The advantages and disadvantages of bioorthogonal proteins

Groenewold, G.J.M.

Chapter 5

Summary and future prospects

Abstract

This Chapter describes various future applications of bioorthogonal protein expression, such as its use to synthesize bioorthogonal fluorescent proteins, and glycosylated antigen libraries.

The aim of this thesis was to develop bioorthogonal antigenic proteins and to use these as tools to study antigen processing, presentation and T cell activation.

In **Chapter 1** an introduction to the field of protein modification was given. This chapter focused on the synthesis of proteins with bioorthogonal ligation handles; in particular those that can be incorporated via modified protein expression systems.

Chapter 2 challenged the production and purification of model protein ovalbumin with the incorporation of the unnatural amino acids azidohomoalanine (L-Aha) and homopropargylglycine (L-Hpg). The metabolic labeling of the protein with L-Aha was indeed possible in BL21::MetA. Successful purification of the protein enabled the study of differences between the azidylated and the wild type version of this protein and gives one the possibility to use this modified antigenic protein for antigen presentation studies.

In **Chapter 3** the bioorthogonal incorporation of L-Aha and L-Hpg into the immunological model protein tetanus toxin C fragment (TTCF) was presented. A series of mutants of the protein was made, their ability to activate TTCF-specific T cell clones was assessed, as well as the effect of the bioorthogonal modifications on T cell activation. The latter were shown to be tolerated by the T cell receptor. This scope of this tolerance was broadened to the MHC-II restricted epitope of ovalbumin, which was assessed using a series of peptide antigens.

In **Chapter 4** horseradish peroxidase (HRP) was explored as new tool for immunohistology. HRP was subjected to diazotransfer conditions and the resulting HRP-N_3 was used in dot blot and immunoblot experiments. These were successfully performed both on purified alkylated protein and alkylated bacteria. Furthermore, a ligation reaction between the peroxidase and an alkylated activity-based probe (ABP) was presented. However, this should be further optimized, as the detection limit was reached when this method was applied in cellular ABPP studies.

With this broadening of the expertise to produce large amounts of recombinant bioorthogonal proteins, some new approaches can be envisaged. The first steps towards some of these are described below, but a thorough assessment should be performed in

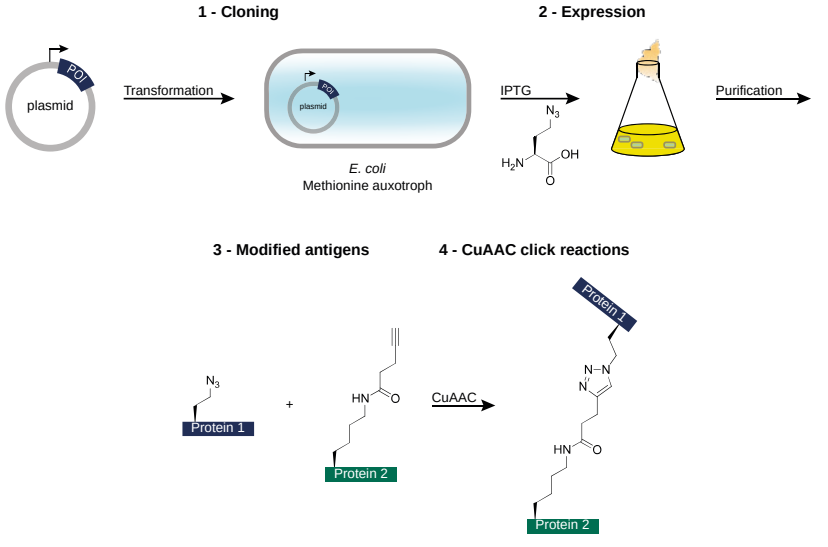

Figure 1. General strategy for the ligation of two proteins. Individual steps subject of here-described studies are the transformation of pEH101 to methionine-auxotrophic *E. coli* B834 (**1**); induced expression in the presence of the methionine analogue azidohomoalanine and subsequent purification (**2**); Combining this with the alkyne modified β-casein (**3**); and the subsequent ligation reaction, resulting in a protein-protein ligation (**4**).

future experiments.

5.1 Bioorthogonal chemistry for protein-protein ligation

The first use of this approach is to ligate two proteins together. Normal ligations rely either on the introduction of a sortag – an LPXTG tag which can be used for ligation reactions using the Sortase A enzyme – [1], or on the random ligation between cysteine (Cys) and lysine (Lys) residues. The former method is relatively low yielding and requires genetic modification of the protein; as well as a free N- and C-terminus. The most common method of Lys/Cys ligation suffers from poor control reactions, polymerization and homomultimerization. It was thus envisaged that bioorthogonal ligation reactions could be used to prevent homodimerization, whilst still getting high enough yields. This was done using the approach outlined in Figure 1: one protein is modified with an alkyne handle and the other with an azide handle and the two are ligated together using the copper(I)-catalyzed azide alkyne cycloaddition (CuAAC) [2]. This approach would give

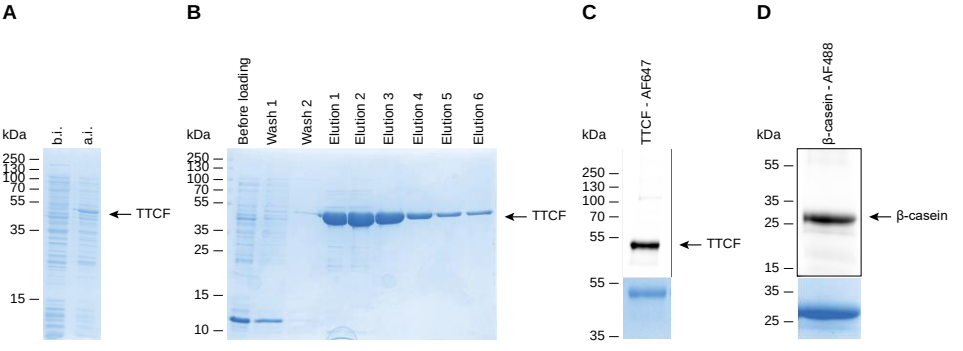

Figure 2. Expression of TTCF-Aha and fluorescent analysis of modified TTCF and β-casein. A) The before and after induction (b.i. and a.i. respectively) samples showing the expression of TTCF-Aha. B) Ni-NTA purification of TTCF-Aha, of which fractions 1-6 resulted in >95% pure protein and were subsequently used for C) fluorescent analysis by ligation to AF647 alkyne. D) Fluorescent analysis of β-casein-alkyne by ligation to AF488 azide. A-D) Proteins were resolved in a 10% or 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

far better control over the protein:protein ratio and preclude the aforementioned homodimerization.

With this aim in mind, two different model proteins, TTCF and β-casein, were modified with either an azide or alkyne handle. The former was made using the recombinant expression and purification protocols outlined in **Chapter 3** (Figure 2A and B). The second protein**,** β-casein, was chemically modified with an alkyne handle using pentynoic acid *N*-hydroxysuccinimide (HOSu) ester as a quick method for achieving this reagent. For this, 100 µM β-casein in 0.1 M $NaHCO_3$ pH 8.6 was combined with 1 eq. of pentynoic acid HOSu ester and this coupling took place overnight at 4˚C. Subsequently, the reaction was desalted and buffer exchanged against 100 mM HEPES pH 8.4.

The accessibility of the ligation handles was first assessed using fluorophores with the opposite ligation handle, Alexa Fluor 647 (AF647) alkyne (for ligation to TTCF) and AF488 azide (for ligation to β-casein) under CuAAC-conditions. The resulting in-gel fluorescence showed that both proteins were ligated to the fluorophore (Figure 2C and D).

It was next assessed if it was possible to ligate the two proteins using the CuAAC reaction. For this, β-casein-azide and TTCF-alkyne were ligated in a 1:0.5 or in a 1:1 molar ratio in the presence of ligation mixture containing either 0, 1 or 5 eq. of Cu-

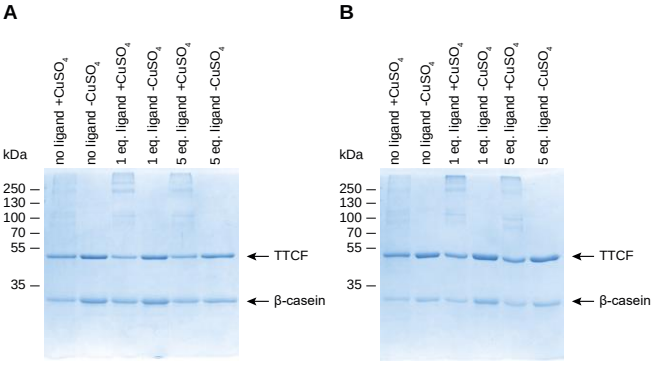

Figure 3. Ligation reaction between β-casein-alkyne and TTCF-azide. This reaction was performed in a A) 1:0.5 or B) 1:1 molar ratio in the presence of 0, 1 or 5 eq. of THPTA ligand. The 1:1 molar ratio showed a higher likelihood of aggregation of the proteins. A-B) Proteins were resolved in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

stabilizing THPTA ligand, in presence/absence of $CuSO_4$ for 15 min at rt. SDS-PAGE analysis of the reaction showed that the ligation efficiency was independent of the protein ratio and of the ligand concentration, but – as expected – only proceeded in presence of $CuSO_4$ (Figure 3A and B). It was, however, observed that an increase in the concentration of ligand present in the ligation mixture led to an increase in higher molecular weight bands (Figure 3), likely due to the presence of multiple ligation handles in both proteins. However, this approach does offer the opportunity to produce protein-protein heterodimers without the presence of homodimer contaminants.

5.2 The importance of protein glycosylation in antigen presentation

A second area in which the high yielding production of bioorthogonal proteins can be of interest is in producing homogeneous glycoforms of these proteins [3]. As for the protein dimerization reactions described above, the common approaches to produce such proteins – such as enzymatic glycan remodeling [4, 5] or lysine modification [6], are either limited in the nature and sites of modification and/or result in heterogeneous mixtures in terms of modified sites. This can have extensive immunological consequences, probably best exemplified by the disagreements regarding the role of the mannose receptor (MR) in enhancing antigen cross-presentation. Burgdorf *et al.* published the observation that glycosylated Ova-antigen can be internalized via the MR

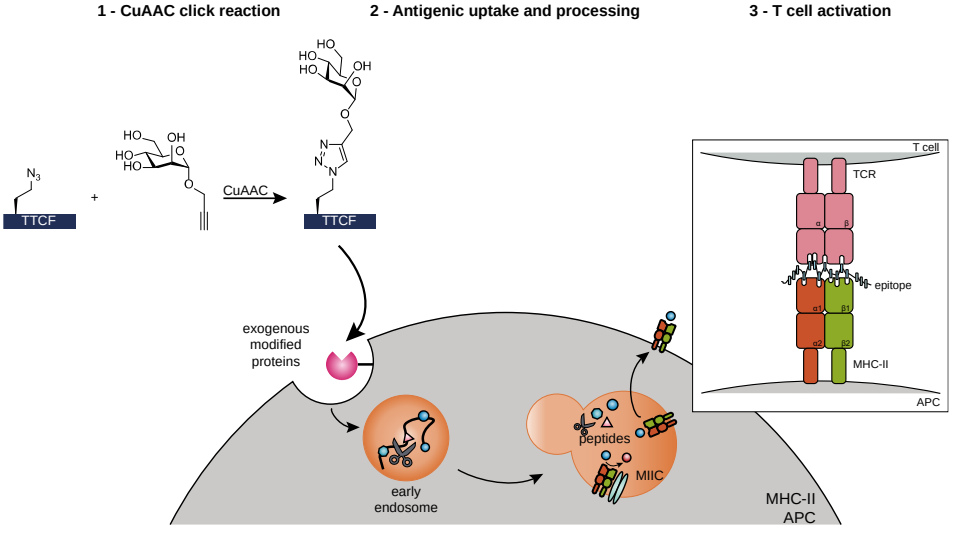

Figure 4. General strategy for MHC class II antigen presentation using glycosylated proteins. Individual steps subject of here-described studies are the azidylation of TTCF and the glycosylation of this protein by for instance alkylated mannose (**1**). This modified protein was next subject to antigenic uptake and processing, before T cell activation by means of IL-2 production was measured (**2** and **3**).

leading to routing of this antigen to a 'cross-presentation enabled'-endosome, leading to enhanced CD8 T cell activation [7]. However, other studies of the same system [8] showed quite the opposite effect for the same system. The difference can only be rationalized by the fact that different glycoform mixtures and modifications were used in these systems and that these have differential interactions with the immune lectins found on APCs.

The MR receptor has several binding domains which are able to recognize several carbohydrate moieties, besides mannose monosaccharides. Examples of these are fucose, glucose, *N*-acetyl glucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), mannose disaccharides, and more complex trisaccharides such as Lewis^X and tri-GlcNAc [9-12]. For the mannose disaccharides the α1-6, and α1-3 linkages are preferred over α1-2 and α1-4 linkages [9, 10].

The effect of the trisaccharides was evaluated by the group of Van Kooijk, using ovalbumin as a model antigen [13]. In this study, they modified ovalbumin with either Lewis^A or tri-GlcNAc and illustrated that both glycosylated proteins showed enhanced

binding to the MR compared to the wild type protein. Furthermore, glycosylation of the protein showed an increased MHC class I-mediated cross presentation.

With the aim to analyze how glycosylation influences MHC presentation and T cell activation – as depicted in Figure 4 – the bioorthogonal glycosylation of antigens was attempted – much along the lines of the previously described strategy [14]. For this, it was decided to use an azidylated TTCF (produced as earlier described in Chapter **2** and section **5.1**), which was ligated to different alkylated glycans using the CuAAC [2, 3].

The azidylated protein was next modified with three different alkylated sugar moieties which are known to bind the mannose receptor, namely propargylated GlcNAc [9], propargylated mannose [9] and propargylated 4-SO_4-GalNAc [15]. For this, a literature protocol was followed, with minor adjustments [3]. In short: the azidylated protein was combined with the corresponding glycan and buffered with sodium phosphate pH 8.3. The reaction – under inert conditions – was started by the addition of a Cu(I) solution. After brief vortexing, the reaction was left for 5 min at rt. After two more additions of the Cu(I) solution, the protein was immediately desalted and buffer exchanged to PBS.

With the aim to analyze if every azide moiety of the protein was modified with the respective glycan, it was decided to expose the unmodified protein (Met), the azidylated protein (Aha) and the three resulting glycosylated proteins to a CuAAC ligation mixture containing AF647 alkyne. SDS-PAGE-fluorimetry showed no fluorescent signal for the glycosylated proteins and the negative control (Met), whereas the positive control (Aha) did show the AF647 signal (Figure 5A). It was therefore assumed that every L-Aha moiety in the protein was modified with the sugar residue. However, in the future this must be confirmed, for instance using mass spectrometry analysis.

Next, the immunogenicity of these modified proteins was analyzed. For this, the glycosylated proteins were given to bone marrow derived dendritic cells (BMDCs), which acted as APCs in this experiment. After 4 hours of pulse, the cells were washed twice with PBS and the TTCF-specific 2F2 T cell hybridoma was added. After overnight chase, T cell activation was measured via an IL-2 readout. This showed no significant difference between the glycosylated proteins (Figure 5B).

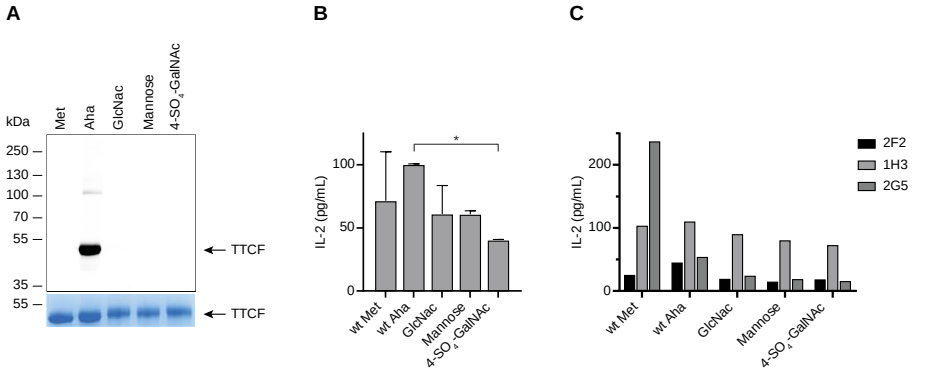

Figure 5. T cell activation by glycosylated proteins. A) Representative in-gel fluorescence after AF647 alkyne ligation to the differently derived azidylated or glycosylated proteins. Proteins were resolved in a 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue G-250. B) BMDCs were pulsed for 4 hours with different concentrations of TTCF wild type and glycosylated versions, before being presented to T cell hybridoma 2F2. The produced IL-2 concentration was not significantly different for any of the glycosylated proteins. C) The same experiment as described for B) presented to T cell hybridomas 2F2, 1H3 and 2G5. 2G5 did not produce IL-2 when using the modified protein. Data was expressed as mean (± SD) (N = 1 or N = 2, n = 3). Statistical analysis was performed using Tukey's multiple comparisons test. * $P \leq 0.05$.

In addition, it was analyzed whether the T cell activation of glycosylated proteins was epitope specific. Therefore, an initial study was performed in which besides the activation of the 2F2, also the activation of the 1H3 and the 2G5 T cell hybridomas was measured. This showed that the IL-2 levels of both the 2F2 and 1H3 T cells remained mostly unaltered upon activation when using the glycosylated proteins (Figure 5C). However, in line with the expectations (see **Chapter 2**), the 2G5 epitope showed no T cell activation when any of the modified proteins was used.

The experiments described in this section showed that glycosylation of TTCF does not alter the MHC class II antigen response. This was earlier reported for Ova-sulfo-Le^A and Ova-tri-GlcNAc [13], however for these glycoproteins it was shown that MHC class I antigen presentation was enhanced. Therefore, it is recommended to evaluate the glycosylation of recombinant ovalbumin in an antigen presentation assay, using both OT-I and OT-II as responding T cells.

5.3 Bioorthogonal fluorescent bacteria

Fluorescent proteins have been transformative in the study of living systems [16, 17]. GFP is the most commonly used fluorescent protein, exciting mostly at 395 nm, and

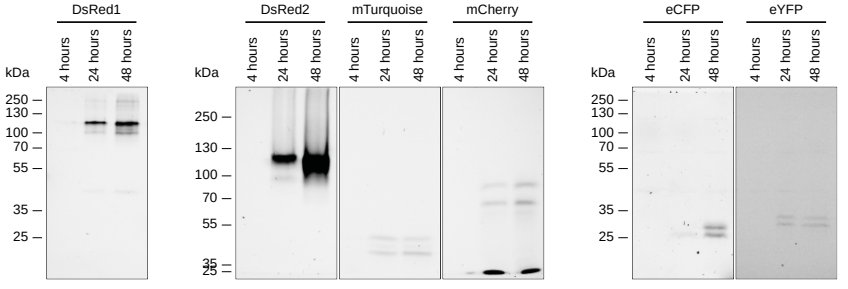

Figure 6. In-gel fluorescence of the constitutively expressed fluorophores. In-gel fluorescence of DsRed1, DsRed2, mTurquoise, mCherry, eCFP and eYFP after 4, 24 and 48 hours of culture growth. Proteins were resolved in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

emitting at 509 nm [16]. In the study of antigen processing, they can also serve as excellent markers: the loss of fluorescence can be used to determine whether a protein or bacterium is degraded [18]. After this degradation, however, the protein fragments are invisible. It has previously been shown that bioorthogonal amino acids incorporated into recombinant proteins served as excellent markers to study precisely this later degradation and that combining these two types of reagents would yield an informative tool to study degradation: The presence of both fluorescent signals in a cell could indicate the presence of intact fluorescent protein in a particular organelle, whereas the presence of only the bioorthogonal label would indicate protein degradation [19, 20].

Within the framework of studying the degradation of bacteria by APCs, a variety of fluorescent protein-producing bacteria – also capable of BONCAT labeling – were produced. For this, expression vectors for DsRed1, DsRed2, mTurquoise, mCherry, eCFP and eYFP, were produced and expression of the fluorescent proteins was first assessed in *Escherichia coli* (*E. coli*) B834(DE3). For this, the genes coding for each of the aforementioned fluorophores: DsRed1, DsRed2_S4T, mTurquoise, mCherry, eCFP and eYFP were cloned behind the hns promoter as earlier described by Van Elsland *et al.* [18]. Next, the resulting plasmids were transformed into *E. coli* B834 and protein expression was monitored. For this, *E. coli* B834 bearing the plasmids were grown in LB medium and after 4, 24 and 48 hours of growth samples were taken. SDS-PAGE analysis showed that all of the mentioned fluorophores were constitutively expressed (Figure 6). DsRed1 and DsRed2, mCherry and eYFP needed at least 24 hours to become fluorescent, whereas

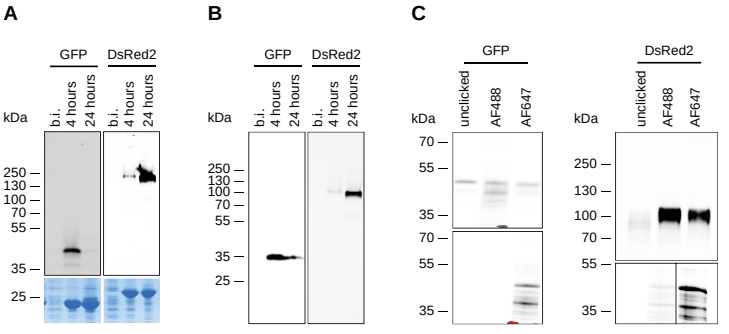

Figure 7. In-gel fluorescence of the recombinant fluorophorescent proteins. A) In-gel fluorescence of GFP and DsRed2 expressed in LB medium and Coommassie staining (lower panel). B) In-gel fluorescence of GFP and DsRed2 expressed in the presence of L-Aha. C) Left: Fluorescence analysis of GFP: AF488 analysis in the upper panel and AF647 analysis in the lower panel. Right: Fluorescence analysis of DsRed2: AF550 analysis in the upper panel, AF488 in the left lower panel and AF647 in the right lower panel. A-C) Proteins were resolved in a 10% SDS-PAGE and stained with Coomassie Brilliant Blue G-250.

mTurquoise and eCFP already showed the emergence of fluorescence after 4 hours. Of note is the visualization of both DsRed1 and DsRed2 in its tetrameric form [21, 22] and mCherry in both its monomeric and weak dimeric form [23]. With the fluorescence of the constitutively expressed proteins confirmed, the constructs can now be used in for instance lysosomal degradation experiments.

Besides the constitutively expressed fluorescent proteins, it was also decided to prepare two inducible constructs, containing either DsRed2_S4T or GFP_A206K. Both were expressed in *E. coli* with and without the incorporation of L-Aha using the standard protocol (Chapter **2** and **3** and sections **1** and **2)**. Fluorescence analysis of the expression samples showed the presence of GFP and DsRed2 when grown in LB medium (Figure 7A) and when expressed in the presence of L-Aha (Figure 7B). Coomassie staining confirmed expression and revealed that DsRed2 is only fluorescent in its tetrameric folding as described in literature [21].

Next, both cell lysates containing wild type and azidylated GFP and DsRed2 were subjected to a CuAAC ligation mixture containing either AF488 alkyne or AF647 alkyne. The resulting fluorescent image showed as expected an overlap between AF488 and GFP (Figure 7C; left). Surprisingly, no overlap was seen between AF647 and GFP. In the presence of the reducing conditions of the CuAAC ligation mixture, GFP diffused in

multiple protein bands. For DsRed2, the tetrameric protein did not ligate to the fluorophores, whereas the monomeric version did ligate to both (Figure 7C; right). In future, the ligation reactions should be confirmed using either LC-MS/MS and/or Native PAGE analysis.

Acknowledgements

Dimitris Poulcharidis is kindly thanked for his synthesis of pentynoic acid HOSu ester and azidohomoalanine. Propargyl GlcNAc and THPTA are kindly synthesized by Ward Doelman. Lars Verschoor synthesized propargyl 4-SO_4-GalNAc. Niels Reintjens is kindly thanked for his synthesis of propargyl mannose. Julia Pols and Joni van Rijn are kindly thanked for cloning the TTCF M1149I mutant and the colour library respectively.

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. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) materials were purchased at Bio-Rad.

Solutions

PBS contained 5 mM KH_2PO_4, 15 mM Na_2HPO_4, 150 mM NaCl, pH 7.4. 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

Chemical modification of β-casein

Dissolve pentynoic acid HOSu ester to a concentration of 100 mM in DMSO (19.5 mg/mL). Dissolve β-casein to a concentration of 100 µM (2.4 mg/mL) in 0.1 M $NaHCO_3$ pH 8.6 and add 1 eq. of pentynoic acid HOSu ester. After overnight coupling at 4˚C, the reactions were desalted over a Zeba Spin Desalting Column (Thermo Scientific), 40 kDa MWCO according to manufacturer's protocol and simultaneously buffer exchanged against 100 mM HEPES pH 8.4. The modified protein was stored at -20˚C until further use.

Strains and plasmids

E. coli strains XL10 and B834(DE3) were used as cloning and expression strains respectively. Plasmid pEH101 containing full-length *TTCF* with a linker sequence was a gift from the Watts lab [24] and was used for the expression of 10His-TTCF and contained the IPTG-inducible T7 promoter.

Cloning

TTCF

pEH101 containing full-length *TTCF* with a linker sequence was a gift from the Watts lab [24]. To obtain pET16b_10His-TTCF, the DNA fragment encoding TTCF was 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).

Table 1: List of primer sequences

#	primer name	sequence 5' -> 3'
p1	TTCF_fwd	CGCAAAATTCCCTCTAGAATAATTTTTG
p2	TTCF_rev	GTTAGCAGCCGGATCCTTAGTCGTTGG
p3	T7_GFP_fwd	GGCGGCCGTCTCCCATGAGTAAAGGAGAAGAAC
p4	T7_GFP_rev	GGCGGCGGATCCTTATTTGTATAGTTCATCC
p5	T7_DsRed2_S4T_fwd	GGCGGCCGTCTCCCATGGCCTCCACCGAGAACG
p6	T7_DsRed2_rev	GGCGGCCGTCTCGGATCCTTTATCTAGATCCGGTGG
p7	hns_DsRed1_fwd	GGCGGTATGCATATGGTGCGCTCCTCC
p8	hns_DsRed1_rev	GGCGGTACGCGTCTACAGGAACAGGTG
p9	hns_DsRed2_S4T_fwd	GGCGGTATGCATATGGCCTCCaCCGAG
p10	hns_DsRed2_rev	GGCGGTACGCGTTTATCTAGATCCGG
p11	hns_mCherry_fwd	GGCGGTATGCATATGGTGAGCAAGGGC
p12	hns_mCherry_rev	GGCGGTACGCGTCTAGACTCGAGATCTG
	hns_mTurqouise_fwd	
p13	hns_eCFP_fwd	GGCGGTATGCATATGGTGAGCAAGGGC
	hns_eYFP_fwd	
p14	hns_mTurqouise_rev	
	hns_eCFP_rev	GGCGGTACGCGTTTACTTGTACAGCTC
	hns_eYFP_rev	

Inducible fluorophores

To obtain pET16b_GFP and pET16b_DsRed2_S4T, the DNA fragments encoding the fluorophores were amplified by PCR. Using this PCR reaction, DsRed2 was mutated to DsRed2_S4T, to enhance fluorescent signal [25]. The resulting fragments were ligated into the pET16b vector using the NcoI and BamHI restriction sites. All sequences were verified by Sanger sequencing (Macrogen).

Constitutively expressed fluorophores

To obtain the constitutively expressed constructs containing DsRed1, DsRed2_S4T, mTurquoise, mCherry, eCFP and eYFP, the DNA fragments encoding the fluorophores were amplified by PCR. Using this PCR reaction, DsRed2 was mutated to DsRed2_S4T, to enhance fluorescent signal [25]. The resulting fragments were ligated into the hns_GFP vector [18] using the NsiI and MluI restriction sites. All sequences were verified by Sanger sequencing (Macrogen).

Bacterial growth and protein expression

Expression of TTCF, GFP and DsRed2_S4T

An overnight culture of B834 containing the corresponding plasmid was diluted 1:100 in LB medium containing 50 µg/mL ampicillin (and 1% glucose in case of TTCF).

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 for 4 hours or 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. The before and after induction samples were suspended in 1* Laemmli buffer and the protein fractions were combined with 4* Laemmli buffer and all resolved in a 10% SDS-PAGE 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) in case of the fluorescent proteins. Coomassie staining (Coomassie Brilliant Blue G-250) was used for protein analysis.

Expression of azidylated TTCF, GFP and DsRed2_S4T

An overnight culture of B834 containing the corresponding plasmid was diluted 1:100 in LB medium containing 50 µg/mL ampicillin (and 1% glucose in case of TTCF). 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. The before and after induction samples were suspended in 1* Laemmli buffer and the protein fractions were combined with 4* Laemmli buffer and all resolved in a 10% SDS-PAGE 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) in case of the fluorescent proteins. Coomassie staining (Coomassie Brilliant Blue G-250) was used for protein analysis.

Constitutive expression of DsRed1, DsRed2_S4T, mTurquoise, mCherry, eCFP and eYFP

An overnight culture of B834(DE3) containing the corresponding plasmid was diluted 1:100 in LB medium containing 50 µg/mL ampicillin. Cells were grown at 37˚C, 180 rpm and samples were taken after 4, 24 and 48 hours of incubation.

The samples were suspended in 1* Laemmli buffer and the protein fractions were combined with 4* Laemmli buffer and all resolved in a 10% SDS-PAGE along with PageRuler™ Plus Protein Marker (Thermo Scientific), before scanning Cy2, Cy3 and Cy5 multichannel settings (532/528, 605/50 and 695/55 filters, respectively; ChemiDoc™ MP System, Bio-Rad). Coomassie staining (Coomassie Brilliant Blue G-250) was used for protein analysis.

Protein purification

Purification of azidylated TTCF

Adjusted from Antoniou *et al.* (2000) [24]**.** A 1 L 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 proteins (10000 rcf, 15 min, 4˚C). The supernatant was filtered over a 0.2 µm filter (Filtropur S, Sarstedt) and loaded on a 2 mL His-column (Ni-NTA Agarose; Qiagen). After loading at 4˚C for 2 hours, the column was washed twice with 2.5 CV of IMAC25 buffer (100 mM Tris-HCl pH 8.0, 500 mM NaCl, 25 mM imidazole), before eluting with 7 CV at 200 mM imidazole. The protein fractions were monitored by A_{280} values and resolving samples on 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 with an A_{280} value over 0.1 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.

Alternatively, the lysed cells were 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. Subsequently followed by the protocol described above.

Ligation reactions

Protein-protein

β-casein-alkyne and TTCF-Aha were ligated via CuAAC reaction. 0.5 or 1 eq. of TTCF-Aha was diluted in 100 mM HEPES pH 8.4 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 1 eq. of β-casein-alkyne in 88 mM HEPES pH 7.2, final concentration in click mix). After 15 min ligation 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). Coomassie staining (Coomassie Brilliant Blue R-250) was used to examine protein ligation.

Protein-glycan

Adjusted from van Kasteren *et al.* (2007) [3]. The reaction should be executed under inert conditions and therefore nitrogen was bubbled through each solution before use.

Dissolve ligand (39.1 mg/mL in MQ:acetonitrile 1:1, 90 mM) and CuBr (10 mg/mL in acetonitrile, 70 mM). Combine prior to addition in a 5:6 ratio respectively. Dilute the protein to 2 mg/mL in PBS (500 µL). Add a dissolved glycan solution (250 µL, 31.2 mM, 50 eq. in MQ water) and 100 µL of sodium phosphate, pH 8.3. To this a Cu(I)/ligation solution (25 µL) was added and after 10 sec of vortexing, left for 5 min at rt. This was repeated another two times using a freshly prepared Cu(I)/ligation solution. After the third time, the glycosylated protein was immediately buffer exchanged over a Zeba Spin Desalting Column (Thermo Scientific), 7 kDa MWCO according to manufacturer's protocol and simultaneously buffer exchanged against PBS. Protein concentrations were determined using Quick Start™ Bradford Protein Assay (Bio-Rad) and the modified proteins were stored at 4˚C or flash-frozen and stored at -80˚C until further use.

Protein-fluorophore

Proteins were ligated via CuAAC reaction to AF647-alkyne. The protein was diluted to a concentration of 0.5 µg/µL in 100 mM HEPES pH 7.2 or MQ water 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). After 1 h ligation at rt in the dark, the reaction

was quenched by the addition of 4* Laemmli buffer. Samples were resolved in a 7.5% or a 10% SDS-PAGE gel along with PageRuler™ Plus Protein Marker (Thermo Scientific). Coomassie staining (Coomassie Brilliant Blue G-250) was used as protein loading control.

Cell culture

General

T cell hybridomas 2F2, 1H3 and 2G5 were a kind gift of C. Watts and were tested on regular basis for mycoplasma contamination. Cultures were discarded after 2 months of use. The cells 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), 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.

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 RPMI 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.

Antigen presentation 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 peptides or proteins at indicated concentrations. The cells were incubated with 0.1 mg/mL (final conc.) of 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 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 hybridoma was measured by IL-2 readout using an ELISA assay according to manufacturer's protocol (Invitrogen).

Statistical analysis

Replicates shown represent biological replicates and data represent means ± SD. All statistical analysis was determined using GraphPad Prism® 8 or Microsoft Excel 2016. **** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; NS if $P > 0.05$.

References

1. Popp, M.W., Antos, J.M., Grotenberg, G.M., Spooner, E. and Ploegh, H.L. (2007). Sortagging: A versatile method for protein labelling. *Nat. Chem. Biol. 3,* p.707-708
2. Agard, N.J., Prescher, J.A. and Bertozzi, C.R. (2004). A strain-promoted [3+2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J. Am. Chem. Soc. 126,* p.15046-15047
3. van Kasteren, S.I., Kramer, H.B., Gamblin, D.P. and Davis, B.G. (2007). Site-selective glycosylation of proteins: Creating synthetic glycoproteins. *Nat. Protoc. 2,* p. 3185-3194.
4. Moremem, K.W., Tiemeyer, M. and Nairn, A.V. (2012). Vertebrate protein glycosylation: Diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol. 13,* p.448-462
5. Ploeg, H. and Neefjes, J.J. (1990). Protein glycosylation. *Curr. Opin. Cell Biol. 2,* p.1125-1130
6. Oliver, C.M., Melton, L.D. and Stanley, R.A. (2006). Creating proteins with novel functionality via the Maillard reaction: A review. *Crit. Rev. Food. Sci. Nutr. 46,* p.337-350
7. Burgdorf, S., Kautz, A., Böhnert, V., Knolle, P.A. and Kurts, C. (2007). Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science. 316,* p.612-616
8. Segura, E., Gupta, N., Albiston, A.L., Wicks, I.P., Chai, S.Y. and Villandangos J.A. (2010). Reply to Burgdorf *et al.*: The mannose receptor is not involved in antigen cross-presentation by steady-state dendritic cells. *Proc. Natl. Acad. Sci. U.S.A. 107,* E50-51
9. van Die, I. and Cummings, R.D. (2017). The mannose receptor in regulation of helminth-mediated host immunity. *Front. Immunol. 8,* 1677
10. Kéry, V., Křepinský, J.J.F., Warren, C.D., Capek, P. and Stahl, P.D. (1992). Ligand recognition by purified human mannose receptor. *Arch. Biochem. Biophys. 298,* p.49-55
11. Leteux, C., Chai, W., Loveless, R.W., Yuen, C-T., Uhlin-Hansen, L., Combarnous, Y., Jankovic, M., Maric, S.C., Misulovin, Z., Nussenzweig, M.C. and Feizi, T. (2000). The cysteine-rich domain of the macrophage mannose receptor is a multispecific lectin that recognizes chondroitin sulfates A and B and sulphated oligosaccharides of blood group Lewis^a and Lewis^x types in addition to the *N*-glycans of lutropin. *J. Exp. Med. 191,* p.1117-1126
12. Liu, Y., Chirino, A.J., Misulovin, Z., Leteux, C., Feizi, T., Nussenzweig, M.C. and Bjorkman, P.J. (2000). Crystal structure of the cysteine-rich domain of mannose receptor complexed with a sulphated carbohydrate ligand. *J. Exp. Med. 191,* p.1105-1115
13. Singh, S.K., Streng-Ouwehand, I., Litjens, M., Kalay, H., Burgdorf, S. Saeland, E., Kurts, C., Unger, W.W. and van Kooijk, Y. (2011). Design of neo-glycoconjugates that

target the mannose receptor and enhance TLR-independent cross-presentation and Th1 polarization. *Eur. J. Immunol. 41,* p.916-925

14. Dieterich, D.C., Link, A.J., Graumann, J., Tirrell, D.A. and Schuman, E.M. (2006). Selective identification of newly synthesized proteins in mammalian cells using biorthogonal noncanonical amino acid tagging (BONCAT). *PNAS. 103,* p.9482-9487
15. Liu, Y., Misulovin, Z. and Bjorkman, P.J. (2001). The molecular mechanism of sulphated carbohydrate recognition by the cysteine-rich domain of mannose receptor. *J. Mol. Biol. 305,* p.481-490
16. Snapp, E. (2005). Design and use of fluorescent fusion proteins in cell biology. *Curr. Protoc. Cell Biol.* Chapter: Unit-21.4
17. Dunn, K.W., Kamocka, M.M. and McDonals, J.H. (2011). A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol. Cell Physiol. 300,* c.723-742
18. van Elsland, D.M., Bos, E., de Boer, W., Overkleeft, H.S., Koster, A.J. and van Kasteren, S.I. (2016). Detection of bioorthogonal groups by correlative light and electron microscopy allows imaging of degraded bacteria in phagocytes. *Chem. Sci. 7,* p.752-758
19. van Elsland, D.M., Pujals, S., Bakkum, T., Bos, E., Oikonomeas-Koppasis, N., Berlin, I., Neefjes, J., Meijer, A.H., Koster, A.J., Albertazzi, L. and van Kasteren, S.I. (2018). Ultrastructural imaging of salmonella-host interactions using super-resolution correlative light-electron microscopy of bioorthogonal proteins. *Chembiochem. 19,* p.1766-1770
20. Araman, M.C., Pieper-Pournara, L., van Leeuwen, T., Kampstra, A.S.B., Bakkum, T., Marqvorsen, M.H.S., Nascimento, C.R., Groenewold, G.J.M., Wulp, W., Camps, M.G.M., Overkleeft, H.S., Ossendorp, F.A., Toes, R.E.M. and van Kasteren, S.I. (2019). Bioorthogonal antigens allow the unbiased study of antigen processing and presentation. *BioRxiv.* p.439323
21. Bevis, B.J. and Glick, B.S. (2002). Rapidly maturing variants of the discosoma red fluorescent protein. *Nat. Biotechnol. 20,* 83-87
22. Wall, M.A., Socolich, M. and Ranganathan, R. (2000). The structural basis for red fluorescence in the tetrameric GFP homolog DsRed. *Nat. Struct. Biol. 7,* p.1133-1138
23. Suzuki, T., Arai, S., Takeuchi, M., Sakurai, C., Ebana, H., Highashi, T., Hashimoto, H., Hatsuzawa, K. and Wada, I. (2012). Development of cysteine-free fluorescent proteins for the oxidative environment. *PLoS One. 7,* e.37551
24. Antoniou, A.N., Blackwood, S-L., Mazzeo, D. and Watts, C. (2000). Control of antigen presentation by a single protease cleavage site. *Immunity. 12,* p.391-398
25. Sörenson, M., Lippuner, C., Kaiser, T., Mißlitz, A., Aebischer, T. and Bumann, D. (2003). Rapidly maturing red fluorescent protein variants with stronly enhanced brightness in bacteria. *FEBS lett. 552,* p.110-114