimally perturbed, yet leave the protein traceable even during extensive degradation. The approach relies on the use of amino acids that carry click handles that are so small that they can be incorporated in the nascent protein backbone in place of natural amino acids. This technique, called BONCAT (refer to Section 2.4.2), could thus yield proteins in which certain amino acids are substituted by isosteric, isocoulombic and isopolar counterparts, that can be used for their detection (Figure 3.2).

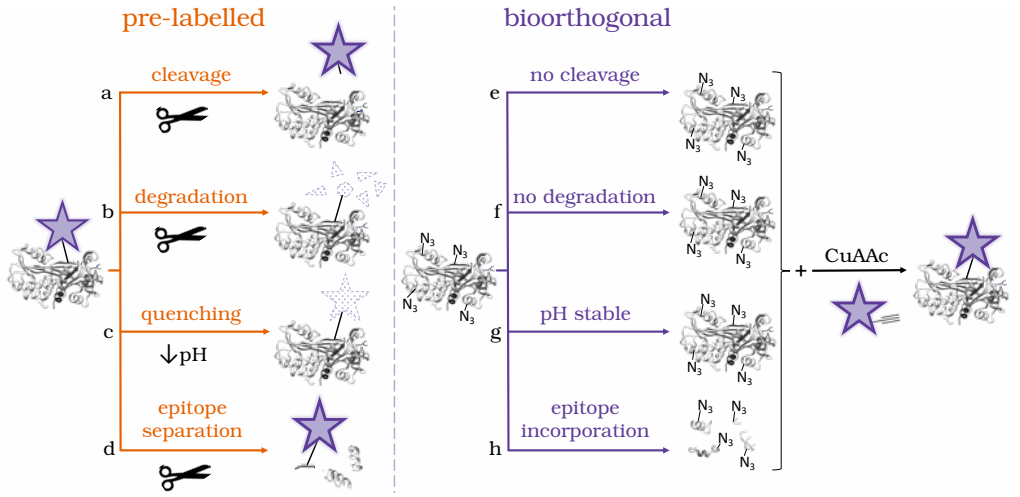


Figure 3.1: Comparison of commercial pre-labelled reporter antigens with bioorthogonal antigens. The use of reporter antigens pre-labelled with e.g., fluorophore can lead to signal biases. (a) Degradation of the linker connecting protein and reporter, (b) degradation of the reporter itself, (c) quenching of the reporter by low pH, (d) degradation of the protein leaving the reporter being attached to only one protein fragment. Bioorthogonal antigens do not suffer from these biases, as (e) the chemical handle is protease resistant, (f) allows retrospective label attachment, (g) is pH stable and (h) allows multiple label incorporation or the targeted incorporation into epitopes.

For the study of antigen processing, these bioorthogonal moieties have the advantage of a high stability, even to the conditions found in the endo-lysosome[175]. This chapter explores the use of bioorthogonal protein antigens in antigen presentation. For this, the bioorthogonal antigen must first be produced, for which two options are available that are rooted in adaptation of biological protein synthesis: BONCAT[185] and amber codon suppression[198], which are both described in Section 2.4.2. In this chapter, the use of BONCAT was chosen for two reasons: first of all, this approach yields proteins carrying multiple bioorthogonal sites in place of all methionines. Secondly, the approach is easy in terms of optimization: conventional *E. coli* expression plasmids can be used in Met-auxotrophic strains[210]. These *E. coli* strains are unable to synthesize methionine, but can incorporate UAAs that are substrates of methionine aaRS in an efficient manner. It was chosen to express the antigens using the UAAs Aha or Hpg[183], as these can be ligated readily using the Staudinger ligation, CuAAC or SPAAC (refer to Section 2.4.1).

3.2 Results

In order to explore the feasibility of bioorthogonal antigens as an immunological tool, the focus was first to put on developing reagents based on Ova as model antigen. This albumin from chicken egg is one of the most established immunological models. A multitude of reagents and methods have been developed on the base of this protein, which is readily available as a reference reagent. Using Ova as a model would thus allow the robust evaluation of the approach in the context of this extensive body of

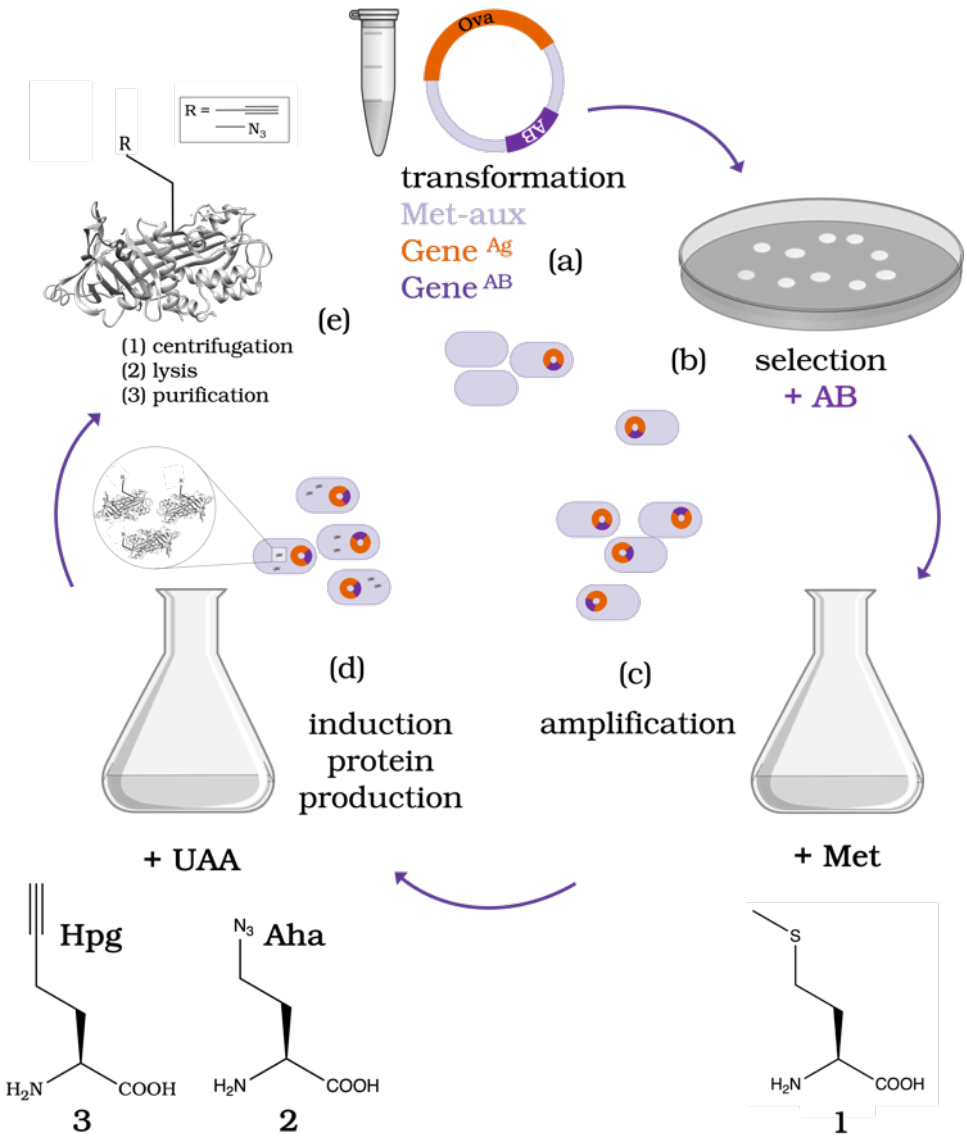
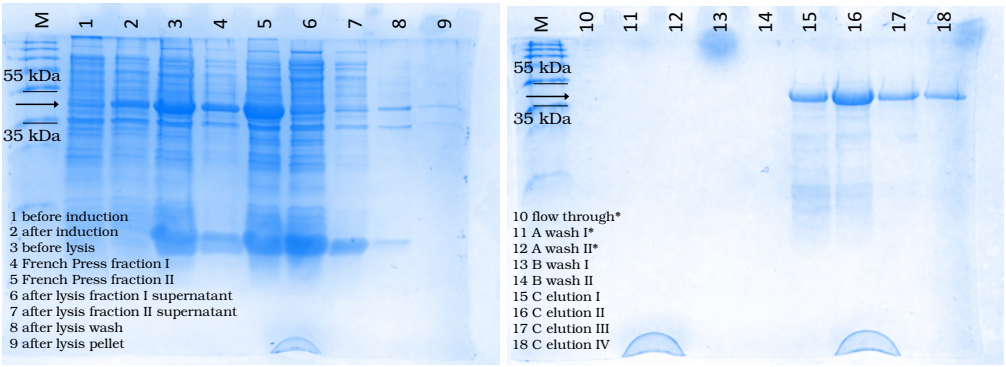
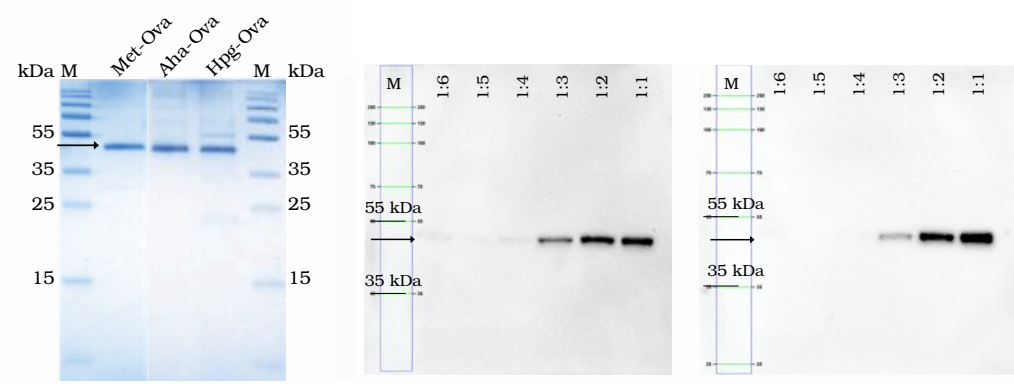


Figure 3.2: Scheme of BONCAT method used in combination with metabolic labelling. Experimental circuit showing the entire protein production process from the (a) transformation of Met-auxotroph bacteria with a plasmid encoding the gene of the antigen and a gene allowing antibiotic tolerance, (b) selection of plasmid positive bacteria by antibiotic pressure, (c) amplification of bacteria to desired culture size with supplemented Met, (d) induction of protein production and replacement of Met by the desired UAA. (e) (1)-(3) downstream processing and purification to obtain the pure protein of interest. (**1**) Met = methionine, Ag = antigen, Ova = ovalbumin, AB = antibiotic, UAA = unnatural amino acid e.g., (**2**) Aha= azidohomoalanine or (**3**) Hpg= homopropargylglycine.

recent work. Thus, an Ova expression system was therefore developed and used to produce bioorthogonal Ova, which was then used in preliminary immunological studies.



(a) protein production and purification



(b) purified protein

(c) protein identification

Figure 3.3: Protein purification and identification of recombinant ovalbumin and recombinant bioorthgonal ovalbumin variants. (a) SDS-PAGE of protein production showing total bacterial lysate before and after specific protein induction (left), SDS-PAGE of different fractions of the protein purification (right). *samples were analysezed on a separate gel due to their high protein content. (b) SDS-PAGE of purified bioorthogonal proteins Met-Ova, Aha-Ova and Hpg-Ova. (c) Western blot of purified protein samples after induction applied on SDS-PAGE in a dilution series. Detection via an ovalbumin-specific mAb serves as proof that overexpressed protein is indeed Ova. Ova indicated by the black arrow (→) at a height of 42 kDa. mAb = monoclonal antibody.

3.2.1 Expression of bioorthogonal antigens

Three variants of Ova were produced from the plasmid pCMG27 containing Ova fused to a Hexa-His-Tag (His_6) for purification with a tobacco etch virus (TEV)-cleavage site (a kind gift from Dr. N. del Cid and Prof. Dr. M. Raghavan[211]): a wt (Met-Ova) and two bioorthogonal variants containing Aha (Aha-Ova) in place of Met or containing Hpg (Hpg-Ova). All variants were expressed in the *E. coli* met-auxotrophic strain B834[210].

This expression host, which was first described for the expression of proteins containing selenomethionine[188], was used by the Tirrell group[187] to incorporate bioorthogonal amino acids, after which the Davis-group[212] optimized the system to enable near quantitative incorporation of non-canonical amino acids (ncAAs).

After introduction of the expression plasmid containing the gene of interest under the T7 promotor controlled by the *Lac*-operon (LacZ), the bacteria were first expanded in Met-containing medium to the appropriate optical density. Collection of the pellet by centrifugation and resuspension in Met-free medium, followed by the addition of either methionine or the bioorthogonal amino acids Aha or Hpg[186,187] prior to induction with isopropylthiogalactoside (IPTG) enabled expression of the proteins with these amino acids at the Met-sites[190]. After expression of 16-24 h, SDS-PAGE analysis comparing the whole cell lysate before and after the induction confirmed the presence of an overexpressed protein at a position corresponding to the size of denatured Ova protein. Purification by nickel affinity chromatography yielded the proteins in the range of 1 mg/L for Met-Ova and 0.5 mg/L for Aha-Ova and Hpg-Ova. SDS-PAGE analysis showed the production of Ova as a single band running at an apparent molecular weight of ~42 kDa, which is in line with the reported molecular weight of 42.8 kDa (UniProtKB: P01012, OVAL_CHICK[213]) with purity of > 90 %. Western blot against the His_6-tag or using an anti-Ova polyclonal antibody confirmed the identity of the Ova protein (Figure 3.3).

3.2.2 Characterization of bioorthogonal antigens

Circular dichroism spectroscopy (CD) was used to assess protein folding (Figure 3.4[214]) and it was confirmed that Met-Ova as well as the two bioorthogonal versions were mainly *a*-helical shown by the two minima at ~220 nm and ~208 nm and a maximum at 192 nm[215].

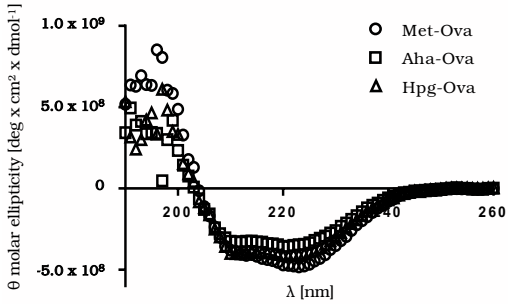
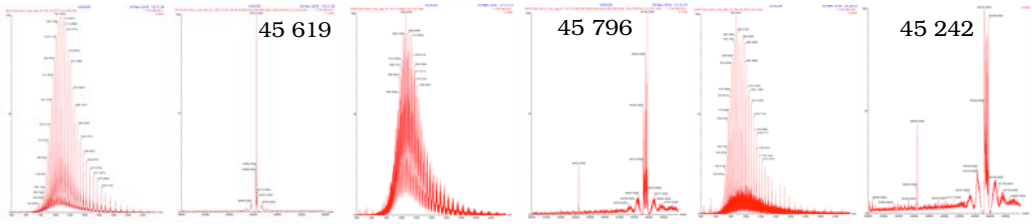


Figure 3.4: Protein characterization by circular dichroism. Circular dichroism of purified bioorthogonal antigens for analysis of correct secondary structure of the protein after production and purification. Met-Ova (dot), Aha-Ova (square), Hpg-Ova (triangle).

MS analysis of the proteins showed it to be 45,796 kDa for Aha and 45,242 kDa for Hpg with several peaks shown with mass differences[216], indicating the incomplete incorporation of Aha and Hpg due to leaky expression during the growth phase (Figure 3.5[214]). One aspect, that must be considered generally in this approach is the lack of glycosylation due to the prokaryotic expression system. As Ova is normally glycosylated[217,218], this does alter the structure. However, it does simplify the system



(a) Met-Ova **(b)** Aha-Ova **(c)** Hpg-Ova

Figure 3.5: Characterization of bioorthogonal antigens by mass spectrometry. (a) ToF-MS analysis of Met-Ova, expected mass: 45 618.7 Da, observed mass: 45 619 Da; (b) ToF-MS analysis of Aha-Ova, expected mass (with Met): 45 534 Da, observed mass: 45 796 Da; (c) ToF-MS analysis of Hpg-Ova, expected mass (with Met): 45 245 Da, observed mass: 45 242 Da. Background signal refers to internal background noise due to low ionization energy.

due the removal of any glycan-lectin interactions in the immune response[219]. This is, however, still a limitation of the approach in its current form.

3.2.3 Visualization of bioorthogonal protein antigens by fluorophore attachment

With both Met-Ova and the two bioorthogonal Ova-variants in hand, their visualization was attempted using the copper-catalysed Huisgen cycloaddition (ccHc)-reaction using a Cu(II)- and reducing agent strategy[220]. After click ligation of fluorophore **4** (Aha-Ova, Figure 3.6) with Aha-Ova the protein mixture was denatured, run on SDS PAGE and the resulting wet gel slab was scanned at 480 nm. The same was performed after click ligation of fluorophore **5** with alkyne-modified Ova (Hpg-Ova, Figure 3.6). This was done using the whole bacterial cell lysate (refer to Figure 3.7) as well as the purified recombinant Ova analogues. It was shown that only in the lanes were samples hat undergone successful click ligation to the respective alkyne (**4**) or azide (**5**) fluorophore a fluorescent signal could be visualized by bioorthogonal chemistry.

3.2.4 Intracellular degradation of bioorthogonal antigens in antigen presenting cells

It was next explored whether the bioorthogonal Ova-variants could be used to show the degradation in live APCs (Figure 3.8).

As the antigen and its breakdown fragments have to be selectively detected within the APC-lysates, only fluorophore or bioorthogonal antigens can be used for this purpose (Figure 3.9).

To test the degradation of these antigens, D1 APCs[141] were pulsed with antigen for 2 h (Aha-Ova, Hpg-Ova). The cells were then washed and chased for the indicated time. After fixation, the cells were lysed and fluorophores introduced by a ccHc-reaction with AlexaFluor647-azide or -alkyne. Labelled lysates were then separated by SDS-PAGE and the fluorescent signal measured by fluorescence densitometry. In contrast to the proteolytic stability of the proteins in isolated lysosomes, the live cells succeeded in degradation of all variants. For all samples a decrease in the band intensity of intact Ova

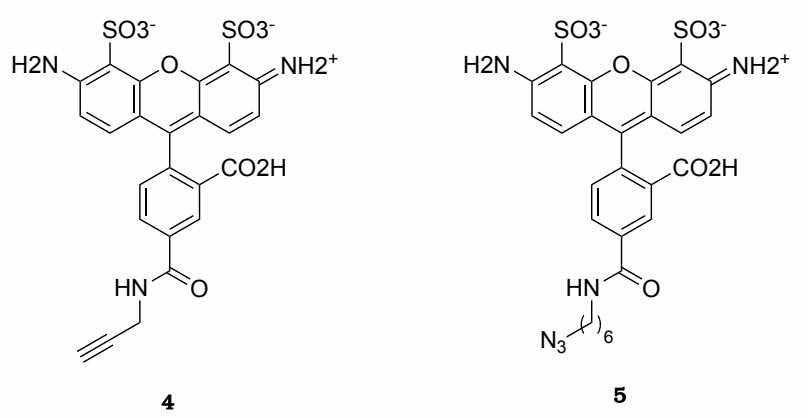
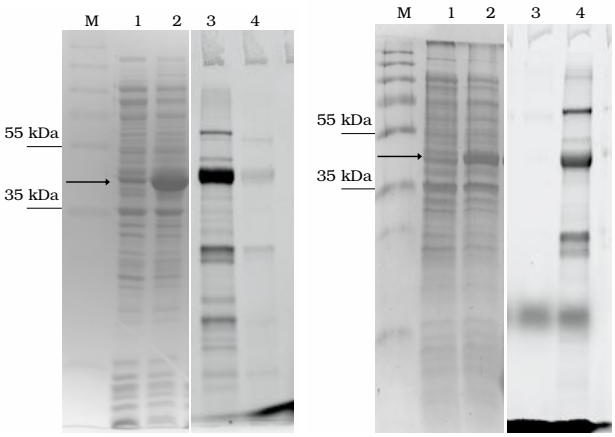


Figure 3.6: Chemical structure of functionalized alkyne- and azide-fluorophore. **4** AlexaFluor488-alkyne, **5** AlexaFluor488-azide.



(a) Aha-Ova **(b)** Hpg-Ova

Figure 3.7: Visualization of bioorthogonal antigens by fluorescence densitometry of crude bacterial whole cell lysate. SDS-PAGE of (a) click-ligated Aha-Ova and (b) Hpg-Ova showing fluorescence densitometry of bioorthogonally labelled proteins contained in *E. coli* whole cell lysate on the right hand side and Coomassie staining as control for whole protein content on the left hand side. (M) marker, (1) Coomassie staining, before induction, (2) Coomassie staining, after induction, (3) bioorthogonal labelling with **4**, (4) bioorthogonal labelling with **5**. Ova protein is indicated by the black arrow (→) at ~42 kDa.

was observed, but no intermediate-sized fragments. Small peptides were not observed, because, if present in the sample, they would have eluted from the gel. The excess of free fluorophore displayed the same gel permeance as the smaller peptide fragments, so all signal <3 kDa was obscured. Attempts at removing the excess fluorophore also resulted in the loss of the smaller fragments.

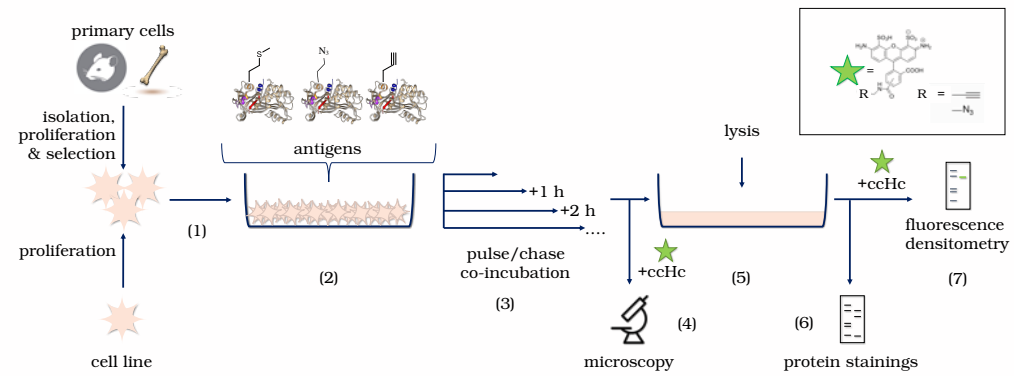


Figure 3.8: Workflow scheme of intracellular degradation of protein antigens. 1) Seeding of isolated and selected primary cells or an antigen presenting cell line at the respective conditions. 2) Addition of bioorthogonal antigens at indicated concentrations. 3) Pulse-chase regimen of co-incubation for different periods of time to follow degradation. 4) Readout time point for assays using intact cells e.g., confocal microscopy with or without performance of ccHc-reaction on fixed cells. 5) Addition of lysis buffer to release degradation products from the cell interior. 6) Readout time point of assays using whole cell lysate e.g., SDS-PAGE. 7) Readout time point for assays using fluorescent tags e.g., fluorescence densitometry after performance of ccHc-reaction on cell lysate.

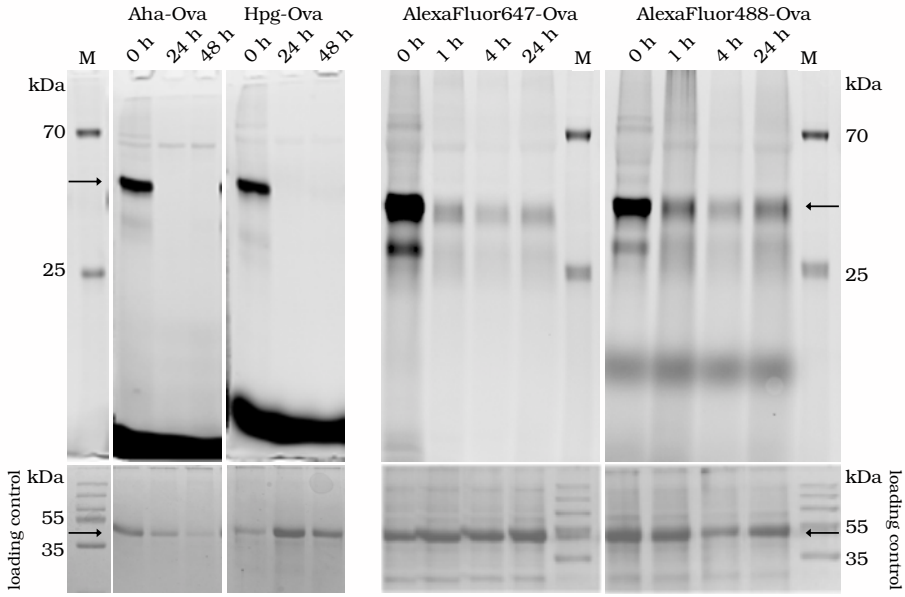
3.2.5 Antigen presentation of bioorthogonal antigens

The main envisaged advantage of using bioorthogonal antigens to study antigen presentation would be that they behave similar in terms of processing and presentation to their native counterparts. In order to assess whether this is actually the case, the bioorthogonal proteins were tested in a T cell co-culture assay consisting of an antigen presenting cell and a T cell specifically recognizing the presented antigen fragments. Incubation of DCs as APCs with either Met-Ova, Aha-Ova or Hpg-Ova as antigens showed, that all bioorthogonal variants of the antigen could indeed activate T cells to a similar extent (Figure 3.10[214]).

The experiments were performed using the control peptides, the dominant CD8a^+ T cell epitope (256-264, SIINFEKL, SL-8) and a larger peptide incorporating the SIINFEKL epitope that has been used in related antigen presentation studies hereafter termed 'long peptide' (LP, residues 247-264, DEVSGLEQLESIINFEKL, DL-18) to measure overall responsiveness of the T cells. All experiments gave significant T cell activation to a level equal to Met-Ova above 0.5 mg/ml of protein. T cell activation proved not very high, although Ova is a common antigen used as reference for immunogenicity studies due to its extensive characterization[221,222]. The T cell response proved independent of the UAA incorporated into the protein.

3.2.6 Comparison with fluorescently pre-labelled ovalbumin

In this and the preceding chapters fluorophore labelled antigens were described to have several disadvantageous properties. This applies especially with regards to their application in intracellular studies. Introduction of bioorthogonal antigens, as an alternative



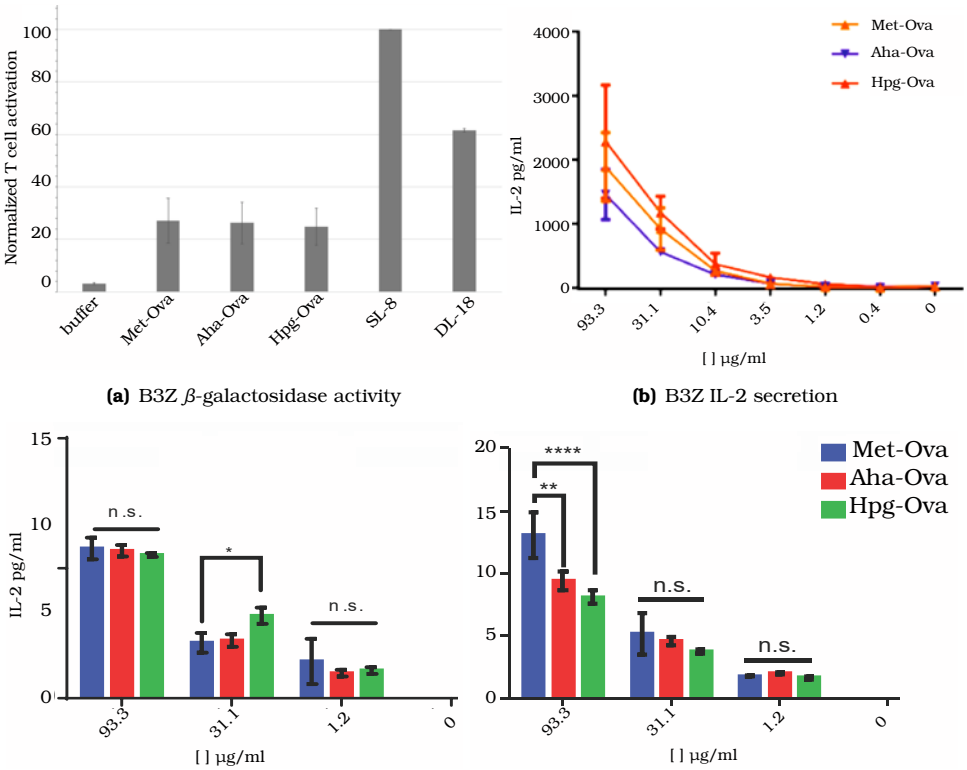
(a) bioorthogonal ovalbumin **(b)** purchsed ovalbumin

Figure 3.9: Fluorescence densitometry of bioorthogonal and purchased ovalbumin variants. D1 antigen presenting cells incubated with 10 μM of bioorthogonal Ova or fluorophore pre-modified analogues. (a) Aha-Ova and Hpg-Ova were chased for 0 h, 24 h and 48 h with D1 cells as APCs. Bioorthogonal ligation was performed on the cell lysates, which were analysed by SDS-PAGE and subsequent fluorescence densitometry measurement. (b) AlexaFluor488-Ova and AlexaFluor647-Ova were chased for 0 h, 1 h, 24 h and 48 h with D1 cells as APCs. The resulting lysates were analysed by SDS-PAGE and subsequent fluorescence densitometry measurement. Ova protein is indicated by the black arrow (→).

tool to commercially available fluorophore pre-labelled antigens, opens up promising advantages. In the following examples of direct comparison between the commercial product with the self-made bioorthogonal antigens are elaborated. Direct comparison of both antigens was performed in different assay settings. In a first experiment the classical AlexaFluor488-modified Ova protein was compared to the bioorthogonal antigens to determine, if they can be visualized after uptake by primary murine bone BMDCs (Figure 3.11). For this AlexaFluor488-Ova as positive control and Aha-Ova and Hpg-Ova as bioorthogonal proteins were co-incubated 1 h (pulse, with protease inhibitor) with primary murine bone marrow dendritic cells and then chased for 1 h followed by bioorthogonal ligation.

3.2.6.1 Biophysical characterization of fluorescently pre-labelled ovalbumin

Native page and CD of bioorthogonal Ova variants (Figure 3.12a) in direct comparison to AlexaFluor647- and AlexaFluor488-Ova confirmed structural differences. Native page analysis showed migration behaviour of Met-Ova, Aha-Ova and Hpg-Ova, whilst showing altered migration of fluorophore pre-modified Ova-analogues compared to wt-



(a) B3Z β-galactosidase activity

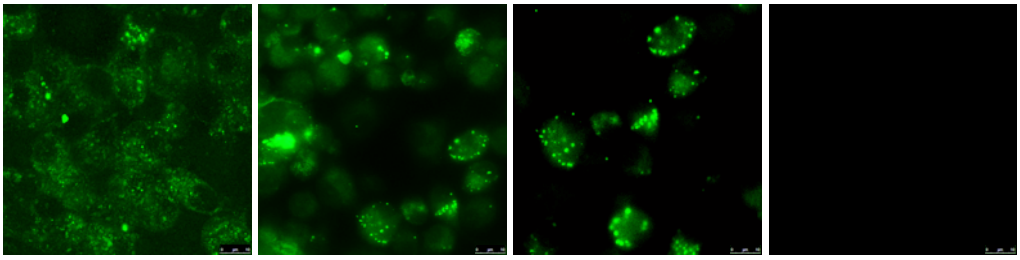
(b) B3Z IL-2 secretion

(c) OT-I IL-2 secretion

(d) OT-II IL-2 secretion

Figure 3.10: T cell activation following antigen presentation by antigen presenting cells. Antigen presentation of Met-Ova, Aha-Ova and Hpg-Ova in a co-culture assay using D1 cells as APCs and B3Z T cell hybridoma as T cells. Protein antigens were added 0.5 mg/ml or as indicated and co-cultured with D1 cells for uptake in a pulse-chase regiment for 1 h pulse and 5 h chase. T cells were added and left to get activated for 17-24 h. At the end point activation of T cells was measured by (a) absorption of β-galactosidase substrate conversion at 570 nm and (b) concentration of secreted IL-2 is determined by ELISA in relation to the assay internal standard curve using recombinant IL-2. n = 3 independent experiments with at least two experimental (N = 2) replicates. Antigen presentation of Met-Ova, Aha-Ova and Hpg-Ova in a co-culture assay using BMDCs as APCs (c) $CD4^+$ (OT-II) and (d) $CD8a^+$ (OT-I) T cells. Protein antigens were added as indicated and co-incubated with BMDCs for 4 h, after which excess antigen was washed away before co-culture with $CD8a^+$ or $CD4^+$ T cells isolated from mouse spleen. After 24 h, activation of T cells was measured by IL-2 ELISA. Asterisks refer to given P values, * $p < 0.05$, ** $p < 0.005$, **** $p < 0.00005$. Group mean values were analyzed by two-way analysis of variance with Tukey *post hoc* significant difference test using GraphPad Prism 6.0. Data are represented as means ± SD. n = 3 independent experiments with at least two experimental (N = 2) replicates.

Ova. CD showed similar structural behaviour of the bioorthogonal variants. While the Aha-Ova- and Hpg-Ova showed similar ellipticity (∂)[215] to wt-Ova, indicating a similar structural stability, fluorophore pre-modified Ova-analogues (Figure 3.12b[223]) showed a different structure.



(a) AlexaFluor488-Ova **(b)** Aha-Ova **(c)** Hpg-Ova **(d)** cells only

Figure 3.11: Visualization of cellular uptake of bioorthogonal proteins by antigen presenting cells using confocal microscopy. Cellular uptake of bioorthogonal Ova by primary murine bone marrow dendritic cells. For (a) positive control (AlexaFluor488-Ova), (b) Aha-Ova, (c) Hpg-Ova, protein was co-incubated 1 h with the APCs and then chased for 1 h before labelling by bioorthogonal ligation and visualization using an Leica SP5. Confocal microscopy images after 1 h chase. (d) cells only, no added proetin, no co-stainings were performed. Scale bar = 10 μm.

3.2.6.2 T cell activation by fluorescently labelled ovalbumin

BMDCs were incubated for 4 h with antigens, after which excess antigen was washed away before co-culture with OT-I (Ova-specific CD8a^+ T cells) or OT-II (Ova-specific CD4$^+$ T cells) T cells isolated from mouse spleen (refer to Chapter 3.4.1 Animals). After 24 h, activation of T cells was measured by IL-2 ELISA[224,225]. This assay showed that Aha-Ova and Hpg-Ova variants induce similar CD8a^+ T cell activation compared to Met-Ova. CD4$^+$ T cell activation differed significantly only at the highest Ova-concentration; with the difference in presentation wearing off at the lower concentrations. AlexaFluor647- and AlexaFluor488-Ova were also used to activate OT-I and OT-II T cells, however the experimental variation between replicates was extensive, with the difference between Ova and the fluorophore modified variants varying over two orders of magnitude between repetitions for both OT-I and OT-II activation assays (examples shown in Figure 3.13[223]).

3.2.6.3 Visualization of fluorescently pre-labelled ovalbumin

The fate of one of the bioorthogonal antigens, Aha-Ova, as well as AlexaFluor647-Ova was visualized by confocal microscopy (Figure 3.14[223]).

For this, the dendritic cell lines DC2.4[140] (Figure 3.13a) or D1[141] were pulsed with the protein or with 10 mM $NaHCO_3$ buffer as a negative control (< 5 % v/v) for 2 h in presence of lipopolysaccharides (LPS)[226] to allow for maturation of the endosomes to a late endo-lysosomes[227]. Only AlexaFluor647-Ova localized partly to (late) LAMP-1$^+$ vesicles at this time point, whereas Aha-Ova showed no overlap with LAMP-1. Increased uptake of fluorophore-modified antigen was observed in both the DC2.4 and the D1 cell line. This suggests a possible interference of the fluorophore handle on the routing of the antigen (Figure 3.14b), perhaps due to the charge and lipophilicity-induced destabilization of the antigen structure for AlexaFluor647-Ova. These data suggest that bioorthogonal antigens are suitable reagents for the study of antigen processing and presentation, due to the similar structure and biophysical behaviour compared to wt-Ova, combined with the ability to introduce a detectable group at the end of the

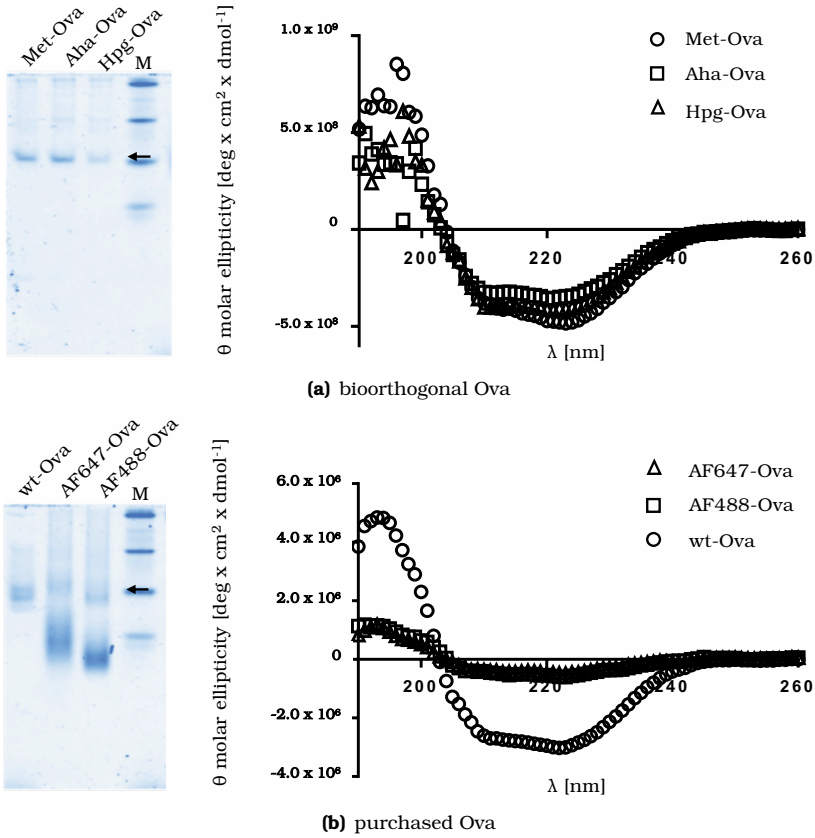


Figure 3.12: Native polyacrylamide gel electrophoresis and circular dichroism of bioorthogonal and purchased ovalbumin variants. Biophysical characterization of bioorthogonal antigens via native polyacrylamide gel electrophoresis (left) and circular dichroism spectroscopy (right) of (a) bioorthogonal Ova and (b) purchased Ova variants. Met-Ova (triangle), Aha-Ova (circle), Hpg-Ova (square), wt-Ova (triangle), AlexaFluor647-Ova (AF647-Ova, circle), AlexaFluor488-Ova (AF488-Ova, square). M = marker.

biological experiment.

3.3 Discussion and conclusion

The above-mentioned examples show that the nature of AlexaFluor488-Ova, used originally as a common tracing reagent in immunology, affects its cellular uptake, intracellular processing and presentation in common antigen presenting cells. Similar results were also obtained for other fluorescent Ova constructs. The first set of experiments discussed in this chapter entails studies towards the production and quality control of bioorthogonal antigens. These are then applied as bioorthogonal antigens in common immunological experimental settings. The last block of experiments shows side by side comparisons of standard commercial reagents used in commonly accepted immunological assays, as described in literature, with their bioorthogonal counterparts. From the

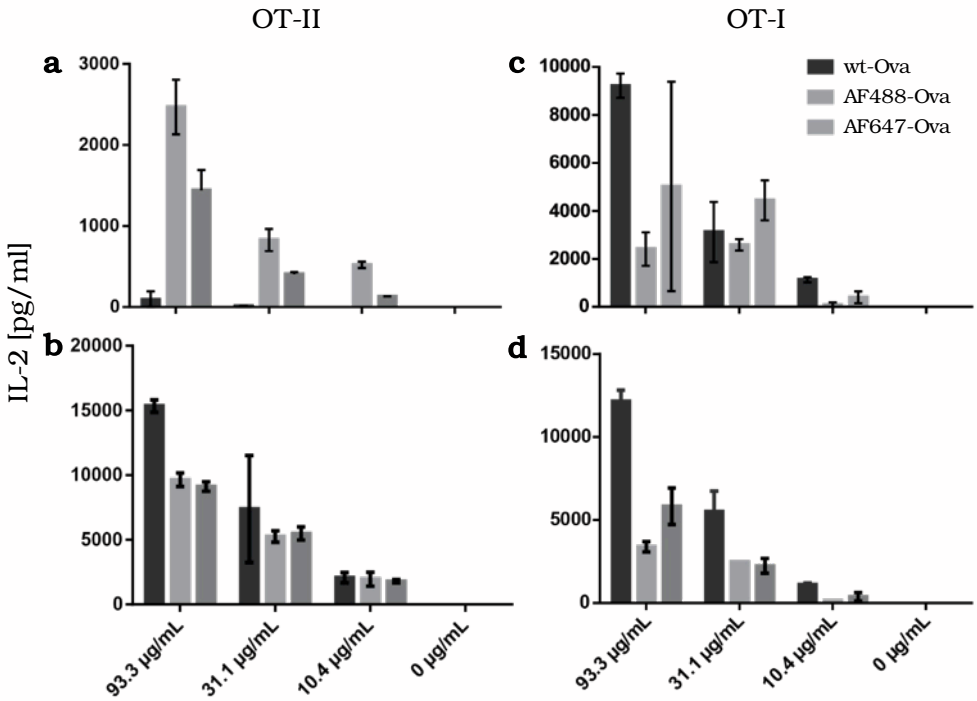
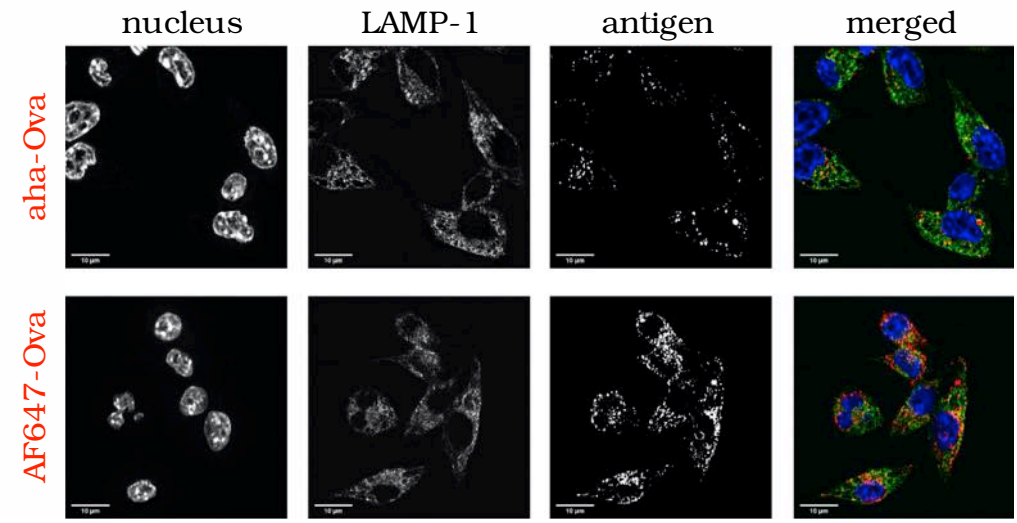


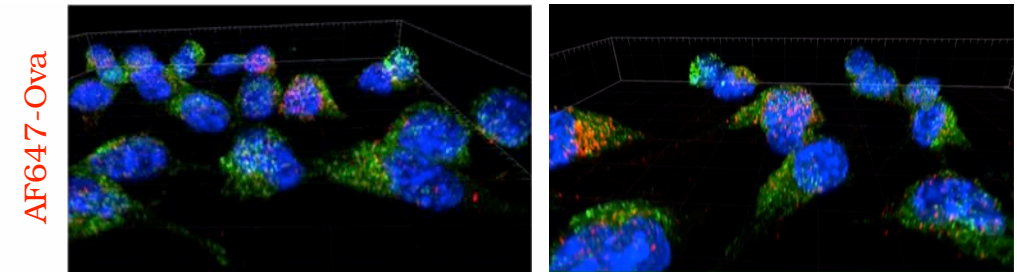
Figure 3.13: T cell co-culture assay replicates of purchased fluorescent and non-fluorescent ovalbumin variants. Influence of fluorophore modification of Ova on the activation of antigen specific $CD4^+$ (OT-II, left) and $CD8a^+$ (OT-I, right) T cells. Examples of ELISA (IL-2) replicates for (a)-(b) $CD4^+$ T cell or (c)-(d) $CD8a^+$ T cell proliferation after feeding BMDCs with glycosylated commercially available Ova (Ova, dark grey) and fluorophore modified Ova (light grey, AlexaFluor488-Ova; grey, AlexaFluor647-Ova). Data are represented as means ± SD of three technical replicates.

results described in this chapter it can be concluded that bioorthgonal antigens have their advantages, but also their limitations. Their production is relatively laborious, although their production follows mainly well-established standard techniques with the possibility for large(r) scale production. Recombinant proteins produced in a prokaryotic expression system delivers non-glycosylated proteins mostly, which as is the case here may be different from their native counterparts. Although bacterial strains have been developed to express certain forms of glycosylation[228], these are often not exactly emulating those in wildtype, mammalian proteins.

Another limitation of the technique chosen for the integration of the chemical handles is that the UAA are integrated on a global molecular basis. Therefore, a protein with many chemical handles is produced. As incorporation efficiency is usually around 90 %, it is not known, which Met-sites of the protein were replaced with the UAA, how the protein structure changed exactly due to the incorporation without further extensive analysis. The UAAs used are not identical in structure, and contain different chemical functionalities with different hydrogen bonding capacities (for example). This is enough to yield a small change in proteolytic susceptibility. However, these changes are minor compared to those of all other available detectable reagents and their exploration is



(a) Aha-Ova versus AlexaFluor647-Ova



(b) AlexaFluor647-Ova

Figure 3.14: Confocal microscopy of bioorthogonal and fluorophore pre-labelled ovalbumin in dendritic cells. DCs were incubated for 2 h (pulse) with Aha-Ova or AlexaFluor647-Ova (AF647-Ova). Cells were fixed with 0.5 % PFA and processed for immunofluorescence imaging by co-staining with lysosomal-associated membrane protein 1 (LAMP-1, green in merged image) as a lysosomal marker and DAPI (blue in merged image) for the nucleus. Aha-Ova was stained using ccHc with AlexaFluor647-alkyne fluorophore (red in merged image). Scale bar is 10 μm. (a) upper panel: imaging of Aha-Ova, lower panel: imaging of AlexaFluor647-Ova (b) Screenshot of a 3D-view of DCs treated with AlexaFluor647-Ova highlighting partial overlap (in yellow) of the lysosomal marker (green) and the fluorophore-modified Ova (red). PFA = paraformaldehyde

subject of elaboration in the following chapters.

In conclusion it can be said that the results shown above represent an early effort of the exploration of bioorthogonal antigens as viable alternative for the investigation of intracellular protein processing and presentation. In order to produce viable results, further experiments have to be done to match existing assay standards.

3.4 Materials and methods

3.4.1 Materials

Chemicals

Chemical reagents for buffer preparation and chemical synthesis were purchased from Acros (Belgium), Chem-Lab (Belgium), Honeywell Riedel-de Haën (Germany), Merck (The Netherlands), Novabiochem (The Netherlands), Sigma Aldrich (The Netherlands), Sigma Life Sciences (The Netherlands) or Sphaero Hispanagar (Spain) and used without further purification unless stated otherwise.
Ni-NTA was purchased from Qiagen (ref 145022017). Acetic anhydride and Amicons® ultra centrifugal filters were obtained from Merck. Pyridine, phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma. Chlorophenol red-β-D-galactopyranoside sodium salt was purchased form Calbiochem. 3-[(3-Cholamidopropyl)dimethyl- ammonio]-1-propanesulfonate hydrate (CHAPS) (ref SC-29088) was obtained from Santa Cruz. Dithiothreitol (DTT) was obtained from Biochemica Applichem. 2-(N-morpholino)-ethanesulfonic acid (MES) from Acros Organics. CFSE was purchased from Invitrogen. PFA for microscopy in EM-grade purity was purchased from Electron Microscopy Science (ref 157-8). Imidazole was purchased from ACS (ref 1.04716.0250). IGEPAL was purchased from Sigma (ref I3021). AlexaFluor488 ™ azide, AlexaFluor488 ™ alkyne, AlexaFluor647 ™ alkyne, AlexaFluor647 ™ azide where purchased from Thermo Fisher Scientific.

Antibodies

Polyclonal rabbit anti-His IgG antibody was obtained from Dako (ref P0448). Polyclonal goat anti-rabbit IgG-HRP (H/L) antibody was obtained from BioRad Laboratories Ltd. (ref 170-6515). Polyclonal anti-Ova antibody, was obtained from LifeSpan BioSciences (ref LS-C59287). Rat anti-mouse LAMP-1-AlexaFluor647 ™ or -AlexaFluor488 ™ was obtained from Biolegend (ref 121609, ref 121608, clone 1D4B). Mouse anti-chicken ovalbumin monoclonal antibody was obtained from Sigma (ref A6075, clone OVA-14). Streptavidin HRP-conjugated goat anti-mouse IgG secondary antibody was obtained from Santa Cruz Biotechnology (ref sc-2005).

Proteins

Ova wildtype protein (UniProt P01012, PDB 1OVA) was obtained from Worthington (ref LS-003054). Lysozyme was purchased at Amresco (EC 3.2.1.17, CAS 9001-63-2, ref 0663-5G). Benzonase®nuclease (EC 3.1.30.2, CAS 9025-65-4) was purchased from Sigma Aldrich (ref E1014-25KU). RNase-A from bovine pancreas (EC 3.1.27.5, CAS 9001-99-4) were purchased from Sigma Aldrich (ref R6513). DNase-I from bovine pancreas (EC 3.1.21.1) were purchased from Roche (ref 11284932001). Collagenase-D from *C. histolyticum* (EC 3.4.24.3) was purchased from Roche/Sigma Aldrich (ref COLLD-RO, 11088858001). Ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor was purchased from Roche (ref 11836170001). Recombinant mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF) was purchased from Peprotech (ref 315-03) or from ImmunoTools (ref 12343125). Ova-AlexaFluor488 ™ or -AlexaFluor647 ™ conjugates were obtained from Thermo Fisher Scientific (ref O34781 and O34784).

Media and Buffers

SelenoMethionine Medium Base plus Nutrient Mix, Methionine deficient medium for bacterial culture, were purchased at Molecular Dimensions (CalibreScientific, ref MD12-501 and MD12-502); Dulbecco's Modified Eagle Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM), Roswell Park Memorial Institute Medium 1640 (RPMI 1640), fetal calf serum (FCS) and HyClone™ FCS (FCS HI) were purchased at Sigma Aldrich - FCS was heat-inactivated in-house; Glutamax™ (ref 35050-038), trypsin (ref 15090-046), β-ME, sodium pyruvate, minimal essential medium (MEM) and non-essential amino acids (neAA, 100x) were purchased from GIBCO;

Penicillin (pen) and streptomycin (strep) antibiotic solution, 1 M Hepes, 1 M Tris base, trypsin (EC 3.4.21.4, CAS 9002-07-7) with EDTA, phosphate-buffered saline (PBS), EDTA in PBS, monosodium phosphate (MSP) buffers e.g., 50 mM NaH_2PO_4 5 M NaCl, 5 M HCl, 5 M NaOH, 87 % glycerol, Coomassie Brilliant Blue (CBB) solution and 4x concentrated Lämmli buffer as sample buffer (SB) were prepared by hand in-house.

Animals

Animals were obtained from Envigo Inc. (Horst, the Netherlands), eight- to twelve-week old, specific pathogen-free male or female C57Bl/6NHsd mice and kept at the AAALAC-certified institutional animal facility under specified pathogen-free conditions. Animals were co-housed in cages using compressed sawdust as bedding, under controlled conditions of temperature, humidity and light (12-hour light, 12-hour dark cycles). Standard rodent diet and water were provided *ad libitum* and mice were provided with nesting material and enrichment. Animal well-being was checked daily. All measures were taken to minimize pain, distress and suffering and all procedures were performed by trained personnel. OT-I mice (CD8a^+ T cell transgenic mice expressing a Va2Vβ5 T cell receptor recognizing the Ova-derived T cell epitope SIINFEKL (ovalbumin residues 257-264) in the context of H-2Kb) and OT-II mice (CD4$^+$ T cell transgenic mice expressing a T cell receptor recognizing the Ova-derived T_h cell epitope ISQAVHAAHAEINEAGR, ovalbumin residues 323-339, in association with I-Ab)[229] were bred and kept at the Leiden University Medical Center animal facility or at the Leiden Advanced Drug Research Centre (kindly provided by Dr. Bram Slutter) under specific pathogen-free conditions. Mice were sacrificed at 8-12 weeks of age.

Other

Bacterial methionine auxotrophic strain (*E. coli* B834(DE3), met-aux, Genotype: F- ompT hsdSB (rB- mB-) gal dcm met(DE3)) was bought from Novagen (ref 69041). Vector construct pMCSG7[230] was kindly provided by Dr. N. del Cid (Principle Scientist at Tentarix Biotherapeutics, San Diego, California) and Prof. Dr. M. Raghavan (Professor at Medical School, University of Michigan). Mammalian protease inhibitor cocktail was used from AMRESCO (ref M250). EDTA-free protease inhibitor was purchased from Roche (ref 11836170001). Dynabeads™ Untouched™ Mouse T cell Kit (ref 11413D) and ELISA kit were purchased from Invitrogen™ (ref 88-7024-22). DAPI was obtained from Abcam (ref ab104139). Multi-well microscopy slides were purchased from IBIDI (ref 81201 with ref 10811). Spin columns for MS sample clean-up were from BioRad (ref 732-6221). 70 μm cell strainer was obtained from BD (ref 352350). Other plastic

ware for cell culture was purchased from Sarstedt. Silver staining kit was obtained from SilverQuest™ (ref LC6070). Enhanced chemiluminescent (ECL)™ Cytiva Amersham™ Pharmacia Biotech kit was purchased from GE Healthcare (ref RPN2235), or Merck - Sigma Aldrich (GERPN2235).

3.4.2 Methods

Protein production of ovalbumin

The gene for the Met-Ova (*G. gallus*) full length protein was cloned into the vector construct pMCSG7[230] as described elsewhere[211,231]. The construct was kindly provided by Dr. N. del Cid (Principle Scientist at Tentarix Biotherapeutics, San Diego, California) and Prof. Dr. M. Raghavan (Professor at Medical School, University of Michigan). The plasmid transformed into the methionine auxotroph expression strain *E. coli* B834 (DE3)[210]. The construct contained the following N-terminal sequence: MHHHHHHSS-GVDLGT**ENLYFG**SNA sequence with the start codon (M, Met), purification tag (H_6) for Ni-NTA purification and the TEV-cleavage site (bold) for removal of the tag after production.

A single colony of *E. coli* B834 transformed with pMCSG7-Ova was selected after growth at 37 °C on lysogeny broth (LB) agar plates supplemented with ampicillin (amp, 50 μg/mL) and used to inoculate 2 mL LB medium containing amp. The culture was grown over night at 37 °C and under constant shaking (180 rpm). For protein expression, the culture was diluted 50 times in supplemented LB medium (1 v/v % glucose, amp) and grown at 37 °C until reaching an optical density at 600 nm (OD_{600}) ~0.6, approximately after 3 h. Protein expression was induced with 1 mM IPTG after medium exchange (LB Medium without glucose). Expression was continued at 29 °C for 4 h, 180 rpm. The culture was harvested by centrifugation (4 °C, 30 min, 4000 x g). The supernatant was discarded and the pellet either snap frozen in liquid nitrogen for short term storage or lysed immediately for further use.

Expression of bioorthogonal Ova protein (Aha-Ova and Hpg-Ova) was performed as described for the Met-Ova protein with the following changes. After reaching the optimal OD_{600} for the pre-culture, the medium was exchanged for a methionine deficient medium and the culture was left to grow for another 30 min at 37 °C. Then the medium was supplemented with UAA, namely, Aha (0.4 mM) or D-/L-Hpg (0.8 mM) and expression was induced by addition of 1 mM IPTG. Expression and harvest were continued as described for Met-Ova.

Protein purification of ovalbumin

Protein purification was adapted mainly from Lechner *et al.* (2016)[232]. After harvest by centrifugation for the bacterial pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10 % glycerol, 1 mg/ml lysozyme, 1 tablet of EDTA-free protease inhibitor, 5 mM β-ME, 250 U benzonase per 100 ml culture volume, pH 8.0, *opt.* DNase-I and RNase-A according to manufacturer's instructions). Cells were lysed by high-pressure homogenization using a French Press (Stansted, 1.9 kbar). Cell debris was removed by centrifugation for 30 min at 4 °C and 15,000 x g. The resulting supernatant was incubated with Ni-NTA resin, previously equilibrated with purification buffer A according to manufacturer's instructions. Protein binding was enabled by

gentle rocking of the resin for 30 min at 4 °C. The resin containing liquid was filled in an appropriate empty column, the flow-through was collected and the resin washed twice with 5 column volumes (CV) of purification buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) followed by two washes with 5 CV precooled purification buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM - 50 mM imidazole, pH 8.0). Finally, bound protein was eluted with four times 1 CV of purification buffer C (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0). As an alternative to the Ni-NTA resin, an ÄKTA protein purification system from GE Healthcare in combination with a pre-packed 1-5 ml HisTrap HP column was used following the same protocol. All collected fractions were tested on SDS-PAGE.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

For SDS-PAGE analysis all samples were heated for 5 min at 95 °C. Samples with bioorthogonal labelling mixtures were exempted from heating to prevent unspecific labelling. After a quick spin and manual resuspension 20 μl of each sample was loaded onto 1.5 mm, 15 % SDS-PAGE gel and run for ~50 min at constant 250 V in the dark, if necessary. For western blots SDS-PAGE gels with a thickness of 0.75 mm were used for better protein transfer onto the polyvinylidene difluoride (PVDF) membrane. Subsequently, measurements or stainings were performed as indicated. Fluorescence densitometry was measured at the appropriate wavelengths, after which a Coomassie staining or silver staining was performed.

Native polyacrylamide gel electrophoresis

Native PAGE was performed as described by Arndt *et al.* (2012)[233] ImageLab 5.2 software from Biorad.

Western blot

For western blot analysis SDS-PAGE was performed as described above and the protein transferred onto PVDF membranes using a Trans-Blot Turbo Transfer System from BioRad. Ova was detected in the total cell lysate or the elution fractions by western blotting with a mouse anti-chicken ovalbumin mAb, a streptavidin HRP-conjugated goat anti-mouse IgG secondary antibody and an ECL kit.

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 (sec) for each scan in a 1 mm quartz cuvette at 1 nm resolution were acquired at room temperature (rt) and were averaged. For processing of raw data, GraphPad Prism 7.0 was used. The spectra are given in molar ellipticity (∂).

Protein mass spectrometry

Whole protein MS was performed by dissolving 10 μM of the pure target protein in 20 mM $NH_4CH_3CO_2$ (0.1 ml per sample) and subsequent clean-up using a spin column. Tris(2-carboxyethyl)phosphine (TCEP) or 5 mM DTT was added depending on the respective protein to reduce thiols.

Synthesis of L-azidohomoalanine

For the synthesis of L-azidohomoalanine a modified protocol from Zhang *et al.* (2010)[231] has been performed. The purity of the compound has been analysed via ^{1}H-NMR and liquid chromatography-mass spectrometry (LC-MS). All spectroscopic data was in agreement with previous results, ^{1}H-NMR (D_2O), 400 MHz: δ [ppm] = 4.05 (t, 1H, α-CH), 3.55 (t, 2H, γ-CH2), 2.15 (m, 2H, β-CH2).

Synthesis of D-/L-homopropargylglycine

The synthesis of D-/L-homopropargylglycine has been described elsewhere[234]. The purity of the compound has been analysed via ^{1}H-NMR and LC-MS. All spectroscopic data was in agreement with previous results, ^{1}H-NMR (D_2O), 400 MHz: δ [ppm] = 4.12 (t, 1H, α-CH), 2.3-2.12 (m, 1H), 2.11-2.0 (m, 1H).

Bioorthogonal click chemistry

For the bioorthogonal labelling mixtures 10 mM sodium ascorbate was added to 1 mM $CuSO_4$, both diluted in water. Followed by the addition of 1 mM TTMA[235] or THPTA[236] in DMSO, 100 mM Hepes,pH 8.4 and 2.5-5 μMof a 2 mM solution of the respective Cy5-alkyne or TAMRA-azide fluorophore in DMSO to the cocktail. Alternatively, a labelling mixture with reduced cytotoxicity was used with 1 mM $CuSO_4$, 10 mM sodium ascorbate, 1 mM THPTA ligand and 10 mM aminoguanidine (pimagedine, CAS 79-17-4) final concentration with the same buffer conditions. Please note, upon addition of sodium ascorbate to $CuSO_4$, a color change from transparent blue to brown (reduction of Cu(II) into Cu(I)) and finally to yellow should occur.

Intracellular antigen degradation

Antigen presenting cells of choice were harvested and counted as usual and seeded in a 48 wells plate (0.2 x 10^6 cells/well). The cells were incubated for 1 h (pulse) with the indicated concentration of protein and washed 3 times with warm culture warm medium to remove excess protein. In case of a chase experiment cells were incubated for the indicated time at normal culture conditions (chase). Before addition of 50 μl lysis buffer (50 mM NaCl, 0.25 % CHAPS, 50 mM Tris pH 7.5, mammalian protease cocktail, 0.25 U benzonase) the cells were washed 3 x with PBS to remove medium residuals. The plates were frozen in liquid nitrogen and stored at -20°C until further processing. Samples were thawed slowly on ice and protein concentration of the samples was measured. Click chemistry was performed, if anticipated for the respective samples by adding the click mixture in a 1:1 (v/v) ratio to samples or by diluting 1:1 (v/v) with a respective buffer instead of the fluorophore as a control. Chemical ligation was done for 30 min in the dark at rt. To stop the reaction, sample buffer

was added and the samples were subsequently treated as stated above (Chapter 3.4.2). For analysis 10-20 μg of total protein was mixed with SDS loading buffer for analysis by SDS-PAGE. Fluorescence densitometry, western blot and Coomassie staining were performed subsequently according to the experimental setting.

Beta-galactosidase antigen presentation assay

DCs of choice were harvested as described and plated in 96-well tissue-culture treated microtiter plates (50,000 cells/well) in complete IMDM (without GM-CSF in case of BMDCs) and allowed to adhere at 37 °C and 5 % CO_2 for 1 h prior to the addition of proteins at the indicated concentrations. DCs were incubated with the proteins for the indicated times, followed by a wash with complete IMDM to remove excess antigen solution, which was not taken up by the cells. Peptide controls were incubated as follows: 1 h for immuno-dominant minimal epitopes, SL-8, 10-20 μM; 3 h for synthetic long peptides e.g. DL-18, 30-40 μM. The T cell hybridoma B3Z cells were checked for viability and cell density and added to the plate with the pulsed dendritic cells 100 μL /well in complete IMDM (50,000 cells/well)[156]. The DCs and T cells were co-cultured for 17 h at 37 °C. Subsequently, the cells were spun down at 1500 rpm for 3 min and supernatant was collected and saved. Stimulation of the B3Z hybridoma was measured by a colorimetric assay using CPRG as a substrate as described[237] or by measuring the IL-2 concentration by ELISA in the saved supernatant.

Interleukin-2 enzyme-linked immunosorbent assay

As a readout for T cell activation, IL-2 secretion was measured by ELISA in the supernatant of T cell co-cultures 20 h after their addition to antigen presenting cells. Usually, one or two different dilutions of the pure supernatant were used obtain results within the dynamic range of the assay. ELISA was performed according to manufacturer's instructions.Assays were performed in duplicates.

Confocal microscopy, monocolour

APCs were cultured as described for each cell type respectively. Following harvesting, 0.1 x 10^6 cells/well were seeded in a tissue culture treated multi-well slide in complete IMDM medium and were left to attach. Cells were incubated with the indicated concentrations of protein at 37 °C and 5 % CO_2 for indicated amounts of time. For controls protease inhibitor cocktail was used to prevent degradation, if indicated. Then the medium was removed and the cells were washed twice gently with PBS. All subsequent steps were performed at rt. For fixation, the washed cells were covered with 4 % PFA for 15 min. Wells were washed twice with PBS (pH 7.4). Blocking with simultaneous permeabilization was done using block/perm buffer (PBAS: PBS pH 7.4 with 1 w/v % BSA and 0.5 w/v % saponin) for 10 min followed by the staining with the bioorthogonal labelling mixture (1 mM $CuSO_4$, 10 mM sodium ascorbate, 1 mM THPTA ligand[236], 10 mM aminoguanidine, 100 mM Hepes, pH 8.4, AlexaFluor488 azide 5 μM) at rt for 1 h in the dark. After extensive washing, 3 times with 1 w/v % PBA and, if necessary, with methanol (MeOH) for removal of residuals from the ligation reaction, anti-LAMP-1 staining was performed according to manufacturer's instructions in PBSS for 1 h in the dark. Afterwards, the PBA washing was repeated 3 times for 10 min. All cells were

counter stained for 1 min with Hoechst (0.5 μg/mL) in PBS with Tween-20 (PBST-20) followed by 3 times washing with PBS. In case silicon chamber was removed mounting for immunofluorescent imaging was done using glycerol/DABCO.

Major histocompatibility class-I or class-II restricted antigen presentation assay

BMDCs were seeded (50,000/well, medium described above) in treated cell culture 96-well plates (flat bottom) and left at 37 °C for 2 h to allow adhesion. Then, cells were incubated with different concentrations of proteins, ranging from 0.4 to 93.3 μg/mL, for 6 h. Stock solutions of proteins were prepared in 10 mM $NaHCO_3$ and DMEM complete medium. The class-II epitope of Ova (T_h cell epitope ISQAVHAAHAEINEAGR) or the class-I epitope of Ova (CTL epitope SIINFEKL), were used as a positive control for antigen presentation. After the period of incubation, cells were washed with warm IMDM and $CD4^+$ OT-II T cells or $CD8a^+$ OT-I T cells were added (50,000 cells/well, RPMI medium supplemented with 10 % heat inactivated FCS, 2 mM Glutamax™, pen 50 U/mL and strep 50 μg/mL, 20 μM of β-ME). As a read out for T cell activation, IL-2 secretion (ELISA) was measured in the co-culture supernatant 20 h later or CFSE positivity was determined by flow cytometry. Assays were performed in duplicates.

Confocal microscopy, multicolour

Differentiated D1 cells were seeded in a tissue culture treated, polylysine coated 96-well microtiter plate at 5 x 10^4 cells/well and were left to attach 3 h. Bioorthogonal antigens were diluted to a final concentration of 2 mg/mL, of which 50 μg was added to each well bearing 200 μL of cell suspension over 2 h (pulse) and were chased for 0, 2, 4 and 24 h at 37 °C, 5 % CO_2 under humidified air. Cells were washed with PBS before fixing with cold 2 % PFA in PBS for 30 min at rt or with 0.5 % PFA in PBS over night. After fixation, cells were again washed with PBS, then with the quenching buffer (20 mM glycine in PBS) and permeabilised with staining buffer (0.1 % IGEPAL, 1 % BSA in PBS) for 20 min at rt. After washing with PBS and subsequently with 100 mM Hepes (pH 8), bioorthogonal antigens were incubated 1 h with click mix (1 mM $CuSO_4$, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM aminoguanidine, 100 mM Hepes pH 8 and 5 μM AlexaFluor647-azide or -alkyne). Upon CuAAC, cells were washed first with 100 mM Hepes (pH 8), then with PBS, then incubated for 30 min with 1 % BSA in PBS and stained with AlexaFluor488 LAMP-1 antibody (1:200 dilution, staining in 1 % BSA, 0.1 % tween-20 in PBS) for 1 h. Cells were washed three times with PBS before addition of fluoroshield-mounting medium containing DAPI and samples were imaged with a Leica SP (63 x oil lens, N.A.=1.4) or Andor Dragonfly confocal microscope.

Cell culture

Generation of bone marrow derived dendritic cells and culture

BMDCs were generated from B57BL/6 mouse bone marrow essentially as described[238,239] with some modifications. Bone marrow was flushed from femurs and tibia and cells cultured in 150 mm non-adhesive plates at ~10x10^6/well in IMDM supplemented with 8 % heat-inactivated fetal calf serum, 2 mM Glutamax™, 20 μM β-ME, pen 50 U/mL and strep 50 μg/mL in the presence of 20 ng/mL GM-CSF. Fresh medium with GM-CSF was added on day 3. On day 4 and day 7 cells were split using 4 mM EDTA in PBS.

The cells were generally used on day 9 or day 10. Cells were phenotyped on day 9 for CD11c, CD86, MHC-I, CD4 or CD8*a*, MHC-II, CD45.1 by flow cytometry.

Dendritic cell line D1

Dendritic cell line (D1)[141,240] cells were cultured in non-tissue culture treated 150 mm plates in IMDM medium supplemented with 10 % Hyclone FCS (HI) (pre-tested for baseline T cell activation), 2 mM Glutamax™, 50 μM β-ME, 100 U/mL pen and 30 % R1 cell supernatant (10-20 ng/ml GM-CSF containing culture supernatant from NIH/3T3 fibroblasts, purified[141]) at 37 °C, 5 % CO_2 in humidified air. Cells were split every 3-4 days using 2 mM EDTA in PBS and grown until maximally $1x10^6$ cells/ml.

Dendritic cell line DC2.4

Murine C57BL/6 derived dendritic cell line DC2.4 was a kindly provided by Prof. Dr. K. Rock (University of Massachusetts Medical School)[140] and was cultured in IMDM medium supplemented with 10 % FCS (HI), 2 mM Glutamax™, 100 U/mL pen, 100 μg/mL strep, 50 μM β-ME, 10 mM Hepes, 1 mM sodium pyruvate, non-essential amino acids or RPMI (incl. Hepes) supplemented with 10 % FCS, Glutamax™, 200 U/mL pen, 200 μg/mL strep, 50 μM β-ME, 1 mM sodium pyruvate, non-essential amino acids. Cells were detached using 0.25 % pre-warmed trypsin with EDTA and split every 2-3 days and grown until 70-80 % confluence for maximally 15 passages. Culture conditions: 37 °C, 5 % CO_2 humidified air.

Macrophage cell line Raw 264.7

The murine macrophage cell line RAW 264.7[241] was grown and cultured in DMEM medium supplemented with 10 % FCS, 2 mM Glutamax™ and pen 100 U/ml, strep 50 μg/ml. Cells were detached using a cell scraper, split every 2-3 days and grown to 70 % confluence for maximally 15 passages. Culture conditions: 37 °C, 5 % CO_2 humidified air.

Monocyte cell line THP-1

The human monocytic cell line THP-1[242] was grown and cultured in DMEM medium supplemented with 10 % FCS, 2 mM Glutamax™ and pen 100 U/ml, strep 50 μg/ml. Preparations for an experiment whereby monocyte to macrophage transition was required, involved seeding cells in DMEM medium lacking pen-strep and the over night incubation with 1 μM PMA that ensured the differentiation of the monocytes into macrophages. Culture conditions: 37 °C, 5 % CO_2 humidified air.

T cell line B3Z-hybridoma

The $OVA_{257-264}$-specific, H-2K*b*-restricted CTL hybridoma, B3Z[156] was cultured in IMDM medium supplemented with 10 % FCS, 2 mM Glutamax™, 0.25 mM β-ME, 200 U/mL pen, 200 μg/mL strep in the presence of 500 μg/ml hygromycin B every alternating week[243]. Cells were split every 2-3 days and grown until the cell concentration reached a maximum of $1x10^6$ cells/ml. Culture conditions: 37 °C, 5 % CO_2 humidified air.

Primary transgenic ovalbumin specific T cells

Antigen presentation of Ova *in vitro* to primary T cells was determined by activation of isolated lymphocytes of Ova-specific transgenic OT-I (CD8a^+) or OT-II (CD4$^+$) mice, transgenic for the TCR recognizing the Ova epitope SIINFEKL bound to the MHC-I (H-2K*b*, H-2q haplotype[244,245]) or the epitope ISQAVHAAHAEINEAGR[246,247] bound to the MHC-II. Spleens from OT-II T cell receptor transgenic mice were harvested and homogenized using a 70 μm cell strainer. Untouched CD4$^+$ T cells were isolated with a murine-specific T cells isolation kit from Miltenyi for CD4$^+$ T cell negative selection, according to the manufacturer's instructions. CD4$^+$ T cells purity was typically higher than 85 % as previously described[120,248]. For analysis of T cell proliferation, cells were either stained with CFSE the dilution of which was analysed by flow cytometry after four days of culture or the supernatant of the co-culture was removed after 20 h of culture for analysis by murine IL-2 specific ELISA.