The advantages and disadvantages of bioorthogonal proteins

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Chapter 3

T cell activation by bioorthogonally labelled tetanus toxin C fragment

Abstract

In this Chapter the bioorthogonal expression and initial immunological evaluation of the antigen tetanus toxin C fragment was assessed. A mutant library of the protein was made that contained a bioorthogonal amino acid at every position in the known MHC-II restricted epitopes. The effect of these mutations on antigen processing and T cell activation was analyzed.

Introduction

Antigen presentation plays a vital role in the T cell immune response. To induce this response, proteins are cleaved into peptide fragments, which are presented on either the major histocompatibility complex (MHC) class I or MHC class II [1, 2]. Peptides presented via MHC class II are presented to CD4+ T helper cells [3]. These particular T cells are involved in immunity and inflammation and, depending on their cytokine production, can be classified into different subtypes [4, 5], such as Th1, Th2, Treg and Th17. Their activation is therefore pivotal for many branches of the adaptive immune response [6-11].

To obtain an MHC class II antigen induced T cell responses, peptides arising from exogenous proteins need to be internalized by professional antigen presenting cells (APCs) – expressing the MHC class II molecules – via for instance endocytosis [12]. After this, proteins are enclosed in endosomes and processed into peptide fragments, which will encounter the MHC class II molecules. These are concurrently transported to the MHC class II loading compartment (MIIC) (Figure 1A - step 1 and 2), where the peptides can be loaded onto the open-ended groove of MHC class II molecules. MHC class II molecules bind a core epitope of nine amino acid residues – with strong anchor points at p1, p4, p6 and p9 –, but can hold peptides of ~13-25 residues [13-15]. Next, the complex is transported to the plasma membrane (Figure 1A - step 3), where the epitope is recognized by a T cell receptor with a possible antigenic reaction of the T cell as the result [2, 16].

As described above, antigens are taken up by a professional APC, processed and subsequently loaded onto a newly synthesized MHC class II molecule [17]. This, however, is the route that the majority of the proteins will follow [18]. In some cases, the antigenic protein can bind directly – or after partially unfolding – to these molecules [18, 19]. The mainly intact proteins will not bind to the newly synthesized MHC class II molecules, but will bind instead to the ones recycled from the cell surface and/or from instable formed complexes [18, 20, 21].

Some of the particulars of MHC class II antigen processing remain unknown. For example, it is known that peptide cleavage plays an important role in T helper cell activation, but the order of cleavage/binding is still under debate: does the protein bind

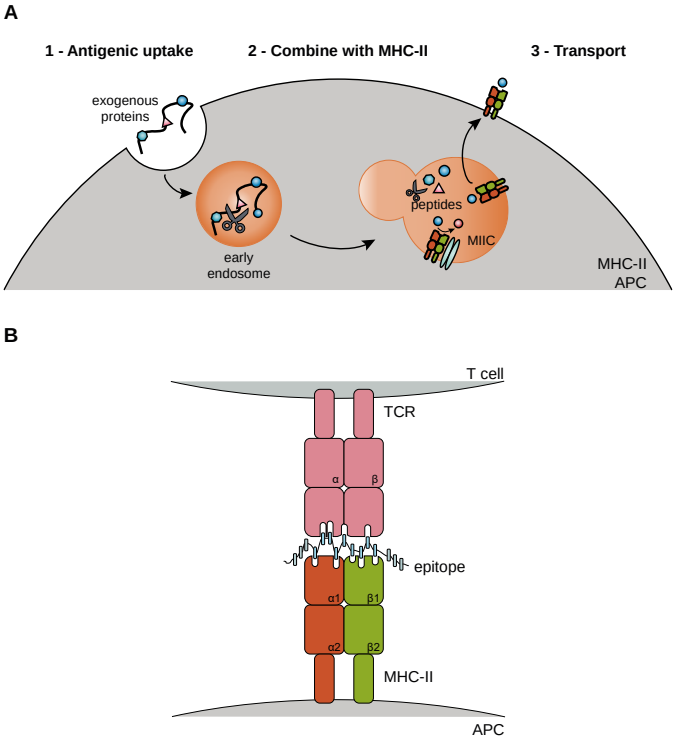

Figure 1. Schematic overview of MHC class II antigen presentation. A) Schematic overview of the uptake of an antigen, which is cleaved by peptides before being loaded in the MIIC complex on MHC class II molecules. The combined MHC class II peptide complex is transported to the plasma membrane. B) At the plasma membrane a T cell can recognize the epitope and will become activated.

the MHC class II molecule first and is trimmed afterwards? Or is the protein processed first and the liberated peptides then loaded (as depicted in Figure 1A) [22]. A recent study of Roche *et al.* shows that the order of processing and loading may be dependent on the activation-status of the APC [23]. A second parameter that remains enigmatic is that the *in vitro* binding affinity of both the peptide for the MHC class II, and that between the T-cell receptor and the peptide-MHC-II complex does not correlate to immunogenicity [24]. This suggests that other factors – either following T cell recognition or that take place during peptide loading are essential determinants for immunogenicity. For instance, the open-ended nature of the peptide binding groove of MHC class II makes it possible that overhanging amino acids in the flanking region of the core epitope are involved, and that instead of a single peptide epitope an array of peptides differing in the length/nature of the overhang are presented [25].

To study MHC class II antigen processing and presentation, model proteins are often used. One such model protein is tetanus toxin C fragment (TTCF; residues 865-1315 of tetanus toxin) [26, 27], which has been earlier used to gain knowledge about the adaptive immune response [25, 27]. Presentation of this protein only requires limited processing by asparagine-specific endopeptidase (AEP) [19, 28, 29]. Antigen presentation can be traced using fluorescent antigen variants to correlate a visual image to the overall immune outcome. However, it has been shown that this strongly reduces antigen presentation for ovalbumin [30].

To prevent this problem, it was postulated that a small ligation handle – such as an azide or an alkyne moiety – inside the TTCF epitope would result in presentation of an antigen closer in structure to the wild type one, yet allowing detection of the antigen using click chemistry (see **Chapter 1**) – a postulate that was investigated in research described in this Chapter. For this, a strategy developed by Tirrell and co-workers was selected, where a model protein is expressed with non-canonical amino acids, allowing for i.e. the bioorthogonal non-canonical amino acid tagging strategy (BONCAT) [31]. In this method, an amino acid auxotrophic bacterial strain is used for protein expression. Furthermore, the canonical methionine is removed from culture media and supplemented with the isosteric azidohomoalanine [32] instead using the natural methionine tRNA synthetase for incorporation [33].

In this Chapter, it is tested whether or not it is possible to create a protein in which an amino acid in the epitope of interest is replaced with an L-azidohomoalanine (L-Aha) residue to allow the tracking of the epitope all the way from uptake to presentation on MHC-II. Furthermore, it is investigated if these mutated proteins will still be processed in an APC [30] and are able to induce T cell activation. This Chapter will conclude with general observations on the obtained results and will end with recommendations for future experiments.

Results

The aim of this Chapter is to follow antigen processing from uptake to MHC class II presentation and subsequent T cell activation using a family of bioorthogonal variants

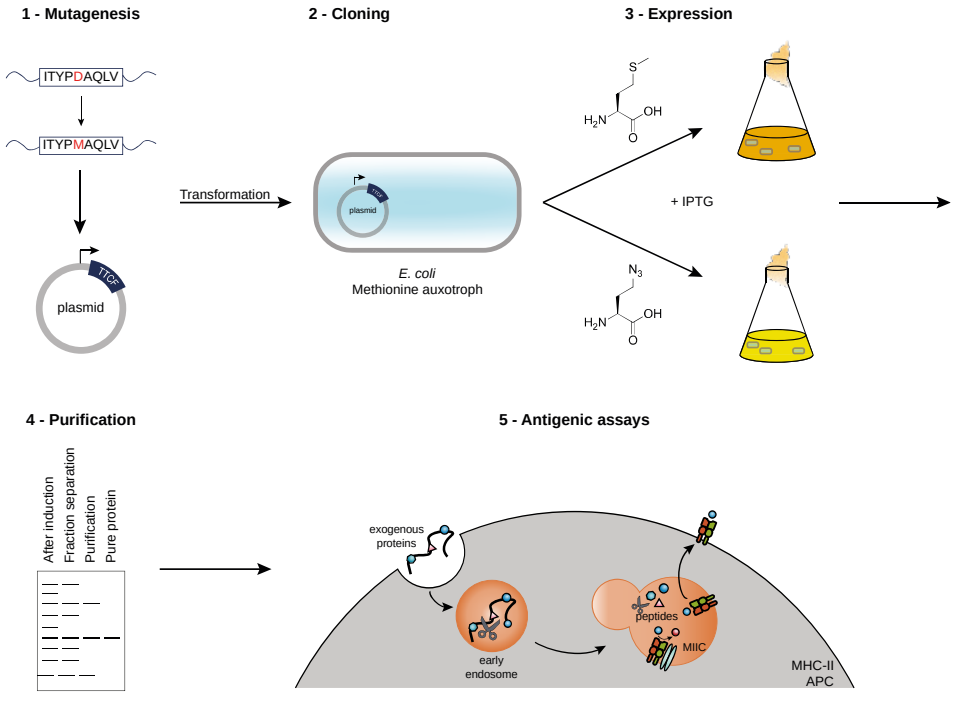

Figure 2. Schematic overview of the workflow. Experimental setup describing the mutagenesis of *TTCF* of which the resulting plasmid was transformed into the *E. coli* auxotroph B834(DE3). The protein was expressed in either LB medium or in SelenoMet medium to incorporate L-Met or L-Aha respectively. The protein was purified, before using the resulting TTCF (mutant) proteins in antigen presentation assays.

of TTCF (Figure 2), where each position in the predicted core epitope was mutated to methionine (L-Met) to allow expression with L-azidohomoalanine at these sites.

2.1 Epitope mutation and protein expression

Multiple T cell hybridomas, specific for different epitopes of the model protein TTCF, have been reported by the group of C. Watts [27]. In this Chapter the 2F2 hybridoma was used [27], which recognises a peptide spanning the residues 900 - 916 of tetanus toxin (SGFNSSVITYPDAQLVP; the predicted core epitope is underlined) (PDB: 1A8D; Figure 3A).

Core epitope mutants were therefore made from the previously reported pEH101 plasmid containing *TTCF* C-terminally ligated to a ten-histidine (His)-tag, kindly provided by C. Watts [34]. This resulted in the generation of nine mutant genes, each of

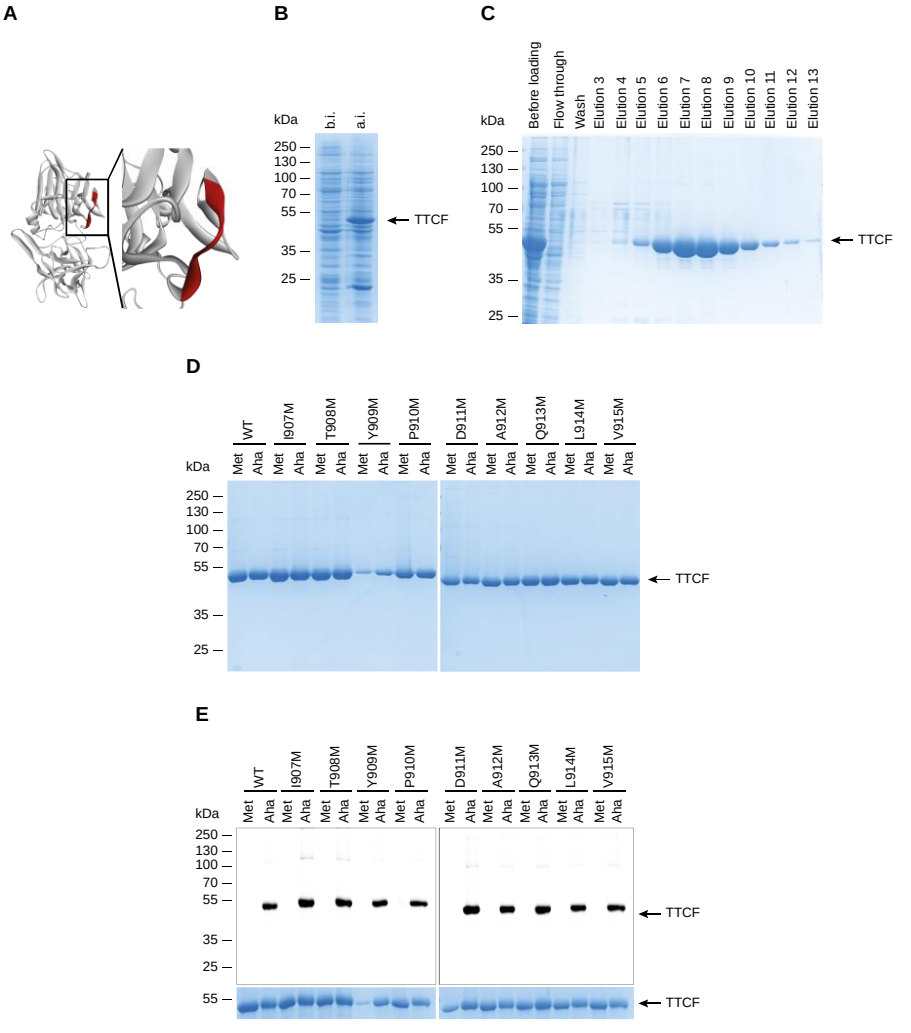

Figure 3. The use of the 2F2 epitope and its mutants. A) Crystal structure of TTCF (PDB: 1A8D) with in red the position of the epitope in the protein; visualized in Discovery Studio. B) Representative picture of the before and after induction samples of TTCF Aha. C) Representative picture of the purification of TTCF Aha. D) The purified proteins, all with a purity of >98% as analyzed by SDS-PAGE. E) The resulting azide-containing proteins were ligated to AF647 alkyne (upper panel) with as control the coomassie staining of all the proteins. B-E) Proteins were resolved in a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue G-250.

which was cloned into the multiple cloning site of vector pET16b and transformed into *E. coli* B834(DE3) [35]. Subsequent expression in LB medium gave overexpression of a protein as revealed by the emergence of a band at 53 kDa after SDS-PAGE. This protein

Table 1: List of expressed proteins

Mutation	Met incorporation	Aha incorporation
-	ITAPDAQLV	ITAPDAQLV
I907M	MTAPDAQLV	AhaTAPDAQLV
T908M	IMAPDAQLV	IAhaAPDAQLV
Y909M	ITMPDAQLV	ITAhaPDAQLV
P910M	ITAMDAQLV	ITAAhaDAQLV
D911M	ITAPMAQLV	ITAPAhaAQLV
A912M	ITAPDMQLV	ITAPDAhaQLV
Q913M	ITAPDAMLV	ITAPDAAhaLV
L914M	ITAPDAQMV	ITAPDAQAhaV
V915M	ITAPDAQLM	ITAPDAQLAha

proved present in the after induction sample (Figure 3B), and could be purified using the 10His-tag with Ni^{2+} affinity chromatography [34] (Figure 3C).

Expression with the bioorthogonal amino acid L-Aha was next attempted. For this the cells were grown in SelenoMet-medium that was depleted of L-Met and augmented with L-Aha, thus conditions that are expected to yield proteins in which all (or most) Met-residues are expected to be replaced by this azide-containing counterpart [36]. The cells were incubated overnight after IPTG induction and all mutants showed – as displayed by SDS-PAGE analysis – the appearance of a robust band for overexpression at the predicted 53 kDa. Purification as before yielded the protein with >98% purity (Figure 3D) as determined by SDS-PAGE. The thus obtained twenty TTCF and mutant TTCF proteins, containing either eight (wild type) or nine internal (mutants) L-Met or L-Aha residues are summarized in Table 1.

2.2 TTCF-Aha ligations

It was next determined whether these proteins were reactive under a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) also called a Cu(I)-catalyzed Huisgen cycloaddition conditions (ccHc) towards Alexa Fluor 647 (AF647) alkyne. Separation by SDS-PAGE of the thus reacted proteins and subsequent in-gel fluorescence showed that all the azidylated proteins had reacted with AF647 alkyne, whereas their methionine containing counterparts had not (Figure 3E).

2.3 TTCF degradation

Next, processing of the modified TTCF proteins by antigen presenting cells (APCs) was assessed [30]. For this bone marrow derived dendritic cells (BMDCs) were pulsed with the antigens and chased for 0, 1, 4 or 24 hours. Afterwards, the cells were

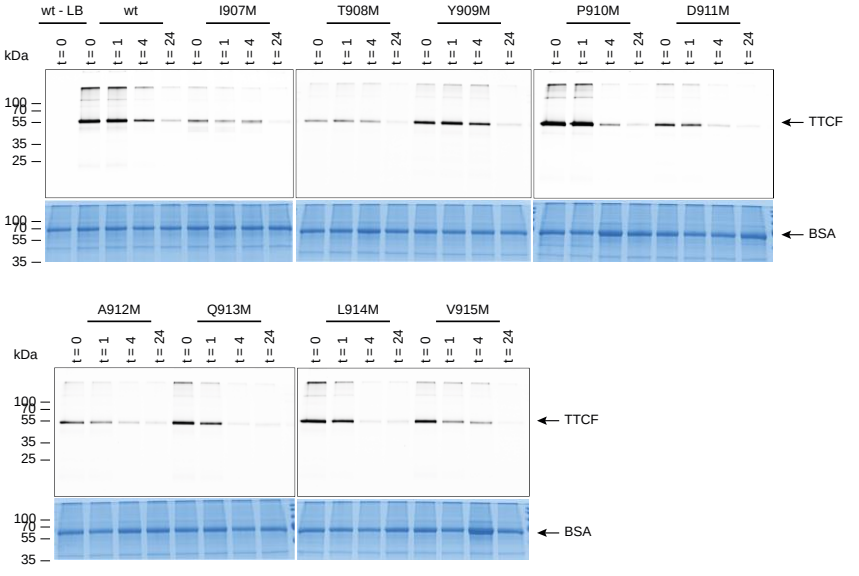

Figure 4. Degradation assay of TTCF by BMDCs. The different azide-containing proteins were used to pulse BMDCs for 1 hour, after which a chase of 0, 1, 4 or 24 hours took place. Cells were lysed together with the wild type protein containing L-Met as negative control, each of the protein samples showed degradation over time as visualized after ligation to AF647 alkyne. Proteins were resolved in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

washed, lysed and subjected to the above mentioned CuAAC prior to SDS-PAGE separation and in-gel fluorescence analysis (Figure 4). From these experiments it appeared that after 4 hours the level of intact azidylated protein was reduced by >25% and >50% after a 24 hours chase (Figure 4). For some of the mutants, a lower uptake was observed. Surprisingly, all the mutants degraded over time with no observation of intermediates, a process also observed by Araman *et al.* [37]. This could either be because the proteins are rapidly degraded after initial cleavage or because the azide handle is reduced over time. The latter was disproved in a previous study by Bakkum *et al.* [38].

Protein degradation within the first hour of pulse is thought to be related to destructive antigen processing [36]. This is associated with rapid release of the chain-derived class II-associated invariant chain peptide (CLIP), whereby the protein can be loaded into the MHC class II molecule on the cell surface or in the early endosome [39, 40]. This process could lead to different epitopes, a process which could lead to

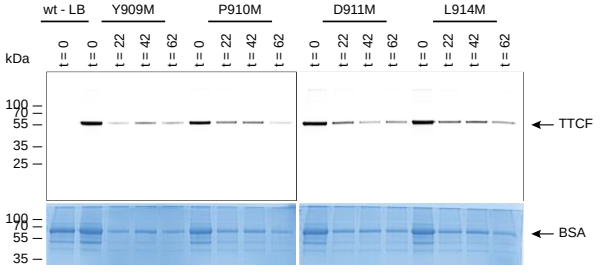

Figure 5. Pulse assay of TTCF to BMDCs. The randomly chosen azide-containing proteins were used to pulse BMDCs for 1 hour. Cells were lysed together with the wild type protein containing L-Met as negative control, none of the samples showed degradation over time as visualized after ligation to AF647 alkyne. Proteins were resolved in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

suboptimal T cell presentation. To analyze the rate of protein degradation during the first hour of antigen processing, the cells were pulsed with selected mutant proteins for 22, 42 and 62 minutes. The cells were washed, lysed and subjected to a ligation mixture containing AF647 alkyne as described for the antigen degradation experiment. The normalized in-gel fluorescence showed no decrease in fluorescent signal at these shorter pulse times (Figure 5).

2.4 T cell activation by Aha-epitopes

It was next determined whether the in-epitope mutations affected activation of the 2F2-hybridoma. BMDCs were therefore pulsed for 4 hours with each of the TTCF proteins (0.1 mg/mL final concentration), washed twice with PBS and incubated with 2F2 overnight. T cell activation – as analyzed via an IL-2 ELISA – showed that mutating each of the amino acid residues individually to methionine in this ITYPDAQLV epitope (as depicted in Table 1) still resulted in T cell activation (Figure 6A). In addition, when this assay was performed using the proteins containing L-Aha residues, IL-2 production was observed (Figure 6B). To more precisely study this phenomenon, presentation efficiency of a minimal synthetic epitope modified with a bioorthogonal group was analyzed. No antigen presentation was observed for this 2F2 epitope.

In order to assess the effect of modification in the peptides with bioorthogonal groups on only the presentation, a library of peptides based on the well-studied OT-II epitope (ISQAVHAAHAEINEAGR (Figure 7A)) of ovalbumin was synthesized. Different

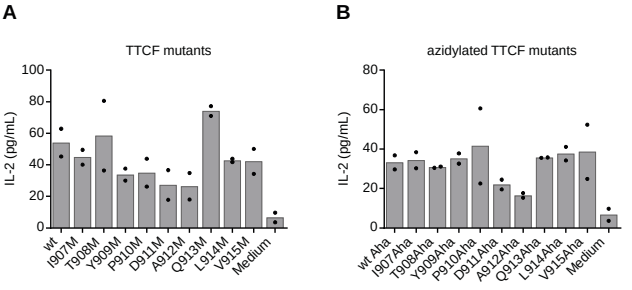

Figure 6. Antigen presentation of TTCF epitope mutants by BMDCs to 2F2 T cell hybridomas. A) BMDCs were pulsed for 4 hours with either wild type TTCF or a mutant in which a point mutation to methionine was made in the epitope. Followed by 20 hours presentation to the T cells (N = 2, n = 3). B) As for A), but containing an azidohomoalanine residue at each of the methionine positions (N = 2, n = 3).

residues of the predicted core epitope – AAHAEINEA – were mutated to L-Aha [41]. The immunogenic properties of the wild type and resulting mutant peptides were evaluated with an antigen presentation assay, using A20 and the Ova MHC-II specific T cell line DO11.10 [42].

In order to obtain antigen presentation, the I-Ad-positive B cell line A20 was pulsed for 4 hours with each of the long ova peptides prior to the addition of the DO11.10 cells for a 20 hour chase period. T cell activation was subsequently analyzed via an IL-2 ELISA assay. This showed that peptides containing mutations at positions 1 and 8 still activated the T cells, whereas antigen presentation assays with mutations at positions 2, 3, 5 and 7 did not (Figure 7C). In line with literature data, mutating position 5 – facing towards the TCR – did not show any response. Both positions capable of inducing T cell responses after mutation are positioned towards the T cell receptor (TCR) [41]. With mutant position 8 giving a higher initial response, it was decided to continue with this peptide for future ligation reactions.

To determine to what extent chemical modifications are allowed within the epitope, it was decided to react the azidylated p8 peptide (ISQAVHAAHAEINAhaAGR) with a biotin-phosphine (ISQAVHAAHAEINbiotinAGR) and the resulting peptide was used for antigen presentation studies (Figure 7B). This showed a decrease of approximately 50% activation as compared to the azidylated mutant peptide p8 (Figure 7D).

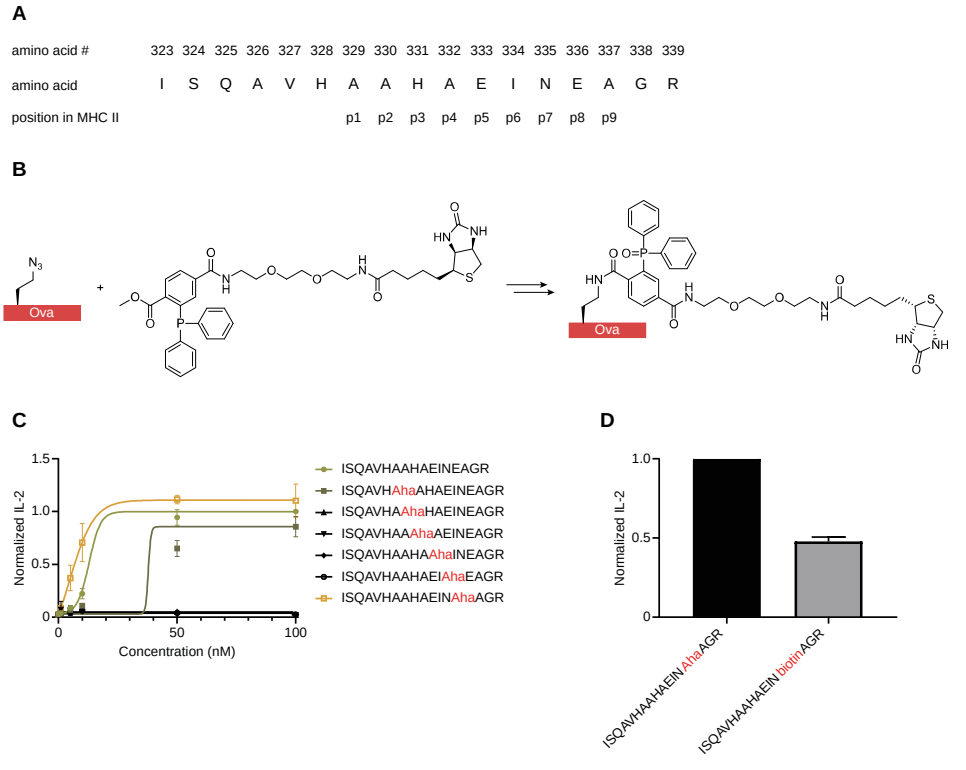

Figure 7. Antigen presentation of Ova peptides by A20 to DO11.10. A) Amino acid sequence of the ovalbumin MHC class II ISQAV long peptide. B) Schematic reaction of azidylated ovalbumin with biotin-phosphine. C) A20s were pulsed for 4 hours with either a wild type or an azidylated mutant peptide, which was followed by 20 hours presentation to the T cells (N = 3, n = 2/3). D) As for C), but using a mutant peptide and its biotinylated counterpart (N = 2, n = 2/3). All T cell activation studies were analyzed by ELISA IL-2 readout

Next, it was attempted to use ISQAVHAAHAEINAhaAGR in an immunoprecipitation assay [43, 44]. For this, A20s were pulsed for 24 hours with either the unmodified peptide, mutant p8 peptide or the p8 mutant peptide, pre-modified with biotin (using a Staudinger ligation reaction). After this chase period, the cell lysate containing the mutant p8 peptide was subjected to a Staudinger ligation using biotin-phosphine (2 hours at 37˚C). All cell lysates were then immunoprecipitated using streptavidin-coated beads. Subsequent Western blot analysis of the eluate showed no detectable MHC-II for the unmodified peptide and the pre-modified peptide, but did show signal when staining for the β-chain of MHC class II in the elution fractions (Figure 8). This confirms the suitability of the p8 modified antigens as a ligation reagent.

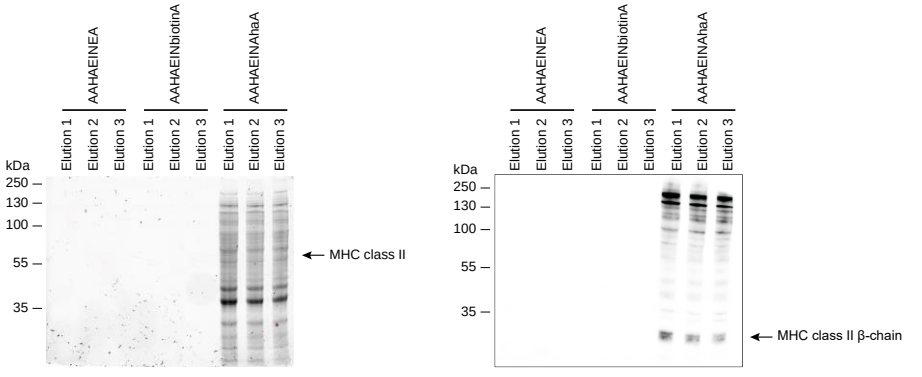

Figure 8. MHC class II immunoprecipitation. Left: 10% TGX Stain Free gel containing the elution fractions of the three used peptides: the wild type peptide (AAHAEINEA), the premodified peptide (AAHAEINbiotinA) and the azidylated peptide (AAHAENAhaA) subjected to a Staudinger ligation with biotin-phosphine in cell lysate. Right: The corresponding Western blot, using an MHC Class II (β-chain) antibody.

Discussion and conclusion

In this Chapter it was shown that it was possible to produce antigens with the incorporation of L-Aha, which could be followed in antigen degradation experiments. Furthermore, subsequent antigen presentation studies showed that these azidylated proteins still induced T cell activation. Due to issues with the IL-2 levels of the TTCF antigens and difficulties with expressing azidylated Ova proteins (in more details discussed in **Chapter 2**) it was chosen to perform experiments using both the TTCF proteins and Ova peptides.

One area in which these reagents could be of future interest would be in providing reagents that could block T cell activation (Figure 9A-C). As the azides can be ligated with bulky groups under physiological conditions, the ligation of a bulky group could be used as a stop-signal for T cell activation (as shown for the Ova p8 peptide). In a preliminary experiment, it was opted to use the incorporated azide handle in the TTCF epitope and ligate a bulky group to this position after antigen processing in order to prevent the T cell receptor to recognize this. However, an initial experiment in which the azides were ligated with either biotin-phosphine, bicyclononyne (BCN)-biotin, dibenzocyclooctyne (DBCO)-AF647, or reduced to an amine with tris(2-carboxyethyl)phosphine (TCEP) did not induce a detectable blockade of T cell recognition (Figure 9D). The reasons for this

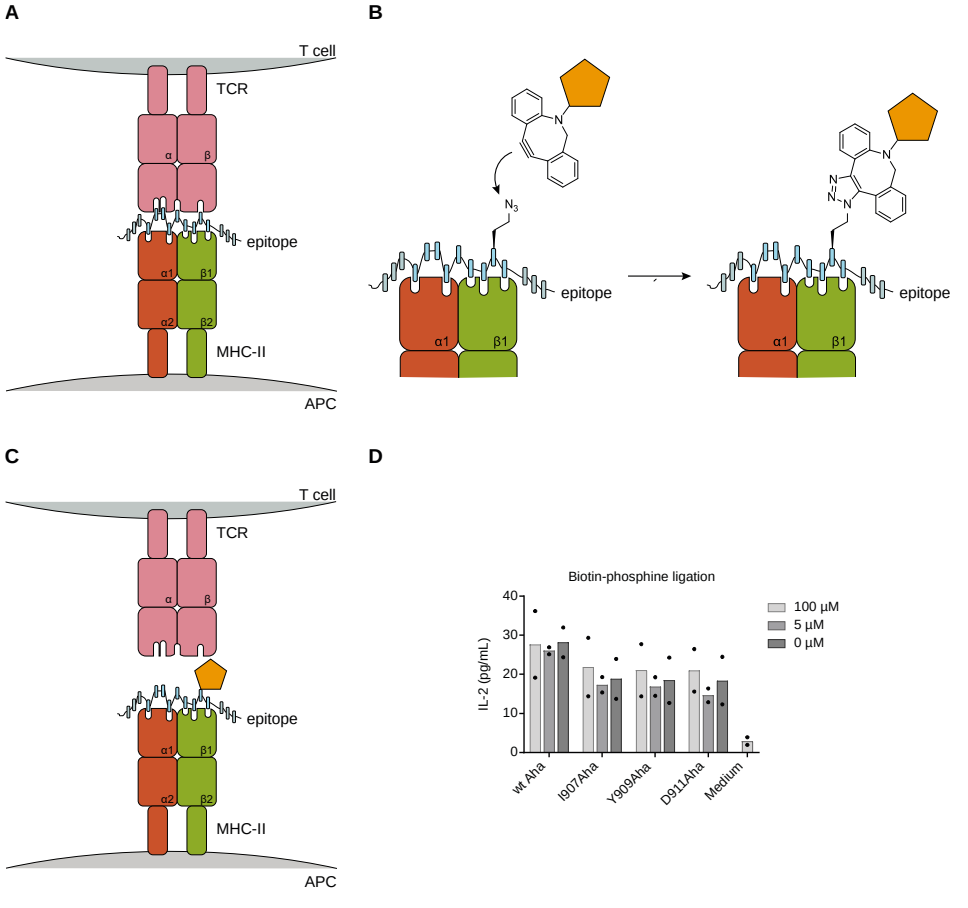

Figure 9. Antigen presentation of TTCF by BMDCs to 2F2 T cell hybridomas. A) At the plasma membrane a T cell can recognize the epitope and will become activated. This activation might be blocked by ligation to a B) bulky group on the presentation site. Which results in a block of C) recognition by the T cell receptor. D) BMDCs pulsed with different proteins, which were subjected to biotin-phosphine and subsequent overnight antigen presentation (N = 2, n = 3). T cell activation studies were analyzed by ELISA IL-2 readout.

could be either a broad tolerance at the tested positions, or inefficient ligation reactions. However, due to time constraints this could not be further investigated.

Acknowledgements

Mikkel Marqvorsen is kindly thanked for his help with extracting BMDCs. Nico Meeuwenoord synthesized the SGFNSSVITYPDAQLVP peptide. Ward Doelman is kindly thanked for his synthesis of all Ova peptides and THPTA. Isa van der Vlugt worked on *TTCF* gene modifications and protein expression.

Materials and methods

General

All chemicals and reagents were purchased at Sigma-Aldrich, Alfa Aesar, Acros, Merck or VWR, unless stated otherwise. Reagents were used without further purification. SDS-PAGE, TGX Stain Free and Western blot materials were purchased at Bio-Rad. Cloning reagents were ordered at Thermo Fisher Scientific. DNA primers were ordered at Sigma-Aldrich or Integrated DNA Technologies. Protease inhibitors were obtained from Roche. Peptides were in-house synthesized containing a C-terminal amide and characterized by NMR and/or LC-MS. Cell culture disposables were from Sarstedt.

Solutions

PBS contained 5 mM KH_2PO_4, 15 mM Na_2HPO_4, 150 mM NaCl, pH 7.4 and PBST was PBS supplemented with 0.05% Tween-20. TBS contained 50 mM Tris-HCl, 150 mM NaCl and TBST was TBS supplemented with 0.05% Tween-20. Laemmli sample buffer 4* contained 60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.01% (v/v) bromophenol blue.

Compound synthesis

Imidazole-1-sulfonyl azide hydrogen sulfate

As described by Potter *et al.* [45]. A 250 ml round-bottomed flask with a stirring bar was brought under nitrogen atmosphere and cooled to 0˚C. Sodium azide (5.2 g, 80 mmol, 1.0 eq.) was added before the addition of dry EtOAc (80 mL). After adding SO_2Cl_2 (6.5 ml, 80 mmol, 1.0 eq.) over 10 min the reaction was slowly warmed to rt and left stirring under an inert atmosphere overnight. The reaction mixture was then recooled to 0˚C, before the addition of imidazole (10.9 g, 160 mmol, 2.0 eq.) and the resulting suspension was stirred for 3.5 h at 0˚C. The reaction mixture was basified with saturated aqueous $NaHCO_3$ (150 mL). The organic layer was separated from the aqueous layer, washed with H_2O and dried with $MgSO_4$. The suspension was filtered and cooled to 0˚C. Concentrated H_2SO_4 (4.4 ml, 80 mmol, 1.0 eq.) was added over 5 min. The mixture was warmed to rt and stirred until a precipitate had formed, which was filtered and washed with EtOAc. The remaining white crystals were dried under vacuum to afford the title compound pure (12.8 g, 47 mmol, 59%).

Boc-Dab-COOH

As described by More and Vince [46]. At 4˚C the solution of *N*-Boc-L-glutamine (27.9 g, 113 mmol, 1.0 eq.) in THF (0.27 L) and water (68 mL) was combined with (Diacetoxyiodo)benzene (39 g, 136 mmol, 1.2 eq.). After 6.5 h of stirring, the mixture was evaporated to dryness, dissolved in H_2O (0.13 L) and washed four times with EtOAC (100 mL). The aqueous layer was evaporated to dryness and 3x co-evaporated with toluene and dried under vacuum to afford a pure compound (14.6 g, 67 mmol, 59%).

(S)-2-amino-4-azidobutanoic acid (L-azidohomoalanine)

Adjusted from Zhang *et al.* [47]. Boc-Dab-COOH (4.97 g, 23 mmol, 1.0 eq.) was suspended with K_2CO_3 (8.03 g, 58 mmol, 2.5 eq.) and $CuSO_4.5H_2O$ (59 mg, 0.23 mmol, 0.01 eq.) in MeOH (125 mL). To a stirred suspension, imidazole-1-sulfonyl azide hydrogen sulfate (6.23 g, 23 mmol, 1.0 eq.) was added and the mixture was stirred overnight at rt. Upon completion on TLC, the mixture was concentrated and diluted in EtOAC (100 mL). The organic layer was washed with 1% HCl (500 mL) twice and H_2O (500 mL) once. The crude was purified by column chromatography and fractions with over 90% purity as confirmed by NMR data were treated with 20% TFA in DCM for 4 h at rt. Upon completion on TLC, DCM was evaporated and the resulting oil was 2x co-evaporated with H_2O. The product was dissolved in MQ water and lyophilized 3 times to obtain the product (3.7 g, 14 mmol, 61%).

Tris-hydroxypropyltriazolylmethylamine (THPTA)

A two-step synthesis as described by Hong *et al.* [48].

Strains and plasmids

E. coli strains XL10 and B834(DE3) were used as cloning and expression strains. Plasmid pET16b was used for the expression of 10His-TTCF and its mutant variants and contained the IPTG-inducible T7 promoter.

Cloning

pEH101 containing full-length *TTCF* with a linker sequence was a gift from the Watts lab [18]. To obtain pET16b_10His-TTCF, the DNA fragment encoding TTCF was

Table 3: List of primer sequences

#	primer name	sequence 5' -> 3'	#	primer name	sequence 5' -> 3'
p1	TTCF_fwd	CGCAAAATTCCCTCTAGAATAATTTTTG	p11	TTCF_D911M_fwd	CATATCCAATGGCTCAATTGG
p2	TTCF_rev	GTTAGCAGCCGGATCCTTAGTCGTTGG	p12	TTCF_D911M_rev	CCAATTGAGCCATTGGATATG
p3	TTCF_I907M_fwd	CCTCTGTTATGACATATCC	p13	TTCF_A912M_fwd	CATATCCAGATATGCAATTGGTGCC
p4	TTCF_I907M_rev	GGATATGTCATAACAGAGG	p14	TTCF_A912M_rev	GGCACCAATTGCATATCTGGATATG
p5	TTCF_T908M_fwd	CTGTTATCATGTATCCAGATG	p15	TTCF_Q913M_fwd	CCAGATGCTATGTTGGTGCCGG
p6	TTCF_T908M_rev	CATCTGGATACATGATAACAG	p16	TTCF_Q913M_rev	CCGGCACCAACATAGCATCTGG
p7	TTCF_Y909M_fwd	GTTATCACAATGCCAGATGCTC	p17	TTCF_L914M_fwd	GATGCTCAAATGGTGCCGGGC
p8	TTCF_Y909M_rev	GAGCATCTGGCATTGTGATAAC	p18	TTCF_L914M_rev	GCCCGGCACCATTTGAGCATC
p9	TTCF_P910M_fwd	CACATATATGGATGCTCAATTGG	p19	TTCF_V915M_fwd	GTTGATGCCCGGCATCAATTGAG
p10	TTCF_P910M_rev	CCAATTGAGCATCCATATATGTG	p20	TTCF_V915M_rev	CTCAATTGATGCCGGGCATCAAC

amplified by PCR from the pEH101 plasmid. This fragment was ligated into the pET16b vector using the XbaI and BamHI restriction sites. All sequences were verified by Sanger sequencing (Macrogen).

Mutations of TTCF were created using site-directed mutagenesis. The resulting DNA fragment was ligated into the pET16b vector as described for the wild type gene.

Bacterial growth and protein expression

Expression of TTCF

An overnight culture of B834 containing pET16b_TTCF (earlier cloned from the pEH101 plasmid, kindly provided by prof. C. Watts) [34], or one of the mutants, was diluted 1:100 in LB medium containing 50 µg/mL ampicillin and 1% glucose. Cells were grown at 37˚C, 180 rpm to an OD_{600} of ~0.8 and sedimented (3428 rcf, 15 min, 4˚C) before being resuspended in LB medium containing 50 µg/mL ampicillin. The culture was induced with IPTG (1 mM final concentration) and expression took place ON at 30˚C, 130 rpm. After overnight expression, cells were pelleted and washed once with PBS. Pellets were stored at -80˚C until protein purification.

Expression of azidylated TTCF

An overnight culture of B834 containing pET16b_TTCF (earlier cloned from the pEH101 plasmid, kindly provided by prof. C. Watts) [34], or one of the mutants, was diluted 1:100 in LB medium containing 50 µg/mL ampicillin and 1% glucose. Cells were grown at 37˚C, 180 rpm to an OD_{600} of ~0.8 and sedimented (3428 rcf, 15 min, 4˚C) before being washed twice with SelenoMet medium (Molecular Dimensions). Cells were resuspended in SelenoMet medium containing 50 µg/mL ampicillin and depleted for 30 min at 37˚C, 180 rpm before 30 min depletion at 30˚C, 130 rpm. After this azidohomoalanine (L-Aha TFA salt; 72 mg/L) was added to the culture and 15 min later

the culture was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM final concentration) and expression took place ON. After overnight expression, cells were pelleted and washed once with PBS. Pellets were stored at -80˚C until protein purification.

Protein purification and analysis

Purification of TTCF and azidylated TTCF

Adjusted from Antoniou *et al.* (2000) [34]**.** A 1L culture pellet was resuspended in 15 mL lysis buffer containing 100 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mg/ml lysozyme, 250 U benzonase. Lysis took place for 20 min at rt, before separating the soluble from the insoluble protein (10000 rcf, 15 min, 4˚C). The supernatant was filtered over a 0.2 µm filter (Filtropur S, Sarstedt) and loaded on a 5 mL His-column (Ni-NTA Superflow Cartridge; Qiagen). After loading, the column was washed with 5 CV of IMAC25 buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 25 mM imidazole), before eluting with a 15 CV gradient 25 - 500 mM imidazole. The protein fractions were monitored by resolving samples in a 10% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue G-250) revealed the purity of the protein. The fractions containing the most pure protein were combined and extensively dialysed (6 - 8 kDa MWCO, 3.3 mL/cm, FisherBrand or 12 - 14 kDa MWCO, 2 mL/cm, Spectra/Por) against PBS. The protein was concentrated over an amicon spin filter 10 kDa MWCO, before being aliquoted, flash frozen and stored at -80˚C until further use.

Cell culture

General

A20s and the T cell hybridoma 2F2 were a kind gift of C. Watts and were tested on regular basis for mycoplasma contamination. Cultures were discarded after 2 months of use. A20s were cultured at 37˚C under 5% CO_2 in RPMI 1640 (containing 25 mM HEPES) supplemented with stable glutamine (2 mM), heat inactivated fetal calf serum (10% v/v; Biowest), β-mercaptoethanol (50 µM), penicillin (200 IU/mL; Duchefa) and streptomycin (200 µg/mL; Duchefa). Cells were passaged every 2 - 3 days. The 2F2 T cell hybridoma was cultured at 37˚C under 5% CO_2 in RPMI 1640 (containing 25 mM HEPES)

supplemented with stable glutamine (2 mM), heat inactivated fetal calf serum (10% v/v; Biowest), pyruvate (1 mM), NEAA (1x), β-mercaptoethanol (50 µM), penicillin (200 IU/mL; Duchefa) and streptomycin (200 µg/mL; Duchefa). Cells were passaged every 2 - 3 days.

The MHC class II Ova (323-339) specific T cell DO11.10 was a kind gift of F. Ossendorp. The cells were cultured at 37˚C under 5% CO_2 in IMDM supplemented with stable glutamine (2 mM), heat inactivated fetal calf serum (10% v/v; Biowest), β-mercaptoethanol (50 µM), penicillin (200 IU/mL; Duchefa) and streptomycin (200 µg/mL; Duchefa). Cells were passaged every 2 - 3 days.

BMDCs were isolated from the bone marrow from the tibia and femurs of a C57BL/6 mice. Cells were cultured at 37˚C under 5% CO_2 in IMDM medium supplemented with stable glutamine (2 mM), heat inactivated fetal calf serum (10% v/v; Biowest), β-mercaptoethanol (20 µM), GM-CSF (20 ng/mL, Immunotools), penicillin (200 IU/mL; Duchefa) and streptomycin (200 µg/mL; Duchefa). Cells were passaged on day 4 and day 7. Generally cell experiments were performed on day 7 or 8.

Degradation assay

BMDCs (50000 cells/well) were seeded in a 96-well tissue-culture treated microtiter plate. Adherence was allowed at 37˚C under 5% CO_2 for at least 1 h prior to the addition of 100 µg of protein. After 1 h cells were sedimented and protein was chased for 0, 1, 4 or 24 h at 37˚C under 5% CO_2. Subsequently, cells were sedimented by centrifugation (360 rcf, 5 min, rt) and stored at -20˚C before fluorescent analysis.

Pulse assay

As described for degradation assay with the following adjustments. Protein was pulsed for 0, 22, 42 and 62 min. Cells were sedimented by centrifugation (360 rcf, 5 min, 4˚C) and the pellets were stored at -20˚C before fluorescent analysis.

Antigen presentation assay – TTCF proteins

BMDCs (50000 cells/well) were seeded in a 96-well tissue-culture treated microtiter plate. Adherence was allowed at 37˚C under 5% CO_2 for at least 1 h prior to the addition of peptides or proteins at indicated concentrations. The cells were incubated with the

antigens for 4 hours, followed by 2 times washing with PBS. The corresponding T cell hybridoma (50000 cells/well) was added to the pulsed BMDCs and cells were co-cultured for 17 h or 20 h for antigen recognition and IL-2 production by the T cells at 37˚C under 5% CO_2. After overnight incubation, cells were sedimented by centrifugation (360 rcf, 5 min, rt) and supernatant was transferred to a new 96-wells plate. Stimulation of the T cell hybridoma was measured by IL-2 readout using an ELISA assay according to manufacturer's protocol (Invitrogen).

Antigen presentation assay – Ova peptides

A20s (50000 cells/well) were seeded in a 96-well tissue-culture treated microtiter plate. Adherence was allowed at 37˚C under 5% CO_2 for at least 1 h prior to the addition of the peptides at indicated concentrations. The cells were incubated with the antigens for 4 hours, followed by the addition of DO11.10 (50000 cells/well). Subsequently, the cells were co-cultured for 20 h for antigen recognition and IL-2 production by the T cells at 37˚C under 5% CO_2. After overnight incubation, cells were sedimented by centrifugation (360 rcf, 5 min, rt) and supernatant was transferred to a new 96-wells plate. Stimulation of the T cell was measured by IL-2 readout using an ELISA assay according to manufacturer's protocol (Invitrogen).

Antigen presentation blocking assay – TTCF proteins

BMDCs (50000 cells/well) were seeded in a 96-well tissue-culture treated microtiter plate. Adherence was allowed at 37˚C under 5% CO_2 for at least 1 h prior to the addition of 0.3 mg/mL of protein and 1 ng/µL lipopolysaccharide (LPS). After 6 h of pulse, cells were sedimented and washed once with PBS. Protein was chased in BMDCs overnight in medium containing LPS. After 18 h the cells were sedimented (360 rcf, 5 min, rt) and washed once with PBS. Subsequently, cells were fixed on ice with 50 µL ice cold 0.2% glutaraldehyde. After 60 sec the reaction was quenched by the addition of 200 µL of ice cold BMDC medium. Cells were sedimented by centrifugation (300 rcf, 5 min, rt) and washed once with 100 µL PBS. Subsequently biotin-phosphine was added at indicated concentrations for 2 hours at 37˚C under 5% CO_2 in BMDC medium. The cells were washed once with PBS before the 2F2 T cell hybridoma (50000 cells/well) were added to the pulsed BMDCs and co-cultured for 20 h 2F2 T cell hybridoma at 37˚C under 5% CO_2.

After overnight incubation, cells were sedimented by centrifugation (360 rcf, 5 min, rt) and supernatant was transferred to a new 96-wells plate. Stimulation of the T cell hybridoma was measured by IL-2 readout using an ELISA assay according to manufacturer's protocol (Invitrogen).

Fluorescent analysis

Fluorescent analysis of azidylated TTCF

TTCF-Aha and its mutants were characterized by ligating Alexa Fluor 647 (AF647; Invitrogen) alkyne to the azide click handle. This was done via CuAAC reaction, 2.5 µg protein was combined 2:1 (v/v) with click mix (containing 3 mM copper sulfate, 30 mM sodium ascorbate, 3 mM THPTA ligand, 30 mM aminoguanidine-HCl and 14 µM AF647-alkyne in 88 mM HEPES pH 7.2, final concentration in click mix) reaction for 1 hour at rt in the dark. The reaction was quenched by the addition of 4* Laemmli buffer. Samples were resolved in a 10% SDS-PAGE gel along with PageRuler™ Plus Protein Marker (Thermo Scientific), before scanning Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively; ChemiDoc™ MP System, Bio-Rad). Coomassie staining (Coomassie Brilliant Blue G-250) was used for correct protein loading.

Degradation and pulse assay

Cells were lysed in 50 µL lysis buffer (100 mM HEPES pH 7.2, 50 mM NaCl, 1x EDTA free protease inhibitor (Roche), 0.25% CHAPS, 25 U benzonase) for 30 min on ice. Protein concentration was measured by Qubit according to manufacturer's protocol (Invitrogen). The cell lysate was diluted to 1.2-1.3 mg/mL in 20 µL and combined 2:1 (v/v) with click mix (containing 3 mM copper sulfate, 30 mM sodium ascorbate, 3 mM THPTA ligand, 30 mM aminoguanidine-HCl and 14 µM AF647-alkyne in 88 mM HEPES pH 7.2, final concentration in click mix) to react the remaining azidylated protein for 1 hour at rt in the dark. The reaction was quenched by the addition of 4* Laemmli buffer. Samples (30 µL, 18-20 µg of total lysate) were resolved in a 15% SDS-PAGE gel along with PageRuler™ Plus Protein Marker (Thermo Scientific), before scanning Cy3 and Cy5 multichannel settings (605/50 and 695/55 filters, respectively; ChemiDoc™ MP System, Bio-Rad). Coomassie staining (Coomassie Brilliant Blue G-250) was used for correct protein loading.

Immunoprecipitation

$5.0 \cdot 10^7$ A20 cells were seeded ($1 \cdot 10^6$/mL medium) in a culture flask and left for 1 hour before a 24 h incubation with either ISQAVHAAHAEINEAGR, ISQAVHAAHAEINAhaAGR or ISQAVHAAHAEINbiotinAGR (100 nM). Cells were then washed twice with PBS and subjected to a freeze/thaw cycle. Cells were lysed in lysis buffer containing 0.25% deoxycholate, 0.5% IGEPAL CA-630, 1 mM EDTA, 1 mM PMSF, Roche EDTA free protease inhibitor in PBS for 1 hour, rotating at 4˚C and the solubilized proteins were separated by centrifugation (15000 rcf, 15 min, 4˚C). The cell lysate containing ISQAVHAAHAEINAhaAGR was next reacted with biotin-phosphine (2 hours at 37˚C) and the resulting peptide was purified over a Zeba spin desalting column. The cleared lysates were diluted to 30 mg/mL in lysis buffer and to 5 mg/mL in PBS, before being subjected to immunoprecipitation using Dynabeads™ MyOne™ Streptavidin C1 magnetic beads. After binding for 1 hour at 4˚C and a subsequent binding at rt for 30 min, the beads were washed with buffer A (150 mM NaCl in 20 mM Tris-HCl, pH 7.4), buffer B (400 mM NaCl in 20 mM Tris-HCl, pH 7.4), buffer A and buffer C (20 mM Tris-HCl, pH 8.0). Subsequently the protein was eluted by suspending the beads in 3x 50 µL 1* Laemmli sample buffer (5 min at 65˚C).

Western blot analysis

Samples were resolved in a 10% TGX Stain Free gel along with PageRuler™ Plus Protein Marker (Thermo Scientific) and transferred onto a PVDF membrane by Trans-Blot Turbo™ Transfer system directly after scanning. Membranes were washed with TBS and TBST and blocked with 5% BSA in TBST at rt for 1 h. Subsequently, the membranes were incubated with primary antibody in 5% BSA in TBST (1 h at rt) Membranes were washed 3x with TBST and incubated with matching secondary antibody in 5% milk in TBST (1 h at rt). Subsequently washed three times with TBST and once with TBS. Membranes were developed with luminol (10 mL of 1.4 mM luminol in 100 mM Tris, pH 8.8 + 100 µL of 6.7 mM p-coumaric acid in DMSO + 3 µL of 30% (v/v) H_2O_2) [49] and chemiluminescence was detected on the ChemiDoc™ MP System in the chemiluminescence channel and the protein marker was visualized with Cy3 and Cy5 settings.

Primary antibodies: monoclonal InVivoMAb anti-mouse MHC Class II (β-chain), clone KL277 (1:1000, BioXCell, BE0140)

Secondary antibody: Rabbit Anti-Armenian hamster IgG H&L (1:5000, Abcam, ab5745).

Graphical analysis

All analysis was determined using GraphPad Prism® 6 and 8 or Microsoft Excel 2016.

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