Immunochemical approaches to monitor and modulate the adaptive immune system

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Production and thermal exchange of conditional peptide-MHCI multimers

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Abstract

Cytotoxic CD8+ T cells mediate cellular immunity through recognition of specific antigens presented by MHC class I on all nucleated cells. Studying T cell interactions and responses provides invaluable information on infection, autoimmunity and cancer. Fluorescently-labeled multimers of MHCI can be used to quantify, characterize and isolate specific CD8+ T cells using flow cytometry. In this unit we describe the production and use of conditional MHCI multimers that can be loaded with peptides of choice just by incubating them with the desired peptide at a defined temperature. These multimers are folded with a template peptide that forms a stable complex at low temperature, but dissociates at a defined elevated temperature. Using this technology multiple MHCI multimers can be generated in parallel, to allow staining and isolation of large sets of antigen-specific CD8+ T cells, especially when combined with barcoding technologies.

Introduction

Major histocompatibility class I (MHCI) molecules complexed with antigenic peptides and multimerized on a streptavidin backbone are the classical reagents to visualize, characterize and isolate antigen-specific CD8+ T cells[1]. By labeling peptide-MHCI (pMHCI) multimers with a fluorophore they can be used for analysis and isolation of CD8+ T cells specific for a given antigen using flow cytometry. Many

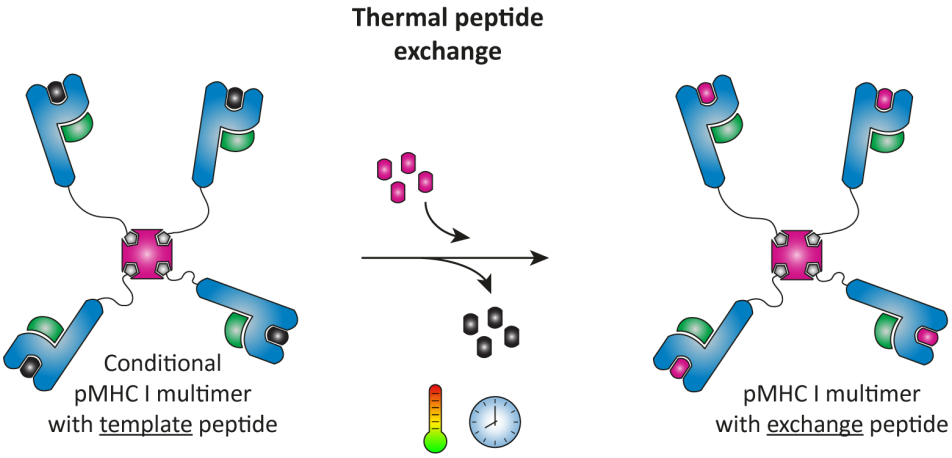

Figure 1. **Schematic representation of thermal peptide exchange on MHCI multimers.** Conditional MHCI monomers are folded with a low-affinity peptide, multimerized using streptavidin and stored at -80°C. To induce exchange conditional pMHCI multimers are warmed up in the presence of a peptide of interest.

different specificities can be identified in parallel using combinatorial coding, mass cytometry or DNA barcoding technologies[2-4]. Conventional production of pMHCI complexes is a laborious process: for every T cell specificity a new pMHCI complex with a different peptide has to be produced, as MHCs are unstable without peptide and can therefore not be folded empty[5]. We have recently reported a peptide exchange technology that allows generation of a large batch of pMHCI multimers and exchange of the peptide using thermal dissociation (Fig. 1)[6]. We have established exchange conditions for the most common human MHCI allele in the Caucasian population, HLA-A*02:01, and the murine allele H-2K^b. The design of template peptides suitable for thermal exchange on other MHCI alleles requires careful selection of proper peptides that dissociate under low-temperature conditions and the generation of conditional multimers for those alleles is anticipated in the near future.

In Basic Protocol 1 of this unit we describe the production of conditional pMHCI monomers and in Basic Protocol 2 we describe the validation of thermal dissociation and peptide-mediated stabilization. The procedures for multimerization and exchange are described in Basic Protocols 3 and 4. In the support protocols we describe the expression and purification of MHCI heavy chain and β2m inclusion bodies, folding of β2m and determination of the biotinylation efficiency.

BASIC PROTOCOL 1: FOLDING, BIOTINYLATION AND PURIFICATION OF CONDITIONAL pMHCI MONOMERS

The procedure for folding and biotinylation of MHCI complexes is based on previously described protocols, with some adaptations[7-9]. Conditional complexes are produced from MHCI heavy chain inclusion bodies, prefolded β2m (described in Support Protocol 2) and a template peptide (IAKEPVHGV for HLA-A*02:01 or FAPGNAPAL for H-2K^b). The MHCI heavy chain contains a 15-amino acid C-terminal recognition sequence for the BirA biotin ligase, which enzymatically conjugates a biotin molecule to the lysine in that sequence. The degree of biotinylation of pMHCI monomers following Basic Protocol 1 should be (near) complete, but it is recommended to determine the degree of biotinylation for each batch of pMHCI as described in Support Protocol 3. The present protocol describes a 50-ml folding reaction, but can be scaled up as desired. Alternative steps for large-scale folding reactions are mentioned when applicable.

Materials

Denaturing buffer (8 M urea/100 mM Tris•Cl, pH 8)
MHCI heavy chain inclusion bodies (Support Protocol 1)

Folding buffer (see recipe)
Template peptide: IAKEPVHGV for HLA-A*02:01; or FAPGNAPAL for H-2K^b (commercial sources or prepared through standard solid-phase peptide synthesis)
Dimethyl sulfoxide (DMSO)
Prefolded β2m (Support Protocol 2)
Milli-Q water
MHC buffer (300 mM NaCl/20 mM Tris•Cl, pH 8)
Biotinylation solution (see recipe)
Glycerol
Liquid nitrogen (for freezing)

1.5-ml reaction tubes
Rotator
50-ml conical tubes
Ice bucket with ice
Sonicator
Microcentrifuge
Syringe and 0.22- and 0.45-µm syringe filters
Water bath at 10°C
30-kDa MWCO centrifugal concentrators, 0.5- and 15-ml (e.g., Amicon Ultra centrifugal filters; Merck Millipore)
Illustra NAP-10 column (GE Healthcare)
Spin-X centrifuge tube filters 0.22 µm (Corning)
FPLC system with gel-filtration column (e.g., Superdex 75 10/300 (GE Healthcare))
PCR tubes or 1.5-ml polypropylene screw cap microcentrifuge tubes (Sarstedt)
Additional reagents and equipment for SDS gel electrophoresis and staining of proteins[10,11] and gel-filtration chromatography[12]

Folding of pMHCI complexes

1. Prepare 500 µl fresh denaturing buffer.
2. In a 1.5-ml reaction tube dissolve ~2.5 mg of MHCI heavy chain inclusion bodies (Support Protocol 1) in 500 µl denaturing buffer. Rotate at room temperature for at least 2 hr and preferably overnight to ensure complete dissolution.
3. Set up 50 ml folding buffer in a 50-ml conical tube, rotate ~15 min at RT, and then cool on ice for 1-1.5 hours.
4. In the meantime, dissolve 3 mg of template peptide in ~500 µl DMSO and sonicate 10-15 min.

Peptides that contain hydrophobic amino acids dissolve poorly in polar solvents, such as PBS or water. Therefore, it is recommended to dissolve peptides in DMSO and store them as 10-mM stocks at -20°C. Sonication of freshly prepared solutions, preferably in a warm water bath, improves solubility.

5. Add template peptide solution to 50 mL of folding buffer (60 µM final concentration).
6. Thaw a 1.2-mg aliquot of prefolded β2m (Support Protocol 2).
7. Microcentrifuge β2m and MHCI heavy chain for 2 min at 16,000 × *g* , save 1 µl of each supernatant at -20°C as a reference for SDS-PAGE analysis (step 18), and add the remainder of each supernatant to the folding buffer containing template peptide (final concentrations: 6 µM β2m and 3 µM MHCI heavy chain).
8. Filter the folding solution through a 0.22-µm filter using a syringe and leave in a 10°C water bath for 4-5 days.

Large-scale reactions can be filtered using a bottle-top filter.

Biotinylation of pMHCI complexes

NOTE: Folded complexes will dissociate at elevated temperatures, so from this point, keep all solutions and reagents on ice and centrifuges at 4°C!

9. Sediment aggregates in the folding solution by centrifugation for 10 min at 4,000 × *g*, 4°C, and filter supernatant through a 0.45-µm filter using a syringe.

Depending on the purity of the inclusion bodies some protein aggregates may form during folding. Removing precipitates by centrifugation and filtering prevents obstruction of the filters used for concentration.

10. Wash a 15-ml 30-kDa MWCO centrifugal concentrator first with Milli-Q water and then with MHC buffer by centrifugation for 10 min each at 4,000 × *g*, 4°C. Add the fitlered folding reaction (from step 9) and concentrate to ≤1 ml by centrifugation for 10 min at 4,000 × *g*, 4°C.

For concentration of large-scale reactions use a 30 kDa MWCO PES Vivaflow 200 protein concentrator system (Sartorius), driven by a peristaltic pump.

11. In a cold room, recover concentrated sample and exchange folding buffer for MHC buffer using a NAP-10 column: wash the column 3 times with 1 ml MHC buffer, apply sample, and elute with an additional 1 ml MHC buffer.
12. Filter concentrated folding reaction through a SpinX centrifuge tube filter by centrifugation for 2 min (or longer if necessary) at 16,000 × *g*, 4°C.
13. Prepare biotinylation solution (see recipe) on ice, and add 1 ml biotinylation

solution to the 1 ml pMHCI solution. Incubate overnight at 4°C, preferably on a rotator.

The enzymatic activity of BirA biotin ligase is low at 4°C and therefore the biotinylation reaction requires overnight incubation (~16 h).

Purification of biotinylated pMHCI

14. Sediment aggregates in the folding solution by centrifugation for 10 min at 4,000 × *g*, 4°C.

    Some precipitation may form overnight. Sedimentation prevents obstruction of the filters used for concentration.

15. Wash a 0.5-ml 30-kDa MWCO centrifugal concentrator with Milli-Q water and then with MHC buffer. Concentrate biotinylation reaction to ~500 µl by centrifugation for 10 min at 16,000 × *g*, 4°C.
16. Filter concentrated folding reaction through a SpinX centrifuge tube filter by centrifugation for 2 min (or longer if necessary) at 16,000 × *g*, 4°C.
17. Purify biotinylated pMHCI complexes by gel-filtration chromatography at 4°C, for example, using an FPLC system equipped with a Superdex 75 10/300 column (GE Healthcare).

    One 50-ml folding reaction typically yields between 0.1 and 2 mg of folded complex depending on the peptide and MHCI allele. The total volume can be concentrated to ~500 µl for one injection on an S75 10/300 column. Larger-scale reactions yield higher protein quantities and should be purified in multiple 500-µl runs or using a larger column, such as an S75 16/600.

18. Analyze fractions using SDS-PAGE. For reference include a protein standard, such as SeeBlue™ Pre-stained Protein Standard, and the reference MHCI heavy chain and β2m samples (set aside in step 7). Figure 2 shows a typical FPLC chromatogram and corresponding gel.

    We typically run our complexes on a 10% Bis-Tris gel for 30 min at 200V in MES buffer. On a denaturing gel, the complex dissociates and two bands will be visible: one at ~36 kDa corresponding to the heavy chain, and one at ~10 kDa, corresponding to β2m. The peptide is too small to visualize on gel.

19. Pool fractions that contain pMHCI, and concentrate to 2-5 mg/ml using a 15-ml 30-kDa MWCO centrifugal concentrator (prewashed with Milli-Q water and MHC buffer).

    pMHCI complexes are more stable at higher concentrations.

The concentration can be measured using a Nanodrop spectrophotometer and the Lambert-Beer Law: $c = A/\varepsilon \times L$. The extinction coefficient (ε) at OD_{280} can be estimated from the number of tryptophans (W) and tyrosines (Y) in the protein sequence according to the following formula: $\varepsilon = (nW \times 5{,}500) + (nY \times 1{,}490)$.

20. Determine the volume of the sample and add glycerol to a final concentration of 15%.

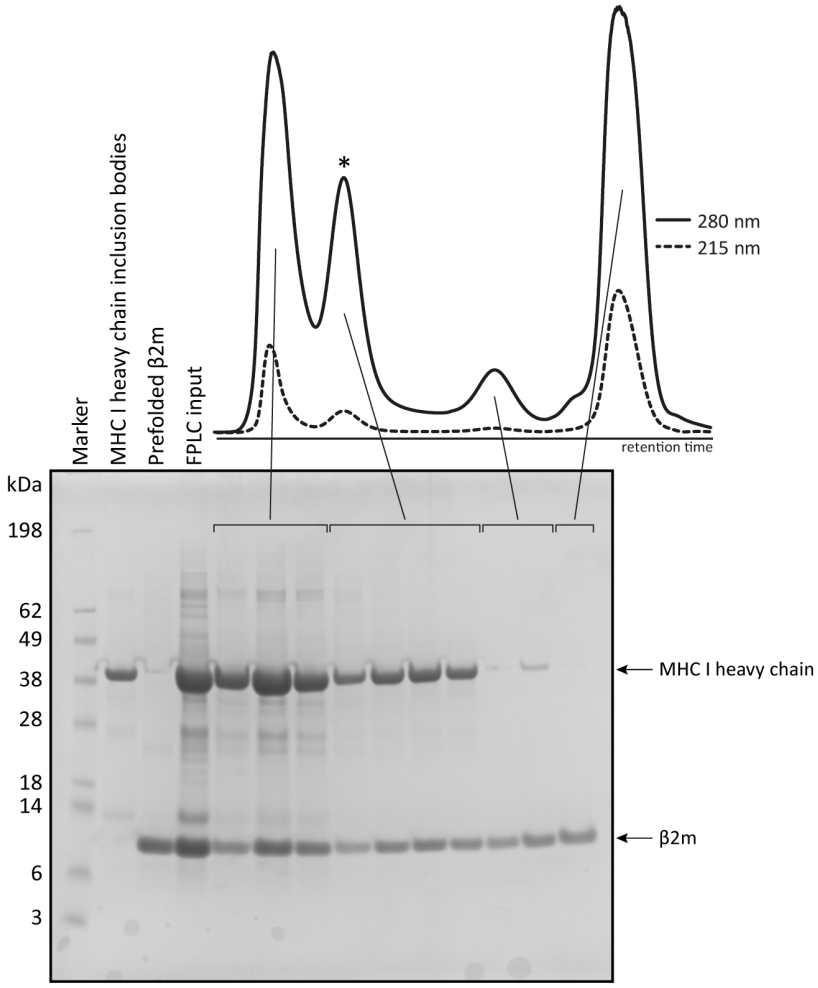

Figure 2. **SDS-PAGE analysis (10% Bis-Tris gel in MES running buffer) of a typical pMHCI purification using FPLC.** The first peak in the chromatogram contains mostly aggregates, whereas the second peak (marked with an asterisk) contains properly folded, pure pMHCI complexes, visible on this denaturing gel as two separate bands for the heavy chain (~36 kDa) and β2m (~10 kDa). Later peaks contain free heavy chain and free β2m. A protein marker, MHCI heavy chain inclusion bodies and prefolded β2m are included for reference.

Glycerol is added as a cryoprotectant. It forms strong hydrogen bonds with water molecules, thus preventing the formation of ice crystals that can damage proteins.

21. Aliquot the sample into PCR tubes or 1.5-ml polypropylene screw cap tubes, depending on the desired volume. Snap-freeze aliquots in liquid nitrogen and store at -80°C.

    We typically store aliquots of 5, 10 and 25 µl. Frozen conditional pMHCI monomers can be stored at -80°C for at least a year. We recommend validation of the exchange performance (Basic Protocol 2) before moving on to multimerization.

BASIC PROTOCOL 2: CONFIRMING THERMAL EXCHANGE PERFORMANCE OF CONDITIONAL pMHCI MONOMERS BY GEL FILTRATION HPLC

For each batch of conditional pMHCI monomers, the thermal exchange performance should be validated. This protocol describes the use of gel filtration HPLC to confirm exchange at pre-established conditions, but can also be used to test additional exchange times and temperatures. When incubated at a higher temperature without peptide, the pMHCI monomer peak should disappear (Fig. 3, magenta line compared to black line), but in the presence of an exchange peptide the complex is stabilized and the peak should remain visible (Fig. 3, green line). For efficient stabilization the exchange peptide should have a higher affinity for its corresponding MHCI than the template peptide (<4,000 nM for H-2K^b-FAPGNAPAL and <7,288 nM for HLA-A*02:01). A link to an affinity prediction tool is provided in Internet Resources.

Materials (also see Basic Protocol 1)

Conditional pMHCI monomers (Basic Protocol 1)

Cold phosphate-buffered saline (PBS, pH 7.4, tablets reconstituted in 500 ml demineralized water; Gibco)

10 mM exchange peptide in DMSO stock, higher affinity than the template peptide (e.g., cytomegalovirus peptide NLVPMVATV for HLA-A*02:01 or ovalbumin peptide SIINFEKL for H-2K^b)

HPLC system with gel filtration column (e.g., 300 × 7.8 mm BioSep SEC-s3000, Phenomenex, cat. no. 00H-2146-K0)

PCR machine, Thermoblock or incubator

Testing the stability of pMHCI monomers at room temperature

1. Thaw conditional pMHCI monomers on ice. Typically, a 5 µl aliquot of 2-5 mg/ml pMHCI is enough for four to ten 100-µl injections of 0.5 µM pMHCI monomers.
2. In a 1.5-ml screw-cap microcentrifuge tube, dilute conditional pMHCI monomers to a 0.5 µM in PBS.

   We typically prepare 10% extra to allow for variance when drawing up the sample for injection.

3. Sediment aggregates by centrifugation for 1 min at 16,000 × *g*, room temperature.
4. Analyze the sample by HPLC using a gel filtration column, such as a 300 × 7.8 mm BioSep SEC-s3000 column (Phenomenex) with PBS as running buffer.

   *Analysis of this sample provides information on the stability of the conditional complex at room temperature. Expect to see a sharp peak when injecting HLA-A*02:01-IAKEPVHGV, but no peak when injecting H-2K^b-FAPGNAPAL, which is unstable at room temperature (see Fig. 3, pMHCI input; black lines).*

Analysis of peptide-mediated stabilization of pMHCI monomers post thermal exchange

5. Prepare 0.5 µM pMHCI in PBS containing 50 µM exchange peptide to confirm stabilization of the exchanged complex.
6. Incubate at established exchange conditions in a PCR machine, Thermoblock or incubator (3 hr at 32°C for HLA-A*02:01; 5 min at room temperature for H-2K^b).
7. Sediment aggregates by centrifugation for 1 min at 16,000 × *g*, room temperature.
8. Analyze by gel-filtration HPLC.

   When incubated with an exchange peptide, the MHC monomer peak should be at least as high as the input peak.

Thermal dissociation of conditional pMHCI monomers

9. Prepare a 0.5 µM pMHCI solution in PBS.
10. Incubate at established exchange conditions in a PCR machine, Thermoblock or incubator (3 hours at 32°C for HLA-A*02:01 or 5 min at room temperature for H-2K^b).
11. Sediment aggregates by centrifugation for 1 min at 16,000 × *g*, room temperature.
12. Analyze by gel-filtration HPLC.

*At the optimal exchange conditions (3 hr at 32°C for HLA-A*02:01, and 5 min at room temperature for H-2K^b) the monomer peak should virtually disappear when incubated without peptide (Fig. 3).*

BASIC PROTOCOL 3: MULTIMERIZATION OF CONDITIONAL pMHCI MONOMERS

The low affinity of a T cell receptor (TCR) for pMHCI monomers enables sequential activation of multiple T cells by one pMHC in vivo. Through multimeric binding, the avidity of pMHC binding to TCRs becomes sufficiently high to stably label specific CD8$^+$ T cells for visualization and isolation[13]. Therefore, pMHCI monomers are biotinylated for multimerization on streptavidin in order to create tetrameric complexes. In addition, labeled streptavidin can be used to incorporate desired

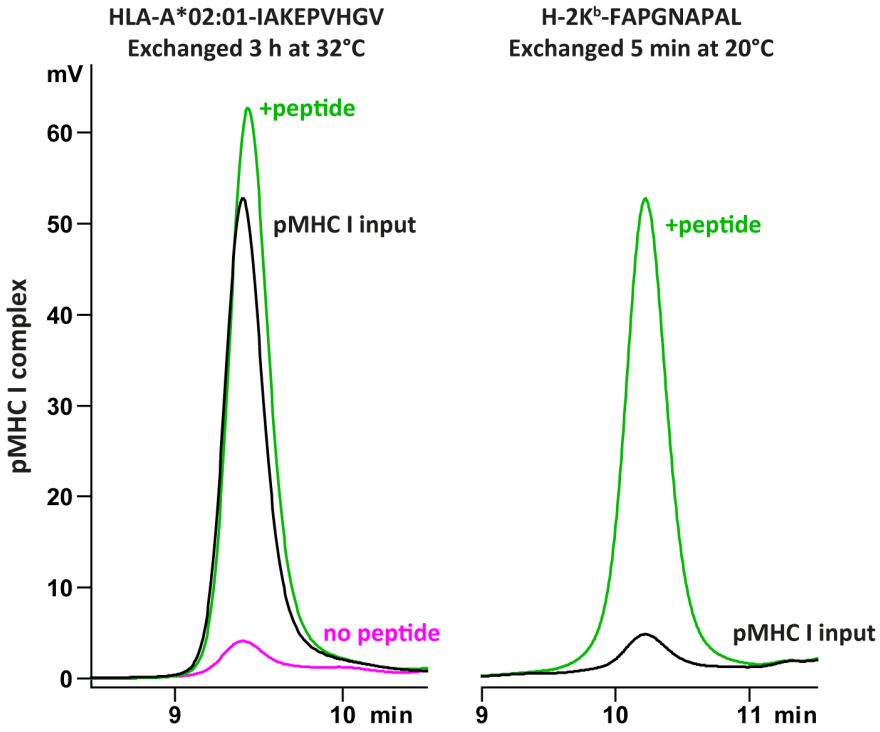

Figure 3. **Overlay of typical gel filtration HPLC chromatograms that confirm thermal exchange of conditional HLA-A*02:01 monomers (left) and H-2K^b monomers (right).** When incubated without peptide the HLA-A*02:01 monomer peak (0.5 µM) disappears (no peptide, magenta line), indicating dissociation, whereas in presence of a high-affinity peptide (50 µM) the complex remains stable (+peptide, green line) compared to nonincubated monomers (pMHCI input, black line). H-2K^b-FAPGNAPAL dissociates at room temperature (pMHCI input black line), which can be 'rescued' by addition of a high-affinity peptide (+peptide, green line).

fluorophores. Allophycocyanin (APC) and phycoerythrin (PE) are typically used, but other fluorophores have also been successfully used in combination with pMHCI multimers. This protocol describes the preparation of 80 µl pMHCI multimer solution (0.625 µM final), but the reaction can be scaled depending on concentration and volume of pMHCI aliquots.

Materials (also see Basic Protocols 1 and 2)

Biotinylated conditional pMHCI monomers (Basic Protocol 1)

1 mg/ml APC-conjugated streptavidin (SA-APC; Thermo Fisher Scientific, Invitrogen, cat. no. S868) or 1 mg/ml PE-conjugated streptavidin (SA-PE; Thermo Fisher Scientific, Invitrogen, cat. no. S866)

Cold glycerol

NOTE: Folded complexes will dissociate at elevated temperatures, so keep all solutions and reagents on ice and (micro)centrifuges at 4°C!

1. On ice, dilute biotinylated conditional pMHCI monomers in cold PBS to a concentration of 5 µM.

2a. If making APC-labeled multimers: Add 53.6 µl cold PBS to 10 µl of pMHCI monomers.

2b. If making PE-labeled multimers: Add 52.4 µl cold PBS to 10 µl of pMHCI monomers.

3. Add either 1.4 µl SA-APC or 2.6 µl SA-PE. To ensure saturation of all four biotin-binding sites, add streptavidin conjugates stepwise. For example, three 0.47-µl additions of SA-APC or three 0.87-µl additions of SA-PE at 5-min intervals.

   To saturate all four of streptavidin's binding sites, 0.125 µM streptavidin should be added to 0.5 µM pMHCI monomer, corresponding to 2 µg of SA-APC (molecular weight ~160 kDa) or 3.75 µg of SA-PE (molecular weight ~300 kDa) per 100 µl pMHCI solution. To ensure binding sites are fully saturated, we add 70% of these amounts for a ratio of ~6 pMHCI monomers to 1 streptavidin. With an excess of streptavidin not all binding sites would be saturated, resulting in the formation of lower order MHCI multimers that poorly bind T cells due to lower avidity. Ideally a little residual MHCI monomer remains present, ensuring full saturation of streptavidin.

4. Add 15 µl cold glycerol and mix well.

5. Aliquot into PCR tubes or 1.5-ml polypropylene screw cap tubes, depending on the desired volume. Snap-freeze aliquots in liquid nitrogen and store at –80°C.

   We typically prepare 8-µl aliquots. Frozen pMHCI multimers can be stored at –80°C for at least a year.

BASIC PROTOCOL 4: THERMAL PEPTIDE EXCHANGE ON CONDITIONAL pMHCI MULTIMERS

This protocol describes the thermal exchange of conditional pMHCI multimers for any number of desired peptides in parallel. Conditional multimers are temperature-labile and should be kept on ice until a peptide is added. For efficient stabilization the exchange peptide should have a higher affinity for its corresponding MHCI than the template peptide (<4,000 nM for H-2K^{b}-FAPGNAPAL and <7,288 nM for HLA-A*02:01). One 10 µl aliquot of exchanged multimer is typically enough to stain ten to twenty samples, each containing 1,000,000 peripheral blood mononuclear cells (PBMCs).

Materials (also see Basic Protocols 1 to 3)
10 mM exchange peptide(s) stock solution
Conditional pMHCI multimers (Basic Protocol 3)

1. Dilute 10 mM exchange peptide stock(s) to 250 µM in PBS.

   Hydrophobic peptides do not readily dissolve in PBS. Therefore it is recommended to dissolve peptides in DMSO and store them as 10-mM stocks at -20°C.

2. Take an 8-µl aliquot of MHCI multimer from freezer and immediately place on ice.

   Conditional MHCI multimers may dissociate at room temperature and should be kept cold prior to exchange, so make sure to keep them on ice when moving them from freezer to bench. Especially H-2K^{b}-FAPGNAPAL is prone to rapid dissociation and should remain frozen until an exchange peptide is added.

3. Add 2 µl 250 µM exchange peptide solution to frozen 8 µl MHCI multimer. As the mixture thaws, briefly pipette up and down to mix.
4. Briefly spin and incubate exchange reactions in a PCR machine, Thermoblock or incubator at defined temperature and time to induce exchange.

   *H-2K^{b}-FAPGNAPAL readily exchanges within 5 min at room temperature. Exchange of HLA-A*02:01-IAKEPVHGV multimers is complete after a 3-hr incubation at 32°C.*

5. Briefly spin the tubes. The exchanged multimers are now ready for staining of T cells.

   Exchanged multimers can be stored at 4°C and used for at least a week

without loss of function. They can typically be diluted 1:40 dilution to stain 1,000,000 PBMCs in 40 µl of FACS buffer.

SUPPORT PROTOCOL 1: BACTERIAL EXPRESSION AND PURIFICATION OF MHCI HEAVY CHAIN AND β2M INCLUSION BODIES

The procedure for bacterial expression and purification of MHCI heavy chain and β2m inclusion bodies is based on protocols described previously[7-9]. Both proteins can be expressed in parallel following the same steps. This protocol describes expression in 2 L and can be scaled up or down accordingly.

Materials

Competent *E. coli* strain BL21 (DE3) (Novagen, cat. no. 69450)
MHCI heavy chain and human β2m expression constructs (see recipe)
Liquid LB medium (sterilized, e.g., BD Difco™ LB Broth, cat. no. 244620)
Ampicillin (Roche Diagnostics)
1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) in de-ionized water
Lysis buffer (see recipe)
10 mg/ml lysozyme (Roche Diagnostics) in lysis buffer
1 M $MgCl_2$ in de-ionized water
1 M $MnCl_2$ in de-ionized water
10 mg/ml DNase I stock (see recipe)
Detergent buffer (see recipe)
Wash buffer (see recipe)

Incubator (shaking and stationary)
LB agar plate containing 50 µg/ml ampicillin
Sterile pipette tip
Inoculation tube (with foil or cap)
Spectrophotometer and cuvettes
2-L Erlenmeyer flasks
High-speed centrifuge and 250-ml to 1-liter buckets
Sonicator

Protein expression in E. coli

1. Express MHCI heavy chains and β2m separately. Transform 100-200 ng plasmid DNA (MHCI heavy chain or β2m) into 50-100 µl competent *E. coli* cells in a reaction tube for 30 min on ice, 2 min at 42°C, 5 min on ice, respectively.
2. Add 500 µl LB medium and incubate 30-60 min at 37°C with shaking.
3. Plate 200 µl of inoculate on an LB agar plate containing 50 µg/ml ampicillin,

and incubate overnight at 37°C.

The LB plate can be stored at 4°C for up to 4 days.

4. Use sterile pipette tips to select two single colonies from the LB plate, and drop each tip into an inoculation tube containing 10 ml liquid LB medium with 50 µg/ml ampicillin. Cover the tube loosely with foil or a cap that is not air tight and incubate ~6 hr with shaking to an OD_{600} of 0.8, and then store at 4°C overnight.
5. Add the 10-ml inoculates to 2L of liquid LB medium containing 50 µg/ml ampicillin. Divide between four 2-L Erlenmeyer flasks and incubate the cultures at 37°C with shaking to an OD_{600} of 0.6.

   The cultures should reach an OD_{600} of 0.6 in 3 to 4 hr. Bacteria grow exponentially, so check regularly.

6. Take a 1-ml sample of the culture for SDS-PAGE analysis. Pellet bacteria by centrifugation for 10 min at 12,000 × *g*, 4°C. Discard supernatant ans store pellet at -20°C.
7. Induce protein expression by adding 200 µl of 1 M IPTG to each Erlenmeyer flask containing 500 ml *E. coli* cell culture (fincal concentration, 0.4 mM IPTG).
8. Incubate ~4 hr at 37°C with shaking.
9. Take a 0.5-ml sample for SDS-PAGE analysis. Pellet bacteria by centrifugation for 10 min at 12,000 × *g*, 4°C. Discard supernatant ans store pellet at -20°C.
10. Harvest the remainder of the induced bacteria by centrifugation for 15 min at 4,000, 4°C. Suspend the cell pellet(s) 25 ml lysis buffer per 2-L culture, transfer the suspension to a 50-ml conical tube. Store suspended cells at -80°C for at least a year or -20°C for a few days.

Isolation and purification of inclusion bodies

11. Thaw the bacteria from 2 L culture.
12. Once the suspension is thawed, add 2.5 ml lysozyme (10 mg/ml in lysis buffer), and incubate 20 min on ice or on a rotator in a cold room.

    The solution must become viscous before proceeding.

13. Add the following:
    275 µl 1 M $MgCl_2$ stock (10 mM final)
    27.5 µl 1 M $MnCl_2$ stock (1 mM final)
    27.5 µl 10 mg/ml DNase I (10 µg/ml final)

14. Incubate 30 min at room temperature.

    The solution must become fluid.

15. Sonicate at 50% for 2 min with 20 s on, 20 s off intervals.
16. Centrifuge lysates for 10 min at 12,000 × *g*, 4°C, and discard the supernatant.
17. Add 25 ml detergent buffer, and sonicate at 30% for 30 2 with 10 s on, 10 s off intervals.
18. Centrifuge lysate 10 min at 12,000 × *g*, 4°C, and discard the supernatant.
19. Add 20 ml wash buffer, and sonicate at 30% for 30 s with 10 s on, 10 s off intervals.
20. Centrifuge lysate 10 min at 12,000 × *g*, 4°C, and discard the supernatant.
21. Repeat steps 19 and 20 twice.
22. Add 20 ml wash buffer without Triton, and sonicate at 30% for 30 s with 10 s on, 10 s off intervals.
23. Centrifuge lysate 10 min at 12,000 × *g*, 4°C, and discard the supernatant.
24. Repeat steps 22 and 23.
25. Suspend inclusion bodies in 10 ml wash buffer without Triton, and measure the protein concentration, e.g., using the Bradford assay.

    Depending on the construct, expression yields are between 50 and 250 mg/l with a protein purity of 90-98%.

26. Prepare desired aliquots, and pellet inclusion bodies by centrifugation for 5 min at 16,000 × *g*, room temperature, and discard the supernatant.

    We recommend freezing aliquots of 2.5 mg (or multiples thereof), since we typically use 2.5 mg inclusion bodies per 50-ml folding reaction. Inclusion bodies can be stored at -80°C for at least a year.

SUPPORT PROTOCOL 2: FOLDING OF HUMAN β2M

Human β2m is used for the production of both human and murine MHCI complexes, because of its higher stability compared to its murine counterpart. Using prefolded β2m for folding of pMHCI ensures stabilization of MHCI and increases folding yields compared to using β2m inclusion bodies.

Materials

Denaturing buffer (8 M urea/100 mM Tris•Cl, pH 8)
Purified human β2m inclusion bodies (Support Protocol 1)
Phosphate-buffered saline (PBS, pH 7.4, tablets (Gibco) reconstituted in 500 ml demineralized water)
10 mM Tris•Cl (pH 7) in PBS
Dialysis tubing (10 kDa MWCO) and large beaker or bucket

1. Suspend pelleted β2m inclusion bodies to 3 mg/ml in freshly-prepared denaturing buffer.
2. Transfer the solution to a dialysis tube, and dialyze overnight against 2 L 10 mM Tris•Cl (pH 7) in PBS at 4°C.
3. The next day, dialyze against two changes of fresh buffer, 4 hr each.

   During dialysis, some β2m will precipitate, that can be collected, dissolved in fresh denaturing buffer and dialyzed in a new dialysis tube for increased protein yield.

4. Transfer dialyzed, folded β2m to a 1.5-ml reaction tube and sediment insoluble material by centrifugation for 20 min at 12,000 × *g*, 4°C.
5. Analyze 10 µl of supernatant and samples from steps 6 and 9 of Support Protocol 1, bySDS-PAGE.

   Suspend pellets from step 6 and 9 of Support Protocol 1 in 100 µl sample buffer, and analyze 10 µl each.

6. Determine the concentration of the folded β2m protein, e.g., using a Bradford assay.
7. Prepare desired aliquots of folded β2m, snap freeze, and store at -80°C.

   A typical 50-ml pMHCI folding reaction requires one aliquot of 1.2 mg β2m.

SUPPORT PROTOCOL 3: DETERMINATION OF THE BIOTINYLATION EFFICIENCY

This protocol describes how to use HPLC to determine the degree of biotinylation of MHCI monomers. Each batch of pMHCI monomers and preferably also each batch of streptavidin should be tested. MHCI multimers will form by addition of streptavidin, and the height of the monomer peak in the chromatogram will decrease with increasing ratios of streptavidin (Fig. 4). Generally, 90-95% of pMHCI monomers will be biotinylated. When testing highly unstable complexes, such as H-2K^b-FAPGNAPAL, exchange peptide should be added for stabilization. If samples can be measured on a cooled (4°C) HPLC system, the peptide can be omitted.

Materials

Conditional pMHCI monomers (Basic Protocol 1)

Exchange peptide (of higher affinity than the template peptide; e.g., cytomegalovirus peptide NLVPMVATV for HLA-A*02:01 or ovalbumin peptide SIINFEKL for H-2K^b)

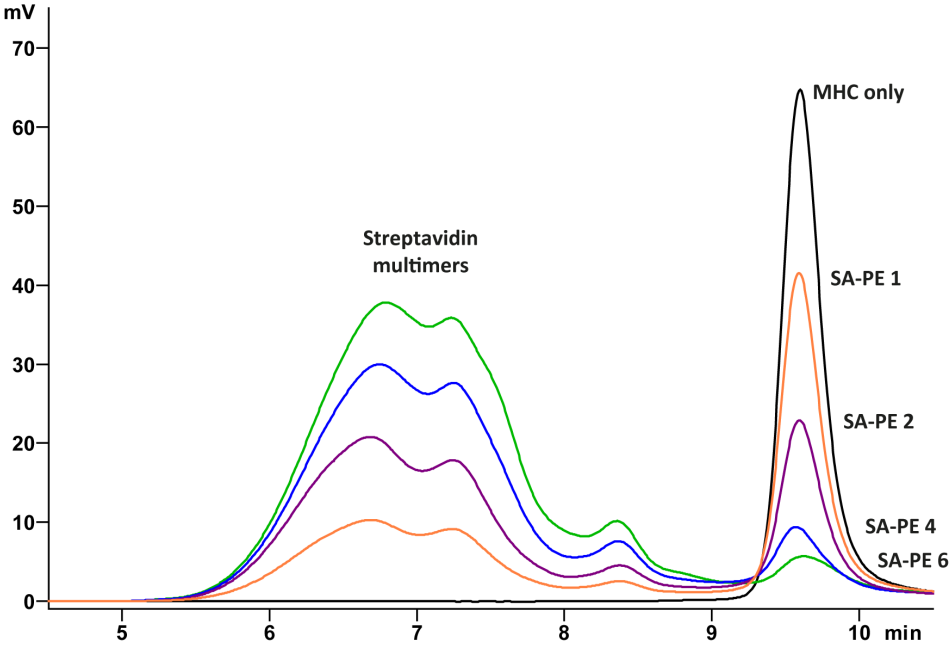

Figure 4. **Overlay of HPLC chromatograms that confirm biotinylation of pMHCI monomers.** When incubated with increasing ratios of PE-conjugated streptavidin (SA-PE) the monomer peak decreases, whereas the streptavidin multimer peak increases, indicating the formation of pMHCI multimers.

1. Prepare five samples in 1.5-ml Sarstedt tubes to determine the biotinylation efficiency in 100 µl pMHCI monomer solution (0.5 µM). Prepare each sample fresh before analysis.

   Add the components in the order listed. Keeping to this order will ensure the peptide is in solution and available for exchange and stabilization of MHCI. We typically prepare 10% extra to allow for variance when drawing up the sample for injection into the HPLC.

   PE can be bleached by UV light. Keep the SA-PE on ice and away from light as much as possible.

	pMHCI only	SA-PE 1	SA-PE 2	SA-PE 4	SA-PE 6
PBS	Fill to 100 µl final volume				
Exchange peptide	Optional, 50 µM final				
pMHCI monomers	0.5 µM final				
SA-PE (µl)	-	1	2	4	6

2. Incubate the sample on ice in the dark for 5 min to allow all biotinylated monomers to bind.
3. Sediment aggregates by centrifugation for 1 min at 16,000 × *g*, room

temperature.

4. Analyze each sample by HPLC using a gel filtration column (e.g., 300 × 7.8 mm BioSep SEC-s3000 column (Phenomenex) with PBS as running buffer.

   By adding sequential ratios of SA-PE the degree of biotinylation of pMHCI monomers can be determined.

Reagents and solutions

Biotinylation solution (1 ml)
40 µl 5 mM D-biotin in 100 mM NaP, pH 7.5 (0.2 mM final concentration)
40 µl 0.5 M ATP in 1 M Tris•Cl, pH 9.5 (20 µM final concentration)
1.5 µg BirA biotin ligase (commercial sources, such as Avidity)
200 µl 10× ligase buffer (50 mM $MgCl_2$ in 0.2 M Tris•Cl, pH 7.5; 10 mM final $MgCl_2$ concentration)
80 µl cOmplete EDTA-free Protease Inhibitor Cocktail (1 tablet in 2 ml Milli-Q water)
640 µl Milli-Q water
Prepare fresh

Detergent buffer
0.2 M NaCl
1% (w/v) sodium deoxycholate monohydrate
1% (v/v) Nonidet P-40 substitute
20 mM Tris•Cl (pH 7.5)
2 mM EDTA
Store up to one year at room temperature

DNase I stock solution, 10 mg/ml
10 mg/ml DNase I (Roche Diagnostics)
50% (v/v) glycerol
150 mM NaCl
Store up to 1 year at -20°C

Folding buffer (50 ml)
4.2 g L-arginine-HCl (400 mM final concentration)
5 ml 1 M Tris•Cl, pH 8 (100 mM final concentration)
0.2 ml 0.5 M EDTA (2 mM final concentration)
5% (v/v) glycerol
Adjust to 47 ml with Milli-Q water
Filter sterilize through a 0.22-µm filter, and store at 4°C for at least a few weeks

Immediately before folding reaction, add:
76.8 mg or 2.5 ml 100 mM reduced glutathione (5 mM final concentration)
16.4 mg or 0.5 ml 50 mM oxidized glutathione (0.5 mM final concentration)
0.5 tablet cOmplete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics)
Adjust to 50 ml total with Milli-Q water if necessary

Lysis buffer
50 mM Tris•Cl (pH 8)
25% (w/v) sucrose
1 mM EDTA
Filter sterilize and store at 4°C for up to 1 year

Triton wash buffer
50 mM Tris•Cl (pH 8)
100 mM NaCl
1 mM EDTA (pH 8)
0.5% (v/v) Triton X-100
Filter sterilize and store at 4°C for up to 1 year

COMMENTARY

Background Information
The ability to distinguish between healthy and infected or mutated cells is crucial for maintaining the balance between immunity and tolerance. This immune recognition is mediated by T cells, the key players of the highly specific adaptive arm of immunity. By displaying peptides derived from intracellular proteins on their surface, all nucleated cells can provide cytotoxic $CD8^+$ T cells with a glimpse of the ongoing processes inside the cell. Upon recognition of a non-self (i.e. viral or mutated) peptide $CD8^+$ T cells become activated, resulting in proliferation and killing of the target cell. After clearance of the infection or cancer most $CD8^+$ T cells disappear, but some remain to become memory T cells. The memory response is much faster than the first response and ensures that the infection will be rapidly cleared in case of re-infection with the same pathogen.

The molecules responsible for presentation of intracellular peptides are major histocompatibility complex class I (MHCI) molecules, heterotrimeric complexes that consist of an immunoglobulin (Ig)-like heavy chain, beta-2 microglobulin (β2m) and the peptide that resides in a binding groove formed by two α helices in the heavy chain. MHCI typically binds peptides of 8-10 amino acids that have been generated by proteasomal processing. These peptides fit in a peptide-binding groove that is closed at two ends, thus fixing the length of peptides.

Longer peptides can bind with the two ends of the peptide fixed and the center of the peptide bulging out of the binding groove. Which peptides bind is determined by the interactions between the amino acid side chains of the peptide and the binding pockets present in the MHC's specific peptide-binding groove. The MHC heavy chain is highly polymorphic, which means that many variants exist due to mutation, recombination and gene conversion. For this reason, MHCI (and MHCII, the other predominant polymorphic protein class) are the major transplantation antigens. Most of the polymorphisms are found in the DNA regions that code for the binding groove and therefore the location and nature of the binding pockets differs between MHCI subtypes. As a consequence each subtype (allele) has its preferred peptide motifs. Every individual has three MHC class I heavy chain genes that in human are named HLA-A, HLA-B and HLA-C. Since these can differ between parents, each human individual expresses three to six different allotypes. This collection of HLAs provides broad protection against intracellular pathogens, since different peptide fragments of their proteins can be presented by different MHCI alleles.

Cytotoxic T cells distinguish between self and non-self peptides through their T cell receptors (TCRs). These TCRs are highly diverse and recognize only specific peptide-MHCI (pMHCI) combinations. The frequency of a specific T cell in circulation is typically low if it has never encountered its cognate antigen and therefore analyzing T cell frequencies in blood or tissue samples provides valuable information on an individual's immune status. In addition, $CD8^+$ T cells are potent targets for immune therapy due to their cytotoxic activity directed only at infected or mutated cells. Characterizing and visualizing $CD8^+$ T cell responses using MHCI multimers enables the study of antigen-specific T cell populations and the efficacy of immune intervention strategies.

To facilitate parallel production of multimers with different specificities a number of peptide exchange technologies have been developed. These methods allow the folding of one large batch of pMHCI monomers and the exchange of the template peptide using chemicals or dipeptides as catalysts, or by cleaving a UV-labile peptide[14-18]. We have recently developed an exchange technology that does not rely on chemicals or UV that can damage the protein, but instead uses temperature to induce exchange (Fig. 1)[6]. Our method was based on the finding that MHCI on-rates of peptides with various affinities are comparable at a range of temperatures, but off-rates increase with temperature[19]. For both HLA-A*02:01 and $H\text{-}2K^b$ we have designed template peptides with an affinity that is high enough for efficient folding at 4°C, but low enough for dissociation at elevated temperatures. This novel exchange technology is superior to preceding techniques in its potential for peptide exchange on MHC multimers, reducing pre-staining handling time even further.

Critical Parameters and Troubleshooting

The folding buffer is an aqueous solution and therefore template peptides with poor water solubility may precipitate, as may the peptides used for exchange, which is performed in PBS. We therefore recommend to use only peptides from stocks in DMSO, preferably sonicated before use, both for folding and exchange. Filtering the folding buffer removes precipitates, thus increasing folding yields.

The conditional monomers and multimers produced through this protocol are sensitive to elevated temperatures. Therefore as soon as they are folded, pMHCI monomers and other reagents used should be kept on ice and (micro)centrifuges should be kept at 4°C. Peptides used for folding should be very pure (>99%). Since the peptides used for thermal exchange have a low affinity for MHCI any impurity in the form of a peptide may result in folding of anincorrect complex. Presence of a truncated higher-affinity peptide may result in a stable complex not suitable for thermal exchange. By checking the exchange performance of every batch of pMHCI monomers this undesirable stabilization can be discovered timely. Likewise, we recommend to determine the degree of biotinylation for every batch of biotinylated pMHCI. Failure to saturate all streptavidin binding sites results in lower order multimers (trimers or even dimers) that may poorly bind TCRs due to lower avidity. This would result in decreased staining efficiency and a higher background signal.

The efficiency of exchange is related to the affinity of the exchange peptide. Lower-affinity peptides are less potent in stabilizing MHCI at elevated temperatures and therefore exchange for low-affinity peptides may be less efficient at the exchange conditions.

Understanding Results

The efficiency of the MHCI folding reaction is dependent on the peptide used for folding; in general folding with higher affinity peptides results in a higher yield. Since the template peptide should have a low affinity to allow temperature-mediated exchange, folding yields are expected to be low. We have previously observed yields of ~25-30% for HLA-A*02:01-IAKEPVHGV (~800-1000 µg from a 50-ml folding reaction) and ~2-5% for H-2K^{b}-FAPGNAPAL (~70-170 µg from a 50-ml folding reaction).

Time Considerations

Folding, biotinylation and purification of pMHCI monomers (Basic Protocol 1) takes 5-6 days, of which 3-4 days are merely incubation time and can be spent otherwise. Monomers can be stored in a -80°C freezer until tested (Basic Protocol 2) or multimerized (Basic Protocol 3), which only takes little time. This is not different from conventional MHCI multimer production. Peptide exchange (Basic Protocol 4) takes only minutes for multimers of H-2K^{b}-FAPGNAPAL and 3 hours

for multimers of HLA-A*02:01-IAKEPVHGV. Bacterial expression of MHCI heavy chains and β2m (Support Protocol 1) takes about a week, including folding of β2m (Support Protocol 2). These procedures need to be executed only occasionally, since large batches can be produced and stored for later use. Determining the biotinylation efficiency (Support Protocol 3) takes a few hours, depending on the HPLC system and column used.

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Internet Resources

http://www.cbs.dtu.dk/services/NetMHC/
Web site used to predict peptide-MHCI binding affinities.