Structural and Biophysical Insights into the TCRαβ Complex in Chickens

Lijie Zhang, Yanjie Liu, Geng Meng, Ruiying Liang, Bing Zhang, Chun Xia

xiachun@cau.edu.cn

HIGHLIGHTS

Structural analysis of the overall architecture of the chicken TCRαβ was completed

The positively charged cleft between the CDR3s might accommodate acidic side chains

The changes in the C domains of ch-TCRαβ may impact the assembly of TCR-CD3 complex

The distinct topology of chicken TCR Cβ domain coevolved with CD3 heterodimers

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Structural and Biophysical Insights into the TCRαβ Complex in Chickens

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SUMMARY
In this work, chicken HPAIV H5N1 epitope-specific TCRαβ (ch-TCRαβ) was isolated and its structure was determined. The Cα domain of ch-TCRαβ does not exhibit the typical structure of human TCRαβ, and the DE loop extends outward, resulting in close proximity between the Cα domain of ch-TCRαβ and CD3εδ/γ. The FG loop of the Cβ domain of ch-TCRαβ is shorter. The changes in the C domains of ch-TCRαβ and the difference in chicken CD3εδ/γ confirm that the complexes formed by TCRαβ and CD3εδ/γ differ from those in humans. In the chicken complex, a positively charged cleft is formed between the two CDR3 loops that might accommodate the acidic side chains of the chicken pMHC-I-bound HPAIV epitope intermediate portion oriented toward ch-TCRαβ. This is the first reported structure of chicken TCRαβ, and it provides a structural model of the ancestral TCR system in the immune synapses between T cells and antigen-presenting cells in lower vertebrates.

INTRODUCTION
Specific T cell and antibody immunities are the two main lines of defense against viral, bacterial, and parasitic infections in jawed vertebrates. T cells recognize the antigenic peptides of these pathogenic microorganisms presented by major histocompatibility complex (MHC)-I/II bound to the peptide (pMHC-I/II) on antigen-presenting cells (APCs) through T cell receptors (TCR) and then trigger a specific T cell immune response by a series of signaling events (Davis and Bjorkman, 1988; Kaufman, 2018; Rudolph et al., 2006). The key link is that TCR and pMHC-I/II, the related co-receptors CD3 and CD8/CD4, and other costimulatory receptors, complete the first activation event in the immune synapses (Adams et al., 2016; Li et al., 2013; Sibener et al., 2018; Taniuchi, 2018). TCRs are heterodimers on the T cell surface composed of α- and β- or γ- and δ-chains linked by disulfide bonds. Each TCR chain consists of variable and constant Ig-like domains, followed by a transmembrane domain and a short cytoplasmic tail (Fields et al., 1995; Garcia et al., 1996). In addition, TCR binds to pMHC-I/II through the complementary-determining region (CDR) in its variable domain. To date, the complete TCRαβ structure (Garcia et al., 1996; Kjer-Nielsen et al., 2002), TCRαβ-pMHC complex (Reinherz et al., 1999; Stewart-Jones et al., 2003; Tyan et al., 2005), and TCRαβ-CD3 complex from mammals have revealed a variety of ways for TCR to specifically recognize antigens (Dong et al., 2019). However, how these central immune protein molecules interact in the immune synapses has not been fully clarified by structural biology, and only some reasonable inferences are available (Li et al., 2013). It is worth noting that although these structures of pMHC-I, pMHC-II, and CD3 in nonmammals have been solved (Berry et al., 2014; Chen et al., 2017; Koch et al., 2007; Wu et al., 2017; Zhang et al., 2020a), the structure of TCR is still unknown. This lack of information particularly hinders the understanding of immunology in lower vertebrates.

A series of achievements have been made in the study of chicken TCR. Chicken TCR studies began with the demonstration of the TCR-CD3 complex on the surface of chicken T cells by using monoclonal antibodies (Chen et al., 1986). Subsequently, three TCRs (TCR1, TCR2, and TCR3) were identified and found to be successively expressed during the development of chicken T cells (Chen et al., 1989; Lahti et al., 1988). Chicken TCR1 is TCRγδ, and both TCR2 and TCR3 are TCRαβ. The α chains of chicken TCR2 and TCR3 are identical, but the variable regions in the β chain are different and are encoded by the Vβ1 and Vβ2 genes, respectively (Tjoelker et al., 1990). Each of the chicken TCR chains is encoded by a separate combination of genes (Cooper et al., 1991; Shigeta et al., 2004; Tjoelker et al., 1990). For example, the chicken α chain is encoded by TCR-V genes, TCR-J genes, and TCR-C genes, whereas β chains are encoded by these three genes as well as by various TCR-D genes. In addition, the chicken TCRγδ gene locus is very simple compared with that of mammals; it contains only two major Vβ gene families (Vβ1 and Vβ2) and one D-J-C gene cluster that includes one D gene, four J genes, and one C gene (Shigeta et al., 2004). The main chicken TCRα gene
The region includes the Vα1 family (41 genes), the Vα2 family (19 genes), 48 Jα genes, and one Cα gene (Chen et al., 1996; Kubota et al., 1999; Parra and Miller, 2012). Surprisingly, chickens lack distinct CD3γ and CD3δ subunits but express a hybrid CD3δ/γ protein that shares equal homology with mammalian CD3δ and CD3γ (Gobel and Dangy, 2000). The chicken CD3δ/γ molecule displays significant differences from the human and mouse CD3 molecules in both its packing orientation and its dimer interface (Berry et al., 2014). During chicken T cell activation and signaling, the key molecules pMHC-I/II, CD8, and CD3 have been known. However, knowledge of the other key molecules TCRαβ and pMHC-I/II is still lacking, and this prevents us from achieving a deep and systematic understanding of chicken immunology.

In this study, the crystal structure of chicken TCRαβ (ch-TCRαβ) was determined. The Cα domain of ch-TCRαβ does not exhibit the structure typical of human TCRαβ. The extension of the DE loop of the Cα domain, the shortening of the FG loop of the Cβ domain, and the difference in the packing orientation of CD3δ/γ confirm that the complexes formed by chicken TCRαβ (ch-TCRαβ) and CD3δ/γ are very different from those of humans. In ch-TCRαβ, there is a positively charged cleft between the two CDR3 loops that might accommodate the acidic side chains of the MHC-bound peptide intermediate portion oriented toward ch-TCRαβ. These results provide key guidance for TCR-based T cell recognition, and provide key knowledge of the molecular anatomy between T cells and APC in the immune synapses of chickens.

**RESULTS**

**Epitope-Specific TCRαβ Genes in CD8+ T Cells**

Tetrameric pBF2*1501 complexes constructed for the PA123-130 peptide are shown in Figure S1. After immunizing B12.5 haplotype chickens with PA123-130 peptide, flow cytometry analysis showed that the percentage of CD8+ T cells specific to the PA123-130 peptide was significantly greater in the immunized group than in the control group (Figure 1). The percentage of CD8+ T cells was 5.13%–10.70% for the peptide-immunized animals. The cells in the double-positive areas of the control group were scattered and nonspecific, accounting for 0.50%–0.97% of the total cells. Statistical analysis confirmed that the percentages of PA123-130 epitope-specific CD8+ T cells were significantly higher in the peptide-immunized group than in the control group (p < 0.01). The data are shown as the mean ± SEM. **p < 0.01 by unpaired t test.

![Figure 1. Detection of CD8+ Tet+ T Cells from Immunized and Control B15 Chickens](image)

(A and B) (A) Peripheral blood mononuclear cells (PBMCs) were isolated from each of 12 chickens (4 chickens in each group) and stained with both the PE-labeled pBF2*15 tetramer (Tet) that incorporated the peptide PA123-130 and an fluorescein isothiocyanate-labeled anti-CD8 monoclonal antibody. Flow cytometry was performed to detect CD8+ T cells that were specific for the peptide PA123-130 (i.e., CD8+ Tet+ T cells). The data are presented as pseudocolor plots. The percentage of cells in the tetramer and CD8 double-positive areas was 5.13%–10.70% for the peptide-immunized animals. The cells in the double-positive areas of the control group were scattered and nonspecific, accounting for 0.50%–0.97% of the total cells. (B) Statistical analysis confirmed that the percentages of PA123-130 epitope-specific CD8+ T cells were significantly higher in the peptide-immunized group than in the control group (p < 0.01). The data are shown as the mean ± SEM. **p < 0.01 by unpaired t test.
The Architecture of ch-TCRαβ

The extracellular regions of the ch-TCRα and ch-TCRβ genes were subcloned for prokaryotic expression (Table S1). Ch-TCRα and ch-TCRβ formed a heterodimer in vitro (Figure S2). The high-purity ch-TCRαβ was used to screen crystals; this led to the collection of a set of diffraction data at a resolution of 2.1 Å for ch-TCR. However, in the process of structural determination, only the solution of the ch-TCRα chain can be solved, and the solution of the ch-TCRβ chain cannot be obtained by the available methods. Therefore, we performed amino acid mutagenesis of the ch-TCRβ chain, introduced selenomethionine, and collected the diffraction data at a resolution of 1.9 Å for selenomethionine (SeMet)-ch-TCRαβ. The crystal structure of ch-TCRαβ was determined by a combination of molecular replacement and single-wavelength anomalous diffraction (SAD) phasing. The final refinement of the structure generated R/Rfree factors of 0.205/0.236 (Table 1). Crystal structure analysis of ch-TCRαβ showed two molecules in each asymmetric unit (Figure S3). The two molecules were very similar (Figure S3). Apart from certain key features, the topology of the ch-TCRαβ structure is basically the same as that of known TCRαβ structures (Figure 2).

Multiple sequence alignment showed that ch-TCRα shares less than 35% amino acid sequence identity with human and mouse TCRα (Figure 3). The similarity of the Cα domain was less than 23%, whereas the similarity of the ABED sheet was between 22% and 24%, and the similarity of the CFG sheet was no more than 18%; in particular, the similarity with the Cλ domain was between 22% and 24%, and the similarity of the CFG sheet was no more than 18%; in particular, the similarity with the Cκ chain of 2C (PDB: 1TCR) was only 7% (Figure 3). The similarity between the β chains was 35%–46%, higher than that of the α chains. The similarity between Cβ domains was higher than 40%, but there was a large fragment deletion between the F and G strands. The difference in amino acid sequences may indicate that ch-TCRαβ has certain species-specific characteristics.

ch-TCRαβ Structure Reveals a Close Connection with the CD3 Molecule

Superposition of the ch-TCRαβ structure and the solved TCRαβ structures showed the greatest difference in the Cα domain, where the root-mean-square deviation of the Cα-carbon positions was greater than 2.0 Å (Table S2). The Cα domain of ch-TCRαβ showed lower thermal factors (B values) than in the other resolved TCRαβ structures. The most obvious difference between the Cα domain of ch-TCRαβ and that of previously solved TCRαβ structures was in the CFG sheet (Figure 4A). The CFG sheet of the Cα domain consisted of a regular IgSF-C1 domain. Hydrogen bonds between the main chains of the C, F, and G strands maintained the standard CFG sheet conformation (Figure 4B). The Cα domains of human and mouse TCRαβ contain unusual secondary structures that are highly divergent from the standard IgSF-fold (Figure 4C). Additionally, the F segment in human and mouse TCRαβ formed an unusual one-turn mini-helix that lacked hydrogen bonds to the neighboring strands (Figure 4D). There was no mini-helix in the Cα domain of ch-TCRαβ, and the position that the mini-helix would have occupied instead contained the regular F strand (Figure 4A). The Cα domain of ch-TCRαβ was identical to that of the previously solved TCRαβ; the two sheets were far apart, and the connection was looser than that of the classic IgSF-C1-fold (Figure S4). It is worth mentioning that the Cβ domain of TCRγδ also retained the classical IgSF-fold similar to that of ch-TCRαβ. These characteristics indicate that the Cα domain of ch-TCRαβ adopts a conformation intermediate between that of human and mouse TCRαβ and classical IgSF-C1.

The ABED sheet was similar to the corresponding sheet in IgSF-C1 domains and other TCRαβ structures, but there were also some differences, mainly in the region between the D and E strands (Figure 4E). Compared with those of previously reported TCRαβ structures, the D and E strands of ch-TCRαβ were longer. The direction of the DE loop between the D and E strands changed, and the loop extended farther outward, playing a key role in binding to the CD3 molecule. At certain matching positions in the DE loop, the distance between the superposed ch-TCRαβ and human TCR-CD3 complex structures was very short, even constituting close contact (Figure 4F). Because chicken CD3εδγ has a unique packing orientation and dimer interface compared with mammalian CD3εγ and CD3εδ, ch-TCRαβ and ch-CD3 may enable the formation of a signaling complex that differs from the complex found in humans. Amino acid sequence alignment showed that the deletion of amino acids 148–152 and the insertion of amino acids 159–162 in ch-TCRαβ might have caused the shift in the DE loop. Apart from a few amino acid substitutions, the Cα domains of TCRαβ were conserved in chickens. This feature was common in chickens and is considered a species characteristic.
Short FG Loop of the Cβ Domain in ch-TCRαβ Caused by 12-Residue Deletion

Superposition of ch-TCRαβ with the previously solved TCRαβ structures revealed that there were also large differences in the Cβ domain (Figure 5A). First, the FG loop of the Cβ domain of ch-TCRαβ was shorter (Figures 5A and 5B). The Cβ domains of mice and humans contain an elongated 12-residue FG loop that extends out to the side of the Cβ domain and adopts a roughly similar conformation in the two species. Sequence alignment of the FG loop regions of the Cβ domains of various species showed that whereas the 12-residue insertion is well conserved among mammals, nonmammals do not have this 12-residue insertion within the FG loop (Figure 5C). In αβ T cells, the FG loop is important for development, thymic selection, and T cell function and has recently been shown to be important for sustained TCR-MHC binding and T cell signaling (Brazin et al., 2015; Wang, 2020). It has also been suggested to be important for CD3ε binding. The unique structure of the ch-CD3 molecule suggests that ch-TCRαβ with a short FG loop may

|                        | ch-TCR     | SeMet ch-TCR |
|------------------------|------------|-------------|
| Data collection        |            |             |
| Space group            | P1211      | P 1 21 1    |
| Cell dimensions        |            |             |
| a, b, c (Å)            | 77.43, 83.097, 82.416 | 76.48, 77.40, 80.60 |
| α, β, γ (°)            | 90, 117.716, 90 | 90.00, 116.02, 90.00 |
| Wavelength (Å)         | 0.97923    | 0.97923     |
| Absorption (Se)        | Peak       |             |
| Resolution (Å)         | 29.29–2.091 (2.166–2.091) | 77.40–2.1 (2.15–2.10) |
| Total reflections       | 382,365    | 378,197     |
| Unique reflections      | 54,200     | 49,681      |
| Rsym or Rmergeb        | 0.114(0.860)b | 0.092(0.646) |
| Vol                     | 15.1(3.5)c | 14.9(3.3)   |
| Completeness (%)        | 99.4 (97)b | 99.9 (99.9)b |
| Redundancy              | 7.1(5.6)   | 7.6 (7.7)   |
| Refinement              |            |             |
| No. Reflections        | 52,142     |             |
| Rwork/Rfree (%)         | 20.5/23.6  |             |
| RMS deviations         |            |             |
| Bond lengths (Å)        | 0.02       |             |
| Bond angles (°)         | 1.97       |             |
| Average B factor        | 50.16      |             |
| Ramachandran plot quality |            |             |
| Most favored region (%) | 94.91      |             |
| Allowed region (%)      | 5.08       |             |
| Disallowed (%)          | 0.00       |             |

Table 1. Data Collection and Refinement Statistics

*Values in parentheses represent the highest resolution shell.
*bRmerge = Σ[I(hkl)–<I(hkl)>]/Σ[I(hkl)], where I(hkl) is the observed intensity and <I(hkl)> is the average intensity recorded in multiple measurements.
*cR = Σ||Fobs|-k|Fcalc||/Σ|Fobs|, where Rfree is calculated for a randomly chosen 5% of reflections and Rwork is calculated for the remaining 95% of reflections used for structure refinement.

Short FG Loop of the Cβ Domain in ch-TCRαβ Caused by 12-Residue Deletion

Superposition of ch-TCRαβ with the previously solved TCRαβ structures revealed that there were also large differences in the Cβ domain (Figure 5A). First, the FG loop of the Cβ domain of ch-TCRαβ was shorter (Figures 5A and 5B). The Cβ domains of mice and humans contain an elongated 12-residue FG loop that extends out to the side of the Cβ domain and adopts a roughly similar conformation in the two species. Sequence alignment of the FG loop regions of the Cβ domains of various species showed that whereas the 12-residue insertion is well conserved among mammals, nonmammals do not have this 12-residue insertion within the FG loop (Figure 5C). In αβ T cells, the FG loop is important for development, thymic selection, and T cell function and has recently been shown to be important for sustained TCR-MHC binding and T cell signaling (Brazin et al., 2015; Wang, 2020). It has also been suggested to be important for CD3ε binding. The unique structure of the ch-CD3 molecule suggests that ch-TCRαβ with a short FG loop may
bind ch-CD3 in different ways, and ch-TCRαβ may have different signaling mechanisms. In fact, the insertion of 12 residues occurs only in the Cγ domain of TCRγδ and in the mammalian Cβ domain of TCR but not in other IgSF structures.

Interactions within the ch-TCRαβ Heterodimer

Although the conformation of the C regions was significantly altered, it remained relatively conserved at the interface between Cα and Cβ. The Cα-Cβ interface was highly polar, with a skewed distribution of acidic residues on the Cα domain and basic residues on the Cβ domain and the presence of many other polar residues, and included hydrogen bond interactions between polar residues on the Cα domain and charged residues on the Cβ domain, or vice versa (Figure 5D). The Cα-Cβ interface was stabilized by many hydrogen bonds and van der Waals interactions. Because there is no long FG loop, the buried area between Cβ-Vβ domains was small. The binding angle between the Cβ and Vβ domains was similar to that of the resolved TCRαβ structures. Because the F and G strands of the Cβ domain of ch-TCRαβ were longer than those in the other TCRαβ structures and the N terminus of the G strand was closer to the Vβ domain, two hydrogen bonds formed between β211Gln and β6Ser/β7Ile, but no other hydrogen bonds existed. There was no hydrogen bond at the Vα-Cα interface, and the angle and stability of the elbow region were maintained mainly by van der Waals interactions.

Conformation of the CDR Loops of ch-TCRαβ

The CDR loops in the ch-TCRαβ structure were labeled and analyzed according to the accepted division of CDRs and the solved human TCRαβ structures (Figures 6A and 6B). The CDR1s and CDR2s of ch-TCRαβ were relatively invariant germline genetic coding components that were derived directly from different variable region gene segments (Figure 6C); the CDR3s of ch-TCRαβ were highly variable components obtained by the random addition and deletion of nucleotides during gene rearrangement and by the random combination of numerous linker gene fragments and the variable gene fragments V(D)/J (Figure 3).

The CDR1β loop of ch-TCRβ was stabilized predominantly by hydrogen bonds between β22Gln, β26His, and β27Asp located on the CDR1β loop and residues located on the HV4β and CDR3β loops (Figure 6D). The CDR2β loop of ch-TCRβ linked the C and C′ strands, which were the edge strands of two sheets. The CDR2β loop of ch-TCRβ was stabilized by intraloop hydrogen bonds. β65Arg and the residues of the CDR1β and CDR2β loops of ch-TCRβ formed a network of five hydrogen bonds that played an important role in maintaining the stability of the CDR1β and CDR2β loops. The CDR1α loop of ch-TCRα was stabilized by hydrogen bonds formed by the backbone atoms of the residues located in the CDR1α loop and the side chains of the residues located in the CDR3α and HV4 loops. The CDR2α loop of ch-TCRα consisted of five residues with no intraloop interaction. There was only a hydrogen bond between the two CDR3 loops of ch-TCRαβ, so that the two CDR3s loops tended to face each other (Figure 6E). The CDR loops of TCRαβ are highly flexible, and these analyses only represent the conformations in that structure. The buried surface
area at the Vα-Vβ interface of ch-TCRαβ was calculated analytically to be 1,403 Å². Hydrophobic bonds were the dominant interaction forces between the Vα and Vβ domains of ch-TCRαβ (Figure 6E).

Specificity of the CDR Loops of ch-TCRαβ

Superposing the CDR loops of ch-TCRαβ on previously solved TCRαβ structures showed that the CDR1β and CDR2β loops of ch-TCRαβ were consistent with those of previously solved Vβ structures (Figure 7A). The CDR1β loop of ch-TCRαβ contained the relatively conserved loop-stabilizing Gln at position 22. Gln and His at positions 22 and 26 were conserved, as they are in many mouse and human CDR1 loops and played a similar structural role of stabilizing the center of the turn. Hence, these CDR1β structures probably represent canonical structures for most Vβ chains. The CDR2β loop of ch-TCRαβ was stabilized by the intraloop hydrogen bonds. Unlike the CDR1β and CDR2β loops, the conformations of the CDR1α and CDR2α loops differed from those of the previously solved TCRαβ structures (Figure 7B). The main reason for this was the difference in the length of CDR1α and the difference in the intraloop interactions.

Immune Synapses between T Cells and Antigen-Presenting Cells

In the immune synapses between T cells and APC, TCRαβ recognizes pMHC-I/II. It should be noted that the CDR loops. The peptide and MHC bind to TCR V regions via amino acids in their CDR loops. The CDR1β
and CDR2β loops of TCR recognize and bind to the α1 helix of MHC-I. Asp and Tyr at positions 27 and 28 of ch-TCRβ and Tyr at position 47 of ch-TCRβ potentially bind to the MHC-I α1 helix. J47Tyr was conserved in most species and was positioned over a similar site on the MHC-I α1 helix. The CDR1α and CDR2α loops of TCR recognize and bind to the α2 helix of MHC-I. Tyr at position 28 and Val and Lys at positions 48 and 49 of
ch-TCRβ also potentially bound to the MHC-I α2 helix. Most of the previous TCRαβ structures show a cleft between the two CDR3s loops; this cleft accommodates the side chains of the intermediate portion of the MHC-bound peptide, which is oriented toward TCRαβ. Superposing the CDR3 loops of resolved TCRαβ structures indicated that the two CDR3 loops of ch-TCRαβ were long and tended to face each other (Figures 6 and 7). In ch-TCRαβ, a positively charged cleft was formed between the two CDR3 loops that might accommodate the side chains of acidic residues of PA123-130 (Figures 7C and 7D). Detailed interaction residues and docking angles between pMHC-I/II and ch-TCRαβ require structural data to be obtained.

**DISCUSSION**

This article elucidates the first specific TCRαβ structure for chicken BF2*1501 presenting the avian influenza virus epitope PA123-130 and therefore has special significance in two ways: it reveals the characteristics and significance of the ch-TCRαβ structure and makes it possible to build a model of the interaction among TCRαβ, CD3, and pMHC-I/II molecules at immune synapses.

First, multiple sequence alignment showed that ch-TCRβ and ch-TCRα share only 35%–46% and 26%–31% amino acid identity with human and mouse TCRβ and TCRα, respectively, and consistent with this, the overall architecture of ch-TCRαβ has certain species-specific characteristics. This first structure of the chicken TCRαβ provides a structural model of the ancestral TCRαβ in lower vertebrates.

The Cα domain of ch-TCRαβ is a classic IgSF-C1 fold composed of seven antiparallel β-strands, in contrast to previously solved TCR structures, which are highly divergent from the standard IgSF-fold. The change causes the Cα domain of ch-TCRαβ to present different molecular surfaces to the CD3 subunits and to other cell surface molecules. The D and E strands of the Cα domain of ch-TCRαβ are longer, and the DE loop extends farther...
outward and plays a key role in binding to the CD3 molecule. Another interesting feature is that the FG loop of the Cβ domain of ch-TCRαβ is shorter (Figure 4). In the mouse and human Cβ domains, the elongated 12-residue FG loop extends out to the side of the domain (Wang and Reinherz, 2012). The FG loop is important for development, thymic selection, and T cell function and has recently been shown to be important for sustained TCR-MHC binding during TCR-pMHC interactions and T cell signaling (Brazin et al., 2015).

The immune synapses in which a specific TCR interacts with pMHC-I/II are formed on the surface of T cells and APC; within the synapse, the V region of TCR recognizes the peptide and the binding region of pMHC-I/II and receives important signals in T cell activation (La Gruta et al., 2018). Subsequently, the C region, the
transmembrane region, and the intracellular region of the TCR bind to the CD3 molecule and transmit the signal downstream (Dong et al., 2019). Based on the structure of the human TCR-CD3 complex (Dong et al., 2019), it was found that when the chicken δ/γ chain was superposed with the human δ chain, the ch-TCRα chain was oriented toward CD3ε/δ and the distance between the superposed ch-TCRα chain and the CD3ε/δ/γ chain was very small, even suggesting overlap; this was not a reasonable combination. When the ε chains of chicken and human were superimposed, the distance between the ch-TCRα chain and the CD3ε/γ chain was reasonable, but the binding surface differed from that of the TCR-CD3 complex in humans. It is also possible that the CD3ε and CD3δ/γ chains of chicken do not function in the same manner as the human ε and δ chains but are instead employed in a completely novel way. There are elongated FG loops in the TCR Cβ domains and separately expressed CD3δ and γ chains in mammals, whereas short FG loops and hybrid CD3δ/γ chains are common in nonmammals; this indicates that the distinct topology of CD3 heterodimers coevolved with TCR Cβ domains to optimize the quaternary TCR structure for pMHC-I/II-triggered TCR activation (Brazin et al., 2015; Kim et al., 2010). Therefore, it is certain that the combination of chicken TCRαβ and CD3εδ/γ differs from that of humans. The more highly conserved chicken γ chain can replace its mouse counterpart in γ-deficient T cells and send signals downstream (Gobel and Bolliger, 1999). Assembly of the TCR-CD3 complex is mediated by its ECDs, CPs, and transmembrane helices. The CP and transmembrane interactions play a major role in the assembly.

Figure 7. Structural Analysis of the CDR Loops of ch-TCRαβ
(A) Top view of the CDR loops shown on the surface.
(B) Top view of the CDR loops shown as polarity. Red indicates negative polarity, and blue indicates positive polarity.
(C and D) The CDR1, CDR2, and CDR3 loops of the α and β chains were analyzed and compared in detail. The color of the CDR loops of ch-TCRαβ is as in Figure 2. The CDR loops of other resolved TCRαβ structures are shown in gray.
of the TCR-CD3 complex, whereas the ECD interactions are secondary (Call et al., 2002). The correct binding conformation between ch-TCR and ch-CD3 molecules has not yet been determined, and the structure of the complex must be resolved to clarify its actual conformation.

When interacting with MHC molecules, the greatest difference observed was that the two CDR3s of ch-TCR were bound by only a single hydrogen bond between the two loops; thus the two CDR3s tended to face each other. In ch-TCRβ, a positively charged cleft that might accommodate the side chains of acidic residues of PA123-130 was formed between the two CDR3 loops (Figures 6C and 6D, Figure 7). Detailed interaction residues and docking angles between pMHC/II and ch-TCRβ require structural data to be obtained.

Limitations of the Study
In this study, we provided a detailed structural analysis of the overall architecture of the chicken TCRβ that served as a molecular basis for a comparative investigation with other TCRβ structures from other distant species, and in the context of the immune synapse. Here, the study showed that the DE and FG loop in the chicken TCRβ were longer and shorter, respectively, than the mammalian counterparts, suggesting that it may impact in overall assembly of the chicken immune synapse. However, if we can obtain the chicken TCR-MHC structure further, this will greatly strengthen the article immensely.

Resource Availability
Lead Contact
All relevant data and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Chun Xia (xiachun@cau.edu.cn).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
The coordinates and structure factors generated in this study have been deposited to the Protein DataBank under accession number 6LIR.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101828.

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AUTHOR CONTRIBUTIONS
C.X. designed the study and supervised the project; L.Z., Y.L., R.L., and B.Z. performed experiments; L.Z. performed the data analysis; L.Z. and G.M. solved the structure; C.X. provided guidance on data analysis; C.X. and L.Z. wrote the paper.

DECLARATION OF INTEREST
The authors declare no competing interest.

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Supplemental Information

Structural and Biophysical Insights into the TCRαβ Complex in Chickens

Lijie Zhang, Yanjie Liu, Geng Meng, Ruiying Liang, Bing Zhang, and Chun Xia
Figure S1. Production of the pBF2*1501 tetramer (Related to Figure 1). (A) The pBF2*1501-BSP complexes (solid line) were purified by chromatography on a Superdex 200 size-exclusion column and biotinylated using the BirA enzyme. The biotinylated pBF2*1501 complexes (dashed line) were subsequently purified using the same column. Peaks 1 and 2 represent the correctly refolded pBF2*1501-BSP complex and the biotinylated pBF2*1501 complex, respectively. The efficiency of purification of the complex was tested via SDS-PAGE. (B) Evaluation of tetramerization efficiency via SDS-PAGE. Lane 1 is untetramerized pBF2*1501 monomer, lane 2 is pBF2*1501 tetramer, and lane 3 is PE-labeled streptavidin.

Figure S2. Assembly of the chicken TCRαβ heterodimer (Related to Figure 2). Ch-TCRα and ch-TCRβ chains were expressed and co-refolded in vitro. The ch-TCRαβ heterodimer curve is shown in blue, and the NaCl concentration curve is shown in red. The insets show the SDS-PAGE (15%) analysis of the peak that is labeled on the curve. Lane M contains molecular mass markers (labeled in kDa).
Figure S3. Overall structure of chicken TCRαβ (Related to Figure 2). (A) Two molecules (M1 and M2) in an asymmetric unit are shown in cartoon representation. The ch-TCRαβ molecules are packed “head to tail”. The α chain of the M1 molecule is shown in yellow-orange, the β chain of the M1 molecule is shown in cyan, and the α chain and the β chain of the M2 molecule are shown in light pink and in gray, respectively. (B) Superposition of M1 and M2. The structures of the M1 molecule were very similar to those of the M2 molecule, and the RMSD for all of the Ca atoms in the two molecules was 0.3895.

Figure S4. Connection between the ABED and CFG sheets of the Ca domain of ch-TCRαβ (Related to Figure 4). Superposition of the Ca domain of ch-TCRαβ and the Ca domain of 2C-TCRαβ (A) C domain of the antibody light chain (PDB: 1MLC) (B) and Cγ domain of TCRγδ (C). The Ca domain of ch-TCRαβ is shown in yellow-orange, the C domain of the antibody light chain is shown in gray, and the Cγ domain of TCRγδ is shown in slate.

Table S1. PCR primers specific for chicken TCR genes (Related to Figure 2).

| Primers                      | Sequence (5’-3’)                        |
|------------------------------|-----------------------------------------|
| Ch-TCRa-cloning-forward primer | ATGGATTTTGTGAGTTTGCTTCTTGTATCTTTCA      |
| Ch-TCRa-cloning-reverse primer  | TCATTGGATTATTTCCACATAAG                 |
| Ch-TCRβ-cloning-forward primer  | ATGTGGACAATTTTGGTGCAATGGTCTTGG          |
| Ch-TCRβ-cloning-reverse primer  | CTAGTACATTTTCTGTACACAAAGCATC           |
| Ch-TCRa-expression-forward primer | CCGGAATTCAATCTGAGGTGCAGCAGGAGCGTCG     |
| Ch-TCRa-expression-reverse primer | CCGCCTGAAGTGGTTTCAGTTCTATCTGTTGG     |
| Ch-TCRβ-expression-forward primer | CCGGAATTCAATCTGAGGTGCAGCAGGAGCGTCG     |
| Ch-TCRβ-expression-reverse primer | CCGCCTGAAGTGGTTTCAGTTCTATCTGTTGG     |
| Ch-TCRβ (L141M) -forward primer  | AGAAGAAGAAGGCGCAATGGATAGCTGGCTGCTCTGTCTTTC |
| Ch-TCRβ (L141M) -reverse primer  | AACCAGAGGCGCCAGGCAATCCATTGTGGCCTCTCTTCTTCTTTC |
### Table S2. The RMSD values for main chain superposition of ch-TCRαβ and other TCRαβ molecules that have been resolved (Related to Figure 2).

| number | PDB   | Overall | α chain | α1 domain | α2 domain | β chain | β1 domain | β2 domain |
|--------|-------|---------|---------|-----------|-----------|---------|-----------|-----------|
| 1      | 1TCR  | 1.9559  | 1.8893  | 1.4080    | 2.1689    | 1.9175  | 0.7611    | 0.7754    |
| 2      | 3QH3  | 1.5960  | 1.8408  | 1.3602    | 2.4946    | 1.7031  | 0.7789    | 0.9415    |
| 3      | 2VLm  | 2.5061  | 1.8935  | 1.2091    | 2.5604    | 2.7177  | 0.9901    | 0.9922    |
| 4      | 3VXQ  | 2.0579  | 1.8767  | 1.4918    | 2.6762    | 1.8406  | 1.3183    | 0.8512    |
| 5      | 3VXT  | 2.2930  | 1.8406  | 1.3447    | 2.4408    | 2.4727  | 0.7940    | 0.9564    |
| 6      | 2NW2  | 1.8554  | 1.8741  | 1.3447    | 2.4060    | 1.7797  | 0.7732    | 0.9647    |
| 7      | 1KGC  | 2.0998  | 1.7001  | 1.5452    | 2.4575    | 2.0937  | 1.3099    | 0.9623    |
| 8      | 3SKN  | 2.1018  | 1.8008  | 1.3725    | 2.3780    | 2.1472  | 1.1740    | 1.1004    |
| 9      | 2BNu  | 2.3448  | 1.9808  | 1.1569    | 2.4623    | 2.4649  | 0.7616    | 0.9231    |
| 10     | 3DX9  | 2.4777  | 2.2047  | 1.5888    | 2.6606    | 2.3227  | 1.5093    | 0.9195    |
| 11     | 1UTP  | 2.3870  | 2.0550  | 1.3006    | 2.1924    | 2.2512  | 1.4052    | 1.0493    |
| 12     | 3QEU  | 2.0177  | 1.8309  | 1.4561    | 2.4347    | 2.0975  | 0.7782    | 0.9986    |
| 13     | 4JFh  | 2.2425  | 2.0359  | 1.3476    | 2.7692    | 2.3422  | 1.6909    | 0.9387    |
| 14     | 5NMD  | 2.1216  | 1.5539  | 1.1297    | 2.3246    | 2.5111  | 1.4310    | 0.8468    |
| 15     | 6AT6  | 2.0588  | 1.9343  | 1.4932    | 2.0293    | 1.9278  | 0.8368    | 1.0012    |
| 16     | 4GG8  | 2.5761  | 2.3022  | 1.6889    | 2.5064    | 2.2523  | 1.4854    | 1.0387    |
| 17     | 4E42  | 1.9533  | 2.0729  | 1.3151    | 2.5873    | 1.6488  | 1.3142    | 1.1269    |
| 18     | 2IAL  | 2.3207  | 1.6652  | 1.3925    | 2.5648    | 2.1434  | 0.8834    | 0.9980    |
| 19     | 4GKZ  | 2.2049  | 2.0421  | 1.5538    | 2.4580    | 2.1372  | 0.6114    | 0.9914    |
| 20     | 3QFf  | 1.8832  | 2.2017  | 1.3307    | 2.6464    | 1.6848  | 1.2135    | 1.0840    |
| 21     | 6CHI  | 1.9029  | 1.9978  | 1.6518    | 2.6505    | 1.5212  | 1.3788    | 0.9390    |
| 22     | 2Q86  | 1.6731  | 1.7162  | 1.0898    | 2.1142    | 1.6848  | 0.7379    | 0.9790    |
| 23     | 3AXL  | 2.2069  | 1.9374  | 1.0927    | 2.4578    | 2.1292  | 0.9373    | 1.0110    |
| 24     | 2EYS  | 2.4755  | 2.1096  | 1.2196    | 2.3824    | 2.3708  | 0.9767    | 0.9861    |
| 25     | 3TYF  | 1.7811  | 1.9347  | 1.34      | 2.1313    | 1.5448  | 1.0415    | 1.0140    |
| 26     | 4E6   | 2.1232  | 1.7456  | 1.4341    | 2.3432    | 1.9363  | 1.2851    | 0.9904    |
| 27     | 4DZB  | 2.0890  | 2.1617  | 1.2290    | 2.3234    | 1.8788  | 1.4587    | 1.0056    |
| 28     | 4MNH  | 2.8916  | 2.1655  | 1.6232    | 2.4022    | 2.7404  | 1.9961    | 0.9140    |
| 29     | 4LFH  | 2.4674  | 2.0838  | 1.4420    | 2.3895    | 2.4516  | 1.8655    | 1.5204    |
| 30     | 1HXM  | 3.0576  | 2.7025  | 1.6079    | 2.6080    | 2.4617  | 1.5034    | 1.6092    |

Numbers 1 to 15 are TCR molecules associated with MHC-I molecules; numbers 16 to 21 are TCR molecules associated with MHC-II molecules; numbers 22 to 27 are innate-like TCR; and numbers 28 to 30 are TCRγδ.
Transparent Methods

Sorting epitope-specific CD8⁺ T-cells. According to the peptide-binding motif (X-R-X-X-X-X-Y) of the BFY*15:01 allele of B15-haplotype chickens, the peptide REVHTYY (located at positions 123-130 of the PA protein, and named PA123-130) was derived from the PA protein of the highly pathogenic avian influenza (HPAIV) H5N1 virus (Li et al., 2020; Walwyn et al., 2006). PA123-130 was synthesized and purified to 90% purity by reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry (SciLight Biotechnology LLC, Beijing, China). PE-labeled pBF2*15:01 tetramers for the detection of PA123-130-specific CTLs were constructed as previously reported (Li et al., 2020). Subsequently, according to our previously reported method, PA123-130 peptide plus adjuvant was used to immunize B15 haplotype specific pathogen-free (SPF) chickens (BF2*15:01), and PA123-130 peptide-specific T-cells were sorted (Li et al., 2020). A total of 8 seven-day-old B15 lineage SPF chickens were divided into two groups with 4 chickens in each group. One group was immunized with PA123-130 plus adjuvant, and the other group was the control group. Each animal received a total of 3 immunizations at 7-day intervals. Seven days after the third immunization, peripheral blood lymphocytes (PBMC) were isolated from blood collected from the animals’ wing veins, and 2 × 10⁶ cells from each individual were stained with PE-labeled tetramers and an FITC-labeled mouse anti-chicken CD8α monoclonal antibody (SouthernBiotech, USA). After staining, PA123-130 peptide and CD8 double-positive T-cells were detected and sorted by flow cytometry (FACSAria, BD, USA). The sorted cells were suspended in RPMI1640 medium containing 10% fetal bovine serum and counted again. More than 10⁶ cell events could be used to extract mRNA.

Cloning TCRα and TCRβ chains. Total RNA was extracted from the PA123-130-specific CD8⁺ T-cells using TRizol reagent, and RNA concentrations were measured via spectrophotometry. After confirmation of the integrity of the RNA by analysis on a 1.5% (w/v) agarose gel, it was reverse-transcribed into cDNA using the ExScript RT Reagent Kit (TaKaRa Biotechnology, China). Two pairs of primers were designed based on the sequences of the two known TCR genes (U-EF554736 and U-EF554782) and used to amplify full-length TCRα and TCRβ sequences by the polymerase chain reaction (PCR) (Table S1). Amplifications were performed in a reaction volume of 50 μl. The reaction contained 100-200 ng of cDNA, 25 pmol of each primer, 4 μl of 2.5 mM dNTP, 10× PCR buffer with 15 mM MgCl₂, and 1 unit of HiFi DNA polymerase (Transgen Biotech, China). The thermal cycling conditions were as follows: initial denaturation at 98 °C for 5 min, followed by 32 cycles of 98 °C for 10 s, 60 °C for 15 s, and 72 °C for 1 min and final extension at 72 °C for 10 min. The PCR products were inserted into the pMD-18T vector (TaKaRa Biotechnology, China), and positive clones were selected and sent to BioSune (Shanghai, China) for sequencing. The sequences have been submitted to GenBank (National Center for Biotechnology Information: https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers GenBank: MN646854, MN646855.

Protein preparation. As described above, the ch-TCRα gene (encoding extracellular residues 1-218) and the ch-TCRβ gene (encoding extracellular residues 1-238), with the addition of both EcoR I and Xho I sites, were cloned by PCR (Table S1). The PCR products were sequenced, ligated into the prokaryotic expression vector pET21a (Novagen) at the EcoRI and XhoI restriction sites, and transformed into Escherichia coli (E. coli) strain BL21 (DE3). Recombinant ch-TCRα and ch-TCRβ were expressed in inclusion bodies and induced by 0.5 mM isopropyl-β-D-thiogalactoside. To calculate the correct phase by the SAD method, site-directed mutagenesis was performed to construct the ch-TCRβ chain containing the L141M mutation using overlap PCR with the Fast Mutagenesis System Kit (TransGen Biotech, China) (Table S1). The mutant ch-TCRβ recombinant plasmid was then transformed into the methionine-auxotrophic E. coli strain B834 (DE3) for the expression of SeMet-substituted ch-TCRβ proteins (SeMet-ch-TCRβ). SeMet-ch-TCRβ was prepared as previously described (Chen et al., 2018; Hendrickson et al., 1990).

Assembly of antigen-specific ch-TCRαβ heterodimers. For in vitro refolding, purified ch-TCRα and SeMet-ch-TCRβ inclusion bodies were dialuted at a ratio of 1:2 in a refolding solution containing 100 mM Tris-HCl, 5 M guanidinium chloride, 40 mM L-arginine-HCl, 2 mM EDTA, 5 mM reduced glutathione and 0.5 mM oxidized glutathione, pH 8.0. After stirring for 1 hour at 4 °C, the refolding solution was dialyzed to eliminate the Gua-HCl. The folding solution was concentrated and purified by Resource Q anion-exchange chromatography (GE Healthcare, China) as previously described (Chen et al., 2017). Ch-TCRα and ch-TCRβ were refolded and purified as described above for ch-TCRα and SeMet-ch-TCRβ.

Crystallization and data collection. Purified SeMet-ch-TCRαβ and ch-TCRαβ heterodimers were
concentrated to 4 mg/mL and 8 mg/mL, respectively, in a buffer containing 20 mM Tris-HCl (pH 8.0) and 50 mM NaCl for crystallization. After mixing with the reservoir buffer at a 1:1 ratio, the concentrated ch-TCRαβ heterodimers were crystallized by the sitting-drop vapor diffusion method at 18 °C. Index, Crystal Screen I/II, and Crystal Screen Cryo I/II Kits (Hampton Research, Riverside, CA) were used to screen for optimal crystal growth conditions. After 7 days, SeMet-ch-TCRαβ crystals were obtained with solution No. 22 from the PEG/Ion 2 Kit (0.2 M tribasic ammonium citrate pH 7.0, 20% w/v polyethylene glycol 3350) (Hampton Research), and ch-TCRαβ crystals were obtained with solution No. 45 from the PEG/Ion Kit (0.2 M tribasic lithium citrate tetrahydrate, 20% PEG3350) (Hampton Research). The preliminary screening conditions were further optimized manually using graded variations in the concentrations of precipitant and salt in hanging drop vapor diffusion experiments at 4 °C and 18 °C. SeMet-ch-TCRαβ crystals and ch-TCRαβ crystals were obtained under optimized conditions at 18 °C (0.22 M tribasic ammonium citrate pH 7.0 with 18% w/v polyethylene glycol 3350 and 0.25 M tribasic lithium citrate tetrahydrate with 24% PEG 3350, respectively). Prior to data collection, all crystals were cryoprotected in reservoir buffer containing 30% (v/v) glycerol and flash-cooled at 100 K. The 1.9 Å diffraction data of the SeMet-ch-TCRαβ crystal and the 2.1 Å diffraction data of the ch-TCRαβ crystal were collected at 100 K. Data collection was performed at the Shanghai Synchrotron Radiation Facility using beamline BL17U at 0.97923 Å with an ADSC 315 CCD detector (Shanghai, China) (Wang et al., 2016). The collected intensities were indexed, integrated, corrected for absorption, scaled, and merged using the HKL3000 package (Minor et al., 2006).

**Structure determination and refinement.** The data collection and refinement statistics are summarized in Table 1. Structural determination was performed using the SAD method with selenomethionine as an anomalous signal as previously reported (Liu et al., 2012). Briefly, the expected heavy atoms were determined by SHELXD (Schneider and Sheldrick, 2002), and the initial phases were then determined using Phaser (McCoy, 2007). Density modification was performed by DM (Cowtan and Main, 1996). Approximately 90% of the ch-TCRαβ residues were traced automatically by ARP/Warp (Perrakis et al., 2001). The structure of ch-TCRαβ was solved by molecular replacement with Phaser in the CCP4 package and SeMet-ch-TCR as the search model (Collaborative Computational Project, 1994). Extensive model building and restrained refinement were performed with COOT (Emsley and Cowtan, 2004) and REFMACS (Murshudov et al., 1997), respectively. Refinement cycles were performed using the phenix.refine program in the PHENIX package (Adams et al., 2002) with isotropic ADP refinement and bulk solvent modeling. The stereochemical quality of the final models was validated using the PROCHECK program (Laskowski et al., 1993).

**Data analysis.** The SignalP 3.0 Server was used to predict the presence and location of signal peptide cleavage sites (Almagro Armenteros et al., 2019). Structural illustrations were generated using PyMOL (http://www.pymol.org/) and UCSF Chimera (http://www.cgl.ucsf.edu/chimera/). The isotropic B factor was calculated using the equation B=8π2<μ2>. Solvent-accessible surface areas were calculated using the PDBePISA webpage (http://www.ebi.ac.uk/pdbe/pisa/picite.html). Protein amino acid sequences were compared using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Chojnacki et al., 2017). The coordinates and structure factors generated in this study have been submitted to the Protein Data Bank (https://deposit-pdbj.wwpdb.org/deposition/) under accession number 6LIR.
Supplemental References

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