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Construction and functional test of a chicken MHC-I (BF2*15)/peptide tetramer

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Abstract

The major histocompatibility complex class I (MHC class I) peptide tetramer is a sensitive and valuable tool to evaluate antigen-specific cytotoxic T lymphocytes (CTLs) of many animal species. To date, no chicken MHC class I peptide tetramer has been reported. In this report, we describe construction and functional evaluation of a chicken MHC-I (BF2*15)/peptide tetramer. To construct the chicken MHC class I peptide tetramer, genes of the chicken MHC-I α chain (BF2*15) and β2 microglobulin (Chβ2m) were synthesized by RT-PCR from the total RNA of PBMCs and the signal sequences were deleted. The BF2*15 was then fused with the BirA substrate peptide (BSP) sequence at the C terminus. Next, the synthesized PCR products of BF2*15 and Chβ2m were cloned into the expression vector pET-28a (+) and expressed in *Escherichia coli* strain BL21 (DE3). Highly purified BF2*15-BSP heavy chain and Chβ2m were obtained by a Ni^{2+} NTA column affinity purification, yielding approximately 1.6 mg of BF2*15-BSP and 2.4 mg of Chβ2m per 1 g of the pelleted bacteria. The purified BF2*15-BSP heavy chain and Chβ2m were refolded with synthetic peptide originated from infectious bronchitis virus nucleoprotein (IBV $N_{71–78}$) in refolding buffer to generate the monomer of BF2*15/peptide complex. The monomer was then biotinylated and tetramerized using PE-labeled streptavidin. Upon functional evaluation of the construct by using flowcytometry, we observed that 3.65% of CTLs were specific to IBV nucleoprotein. This demonstrates that the CTL response of IBV-infected chicks could effectively be evaluated using the prepared MHC-I BF2*15/peptide tetramer.

Keywords: Chicken MHC class I; BF2*15-BSP and β2 microglobulin genes; BF2*15/peptide tetramer

1. Introduction

The classical MHC class I is a membrane surface protein found on virtually all cells in the body, and its main function is to bind antigenic peptides and present them on the surface of virally infected or tumor cells (Kindt et al., 2007). CTLs recognize antigenic peptides, in the context of MHC class I molecules on the cell surface, viatheir TCR and, upon recognition, lyse these target cells. Therefore, it is important to quantitatively measure antigen-specific T-cells accurately and promptly. Until the development of MHC-I tetramer technology, the main techniques for functional and quantitative measurement of antigen-specific T-cells were limiting dilution assay (LDA), enzyme-linked immunospot assay (ELISPOT), and intracellular cytokine staining (ICS) in conjunction with flow cytometry (Taswell, 1981; Jung, 1993; Scheibenbogen et al., 1997, 2000).

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Altman et al. (1996) first described the use of HLA (human leucocyte antigen)-peptide tetrameric complexes to directly visualize antigen-specific CTLs by flow cytometry. This technique is not dependent on proliferation of the cells and therefore allows the direct quantification of antigen-specific CTLs without *in vitro* manipulation (Meidenbauer et al., 2003). To date, this technique has been applied successfully to study cell-mediated immunity on human (HLA), mouse (H-2, mouse MHC), macaque (MAMU, macaque MHC), chimpanzee (PATR, chimpanzee MHC), horse (ELA, equine leucocyte antigen), and pig (SLA, swine leucocyte antigen) (Dunbar et al., 1998; Donahoe et al., 2000; Kalergis et al., 2000; Oleksiewicz et al., 2002; Skinner et al., 2000; Meidenbauer et al., 2003; Mealey et al., 2005).

The chicken MHC class I (BF2 and Rfp-Y) gene sequences have been previously reported (Kaufman et al., 1992; Briles et al., 1993; Miller et al., 1994). Moreover, the genomic structure of Leghorn chicken MHC has also been reported by Kaufman et al. (1999). Within a 44 kb DNA segment, there are two loci-encoding class I heavy chains named BF1 and BF2. The BF2 locus is dominantly expressed, whereas the BF1 locus is less expressed and has a lower mRNA transcript (Kaufman et al., 1999; Miller et al., 2004). However, despite this knowledge of the MHC-I sequence and genomic structure, no chicken MHC-I tetramer has been reported. In this communication, we report the first construction and functional evaluation of a chicken MHC-I/peptide tetramer.

2. Materials and methods

2.1. Peptide

Boots et al. (1991) reported the first defined coronavirus T-cell epitope which is located in the amino acid sequence 71–78 of the infectious bronchitis virus nucleoprotein (IBV $N_{71–78}$). The IBV $N_{71–78}$ (WRRQARYK) derived from IBV H52 strain was synthesized at GL Biotech Co. (Shanghai, China) purified to purity >95% by FPLC.

2.2. Chickens

All BF2*15 white Leghorn SPF (specific pathogen free) chickens used in this study were obtained from the Experimental Animal Center of Harbin Veterinary Research Institute, and housed in isolator cages.

2.3. Virus

The IBV H52 strain was titrated and stocked in our laboratory.

2.4. Cloning the BF2*15 and Chβ2m gene from chicken PBMCs

Total RNA was extracted from PBMCs (peripheral blood mononuclear cells) with TRIZOL (Invitrogen, San Diego, CA) from a SPF chicken. The full-length BF2*15 and Chβ2m cDNA were cloned by RT-PCR. Oligonucleotide primers were designed according to the entire coding region of BF2*15 and Chβ2m cDNA sequences reported in GenBank (accession numbers: **L28958** and **M84767**); the sequences of forward and reverse primers of the BF2*15 and Chβ2m were as follows: BF2*15 forward: 5′-TGC AGC GGT GCG AGG CGA T-3′, BF2*15 reverse: 5′-TTA TTT CAC AGG AAG CAG TGC-3′; Chβ2m forward: 5′-ACA GCG GAG CCA TGG GGA A-3′, Chβ2m reverse: 5′-ATC CCG GGC ACA GCT CAG A-3′. Reverse transcription reaction was conducted at 42 °C for 2 h using AMV Reverse Transcriptase XL (TaKaRa, Dalian, China). PCR amplifications were performed using the LA-Taq DNA polymerase system (TaKaRa, Dalian, China) under the following conditions: starting at 95 °C for 5 min for denaturation, followed by 35 cycles at 94 °C for 1 min, at 60 °C for 1 min, at 72 °C for 1 min, and then at 72 °C for 10 min for extension. The PCR products were inserted into the pMD18-T vector (TaKaRa, Dalian, China) according to the manufacturer's instructions. The positive clones with the correct sequence were named as pMD-BF2*15 and pMD-Chβ2m. The signal peptides of BF2*15 and Chβ2m genes were predicted with SignalP (Bendtsen et al., 2004) and the sequence of the signal peptide in the BF2*15 gene was deleted. In addition, a 15-amino acid residue substrate peptide for BirA-dependent biotinylation was fused to the COOH terminus of BF2*15 through the *Eco*RI and *Hin*dIII site adapters with the forward primer 5′-GGA ATT CAT GGG GCC GTG CGG GG-3′ and the reverse primer 5′-GCG CAA GCT TTT AAC GAT GAT TCC ACA CCA TTT TCT GTG CAT CCA GAA TAT GAT GCA GGA TGG AGG GGT TGC TCC CGG G-3′, using the pMD-BF2*15 plasmid as the template. The signal peptide of the Chβ2m gene was also deleted using *Eco*RI and *Hin*dIII site adapters with the forward primer 5′-GGA ATT CAT GGG GAA GGC GGC GGC-3′ and the reverse primer 5′-GCG CAA GCT TTT AGA ACT CGG GAT CCC A-3′, using the pMD-Cβ2m plasmid as the template. The amplified DNAs were digested with

*Eco*RI and *Hin*dIII, and ligated into prokaryotic expression vector, pET-28a (+) (Novagen, Madison, WI) at the *Eco*RI/*Hin*dIII site. Clones with the correct inserts were identified using PCR and restriction analysis of vectors carrying the recombinant DNA. Finally, the nucleotide sequences of the selected clones were confirmed by DNA sequence analysis. The positive clones were named as pET-BF2*15-BSP and pET-Chβ2m.

2.5. Expression of BF2*15-BSP and Chβ2m

For protein expression, recombinant plasmids pET-BF2*15-BSP and pET-Chβ2m were transformed into *Escherichia coli* strain BL21 (DE3) and plated on LB-agar supplemented with kanamycin (50 μg/ml). The transformants were grown overnight at 37 °C in LB supplemented with kanamycin (50 μg/ml) and the bacteria were subcultured the following morning at 37 °C. IPTG (1 mM) was added when the cultures reached $OD_{600} = 0.6$. Four hours after induction, the samples were harvested by centrifugation at 8000 rpm (F0685 rotor, Beckman) for 10 min and resuspended with 50 mM Tris–HCl (pH 8.0), sonicated, and spun down. The inclusion bodies were washed twice with 50 mM Tris–HCl (pH 8.0) and rinsed with 20 mM Hepes (pH 8.0), 8 M urea, and 50 mM NaCl.

2.6. SDS-PAGE and Western-blotting assay

The procedure used for the 12% tricine-SDS-PAGE has been described (Schagger and Jagow, 1987). The protein bands were visualized by staining with Coomassie brilliant blue (CBB). The quantity of the products was calculated by comparison of the density to that of the protein molecular mass standards (concentrations of the markers are described in the manufacturer's manual, Ferments UAB, Lithuania). For Western blot analysis, the proteins on the gel were blotted onto a NC membrane (Pall-Gelman, NY, USA). The membrane was blocked for 1 h at room temperature with phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) (Sigma, Louis, MO, USA), then incubated for 1 h with 1:5000 diluted anti-His6 monoclonal antibody (Novagen, Madison, WI, USA), followed by incubation with an HRP-conjugated anti-mouse IgG reagent (Sigma, Louis, MO, USA). Bound antibody was visualized using diaminobenezidene (DAB).

2.7. Protein purification

The inclusion body fraction which was solubilized in 20 mM Hepes (pH 8.0), 8 M urea, and 50 mM NaCl was loaded directly onto 2 ml Ni–NTA agarose beads (Novagen), which had been equilibrated with binding buffer (20 mM Tris–HCl (pH 8.0), 500 mM NaCl, and 5 mM imidazole). Before loading the lysate onto the Ni–NTA agarose beads, the concentration of imidazole (pH 8.0) was added up to 10 mM. The beads were rotated with the inclusion body fraction for 2 h at 4 °C, unbound protein was collected, and the beads were washed with 20 volumes of wash buffer (20 mM Tris–HCl (pH 7.9), 0.5 M NaCl, 20 mM imidazole, 8 M urea, 10% glycerol, and 0.2 mM PMSF). His6-tagged protein was eluted with the wash buffer containing 500 mM imidazole. The concentrations of purified BF2*15-BSP and Chβ2m were determined using the Bradford method with BSA as a standard.

2.8. Construction of monomeric and tetrameric BF2*15-peptide complexes

Construction of monomeric and tetrameric BF2*15-peptide complexes were carried out according to the protocol described by Dirk H. Busch (http://www.mikrobio.med.tu-muenchen.de/forschung/projekte/index.htm). Briefly, BF2*15-BSP heavy chain and Chβ2m were refolded with synthetic peptide IBV $N_{71–78}$ in refolding buffer, consisting of 100 mM Tris–HCl (pH 8.0), 400 mM L-arginine, 2 mM EDTA, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, and 0.5 mM phenylmethysul-fonylfluoride (PMSF) per 500 ml. The refolding mixture was stirred and incubated at 10 °C for 48 h and then directly concentrated and desalted using ultrafiltration system and micro spin tubes (AMICON, USA). The folded product was then subjected to enzymatic biotinylation by BirA enzyme (Avidity Denver Co. USA) at 25 °C overnight. After biotinylation, the sample was run over a gel filtration column to purify the BF2*15/peptide fraction. The tetrameric complexes of biotinylated BF2*15/peptide were produced by mixing purified biotinylated monomer with PE-labeled streptavidin at a molar ratio of 4:1. Then the resulting BF2*15/peptide tetramer was further purified with 100 kDa MWCO spin tube (AMICON, USA) and stored at 4 °C in PBS with a cocktail of protease inhibitors: 1 mM pepstatin, 1 mM leupeptin, 0.001% NaAzide, and 1 mM EDTA.

2.9. Isolation of PBMCs

PBMCs from five infected or five uninfected SPF chicks were used as the source of effector cells for flow cytometry assay. The chicks were inoculated at 3 weeks by the nasal–oral routine with $10^{5.5}$ egg infectious doses

(EID_{50}) of the IBV H52 strain. Anticoagulated blood from chicks was collected at 10 days post-inoculation (p.i.) as described elsewhere (Seo and Collisson, 1997). PBMCs were isolated by centrifuging 5 ml of the anticoagulated blood for 20 min at 1500 rpm through 5 ml of Ficoll-Hypaque gradient (Histopaque, Sigma, USA). Viable cells were collected from the interface and washed three times with PBS (pH 7.2).

2.10. CTL detection with BF2*15-peptide tetramer

The PBMCs, from each group, were pooled and subjected to flow cytometry to detect the antigen-specific CTL. For immunofluorescence staining, 10^6 cells were incubated with anti-chicken CD8 monoclonal antibody (Southern Biotech, Birmingham, USA) for 30 min at 4 °C and then washed three times in PBS containing 0.1% bovine serum albumin (BSA). An FITC-conjugated F(ab')2 fragment of goat anti-mouse IgG (Southern Biotech, Birmingham, USA) was used as the second antibody, and the cells were washed three times. The cells were then stained with 25 μl of 1:100 diluted PE-labeled BF2*15-peptide tetramer at 37 °C for 30 min and then washed twice. Stained cells were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). All flow staining and FCM assays were repeated in at least three independent experiments. The fluorescence intensity (FI) of cells was analyzed with CELLQuest software (Becton Dickinson).

3. Results

3.1. Cloning of BF2*15 gene and Chβ2m gene

The RT-PCR products of BF2*15 and Chβ2m-encoding genes were separated by electrophoresis in 1% agarose gel. Specific bands at positions corresponding to about 1000 bp and 360 bp were detected, which was in accordance with expectation. The BF2*15 and Chβ2m genes were sequenced and the results showed that they were C + G rich (65.12% and 65.56%, respectively). Additionally, the first 63 bp sequences of both genes, with 84.13% and 76.19% C + G, respectively, encode the signal peptides. Also, 11 of 21 amino acid residues in each signal peptide were strongly hydrophobic.

3.2. Expression and identification of recombinant proteins

The SDS-PAGE analysis showed that the transformed cells with pET-BF2*15-BSP produced a large amount of the protein at a mass of about 38 kDa (Fig. 1, lane 2). Similarly, the analysis of Chβ2m showed that there was a protein expression at position appropriate for a mass of about 15 kDa (Fig. 1, lane 6). The soluble analysis showed that the BF2*15-BSP and Chβ2m were located mainly in the insoluble fraction in the cells as inclusion bodies. The molecular mass, showed by the mobility of the recombinant proteins observed by SDS-PAGE, was in accordance with that predicted from the amino acid sequence.

Western blot assay showed that there was strong hybridized signal where the expressed proteins of each of the two constructions localized, while there was no such signal at the corresponding position in the negative control cells.

3.3. Purification of the recombinant proteins

Using the pET-28a (+) vector, we introduced a His6-tag at the NH_2 terminus of BF2*15-BSP and Chβ2m. Thus, the protein can be purified using Ni–NTA affinity material. Both BF2*15-BSP and Chβ2m proteins were purified using Ni–NTA agarose columns under denaturing conditions. Fractions of eluted proteins were analyzed by SDS-PAGE and identified after staining with CBB. The analysis revealed that single-step elution using standard conditions resulted in above 95% purity of both protein fractions and their molecular masses were about 38 kDa and 15 kDa, respectively, by a comparison of the protein molecular weight standards (Fig. 1). Typically, about 1.6 mg of BF2*15-BSP and 2.4 mg of Chβ2m were recovered from 1 g of pelleted cells measured by BCA assay.

3.4. Detection of CTL with soluble BF2*15-peptide tetramer in vitro

BF2*15-BSP heavy chain and Chβ2m were refolded with IBV $N_{71–78}$ in refolding buffer to generate the monomer of BF2*15-peptide complex. Determined by BCA assay, 3 mg BF2*15-peptide monomer was recovered after the gel filtration. The monomer was biotinylated, and tetramerized with PE-labeled streptavidin at a molar ratio of 4:1. After further purification with 100 kDa MWCO spin tube, 2.5 mg/ml PE-labeled BF2*15-peptide tetramer were obtained. In order to confirm whether the BF2*15-peptide tetramer is able to bind to specific CTLs, the PBMCs from either IBV H52 infected or uninfected chickens were stained with the prepared PE-labeled BF2*15-peptide tetramer, and subjected to analysis by flow cytometry. The frequency of tetramer stained CTLs 10 days post-infection was

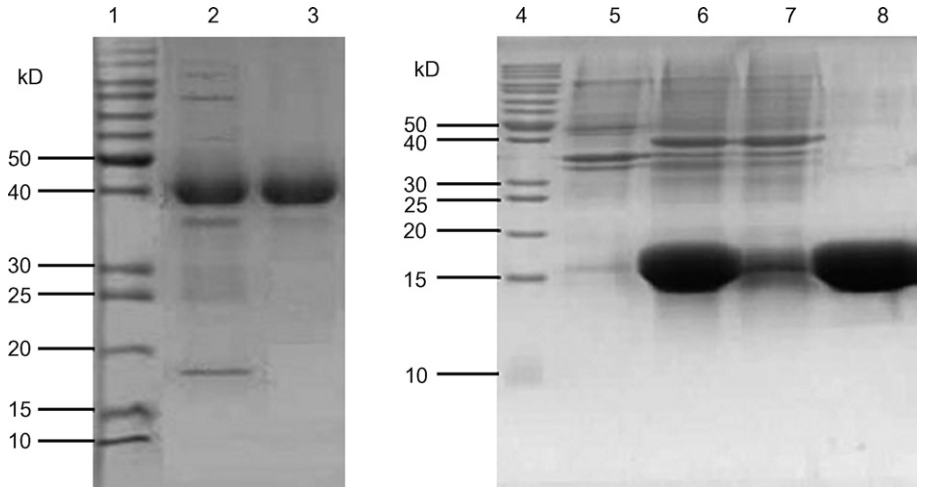

Fig. 1. Purification of recombinant BF2-BSP and Chβ2m. Lanes 1 and 4: protein marker (10–200 kDa); lane 2: unpurified recombinant BF2-BSP; lane 3: purified recombinant BF2-BSP protein; lane 5: pET-28a (+) vector expressed in BL21 (DE3); lane 6: recombinant Chβ2m expressed in BL21 (DE3); lane 7: target protein (Chβ2m) was removed from the sample; lane 8: target protein (Chβ2m) eluted from His-Bind Resin.

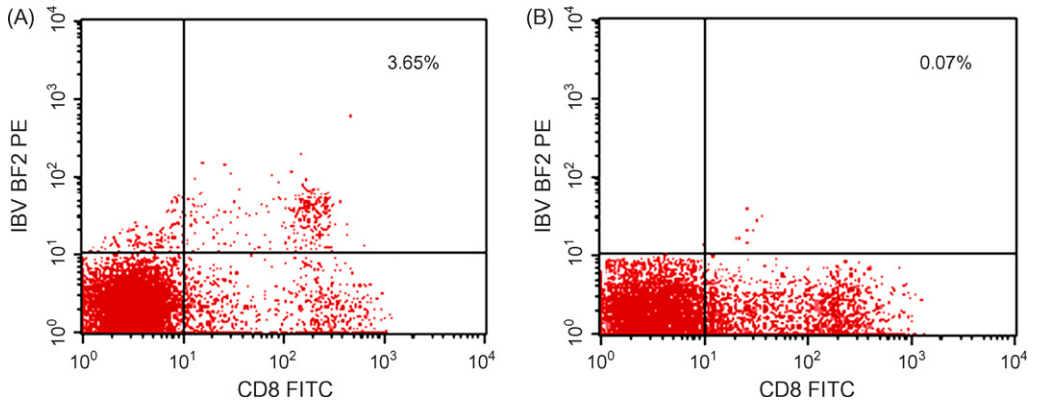

Fig. 2. Determination of specific CTL. Specific CTL was detected by BF2*15/peptide tetramer and anti-chicken CD8 mAb (followed by incubation with FITC-conjugated goat anti-mouse IgG) in PBMC of chicken with IBV infection (A) or without IBV infection (B). CTL frequencies were 3.65% (A) and 0.07% (B), respectively.

3.65% (Fig. 2A), while the uninfected control was only 0.07% (Fig. 2B). These results demonstrate that the prepared BF2*15-peptide tetramer can be used for detection of specific CTLs in chickens.

4. Discussion

$CD8^+$ T-cells mediate defense against primary infection of microbial pathogens and also provide long-term protective immunity (Serbina and Pamer, 2003). Quantitative analyses of antigen-specific T-cells have provided important information on the course of immune responses (Owen et al., 1982; Maier et al., 1986; Murali-Krishna et al., 1998). Before the development of the MHC-I tetramer technology, the main techniques for functional and quantitative measurements of antigen-specific T-cells were LDA, ELISPOT, and ICS in conjunction with flow cytometry (Taswell, 1981; Jung, 1993; Scheibenbogen et al., 1997, 2000).

LDA requires at least 6–7 days *in vitro* culture of the T-cells of interest in conjunction with cytolytic testing (Taswell, 1981). This method is notoriously subject to variability, which probably reflected the frequency of specific cells, their expansion potential *in vitro*, and the ability to lyse appropriate targets (Rehermann et al., 1996a,b). Using ELISPOT, some peptide-specific T-cells, which either secrete cytokines levels below the assay's detectable limit or have cytokine profiles outside of those being assayed, may not be detected. Lastly, intracellular cytokine staining of peptide-stimulated

T-cells usually leads non-viablity of the T-cells being assayed (Jung, 1993). The advent of peptide-MHC tetrameric complexes heralds a new era in the study of antigen-specific T-cells and their role in viral infections.

Because of low affinity and fast off-rates of the MHC-peptide ligand to the TCR, direct staining of T-cells seemed unachievable. Altman et al. (1996) constructed soluble MHC class I/peptide molecules linked together to form tetramers that were able to bind to the matching TCR with higher avidity than the sum of the single monomeric affinities.

This stable structure allows enumeration of antigen-specific T-cells without prior *in vitro* expansion, in addition, to phenotypical analysis using flow cytometry and functional studies on tetramer sorted T-cell clones (Altman et al., 1996). Additionally, the MHC class I tetramer staining can also be used *in situ* to visualize antigen-specific $CD8^+$ T-cells directly in tissues, leaving their spatial relationship to other cells intact (Haanen et al., 2000; Skinner et al., 2000).

To construct the chicken MHC class I peptide tetramer, the BF2*15-BSP and Chβ2m were expressed in prokaryotic expression system and purified on the denatured condition. Then the two subunits refolded to form a BF2*15/peptide monomeric complex by dilution method in the presence of an antigenic peptide IBV $N_{71–78}$. We adopted a procedure established by John Altman (NIH Tetramer Facility, www.niaid.nih.gov/reposit/tetramer/index.html) into our protocol. The MHC complexes, in the refolding buffer, were directly concentrated instead of dialysing. This approach allows us to reuse the flowthrough, which contains most of the peptide, for a second (or even third) refolding. The monomeric complex was tetramerized by binding to fluorescently labeled streptavidin to generate BF2*15/peptide tetramer. The results of flow cytometer analysis demonstrate that the prepared BF2*15-peptide tetramer can be used for detection of IBV nucleoprotein-specific CTLs in chicks.

In this paper we have described our construction of a chicken MHC-I tetramer, which can used to evaluate the antigen-specific CTLs. When the T-cells interact with the target cells, the T-cells recognize both the antigen displayed in cleft and MHC molecules. So, in this experiment, the positive signal detected in the two-color flow cytometry assay was enough to illustrate the peptide-specific CTL. However, it could be better to understand the peptide-specific CTL if there are two more controls, that is, to use the chickens with different MHC types and chickens infected with a different virus.

The chicken MHC-I tetramer constructed in this paper can be used in wide variety experiments. Besides its potential use in the study of the overall evaluation of the T cell immune response, this tetramer can also be used to track the kinetics of the IBV-specific CTL response. Also, the BF2 tetramer can be used for the evaluation on CTL response specific to other chicken viruses by replacing the peptide in the peptide-binding groove of BF2 during refolding procedure. Additionally, few BF2-restricted T cell epitopes have been identified, thus far. The prepared BF2*15-peptide tetramer can be used as an effective tool for mapping the BF2-restricted T cell epitopes. Once more BF2-restricted T cell epitopes have been mapped, this BF2*15-peptide tetramer can be more effectively used to investigate the CTL response to viral infection in the chicken.

In summary, the generation of BF2*15/peptide tetramer lays the foundation for further study on the characteristic of antigenic peptide presented by BF2*15, and provides a valuable tool for the research on cell-mediated immune of virus-related diseases in chicks.

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