Bioorthogonal Antigens

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Tools for studying antigen processing and cross-presentation

Published as part of: Joanna B. Pawlak and Sander I. van Kasteren. eLS. 2017, in press.

2.1 Introduction

The adaptive immune system is the branch of the immune system that has evolved to mount a tailored immune response against specific antigens. The subset of adaptive immune cells called cytotoxic $CD8^+$ T lymphocytes (CTLs) are arguably the key mediator in eliminating two specific diseases, namely viral infections and cancers. Despite their apparent different origin (although some cancers are caused by viruses), they share a feature that renders them susceptible to attack by CTLs: both the genetic alterations that are a hallmark of cancer[1] and the extensive transcription program induced by a virus infection using host cell ribosomes, result in the presence of mutated proteins in the cytosol, which in turn can be processed in the MHC-I pathway to result in the appearance of *neoepitopes* on the cell surface. Unlike the peptides that are present during health, the CTLs capable of recognizing these neoepitopes have not been eliminated through central tolerance mechanisms and, once activated, could kill the virus infected or tumor cell upon recognizing its cognate neoepitope.

However, herein lies the conundrum of cross-presentation: the CTL has to be activated by an antigen presenting cell (likely a subset of dendritic cell, called the $CD8^+$ DC) which, especially in the case of tumors, does not produce the same neoepitope as the tumor cell. These cells must thus acquire the mutated proteome

from the tumor exogenously and present it on its own MHC-Is, which are normally reserved for the presentation of peptides from cytosolic sources. This process called antigen cross-presentation – discovered over 40 years ago[2] – presents a complex trafficking problem that can be summarized as follows: "how does antigen that has been taken up and compartmentalized into the endo-lysosomal system encounter the MHC-I loading machinery that resides in the endoplasmic reticulum?".

2.2 Pathways of antigen cross-presentation

Due to the pivotal importance of cross-presentation in the anti-viral and anti-tumor immune responses, it has been the subject of intensive research and different solutions to the above problem have been presented in the literature since its inception. At present, a spectrum of potential routes has been reported that are bookended by two suggested general paths: the cytosolic and vacuolar pathways of antigen cross-presentation[3].

The key feature of the cytosolic pathway is that following uptake, antigen is routed via the cytosol where it intersects with the conventional MHC-I peptide processing and loading pathway: internalized exogenous antigens are exported from the phagosome to the cytosol early after uptake, where they are degraded into short peptides by the proteasome and transported into the endoplasmic reticulum (ER) by transporter associated with antigen processing 1 (TAP1) where they are loaded on MHC-I molecules and finally transported to the cell surface[4]. However, the identity of molecular mechanism responsible for antigen export from endosomes and phagosomes to the cytosol still remains to be fully elucidated. A few possible models for antigen export to the cytosol have been proposed by different groups. One of these models describes a direct fusion of phagosomes with the ER membrane (ER-phagosome fusion) allowing for the ER-proteins to merge into phagosomes. This offers a possible explanation for a presence of the ER-resident proteins in phagosomes. Subsequently, it is believed that these ER-associated proteins such as Sec61 and p97 act as an antigen translocon into the cytosol[4c, 5]. As an alternative to this model, involvement of Derlin-1 known as degradation in ER protein 1 instead of Sec61 in the ER-phagosome fusion model has been proposed[6] (Figure 1, left panel). Yet another variant of the cytosolic pathway has been proposed: after proteasomal proteolysis the antigens are imported back into the phagosome via TAP1 transporters residing on the phagosomal surface rather than the ER, where they are recruited by the ER-derived molecule - SNARE-protein Sec22b[7]. Back in the phagosome the peptides are loaded onto MHC-I molecules by a yet unidentified loading machinery and ultimately transported to the cell surface for sampling by the $CD8^+$ T cells[8] (Figure 1, left panel).

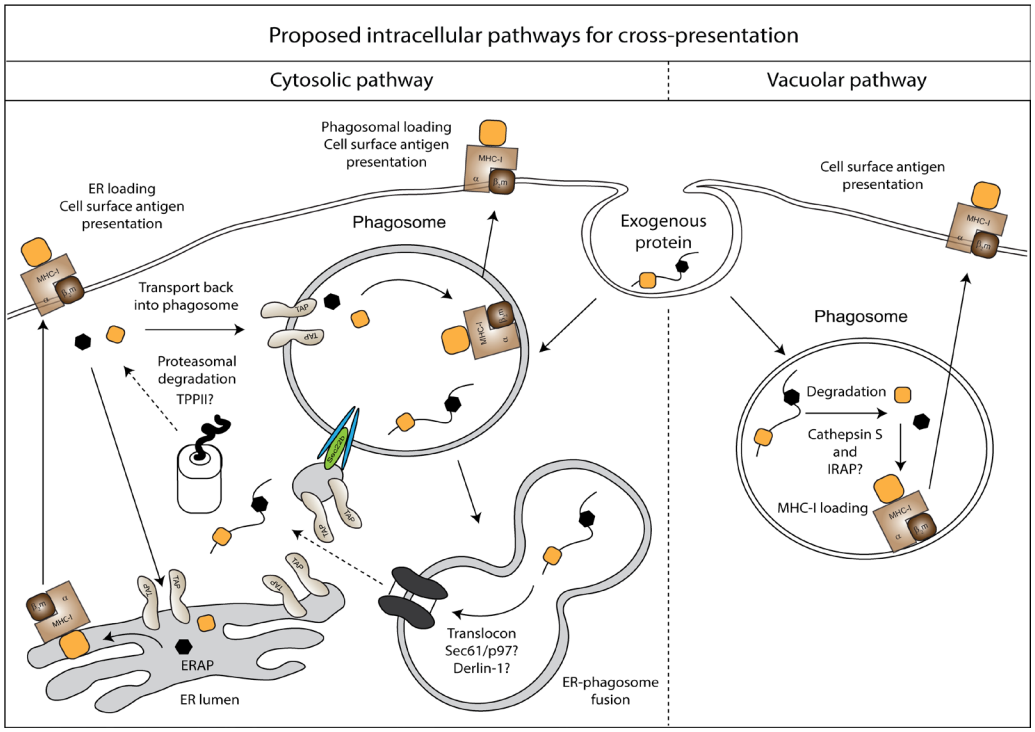

Figure 1. *Schematic representation of the intracellular pathways operating throughout the cytosolic and vacuolar cross-presentation pathways (gap junctions mediated peptide transfer not shown).*

Within the cytosolic pathway(s), it is generally believed that the proteolytic steps required for the liberation of the epitope peptides takes place outside the phagosome in which they have been taken up. It has, for example, been shown that raising the endosomal pH using chloroquine (which is postulated to lower protease activity in this compartment)[9] enhanced the export of antigen into the cytosol. Instead, the key cleavages of peptides are believed to be executed by the constitutive proteasome[10] or immunoproteasome[11], the latter of which is expressed mainly in dendritic cells and it is induced by proinflammatory cytokines[12]. Further trimming of the precursor peptides (peptides with *N*-terminal extensions) usually takes places after the peptides are transported to the ER but can also occur in the cytosol by cytosolic aminopeptidases such as tripeptidyl peptidase II (TPPII)[13]. TPPII has both endo and exopeptidase activity and unlike most other aminopeptidases it can trim long (>14 amino acids) as well as short peptides (<14 aa)[14]. There are many other cytosolic aminopeptidases such as leucine aminopeptidase (LAP)[15], bleomycin hydrolase (BH) and pyromycin-sensitive aminopeptidase (PSA)[16] however it is not known which ones other than TPPII contribute to antigen cross-presentation[17].

Aminopeptidases present in the ER are referred to as ER aminopeptidases (ERAP) or ER-associated aminopeptidase (ERAAP)[18]. ERAP trims peptides from the *N*-terminus

until they reach a size of 9 or 8 amino acids[19]. It has been hypothesized that ERAP may also be able to trim the peptides that are already bound to MHC-I however limited evidence exists to prove or disprove this hypothesis[19a].

In contrast, the evidence for the vacuolar route suggests that internalized exogenous antigens do not necessarily require departure from their initial uptake vesicle. Instead, antigens are directly degraded into peptides in the phagosomes and loaded onto MHC-Is, which have either been actively recruited or co-internalized from the cell surface during uptake[20] (Figure 1, right panel). After this loading has taken place, the MHC-Is containing new cross-presented peptides are recycled to the cell surface[21]. In this latter case, as well as in the hybrid pathway in which peptides are transported back into the cytosol, the machinery responsible for peptide editing remains poorly understood: how peptides are chosen for MHC-I loading in an environment more acidic than the ER is not known[4b, 22]. One potential protein capable of peptide editing *in vitro*[23] is TAPBPR, a homologue of tapasin[24] which was found to be highly expressed in purified phagosomes of cross-presenting cells. Importantly, unlike tapasin, TAPBPR does not bind any conventional ER-based peptide loading proteins, nor is it retained in the ER (the principal compartment for conventional MHC-I peptide loading)[23c]. Taken together these findings imply that TAPBPR could be one of the peptide editors in vacuolar antigen cross-presentation.

In the vacuolar pathway, the epitope peptides are generated in the phagosome itself however by which proteases is not fully known and understood. It is hypothesized that the cysteine proteases, such as cathepsin S and insulin-regulated aminopeptidase (IRAP) are the key proteases involved in a generation of these peptides[25]. IRAP is a homologue of ERAP, it also trims peptides from the *N*-terminus but it does not stop when they reach size of 9 or 8 amino acids but instead can generate peptides shorter than 8-mer[19c, 26]. Unlike other cathepsins which are active at the acidic pH, cathepsin S is strongly active at the neutral pH which is believed to be present in cross-presenting vacuoles implying that it could be able to generate 8-9mers peptides in that particular environment[27]. Protease activity is thus crucial for generating an appropriate peptide length necessary for an efficient binding to MHC molecules[28]. On the other hand an over-activity may be responsible for a too rapid degradation of peptides before it can be loaded on MHC molecules, or can escape the endo-lysosomal system for cross-presentation[29].

An alternative mechanism for antigen cross-presentation: gap junction mediated peptide transfer has been presented where peptides can be transferred from the cytosol of one cell into the cytosol of its neighbor through gap junctions[30]. Gap junctions are non-specific intercellular channels that allow passive diffusion of molecules (MW~1800). Once transferred, the peptides enter the MHC-I antigen

presentation pathway that results in cytotoxic T cell recognition of these innocent neighboring cells. That would mean that the cells can be recognized and killed by the CTL before the actual infection would take a place and thus prevent the spread of the infection itself.

As all of the above proposed models of intracellular cross-presentation may indicate, the biology of cross-presentation is still likely incompletely understood. This chapter focuses on two main topics in regard to the availability of molecular tools/assays for studying intracellular antigen trafficking and presentation.

2.3 Approaches for studying antigen presentation

The stalwart reagent for measuring cross-presentation activity has been the use of genetic techniques and the use of epitope-specific T cells and T cell clones. These very sensitive cells – capable of recognizing as few as 1-3 peptide-MHC-I complexes per target cell[31] – allow the facile quantification of specific peptides on the cell surface[32], as their activation is likely dependent on the concentration of presented peptide on the APC-surface.

Most commonly used are T cells directed towards the dominant epitope of the ovalbumin protein spanning residues 257-264 (SIINFEKL) in the context of H2-K^b[33]. The development of transgenic mice producing only T cells against this epitope allowed the isolation of large numbers of primary T cells capable of *in vitro* detection of this specific epitope. The use of these cells is very widespread in the study of cross-presentation. It has allowed for the identification of potential contributing proteins and factors to the cross-presentation pathway. For example, the essential role of the proteasome in cross-presentation was discovered by Rock and co-workers when they used these OT-I cells in combination with proteasome inhibitors to show that inhibition of the proteasome abolished cross-presentation, but not MHC-II restricted presentation[20d, 34].

Similarly, also TCR transgenic mice (OT-II) that produce MHC-II restricted, ovalbumin residues 323-339 (ISQAVHAAHAEINEAGR), specific $CD4^+$ T cells (OT-II), are available and used for MHC-II antigen presentation studies[35].

The on-surface quantification of specific peptides in MHC-complexes received a further boost by the development of immortal T cell clones – especially those that had incorporated β-galactosidase under the IL-2 promoter. The Shastri group produced immortal T cell hybridomas specific for SIINFEKL-MHC-I complex ($OVA_{257-264}$-H2-K^b) to quantify as a measurement of T cell response, the amount of generated SIINFEKL epitopes at the cell surface after ovalbumin processing by the APCs[36]. The T cell hybridomas (B3Z) were generated by transfecting a bacterial β-galactosidase gene (*lac*Z)-inducible cell line (Z.8) with the nuclear factor of activated T cells (NFAT)-

element of the human interleukin 2 (IL-2) enhancer-*lac*Z reporter construct and subsequently by fusing the Z.8 with B3 cells (cytotoxic T cell clone specific for OVA/MHC-I ligand)[32, 37]. Those B3Z T cells hybridomas will thus when activated not only produce *lac*Z but also secrete the IL-2. The generated SIINFEKL-MHC-I complexes can be evaluated in the context of T cell activation (*lac*Z assay) through monitoring of β-galactosidase mediated conversion of a fluorogenic or chromogenic substrates or by measuring the IL-2 secretion by colorimetric assays[38]. The advantages of these cells were the quick read out and the sustained *in vitro* growth of these cells, eliminating the need for maintaining. The B3Zs were shown to be capable of detecting pMHC-I complexes after incubation with 20pM of peptide[39], which – whilst two orders of magnitude less that for the OT-I cells, is still very sensitive. This approach has thus been translated to the development of many other *lac*Z inducible T cell hybridomas specific for other pMHC-I complexes and are available against, for example, virus infected cells or tumor antigens[37, 40]. A very recent boost to the field has been the reverse determination of a TCR-ligand. Using the known specificities for given MHCs and peptides from a large number of TCRs, Glanville *et al.* could find paratope hotspots that would allow the identification of TCR-specificity[41]. In the future this may assist in the rational design of TCRs without the need to invoke and isolate T cells with a given affinity.

A reductionist approach (not requiring T cells or hybridoma) has also been developed, namely in the form of T cell receptor (TCR)-like antibodies specific for a given pMHC-I complex[42]. Porgador *et al.* produced a monoclonal antibody specific for MHC-I bound to ovalbumin peptide $OVA_{257-264}$ (SIINFEKL) complex (25-D1.16) with a limit detection approaching that of T cells (approximately 20pm peptide)[43]. This antibody conjugated to a fluorophore allows for direct quantification of SIINFEKL-MHC-I at the cell surface (direct binding of SIINFEKL and after ovalbumin processing) using flow cytometry as well as visualization of intracellular trafficking of this complex using confocal microscopy. Moreover the antibody can serve as a reporter to identify the *in situ* localization of antigen presenting cells bearing SIINFEKL-MHC-I complexes.

There are, however, two major limitations to the use of T cell-based reagents in the study of cross-presentation. The first one is that – by virtue of only the final stages of the process being detected – the underlying mechanisms can only be revealed indirectly. The second problem is that of bias: only those epitopes against which T cells have been identified and cultured can be detected but no information is given on other epitopes.

Evidence for the diversity of peptides capable of binding MHC-Is came from the pioneering work by Rammensee and co-workers who provided insight into the properties of the MHC-I 'ligandome' using an approach based on elucidation and identification[44]. Using a workflow that initially consisted of the immuno-precipitation

of MHC-complexes (from 10 billion cells) followed by Edman-degradation of the peptides, it was found that all positions of the bound peptide were highly varied [45] at all positions, except the two anchor residues. At these points, very few amino acid types were identified using this approach, confirming the importance of these anchors to MHC-I binding. The advent of mass spectrometry added to the richness of the approach: rather than using Edman degradation for peptide identification and sequencing, LC-MS-MS did allow identification of specific MHC-I-bound peptides[46] from a tumor cell line (SW1116). By this approach, sensitivities <10 fM could be achieved, which corresponded to the detection of peptides carrying 8 copies per cell. However, 3 billion cells were needed to achieve this, which is beyond the growth range of many cell lines. However, with the advent of more sensitive MS-MS techniques, the cell numbers needed to provide full coverage have dropped and the approach has now been used, for example, to quantify the number of spliced peptides on the MHC-I ligandome (made from the proteasome catalyzed re-ligation of peptide fragments)[47], to show the contribution of peptides of non-canonical reading frames to antigen presentation[48], and the role of specific proteases, such as ERAAP, to the peptidome[49]. The diversity of the MHC-I-bound peptides over the course of a developing cancer has even recently been reported and the changes in these peptides longitudinally have shown the potential for T cell mediated clearance – even that based on non-neoepitopes[50]. It was also discovered using this approach that post-translationally modified peptides (for example those modified with O-GlcNAc) were presented by cells providing a potential added layer of the complexity of the immune surveillance. The limitations of the technique lie in that, even with ever advancing mass spectrometry, the underlying immunoprecipitation means that it cannot be readily determined from where in the cell the peptide-MHCs have originated, nor can it be excluded that by disrupting the membranes in the cell peptides are exchanged in the MHC-I during the isolation process. Cell-surface acid elution of peptides can prevent this, but does require more cells. Despite these limitations, the use of mass spectrometry has provided major new insights into the peptides and proteins that are presented on cells in health and disease and are beginning to give us a molecular understanding of T cell recognition.

2.4 Approaches for studying intracellular antigen routing

The mechanistic elucidation of cross-presentation has proven difficult, especially due to the complex nature of intracellular routing the antigen can take. Some elegant approaches have been reported to study this subcellular routing, especially in combination with genetic techniques. Two that will be highlighted here are reporter proteins and fluorophore modified antigens.

The reporter proteins rely on intrinsic enzyme (or fluorescence) functionality to detect their presence in subcellular fractions. For example, horseradish peroxidase (HRP) was used by Watts and colleagues[51] to show that the internalized antigens were released into cytosol[52] by using fluorogenic substrates to detect intact protein in the cytosol after macropinocytosis[52a, 53]. One downside to the use of HRP turned out to be that it stimulated its own uptake, because of which skewing of these results could not be excluded[52a, 54].

Ackerman *et al.* used a luciferase enzyme to study cytosolic entry of protein[4c]. Luciferases make up a class of oxidative enzymes that catalyze the oxidation of luciferin in the presence of ATP and oxygen to produce bioluminescence[55], making them one of the most sensitive reporter proteins available. The luciferase reporter assay has, for example been used to study antigen retranslocation into phagosomes[56] using a latex-bead retrieval approach. Isolated phagosomes were incubated with the cytosolic fraction of a cell either in absence or with presence of ATP and luciferase activity was observed only in the phagosomes that were incubated with ATP - containing cytosols and it served as an indication of a successful export of internalized antigen from phagosomes.

Lin *et al.*[57] used a 'reporter protein' in a different manner: to detect cells capable of cross-presentation *in vivo*, horse cytochrome c protein was used as a model antigen. Cytochrome c (cyt c) is an oxidase enzyme found in the mitochondrion of eukaryotes[58]. It is relatively small (~12 kDa) and soluble, features that make the cytosolic transfer in cells possible[59]. Cyt c when released from mitochondrion can evoke programmed cell death (apoptosis)[60]. Lin *et al.* exploited the fact that only the cytochrome c from higher eukaryotic organisms can initiate apoptosis in mammalian cells[61]. They injected mice with either horse or yeast cyt c and observed apoptosis only in cells that were exposed to horse cyt c and that were capable of cyt c uptake and cytosolic transfer. Using flow cytometry they were able to quantify the relative proportion and numbers of various types of splenic cells that survived the cyt c exposure and hence, by negative difference, could determine which splenic DC cell subtypes are the most efficient in cytosolic transfer.

This assay was also used by Cebrian *et al.*[62] to compare cross-presentation efficiency via the cytosolic route in two cell lines (DCs derived from wildtype mice and from Sec22b knockdowns). They showed that Sec22b as a vesicle trafficking protein is required for efficient export of antigens to the cytosol by measuring the amount of apoptosis in cells that have been incubated with cyt c. It was shown that apoptosis was decreased in cells lacking the Sec22b indicating that it is crucial for reporter export to the cytosol. The same group also generated mice bearing a conditional DC-specific mutation in the Sec22b gene and showed that Sec22b-dependent cross-presentation in DCs is required to induce anti-tumor immune responses *in vivo*[63].

Cebrian *et al.*[62] also developed, a new method which they adapted from Ray *et al.*[64] to measure the cytosolic export of antigens. They used coumarin-cephalosporin-fluorescein (4)-acetoxymethyl (CCF4-AM) substrate that is lipophilic and readily cell permeable[7]. When taken up by cells the substrate is converted into its negatively charged form (CCF4) which accumulates in the cytosol. CCF4 is also a Fluorescence Resonance Energy Transfer (FRET) substrate that consists of a cephalosporin core linking 7-hydroxycoumarin to fluorescein which together act as fluorescent probes/reporters for FRET assay. Cebrian *et al.*[62] measured antigen export from endocytic compartment into cytosol as follows first dendritic cells (DCs) were loaded with FRET substrate of β-lactamase (CCF) that after cellular uptake accumulates in the cytosol. Then the cells were exposed to β-lactamase which when transported to cytosol cleaves CCF4 resulting in decreased ratio of fluorescein (acceptor fluorophore) over coumarin (donor fluorophore)[65]. Thus, a loss of FRET signal at 535 nm and increased signal at 450 nm (Figure 2). Finally, the β-lactamase serves as a model antigen and its export to the cytosol can be detected by calculating ratiometric values between the 450 and 535 signals (450:535) using flow cytometry[65b, 66]. The bigger the ratio values the more increased export of the β-lactamase to the cytosol.

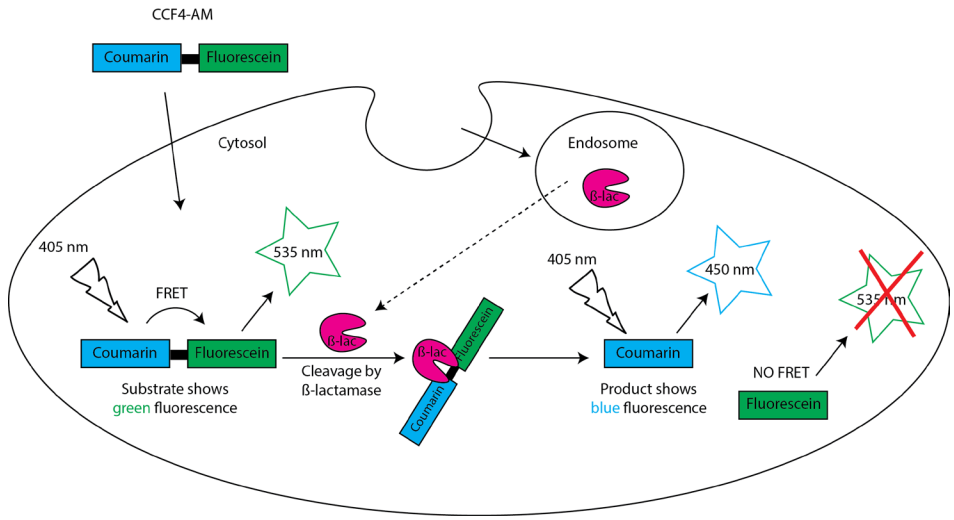

Figure 2. *Schematic representation of the FRET based-β-lactamase assay used to evaluate endosomal export to the cytosol.*

Recently, another assay available for measuring antigen export to cytosol but based on galectin-3 was presented by the van den Bogaart group. Galectin-3 (Gal-3) belongs to a family of beta-galactoside-binding proteins that have an affinity for beta-galactosides[67]. Dingjan *et al.*[68] have transfected cells with galectin-3 conjugated with the fluorescent protein mAzami[69] which is evenly distributed in the cytosol and clusters only when exposed to β-galactoside residues present on glycosylated

proteins[70]. Because these β-galactoside residues are located on glycosylated proteins in the luminal and not the cytosolic side of the endosomal membranes, the recruitment of Gal-3 to these β-galactoside residues could be established only upon endosomal rupture[64]. By co-incubating the cells with OVA-conjugated to Alexa Fluor-647 as an endosomal marker the recruitment of Gal-3-mAzami to OVA-positive endosomes was measured using fluorescence microscopy[71].

Fluorophore-modified antigens have also proven to be valuable reagents for the study of antigen uptake and routing[72]. Ossendorp and colleagues studied kinetics of cross-presentation by conjugating Alexa Fluor-dyes to ovalbumin and presenting it to DCs as either the free, soluble antigen, or in immune complexes with anti-OVA antibodies[72]. Using confocal microscopy and flow cytometry they were able not only to conclude that the antibody bound Alexa Fluor-ovalbumin was taken up much more efficiently than the 'free' OVA but also discovered that this antibody bound exogenous antigen can be conserved for several days within mature dendritic cells in the lysosome-like compartments.

The use of fluorescent protein-antigen fusions such as influenza nucleoprotein (NP) fused with SIINFEKL peptide and enhanced green fluorescence protein (GFP) termed NP-SIINFEKL-EGFP as reported by Princiotta *et al.*[73] to study endogenous antigen processing is however also fraught with danger: fluorescent protein-antigen fusions which can undergo a premature proteolytic degradation and by the very nature of antigen processing – are cleaved from the antigen and can thus only be studied for early events in the process. Chemical fluorophores are relatively large and mostly hydrophobic organic molecules that can alter the properties of the antigen's routing, processing and MHC-loading abilities to the degree that the antigen cannot be found on the cell surface after presentation.

The aim of this thesis is to explore the use of bioorthogonal epitopes to study antigen cross-presentation[74], which in future may provide clearer results to the study of cross-presentation. These are antigens carrying bioorthogonal groups in specific amino acid positions within the epitope region of the antigen that can be reacted selectively within/on the cell using bioorthogonal ligation strategies[75]. Incorporation of bioorthogonal groups into antigens has the advantage over other methods because most of the groups are stable to proteolysis[76] and are small enough to have a minimal impact on routing and loading onto MHC-I molecules[74b, 77]. In the future, these antigens have potential to be applied for imaging of the entire cross-presentation pathway using a single bioorthogonal handle.

2.5 Conclusion

Peptide processing and cross-presentation on MHC-I and –II complexes represent one of the most complex problems in biology. Understanding the manner in which – with a surprising degree of fidelity – peptides are degraded, routed and presented by APCs and host cells remains to be completely understood and an improved understanding of this would lead to the ability to better design vaccines, especially those geared towards the induction tumor/virus targeting CD8 T cells.

2.6 References

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