Immunology

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Bioorthogonal Cleavage and Exchange of Major Histocompatibility Complex Ligands by Employing Azobenzene-Containing Peptides**

*Joanna A. L. Choo, Sock Yue Thong, Jiawei Yap, Wim J. E. van Esch, Manfred Raida, Rob Meijers, Julien Lescar, Steven H. L. Verhelst, and Gijsbert M. Grotenbreg**

Abstract: *Bioorthogonal cleavable linkers are attractive building blocks for compounds that can be manipulated to study biological and cellular processes. Sodium dithionite sensitive azobenzene-containing (Abc) peptides were applied for the temporary stabilization of recombinant MHC complexes, which can then be employed to generate libraries of MHC tetramers after exchange with a novel epitope. This technology represents an important tool for high-throughput studies of disease-specific T cell responses.*

Chemical strategies are increasingly applied to understand and manipulate biological systems. The chemical reactivity of the employed reagents needs to be tuned such that interference with essential biochemical or cellular processes is prevented. Several bioorthogonal reactions have been developed to enable the site-selective conjugation of macromolecules with a myriad of probes (e.g., luminescent dyes and photoresponsive moieties),[1] but the conditional breaking of bonds in the presence of a wide and heterogeneous range of functional groups has received less attention. Cleavable linkers that can be chemoselectively addressed have been deployed in disciplines such as biochemistry, proteomics, and cell biology.[2] One successful application in immunobiology has facilitated the detection of disease-specific T cell responses within large cellular reservoirs. T lymphocytes express a highly diverse range of clonally distributed surface receptors that govern their specificity towards an antigenic peptide fragment associated with a major histocompatibility complex (MHC) on an antigen-presenting cell. Upon recognition, the T cell is tasked to eliminate the virus-infected or tumor cell. Recombinantly produced oligomers of the heterotrimeric MHC protein bind to T cells of corresponding specificity, and the conventional MHC tetramer format has become a cornerstone technology for staining and mapping T cell responses in basic and clinical research on infectious diseases, autoimmunity, cancer, and vaccine development.[3]

Libraries of MHC tetramers have become accessible through the use of synthetic ligands that are released after UV-induced cleavage of the peptide backbone, which allows a novel epitope to refill the evacuated peptide-binding groove.[4] Arrays of peptide-exchanged MHC tetramers enable the interrogation of T cell repertoires regardless of their functional activity. Technical limitations such as low UV penetration, variability in UV irradiation, and the potential for photo- and thermal damage to the protein complexes, highlight the need for alternative modes of cleavage. Chemoselective peptide-exchange methods carry the hazard of compromising the MHC or replacement epitope and risk the loss of antigen recognition. Vicinal diol- or alkanolamine-based conditional ligands, for example, require millimolar periodate concentrations to accomplish complete cleavage, and extended incubation times lead to oxidation of Cys-, Met-, N-terminal Ser-, or Thr- residues.[5] We therefore explored azobenzene-containing (Abc, Z) linkers that are sensitive to sodium dithionite ($Na_2S_2O_4$). The Abc moiety is unaffected by reducing agents common to biological proto-

[*] J. A. L. Choo, Dr. S. Y. Thong, J. Yap, Dr. G. M. Grotenbreg
Department of Microbiology and Immunology Programme
National University of Singapore (NUS)
Life Sciences Institute, #03-05, 28 Medical Drive
117456 (Singapore)
E-mail: grotenbreg@nus.edu.sg
Dr. G. M. Grotenbreg
Department of Biological Sciences
National University of Singapore (NUS)
14 Science Drive 4, 117543 (Singapore)
Dr. W. J. E. van Esch
Division of Reagents, Sanquin Blood Supply
1066 CX Amsterdam (The Netherlands)
Dr. M. Raida
SLING, National University of Singapore (NUS)
Life Sciences Institute
#03-03, 28 Medical Drive, 117456 (Singapore)
Dr. R. Meijers
European Molecular Biology Laboratories
Hamburg Outstation c/o DESY
Notkestraße 85, 22603 Hamburg (Germany)
Dr. J. Lescar
School of Biological Sciences
Nanyang Technological University (NTU)
60 Nanyang Drive, 637551 (Singapore)
Dr. S. H. L. Verhelst
Center for Integrated Protein Science Munich, Lehrstuhl Chemie der Biopolymere, Technische Universität München
Weihenstephanerberg 3, 85354 Freising (Germany)

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cols (e.g., TCEP, DTT) and the fragmentation conditions have been demonstrated to be compatible with biomolecules and living systems.[6] Furthermore, the stereocenter-free building block is accessible from readily available starting materials through a simple and cost-effective synthesis route.

The Abc linker, with its 12 bonds separating the amino and carboxylic acid functionalities, cannot formally be regarded as tetrapeptide isostere (11 bonds, Figure 1a), but it was envisaged to act as a surrogate for four amino acid residues, making allowances for the double bond and aromatic systems counting towards the peptidomimetic backbone. Conditional ligands were designed such that the Abc building block strategically replaced nonessential residues within a parent epitope of high affinity (Tables S1, S2 in the Supporting Information), which improves the likelihood of the resulting Abc ligand binding to and stabilizing the recombinant MHC sufficiently during in vitro refolding and purification.[4b,g] For example, in the HLA-A*11:01-restricted epitope from hepatitis B virus DNA polymerase 110–118 (Table S1), the residues at positions P4–P7 are solvent-exposed, which identifies them as candidates for replacement with Abc.[7] Moreover, the key N- and C-terminal anchor residues Ile (P2) and Lys (P10) were conserved to ultimately furnish the Abc homologue AIM-Z-YPK (**4**), which was obtained through standard Fmoc-based solid-phase peptide synthesis (SPPS; Figure S1 in the Supporting Information). Through applying the same strategy, we obtained a panel of Abc ligands for HLA-A*11:01, HLA-A*02:01, and H2-K^b (Table S1 and Figure S1 in the Supporting Information); which covers the allelic variants of MHC predominantly found in Asian and Caucasian populations, as well as in common murine disease models.

To determine whether binding of the Abc ligands to the MHC product they were designed for was perturbed by the tetrapeptide isostere (Figure 1), we started with a UV-sensitive complex, discharged its peptide cargo through traditional irradiation, and subsequently measured the ability of the Abc ligand (which is inert to photocleavage) to prevent disintegration of the emptied complex by performing MHC stability ELISA (Figure 1b for HLA-A*11:01, Figure S2 for HLA-A*02:01 and H2-K^b. Since the protein requires all subunits to maintain a stable conformation, peptides that rescued the complex were deemed appropriate to produce purified Abc-ligand:MHC molecules (Figure S3). Definitive proof of peptide association, and the molecular details for the binding of the Abc ligand to the MHC, was furnished by X-ray crystallography. The structure of HLA-A*11:01 in complex with AIM-Z-YPK (**4**) was determined to 2.43 Å resolution (Table S3 and Figure S4). The conditional Abc ligand **4** (Figure 1d, cyan) engages the HLA in a manner very similar to that of the parent peptide (Figure 1d, yellow) and occupies the peptide-binding groove by preserving crucial hydrogen bonds and salt bridges formed by the parent peptide through its N- and C-terminal anchor residues (Table S4, Figure S5). The central azobenzene moiety protrudes straight from the groove, is solvent exposed, and sufficiently straddles the four amino acids it was designed to replace. We furthermore observed **4** to occupy two alternative confirmations in the crystal (Figure S6). Optimization of the size-exclusion protocol demonstrated that the two refolded A*11:01 complexes could be separately obtained (Figure S7a,b). These molecular species did not interconvert when left in solution, and they had hydrodynamic volumes of 98 Å and 171 Å, as judged by dynamic light scattering, which likely correspond to the monomeric and dimeric MHCs, respectively (Figure S7c). A second crystal structure of HLA-A*02:01 binding to GLS-Z-RL (**17**) at 2.1 Å resolution essentially displays the same features (but in this case, no alternative conformation for the ligand is observed; Tables S5, S6 and Figures S8, S9).

We next examined how to facilitate rapid and complete Abc-peptide exchange. Exposure of **4** to dithionite indeed resulted in fragmentation towards the expected two aniline products **7** and **8** as confirmed by LC–MS (Figure 2a). By mixing in a stable internal standard **9** at a 1:1 ratio, and interrupting the reaction (ranging from 1 to 5 mM of dithionite) by solid-phase extraction, the kinetics could be tracked by LC–MS analysis (Figure 2b). An incubation period of 5 min with 2.5 mM $Na_2S_2O_{4(aq)}$ was sufficient for the original Abc ligand to fall below the limit of detection, a result indicative of (near) quantitative peptide cleavage in solution.

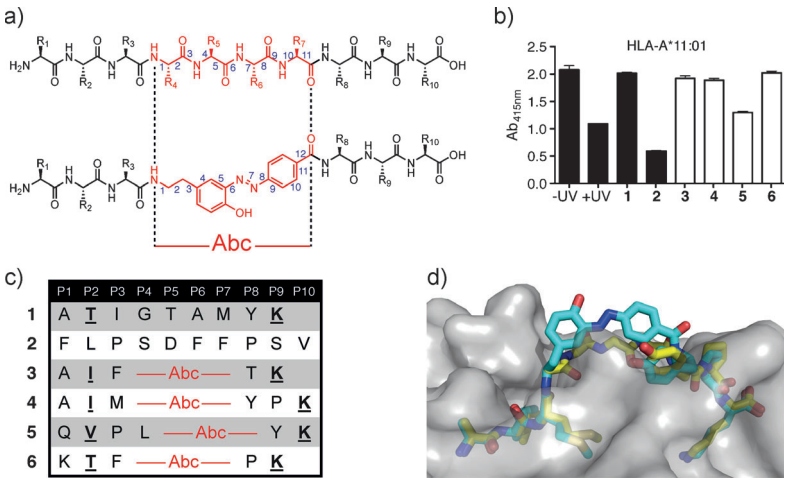

Figure 1. Design of HLA-A*11:01-restricted Abc ligands. a) Replacement of four amino acids residues (11 bonds) with an azobenzene-containing (Abc) tetrapeptide isostere (12 bonds). b) MHC stability ELISA for UV-sensitive A*11:01 molecules, loaded with AIFQSSJTK as previously reported,[4g] were peptide-exchanged with A*11:01-restricted epitope (**1**), A*02:01-restricted epitope (**2**), and A*11:01-restricted Abc ligands (**3** to **6**) upon UV irradiation. MHC molecules before (−UV) and after (+UV) UV irradiation in the absence of rescue peptides were included as controls. c) Sequences of the epitopes (**1** and **2**) and newly synthesized Abc ligands (**3** to **6**) that were used in (b). The position of the Abc moiety in the parent peptides is indicated in red. Anchor residues of the peptides are underlined. d) Overlay of the crystal structures of **4** (cyan; PDB reference ID: 4UQ2, this work) and its parent peptide (yellow; PDB reference ID: 2HN7) in an A*11:01 molecule (grey).

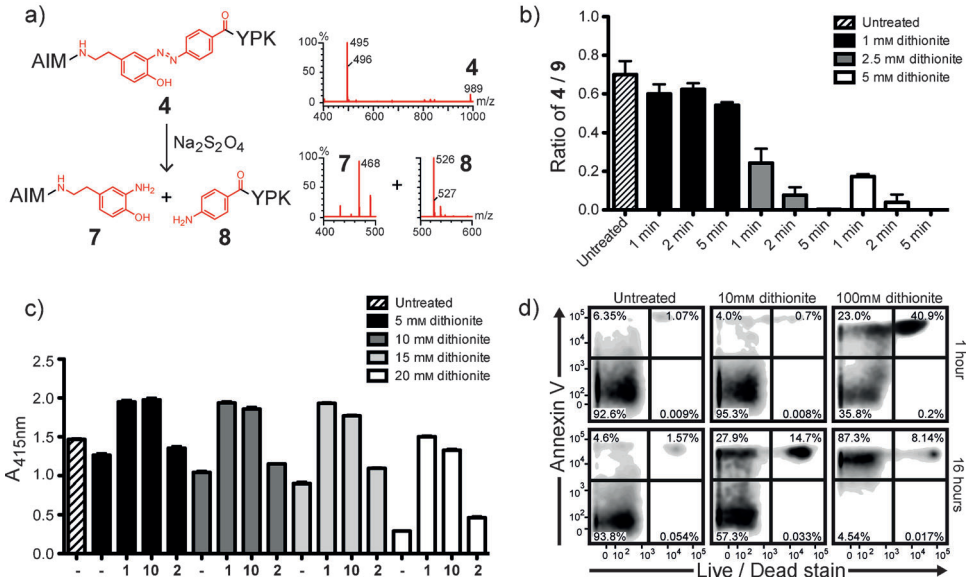

Figure 2. Cleavage kinetics and conditions for HLA-A*11:01-restricted Abc ligand. a) Cleavage of **4** resulted in two aniline products (**7** and **8**) upon the addition of sodium dithionite. Reaction was confirmed using LC-MS. b) **4** is incubated in the presence of **9** at 1:1 ratio with 1, 2.5, or 5 mM of sodium dithionite and the reactions were quenched after 1, 2 or 5 min. The reaction mixtures were analyzed for the presence of intact ABC ligands by using LC–MS. c) Refolded A*11:01 molecules bearing **4** were peptide-exchanged were with A*11:01-restricted peptides (**1** and **10**) and A*02:01-restricted peptide (**2**) in the presence of 5 to 20 mM sodium dithionite. Controls with no peptides (−) added to the MHC molecules were included. Stable MHC molecules were quantified through MHC stability ELISA. d) Freshly isolated peripheral blood mononuclear cells (PBMCs) were incubated with 10 mM (middle column) or 100 mM (right column) sodium dithionite for 1 hour (top row) or 16 h (bottom row) to assess the cellular toxicity of sodium dithionite. Cells were stained with anti-CD8 antibodies, Annexin V, and LIVE/DEAD viability dye, before being analyzed on flow cytometry. Plots shown were gated on the $CD8^+$ population. The numbers in each quadrant represent the percentage of cells relative to the total $CD8^+$ population.

The peptide exchange efficiency (spanning 5 to 20 mM dithionite) was analyzed by ELISA on purified Abc-ligand:MHC complexes with established T cell epitopes (Table S2). Reduction-promoted peptide exchange could be observed at all tested dithionite concentrations (Figure 2c and Figure S10 for A*11:01, Figure S11 for A*02:01, and Figure S12 for K^b). For HLA-A*11:01, the highest signal-to-noise ratio was obtained with ligand **4** at 20 mM $Na_2S_2O_4$, and yet this trend was the reverse for HLA-A*02:01 displaying **17**, thus highlighting that every allelic variant with a tailored Abc ligand will have unique stability characteristics. We further show that disulfide bonds, even in large excess, do not compete with Abc ligands for dithionite, an effect that would lower the effective concentration of the reducing reagent tasked with triggering MHC ligand exchange (Figure S13). The amount of reducing agent was also moderated to prevent toxicity towards cells. It would be preferable if the MHC tetramers of novel specificity could be directly deployed without requiring the removal of any component (i.e., employed reagents or side product) that could unnecessarily lengthen or complicate the peptide exchange and/or staining procedure. Fortunately, both primary and cultured cells of various origins were tolerant to buffered dithionite and showed little sign of apoptosis or cell death at high (10 mM) concentration and prolonged (16 h) exposure (Figure 2d and Figure S14). Balancing the above constraints, we employed 10 mM $Na_2S_2O_{4\ (aq)}$ in the ensuing experiments.

To confirm that our strategy enables the detection of antigen-specific cells from peripheral blood (Figure 3), a short-term expanded T cell line from an A*11:01-carrying donor responsive to Epstein Barr Virus (EBV) antigen ($BRLF1_{134–142}$, **1**, Figure 3b top row) was labeled with MHC tetramers before and after replacement with the canonical epitope (**1**), or an irrelevant peptide (**10**). This established that MHC tetramers generated through chemical- or UV-mediated exchange were equally capable in detecting the frequencies of $CD8^+$ T cells (i.e. 4.16% and 4.03%, respectively) of the correct specificity only and with minimal background. This result could be replicated in an alternative $CD8^+$ T cell line reactive towards Influenza A $M1_{13–21}$ peptide (**10**) when presented by A*11:01 (Figure 3b, bottom row), and reductive exchange of Abc ligands was comparably successful for human HLA-A*02:01 and murine H2-K^b tetramers (Figure S15 and Figure S16, respectively).

Finally, it is vital that cleavage conditions do not alter any functionality on the replacement epitope. Such modifications could pose problems when they occur on critical residues that anchor the peptide to the MHC or are important for T cell receptor engagement, possibly resulting in failure to identify a given T cell population.[5] We therefore compared reductive (10 mM dithionite) with oxidative (0.3 mM periodate) cleavage conditions on well-established T cell epitopes containing said residues. Incubation with periodate predictably cleaved the N-terminal Ser of A*11:01-restricted Influenza A $MP_{13–21}$ epitope (**10**), and (partially) oxidized the Cys and Met of EBV $BMLF\text{-}1_{259–267}$ epitope (**11**), whereas dithionite treatment left the epitopes unaffected (Figure 4 and Figure S17).

In Summary, we have established a truly bioorthogonal and robust strategy for conditional peptide exchange based on a unique panel of chemolabile Abc ligands that can provide functional libraries of T cell labeling reagents for human MHC molecules frequently found in both Asian and Caucasian populations, as well as for murine MHC molecules. The true value of our method lies in the facile epitope replacement, without the need for dedicated UV-irradiation equipment, under conditions that are detrimental to neither the

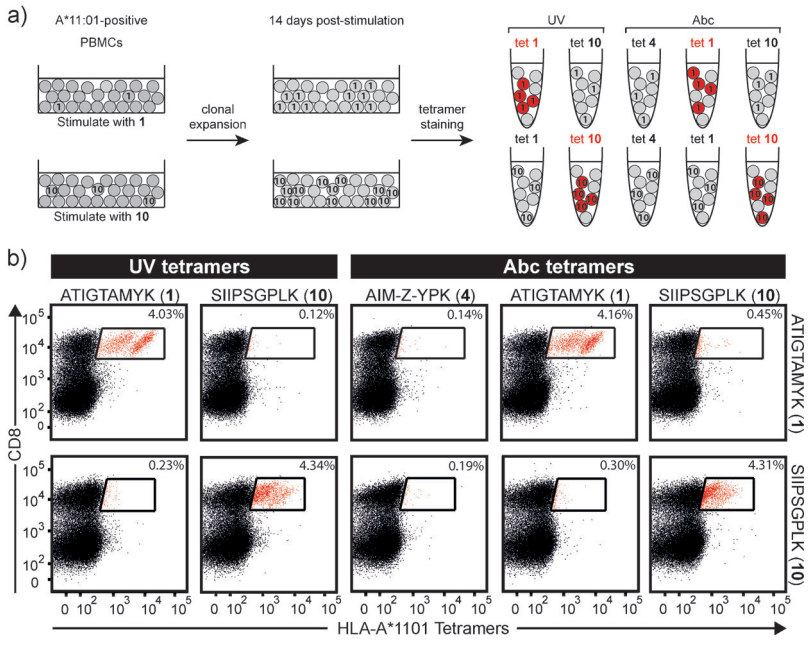

Figure 3. Detection of antigen-specific $CD8^+$ T cells by using A*11:01 MHC tetramers generated from UV-mediated peptide exchange or sodium dithionite mediated peptide exchange. a) A diagram of experimental workflow. Freshly isolated PBMCs from an A*11:01-positive volunteer were stimulated with A*11:01-restricted epitopes EBV $BRLF1_{134-142}$ (**1**, upper row) or Influenza A MP_{13-21} (**10**, lower row) and clonally expanded for 14 days. Antigen-specific $CD8^+$ T cells were then labeled with cognate peptide-bound A*11:01 MHC tetramers (red) and detected by flow cytometry. b) A*11:01-EBV $BRLF1_{134-142}$ (columns 1 and 4), A*11:01-Influenza A MP_{13-21} (columns 2 and 5) and A*11:01-Abc (column 3) tetramers were incubated with the PBMCs to detect $CD8^+$ T cells specific for EBV $BRLF1_{134-142}$ (**1**, upper row) and Influenza A MP_{13-21} (**10**, lower row) 14 days post-stimulation. The numbers in each plot represents tetramer-positive cells as a percentage of the total $CD8^+$ cell population. The plots in columns 1 and 2, and columns 3 to 5 are tetramer staining performed with UV-derived and Abc-derived tetramers, respectively.

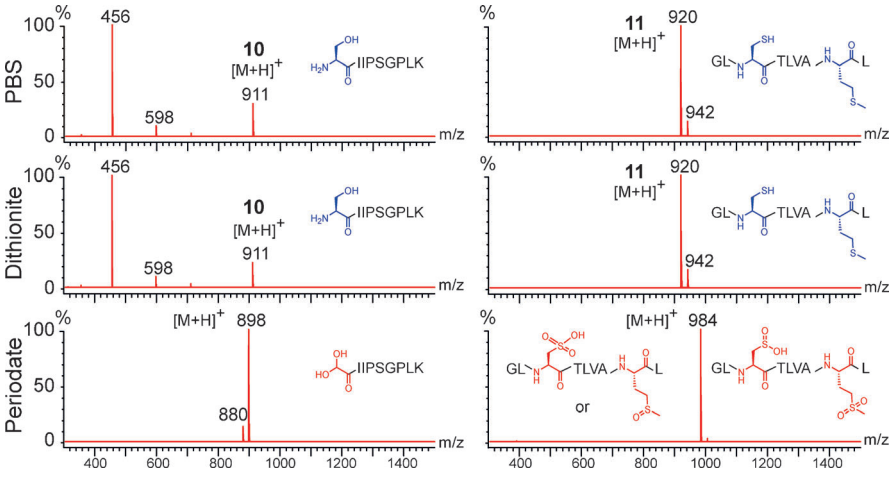

Figure 4. The effects of reductive and oxidative cleavage conditions on epitopes of interest. Mass spectrometry analysis of peptides Influenza A MP_{13-21} (**10**, left column) and EBV $BMLF\text{-}1_{259-267}$ (**11**, right column) after incubation with PBS buffer containing 10 mM sodium dithionite or 0.3 mM sodium periodate. **10** and **11** remained unmodified when incubated in PBS or 10 mM sodium dithionite. The N-terminal serine of **10** was cleaved when incubated in 0.3 mM sodium periodate. Incubation with 0.3 mM sodium periodate resulted in the oxidation of cysteine and methionine residues in **11**. Unmodified and modified residues are shown in blue and red, respectively.

protein, the epitope, nor to the cells. Broad population coverage, through the inclusion of diverse MHC allelic variants, is currently under development since we believe that this will allow the widespread application of this high-throughput method, with which we can tackle the sprawling diversity of biologically relevant T cell populations in both basic research and clinical settings.

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