Bioorthogonal chemistry to unveil antigen processing events

Leeuwen, T. van

3

Bioorthogonal peptide enrichment from complex samples using a rink-amide-based catch-and-release strategy

Introduction

Mass spectrometry-based chemical proteomics can be applied to characterize the nature, function, modifications, and interactions of proteins in their native environment.[1] One of the remaining challenges in this field is the enrichment from complex mixtures of very low abundant proteins of interest. This requires very high efficiency of the retrieval process, and an elution method that is essentially background-free.[2] This becomes particularly poignant in the field of antigen processing and presentation, as here small amounts of antigen taken up by an antigen presenting cells (APC) can make a big difference in the downstream immune response.

The uptake and processing of exogenous antigens and their subsequent loading on MHCs by APCs for activation of T cells is a complex process[3], that is also exquisitely sensitive: as few as 1-2 copies of an antigenic peptide loaded on MHC molecules are sufficient to activate a T cell.[4] The liberation of such peptides from the pathogenic proteins further complicates this. The precise rate and nature of proteolysis during the antigen processing process profoundly affects the efficiency of antigen presentation and T cell activation[5]: certain proteolytic events can result in the liberation of a particular peptide for MHC-loading[6,7], whereas others can lead to its destruction.[8,9]

The precise degradation mechanisms of antigen processing have been studied *in vitro.*[10] This does, however, not recapitulate the topological complexity of the processes that occur within the antigen presenting cell. Following their uptake, antigens are routed through various compartments that contain different protease activities, which process the antigen to peptides. These can then be shuttled to different compartments for MHC-I-restricted presentation (termed cross presentation) or remain in the endo-lysosome for MHC-II-loading.[11] The precise routes and sites of specific proteolytic events remain difficult to investigate, due to a dearth of tools that can identify specific peptide fragments, without affecting their biology.

Recently, “click antigens” were reported as tools to visualize antigen routing and processing in the context of living APCs (*see previous chapters*).[12] These are recombinant antigen proteins containing bioorthogonal functional groups, chemical modalities that are specifically reactive to only their bioorthogonal counterparts.[13] The ligation reactions, termed ‘bioorthogonal reactions’ are chemical reactions that can be performed selectively in biological media. They enable, for example, the selective conjugation of (bio)molecules with detectable groups *after* performing a biological experiment. This means there is less influence of the detectable group on the biology compared to systems where these (for instance, fluorophores) are introduced into the biomolecule before the biological experiments (see earlier chapters). One often-

explored bioorthogonal reaction is the copper-catalyzed azide-alkyne cycloaddition (CuAAC).[14] This reaction involves the formation of a triazole ring by cycloaddition between an azide and a (terminal) alkyne, mediated by copper(I) catalysis. These functional groups do not occur in nature but readily introduced into biomolecules via (bio-)synthetic means. They are often referred to as 'click handles'.

In the case of protein antigens, azide-labelled variants can be produced recombinantly by the replacement of methionine residues by azidohomoalanine (Aha) during the bacterial expression of the protein[15-17], using a technique called bio-orthogonal non-canonical amino acid tagging (BONCAT).[15,18,19] The introduction of this azide-bearing amino acid as a click handle allows for selective targeting and conjugation of the exogenous antigen inside the antigen presenting cell. Previously in this thesis, it was demonstrated that visualization of internalized click antigens can be achieved by CuAAC-mediated fluorophore conjugation.[12] Incorporation of bioorthogonal amino acids is expected to give only minor changes in the protein structure[12,20], and organic azides remain stable inside lysosomes for up to 24 hours, allowing for the imaging of antigen processing even when the antigen is being degraded.[21]

It was therefore hypothesized that click antigens could also be used to study antigen degradation by means of a chemical proteomics approach. Here the bioorthogonal groups would be used to retrieve the bioorthogonal antigens (and their fragments) from APC lysates for mass spectrometric analysis. This could be of great value to the study of antigen degradation in immune cells, providing information on the intactness of an antigen during routing and proteolytic processing inside the APC.

The archetypal approach entails the CuAAC-mediated conjugation of the tagged antigens to biotin, followed by enrichment with avidin-agarose. The biotin-avidin interaction is specific, strong and can only be broken by heating above 70 °C to yield biotin-enriched peptides and proteins for ensuing mass spectrometric analysis.[22] A downside of this method is the unwanted capturing of endogenously biotinylated proteins leading to false positives. The method also suffers from non-specifically bound proteins which are difficult to remove by detergents or high ionic strength washing steps, giving a background that is irreproducible. These drawbacks are of particular concern when considering the very low amounts of antigen that may be present in antigen presenting cells during the processing events. As well, when studying the processing of self-antigens (which can underpin auto-immune disease) any background resulting from endogenous antigens is a complicating factor.

One way to overcome these issues is to remove the biotin-avidin enrichment step by covalently binding the clickable antigens directly to a functionalized solid support, preferentially through a mild, chemo-selective cleavable linker as this would allow the facile release of enriched peptides. The solid support resin beads should have a high chemical stability that permits extremely stringent washing in order to reduce the background signal of non-specifically bound peptides. Previous work in the area of chemical proteomics involved the development of agarose[23,24], sepharose[25], and polyacrylamide based supports.[26] Inorganic supports, in the form of silica[27] and cobalt[28]-based functionalized particles, have also been used for the selective enrichment of bioorthogonally tagged peptides and proteins. Examples of cleavable linkers are levulinoyl esters[29], disulfides[30,31], diazobenzenes[32], acid-cleavable- and photo-cleavable linkers.[33,34] However, in order to retrieve the small amounts of antigen taken up by a dendritic cell for processing and presentation, very high recovery rates are need. The, often, sub-optimal chemical release properties of the above linkers, therefore precludes their use in this context.

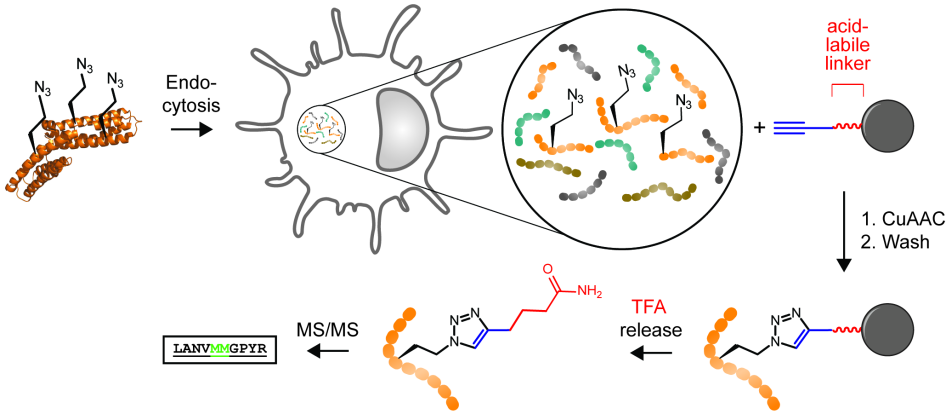


Figure 1. Schematic workflow of Aha-containing peptide enrichment on alkyne-modified poly(ethylene glycol)-based resin. Bioorthogonal antigens are taken up by APCs and processed into peptides. The cells are lysed and Aha-containing peptides are reacted with the alkyne-modified resin via Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC). The resin is then stringently washed with *N,N*-dimethylformamide (DMF) and water (H_2O), followed by peptide retrieval by trifluoroacetic acid (TFA)-mediated release; leaving a triazole with a primary amide moiety on retrieved peptides. Following MS-MS analysis, the modification on peptides allows for facile peptide identification.

In this chapter, an alternative approach to enrich low abundant peptides from complex mixtures is presented. To obtain the required sensitivity, the use of acid-cleavable linkers, which are commonly used in solid phase peptide synthesis, is explored (Figure 1). Commercially available poly(ethylene glycol)-based resins were modified with alkynes and linked via an acid-labile Rink-amide linker as the basis of an azide-selective capture medium. The method presented here proved highly efficient for retrieval of nascent Aha-labelled peptides, most notably in click antigens, and showed greater

efficiency of capture compared to established methods. The methodology is fully bioorthogonal, is compatible with high salt buffers, denaturing conditions, and whole cell lysate to recover peptides present in low nanomolar concentrations. Furthermore, it was shown that after internalization of azido-tagged antigen by dendritic cells, the enrichment method allowed the retrieval of antigen peptides with essentially no background, proving the suitability of the method to study the very rare events that are the likely hallmark of antigen processing in APCs.

Results

3.1 Synthesis and evaluation of alkyne functionalized resins

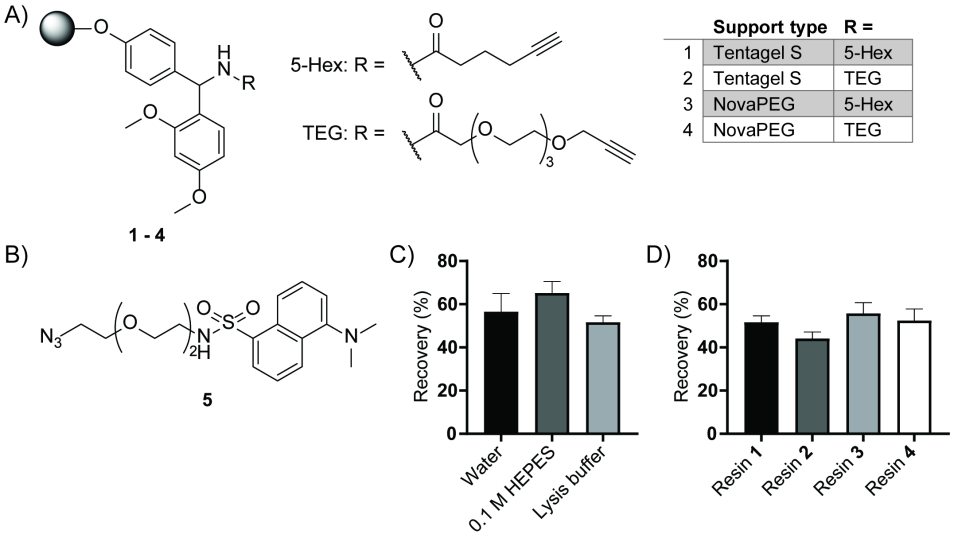
The first aim was to produce an alkyne-modified solid support for the selective enrichment of azide-modified peptides. For this, the resin beads commonly used in solid support peptide synthesis (SSPS) were chosen. While the original polymer support introduced by Merrifield[35] consists of hydrophobic polystyrene material, in later years water compatible supports have been developed, typically consisting of polyethyleneglycol (PEG) chains grafted onto a polystyrene core.[36] More recently solid supports consisting of only PEG have been reported and commercialized.[37] The water-compatibility, chemical stability to pulldown conditions[38], and ready availability of these latter supports, made them excellent candidates for the development of an acid-cleavable retrieval resin.

To facilitate release of the captured peptides from the resin, the chemistries used commonly in the field of peptide synthesis were considered. Liberation of synthesized peptides from a support is typically achieved by acidolysis of an acid-labile linker function. Several different linkers have been developed, some of which requiring high concentrations (50-95%) of trifluoroacetic acid (TFA) in aprotic solvent to mediate cleavage. TFA itself is an excellent solvent for unprotected peptides, and TFA salts are not expected to hinder MS analysis. The two main TFA-labile linker systems used in peptide synthesis are the Rink-amide linker[39], and the para-hydroxybenzyl (PHB)-based linkers.[40] The uncharged carboxamide formed after cleaving the Rink-amide was preferred over the carboxylic acid formed after PHB-cleavage, as its lack of a charge makes it better compatible with positive mode electrospray ionization used in most chemoproteomics protocols.

Two different commercially available, aqueous solvent compatible, solid supports were assessed: Tentagel S and NovaPEG, both of which are pre-functionalized with the Rink-amide linker. These two supports represent two often-used hydrophilic SPPS resins, with Tentagel S being of the PEG-grafted polystyrene variety and NovaPEG consisting of pure PEG-based material. These were derivatized into CuAAC ready supports **1-4** (Figure 2A), and then functionalized with two different alkynes: 5-aminohexynoic acid (5-Hex) and a triethyleneglycol (TEG) terminating in an alkyne.

First, the effectiveness of resin **1** in the retrieval of fluorescent azide **5** from aqueous buffer was evaluated (Figure 2B-C). After CuAAC, followed by washing and release of the clicked molecule with TFA, around 50% of **5** was recovered (as determined by LC-UV analysis). The addition of buffer and other salts to the reaction solvent, in the form of 100 mM HEPES buffer (pH 7.2) or a high salt lysis buffer (8 M urea, 1 M NaCl, 200 mM

TRIS pH 8.0, 4% (w/v) CHAPS detergent), did not diminish this recovery yield. Supports **1**-**4** were evaluated in the same manner (Figure 2D). The samples obtained using resins modified with the TEG-based alkyne (**2**, **4**) showed additional signals in the mass analysis and were therefore not considered applicable for proteomics experiments (Figure S1). The NovaPEG-based support **3** was selected to continue the experiments.



	Support type	R =
1	Tentagel S	5-Hex
2	Tentagel S	TEG
3	NovaPEG	5-Hex
4	NovaPEG	TEG

Figure 2. Overview of the different alkyne-modified resins produced and their evaluation. Structures of alkyne-modified solid supports **1-4** (A). Structure of azide-functionalized dansyl **5** for recovery efficiency determination (B). Evaluation of buffer compatibility using a CuAAC reaction between alkyne-resin **1** and azide **5** (C). Recovery (%) was determined from peak integration of LC-UV using unmodified **5** as an internal standard. Comparison of the efficiency of the recovery of azide **5** using alkyne-resins **1-4** from lysis buffer (D). The recovery (%) was determined as in C. Experiments are the average of n = 3 and displayed as mean ± SD.

3.2 Capture and release of Aha-modified peptides using resin **3**

Next, the recovery of azide-modified peptides using resin **3** was studied. For these experiments, three peptides containing BONCAT-compatible azidohomoalanine (Aha) were synthesized (**6**-**8**, Figure 3A), the sequence of two of which is found in the myelin auto-antigen myelin oligodendrocyte glycoprotein (MOG, peptides **6** and **7**)[41,42] and of one in the RA antigen vinculin (Vin, peptide **8**).[43] These peptides were modified with the fluorescent dansyl-group to aid detection of the peptides by LC-UV. The ability of resin **3** to recover these peptides from lysis buffer was first evaluated. The different peptides were dissolved into the previously described lysis buffer (final peptide concentration 10 µM) and these solutions were added to resin **3**. The CuAAC reaction was performed for two hours, followed by washing with MQ. The captured peptides were then released by treatment with TFA and the recovered peptides were analyzed by LC-UV to determine the recovery efficiency (Figure 3B). The percentage recovery for these three peptides was similar, while the total recoveries here are lower than those found for fluorophore **5** (Figure 2C). The finding that the peptide sequence seems to have little effect on the recovery process, including CuAAC reaction with resin **3**, suggests that the method is unbiased towards peptide identity. Further optimization of the procedure was attempted, such as doubling the amount of resin **3** (2 mg), doubling the amount of THPTA (1 mM) in the CuAAC reaction mixture or extending the CuAAC reaction time to 24h, but no major improvement was seen (Figure S2).

Next, the recovery of the azide-containing peptides from cell lysate was tested by spiking **6**-**8** (final concentration 10 µM) into lysate, before adding resin **3**. The CuAAC reaction was then performed for 2h, followed by washing 3x with MQ, 3x with DMF, and again 3x with MQ to remove non-specific background material. The captured peptides were then released by treatment with TFA and the recovered peptides analyzed by LC-UV to determine the recovery efficiency (Figure 3C). This experiment showed peptide-specific changes in recovery, with the recovery of **6** and **8** increasing with increasing lysate concentrations, and that of **7** decreasing. Pre-trypsinization of the lysate further improved the recovery of all peptides, but it remains to be studied whether this was due to the lower initial lysate concentration (Figure 3C).

Next, non-specific interactions between the resin and the proteins in the mixture were investigated (Figure 3D-E). Washing with only water (Figure 3D) resulted in complex chromatogram upon LC-UV analysis, suggesting the co-elution of multiple a-specific background species. This background was greatly reduced upon washing with DMF (Figure 3E).

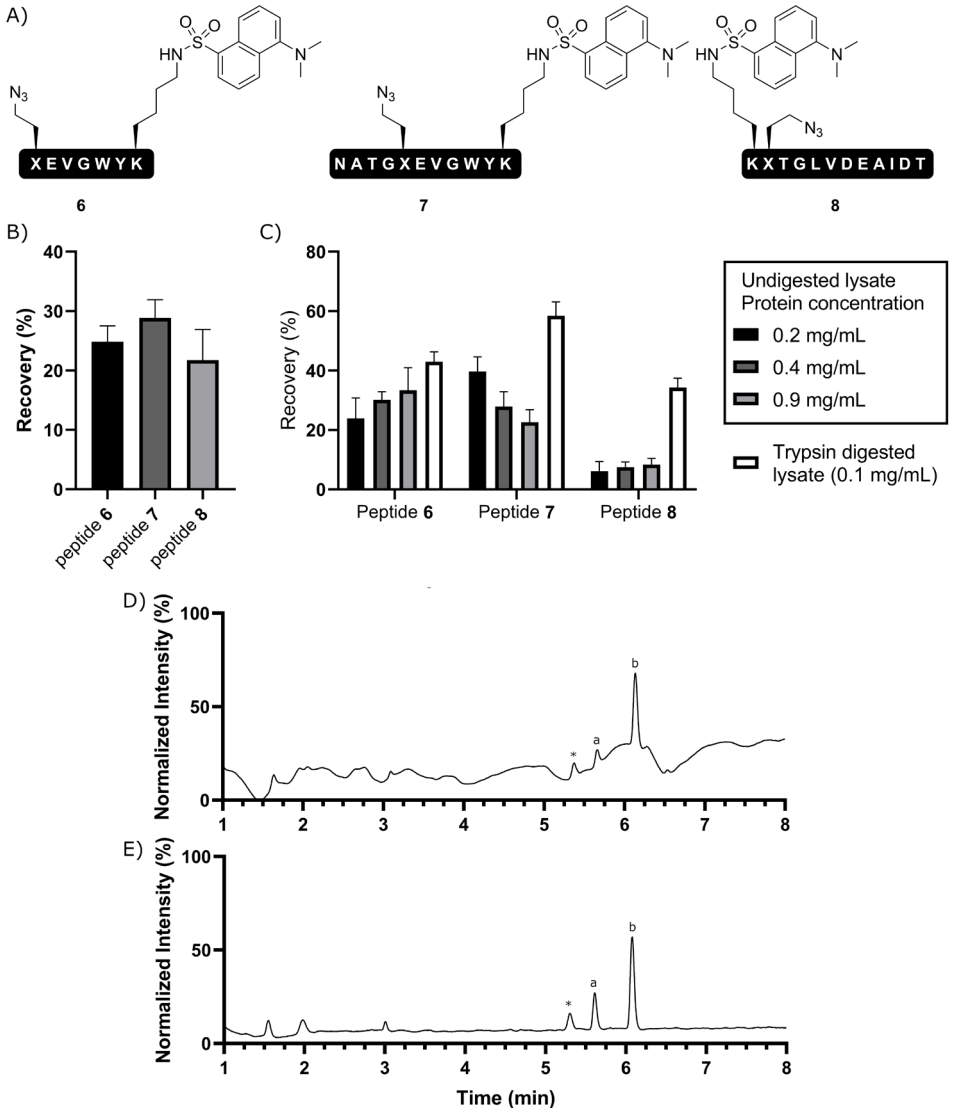


Figure 3. Recovery of dansyl-modified Aha containing peptides from buffer and cell lysates using resin 3. Schematic structure of Aha-containing and dansyl-modified peptides **6**-**8** (A). Recovery of peptides **6**-**8** using alkyne resin **3** (B). Recovery was determined by the area the peak corresponding to the detected peptide on LC-UV, compared to internal standard. Recovery of peptides **6**-**8** (20 µM) from various concentrations of BMDC lysates containing whole proteins, as well as a tryptic digest (C). The specified concentrations refer to the concentration of irrelevant non-labelled lysate proteins. Comparison of partial LC-UV traces of recovered peptide after lysate click and TFA release when using either MQ (D) or DMF (E) to wash the support after CuAAC reaction. [a]recovered peptide [b]internal standard *resin specific impurity.

3.3 Retrieval of Aha-labelled *E. coli* peptides from complex mixtures

Having established the suitability of resin **3** for the pulldown of synthetic peptides, the ability of the approach to retrieve multiple azide-containing peptides from lysate was evaluated. BONCAT-labelled *E. coli* B834(DE3) expressing low levels of the human auto-antigen vinculin (described in chapter 2) was used as a source of azide-containing proteome, as this species can be made to incorporate Aha to near completion.[44] The cells were grown on methionine depleted medium in the presence of Aha to incorporate azides at all methionine sites in newly synthesized proteins. The lysate of these cells and shown to indeed contain CuAAC-reactive proteins (Figure S3).

Next, this Aha-labelled *E. coli* lysate was diluted in lysate of unlabeled bone marrow-derived dendritic cells (BMDCs). The mixtures were digested with trypsin, followed by retrieval of Aha-containing peptides using resin **3**. In this experiment, **3** was directly compared to an alkyne-agarose resin, containing the hydrazine-labile linker, 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde-Alk-agarose). Resin **3** was washed 3x with MQ, then 3x with DMF, and 3x with MQ, whereafter peptides were released by incubation with TFA for 1h. The Dde-Alk-agarose mixture was washed 5x with SDS wash (100 mM Tris, 1% SDS, 250 mM NaCl, 5 mM EDTA, pH 8.0), 5x with 8 M urea/100 mM Tris pH 8.0, and 5x with 20% ACN according to the manufacturer's protocol, whereafter peptides were released by incubation with 200 µL 2% (v/v) hydrazine for 1h. The amounts of resin were adjusted so that equal amounts of available alkyne handles (0.2 µmol) were present in each experiment. Retrieved peptides were measured by MS-MS and identified by PEAKS software (FDR < 1%).

Retrieval with resin **3** yielded 17.5 times more Aha-containing peptides compared to Dde-Alk-agarose (Figure 4). In total, circa 1300 Aha-containing *E. coli* peptides were retrieved from 100 µg Aha-*E. coli* lysate using resin **3**. Dde-Alk-agarose by comparison yielded only circa 70 peptides. Aha-containing peptides emerging from *E. coli* proteins, as well as the low abundant Aha-Vin also expressed by this strain, could be detected from 1 µg *E. coli* lysate spiked into 99 µg of BMDC lysate (Figure 4A-B, Table S1-S2). The selectivity of the protocol was confirmed by the low retrieval of mouse-BMDC sourced peptides (1.6% of total identified; Figure S4).

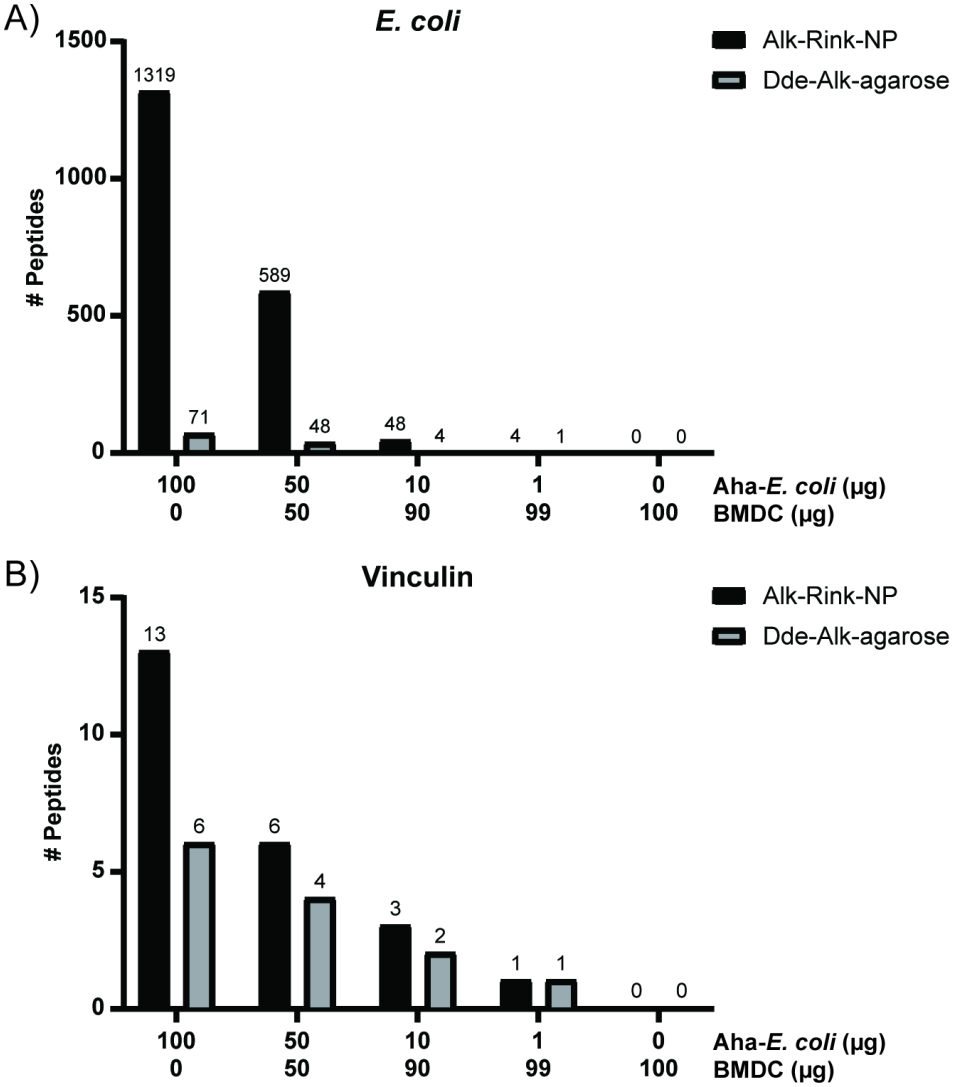


Figure 4. Amounts of Aha-labelled peptides identified after enrichment by resin 3 or Dde-alkyne-agarose from complex mixtures of BMDC and Aha-labelled *E. coli* lysate. The amount of confident (FDR < 1%) nascent Aha-labelled peptides emerging from *E. coli* proteins (A) or vinculin expressed by *E. coli* cells (B) enriched on either resin **3** (black bars) or Dde-Alk-agarose (grey bars).

3.4 Retrieval of Vin-derived Aha-peptides from lysates and live BMDCs

To investigate whether the method could for the envisaged purpose of antigen recovery from APCs, different amounts of an Aha-labelled auto-antigen vinculin (Aha-Vin) were diluted in 100 µg BMDC lysate (Figure 5, Table S3). In theory, five Aha-containing Vin peptides are formed after tryptic digest, which can potentially be enriched from the mixture (Figure 5A). The detection limit of Aha-containing Vin peptides proved to be 1 ng (28 pmol) in 100 µg lysate, as at this concentration at least one tryptic Aha-Vin peptide was identified after enrichment procedure using resin **3** (Figure 5B). Peptide #1 (HMLGEISALTSK) could not be retrieved in any of the experiments, likely due to a missed cleavage of trypsin after adjacent acidic residues.[45] Full coverage of four tryptic Aha-peptides could be achieved by spiking at least 0.1 µg Aha-Vin in lysate (2.8 nmol). Stringent washing of the resin with DMF was proven to be very efficient as no murine or unmodified-Vin peptides were identified from any of the samples.

Finally, it was investigated whether the enrichment method could be applied to study cellular processes as antigen processing by retrieval of auto-antigens from APCs. BMDCs were pulsed with Aha-Vin (1.35 µM) for 2 hours, then washed, and lysed, followed by full proteome digest with trypsin, and enrichment using resin **3** (Figure 5C, Table S4). In this experiment, two out of four potential tryptic Aha-Vin peptides were retrieved without any background of endogenous proteins, proving the suitability to retrieve rare antigen from cells.

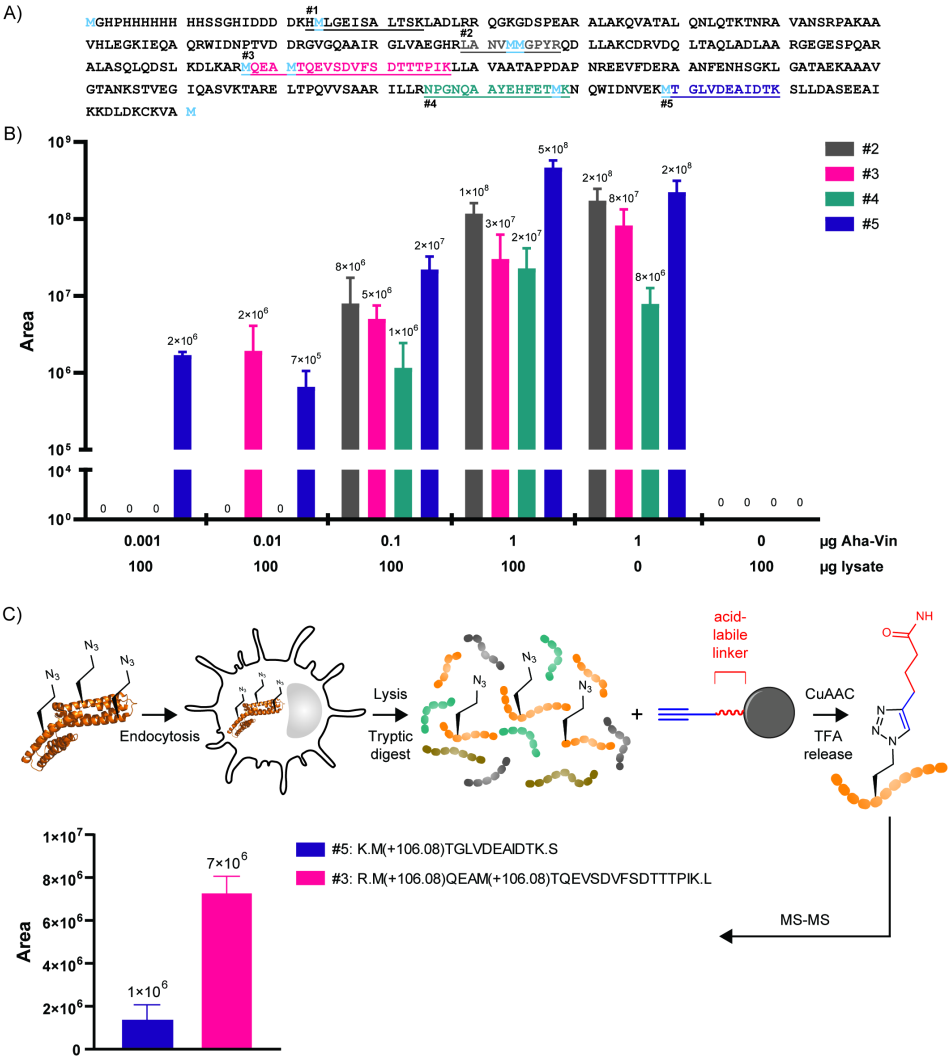


Figure 5. Retrieval of Aha-containing Vin peptides from lysate or live BMDCs (n = 3). Amino acid sequence of vinculin showing five theoretical tryptic peptides numbered 1-5 (A). Retrieval of Aha-containing Vin peptides after spiking different concentrations of Aha-Vin in BMDC lysate (B) or after pulsing live BMDCs with Aha-Vin for 2 hours (C). The peak area (y-axis) of the parent ion of the detected peptide was used as a measure of peptide abundancy. Experiments are the average of n = 3 and displayed as mean ± SD.

Conclusion

In this Chapter, the development of a highly efficient CuAAC-mediated enrichment method for retrieval of low abundant azide-containing peptides from complex mixtures was described. It was shown that by modifying a poly(ethylene glycol)-based resin, commonly used for the solid support synthesis of peptides, with an alkyne functionality, a reagent capable of retrieval of azide-containing peptides could be produced. The high chemical stability of the polymer allowed for stringent washing, which greatly reduced background signal of non-specifically bound molecules. Furthermore, the acid-mediated cleavage reaction is highly compatible with the purification steps required in proteomics workflows. One potential downside of the acid-mediated release is that some acid-labile post-translational modifications (PTMs), like tyrosine sulfation or histidine phosphorylation, may be unintentionally destroyed during the acidic treatment, making this approach unusable in studies focused on detecting these PTMs. Fortunately, many PTMs relevant to autoimmune disorders, like citrullination[46] and/or *O*-glycosylation[47], are known to be stabile to these acidic release conditions.

It was finally also shown that the method could be used for the retrieval of bacterial and protein antigen fragments from dendritic cell lysate, as well as of the recovery of antigen after phagocytosis from the resulting lysate, offering a new reagent to study the processing of these low-abundant proteins in immune cells.

Acknowledgements

Dr. Ward Doelman for synthesis and characterization of resins and peptides, and general collaboration throughout this project. Robin van den Kieboom for contributing to the optimization of resin development and application, and dr. Bobby I. Florea for his general advice and help with MS measurements.

Materials and methods

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 (Alexa Fluor 488 Azide, Alexa Fluor 488 Alkyne, Alexa Fluor 647 Azide, and Alexa Fluor 647 Alkyne) were purchased from Thermo Fisher Scientific.

Cell culture

Bone-marrow derived dendritic cell differentiation
BMDCs were isolated and differentiated as described in chapter 2.

Azidohomoalanine labelling of *E. coli* B834(DE3)

A single colony of *E. coli* B834(DE3) transformed with pET3d-Vin$_{435-741}$ was grown overnight in 10 mL LB augmented with 1% w/v glucose and Ampicillin (50 µg/mL). The next morning, the culture was diluted 1:50 with fresh LB media augmented with 1% w/v glucose and Ampicillin (50 µg/mL) and cells were grown to an OD600 of 0.3-0.5. The resulting culture was then centrifuged (2000 g, 10 min), washed, and resuspended in SelenoMet™ media (Molecular Dimensions, USA) without adding additional methionine. The culture was incubated at 37 °C for 30 min, after which L-azidohomoalanine (Aha, 4 mM final concentration) was added. After 1h, the culture was centrifuged, washed with PBS, and pellets were stored in -80 °C until further use.

Synthesis

Synthesis of alkynyl functionalized NovaPEG-based Rink-amide resin
Functionalization of NovaPEG resin was carried out using typical Fmoc-SPPS methodology. Briefly, 100 mg of NovaPEG Rink amide resin (loading 0.22 mmol/g, Novabiochem) was swelled in 2 mL of DMF for 15 min before the Fmoc-group was removed by treatment of the resin with a 20% (v/v) solution of piperidine in DMF (2 x 5 min), followed by thorough washing of the solid support (5 x 2 mL). 5-hexynoic acid (5 eq, 0.1 mmol, 11 µL) and HCTU (5 eq, 0.1 mmol, 41 mg) were dissolved together in DMF

(200 µL), followed by addition of DiPEA (10 eq, 0.2 mmol, 35 µL). This solution was added to the resin and incubated for 90 min. Completion of the reaction was verified by negative Kaiser test. The resin was drained and washed with DMF (3 x 2 mL), DCM (3 x 2 mL), and methanol (3 x 2 mL) and stored at -20 °C until further use.

Synthesis of (S)-4-azido-2-aminobutanoic acid (azidohomoalanine, H-Aha-OH)
Azidohomoalanine was produced in-house as previously described.[12]

Synthesis of Fmoc-Aha-OH
H-Aha-OH (0.72 g, 5 mmol, 1.0 eq) was dissolved in 1:1 H_2O:dioxane (50 mL). Sodium carbonate (0.53 g, 5 mmol, 1.00 eq.) and Fmoc-OSu (1.69 g, 5 mmol, 1.00 eq.) were added and the solution was stirred at room temperature (r.t.) for 4h, after which TLC (1:9 EtOAc:DCM) showed complete consumption of the starting compound. The dioxane was removed under reduced pressure and the remaining aqueous solution acidified with 1M HCl to pH 2~3. This aqueous solution was then extracted with ethyl acetate and the organic layer was collected, dried over $MgSO_4$ and concentrated. The residue was purified using silica gel column chromatography (10% EtOAc in DCM). Fmoc-Aha-OH was obtained as a pale-blue solid (1.12 g, 3.07 mmol, 61%) **^{1}H NMR** (300 MHz, $CDCl_3$) δ 7.73 (dd, J = 7.4, 1.1 Hz, 2H, Fmoc-Ar), 7.59 – 7.52 (m, 2H, Fmoc-Ar), 7.41 – 7.33 (m, 2H, Fmoc-Ar), 7.28 (tt, J = 7.5, 1.0 Hz, 2H, Fmoc-Ar), 4.47 (d, J = 6.8 Hz, 3H, α-CH, Fmoc-CH_2), 4.20 (t, J = 6.5 Hz, 1H, Fmoc-CH), 3.35 (s, 2H, γ-CH_2), 2.25 – 1.85 (m, 2H, β-CH_2). **^{13}C NMR** (75 MHz, $CDCl_3$) δ 175.5, 156.4 (C=O), 143.9, 143.8 (Fmoc-Cq), 141.6, 128.0, 127.3, 125.1, 120.2, 120.2 (Fmoc-Ar), 67.6 (Fmoc-CH_2), 52.0 (α-CH), 47.9 (γ-CH_2), 47.5 (Fmoc-CH), 31.6 (β-CH_2).

*Synthesis of dansyl-PEG$_2$-azide (**5**)*
Azido-PEG$_2$-amine (27 mg, 0.16 mmol, 2.0 eq) was dissolved in 0.4 mL dry DCM. In a separate flask, dansyl chloride (22 mg, 0.081 mmol, 1.0 eq) was dissolved in 0.8 mL dry DCM and this solution was added dropwise to the dissolved amine. The bright orange solution was stirred overnight at r.t.. The reaction mixture was diluted with 50 mL DCM and washed with 50 mL sat. aq. $NaHCO_3$ and 50 mL brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The residue was purified using column chromatography (1:1 DCM/EtOAc) producing a green, fluorescent oil (27 mg, 0.067 mmol, 85%). **^{1}H NMR** (400 MHz, $CDCl_3$) δ 8.56 (dt, J = 8.5, 1.1 Hz, 1H), 8.33 (dt, J = 8.7, 0.9 Hz, 1H), 8.27 (dd, J = 7.3, 1.3 Hz, 1H), 7.57 (ddd, J = 19.5, 8.6, 7.4 Hz, 2H), 7.21 (dd, J = 7.7, 0.9 Hz, 1H), 5.36 (t, J = 6.0 Hz, 1H), 3.65 – 3.60 (m, 2H), 3.53 – 3.48 (m, 2H), 3.44 – 3.36 (m, 6H), 3.13 (td, J = 5.7, 4.6 Hz, 2H), 2.91 (s, 6H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 152.08, 135.10, 130.51, 130.01, 129.76, 129.52, 128.43, 123.31, 118.97, 115.29, 70.45, 70.37, 70.15, 69.37, 50.69, 45.53, 43.18, 29.81. HRMS calcd for $C_{18}H_{25}N_5O_4S$ $[M+H]^+$: 408.1700, found 408.1699.

Fmoc-SPPS synthesis of peptides

SPPS of peptides was carried out using manual synthesis on a 50 µmol on Tentagel S RAM resin (Rapp Polymere GmbH, Germany) when a C-terminal carboxamide was desired, or on chloro-(2'-chloro)-trityl (CTC) polystyrene resin when a C-terminal carboxylic acid was intended. Fmoc protected amino acids were purchased from either Novabiochem or Sigma-Aldrich. All standard sidechain protection groups were applied, with the exception of Lys(Mmt) used for on-resin fluorophore introduction. Fmoc deprotection was accomplished by repeated treatment with 20% piperidine in DMF (3 + 7 min). To extend the growing peptide chain, 5 eq of Fmoc-amino acid was mixed together with an equimolar quantity of 2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) in DMF at a concentration of 0.5 M, together with 10 eq of diisopropylethylamine (DiPEA). Coupling reactions were carried out for 30-45 min. Fmoc-Lys(Mmt)-OH was coupled using 2 eq of amino acid mixed together with an equimolar quantity of 2-(6-chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) in DMF at a concentration of 0.2 M, together with 4 eq of diisopropylethylamine (DiPEA). These reactions were left to couple for 90 min. Global deprotection and resin cleavage of peptides was accomplished using a 95:2.5:2.5 mixture of TFA/TIS/H_2O for 3h, followed by precipitation from cold diethyl ether (1:9 ratio TFA to ether) and recovery of the precipitate by centrifugation. Crude, tryptophan containing peptides were dissolved in MilliQ water (MQ) a lyophilized overnight in order to remove the residual carboxylate. Preparative reverse phase HPLC on a Waters AutoPurification system (eluent A: H_2O + 0.2% TFA; eluent B: ACN) with a preparative Gemini C18 column (5 µm, 150 x 21.2 mm) yielded the final products. Peptides were characterized using electrospray ionization mass spectrometry (ESI-MS) on a Thermo Finnigan LCQ Advantage Max LC-MS instrument with a Surveyor PDA plus UV detector on an analytical C18 column (Phenomenex, 3 µm, 110 Å, 50 mm × 4.6 mm) in combination with buffers A (H_2O), B (MeCN), and C (1% aq TFA). Quality of crude and purified peptides was evaluated with a linear gradient of 10-90% B with a constant 10% C over 10 min. Preparative reverse phase HPLC on a Waters AutoPurification system (eluent A: H_2O + 0.2% TFA; eluent B: ACN) with a preparative Gemini C18 column (5 µm, 150 x 21.2 mm) yielded the final products. All peptides were purified to at least 95% purity as determined by HPLC-UV analysis.

Chemoselective deprotection of Lys(Mmt) and introduction of the dansyl fluorophore

After the synthesis of the peptide sequence was completed, the resin was washed 3 times with DCM. Chemoselective deprotection of the Mmt group was achieved by addition of a mixture of acetic acid and trifluoroethanol (TFE) in DCM (AcOH/TFE/DCM 1:2:7) to the resin, which was incubated under gentle agitation for 2h. After draining the

resin and thorough washing with DCM, the resin was treated twice with 10% Et_3N in DCM (v:v) for 10 min. Residual Et_3N was removed by washing 5 times with DCM. Dansyl chloride (2 eq.) and DIPEA (4 eq.) were dissolved in DCM (1 mL / 50 µmol resin loading) and added to the resin. The suspension was shaken o.n. at r.t.. The excess solution was drained and the resin was washed 5 times with DCM and 3 times with DMF. Removal of the final Fmoc group and release of the peptide was carried out according to the general SPPS procedure.

Recombinant expression and purification of Aha-vinculin

Aha-vinculin$_{435-741}$ (Aha-Vin) was expressed and purified as previously described.[12]

Preparation of Aha-labelled *E. coli*- and BMDC lysate

Aha-labelled *E. coli* lysate and BMDC lysate were prepared by incubation of cell pellets in lysis buffer (50 mM HEPES pH 7.2, 150 mM NaCl, 8 M UREA, 0.1% SDS, 1% IGEPAL, 1x EDTA-free protease inhibitor, 10 U benzonase) for 1h on ice and subsequent probe sonication (4 x 10 sec, 30% amplitude). Lysates were centrifuged (5 min at 10,000 g) to remove cell debris and protein concentration was measured by Bradford assay. The concentration of lysates was adjusted to 1 mg/mL and stored in -80 °C.

Capture-and-release of fluorophore 5 or peptides 6-8 with alkyne-modified solid supports

1 mg of alkyne-modified resin was transferred to an Eppendorf vial. A 20 µM solution of fluorophore **5** or peptide was prepared in MQ or buffer, which was first degassed for 15 min by bubbling N_2 through the solution. 100 µL of the probe/peptide solution was added to the resin and the vial was shaken for 1h at r.t.. To a separate vial, CuAAC click mix was added in order: 2 µL 0.1 M $CuSO_4$ solution, 2 µL 1.0 M sodium ascorbate solution, 10 µL 0.1 M THPTA solution, and 2 µL 1.0 M aminoguanidine solution. The solution was thoroughly mixed and diluted to 100 µL with MQ or buffer. The click mix was added to the resin suspension and was shaken for 2h at 25°C. The suspension was centrifuged and the supernatant was removed and discarded. The resin was subsequently washed trice by addition of MQ, followed by recovery of the resin by centrifugation, and careful aspiration of the supernatant. Release of the retained molecules was initiated by the addition of 50 µL of release cocktail (TFA/TIPS/MQ 95:2.5:2.5) to the dry resin. The release reaction incubated for 2h at r.t., followed by quenching by the addition of 150 µL MQ, followed by the addition of 2 µL 1 mM

probe/peptide solution as an internal standard. The suspension was centrifuged, the supernatant was collected, and analyzed by LC-MS/UV-VIS (λ = 280 nm, 10-50% B, mass range: 400-2000). Peptide recovery was quantified by the ratio of the area under the UV peak @ 280 nm of the released molecule (identified by MS) and the standard.

For experiments carried out in cell lysate, the labelled peptide was spiked into the lysate as 2 µL of a 10 mM solution. The resin was first incubated with this solution for 1h, followed by initiation of the click reaction as described above.

Peptide enrichment assays

*Enrichment of nascent Aha-labelled E. coli peptides using resin **3***

Samples were prepared by diluting varying amounts of Aha-labelled *E. coli* lysate in BMDC lysate. Mixtures were reduced with DTT (5 mM final) for 15 min at 65 °C and free thiols were alkylated with iodoacetamide (12 mM final) for 30 min at r.t. in the dark. Mixtures were diluted 10x in 100 mM NH_4HCO_3, 1 mM $CaCl_2$, pH 8.0 and proteins were digested by 3 µg trypsin (Sequencing grade, Promega) overnight by shaking at 950 rpm, 37 °C. Trypsin was inactivated by incubation for 5 min at 95 °C. 1 mg alkyne-modified resin was added to the samples and incubated for 1h. Then, CuAAC click mix (1 mM $CuSO_4$, 10 mM sodium ascorbate, 1 mM THPTA ligand, 10 mM aminoguanidine, 100 mM HEPES pH 8.0) was added and samples were incubated for 2h, r.t. at 1000 g. The resin was first washed 3x with MQ, then 3x with DMF, and 3x with MQ by centrifugation at 4000 rpm for 3 min. Samples were incubated with 50 µL TFA for 1h to release peptides and dried by N_2 for 2 min. Eluted peptides were dissolved in 0.5% (v/v) formic acid solution in MQ and desalted over StageTips[48], then dried by SpeedVac (2h at 45 °C), and reconstituted in 30 µL 97:3:0.1 solution (H_2O, ACN, CHOOH) for MS measurements.

Dde-alkyne-agarose enrichment of nascent Aha-labelled E. coli peptides

Mixtures were prepares as described above. 100 µL of Dde-alkyne-agarose (50% slurry, Click Chemistry Tools) was washed with MQ (2 min, 1000 g) and added to the digested lysate mixtures. Then, CuAAC click mix was added and samples were incubated for 2h, r.t. at 1000 g. Samples were transferred to micro-Bio-Spin™ columns (Bio-Rad) and the resin was washed 5x with SDS wash (100 mM Tris, 1% SDS, 250 mM NaCl, 5 mM EDTA, pH 8.0), 5x with 8 M urea/100 mM Tris pH 8.0, and 5x with 20% ACN. Peptides were released by incubation with 200 µL 2% (v/v) hydrazine for 1h at r.t. with agitation. Eluted peptide mixtures were acidified by addition of 5 µL formic acid and desalted over StageTips[48], then dried by SpeedVac (2h at 45 °C), and reconstituted in 30 µL 97:3:0.1 solution (H_2O, ACN, CHOOH) for MS measurements.

Enrichment of Aha-containing Vin peptides from BMDC lysate
Varying amounts of Aha-Vin were spiked in 100 µg BMDC lysate, reduced with DTT (5 mM final) for 15 min at 65 °C, and free thiols were alkylated with iodoacetamide (12 mM final) for 30 min at r.t. in the dark. Samples were diluted 10x in 100 mM NH_4HCO_3, 1 mM $CaCl_2$, pH 8.0 and proteins were digested by 3 µg trypsin (Sequencing grade, Promega) overnight by shaking at 950 rpm, 37 °C. Aha-containing peptides were enriched by CuAAC reaction with 1 mg resin **3** for 2h. The resin was first washed 3x with MQ, then 3x with DMF, and 3x with MQ by centrifugation at 4000 rpm for 3 min. Samples were incubated with 50 µL TFA for 1h to release peptides and dried by N_2 for 2 min. Eluted peptides were dissolved in 0.5% (v/v) formic acid solution in MQ and desalted over StageTips[48], then dried by SpeedVac (2h at 45 °C), and reconstituted in 30 µL 97:3:0.1 solution (H_2O, ACN, CHOOH) for MS measurements.

Enrichment of Aha-Vin peptides from live BMDCs
5 x 10^6 BMDCs were seeded onto 10 cm dishes (non-tissue treated) in 10 mL IMDM supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma, ref# F0804, lot# 015M3344), 2 mM Glutamax™ (GIBCO, ref# 35050-038), 20 µM β-Mercaptoethanol (Gibco, ref# 31350010), 50 IU/mL penicillin, 50 µg/mL streptomycin, and recombinant GM-CSF (20 ng/mL, Peprotech, ref# 315-03). After resting for 2h, cells were pulsed with 1.35 µM Aha-Vin for 2h. Cells were washed with PBS (2x) and harvested by scraping. Cells pellets were collected in Eppendorf tubes and stored in -20 °C until further use.

The cell pellets were lysed in lysis buffer (50 mM HEPES pH 7.2, 150 mM NaCl, 8 M UREA, 0.1% SDS, 0,1% IGEPAL, 1x EDTA-free protease inhibitor, 10 U benzonase) for 30 min at r.t. and subsequent probe sonication (4 x 10 sec, 30% amplitude). Lysates were centrifuged (5 min at 10,000 g) to remove cell debris. Denatured proteins were reduced with DTT (5 mM final) for 15 min at 65 °C and free thiols were alkylated with iodoacetamide (12 mM final) for 30 min at r.t. in the dark. Samples were diluted 10x in 100 mM NH_4HCO_3, 1 mM $CaCl_2$, pH 8.0 and proteins were digested by 3 µg trypsin (Sequencing grade, Promega) overnight by shaking at 950 rpm, 37 °C. Aha-containing peptides were enriched using resin **3** as described above.

LC/MS/MS measurement and data analysis
The desalted peptides solution was separated on an UltiMate 3000 RSLCnano system set in a trap-elute configuration with a nanoEase M/Z Symmetry C18 100 Å, 5 µm, 180 µm x 20 mm (Waters) trap column for peptide loading/retention, and nanoEase M/Z HSS C18 T3 100 Å, 1.8 µm, 75 µm x 250 mm (Waters) analytical column for peptide separation. The column was kept at 40 °C in a column oven. Flow gradient used for analysis was a steep (45 min) gradient of mobile phase A (0.1% formic acid (FA) in ULC-

MS grade water (Biosolve)) and mobile phase B (0.1% FA in ULC-MS grade acetonitrile (ACN, Biosolve)) controlled by a flow sensor at 0.3 µL/min with average pressure of 400-500 bar (5500-7000 psi). Samples were injected (1 µL) on the trap column at a flow rate of 15 µL/min for 9 min with 99%A, 1%B eluent. The gradient was programmed with linear increment to 1% B from t0 to t2 min, 10%B to t5 min, 30%B at t25, 90%B at t26 to t33 and 1%B at t34 to t45 min. The eluent was introduced by electro-spray ionization (ESI) via the nanoESI source (Thermo) using stainless steel Nano-bore emitters (40 mm, OD 1/32", ES542, Thermo Scientific). The QExactive HF was operated in positive mode with data dependent acquisition without the use of lock mass, default charge of 2^+, and external calibration with LTQ Velos ESI positive ion calibration solution (88323, Pierce, Thermo) every 3-5 days to less than 2 ppm. The tune file for the survey scan was set to scan range of 350 – 1400 m/z, 60.000 resolution, 1 microscan, automatic gain control (AGC) of 1e6, max injection time of 50 ms, no sheath, aux or sweep gas, spray voltage ranging from 1.7 to 3.0 kV, capillary temp of 250 °C, and a S-lens value of 80. The sensitive MS method settings were: the survey scan was taken at 120,000 resolution, AGC target of 3e6, maximum IT time of 100 ms, and scan range of 350 to 1400 m/z. For the 10 data dependent MS/MS events the loop count was set to 10 and the general settings were resolution to 15,000, AGC target 1e5, max IT time 50 ms, isolation window of 1.6 m/z, fixed first mass of 120 m/z, and normalized collision energy (NCE) of 28 eV. For individual peaks the data dependent settings were 1.00e3 for the minimum AGC target yielding an intensity threshold of 2.0e4 that needs to be reached prior of triggering an MS/MS event. No apex trigger was used, unassigned, 1^+ and charges > 8^+ were excluded with peptide match mode preferred, isotope exclusion on and dynamic exclusion of 10 sec. In between experiment samples routine wash and control runs were done by injecting 5 µL 97.3.0.1 solution, 5 µL of 10 fmol/µL BSA or enolase digest, and 1 µL of 10 fmol/µL angiotensin III (Fluka, Thermo)/oxytocin (Merck) to check the performance of the platform on each component (nano-LC, the mass spectrometer (mass calibration/quality of ion selection and fragmentation), and the search engine).

Resulting MS RAW. files were analyzed by using PEAKS software 10.0 (Bioinformatic Solutions Inc.) and PEAKSDB search with a focused UniProt identifier database consisting of human vinculin$_{435\text{-}742}$, his-tag vinculin$_{435\text{-}742}$, BSA, yeast enolase, trypsin, avidin, and streptavidin was performed. Error tolerance was set to 10 ppm for the parent mass and 0.2 Da for fragmented ions. Enzyme specificity was set as 'Trypsin, semi-specific' and 30 PTMs per peptide were allowed. Carbamidomethylation (+57.02) was set as a fixed modification and Aha replacement by Met (-4.99) and Met oxidation (+15.99) as variable modifications. The following variable modifications were added per enrichment method: modification of Met to Aha+5Hex (+106.08 Da) for enrichment using resin **3**, Met to Aha-Dde (+50.06) for Dde-alkyne-agarose. FDR was set to 1% and only PTMs with ion intensity > 5% were considered confident. Protein coverage images were obtained

by the software and peptide sequences were combined when found in at least 2/3 replicates.

SDS-PAGE analysis[49]

For SDS-PAGE analysis all samples were heated for 5 min at 95 °C (exception: samples containing click cocktail). 20 µL of each sample was loaded onto a 15% SDS-PAGE gel (0.75 or 1.5 mm) and run for ~70 min at constant 170 V. Subsequently in-gel fluorescence was measured at indicated wavelength filters for Alexa 488, Alexa 647 or Cy5. For imaging the gels, Bio-Rad Chemidoc Imager and ImageLab 5.2 software (Bio-Rad) was used.

Supplementary figures

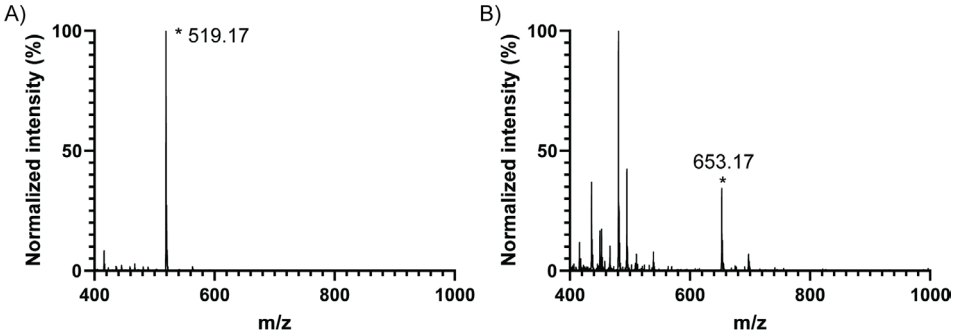


Figure S1. MS analysis of the released product after clicking fluorophore 5 on resin 1 or 2 followed by TFA mediated release. (A) MS spectrum of the released fluorophore from resin **1.** (B) MS spectrum of the released fluorophore from resin **2**. In both cases the mass of the expected product is highlighted.

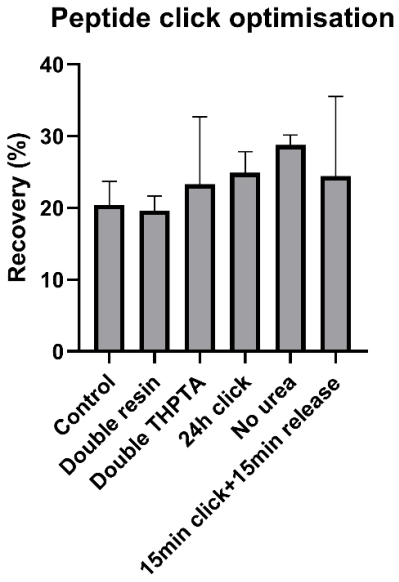


Figure S2. Optimization of the click reaction between resin 3 and peptide 6. Conditions for the click and release were the same as those in Figure 3, with the exception of the indicated modification to the conditions. Double resin = 2 mg of resin 3 used. Double THTPA = Final THPTA concentration 1 mM. 24h click = click reaction run for 24h. No urea = Lysis buffer without 8 M urea. 15 min click + 15 min release = both click-reaction and TFA mediated release shortened to 15 minutes each. Experiments are the average of n = 3 and displayed as mean ± SD.

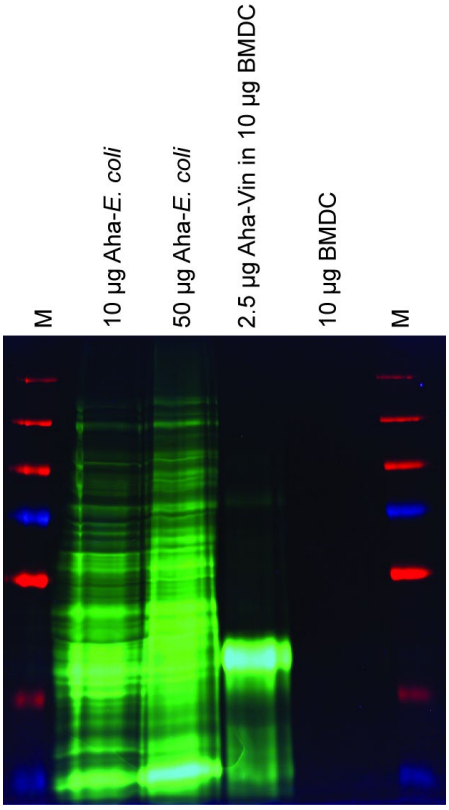


Figure S3. SDS-PAGE fluorescence images showing Aha-incorporation in *E. coli* B834(DE3) and Aha-Vin spiked in unlabeled BMDC lysate. Composite image of channels Cy3, Cy5, and AF488. Aha-labelled proteins were reacted with AF488-alkyne via CuAAC (Green).

Table S1. Aha-containing Vin peptides retrieved on resin 3 from lysate mixtures of Aha-*E. coli* and unlabeled BMDCs.

Aha-*E.coli* (µg): BMDC (µg)	Peptide	-10lgP	ppm	m/z	z	RT (min)	Area	AScore
1 vs 99	R.M(+106.08)QEAM(+106.08)TQEVSDVF SDTTTPIK.L	51.64	-5.4	8.574.209	3	22.85	2.16E6	M1 & M5: Aha+5Hex:1000
10 vs 90	R.LANVM(+106.08)M(+106.08)GPYR.Q	38.17	-4.1	6.823.701	2	17.32	3.06E7	M5: Aha+5Hex:1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVF SDTTTPIK.L	54.63	-3.6	12.856.272	2	23.08	2.36E7	M1 & M6: Aha+5Hex:1000
	K.M(+106.08)TGLVDEAIDTK.S	52.89	-2.9	6.998.650	2	19.60	1.24E8	M1 & M5: Aha+5Hex:1000
50 vs 50	R.LANVM(+106.08)M(+106.08)GPYR.Q	42.04	-2.4	6.823.706	2	17.16	6.42E7	M5 & M6 :Aha+5Hex:1000
	R.LANVM(+106.08)M(+106.08) GPYRQDLLAK.C	53.55	-1.3	10.165.645	2	19.59	2.94E7	M5 & M6: Aha+5Hex:1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVF SDTTTPIK.L	81.20	-1.7	8.574.214	3	22.89	4.06E7	M1: Aha+5Hex:1000
	R.ILLRNPGNQAAYEHFETM(+106.08)K.N	54.08	-0.6	11.696.080	2	19.04	3.36E7	M18: Aha+5Hex:1000
	R.NPGNQAAYEHFETM(+106.08)K.N	90.97	-4.8	9.219.271	2	16.62	2.89E6	M14: Aha+5Hex:1000
	K.M(+106.08)TGLVDEAIDTK.S	57.16	-1.3	4.669.128	3	19.47	3.17E8	M1: Aha+5Hex:1000
100 e coli	K.HM(+106.08)LGEISALTSK.L	37.75	1.9	4.649.255	3	19.37	2.78E6	M2: Aha+5Hex:1000
	R.GLVAEGHRLANVM(+106.08)M(+106.08) GPYRQDLLAK.C	54.44	1.8	9.511.917	3	19.94	4.57E6	M13 & M14: Aha+5Hex:1000
	R.LANVM(+106.08)M(+106.08)GPYR.Q	59.50	-0.3	6.823.705	2	17.21	3.73E8	M5 & M6: Aha+5Hex:1000
	R.LANVM(+106.08)M(+15.99)GPYR.Q	40.55	0.6	6.373.275	2	16.21	2.35E6	M5: Aha+5Hex:14.02, M6: Ox.(M):14.02
	R.LANVM(+106.08)M(+106.08) GPYRQDLLAK.C	67.68	1.2	10.165.648	2	19.68	1.55E8	M5 & M6: Aha+5Hex:1000
	R.LANVM(+106.08)M(+15.99) GPYRQDLLAK.C	39.64	2.7	6.480.175	3	19.37	8.3E6	M5: Aha+5Hex:0, M6: Ox. (M):0
	K.ARM(+106.08)QEAM(+106.08)TQEVSDVF SDTTTPIK.L	88.75	2.4	9.331.359	3	21.72	3.02E6	M3 & M7: Aha+5Hex:1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVF SDTTTPIK.L	94.39	1.3	12.856.294	2	22.82	2.14E8	M1 & M5: Aha+5Hex:1000
	R.M(+106.08)QEAM(+15.99)TQEVSDVF SDTTTPIK.L	76.77	1.6	12.405.861	2	22.72	6.55E6	M1: Aha+5Hex:16.51, M5: Ox.(M):16.51
	R.ILLRNPGNQAAYEHFETM(+106.08)K.N	71.70	1.3	11.696.077	2	19.06	3.49E7	M18: Aha+5Hex:1000
	R.NPGNQAAYEHFETM(+106.08)K.N	89.64	-1.1	9.219.285	2	16.48	3.84E7	M14: Aha+5Hex:1000
	P.GNQAAYEHFETM(+106.08)K.N	63.80	-0.1	8.163.816	2	16.07	3.73E6	M12: Aha+5Hex:1000
	K.M(+106.08)TGLVDEAIDTK.S	64.37	1.0	4.669.128	3	19.47	7.26E8	M1: Aha+5Hex:1000

Table S2. Aha-containing Vin peptides retrieved on Dde-alkyne-agarose from lysate mixtures of Aha-*E. coli* and unlabeled BMDCs.

Aha-*E.coli* (µg): BMDC (µg)	Peptide	-10lgP	ppm	m/z	z	RT (min)	Area	AScore
1:99	K.M(+50.06)TGLVDEAIDTK.S	52.74	-3.9	4.482.378	3	17.73	5.17E5	M1:Aha-Dde:1000
10:90	K.M(+50.06)TGLVDEAIDTK.S	59.12	-0.9	4.482.379	3	17.71	2.13E7	M1:Aha-Dde:1000
	R.M(+50.06)QEAM(-4.99)TQEVSDVF SDTTTPIK.L	58.77	0.5	8.017.245	3	23.40	9.01E6	M1:Aha-Dde:0, M5:Aha:0
50:50	R.M(+50.06)QEAM(-4.99)TQEVSDVF SDTTTPIK.L	91.01	0.7	8.017.246	3	23.41	2.58E7	M1:Aha-Dde:19.86, M5:Aha:19.86
	R.M(+50.06)QEAM(+50.06)TQEVSDVF SDTTTPIK.L	45.29	0.9	8.200.736	3	19.97	1.64E6	M1 & M5:Aha-Dde:1000
	R.NPGNQAAYEHFETM(+50.06)K.N	33.12	-1.7	8.939.170	2	15.75	1.1E6	M14:Aha-Dde:1000
	K.M(+50.06)TGLVDEAIDTK.S	57.39	-2.3	4.482.372	3	17.72	7.65E7	M1:Aha-Dde:1000
100:0	R.M(+50.06)QEAM(-4.99)TQEVSDVF SDTTTPIK.L	81.21	0.5	8.017.245	3	23.41	7.36E7	M1:Aha-Dde:22.37, M5:Aha:22.37
	R.M(+50.06)QEAM(+50.06)TQEVSDVF SDTTTPIK.L	69.19	1.9	8.200.745	3	19.95	5.4E6	M1 & M5:Aha-Dde:1000
	R.M(+50.06)QEAM(+15.99)TQEVSDVF SDTTTPIK.L	61.64	1.0	8.087.186	3	21.42	1.85E6	M1:Aha-Dde:0, M5:Ox.(M):0
	R.NPGNQAAYEHFETM(+50.06)K.N	41.52	-1.4	8.939.172	2	15.76	4.2E6	M14:Aha-Dde:1000
	K.M(+50.06)TGLVDEAIDTK.S	61.48	-1.5	6.718.528	2	17.74	1.4E8	M1:Aha-Dde:1000
	K.M(+50.06)TGLVDEAIDTKSLLDA SEEAIKK.D	32.02	1.4	8.764.703	3	24.20	7.88E5	M1:Aha-Dde:1000

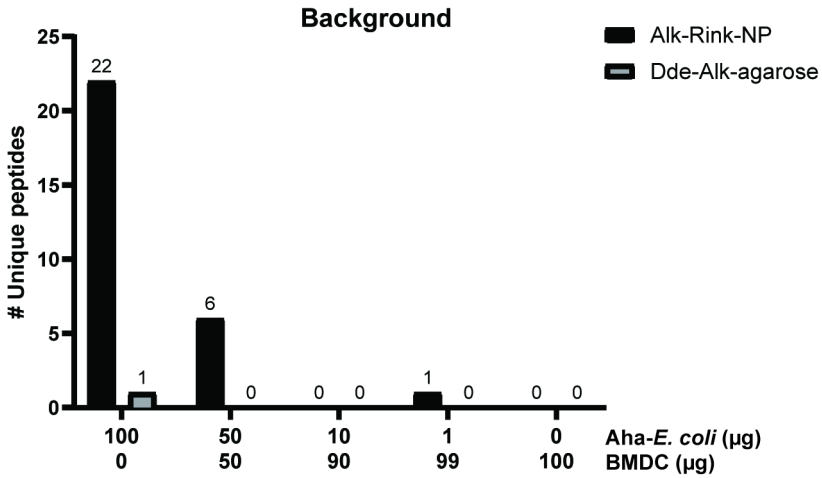


Figure S4. Amount of unmodified peptides identified after enrichment by resin 3 or Dde-Alkyne-agarose from complex mixtures of BMDCs and Aha-labelled *E. coli* lysate. The amount of confident (FDR < 1%) unmodified (background) peptides enriched on either resin **3** (black bars) or Dde-Alk-agarose (grey bars).

Table S3. Retrieved Vin peptides on resin 3 after spiking different amounts of Aha-Vin in BMDC lysate.

Sample	Peptide	-10lgP	ppm	z	RT (min)	Area	AScore
1 ng 1	K.M(+106.08)TGLVDEAIDTK.S	66.38	0.6	2	21.31	1.82E6	M1: 1000
1 ng 2	K.M(+106.08)TGLVDEAIDTK.S	45.39	2.5	2	21.19	0	M1: 1000
1 ng 3	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	65.10	-0.8	3	24.02	0	M1: 1000 M5: 1000
	K.M(+106.08)TGLVDEAIDTK.S	69.18	-1.4	2	21.57	1.57E6	M1: 1000
10 ng 1	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	52.94	0.4	3	23.61	4.03E5	M1: 1000 M5: 1000
10 ng 2	K.M(+106.08)TGLVDEAIDTK.S	52.89	0.4	2	21.09	3.74E5	M1: 1000
10 ng 3	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	69.33	-0.2	3	23.75	9.38E5	M1: 1000 M5: 1000
	K.M(+106.08)TGLVDEAIDTK.S	64.65	-0.3	2	21.00	3.45E6	M1: 1000
100 ng 1	R.LANVM(+106.08)M(+106.08)GPYR.Q	36.69	2.1	2	19.48	3.29E6	M5: 1000 M6: 1000
	R.LANVM(+106.08)M(+106.08)GPYRQDLLAK.C	41.79	0.8	3	23.16	2.88E6	M5: 1000 M6: 1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	69.42	-0.2	3	23.99	6.44E6	M1: 1000 M5: 1000
	R.M(+106.08)QEAM(+15.99)TQEVSDVFSDTTTPIK.L	53.84	-0.2	3	23.84	0	M1: 11.12 M5: 11.12
	R.NPGNQAAYEHFETM(+106.08)K.N	31.97	0.4	3	21.36	2.07E6	M14: 1000
	K.M(+106.08)TGLVDEAIDTK.S	67.92	-0.2	2	21.60	3.11E7	M1: 1000
100 ng_2	R.LANVM(+106.08)M(+106.08)GPYR.Q	35.53	1.2	2	18.50	2.05E6	M5: 1000 M6: 1000
	R.LANVM(+106.08)M(+106.08)GPYRQDLLAK.C	50.51	-0.1	3	22.21	6.66E6	M5: 1000 M6: 1000
	A.NVM(+106.08)M(+106.08)GPYR.Q	37.82	-1.6	2	16.16	3.85E5	M3: 1000 M4: 1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	83.20	-0.2	3	23.83	6.44E6	M1: 1000 M5: 1000
	R.NPGNQAAYEHFETM(+106.08)K.N	29.95	1.0	2	20.54	2.55E5	M14: 1000
	K.M(+106.08)TGLVDEAIDTK.S	75.60	0.8	2	21.19	2.42E7	M1: 1000
100 ng 3	R.LANVM(+106.08)M(+106.08)GPYR.Q	44.81	1.5	2	16.51	1.86E7	M5: 1000 M6: 1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	45.53	1.0	3	21.80	2.19E6	M1: 1000 M5: 1000
	K.M(+106.08)TGLVDEAIDTK.S	65.53	1.5	2	18.45	1.08E7	M1: 1000
1 µg 1	K.HM(-4.99)LGEISALTSK.L	62.95	-8.6	2	27.44	0	M2: 1000
	R.LANVM(+15.99)M(+106.08)GPYR.Q	60.21	3.1	2	17.67	9.32E5	M5: 27.96 M6: 27.96
	R.LANVM(+106.08)M(+106.08)GPYR.Q	55.56	-0.2	2	17.89	1.58E8	M5: 1000 M6: 1000
	R.LANVM(+106.08)M(+15.99)GPYR.Q	45.74	3.1	2	17.67	9.32E5	M5: 10.11 M6: 10.11
	R.LANVM(+106.08)M(+106.08)GPYRQDLLAK.C	69.95	0.9	2	21.65	1.5E8	M5: 100 M6: 1000
	R.LANVM(+106.08)M(+15.99)GPYRQDLLAK.C	62.37	0.2	3	21.52	2.17E7	M5: 17.01 M6: 17.01
	R.LANVM(+15.99)M(+106.08)GPYRQDLLAK.C	55.78	0.2	2	21.43	2.14E7	M5: 10.11 M6: 10.11
	A.NVM(+106.08)M(+106.08)GPYR.Q	36.10	0.1	2	16.17	1.74E7	M3: 1000
	A.NVM(+106.08)M(+106.08)GPYRQDLLAK.C	31.89	2.1	2	20.92	0	M3: 1000
	K.ARMQEAMTQEVSDVFSDTTTPIK.L	59.96	4.5	3	23.70	8.82E5	

	R.M(+106.08)QEAM(+15.99)TQEVSDVF.S	27.86	-0.8	2	22.86	3.75E4	M1: 38.03 M5: 38.03
	R.M(+106.08)QEAM(+15.99)TQEVSDVFSDTTTPIK.L	104.46	0.4	2	23.50	1.82E7	M1: 48.12 M5: 48.12
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	92.64	0.7	2	23.68	1.9E8	M1: 1000 M5: 1000
	R.NPGNQAAYEHFETM(+106.08)K.N	92.44	0.4	2	19.46	8.88E7	M14: 1000
	P.GNQAAYEHFETM(+106.08)K.N	77.64	1.7	2	19.37	2.17E7	M12: 1000
	K.M(+106.08)TGLVDEAIDTK.S	73.02	0.1	2	20.59	5.42E8	M1: 1000
1 µg 2	R.LANVM(+15.99)M(+106.08)GPYR.Q	67.89	1.9	2	18.03	3.83E6	M5: 33.98 M6: 33.98
	R.LANVM(+106.08)M(+106.08)GPYR.Q	60.86	0.0	2	18.34	7.15E7	M5: 1000 M6: 1000
	R.LANVM(+106.08)M(+15.99)GPYR.Q	46.12	1.9	2	18.03	3.83E6	M5: 9.34, M6: 9.34
	R.LANVM(+106.08)M(+106.08)GPYRQDLLAK.C	58.66	-0.3	2	22.35	6.04E7	M5: 1000 M6: 1000
	R.LANVM(+106.08)M(+15.99)GPYRQDLLAK.C	50.58	0.2	3	22.23	7.53E6	M5: 0 M6: 0
	R.LANVM(+15.99)M(+106.08)GPYRQDLLAK.C	41.92	0.2	3	22.23	7.37E6	M5: 0 M6: 0
	A.NVM(+106.08)M(+106.08)GPYR.Q	33.82	-2.2	2	16.17	6.39E5	M3: 1000 M4: 1000
	R.M(+106.08)QEAM(+15.99)TQEVSDVFSDTTTPIK.L	142.31	0.3	2	23.63	5.78E6	M1: 33.81 M5: 33.81
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	111.74	-0.2	2	23.80	5.05E7	M1: 1000 M5: 1000
	R.M(+106.08)QEAMTQEVSDVFSDTTTPIK.L	79.08	0.5	3	24.30	5.85E4	M1: 25.11
	R.NPGNQAAYEHFETM(+106.08)K.N	104.85	0.2	2	20.50	1.18E8	M14: 1000
	P.GNQAAYEHFETM(+106.08)K.N	72.22	1.6	2	20.19	4.53E6	M12: 1000
	K.M(+106.08)TGLVDEAIDTK.S	86.40	0.2	2	20.96	3.35E8	M1: 1000
1 µg 3	K.HM(-4.99)LGEISALTSK.L	61.88	-9.6	2	27.45	0	M2: 1000
	R.LANVM(+15.99)M(+106.08)GPYR.Q	79.79	0.2	2	18.09	7.51E6	M5: 17.01 M6: 17.01
	R.LANVM(+106.08)M(+106.08)GPYR.Q	65.67	-0.2	2	18.32	1.22E8	M5: 1000 M6: 1000
	R.LANVM(+106.08)M(+15.99)GPYR.Q	39.19	0.2	2	18.09	7.51E6	M5: 12.28 M6: 12.28
	R.LANVM(+106.08)M(+106.08)GPYRQDLLAK.C	63.56	-0.6	3	22.19	3.36E7	M5: 1000 M6: 1000
	R.LANVM(+106.08)M(+15.99)GPYRQDLLAK.C	50.71	-0.5	2	22.38	8.69E6	M5: 0 M6: 0
	R.LANVM(+15.99)M(+106.08)GPYRQDLLAK.C	27.63	0.2	3	23.17	0	M5: 0 M6: 0
	A.NVM(+106.08)M(+106.08)GPYR.Q	37.76	-1.3	2	16.15	1.16E7	M3: 1000 M4: 1000
	K.ARMQEAMTQEVSDVFSDTTTPIK.L	83.22	2.1	3	23.96	0	
	R.M(+106.08)QEAM(+15.99)TQEVSDVFSDTTTPIK.L	139.62	0.6	2	23.61	1.65E7	M1: 34.30 M5: 34.30
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	107.62	0.1	2	23.80	6.64E7	M1: 1000 M5: 1000
	R.M(+15.99)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	91.25	0.6	2	23.61	3.76E6	M1: 0 M5: 0
	R.NPGNQAAYEHFETM(+106.08)K.N	120.56	0.3	2	20.39	1.3E8	M14: 1000
	P.GNQAAYEHFETM(+106.08)K.N	89.92	0.2	2	20.34	4.2E7	M12: 1000
	K.M(+106.08)TGLVDEAIDTK.S	91.56	0.1	2	21.13	5.15E8	M1: 1000

Table S4. Retrieved Aha-containing Vin peptides on resin 3 from Aha-Vin-pulsed BMDCs.

Sample	Peptide	-10lgP	ppm	z	RT (min)	Area	AScore
1	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	68.31	0.7	3	23.10	6.86E5	M1: 1000 M5: 1000
2	K.M(+106.08)TGLVDEAIDTK.S	54.66	0.5	2	21.21	7.83E6	M1: 1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	36.74	1.0	3	23.57	2.09E6	M1: 1000 M5: 1000
3	K.M(+106.08)TGLVDEAIDTK.S	53.01	1.1	2	21.61	6.7E6	M1: 1000
	R.M(+106.08)QEAM(+106.08)TQEVSDVFSDTTTPIK.L	40.69	-0.6	3	23.64	1.33E6	M1: 1000 M5: 1000

References

1 Cravatt, B. F., Simon, G. M. & Yates Iii, J. R. The biological impact of mass-spectrometry-based proteomics. *Nature* **450**, 991-1000, (2007).

2 Walther, T. C. & Mann, M. Mass spectrometry–based proteomics in cell biology. *Journal of Cell Biology* **190**, 491-500, (2010).

3 Ludewig, B., Junt, T., Hengartner, H. & Zinkernagel, R. M. Dendritic cells in autoimmune diseases. *Current Opinion in Immunology* **13**, 657-662, (2001).

4 Purbhoo, M. A., Irvine, D. J., Huppa, J. B. & Davis, M. M. T cell killing does not require the formation of a stable mature immunological synapse. *Nature Immunology* **5**, 524-530, (2004).

5 van Kasteren, S. I. & Overkleeft, H. S. Endo-lysosomal proteases in antigen presentation. *Current Opinion in Chemical Biology* **23**, 8-15, (2014).

6 Delamarre, L., Pack, M., Chang, H., Mellman, I. & Trombetta, E. S. Differential lysosomal proteolysis in antigen-presenting cells determines antigen fate. *Science* **307**, 1630-1634, (2005).

7 Delamarre, L., Couture, R., Mellman, I. & Trombetta, E. S. Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis. *The Journal of Experimental Medicine* **203**, 2049-2055, (2006).

8 Manoury, B., Mazzeo, D., Fugger, L., Viner, N., Ponsford, M., Streeter, H., Mazza, G., Wraith, D. C. & Watts, C. Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. *Nature Immunology* **3**, 169-174, (2002).

9 Moss, C. X., Villadangos, J. A. & Watts, C. Destructive potential of the aspartyl protease cathepsin D in MHC class II-restricted antigen processing. *European Journal of Immunology* **35**, 3442-3451, (2005).

10 Hartman, I. Z., Kim, A., Cotter, R. J., Walter, K., Dalai, S. K., Boronina, T., Griffith, W., Lanar, D. E., Schwenk, R., Krzych, U., Cole, R. N. & Sadegh-Nasseri, S. A reductionist cell-free major histocompatibility complex class II antigen processing system identifies immunodominant epitopes. *Nature Medicine* **16**, 1333-1340, (2010).

11 van Kasteren, S., Overkleeft, H., Ovaa, H. & Jacques, N. Chemical biology of antigen presentation by MHC molecules. *Current Opinion in Immunology* **26**, 21-31, (2014).

12 van Leeuwen, T., Araman, C., Pieper Pournara, L., Kampstra, A. S. B., Bakkum, T., Marqvorsen, M. H. S., Nascimento, C. R., Groenewold, G. J. M., van der Wulp, W., Camps, M. G. M., Janssen, G. M. C., van Veelen, P. A., van Westen, G. J. P., Janssen, A. P. A., Florea, B. I., Overkleeft, H. S., Ossendorp, F. A., Toes, R. E. M. & van Kasteren, S. I. Bioorthogonal protein labelling enables the study of antigen processing of citrullinated and carbamylated auto-antigens. *The Royal Society of Chemistry - Chemical Biology* **2**, 855-862, (2021).

13 Bertozzi, C. R. A Decade of bioorthogonal chemistry. *Accounts of Chemical Research* **44**, 651-653, (2011).

14 Wang, Q., Chan, T. R., Hilgraf, R., Fokin, V. V., Sharpless, K. B. & Finn, M. G. Bioconjugation by copper(I)-catalyzed azide-alkyne [3 + 2] cycloaddition. *Journal of the American Chemical Society* **125**, 3192-3193, (2003).

15 Kiick, K. L. & Tirrell, D. A. Protein engineering by in vivo incorporation of non-natural amino acids: control of incorporation of methionine analogues by methionyl-tRNA synthetase. *Tetrahedron* **56**, 9487-9493, (2000).

16 van Hest, J. C. M., Kiick, K. L. & Tirrell, D. A. Efficient incorporation of unsaturated methionine analogues into proteins in vivo. *Journal of the American Chemical Society* **122**, 1282-1288, (2000).

17 Kiick, K. L., Saxon, E., Tirrell, D. A. & Bertozzi, C. R. Incorporation of azides into recombinant proteins for chemoselective modification by the Staudinger ligation. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 19-24, (2002).

18 Kiick, K. L., Weberskirch, R. & Tirrell, D. A. Identification of an expanded set of translationally active methionine analogues in Escherichia coli. *Federation of European Biochemical Societies - Letters* **502**, 25-30, (2001).

19 van Hest, J. C., Kiick, K. L. & Tirrell, D. A. Efficient incorporation of unsaturated methionine analogues into proteins in vivo. *Journal of the American Chemical Society* **122**, 1282-1288, (2000).

20 Lehner, F., Kudlinzki, D., Richter, C., Müller-Werkmeister, H. M., Eberl, K. B., Bredenbeck, J., Schwalbe, H. & Silvers, R. Impact of azidohomoalanine incorporation on protein structure and ligand binding. *ChemBioChem* **18**, 2340-2350, (2017).

21 Bakkum, T., van Leeuwen, T., Sarris, A. J. C., van Elsland, D. M., Poulcharidis, D., Overkleeft, H. S. & van Kasteren, S. I. Quantification of bioorthogonal stability in immune phagocytes using flow cytometry reveals rapid degradation of strained alkynes. *Journal of the American Chemical Society - Chemical Biology* **13**, 1173 - 1179, (2018).

22 Holmberg, A., Blomstergren, A., Nord, O., Lukacs, M., Lundeberg, J. & Uhlén, M. The biotin-streptavidin interaction can be reversibly broken using water at elevated temperatures. *Electrophoresis* **26**, 501-510, (2005).

23 Landi, F., Johansson, C. M., Campopiano, D. J. & Hulme, A. N. Synthesis and application of a new cleavable linker for "click"-based affinity chromatography. *Organic & Biomolecular Chemistry* **8**, 56-59, (2010).

24 Hahne, H., Sobotzki, N., Nyberg, T., Helm, D., Borodkin, V. S., van Aalten, D. M. F., Agnew, B. & Kuster, B. Proteome wide purification and identification of O-GlcNAc-modified proteins using click chemistry and mass spectrometry. *Journal of Proteome Research* **12**, 927-936, (2013).

25 Sibbersen, C., Lykke, L., Gregersen, N., Jørgensen, K. A. & Johannsen, M. A cleavable azide resin for direct click chemistry mediated enrichment of alkyne-labeled proteins. *Chemical Communications* **50**, 12098-12100, (2014).

26 Nessen, M. A., Kramer, G., Back, J., Baskin, J. M., Smeenk, L. E. J., de Koning, L. J., van Maarseveen, J. H., de Jong, L., Bertozzi, C. R., Hiemstra, H. & de Koster, C. G. Selective enrichment of azide-containing peptides from complex mixtures. *Journal of Proteome Research* **8**, 3702-3711, (2009).

27 Wang, S., Xie, W., Zhang, X., Zou, X. & Zhang, Y. Disulfide- and terminal alkyne-functionalized magnetic silica particles for enrichment of azido glycopeptides. *Chemical Communications* **48**, 5907-5909, (2012).

28 Schneider, E. M., Zeltner, M., Zlateski, V., Grass, R. N. & Stark, W. J. Click and release: fluoride cleavable linker for mild bioorthogonal separation. *Chemical Communications* **52**, 938-941, (2016).

29 Geurink, P. P., Florea, B. I., Li, N., Witte, M. D., Verasdonck, J., Kuo, C.-L., van der Marel, G. A. & Overkleeft, H. S. A cleavable linker based on the levulinoyl ester for activity-based protein profiling. *Angewandte Chemie International Edition* **49**, 6802-6805, (2010).

30 Gartner, C. A., Elias, J. E., Bakalarski, C. E. & Gygi, S. P. Catch-and-release reagents for broadscale quantitative proteomics analyses. *Journal of Proteome Research* **6**, 1482-1491, (2007).

31 Hou, Z., Han, X., Wang, Z., Ghazanfar, S., Yang, J. & Liu, H. A terminal alkyne and disulfide functionalized agarose resin specifically enriches azidohomoalanine labeled nascent proteins. *Journal of Chromatography B* **1165**, 122527, (2021).

32 Verhelst, S. H. L., Fonović, M. & Bogyo, M. A mild chemically cleavable linker system for functional proteomic applications. *Angewandte Chemie International Edition* **46**, 1284-1286, (2007).

33 Kim, H. Y., Tallman, K. A., Liebler, D. C. & Porter, N. A. An azido-biotin reagent for use in the isolation of protein adducts of lipid-derived electrophiles by streptavidin catch and photorelease. *Molecular Cell Proteomics* **8**, 2080-2089, (2009).

34 Szychowski, J., Mahdavi, A., Hodas, J. J. L., Bagert, J. D., Ngo, J. T., Landgraf, P., Dieterich, D. C., Schuman, E. M. & Tirrell, D. A. Cleavable biotin probes for labeling of biomolecules via azide−alkyne cycloaddition. *Journal of the American Chemical Society* **132**, 18351-18360, (2010).

35 Merrifield, R. B. Solid Phase Peptide Synthesis. I. The synthesis of a tetrapeptide. *Journal of the American Chemical Society* **85**, 2149-2154, (1963).

36 Bayer, E., Hemmasi, B., Albert, K., Rapp, W. & Dengler, M. Immobilized polyoxyethylene, a new support for peptide synthesis. *Peptides, Structure and Function*, 87-90, (1983).

37 García-Martín, F., Quintanar-Audelo, M., García-Ramos, Y., Cruz, L. J., Gravel, C., Furic, R., Côté, S., Tulla-Puche, J. & Albericio, F. ChemMatrix, a poly(ethylene glycol)-based support for the solid-phase synthesis of complex peptides. *Journal of Combinatorial Chemistry* **8**, 213-220, (2006).

38 Emoto, K., Van Alstine, J. M. & Harris, J. M. Stability of poly(ethylene glycol) graft coatings. *Langmuir* **14**, 2722-2729, (1998).

39 Bernatowicz, M. S., Daniels, S. B. & Köster, H. A comparison of acid labile linkage agents for the synthesis of peptide C-terminal amides. *Tetrahedron Letters* **30**, 4645-4648, (1989).

40 Wang, S.-S. p-Alkoxybenzyl alcohol resin and p-alkoxybenzyloxycarbonylhydrazide resin for solid phase synthesis of protected peptide fragments. *Journal of the American Chemical Society* **95**, 1328-1333, (1973).

41 Reindl, M., Linington, C., Brehm, U., Egg, R., Dilitz, E., Deisenhammer, F., Poewe, W. & Berger, T. Antibodies against the myelin oligodendrocyte glycoprotein and the myelin basic protein in multiple sclerosis and other neurological diseases: a comparative study. *Brain* **122**, 2047-2056, (1999).

42 Araman, C., van Gent, M. E., Meeuwenoord, N. J., Heijmans, N., Marqvorsen, M. H. S., Doelman, W., Faber, B. W., t Hart, B. A. & Van Kasteren, S. I. Amyloid-like behavior of site-specifically citrullinated myelin oligodendrocyte protein (MOG) peptide fragments inside EBV-infected B-cells influences their cytotoxicity and autoimmunogenicity. *Biochemistry* **58**, 763-775, (2019).

43 van Heemst, J., Jansen, D. T., Polydorides, S., Moustakas, A. K., Bax, M., Feitsma, A. L., Bontrop-Elferink, D. G., Baarse, M., van der Woude, D., Wolbink, G. J., Rispens, T., Koning, F., de Vries, R. R., Papadopoulos, G. K., Archontis, G., Huizinga, T. W. & Toes, R. E. Crossreactivity to vinculin and microbes provides a molecular basis for HLA-based protection against rheumatoid arthritis. *Nature Communications* **6**, 6681, (2015).

44 Dubendorf, J. W. & Studier, F. W. Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor. *Journal of Molecular Biology* **219**, 45-59, (1991).

45 Siepen, J. A., Keevil, E.-J., Knight, D. & Hubbard, S. J. Prediction of missed cleavage sites in tryptic peptides aids protein identification in proteomics. *Journal of Proteome Research* **6**, 399-408, (2007).

46 Baka, Z., György, B., Géher, P., Buzás, E. I., Falus, A. & Nagy, G. Citrullination under physiological and pathological conditions. *Joint Bone Spine* **79**, 431-436, (2012).

47 Reily, C., Stewart, T. J., Renfrow, M. B. & Novak, J. Glycosylation in health and disease. *Nature Reviews Nephrology* **15**, 346-366, (2019).

48 Rappsilber, J., Mann, M. & Ishihama, Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature Protocols* **2**, 1896-1906, (2007).

49 Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685, (1970).