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TITLE:

Stability and Structure of Bat Major Histocompatibility Complex Class I with Heterologous β_2-Microglobulin

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SUMMARY

The protocol describes experimental methods to obtain stable major histocompatibility complex (MHC) class I through potential β_2-microglobulin (β_2m) substitutions from different

species. The structural comparison of MHC I stabilized by homologous and heterologous β_2m were investigated.

ABSTRACT

The major histocompatibility complex (MHC) plays a pivotal role in antigen peptide presentation and T cell immune responses against infectious disease and tumor development. The hybrid MHC I complexed with heterologous β_2-microglobulin (β_2m) substitution from different species can be stabilized in vitro. This is a feasible means to study MHC I of mammals, when the homologous β_2m is not available. Meanwhile, it is indicated that mammalian β_2m substitution does not significantly affect peptide presentation. However, there is limited summarization regarding the methodology and the technology for the hybrid MHC I complexed with heterologous β_2-microglobulin (β_2m). Herein, methods to evaluate the feasibility of heterologous β_2m substitution in MHC I study are presented. These methods include preparation of expression constructs; purification of inclusion bodies and refolding of the MHC complex; determination of protein thermostability; crystal screening and structure determination. This study provides a recommendation for understanding function and structure of MHC I, and is also significant for T cell response evaluation during infectious disease and tumor immunotherapy.

INTRODUCTION

The major histocompatibility complex (MHC) exists in all vertebrates and is a set of genes that determines the cell-mediated immunity to infectious pathogens. MHC class I presents endogenous peptides, such as viral components produced upon virus infection, to T cell receptors (TCR) on the surface of $CD8^+$ T cells to mediate cellular immunity and participate in immune regulation[1]. A structural study of MHC I binding to peptides provides information regarding peptide binding motifs and presentation features by MHC I molecules, which plays vital roles in evaluation of $CD8^+$ T cell immune responses and vaccine development.

Since the first crystallization and structural determination of MHC I molecular by Bjorkman et al.[2], the crystal structure analysis of MHC I molecules has greatly promoted the understanding of how peptides bind to MHC I molecules, and helps to understand the interaction of light chains with heavy chains and peptides. A series of follow-up studies indicated that although the genes encoding the light chain is not associated with the MHC, the light chain is a key protein for the assembly of MHC I molecules[3,4]. It interacts with the three domains of MHC class I molecules on multiple surfaces. When the light chain is absent, MHC class I molecules cannot be correctly expressed on the surface of antigen-presenting cells and cannot interact with TCR to exert their immunological functions.

MHC I is comprised of a heavy chain (H chain) and light chain (i.e., β_2-microglobulin (β_2m)), and is assembled through binding to a suitable peptide[5]. The extracellular segment of the H chain consists of α1, α2 and α3 domains[6]. The α1 and α2 domains form the peptide binding groove (PBG). The β_2m chain acts as a structural subunit of the assembly complex in MHC I, stabilizing the conformation of the complex, and is a molecular chaperone for MHC I H chain folding[7-9]. A series of studies have shown that MHC I H chains from various mammals such as

bat (Chiroptera) (Ptal-N*01:01)[10], rhesus macaque (Primates) (Mamu-B*17)[11] (Mamu-A*01)[12] (Mamu-A*02)[13], mouse (Rodentia) (H-2K^{d})[14,15], dog (Carnivora) (DLA-88*50801)[16], cattle (Artiodactyla) (BoLA-A11)[17] and equine (Perissodactyla) (Eqca-N*00602 and Eqca-N*00601)[18] can combine with heterologous β_2m (**Table 1**). These hybrid molecules are often used in structural and functional studies. However, the methodology for the functional and structural study of the hybrid MHC I with heterologous β_2m is not yet summarized. Meanwhile, the structural basis for the interchanged β_2m between different taxa remain unclear.

Herein, the procedure for MHC I expression, refolding, crystallization, crystal data collection and structure determination are summarized. In addition, potential substitutions of β_2m from different species are analyzed through comparing the structural conformation of MHC I stabilized by homologous and heterologous β_2m. These methods will be helpful for further MHC I structural study and CD8$^+$ T cell immune response evaluation in cancer and infectious disease.

PROTOCOL

1. Preparation of expression constructs

1.1. Retrieve the sequences of MHC class I genes (including predicted genes) from bats from the NCBI database.

1.2. Retrieve higher mammal MHC I heavy chain sequences from the Immuno Polymorphism Database (IPD) (www.ebi.ac.uk/ipd/mhc) and the UniProt database (www.uniprot.org).

1.3. To obtain soluble MHC complexes, mutagenize the sequences to remove the cytosolic and transmembrane regions.

1.4. Clone the genes encoding the ectodomains of the bat Ptal-N*01:01 (GenBank no. KT987929[19]) (residues 1–277) and bat β_2m (GenBank no. XP_006920478.1) (residues 1–98) into pET-28a vectors (Novagen, Beijing, China), respectively.

1.5. Insert the optimized (for *Escherichia coli*) sequences were inserted between the *NcoI* and *EcoR1* sites of a modified pET-28a vector.

1.6. Construct human β_2m expression plasmids as previously described[20].

2. Peptide synthesis

2.1. Peptides prediction

2.1.1. As described previously[10], to screen for peptides that may bind to Ptal-N*01:01, predict candidate peptides using the protein bodies of bat-related viruses hendra virus (HeV). To obtain high affinity peptides based on the structural model from the online server,

NetMHCpan 4.0 server (http://www.cbs.dtu.dk/services/NetMHCpan/)[21] and Rosetta FlexPepDock[22] predicted potential binding in the selected peptide fraction. In addition, other software and databases including BIMAS, the Immune Epitope Database (IEDB), NetCTL 1.2, and SYFPEITHI can also be used, all of them integrating prediction of peptide–MHC class I binding affinity, transporter associated with antigen processing (TAP) transport efficiency, proteasomal C-terminal cleavage and half-time of dissociation of peptide–HLA class I molecules in their readouts.

2.1.2. To predict high-binding peptides, use both the NetMHCpan and Rosetta FlexPepDock server.

NOTE: Unfortunately, neither produced predictions matched the experimental data. This may indicate that current MHC binding peptide predictions were not suitable for non-human and non-mouse mammals such as bats, which may have a different manner of peptide binding. Therefore, we need to combine various prediction software and experimental results for comprehensive analysis to obtain high affinity peptides.

2.2. Peptides preparation and preservation

2.2.1. Rather than generating custom peptides, use specialized service providers. Purchase all peptides commercially following peptide prediction tools, which have a higher binding score. The peptide purity was determined to be >95% by HPLC and mass spectrometry.

2.2.2. Store all purchased peptides as freeze-dried powder at −80 °C and dissolve in dimethyl sulfoxide (DMSO) before use.

3. Purification of inclusion bodies

3.1. Transformation of *E. coli*

3.1.1. Transform 10 ng of plasmid containing bat MHC I Ptal-N*01:01 H chain, or human β_2m or bat β_2m into 100 µL of BL21(DE3) *E. coli*. Bathe in ice for 30 min.

3.1.2. Heat shock at 42 °C for 90 s, followed by bathing in ice for 2 min.

3.1.3. Add 800 µL of lysogeny broth (LB: yeast extract, tryptone and NaCl; see the **Table of Materials**) to step 3.1.2 and shake at 200 rotations per minute (rpm) on a rocking platform at 37 °C for 20 min.

3.1.4. Apply 100 µL of bacterial suspension to the plate containing the corresponding antibiotic resistance (ampicillin: 100 ng/mL), which are same as previous constructs.

3.2. Inoculate cultures

3.2.1. Pick a single recently transformed bacterial clone into 3 mL of LB with antibiotic medium (ampicillin: 100 ng/mL) and culture at 37 °C, while shaking at 200 rpm on a rocking platform to activate the bacteria. Cultures can be inoculated late at night, to be ready for induction the next morning.

3.2.2. One night in advance, transfer 500 µL of the activated bacterial stock into 50 mL of LB with antibiotic medium (ampicillin: 100 ng/mL) and incubate overnight at 37 °C, shaking at 200 rpm. For convenience when preparing the next inoculate, freeze the remaining activated bacterial stock in 1 mL aliquots (500 mL of culture with 500 mL of 20% glycerol) at −80 °C until the next preparation is needed.

3.2.3. To generate large amounts of recombinant protein, transfer the bacterial preparation into 2 L of LB with antibiotic medium (ampicillin: 100 ng/mL) at a ratio of 1:100, incubate at 37 °C while shaking at 200 rpm. Culture until an absorbance of 0.6 at 600 nm is achieved. Add 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) (vary the concentration of IPTG for different MHCs, mostly between 0.1 mM-1 mM) to the flask for induction of protein expression.

3.3. Harvest bacteria

3.3.1. Transfer the bacteria to centrifuge bottles and centrifuge at 2000 x *g* for 20 min at 4 °C. All steps from now on should be performed at 4 °C.

3.3.2. Resuspend the bacteria in 60 mL of phosphate buffer saline (PBS) and liberate the expressed recombinant protein by ultrasonic cell disruptor.

NOTE: In general, the program we set on this machine is ultrasonic 6S, interval 12S, 300 W, 99 times).

3.4. Purification of inclusion bodies

3.4.1. Centrifuge the sonicated bacterial suspension at 12,000 x *g* for 30 min. Discard the supernatant and resuspend the pellet in an appropriate volume of washing buffer (0.5% Triton-100, 50 mM Tris pH 8.0, 300 mM NaCl, 10 mM EDTA, 10 mM DTT). Repeat this process once more.

3.4.2. Centrifuge at 12,000 x *g* for 10–20 min. Discard the supernatant and resuspend the inclusion bodies in resuspension buffer (50 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA, 10 mM DTT). Remove a 20 µL sample (inclusion bodies in resuspension buffer) for SDS-PAGE to test the purity of inclusion bodies.

3.4.3. Centrifuge the remaining preparation at 12,000 x *g* for 10–20 min. Discard the supernatant. Weigh the pellet containing the inclusion bodies and add dissolution buffer (6 M Gua-HCl, 10% glycerin, 50 mM Tris pH 8.0, 100 mM NaCl, 10 mM EDTA) to a final concentration of 30 mg/mL. Stir slowly using a magnetic stirrer at 4 °C until the inclusion bodies are dissolved in the dissolution buffer.

3.4.4. Centrifuge 12,000 x *g* for 10–20 min. Discard the supernatant. Store the inclusion bodies at −20 °C or −80 °C.

NOTE: Before proceeding with the purification of inclusion bodies, confirm that the induction and expression of recombinant protein were successful. Run samples from each culture (taken before and after IPTG induction) on a protein gel; 15% SDS-PAGE (SDS-PAGE concentrated gum: H_2O, 30% acrylamide, 1 M Tris-HCl pH 6.8, 10% SDS, 10% APS and TEMED; SDS-PAGE separation gel: H_2O, 30% acrylamide, 1.5 M Tris-HCl pH 8.8, 10% SDS, 10% APS and TEMED; see the **Table of Materials**) for the β_2m, 10% SDS-PAGE for the H chain.

4. Refolding of MHC complex

NOTE: The efficiency of inclusion bodies refolded will affect the yield of protein obtained. Folding competes with polymerization, so it is generally accepted that refolding at low protein concentrations is the most successful method. In this paper, the inclusion bodies concentration is 30 mg/mL.

4.1. Prepare refolding buffer.

4.1.1. Vary the composition of the refolding buffer depending on the protein. For example, the pH, ionic strength, redox conditions and the presence of ligands will affect the refolding results. The most common is to use Tris or HEPES-based buffers at a neutral pH with the NaCl concentration of 50-500 mM, but this also depends on the target protein. The following is the refolding buffer formula used in this article.

4.1.2. Prepare the folding buffer with 100 mM Tris-HCl pH 8.0, 400 mM L-arginine, 2 mM EDTA-2Na.

4.1.3. Cool the buffer to 4 °C in a 250–300 mL flask, and then add 5 mM reduced glutathione (GSH) and 0.5 mM oxidized glutathione (GSSG). Stir slowly using a magnetic stirrer at 4 °C for a further 10–20 min before adding the inclusion bodies and peptides.

4.2. Injection and dilution of MHC H chain and β_2m

NOTE: The temperature at which refolding is performed may vary, although generally, in order minimize aggregation, 4 °C is best. We use dilution to refold the proteins.

4.2.1. Using a needle from a 1 mL syringe, inject 1 mL of hβ$_2$m inclusion bodies or bβ$_2$m inclusion bodies into two refolding buffers (1 liter each), respectively. Inject near the vigorously rotating stirring rod to obtain fast and efficient dilution. β$_2$m refolds relatively easy and remains stable even in the absence of the H chain.

4.2.2. After the β$_2$m has been dissolved in refolding solution, dissolve peptides (5 mg/mL) in DMSO and quickly inject 200 µL into the refolding solution. Stir slowly for 10–20 min before adding the H chain.

4.2.3. Inject the H chain with the same procedure described above for β$_2$m. The H chain is very unstable; therefore, the order of injection is quite important. Inject 3 mL of H chain inclusion bodies into two different refolding buffers (1 liter each) with hβ$_2$m or bβ$_2$m, respectively. The consecutive injection of small aliquots instead of the single application of the whole amount of protein increases the yield of refolded MHC. Allow refolding to proceed at 4 °C for 8–10 h.

4.3. Concentration of refolded protein

4.3.1. Use ultrafiltration in a pressurized chamber with 10 kDa MMCO membrane for concentration of the refolding proteins. This method is convenient and can be combined with buffer exchange. Add exchange buffer (20 mM Tris-HCl, 50 mM NaCl pH 8.0) to the chamber and concentrate to a final volume of 30–50 mL.

4.3.2. Transfer the refolding solution to a centrifuge tube, spin at 12,000 x *g* for 15 min at 4 °C to remove precipitates.

4.3.3. Carefully transfer the supernatant and concentrate further to a final volume of ~1 mL.

4.3.4. Centrifuge at 12,000 x *g* for 10–20 min. Transfer the supernatant to a sterile tube and purify the proteins using a 10/300 GL size-exclusion column.

4.3.5. Collect the samples at the peak and analyze them using SDS–PAGE (15% polyacrylamide gel).

4.3.6. Collect the MHC complex peak and concentrate to a final concentration of 15 mg/mL.

4.3.7. Dilute the complex to 7.5 mg/mL and 15 mg/mL for crystallization.

5. Crystallization, data collection, and processing

5.1. Perform crystallization of complexed MHC and peptide using the sitting drop vapor diffusion technique.

5.2. Screen the Ptal-N*01:01/peptide complexes with commercial crystal kits (e.g., Crystal Screen kit I/II, Index Screen kit, PEGIon kit I/II, and the PEGRx kit).

5.3. Seal the resulting solution and equilibrate against 100 µL of reservoir solution at 4 or 18 °C.

5.4. Observe the crystal growth in culture for 3 days, 1 week, 2 weeks, 1 month, 3 months and 6 months. Use a microscope to see if each drop in the crystal plate has crystals.

NOTE: Ptal-N*01:01/HeV1(bβ_2m) crystals were observed in 0.2 M NaCl, 0.1 M Bis-Tris (pH 5.5), and 25% (w/v) polyethylene glycol 3,350 at a concentration of 7.5 mg/mL. Ptal-N*01:01/HeV1(hβ_2m) crystals were observed in 0.1 M HEPES, pH 7.0, 2% w/v polyethylene glycol 3,350 at a concentration of 7.5 mg/mL.

5.5. For cryogenic protection, transfer the crystals to a storage solution containing 20% glycerol and then cool rapidly in a 100 K gaseous nitrogen stream.

5.6. Collect X-ray diffraction data from the beamline BL19U of the Shanghai synchrotron radiation facility (Shanghai, China).

6. Structure determination and analyses

6.1. With high-resolution structural data, process the strength of the collection and scale using the Denzo program and the HKL2000 software package (https://hkl-xray.com/)[23].

6.2. After choosing a better initial model in the Protein Data Bank (PDB), determine the structures using molecular replacement with the program Phaser MR in CCP4[24] (http://www.ccp4.ac.uk/). The model used was the structure coordinates with Protein Data Bank (PDB) code 5F1I.

6.3. Use the refined X-ray model for the initial joint refinement. Use Phenix refine alone and joint modes for the X-ray alone and the joint neutron and X-ray refinement, respectively.

6.4. After every round of refinement, manually check the model against *Fo − Fc* and *2Fo − Fc* positive nuclear density maps in Coot[25] (https://www2.mrc-lmb.cam.ac.uk/personal/pemsley/coot/). This facilitates the proper placement of waters and certain D atoms. All figures of structures were created by PyMOL (http://www.pymol.org/).

REPRESENTATIVE RESULTS

Previous work reported that the HeV-derived HeV1 (DFANTFLP) peptide was presented by Ptal-N*01:01[10,19]. Herein, the binding capacity of this peptide to Ptal-N*01:01 with homologous bat β_2m (bβ_2m) and heterologous human β_2m (hβ_2m) light chains (**Figure 1C,1D**) was evaluated. Crystals with higher resolution were formed, respectively (**Figure 1E,1F**). A crystal is formed from the Ptal-N*01:01/HeV1 complex, which was formed through

renaturation with bβ$_2$m, and the resolution is 2.31 Å. A crystal is formed from the Ptal-N*01:01/HeV1 complex, which was formed through renaturation with hβ$_2$m, and the resolution is 1.6 Å. The Ptal-N*01:01/HeV1 complex was successfully formed through renaturation with both bβ$_2$m and hβ$_2$m (**Figure 1C,1D**). In this context, we showed that the Ptal-N*01:01/HeV1 complex was not formed without the presence of β$_2$m (**Figure 1A**) and the H-2K^d that fold correctly through the hβ$_2$m (**Figure 1B**).

The structures of Ptal-N*01:01/HeV1/bβ$_2$m and Ptal-N*01:01/HeV1/hβ$_2$m were then analyzed. In the Ptal-N*01:01/HeV1/bβ$_2$m structure, residues R3, H31, Q34, D53, W60, Y63 of bβ$_2$m bound to the H chain residues through bottom of the PBG and residues Q8, Y10, R12, N24, Y28, N98, N99 bound to the α3 domain of the H chain. Similar to the Ptal-N*01:01/HeV1/bβ$_2$m complex, in the Ptal-N*01:01/HeV1/hβ$_2$m structure, conserved residues H31, D53, W60, Y63 of hβ$_2$m which correspond to bβ$_2$m, made contact with the bottom of the PBG and conserved residues Q8, Y10, R12, N24 which correspond to bβ$_2$m bound to α3 domain (**Figure 2A,2B**).

In the overall structures Ptal-N*01:01/HeV1/bβ$_2$m and Ptal-N*01:01/HeV1/hβ$_2$m, the average root-mean-square deviation (RMSD) of residues 1–184 of the H chains (forming the α1α2 PBG) was 0.248 under all C$^\alpha$ atoms superposition (**Figure 3A**). This finding indicated that there was no difference between these two complexes. The conformations of the similar peptides in the complexes with different β$_2$m were then compared. The structure of the peptide alignment showed that the conformations of HeV1 peptides in these two complexes were quite similar (**Figure 3B**). In addition, the structures of gp33(KAVYNFATM) presented by H-2D^b and complexed with mouse β$_2$m (mβ$_2$m) or hβ$_2$m were aligned. The RMSD of the α1α2 PBG of H-2D^b was 0.283 and the overall conformations of peptides in these two structures were also similar (**Figure 3C,3D**). These data indicate that the β$_2$m substitution between bβ$_2$m and hβ$_2$m, and mβ$_2$m and hβ$_2$m do not affect the conformations of presented peptides.

Sequence alignment showed that the amino acids of β$_2$m from different species are highly conserved (**Figure 4**). Following analysis of the β$_2$m from different species showed that most of the amino acids of β$_2$m that were forming the hydrogen bonds with the H chain of MHC I were conserved (**Figure 4, Table 2**). Meanwhile, the diverse residues were also amino acids with similar chemical properties in mammals. However, the key residues involved in β$_2$m binding to the H chain of MHC I showed polymorphisms in chickens, fish and amphibians (**Figure 4**).

Table 1. The various mammals combine with heterologous β$_2$m.

Table 2. Hydrogen bond interactions between heterologous β$_2$m and heavy chain in MHC I of various species.

Figure 1. Purification of the soluble and refolded Ptal-N*01:01/HeV1 complex proteins and photographs of the crystal used for diffraction analysis. The M is molecular weight markers in kDa. The P1 is the aggregates. The P2 is the MHC complex. The P3 is the β$_2$m. (**A**) Ptal-

N*01:01/HeV1 complex was not formed without the presence of β_2m. (**B**) H-2K^d complex was formed through renaturation with hβ_2m. (**C**) Ptal-N*01:01/HeV1 complex was formed through renaturation with bβ_2m. The profile is marked with the approximate positions of the molecular mass standards of 75.0, 44.0, and 13.7 kDa. (**D**) Ptal-N*01:01/ HeV1 complex was formed through renaturation with hβ_2m. (**E**) The crystal is formed from Ptal-N*01:01/HeV1 complex, which was formed through renaturation with bβ_2m. The black arrow represents the crystal used to collect data during X-ray diffraction. (**F**) The crystal is formed from Ptal-N*01:01/HeV1 complex which was formed through renaturation with hβ_2m. The black arrow represents the crystal used to collect data during X-ray diffraction.

Figure 2. Hydrogen bonding between β_2m and heavy chain in hybrid MHC I complexes. Hydrogen bonding between β_2m and H chain in (**A**) Ptal-N*01:01/bβ_2m/HeV1 and (**B**) Ptal-N*01:01/hβ_2m/HeV1 MHC complexes. Hydrogen bond interactions are represented by a black dotted line. The square represents the area zoomed in and shown to the right in the corresponding colored boxes. The red represents that the homologous β_2m and the heterologous β_2m use the same amino acids to form hydrogen bonds with the H chain.

Figure 3. Similar conformation of the MHC complex and the antigenic peptides in hybrid MHC I complexes. (**A**) The superimposition of α1α2 domains of Ptal-N*01:01/bβ_2m (green) and Ptal-N*01:01/hβ_2m (gray). (**B**) The superposition of HeV1 peptide with the superimposition of α1α2 domain of each Ptal-N*01:01 molecule. HeV1 Peptide is represented as pink in Ptal-N*01:01/bβ_2m and as yellow in Ptal-N*01:01/hβ_2m. (**C**) The superimposition of α1α2 domains of H-2D^b /mouse β_2m (mβ_2m) (green) and H-2D^b /hβ_2m (gray). (**D**) The superposition of gp33 peptide with the superimposition of α1α2 domain of each H-2D^b molecule. Peptide gp33 is represented as blue in H-2D^b /mβ_2m and as pink in H-2D^b /hβ_2m.

Figure 4. Structure-based sequence alignment of hβ_2m with β_2m of other species. The black arrows denote β-strands. The residues highlighted in red are completely conserved, and the residues in blue boxes are highly (>80%) conserved. The yellow triangles represent the key amino acids for the interaction between the β_2m and H chains. The sequence alignment was generated using Clustal X[32] and ESPript[33].

DISCUSSION

The construction of a hybrid protein complex through heterologous substitution from different taxa is a common strategy for functional and structural investigations when the homologous complex is not available, such as in the MHC I and its ligands. However, there is limited summarization regarding the methodology and the technology. Herein, the structure of bat MHC I, Ptal-N*01:01, stabilized by bβ_2m or hβ_2m was analyzed. The key amino acids of β_2m binding to Ptal-N*01:01 were found to be conserved between bat and human. Upon further analysis, the key residue involved in β_2m binding to the H chain of MHC I was found to be conserved in mammals but polymorphous in chickens, fish and amphibians. These data indicate that heterologous β_2m substitution is a feasible means by which to study the MHC I of mammals. However, substitution between mammals and birds, fish or amphibians may not be feasible.

Structural studies play pivotal roles in understanding the molecular mechanisms of peptide presentation by MHC I molecules. Heterologous β_2m substitution is commonly used in MHC I structural studies[14,16-18,26-28]. Previous work has shown that in bovine MHC I, N*01801, murine β_2m and bovine β_2m behaved similarly during binding to the α1α2 domains of the N*01801 H chain[17]. Herein, the structure of a single peptide presented by the same H chain of MHC I but different β_2m was analyzed. These data show that the conformations of peptide are similar when stabilized by cross-taxa β_2m, thus indicating that heterologous β_2m substitution does not affect peptide presentation.

Antigen peptides presented by MHC I are recognized by TCR to mediate T cell activation[29]. During viral infection, evaluation of antigen-specific T cell immune responses will greatly improve the understanding of viral infections and host immune responses. The peptide-MHC tetramer is an important technique to evaluate T cell responses; it is a technique to tetramerize the MHC monomer molecule, improve its affinity, and combine it with multiple TCRS on T cells. Tetramers are widely used in research and clinical diagnosis[14,29,30]. Recently, TCR-engineered T cells (TCR-T) have become a hot topic in tumor immunotherapy for their potential efficacy in the treatment of malignant tumors[31]. MHC I tetramer staining is a crucial method for screening specific TCR binding to cancer-related antigen peptides presented by MHC I molecules[29]. Therefore, MHC I tetramer preparation plays a vital role in TCR screening. In addition to binding of the MHC I H chain, β_2m also binds to CD8 on the T cell surface, which may lead to non-specific staining during TCR screening by MHC I tetramer staining. Heterologous β_2m substitution may decrease this non-specific binding of the MHC I tetramer.

However, there are some limitations to the protocol. Firstly, although *E. coli* remains the dominant expression host for recombinant proteins, many post-translational modifications cannot be performed and the expressed protein products form insoluble inclusion bodies. Then, due to the similar substitution of some non-mammalian β_2m with mammalian β_2m, it is not known whether non-mammal β_2m can be replaced by heterologous β_2m to assist and stabilize their MHC structure. In the protocol, we have included a description on our tried analyses using both the NetMHCpan and Rosetta FlexPepDock server. Unfortunately, neither produced predictions matched the experimental data. This may indicate that current MHC binding peptide predictions were not suitable for non-human and non-mouse mammals such as bats, which may have a different manner of peptide binding. Therefore, we need to combine various prediction software and experimental results for comprehensive analysis to obtain high affinity peptides.

In the protocol described here, the key step is that the MHC can be renatured correctly. It is very important to obtain a MHC complex with high refolding efficiency. Therefore, it is crucial to pay attention to select suitable peptides and increase the purity of inclusion bodies.

In conclusion, herein is summarized a protocol for MHC I expression, refolding, crystallization, crystal data collection and structure determination. Furthermore, the feasibility of heterologous β_2m substitution in MHC I study was analyzed. This study provides a sound

reference for understanding MHC I in structural and functional studies. In addition, these data are also significant for evaluation of T cell responses during infectious disease and tumor immunotherapy.

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DISCLOSURES

The authors have nothing to declare.

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Figure 1

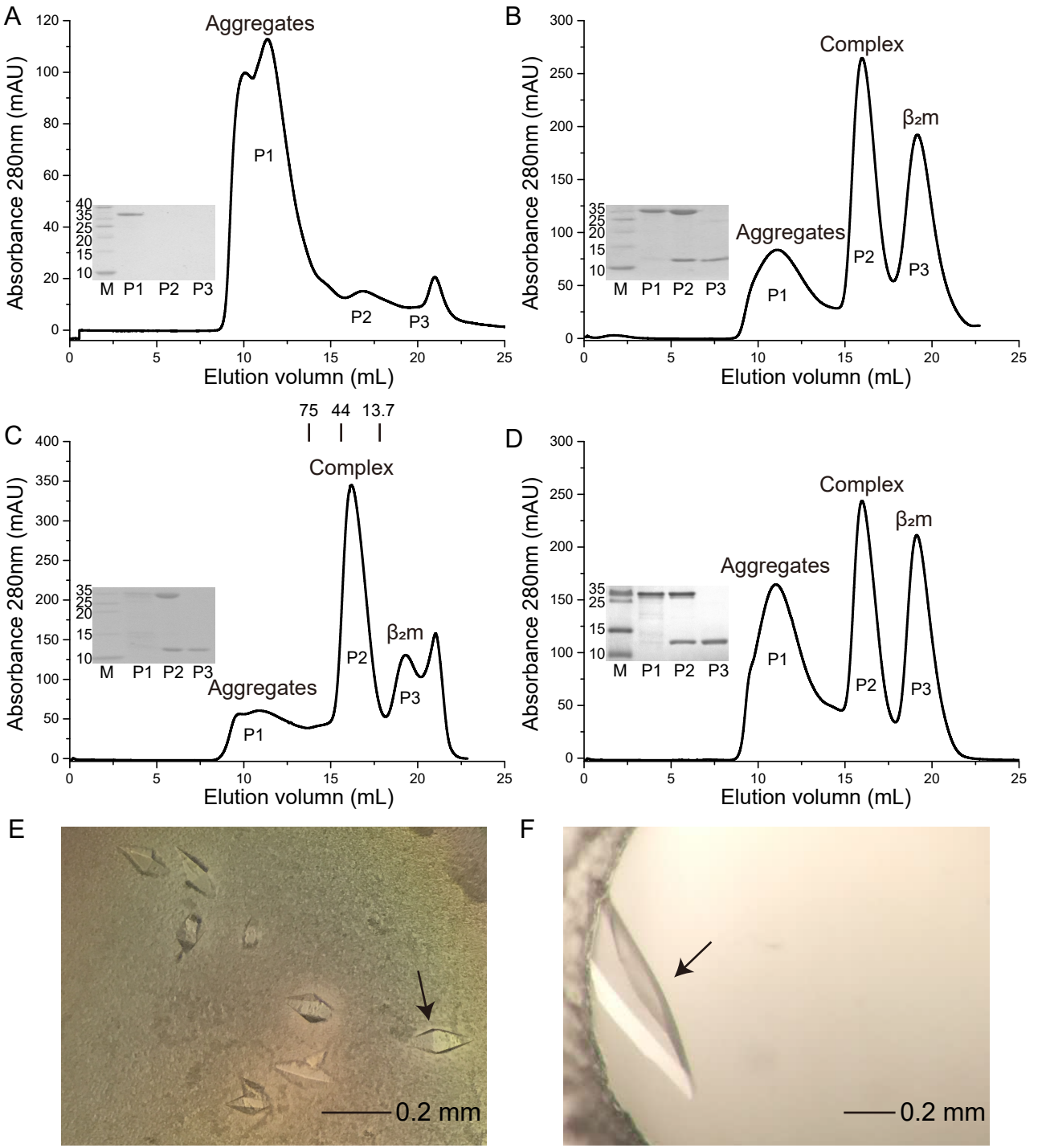
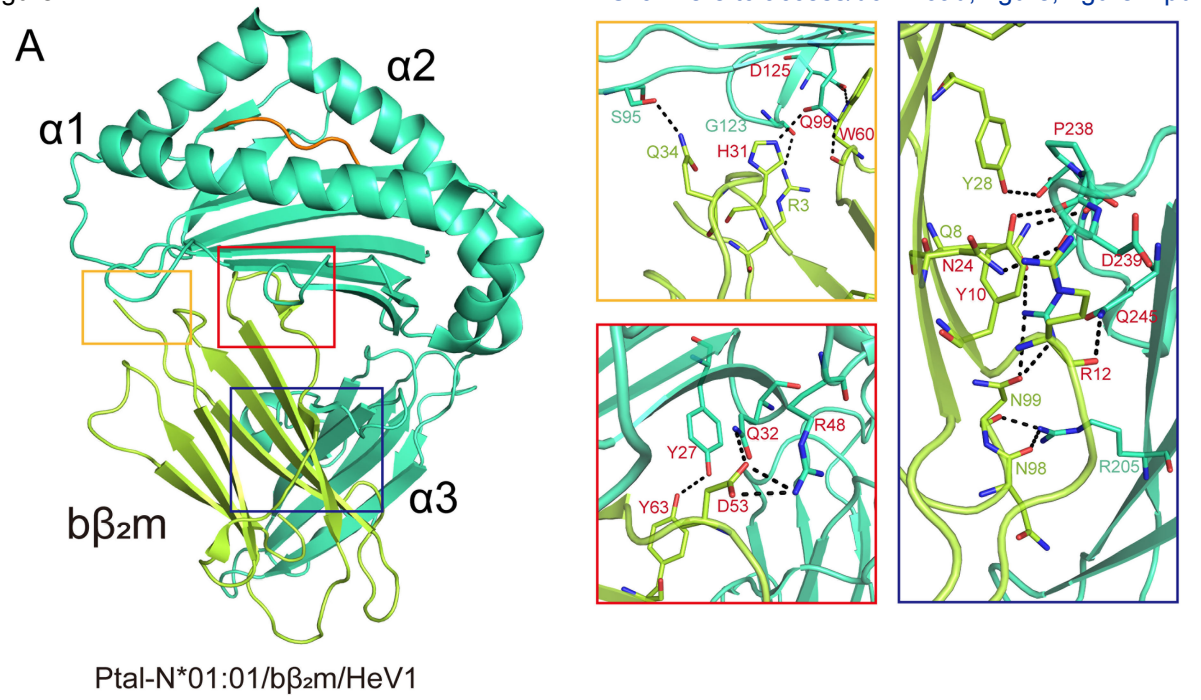
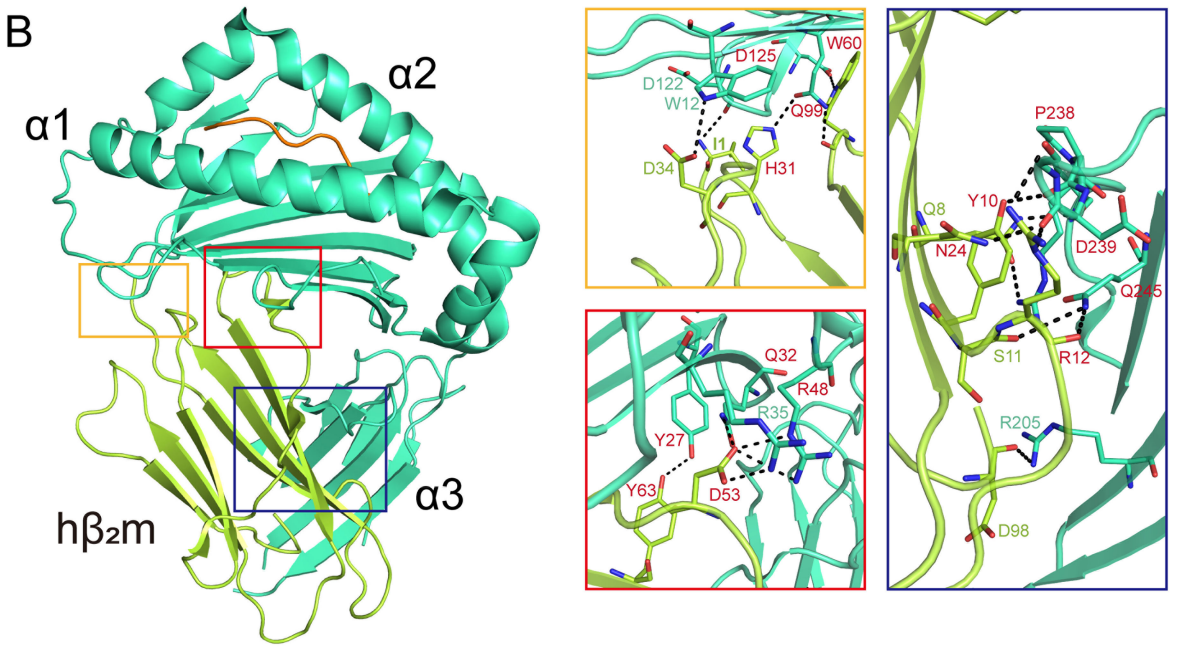


Figure 2

Click here to access/download;Figure;Figure 2.pdf



Ptal-N*01:01/bβ2m/HeV1



Ptal-N*01:01/hβ2m/HeV1

Figure 3

Click here to access/download;Figure;Figure 3.pdf

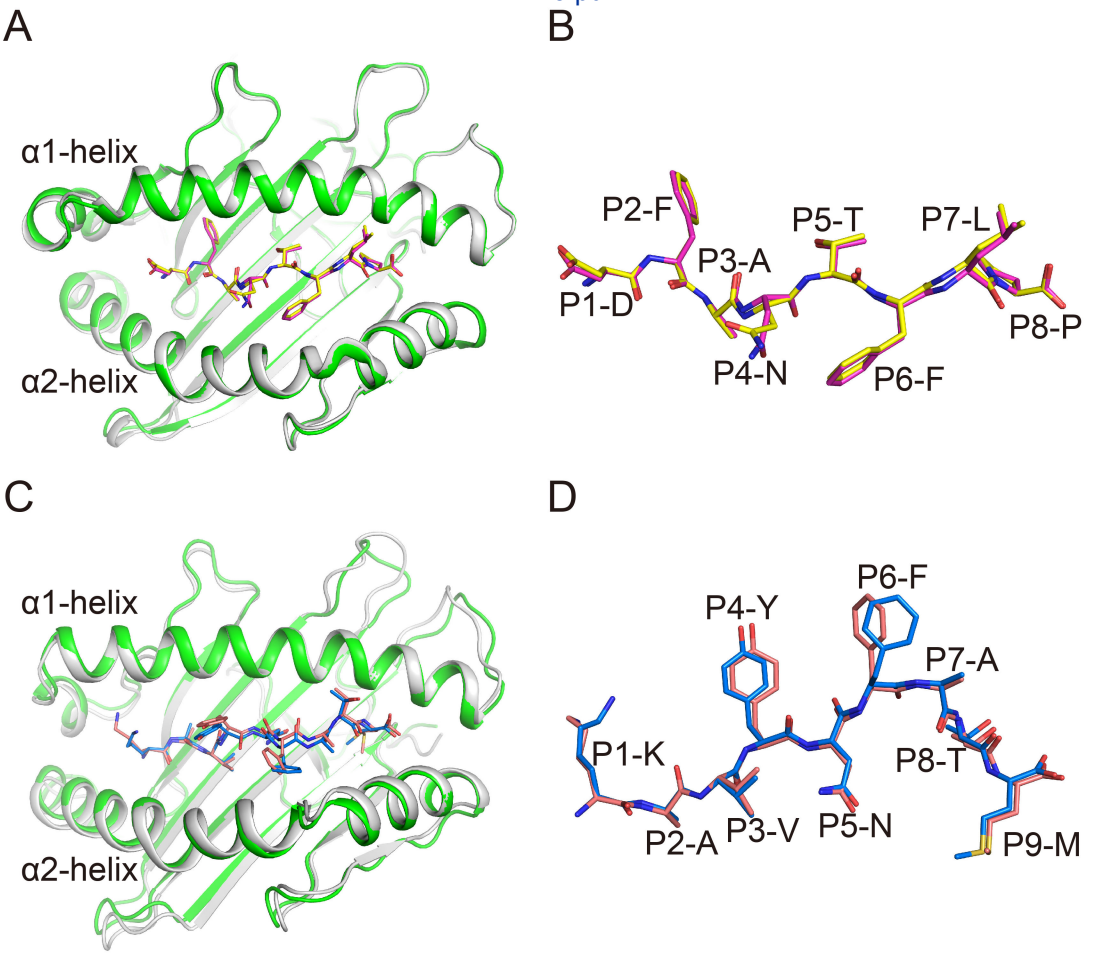


Figure 4

Click here to access/download;Figure;Figure 4.pdf

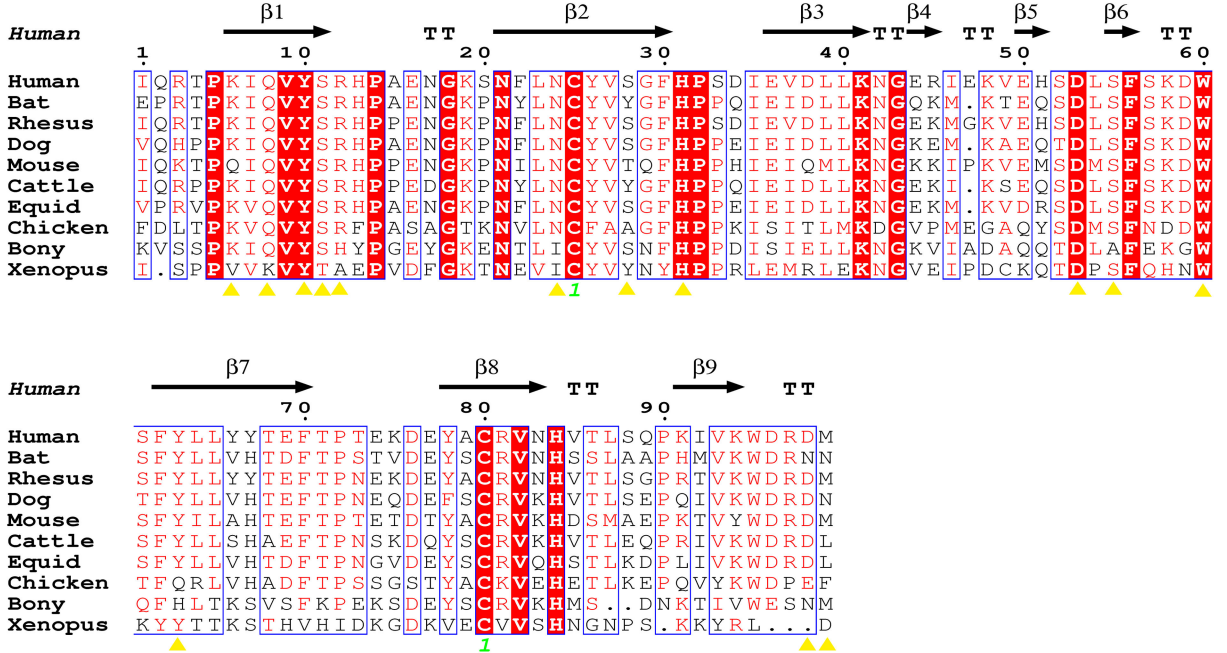


Table 1

MHC molecules	H chain source	Heterologous β_2m source	PDB code	Peptide source	Resolution	References
Ptal-N*01:01	Ptal-N*01:01	human	6K7T	Hendra virus	1.6 Å	PLoS Biol, 2019[7]
Mamu-B*17	Mamu-B*17	human	3RWJ	Simian immunodeficiency virus	2.7 Å	J Immunol, 2011[8]
Mamu-A*01	Mamu-A*01	human	1ZLN	Simian immunodeficiency virus	2.8 Å	J Immunol, 2007[9]
Mamu-A*02	Mamu-A*02	human	3JTS	Simian immunodeficiency virus	2.8 Å	J Virol, 2011[10]
H-2K^d	H-2K^d	human	5GR7	Middle East respiratory syndrome-related coronavirus	2.4 Å	J Immunol, 2017[11]
DLA-88*50801	DLA-88*50801	human	5F1I	self-peptides	2.904 Å	J Immunol, 2016[13]
BoLA-A11	BoLA-A11	mouse	3PWU	Rinderpest virus	1.899 Å	J Virol, 2011[14]
Eqca-N*00602	Eqca-N*00602	mouse	4ZUS	Equine infectious anemia virus	2.6 Å	J Immunol, 2016[15]
Eqca-N*00601	Eqca-N*00601	mouse	4ZUW	Equine infectious anemia virus	2.6 Å	J Immunol, 2016[15]

Table 2

Ptal-N*01:01			Mamu-B*17			DLA-88*50801			H-2K[d]			BoLA-A11		
	human	bat		human	monkey		human	dog		human	mouse		mouse	bovine
H chain	hβ_2m	β_2m	H chain	hβ_2m	β_2m	H chain	hβ_2m	β_2m	H chain	hβ_2m	β_2m	H chain	β_2m	β_2m
W12	D34	/[b]	**Y27**	**Y63**	**Y63**	**Y27**	**S55**	**S55**	**Q32**	**D53**	**D53**	**Y26**	**Y63**	**Y63**
Y27[a]	**Y63**	**Y63**	**Q32**	**D53**	**D53**	**Y27**	**Y63**	**Y63**	**R35**	**D53**	**D53**	**Q31**	**D53**	**D53**
Q32	**D53**	**D53**	**R35**	**D53**	**D53**	**Q96**	**H31**	**H31**	**R48**	**D53**	**D53**	**R34**	**D53**	**D53**
R35	D53	/	R35	D53	/	**Q96**	**W60**	**W60**	**Q96**	**W60**	**W60**	R47	D53	/
R48	**D53**	**D53**	R48	D53	/	**D122**	**W60**	**W60**	**D122**	**W60**	**W60**	R47	D53	/
R48	**D53**	**D53**	**Q96**	**H31**	**H31**	Q32	/	D53	Y27	/	S55	T93	H31	/
S95	/	Q34	**Q96**	**W60**	**W60**	R35	/	D53	Y27	/	Y63	**Q95**	**W60**	**W60**
Q99	**H31**	**H31**	**D122**	**W60**	**W60**	R48	/	D53	S92	/	H34	Y101	K58	/
Q99	**W60**	**W60**	**R202**	**D98**	**D98**	D119	/	V1	D119	/	I1	**D121**	**W60**	**W60**
D122	I1	/	E232	Q8	Y26	R203	D98	/	**R202**	**D98**	**D98**	S91	/	Q34
G123	/	R3	**E232**	**S28**	**S28**	**D233**	**K6**	**K6**	R202	/	D98	Q95	/	H31
D125	**W60**	**W60**	**R234**	**M99**	**M99**	**D233**	**Q8**	**Q8**	E232	Q8	Q29	G119	/	R3
R205	D98	/	**R234**	**M99**	**M99**	**R235**	**Q8**	**Q8**	E232	S28	T28	**R201**	**D98**	**D98**
R205	/	N98	**P235**	**Y10**	**Y10**	R235	M99	N99	E232	/	T28	**E231**	**Q8**	**Q8**
R205	/	N99	**G236**	**R12**	**R12**	**P236**	**Y10**	**Y10**	R234	Q8	M99	**R233**	**Q8**	**Q8**
E235	**Q8**	**Q8**	G236	N24	/	**A237**	**R12**	**R12**	**P235**	**Y10**	**Y10**	R233	M99	L99
E235	/	Y28	G237	R12	/	A237	/	N24	**A236**	**N24**	**N24**	R233	M99	L99
R237	**Q8**	**Q8**	**Q242**	**S11**	**S11**	**Q243**	**R12**	**R12**	A236	/	R12	**P234**	**Y10**	**Y10**
R237	/	N99	**Q242**	**R12**	**R12**	Q243	/	S11	Q242	/	S11	**S235**	**R12**	**R12**
R237	/	N99	**W244**	**M99**	**M99**	W245	/	N99	Q242	/	R12	**S235**	**N24**	**N24**
P238	**Y10**	**Y10**	R234	Q8	/	---	---	---	---	---	---	W243	M99	L99
D239	R12	/	---[c]	---	---	---	---	---	---	---	---	E231	/	Y28
D239	**N24**	**N24**	---	---	---	---	---	---	---	---	---	Q241	/	S11
G240	R12	/	---	---	---	---	---	---	---	---	---	Q241	/	R12
Q245	S11	/	---	---	---	---	---	---	---	---	---	---	---	---
Q245	**R12**	**R12**	---	---	---	---	---	---	---	---	---	---	---	---

[a]The bold type in the table represents that the homologous β_2m and the heterologous β_2m use the same amino acids to form hydrogen bonds

with the H chain.

[b]One of the β_2m does not form a hydrogen bond at this site.

[c]In addition to the hydrogen bond interactions listed above, this species no longer forms hydrogen bonds.

Name	Company	Catalog Number	Comments
10 kDa MMCO membrane	Merck millipore	PLGC07610	
30% Acrylamide	LABLEAD	A3291-500ml*5	
5×Protein SDS Loading	Novoprotein	PM099-01A	
AMICON ULTRA-15 15ML-10 KDa cutoff	Merck millipore	UFC901096	
Ampicillin	Inalco	1758-9314	
APS	Sigma	A3678-100G	
BL21(DE3) strain	TIANGEN	CB105-02	
DMSO	MP	219605580	Wear suitable gloves and eye/face protection. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
DTT	Solarbio	D1070	Gloves and goggles should be worn and operated in a ventilated kitchen. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
EDTA-2Na	KeyGEN BioTECH	KGT515500	
Glycerin	HUSHI	10010618	
GSH	Amresco	0399-250G	
GSSG	Amresco	0524-100G	
Guanidine hydrochloride	Amresco	E424-5KG	
$h\beta_2m$	our lab		Zhang, S. et al. Structural basis of cross-allele presentation by HLA-A*0301 and HLA-A*1101 revealed by two HIV-derived peptide complexes. Mol Immunol. 49 (1-2), 395-401, (2011).
IPTG	Inalco	1758-1400	
L-Arginine Hydrochloride	Amresco	0877-5KG	
NaCl	Solarbio	S8210	

Protein Marker	Fermentas	26614	
SDS	Boao Rui Jing	A112130	
Superdex Increase 200 10/300 GL	GE Healthcare	28990944	
TEMED	Thermo	17919	Gloves and goggles should be worn and operated in a ventilated kitchen.
Tris-HCl	Amresco	0497-5KG	
Triton X-100	Bioruler	RH30056-100mL	
Tryptone	Oxoid	LP0042	
Yeast extract	Oxoid	LP0021	

Thank you for the opportunity to revise the manuscript. Revised portion are shown in the manuscript. The point-by-point reply to all reviewers' comments is listed as following. There are supplemental data uploaded for the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Timely and interesting study of the MHC I complex in mammals using heterologous hybrids to understand MHC structure and function. Approach may prove important for the study of viral infections.

Reply

We thank the reviewer for positive commenting the importance of our work in the context of MHC I complex in mammals using heterologous hybrids to understand MHC structure and function.

Major Concerns:

Inconsistent nomenclature, overall brevity in writing did not provide enough detail for reader to grasp method or repeat protocol; Introduction and Discussion sections too short; Introduction should include background details about the method presented. Discussion lacked synthesis of method.

Reply

We agree with the reviewer's comments. In the new version, we unified the nomenclature, provided more details in the methodology, expanded the introduction (Line45-53) and discussion (Line 293-306).

Minor Concerns:

Misspelled "Histocompatibility" in Title; Poor formatting of tables

Reply

Thanks and we have made the change accordingly (Line 1).

List of additional Comments for JoVE61462:

Line 24: "mean"- change to "means"

Reply:

We have made correction through the manuscript (Line 25).

Lines 50-52: "shown than MHC I chains from various mammals such as bat… rhesus macaque…,mouse…, cattle…and equine." There is inconsistent use of taxonomy between order [e.g. bat (Chiroptera)] and species [e.g. rhesus macaque (Macaca

maltta)]. Also, Latin form is missing. Standardize taxonomic classification and report Latin form in paper.

Reply:

We agree with the review's opinion. We have made a correct classification according to the reviewer's comments (Line 59-62)

Line 52 and 54: do you mean heterologous (rather than heterogenous)

Reply:

We have made correction through the manuscript (Line 62 and 64).

Lines 55-56 and 59: "between different species remain unclear". These are not different species, maybe "taxa" is better word. Authors are not comparing the same taxonomic groups. For example, bat is an order (chiroptera) comprised of many species, while rhesus macaque is an individual species of order primate. Please standardize the taxonomy.

Reply:

We have made correction according to the reviewer's comments (Line 66).

Lines 63-64: There is only one sub-heading so it can be dropped. Also, there is not enough detail given in this section (Preparation of expression constructs) to follow protocol. Please provide more detail.

Reply:

We have made correction according to the reviewer's comments (Line 73-77).

Lines 72-81: Awkward sentence structure for Peptide synthesis section, which needs revision.

Reply

We have modified statement according to the reviewer's comments (Line 102-106).

Lines, 76, 178, 185, 186: Check proper way to cite online server within body of paper, for example name, city and state. Date accessed and link to website generally go to reference list.

Reply:

We have made correction according to the reviewer's comments (Line 89, 216 , 223 , 225).

Line 83: Incomplete sentence: "The purity of the peptide…", which is missing the verb

Reply:

We have made correction through the manuscript (Line 104-105).

Line 91: List LB broth in the Table of Materials

Reply:
We have listed all components according to the reviewer's comments (Line 112)

Line 93: Give medium formulation of plate and the antibiotic used, including concentration of antibiotic. List antibiotic in Table of Materials.
Reply:
We have listed the antibiotic through the manuscript (Line 115, Table of Materials).

Line 94: Clarify statement "Previous constructs contain ampicillin resistance".
Reply:
We have made correction through the manuscript (Line 115).

Lines 96 and 104: List antibiotic used and concentration (include in the Table of Materials).
Reply:
We have listed antibiotic used and concentration (Line 118 and 126).

Line 106: Explain statement "vary the concentration of IPTG for different MHCs". List IPTG in the Table of Materials.
Reply
We have added description (Line 128-129).

Line 112: Explain "ultrasonic 6S, interval 12S, 300W".
Reply
We have added description (Line 134-135).

Line 124: Consider including statement about removing sample for SDS PAGE before freezing the remaining sample away.
Reply
We have listed all components according to the reviewer's comments (Line 141-142).

Lines 127, 161: What is the running buffer for SDS PAGE? List SDS PAGE components in Table of Materials
Reply
We have added description (Line 150-152).

Line 131: What is concentration of Na?
Reply
The ingredient of the medicine is ethylenediaminetetraacetic acid disodium salt, the formula is $C_{10}H_{14}N_2Na_2O_8$, the molecular weight is 336.2, and the concentration we needed is 2 mM.

Lines 139 & 147: "two different refolding buffers (L)" - define "L"

Reply

We have added description (Line 175, 184).

Lines 150-164: Concentration of refolded protein: There is not enough detail given in this section to follow protocol. Please provide more detail.

Reply

Thanks for the reviewer's suggestion. We have provided more detail in describing (Line 155-165 and Line 171-172).

Lines 166-168: Again, details are lacking in the Crystallization section. For example, "Crystallization … was preformed using the sitting drop vapor diffusion technique" Please include the concentration of protein used, amount of reservoir buffer, temperature that the crystals were grown at.

For a second example, "peptide complexes were screened with a Crystal Screen kit…The crystal growth was observed for 3 days…". Please give details of the crystallization screening procedure.

Reply

Thanks for the reviewer's suggestion. We have provided more detail in describing (Line 204-205 and Line 207 and Line 210).

Lines 179, 181, 183 : What is the structure of the model crystal and what is its resolution?

Reply

We have added description (Line 218-219 and Line 230-233).

Line 191: "the crystals with higher resolution were formed" - what are their resolution?

Reply

We have added description (Line 230-233).

Line 194: "The structures of PtaI-N…/hB2m and PtaI-H…/bB2m". List "b" form before "h" form, for consistency, to match the order of presentation in text of paper and of data in figure 1 and figure 2.

Reply:

We have made correction according to the Reviewer's comments (Line 237)

Line 201: " In the overall structures, PtaI-N…/hB2m and PtaI-H…/bB2m". Same consistency issues here.

Reply:

We have made correction according to the Reviewer's comments (Line 244).

Lines 210, 224: same as for lines 194, 201

Reply:

We have made correction according to the Reviewer's comments (Line 253, 266).

Line 210: "do not significantly affect"- there has been no statistical test conducted to determine significance, simply state "do not affect"

Reply:

We have made correction according to the Reviewer's comments (Line 253).

Line 218: "Materials" - is this "Table 3: The Experimental materials"? It doesn't need to be listed twice.

Reply:

The " Materials " was deleted.

Lines 221, 236: " species in common, cross-species" - consider "taxa", authors are not comparing species

Reply:

We have made correction according to the Reviewer's comments (Line 263 and 278).

Line 222: "PD1" - this is first time the abbreviation for program cell death is used. Please define and develop concept to include in discussion.

Reply

Thanks for the reviewer's suggestion. Since MHC class I molecules and PD1 have different binding modes, and there is no specific discussion in this article, we chose to delete according to your comments (Line 264).

Line 240: "The peptide-MHC tetramer is an important technique ". Do the authors mean: "The analysis of peptide-MHC trimer is an important technique"? Figure 1 shows 3 peaks, not four.

Lines 243, 245, 246: "MHC I tetramer" - do the authors mean "trimer"?

Reply

We have added description about the peptide-MHC tetramer (Line 282-284)

Line 335: "Figure Legends" - change to Figure and Table Legends

Reply:

We have made correction through the manuscript (Line 400).

Lines 336-380: List Figures first then Tables, the order as presented in the paper.

Reply:

Thanks for the reviewer's suggestion. We have made correction through the manuscript.

Line 342: "dose" -change to "does"

Reply

We have made correction according to the Reviewer's comments (in the Table 2).

Line 343: "this species", consider "this taxon or molecule" instead

Reply

We have made correction according to the reviewer's comments (in the Table 2).

Line 348: "soluble the refolded" - change to "soluble and refolded"

Reply

We have changed words according to the reviewer's comments (Line 408).

Table 1 - In Header "MHC species" - suggest "MHC molecules". Consider adding another column for Taxa.

Reply

We have made correction according to the reviewer's comments (in the Table 1).

Table 2 - Within columns of table, define "/" and '---" . Also, there are four footnotes that I don't see defined (Y27a, / b, ---c, H-2Kd,).

Reply:

Thanks for the reviewer's suggestion. We have made correction through the manuscript (in the Table 2).

Table 3 - please improve formatting, it is hard to follow compound across all columns of table. Also, the catalog number is missing for Superdex Increase 200 10/ 300 Gl.

Reply

We have made correction according to the Reviewer's comments (in the Table 3).

Figure 1: More information is needed in legend. For example, for A and B give contents of lanes [M is molecular weight markers in kDA, P1-3 are MHC peaks (Ptal-N)]. For C and D, explain why these crystals were chosen for analysis.

Reply

We have made correction according to the Reviewer's comments (Line 409-410).

Figure 2. Please emphasize the similar amino acids in bat and humans that are used for hydrogen bonding. The similarity is not clear in the figure.

Reply

We have emphasized the similar amino acids in bat and humans that are used for hydrogen bonding with same color, and listed in Table 2.

Reviewer #2:

Manuscript Summary:

The authors describe a means of utilizing a well-studied hB2M to facilitate folding and presentation of peptides in the MHC of a heterologous species. They describe well in the introduction the importance of the method, the methods are clear and they highlight the significance of the work and potential utilization of the methodology. This is a rather

troublesome method/procedure and I think it is well suited to JOVE to display the key methodology. Given my understanding of the methodology it seems to be accurate and clear.

Reply:

We appreciate the reviewer's positive feedback on our means of using heterologous β_2m to facilitate folding and presentation of peptides in the MHC.

Major Concerns:

The reviewers should comment on problems with peptide prediction software across species, e.g. given the unique peptide-display properties of bat MHC via NetMHCpan. Also it is well characterized that human MHC-I requires B2M to fold correctly. This hasn't been shown for other species and is merely assumed. The authors should show that bat MHC-I does not fold in the absence of B2M. (This is a simple control but fundamental to the point of the paper). Ideally the authors should show a third species of MHC-I can fold using human B2M as they claim this will work across most mammals (except fishes etc). [This could be shown by a native gel/absorbance etc like Fig 1A, rather the whole way through to attempting crystals].

Reply:

Thanks for the suggestion. In the revised paper, we have included description on our tried analyses using both the NetMHCpan and Rosetta FlexPepDock server (Line298-303). In this context, we have proved that Ptal-N*01:01/HeV1 complex was not formed without the presence of β2m (Figure 1A). And the H-2Kd that fold correctly through the hβ2m (Figure 1B).

Minor Concerns:

In the abstract, "This is a feasible mean to study" should be "This is a feasible means to study".

Reply

Thanks and we have made the change accordingly (Line 25).