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Bioorthogonal antigens as tool for investigation of antigen processing and presentation

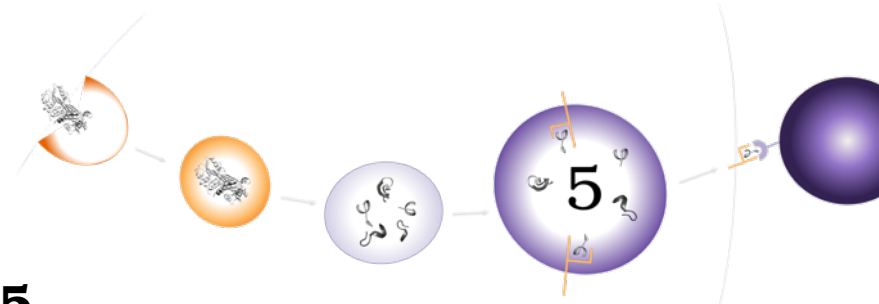
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Chapter 5

Bioorthogonal antigens to study intracellular processing of post-translationally modified antigens

5.1 Introduction

Proteolysis underpins many fundamental biological processes at every developmental stage: from the essential role of the protease fertilin during fertilization[273] to the initiation of cell death through the activation of caspases[274]. It is therefore not surprising that dysregulation of proteolysis is a hallmark in a variety of pathologies: aberrant protease activity has been implicated in the growth and metastasis of tumours[275], auto-immune pathogenesis[111,115], compromised bone desorption[276], and intracellular bacterial persistence[277], to name but a few. The biology of proteases is complex: factors such as their compartmentalized distribution[104], post-translational activation[278], functional redundancy and interplay complicates their study[279], particularly in antigen processing by APCs[280,281]. APCs take up exogenous material, degrade the proteins from these sources and present specific peptides from the degraded protein on their MHCs[282]. Recognition of a specific pMHC by a cognate T cell then leads to the initiation of the adaptive immune response against the source of the peptide[281]. This process is of prime importance in the clearance of exogenous pathogens and cancer, but also for the opposite: the induction of tolerance against innocuous substances and self-tissue[283–285]. As such, understanding of the proteolysis underpinning this phenomenon is essential.

5.1.1 Proteolysis in antigen presenting cells

Proteolysis in APCs is complex: multiple protease and peptidase families (e.g., aspartic, serine and cysteine proteases) are involved in the production of MHC-restricted peptides[38,283,286]. Many of these proteases, particularly those in the endo-lysosomal system, are under tight post-translational control[278]: they are produced as zymogens

that are routed to the vesicles of the endo-lysosomal compartment where they are activated by a combination of the low pH and the removal of inhibitory peptides through the activity of other proteases. The often-promiscuous substrate preference by virtue of their shallow binding grooves[287], attenuation of their activity by endogenous inhibitors[288], changes in pH and radical concentrations are factors that further complicate the study of these enzymes[289].

5.1.2 Structure changes antigenicity

The nature of the antigen (i.e., the substrate) itself also influences proteolysis[290]. For example, a point mutation in the multiple sclerosis auto-antigen myelin basic protein (MBP_{85-99}) prevents its cleavage by the non-papain-like cysteine protease AEP, leading to enhanced presentation to T cells[115]. (De)stabilizing a protein fold can also alter antigenicity: the sequentially identical, but structurally destabilized variants RNase-S and apo-HRP of the stable protein RNase-A and HRP are both poor antigens compared to their stable counterparts. They are rapidly degraded *in vitro* and antigen presentation efficiency is significantly reduced *in vivo*[119]. Stabilizing a fold on the other hand can also reduce its antigenicity, as observed for hen egg lysozyme[291,292].

5.1.3 Post-translational modifications change antigenicity

PTMs on the antigen can alter proteolysis by altering specificity. This can, for example, lead to the appearance of 'neo-autoantigens' - non-thymic peptides, that can drive auto-immune diseases - and has been observed in multiple sclerosis an arginine to citrulline modification in myelin oligodendrocyte glycoprotein (MOG) in B cells prevented destructive epitope processing of such a neo-autoantigen, leading to T cell activation in a marmoset (*C. jacchus*[293]) model of the disease[111].

5.1.4 Detectable reporter proteins

For studying the uptake and degradation of proteins and the subsequent presentation of antigenic peptides derived thereof the use of model proteins is a common approach. This method by default is unable to provide information on the subcellular events that govern processes underpinning this T cell activation. Detectable model proteins, termed reporter proteins, are the obvious alternative to allow detection and have been used to study subcellular routing. Oft-used reporter proteins include lactamases[294], luciferase[295], and HRP[129]. These proteins have proven valuable in the study of early events in the process using enzymatic activation as a biochemical read-out[295]. However, proteolytic degradation of proteins into peptides, required for antigen presentation, will render these reporters catalytically inactive and, hence, precludes their use for obtaining information on later stage events. For the study of processing events, covalent fluorophore-antigen adducts have been used as alternative reporter proteins. These do not provide catalytic activity, but allow visualization through fluorescence for e.g. microscopy. The low molecular weight (~800 Da) and stability to proteolysis of the fluorophores are beneficial traits of these reagents. However, the change in charge, hydrophobicity, shape and structure have been altering the properties of the protein[296,297]. Some showed reduced antigenicity, as observed for example for [121,298,299]. This leads to the hypothesis that the alterations influenced the antigenicity of the proteins which

are therefore not suitable for the study of downstream degradative processes in APCs or the assessment of the resulting immune responses.

5.1.5 Bioorthogonal proteins to study antigen degradation

Bioorthogonal chemistry was assessed here as an approach to study antigen degradation in immune cells circumventing the aforementioned limitations of contemporary techniques (Figure 5.1) [167].

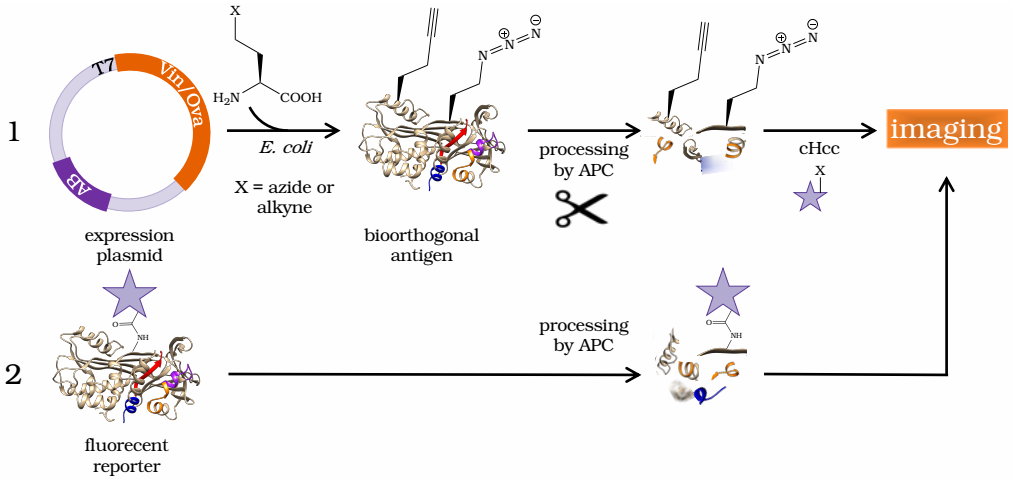


Figure 5.1: Bioorthogonal antigen production from plasmid to assay. Expression of antigenic proteins (Vin/Ova) in the *E. coli* met-auxotrophic strain B834 allows replacement of methionines (x) with azidohomoalanine (Aha, containing an azide) or homopropargylglycine (Hpg, containing an alkyne). Free azide or alkyne groups in the amino acid side chain of bioorthgonal antigens allow the follow up of undisturbed protein degradation by retrospective attachment of chemical tags, such as functionalised fluorophores, using a click-reaction (ccHc). 2) Attachment of fluorophore before degradation resembles how classical fluorescent reporter antigens are used. T7 = promotor, Vin = vinculin, Ova = ovalbumin, AB = antibiotic.

Bioorthogonal chemistry comprises the development and application of selective chemical reactions in the context of the biological milieu [166,179,300]. A small abiotic group, such as an azide or alkyne, is first introduced into a biomolecule and can then - when the biological time course is completed - be selectively modified with a detectable group (e.g., fluorophores or biotin) for visualization and/or identification. The research described in this chapter was based on the premise that a bioorthogonal antigen would be highly suitable for studying degradation events during antigen presentation: the labels introduced into these proteins differ minimally compared to naturally occurring amino acids and bioorthogonal groups are available that are fully stable to the conditions found during endo-/phagosomal maturation [175]. Recombinant proteins with all canonical methionines substituted for either Aha or Hpg can readily be obtained in good yields for this purpose (see Figure 3.2 for details on protein expression) [186,187,301]. In the here-described study bioorthogonal antigens were characterized and explored to follow degradation inside the APC.

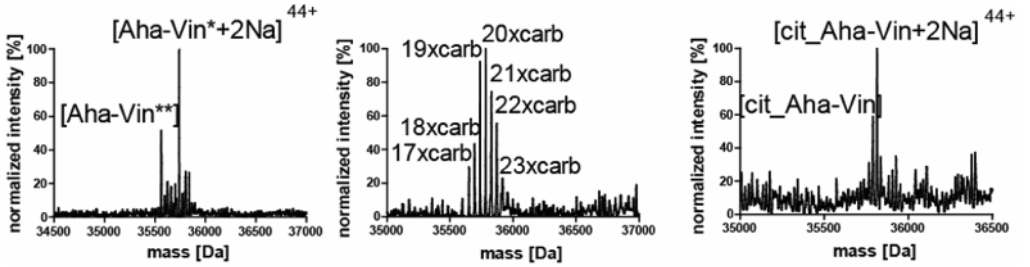
5.2 Results

Met-containing and bioorthogonal variants of the model antigens Ova and Vin were produced as previously described in Chapter 3.2.1 and Chapter 4.2 respectively.

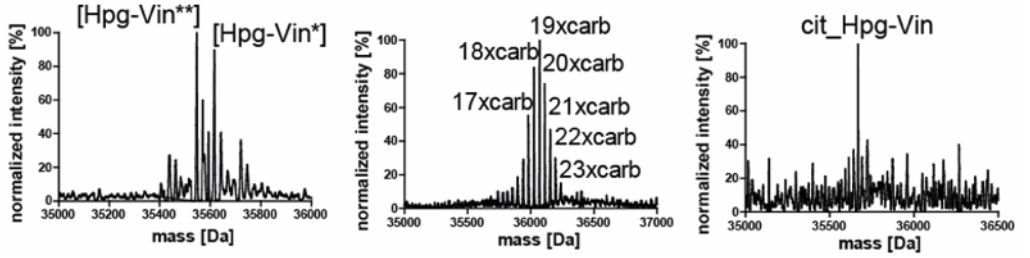
5.2.1 Chemical analysis of the modified bioorthogonal recombinant proteins

These were compared on their physical properties, such as basic structure and stability, as well as molecular mass (Figure 5.2 and Figure 5.3; produced by Dr. M.C. Araman).

Figure 5.2 shows the analysis of bioorthogonal Vin by MS and Figure 5.3 the characterization by CD (for the same characterization data of Ova refer to Chapter 3.2.2).



(a) Aha-Vin

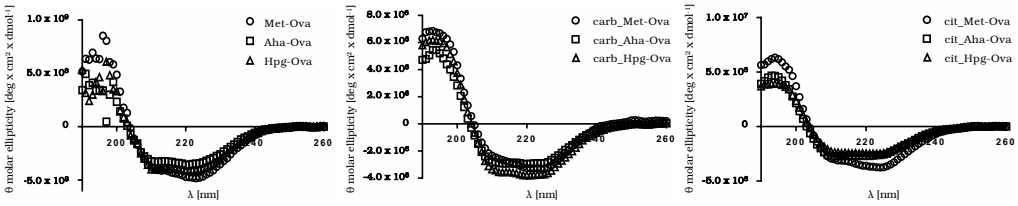


(b) Hpg-Vin

Figure 5.2: Characterizing of bioorthogonal vinculin variants by mass spectrometry. (a) ToF-MS analysis of bioorthogonal Aha-Vin derivatives; from left to right: non-modified Aha-Vin (expected mass: 35715 Da, observed mass: 35 714.6 Da for Aha-Vin ** and 35 759 Da for [Aha-Vin *+2Na]44+), carbamylated Aha-Vin (mixed species from 17x- to 23x-carbamylated Aha-Vin, no traces of non-carbamylated Aha-Vin), citrullinated Aha-Vin (expected mass: 35 734 Da, observed mass: 35 736 Da). (b) ToF-MS analysis of biorthogonal Hpg-Vin derivatives; from left to right: non-modified Hpg-Vin (expected mass: 35 579 Da, observed mass: 35 578.6 Da for Hpg-Vin ** and 35 759 Da for [Hpg-Vin *+2Na]44+), carbamylated Hpg-Vin (mixed species from 17x- to 23x-carbamylated Hpg-Vin, no traces of non-carbamylated Hpg-Vin), citrullinated Hpg-Vin (expected mass: 35 669 Da, observed mass: 35 668 Da).

Characterization by CD revealed that both citrullination and carbamylation alter the secondary structure of the proteins tested, which may affect protease susceptibility. Carbamylation of bioorthogonal Vin increased rigidity around 5-fold as assessed via spectrum minima with similar helicity, compared to their unmodified counterparts. Citrullination of the Vin-derivatives decreased the α-helical content of the protein, which

displays elevated β-sheet and random coil-rich characteristics. carb-Ova had a higher α-helical content and adopted a slight decrease in random coil structure, when compared to its non-modified version. cit-Ova showed a more pronounced negative Cotton effect[302], the optical rotation first decreases and then increases from right to left, compared to the bioorthogonal versions. The percentage distribution of secondary structure elements remained the same as in non-modified versions (for characterization of Vin refer to Chapter 4.2).



(a) non-modified **(b)** carbamylated **(c)** citrullinated

Figure 5.3: Circular dichroism of bioorthogonal ovalbumin variants with and without post-translational modifications. Biophysical characterization of bioorthogonal antigens via circular dichroism (CD) spectroscopy of bacterially expressed bioorthogonal and non-bioorthogonal Ova-analogues for analysis of structure before and after carbamylation and citrullination. (a) non-modified variants Met-Ova, Aha-Ova and Hpg-Ova (b) carbamylated variants carb_Met-Ova, carb_Aha-Ova and carb_Hpg-Ova; and (c) citrullinated variants cit_Met-Ova, cit_Aha-Ova and cit_Hpg-Ova

5.2.2 Intracellular degradation in antigen presenting cells

Because of the unsuccessful use of lysosomal extracts to induce proteolysis, the focus was changed to explore whether the bioorthogonal Ova-variants could be degraded in live APCs instead. Figure 5.4 depicts the assay set-up for these experiments (refer to Figure 3.8 for the basic workflow).

To test the degradation of these antigens, primary murine BMDCs were pulsed with antigen for 1 h (Met-Ova, Aha-Ova and Hpg-Ova without modification, carb_Met-Ova, carb_Aha-Ova and carb_Hpg-Ova; cit_Met-Ova, cit_Aha-Ova and cit_Hpg-Ova, respectively) and analysed by SDS-PAGE followed by fluorescence densitometry (Figure 5.5).

As the antigen and its breakdown fragments have to be selectively detected within the APC-lysates, only fluorophore-labelled or bioorthogonal antigens can be used for this purpose. To test the degradation of these antigens, primary murine BMDC APCs[141] were pulsed with antigen for 1 h (Met-Ova, Aha-Ova and Hpg-Ova or Met-Vin, Aha-Vin and Hpg-Vin, Figure 5.5).

The cells were then washed and chased for either 24 h or 48 h. 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 was visualized after gel electrophoresis (fluorescence densitometry). In live APCs degradation of all variants could successfully be tracked. For all samples a decrease in the band intensity of intact Ova and Vin after the chase-time was observed. However, the uptake of cit-Ova and cit-Vin was less for all variants compared to the non-modified and carbamylated variants despite the addition of equal amounts of protein.

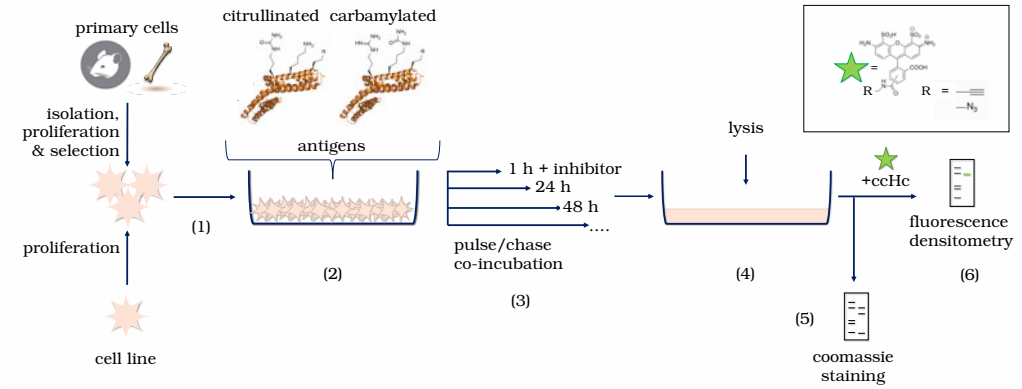


Figure 5.4: Workflow scheme of intracellular degradation of protein antigens. The individual steps involve 1) Seeding of isolated and selected primary cells or an antigen presenting cell line at the respective conditions. 2) Addition of bioorthogonal antigens at indicated concentrations. 3) Pulse-chase regimen of co-incubation for different periods of time to follow degradation. 4) Addition of lysis buffer to release degradation products from the cell interior. 5) Readout of assays using whole cell lysate e.g., SDS-PAGE. 6) Performance of ccHc-reaction on cells or cell lysate and readout using fluorescence densitometry.

Also, no intermediate-sized fragments were observed. The small peptide/amino acid-sized fragments cannot be visualized for the bioorthogonal antigens, as the excess of free fluorophore obscured all bands < 3 kDa (the bottom parts of the gels were for this reason removed prior to fluorescence imaging). In theory, fluorophore labelled bioorthogonal antigens should allow the visualization of low molecular weight species, as protein was purified to remove free fluorophore prior to the biological experiment. However, no such bands were observed for these antigens. This suggests the terminal degradation of fluorophore fragments, which diffuse away or that the fluorophore itself was not resistant to the conditions in the lysosome.

5.3 Discussion and conclusion

In summary, the experiments described in this chapter show that the bioorthogonal antigen variants were useful to track the fate of antigens upon processing by APCs. They were structurally very similar to wildtype antigen; the bioorthogonal groups were stable to the harsh conditions found in the lysosome and the handles were not cleaved or destroyed by proteolysis. This means that - despite the increased complexity by the additional bioorthogonal ligation step - bioorthogonal antigens comprise a useful set of reagents for the more exact study of antigen processing and presentation. Also, genetic manipulation of the immune cells is not required, which makes these reagents compatible with e.g., primary human immune cells. This poses a big advantage from a biological perspective as it opens up a plethora of possibilities for their use in existing biological systems without requiring major adjustments. One example is the fact that side-chains remain available for PTM, allowing the study of the effect of such modifications, such as carbamylation, but also acetylation, methylation and non-enzymatic post-translational modifications, such as glycation on the rate of degradation/presentation in APCs.

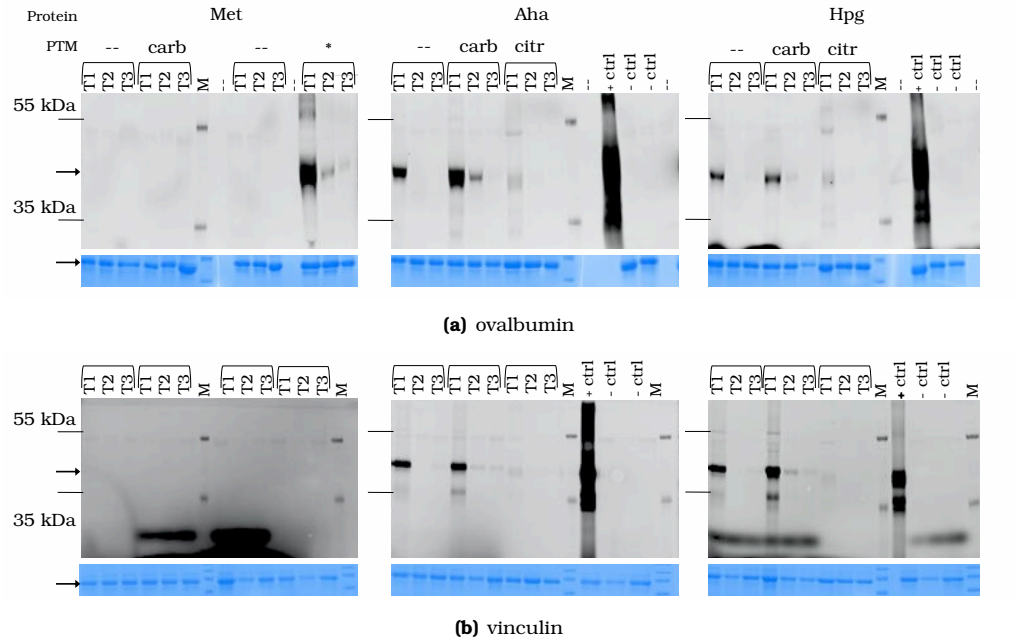


Figure 5.5: Fluorescence densitometry of bioorthogonal ovalbumin and vinculin variants with and without post-translational modification. Primary murine BMDCs incubated with (a) Ova (Met-Ova) or bioorthogonal Ova (Aha-Ova, Hpg-Ova) and (b) Vin (Met-Vin) or bioorthogonal Vin (Aha-Vin, Hpg-Vin). The variants were post-translationally modified with or without post-translational modification over a time course of T_1=1 h, T_2=24 h and T_3=48 h. To analyse degradation of protein over time the cells were lysed after pulse/chase co-incubation. With the resulting lysates a ccHc-reaction was performed and clicked lysates were analyzed by SDS-PAGE and subsequent fluorescence densitometry. Coomassie staining serves as loading control. PTM = post-translational modification, carb = carbamylation, citr = citrullination, - ctrl = negative control: cells with no addition of Ova/Vin, + ctrl = positive control: *in vitro* fluorophore pre-labelled Aha-Ova, Hpg-Ova, Aha-Vin, Hpg-Vin or * = fluorescent AlexaFluor488-Ova (Thermo Fisher Scientific, no additional fluorophore was added), M = protein ladder, empty lane.

5.4 Materials and methods

5.4.1 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. Fluorophores (AlexaFluor488-azide, AlexaFluor488-alkyne, AlexaFluor647-azide, AlexaFluor647-alkyne), were purchased from Thermo Fisher Scientific, the Netherlands.

Animals

Animals originated from Envigo Inc., Huntingdon, United Kingdom.

5.4.2 Methods

Protein production and purification of bioorthogonal proteins

Ova and Vin were produced, isolated and purified as earlier described in Chapter 3.4.2 and Chapter 4.4.2, respectively.

Protein mass spectrometry

Whole protein MS was performed by dissolving 10 μM of the pure target protein in 20 mM ammonium acetate (0.1 ml per sample) and subsequent clean-up using a spin column (BioRad, ref 732-6221). TCEP or 5 mM DTT was added depending on the respective protein to reduce thiols.

Circular dichroism

All recombinant wt and bioorthogonal Vin and Ova variants were characterized via CD spectroscopy[215]. Far UV-CD spectra were recorded using a Jasco J815 CD spectrometer equipped with a Jasco PTC 123 Peltier temperature controller (Easton, MD) between 190-260 nm. A minimum of five spectra with an acquisition time of 70 sec for each scan in a 1 mm quartz cuvette at 1 nm resolution were acquired at rt and averaged. Typical protein concentrations were between 0.1-0.3 mg/mL.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE was performed as earlier described in Chapter 3.4.2.

Bioorthogonal protein degradation intracellular

Differentiated BMDCs were seeded in a tissue culture treated 24-well microtiter plate at 0.2 x 10^6 cells/well in 100-200 μL medium/well and were left to attach for 1 h. The bioorthogonal antigens dissolved in IMDM complete medium were added at the indicated concentrations over 1 h (pulse) at 37°C, 5 % CO_2 under humidified air. Then the medium was aspirated gently, and the cells washed with pre-warmed IMDM medium. Subsequently, cells were pulsed for the indicated times (0- 48 h). The supernatant was then aspirated, and the cells resuspended in 50-100 μL of lysis buffer (100 mM Tris pH 7.5, 50 or 150 mM NaCl, complete protease inhibitor cocktail (EDTA-free), 0.25 % or 0.5 % CHAPS, 250 U Benzonase (Sigma, ref E1014-25KU)) and incubated for 30 min. The resulting samples were normalized by total protein concentration. For this purpose, the lysate was cleared via centrifugation (20000 g, 1 h) and subjected either to Bradford assay (BioRad, the Netherlands, ref 5000001) or Qubit Protein Assay (Thermo Fisher, the Netherlands, ref Q33211) 20 μL of the normalized lysate was then mixed with 20 μL CuAAC-buffer. This buffer was generated via addition of following chemicals in the particular order: $CuSO_4$ in Milli-Q water (100 mM stock concentration, 6.4 mM final concentration), sodium ascorbate in Milli-Q water (1 M stock concentration, 37.5 mM final concentration), followed by TMTA from a DMSO stock (100 mM, 10 μL, 1.3

mM final concentration) and Tris (stock concentration 100 mM, final concentration 88 mM, pH 8.0). Finally, fluorophore-alkyne or -azides were added from DMSO-stocks (stock concentration 2 mM, 1-2 L, 2.5-5 μM final concentration). Please note, upon addition of sodium ascorbate to $CuSO_4$, a colour change from blue to brown (reduction of Cu(II) into Cu(I)) and finally to yellow should occur. Samples were incubated for 30 min at rt under gentile agitation (100-200 rpm) in the dark prior to the addition of Laemmli-buffer and SDS-PAGE analysis after which fluorescence densitometry was measured at the respective wavelengths and the specific protein amount was visualized by Coomassie staining.

Cell culture

Generation of bone marrow derived dendritic cells and culture.

Cells were cultured as earlier described in Chapter 3.4.2.