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The crystal structure of CD8 $\alpha\beta$ in complex with YTS156.7.7 Fab and interaction with other CD8 antibodies define the binding mode of CD8 $\alpha\beta$ to MHC class I

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Summary

The CD8 $\alpha\beta$ heterodimer interacts with class I pMHC on antigen-presenting cells as a co-receptor for TCR-mediated activation of cytotoxic T cells. To characterize this immunologically important interaction, we used mAbs specific to either CD8 α or CD8 β to probe the mechanism of CD8 $\alpha\beta$ binding to pMHCI. The YTS156.7 mAb inhibits this interaction and blocks T cell activation. To elucidate the molecular basis for this inhibition, the crystal structure of the CD8 $\alpha\beta$ immunoglobulin-like ectodomains were determined in complex with mAb YTS156.7 Fab at 2.7 Å resolution. The YTS156.7 epitope on CD8 β was identified and implies that residues in the CDR1 and CDR2-equivalent loops of CD8 β are occluded upon binding to class I pMHC. To further characterize the pMHCI/CD8 $\alpha\beta$ interaction, binding of class I tetramers to CD8 $\alpha\beta$ on the surface of T cells was assessed in the presence of anti-CD8 mAbs. In contrast to YTS156.7, mAb YTS105.18, which is specific for CD8 α , does not inhibit binding of CD8 $\alpha\beta$ to class I tetramers, indicating the YTS105.18 epitope is not occluded in the pMHCI/CD8 $\alpha\beta$ complex. Together, these data indicate a model for the pMHCI/CD8 $\alpha\beta$ interaction which is similar to that observed for CD8 $\alpha\alpha$ in the CD8 $\alpha\alpha$ /pMHCI complex, but in which CD8 α occupies the lower orientation (membrane proximal to the antigen presenting cell), and CD8 β occupies the upper position (membrane distal). The implication of this molecular assembly for the function of CD8 $\alpha\beta$ in T cell activation is discussed.

Keywords

CD8 $\alpha\beta$; TCR co-receptor; antibody complex; T cell; crystal structure

Introduction

The CD8 glycoprotein is essential for the development, activation and biological functions of cytotoxic (CD8⁺) T cells. CD8 comprises two distinct subunits, CD8 α and CD8 β , and is expressed as both $\alpha\beta$ heterodimers and $\alpha\alpha$ homodimers. CD8 $\alpha\beta$ is the most abundant isoform on developing thymocytes and circulatory CD8⁺ T cells.¹ CD8 $\alpha\beta$ interacts with peptide-bound class I major histocompatibility complex (pMHCI) molecules on the surface of antigen-presenting cells to form a tertiary complex with the T cell receptor (TCR) that is a prerequisite

for triggering and activation of cytotoxic T lymphocytes (reviewed in ²). Homodimeric CD8 $\alpha\alpha$ predominates on the surface of intra-epithelial leucocytes (IEL), as well as on Natural Killer cells (NK cells), and has been further observed on $\gamma\delta$ and CD4⁺ T cells.³ CD8 $\alpha\alpha$ also binds pMHCI and this interaction has been characterized from crystal structures of CD8 $\alpha\alpha$ with^{4,5} and without^{6,7} an MHC class I ligand. However, although soluble CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IgSF dimers bind soluble pMHCI with equivalent affinity (11–67 μ M)^{8,9}, CD8 $\alpha\alpha$ fails to compensate for CD8 $\alpha\beta$ in CD8-dependent TCR activation¹⁰ and is approximately 100-fold less effective as a TCR co-receptor.¹¹

Despite the physiological importance of CD8 $\alpha\beta$, information concerning how CD8 $\alpha\beta$ interacts with pMHCI is presently limited to biochemical and mutational analyses,^{12,13,14} and the structural basis for the enhanced co-receptor activity of CD8 $\alpha\beta$ remains unclear.

Crystallographic analysis of a single chain CD8 $\alpha\beta$ immunoglobulin superfamily (IgSF) heterodimer has shown that the CD8 β globular domain adopts an immunoglobulin-like fold that is similar in structure to that of CD8 α and the overall topology of the CD8 $\alpha\beta$ dimer resembles that of CD8 $\alpha\alpha$.¹⁵ The mouse CD8 $\alpha\alpha$ /H-2K^b co-crystal structure⁴ previously revealed a profound asymmetry in the interacting elements of the two CD8 α subunits. A single CD8 α protomer (designated α 1) in the “upper” position, immediately below the α 1/ α 2 peptide-binding platform, accounts for >60% of the total surface area buried upon complex formation and contacts residues in the α 1, α 2 and α 3 domain of pMHCI, as well as β 2-microglobulin (β 2M). The second CD8 α subunit (designated α 2) in the “lower” membrane-proximal position (with respect to the antigen-presenting cell) interacts only with the pMHCI α 3 domain. Structural complementarity in the CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ ligand-binding domains has led to speculation that both CD8 isoforms adopt a common mode of interaction with pMHCI but mutagenesis studies have given rise to fundamentally different models in which CD8 $\alpha\beta$ interacts with pMHCI (H-2K^b) in at least two distinct orientations, such that CD8 β would occupy the position of either CD8 α subunit in the CD8 $\alpha\alpha$ /pMHCI complex.^{12,14} However, the biological significance of such findings is currently unclear.

Monoclonal antibodies (mAbs) provide a means to probe the physiologically relevant interfaces of interacting proteins which avoids the pitfalls of interpreting results from incorrectly folded or non-expressed proteins that are inherent in mutagenesis studies. In this regard, mAbs have been widely used in the investigation of CD8 activity both *in vivo* and *in vitro*. Many CD8-specific mAbs block CD8/pMHCI interaction and inhibit CD8⁺ T cell activation *in vivo* through binding to either CD8 α (YTS105.18, CT-CD8 α , YTS169) or CD8 β (YTS156.7, 53.5.8).^{16,17,18} However, mAb binding does not always abrogate CD8 interaction with pMHCI. Anti-CD8 α mAb 53.6.7 and anti-CD8 β mAb KT112 actually enhance binding of CD8 to class I tetramers.^{18,19,20} Hence, elucidation of the mAb epitopes on the surface of CD8 can provide a means to ascertain which regions of CD8 are occluded and which are exposed upon interaction with pMHCI.

To establish the nature of the interaction between class I MHC and CD8 $\alpha\beta$, we investigated the effects of different antibodies against CD8 $\alpha\beta$ upon pMHCI complex formation. The YTS156.7 mAb is a rat IgG_{2b} against mouse CD8 β which depletes mouse CD8⁺ T cells *in vivo*²¹ and blocks interaction between CD8 $\alpha\beta$ and soluble pMHCI *in vitro*.¹⁰ Numerous studies have employed YTS156.7 to investigate CD8 $\alpha\beta$ -mediated T cell activation and, more recently, to probe the orientation of pMHCI/CD8 $\alpha\beta$ interactions.¹⁴ To determine the molecular basis for YTS156.7 activity, a soluble mouse CD8 $\alpha\beta$ dimer was crystallized in complex with YTS156.7 Fab. The structure enabled identification of the YTS156.7 epitope on the surface of CD8 β and confirmed that the existing crystal structure of a single-chain mouse CD8 $\alpha\beta$ IgSF dimer¹⁵ was indeed properly configured. Location of the YTS156.7 epitope on CD8 β implies that residues in the CDR1 and CDR2-equivalent loops of CD8 β are occluded in the pMHCI/CD8 $\alpha\beta$ complex, either by direct contact with pMHCI, or through steric hindrance.

To determine the disposition of the CD8 α subunit in the pMHCI/CD8 $\alpha\beta$ complex, we investigated the effects of other anti-CD8 mAbs on pMHCI/CD8 $\alpha\beta$ interaction using tetramers of pMHCI. In this way, we could demonstrate that the mAb YTS105.18 epitope on the surface of CD8 α is not occluded in the pMHCI/CD8 $\alpha\beta$ complex. This finding indicates that CD8 α occupies a “lower” position (i.e. more proximal to the antigen-presenting cell (APC)) in the complex, and implies that CD8 β occupies a binding site that is more proximal to the peptide-binding groove of class I MHC (i.e. more distal to the APC and, hence, closer to the T cell).

Results

Overview of the CD8 $\alpha\beta$ /YTS156.7 Fab complex structure

A complex containing the soluble ectodomain of the mouse CD8 $\alpha\beta$ IgSF dimer and mAb YTS156.7 Fab was crystallized in orthorhombic space group $P2_12_12_1$ at pH 6.2 and data collected to 2.7 Å resolution. Preliminary phases were determined by the molecular replacement method and the CD8 $\alpha\beta$ /YTS156.7 Fab structure was refined to 2.7 Å resolution. Data collection and refinement statistics are presented in Table 1. The asymmetric unit of the crystal contains two CD8 $\alpha\beta$ /YTS156.7 Fab complexes (complexes A and B), that are related by a translation along the Y axis. The two complexes are extremely similar and superimposition of all C α atoms gives an rmsd value of 0.77 Å. Subsequent analyses of CD8 $\alpha\beta$ /YTS156.7 will, therefore, be confined to only one of the two complexes in the asymmetric unit (complex A, chain IDs LHAB), unless otherwise stated. The structure of the complex is shown in Figure 1a, and comprises one CD8 $\alpha\beta$ IgSF heterodimer bound to a single YTS156.7 Fab. The refined model contains 659 of the 680 residues that are present in the constructs and includes residues 4–121 of CD8 α , 1–117 of CD8 β , 1–230 of the YTS156.7 heavy chain and 1–214 of the YTS156.7 light chain. Three residues at the amino-terminus of CD8 α as well as residues at the carboxyl-terminus of both CD8 subunits, including the thrombin protease recognition sequence, were not observed in the electron density maps and have not been included in the model.

The refined model has R_{cryst} (23.5%), R_{free} (28.4%) and Ramachandran plot values that are within the ranges commonly observed at this resolution^{22,23} (Table 1). Thr^{L51} incorrectly is assigned to a disallowed region of the Ramachandran plot according to Procheck,²² as it is located within a perfectly acceptable γ -turn that is observed in almost all antibodies.²⁴ $2F_o - F_c$ electron density is clearly defined at the 1 σ level throughout the entire structure, although less-well defined for certain regions of the constant heavy chain (C_H1) and constant light chain (C_L) of YTS156, which have higher overall B values (83.9 Å² and 99.1 Å² for C_H1 and C_L respectively, as compared to 69.7 Å² for the overall complex), due to limited contacts in the crystal. In particular, Pro¹²⁷-Ser¹³⁷ in C_H1 form part of a highly flexible loop that is disordered in most Fab crystal structures.²⁵ Furthermore, the paucity of electron density for the CD and EF loops of C_H1 indicates flexibility in this region of the structure, which lies within a solvent-filled cavity in the crystal.

Conformational flexibility in CD8 $\alpha\beta$ is restricted to a few distinct regions, as indicated by reduced quality of the $2F_o - F_c$ electron density map and higher B values in these regions. A schematic representation of the B values of C α residues in CD8 $\alpha\beta$ is given in supplemental Figure 3. Overall, the electron density in the CD8 β subunit is of higher quality than for CD8 α , which does not contact YTS156.7, and is reflected by a higher B value (79.4 Å² for CD8 α , as compared to 64.6 Å² for CD8 β). The C'C and C'D loops of both CD8 α and CD8 β are highly flexible and exhibit weaker electron density, and correspondingly higher B values. Furthermore, Lys^{a1}-Asn^{a3} that comprise the N-terminus of CD8 α are disordered, as observed in other CD8 structures, and have been omitted from the model.

Structure of the CD8 $\alpha\beta$ heterodimer

The mouse CD8 $\alpha\beta$ IgSF dimer described here represents the crystal structure of a non-covalently associated CD8 $\alpha\beta$ heterodimer, albeit in complex with YTS156.7 Fab. The CD8 α and CD8 β constructs used in expression of our soluble CD8 $\alpha\beta$ both lack the connecting glycopeptides at the carboxyl terminus (31 and 27 residues for CD8 α and CD8 β , respectively) that couple the CD8 globular IgSF ectodomains to the T cell membrane. The CD8 α and CD8 β subunits are representative of the immunoglobulin V-set family, each comprising nine β -strands which form the characteristic anti-parallel, β -sheet. CD8 α and CD8 β heterodimerize through pairing of their respective C' and G strands, such that the CD8 $\alpha\beta$ dimer resembles the antigen binding (Fv) region of antibodies (Figure 1b). Indeed, the B-C, C'-C'' and F-G loops of both CD8 subunits are spatially equivalent to the hyper-variable complementarity-determining region (CDR) 1, 2 and 3 loops of antibodies, respectively. In mouse CD8 α , these loops comprise residues Gly^{a30}-Gly^{a35} (CDR1-equivalent), Tyr^{a55}-Lys^{a62} (CDR2-equivalent) and Val^{a104}-Tyr^{a111} (CDR3-equivalent). The analogous loops on CD8 β comprise Ser ^{β 24}-Ser ^{β 30} (CDR1-equivalent), Trp ^{β 51}-Gly ^{β 56} (CDR2-equivalent) and Thr ^{β 98}-Val ^{β 105} (CDR3-equivalent). The CDR1-equivalent loop of CD8 α (Gly^{a30}-Gly^{a35}) has a higher B value (93.1 Å²), as compared to CD8 α as a whole (79.3 Å²), that indicates greater flexibility. In comparison, the CDR1-equivalent loop in CD8 β forms part of the YTS156.7 epitope and is constrained to a single conformation. The CDR3-equivalent regions of both CD8 α and β subunits are notably short compared with those normally found in antibodies, and the position and orientation of these loops are both fixed in the dimer interface.

Of the three potential N-linked glycosylation sites in CD8 $\alpha\beta$, only one in CD8 α , at Asn^{a70}, shows well-defined electron density for a single N-acetyl glucosamine (GlcNAc) moiety, which occupies the pocket created between the C-C' loop and E-F loop. The amide nitrogen of the GlcNAc hydrogen bonds with the carbonyl oxygen of Glu^{a95}, forming a bridge which stabilizes the relative orientation of the C-C' and E-F loops. Despite a lack of electron density for N-carbohydrates at the other two N-glycosylation motifs in CD8 $\alpha\beta$ (Asn^{a42} and Asn ^{β 13}), enzymic deglycosylation of each CD8 IgSF domain revealed the presence of carbohydrates in both subunits, indicating sugars are, indeed, attached to CD8 β at Asn ^{β 13} (data not shown). This N-glycosylation motif, in the A-B loop of CD8 β , is conserved in both human and mouse and precludes interaction between pMHCI and this region of the molecule. The lack of interpretable electron density for carbohydrates at this site in the CD8 $\alpha\beta$ /YTS156.7 structure indicates a high degree of conformational flexibility and implies that N-carbohydrates do not have a direct influence on the structure of CD8 β *per se*.

The structural aspects of the CD8 $\alpha\beta$ heterodimer have previously been addressed in the crystal structure of a single-chain CD8 $\alpha\beta$ construct, which mimics the physiological form of the molecule, but employs a 29 amino-acid linker to connect the subunits. Comparison of the single-chain CD8 $\alpha\beta$ model with our CD8 $\alpha\beta$ heterodimer structure indicates that they are structurally homologous, and superimpose with rmsd values of 0.85 Å and 1.27 Å for 118 and 115 C α atoms of the α and β subunits, respectively (see supplemental Figure 4). Given that the single-chain CD8 $\alpha\beta$ model (PDB ID 2ATP¹⁵) was not utilized for molecular replacement in structural determination of the CD8 $\alpha\beta$ /YTS156.7 complex structure, a high degree of complementarity between these structures confirms the validity of both models. In both structures, the extended N-terminus of CD8 α projects into the solvent and is highly flexible; as a consequence, Lys^{a1}, Pro^{a2} and Gln^{a3} have been omitted from both models due to lack of interpretable electron density. These same residues are also disordered in the crystal structure of CD8 α in the absence of a class I binding partner,⁶ but are ordered in the interface between one CD8 α subunit and pMHCI in the co-crystal structure.^{4,26} The most notable structural deviations between the two models occur in loop regions of the β subunit, specifically in the C-C' and C''-D loops, which are flexible and exhibit high B values in both structures.

Furthermore, conformational plasticity is indicated by alternative conformations in the solvent-exposed C-C' loop of CD8 β (Gln³³⁸-Asp³⁴²), as well as in the flexible C''-D loops of both CD8 α (Glu⁶⁷-Leu⁷⁴) and CD8 β (Glu⁶¹-Asn⁶⁸) in both structures, consistent with relatively high B values in both models. The B-C (CDR1-equivalent) loop of CD8 β is fixed by interaction with YTS156.7 and has a low B value, in contrast to the equivalent loop in the single-chain structure, which is shifted by 6 Å and exhibits high B values, indicative of significant flexibility in this loop. Importantly, high homology is observed in both the conformation and B values of residues in the F-G (CDR3-equivalent) loops of both CD8 α and CD8 β in both models, suggesting these loops are more ordered and less flexible than the CDR1-equivalent B-C loops.

Despite the different roles of the two isoforms of CD8, the overall structure of the CD8 $\alpha\beta$ IgSF dimer closely resembles that of CD8 $\alpha\alpha$. Comparison with CD8 $\alpha\alpha$,⁶ by superimposition of 112 C α atoms in the CD8 α subunit of each model, illustrates the overall similarity of the two isoforms of the receptor (Figure 2a). Importantly, heterodimerization with the β subunit does not confer conformational changes on CD8 α and the structural features of CD8 α are highly conserved in both isoforms. Furthermore, the dimerization interfaces in both CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ are highly conserved, with equivalent surface complementarity (Sc) values of 0.77 and 0.73, respectively.²⁷ Comparison of the individual CD8 α and CD8 β IgSF domains indicates both subunits are topologically similar, despite low sequence identity (12%).²⁸ Superimposition of 60 corresponding C α atoms from the β -sheets within the Ig cores of both subunits produces an rmsd value of 1.1 Å and reveals striking similarity in the overall architecture of the α and β subunits (Figure 2b). The CD8 β IgSF domain (117 residues) is smaller than that of CD8 α (125 residues) and one of the most profound structural differences is in the length of the A strands, which extend 11 and 5 residues in CD8 α and CD8 β respectively, prior to the characteristic β -bulge. The similar topology of both subunits, together with conservation of the interacting elements within the CD8 α /CD8 β dimerization interface, accounts for the similar architectures of the CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IgSF dimers. Furthermore, spatial and conformational homology in the CDR-equivalent loops of both subunits likely accounts for the similarity in the pMHC1-binding characteristics of both CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ and provides evidence for an equivalent mechanism of interaction.

The YTS156.7 Fab epitope

The YTS156.7 epitope is restricted to the CD8 β subunit and is centered over the D-E loop, roughly at 45° to the plane of the CD8 $\alpha\beta$ dimerization interface (Figure 1a). The Fab interface has a shape complementarity (Sc) value of 0.76,²⁷ which is in the upper range of values reported for antigen-antibody complexes,²⁴ and buries a total surface area of 1,479 Å², incorporating 717 Å² on CD8 β and 761 Å² on YTS156.7.²⁹ Interaction involves all six complementarity-determining regions (CDRs) of the Fab, with the majority (69%) of the buried surface being contributed from the heavy chain, as commonly observed in antibody-antigen interactions.³⁰

The $2F_o - F_c$ electron density map is of excellent quality in the region of the YTS156.7 combining site. YTS156.7 contacts 27 residues on the surface of CD8 β , including five from the B-C (CDR1-equivalent) loop and six from the C'-C'' (CDR2-equivalent) loop (Figure 3). The interface comprises three salt-bridge interactions (Asp^{L92} to Arg^{B77}, Asp^{L92} to Arg^{B78} and Asp^{H32} to Lys^{B27}), and 11 hydrogen bonds, as well as numerous van der Waals contacts, which are predominantly mediated by Tyr^{L32}, Trp^{H52} and Tyr^{H99}. The primary interaction surface centers on Arg^{B77} and Arg^{B78} in the D-E loop of CD8 β , which hydrogen bond with Tyr^{L32} and Thr^{L93}, and salt-bridge to Asp^{L92}. CDR H1 contacts residues in the C-C' loop and CDR H2 inserts within the cleft between the C''/D strands of CD8 β and CDR H3.

Effects of mAbs on CD8 $\alpha\beta$ binding to tetramers of class I pMHCI

To probe the interacting elements between CD8 $\alpha\beta$ and class I pMHC, we evaluated the effects of YTS156.7 and two other anti-CD8 mAbs, YTS105.18 and 53.6.7, on binding of tetramers to OT-1 T cells transgenic for a T cell receptor specific for class I MHC H-2K^b and the SIINFEKL (OVA) peptide. It has been demonstrated that binding of tetramers of H-2K^b-OVA to TCR at the surface of OT-1 splenocytes is dependent upon the participation of CD8,¹⁸ and the OT-1 system is, therefore, ideal for monitoring the effects of mAbs upon CD8/pMHCI interaction. We assessed the effects of mAbs YTS156.7, YTS105.18 and 53.6.7 on T cell activation by measurement of induced Ca²⁺ mobilization following stimulation of OT-1 splenocytes with tetramers of H-2K^b-OVA. YTS105.18 and 53.6.7 are both rat IgG_{2a} mAbs specific for mouse CD8 α . In the absence of mAbs against CD8, exposure of OT-1 cells to tetramers of H-2K^b-OVA gave rise to high levels of calcium flux and subsequent T cell activation, whereas exposure to tetramers of H-2K^b loaded with the control peptide SIYRYYYGL (SIYR) produced no measurable calcium flux (Figure 4a). Incubation of OT-1 T cells with YTS156.7 mAb prior to tetramers of H-2K^b-OVA completely blocked calcium flux, indicating inhibition of pMHCI/CD8 interaction. In contrast, incubation of OT-1 T cells with mAb YTS105.18 had no effect upon pMHCI/CD8 interaction, and exposure of these cells to H-2K^b-OVA tetramers gave rise to high levels of calcium flux, equivalent to that observed for the control cells, indicating that this mAb does not inhibit productive pMHCI/CD8 interactions. Prior incubation of OT-1 T cells with mAb 53.6.7 produced enhanced calcium flux in the presence of H-2K^b-OVA tetramers, suggesting that this mAb induced elevated T cell activation. To exclude the possibility that the observed effects of mAbs on T cell activation were influenced by mAb-mediated co-localization (cross-linking) of surface receptors, calcium flux measurements were repeated in the presence of mAb Fabs. Results obtained for each of the mAb Fabs were equivalent to those obtained for each of the intact IgGs, respectively (Figure 4b), indicating the observed effects of mAbs upon T cell activation are independent of Fc-mediated activities. These data were confirmed by direct measurement of tetramer binding to OT1 splenocytes in the presence of varied concentrations of each of the mAbs (supplemental Figure 5).

Taken together these findings imply that, whilst all three mAbs bind to regions of CD8 that are exposed at the surface of resting T cells, the YTS156.7 epitope is occluded during pMHCI/CD8 $\alpha\beta$ binding, whereas both the 53.6.7 and YTS105.18 epitopes remain exposed. The ability of YTS156.7 Fab to block pMHCI/CD8 interaction, and thereby inhibit T cell activation, is consistent with the renowned biological activity of this mAb *in vivo*. In contrast, the finding that YTS105.18 does not inhibit this interaction is surprising, given that this mAb has been associated with the blockade of T cell activation *in vivo*.^{16,31} However, the molecular mechanism for this inhibition has not been investigated and our *in vitro* data imply that the reported inhibitory effects of this mAb *in vivo* could occur through an indirect mechanism that is dependent upon the organization of molecules within the intercellular contact zone. Specifically, the absence of the antigen-presenting cell membrane and the spatial restraints of the intercellular contact zone in our tetramer-binding experiments represent a fundamental difference that may account for this inconsistency. In this regard, it is also important to note that the inhibitory activity of YTS156.7, conversely, is entirely independent of membrane-associated effects. The finding that mAb 53.6.7 induces an increased level of T cell activation is consistent with previous observations¹⁹; however, the molecular basis for this mechanism has yet to be established.

Mechanism of YTS156.7 activity and orientation of the pMHCI/CD8 $\alpha\beta$ complex

The structural basis for CD8 $\alpha\beta$ /YTS156.7 interaction, together with the biological activity of the YTS156.7 Fab, provides insight to the interaction of CD8 $\alpha\beta$ with pMHCI. Comparison of CD8 $\alpha\beta$ from the single-chain structure with that from our CD8 $\alpha\beta$ /YTS156.7 complex structure

indicates both structures are highly similar and confirms that YTS156.7 does not induce conformational changes in CD8 that may inhibit pMHCI binding. Hence, inhibition of pMHCI/CD8 $\alpha\beta$ by YTS156.7 Fab implies that the YTS156.7 epitope, which includes residues within CDR-equivalent loops 1 and 2, as well as residues in the B, D and E strands of CD8 β (Figure 3), overlaps with the binding site of pMHCI, or that YTS156.7 Fab precludes pMHCI/CD8 $\alpha\beta$ complex formation by steric clash with the pMHCI. Structural similarity in the CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IgSF domain dimers, as well as existing mutagenesis data,¹⁴ suggest that both CD8 isoforms interact with pMHCI in an approximately equivalent manner, such that CD8 $\alpha\beta$ binds to the acidic C–D loop on the side of pMHCI, below the antigen-presenting groove. In the CD8 $\alpha\alpha$ /H-2K^b co-crystal structure, interaction of the two CD8 subunits is asymmetric, with one subunit in an “upper” ($\alpha 1$) position and one subunit in a “lower” ($\alpha 2$) position (Figure 5a). To determine whether our data fit such a mode of binding, we created two models of the pMHCI/CD8 $\alpha\beta$ complex, based on the orientation of CD8 $\alpha\alpha$ in the CD8 $\alpha\alpha$ /H-2K^b structure, in which CD8 β occupies either the $\alpha 1$ or $\alpha 2$ position when bound to pMHCI. Modeling of the interaction in this way indicates that, if CD8 β occupies the $\alpha 1$ position, binding of YTS156.7 would inhibit pMHCI/CD8 $\alpha\beta$ interaction through a substantial steric clash with the pMHCI (Figure 5b). In the alternative model, in which CD8 $\alpha\beta$ binds pMHCI with CD8 β in the $\alpha 2$ position, a clash between the B–C (CDR 2) loop of YTS156.7 V_H and the A–B loop of the pMHCI $\alpha 3$ domain would also inhibit pMHCI/CD8 $\alpha\beta$ interaction (Figure 5c). Both of these models are consistent with the experimentally-determined activity of the YTS156.7 Fab and, hence, the location of the YTS156.7 epitope on CD8 β supports a model in which both CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ interact with pMHCI in an equivalent manner. Furthermore, our finding that mAb YTS105.18 Fab does not inhibit interaction between CD8 $\alpha\beta$ and pMHCI then provides additional information regarding the disposition and orientation of the CD8 α subunit. The experimentally determined effects of YTS105.18 upon pMHCI/CD8 $\alpha\beta$ interaction indicate that, unlike YTS156.7, the YTS105.18 epitope is exposed in the pMHCI/CD8 $\alpha\beta$ complex. The interacting elements between mouse CD8 α and the rat antibody YTS105.18 have been identified in the CD8 $\alpha\alpha$ /YTS105.18 Fab co-crystal structure.⁶ The two potential models for the pMHCI/CD8 $\alpha\beta$ complex, in which CD8 α occupies an $\alpha 1$ or $\alpha 2$ position, respectively, indicate that inhibition of pMHCI/CD8 $\alpha\beta$ interaction by mAb YTS105.18 would occur only if CD8 $\alpha\beta$ binds pMHCI with CD8 α in the $\alpha 1$ position (Figure 5d), whereas YTS105.18 Fab would not block pMHCI/CD8 $\alpha\beta$ interactions in which CD8 α adopts an $\alpha 2$ position (Figure 5e). In summary, the finding that mAb YTS156.7 inhibits binding of soluble tetramers of class I pMHC to CD8 $\alpha\beta$ at the surface of T cells, whereas mAb YTS105.18 does not, indicates that both isoforms of CD8 interact with pMHCI in an equivalent manner, but that CD8 β occupies a position equivalent to the $\alpha 1$ position observed in the CD8 $\alpha\alpha$ /H-2K^b co-crystal structure.

Conclusions

The significance of the CD8 β subunit for CD8 function is well documented, although the molecular basis for the enhanced co-receptor function of CD8 $\alpha\beta$ over that of CD8 $\alpha\alpha$ has yet to be fully elucidated. The extremely low affinity (~ 11 – 67 μ M)^{8,9} of the class I pMHC/CD8 $\alpha\beta$ interaction, coupled with the instability of soluble recombinant CD8 $\alpha\beta$ proteins has rendered analysis of the pMHCI/CD8 $\alpha\beta$ interaction a significant technical challenge. In the absence of a pMHCI/CD8 $\alpha\beta$ co-crystal structure, ongoing efforts have focused on alternative means to determine the nature of the pMHCI/CD8 $\alpha\beta$ interaction. To this end, mutagenesis studies, as well as mAbs against CD8 that inhibit or enhance interactions with pMHCI, have been applied to probe binding of CD8 $\alpha\beta$ with pMHCI.^{12,13,14,19,32} Analysis of the CD8 β -specific inhibition of (CD8⁺) T cell activation and suppression of immune function by mAb YTS156.7 is tantamount to probing the biological significance of the β subunit. Hence, the CD8 $\alpha\beta$ /YTS156.7 co-crystal structure presented here provides valuable information pertaining to CD8⁺ T cell biology. Structural analysis of our physiological CD8 $\alpha\beta$ IgSF dimer confirms

the previously determined structure of a single-chain CD8 $\alpha\beta$ construct and the analysis of the YTS156.7 epitope on CD8 β identifies, or severely restricts the choice of, residues on CD8 β that are involved in interaction of CD8 $\alpha\beta$ with class I pMHC. These findings provide a framework for further studies of CD8 involving mAb YTS156.7.7 interactions both *in vivo* and *in vitro*. Moreover, our data provide insights into the relative orientation of the CD8 α and CD8 β subunits in binding to pMHC. Models of the pMHC/CD8 $\alpha\beta$ complex, based upon the existing CD8 $\alpha\alpha$ /H-2K^b co-crystal structure, illustrate how YTS156.7 inhibits interaction between CD8 $\alpha\beta$ and pMHC through a molecular clash and indicate that both CD8 isoforms do, indeed, adopt a similar mechanism of interaction with pMHC. Furthermore, the finding that the YTS105.18 epitope is not occluded in the functional pMHC/CD8 $\alpha\beta$ complex confirms that CD8 α occupies a lower ($\alpha 2$) position, such that CD8 β occupies an upper ($\alpha 1$) position that places the CD8 β subunit proximal to the T cell membrane. This model for CD8 $\alpha\beta$ /pMHC interaction is consistent with the findings of mutational and functional analyses.¹⁵ Given the structural similarities between the CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IgSF dimers, as well as the convincing support for a model in which both isoforms bind pMHC in an equivalent manner, it is interesting to speculate as to the physiological differences between CD8 $\alpha\beta$ and CD8 $\alpha\alpha$. There is clear evidence that CD8 $\alpha\alpha$ alone can provide some co-receptor activity, albeit less profound than that of CD8 $\alpha\beta$, and given that CD8 $\alpha\alpha$ and CD8 $\alpha\beta$ IgSF dimers bind to soluble class I molecules with equivalent affinity, it is likely that observed differences in the functional activity of the two isoforms of CD8 are primarily mediated by the different binding characteristics of the homodimeric and heterodimeric receptors at the T cell surface. One such difference may result from diversity in the length of the extended stalk region, which is invariably shorter in CD8 β than in CD8 α and likely produces a bowed conformation in the CD8 $\alpha\beta$ stalk at the cell surface. In light of the fact that CD8 α contacts the “lower” site on class I pMHC that is more proximal to the surface of antigen presenting cells, a bowed conformation in the stalk may position the CD8 $\alpha\beta$ IgSF dimer more optimally for interaction with class I pMHC at the surface of antigen presenting cells and may account for the enhanced co-receptor activity over that of CD8 $\alpha\alpha$. On the other hand, the stalk region of homotypic CD8 $\alpha\alpha$ is unlikely to produce a bowed conformation and may position the CD8 $\alpha\alpha$ IgSF dimer less optimally for binding to the side of class I pMHC. This concept is consistent with the observation that the stalk region of CD8 β is critical to the enhanced co-receptor function of CD8 $\alpha\beta$,³³ and may explain the finding that changes to oligosaccharides in the stalk of CD8 β can influence CD8 $\alpha\beta$ function.^{32,34,35} However, in the absence of a detailed analysis of the interacting elements between class I pMHC and CD8 $\alpha\beta$, the molecular basis for the enhanced co-receptor function of CD8 $\alpha\beta$ remains an enigma and must await a pMHC/CD8 $\alpha\beta$ complex crystal structure.

Materials and Methods

Soluble CD8 $\alpha\beta$ expression and purification

Expression constructs for soluble mouse CD8 α and CD8 β were generated as described previously for soluble mouse CD8 $\alpha\alpha$.⁶ Briefly, soluble CD8 $\alpha\beta$ dimers (sCD8 $\alpha\beta$) were expressed in insect cells as a heterodimer of their IgSF domains, comprising residues 1–123 of the mature CD8 α and residues 1–117 of the mature CD8 β subunits. Specific pairing between α and β subunits was achieved by addition of acidic or basic leucine-zipper motifs at the carboxyl terminus of each respective subunit which preclude expression of homodimeric CD8 species which would readily form otherwise. A hexa-histidine motif was also incorporated at the carboxyl terminus of each subunit to facilitate protein purification (see supplemental Figure 1). Both constructs were cloned into the pRMHa3 inducible expression vector, used previously in the expression of soluble CD8 proteins,⁶ and co-transfected at equimolar ratio into *D. melanogaster* SC2 cells. Stable cell lines were maintained in Schneider's media (BioWhittaker/Lonza), containing 500 μ g/ml gentamycin sulphate (G-418) (BioWhittaker/Lonza). Large-

scale cell culture (16 L) was carried out in 2 L disposable roller bottles (Corning) at 28°C, using Insect Express serum-free insect cell medium (BioWhittaker/Lonza). Protein expression was induced with addition of 700 μ M CuSO₄ for three days and sCD8 $\alpha\beta$ was then purified from concentrated supernatant by metal affinity chromatography using Ni-NTA beaded-agarose resin (Qiagen). Subsequent purification was carried out by size exclusion gel filtration chromatography, using an AKTA FPLC system fitted with a HiLoad Superdex 200 16/60 column (GE Healthcare), in 25 mM Hepes, pH 7.8, 150 mM NaCl, and 0.04% sodium azide. The pooled protein fractions were concentrated to 12 mg/ml using a Centricon concentrator device with a 30 kDa MW-cut off (Millipore). Protein concentrations were measured using the Coomassie Plus Bradford assay (Pierce Biotechnology).

Preparation of YTS156.7.7 Fab

The YTS156.7.7 hybridoma was obtained as a kind gift from Dr H. Waldman, Sir William Dunn School of Pathology, University of Oxford, UK. Cells were cultured in RPMI media (Gibco-Invitrogen), containing 5% FCS, 100 mM L-glutamine, 25 mM Hepes pH 7.5 and 100 mM MEM non-essential amino acid solution (Gibco-Invitrogen). Large-scale antibody production was carried out using an Integra CL 1000 cell factory (Integra Biosciences) and IgG was purified from cell-free tissue culture supernatant by affinity chromatography using agarose-beaded protein G (Sigma-Aldrich). The YTS156.7 Fab fragment was isolated by digestion with 10 ng papaya papain (Sigma-Aldrich) protease/mg IgG for 4 hours at 37°C, and purified from the Fc fragment by affinity chromatography using agarose-beaded protein A (Sigma-Aldrich). The amino-acid sequence of the YTS156.7 Fab was determined by RT-PCR, using mRNA extracted from YTS156.7.7 clone (hybridoma) cells and subsequent sequencing of the resulting cDNA.

Purification of the CD8 $\alpha\beta$ /YTS156.7.7 Fab complex

YTS156.7 Fab bound sCD8 $\alpha\beta$ with nanomolar affinity (see Supplemental Figure 2) and the CD8 $\alpha\beta$ /YTS156.7 Fab complex was purified to homogeneity by size exclusion chromatography using an AKTA FPLC fitted with a HiLoad Superdex 200 10/30 column (GE Healthcare) in 25 mM Hepes, pH 7.8, 150 mM NaCl, and 0.04% sodium azide. Subsequent removal of the carboxyl-terminal, leucine-zipper and hexa-histidine motifs from sCD8 $\alpha\beta$ was carried out by digestion with bovine thrombin protease (Calbiochem) using 3 U/mg enzyme for 3 hours at 37°C. Finally, the CD8 $\alpha\beta$ /YTS156.7 Fab complex was purified again by size exclusion gel filtration, using an AKTA FPLC system fitted with a HiLoad Superdex 200 10/30 column (GE Healthcare), in 25 mM Hepes, pH 7.8, 150 mM NaCl, and 0.04% sodium azide.

Crystallization and data collection

Crystallization experiments were carried out using the sitting drop, vapor diffusion method at a protein concentration of 12 mg/ml. Each drop contained 0.45 μ l of protein mixed with an equal quantity of precipitant solution. Crystals of CD8 $\alpha\beta$ /YTS156.7 Fab were obtained from a solution containing 10% polyethylene glycol (PEG 8,000), 0.1 M Na/K phosphate, 0.2 M NaCl, pH 6.2, at 23°C. Crystals were flash cooled to 95 K in a cryo-protectant containing precipitant supplemented with 25% glycerol, and a dataset was collected to 2.7 Å from a single crystal at the Stanford Synchrotron Radiation Laboratory (SSRL) beamline 11-1. Data were integrated and scaled using Denzo and Scalepack, as implemented in the HKL2000 suite.³⁶ CD8 $\alpha\beta$ /YTS156.7 Fab crystallized in space group $P2_12_12_1$, with unit cell dimensions $a = 91.8$ Å, $b = 92.7$ Å, $c = 190.3$ Å, $\alpha = \beta = \gamma = 90^\circ$. Data collection and processing statistics are detailed in Table 1.

Structural determination, refinement and validation of the CD8 $\alpha\beta$ /YTS156.7.7 Fab complex

The CD8 $\alpha\beta$ /YTS156.7 Fab structure was determined by molecular replacement (MR) with the program Phaser.³⁷ Rat mAb YTS105.18 Fab from our CD8 $\alpha\alpha$ /YTS105.18 complex structure (PDB ID 2ARJ⁶) was utilized as a search model for the rat mAb YTS156.7 Fab and the CD8 $\alpha\alpha$ component from the same structure was utilized as a search model for CD8 $\alpha\beta$. Preliminary phases were initially determined for the YTS156.7 Fab. These coordinates were fixed and a subsequent round of MR determined a solution for CD8 $\alpha\beta$, which packed without clashes. The solution was confirmed by rigid-body refinement using the program Refmac-5,³⁸ as implemented in the CCP4 program suite.

Model building, using the program O,³⁹ and energy-minimization refinement, using Refmac-5, was carried out until the refinement converged. R_{cryst} (25.4%) and R_{free} (28.5%) did not improve further due to pseudo-translational symmetry of the molecules in the asymmetric unit. Water molecules were identified from residual density greater than 2.0 in the $2F_o - F_c$ electron density map and were added if within hydrogen-bond distance of the protein.

Validation of geometry and overall quality of the final model was carried out using Molprobit,⁴⁰ as well as the JCSG validation suite, incorporating the programs Procheck,²² and Whatcheck.⁴¹ All structural alignments and RMSD calculations were carried out using the McLachlan algorithm,⁴² as implemented in the program ProFit. Figures were generated using the program MOLSCRIPT⁴³ and Pymol.⁴⁴

Preparation of peptides and MHC tetramers

Peptides OVA (SIINFEKL) and SIYR (SIYRYYYGL) were synthesized by Anaspec (San Jose, CA). Production and purification of the soluble form of H-2K^b was performed as described previously.⁴⁵ Briefly, plasmids encoding the H-2K^b heavy chain with the BirA recognition sequence at the carboxyl-terminus and mouse $\beta_2\text{M}$ were transformed and over-expressed in *D. melanogaster* SC2 cells. The protein was purified by anion exchange chromatography on a Resource-Q column (GE Healthcare) and size exclusion gel filtration using a HiLoad Superdex 200 10/30 column (GE Healthcare). Following biotinylation using BirA (Avidity), the monomeric MHCI complexes (loaded with an endogenous peptide(s) acquired from the expression system⁴⁶) were purified via a second round of size exclusion gel filtration using a HiLoad Superdex 200 10/30 column. The efficiency of biotinylation was assessed by sequential immunoprecipitation with avidin-conjugated beads (Pierce Biotechnology) and was estimated to be 95%. After concentration of the appropriate-sized fractions, biotinylated H-2K^b was mixed with streptavidin (SA)-PE (Biosource Inc) at a 4:1 molar ratio in the presence of a 10-fold excess of added peptide (OVA, SIYR) which was of sufficiently high affinity (~nM) to readily displace the endogenous peptide(s). Finally, tetramers of H-2K^b-peptide complexes were purified on a Superdex 200 PC 3.2/30 column (GE Healthcare).

Tetramer Staining and Calcium flux measurements

Isolated splenocytes from OT-I transgenic mice were treated with 0.165 M NH_4Cl to remove red blood cells and washed in complete medium. For tetramer staining, OT-I splenocytes were first incubated in 5 $\mu\text{g}/\text{ml}$ Fc-Block (BD-Pharmingen) at 4°C for 15 min then stained for 1 hr at 4°C with 50 nM H-2K^b/OVA PE-conjugated tetramers or with H-2K^b/SIYR PE-conjugated tetramers and washed in FACS buffer (PBS 2% FCS, 25 mM Hepes, 2 mM EDTA) before staining with FITC-conjugated anti-CD4 (BD-Pharmingen) and APC-conjugated anti-CD90.2 53.2.1 (eBioscience) mAbs. Lymphocytes were finally washed and re-suspended in FACS buffer and tetramer staining was measured as a function of mean fluorescence. For inhibition experiments, cells were incubated in dilutions of purified anti-CD8 antibodies (anti-CD8 α YTS105.18, anti-CD8 β YTS156.7.7, anti-CD8 α 53-6.7, anti-CD8 β 53-5.8 (BD-Pharmingen) and anti-CD8 α KT15 (Serotec)) in FACS buffer for 15 min at 4°C and then washed prior to

tetramer staining. To measure binding of anti-CD8 antibodies, 50% of the cells were incubated with a PE-conjugated secondary antibody (Donkey anti-Rat IgG, eBioscience) instead of tetramers and washed before subsequent tetramer staining.

For Ca^{2+} mobilization experiments, purified OT-I RAG⁻ splenocytes were then incubated for 30 min at 37°C with 10 µg/ml of the Ca^{2+} -sensitive dye Indo-1 (Molecular Probes) in RPMI medium (Gibco-Invitrogen) containing 1% FCS, 10 mM Hepes and 1% L-Glutamine. Cells were washed 3 times and re-suspended in Ca^{2+} -containing medium (HBSS supplemented with 10 mM Hepes and 1% FBS). Splenocytes were first incubated in 5 µg/ml FcBlock (BD-Pharmingen) at 4°C for 15 min to saturate any unspecific binding sites before staining with PE-conjugated anti-CD4 RM4-5 (BD-Pharmingen) and APC-conjugated anti-CD90.2 53.2.1 (eBioscience) mAbs at 4°C for 15 min to isolate the CD4⁻ T cells. Lymphocytes were finally washed and re-suspended in Ca^{2+} -containing medium. Before stimulation, cells were warmed for 5 min in a 37°C water bath, and then analyzed on an LSR II flow cytometer (BD Biosciences). For stimulation, baseline Ca^{2+} was measured for 1 min, followed by the addition of purified tetramers at a final concentration of 50 nM. Ca^{2+} influx was measured for 5 min. For positive and negative controls, cells were stimulated either with PBS, ionomycin 1 µg/ml (Calbiochem) or 10 µg/ml anti-CD3 2C11.145 (BD-Pharmingen) followed by cross-linking with 50 µg/ml anti-hamster Ig (Jackson ImmunoResearch Labs). For analysis of the effect of anti-CD8 antibodies upon tetramer binding, lymphocytes were pre-incubated at 4°C with the antibodies for 15 min and washed. Ca^{2+} flux results are presented as the ratio of Indo1-Violet (bound to Ca^{2+}) against Indo1-Blue (unbound) over time. All data were acquired on an LSR II flow cytometer (BD Biosciences) and analyzed with the Flow-Jo software (Tree Star). Figures represent signal for CD8⁺ T cells, identified by gating on CD90.2⁺ CD4⁻ T cells.

Protein Data Bank accession code

Coordinates and structure factors for the CD8αβ/YTS156.7 Fab co-crystal structure have been deposited in the RCSB Protein Data Bank with RCSB accession number **RCSB045249** and PDB ID code **3B9K**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Crystal structure of mouse CD8 $\alpha\beta$ in complex with YTS156.7 Fab. (a) Ribbon diagram of the refined model of the CD8 $\alpha\beta$ /YTS156.7 Fab complex (complex A). CD8 α and CD8 β are colored

in green and blue, respectively and YTS156.7 Fab is colored cyan. (b) Ribbon representation of the CD8 $\alpha\beta$ IgSF dimer showing the single carbohydrate moiety at Asn ^{α 42}, as well as the position of asparagine side chains at the potential N-glycosylation sites at Asn ^{α 70} and Asn ^{β 13}. The CDR-equivalent loops of CD8 $\alpha\beta$ are numbered and the YTS156.7 epitope is represented as a molecular surface.

Figure 2.

Comparison of mouse CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ IgSF dimers. (a) Comparison of the CD8 $\alpha\beta$ and CD8 $\alpha\alpha$ IgSF dimers. Superimposition of CD8 $\alpha\beta$ onto CD8 $\alpha\alpha$ from the YTS105.18 complex structure (PDB ID 2ARJ⁶), by aligning the C $^{\alpha}$ atoms in CD8 α , illustrates complementarity in the dimerization interface of both CD8 isoforms and overall structural similarity in the two receptors. CD8 $\alpha\alpha$ is colored maroon and CD8 $\alpha\beta$ is colored green/blue. (b) Stereo representation of the CD8 α and CD8 β IgSF domains, superimposed using the C $^{\alpha}$ atoms in the β -strands of each subunit. CD8 α is colored green and CD8 β is in blue. The N and C termini of each subunit are labeled for clarity.

Figure 3.
The YTS156.7epitope. Expanded view of the molecular surface buried by YTS156.7 on CD8 β , showing the residues that contact YTS156.7. The CDR1 and CDR2-equivalent loops of CD8 β are involved in this interaction.

Figure 4.

YTS156.7.7, but not YTS105.18, blocks MHC class I and CD8 interaction and subsequent T cell activation. (a) Ca²⁺ mobilization in OT-I RAG⁻ splenocytes treated with H-2K^b tetramers loaded with the OVA peptide (H-2K^b/OVA) after pre-staining with mAbs 53.6.7 (red line), YTS105.18 (purple line) or YTS156.7.7 (green line). The tetramers were added at 1 min (arrow) and the sample re-analyzed for a further 4 min. The H-2K^b/OVA tetramer trace is taken from a 5 min time course and overlaid here as a representation of a responding population. Response to the H-2K^b/OVA tetramers in the absence of CD8 antibodies is shown (blue line), as well as response to H-2K^b/SIYR tetramers (black line) as a negative control. All traces indicate the median of the responding population. (b) Equivalent plot to show relative Ca²⁺ mobilization in OT-I RAG⁻ splenocytes treated with H-2K^b/OVA tetramers after pre-staining now with only the Fabs of mAbs 53.6.7 (red line), YTS105.18 (purple line) or YTS156.7.7 (green line). The baselines for all traces are normalized to the response to H-2K^b/OVA tetramers in the absence of any Fabs (blue line).

Figure 5.

Models of the pMHCI/CD8αβ complex, based on the CD8αα/H-2K^b co-crystal structure, illustrate the influence of mAbs YTS156.7 and YTS105.18 and indicate CD8α binds at, or near, the α2 position. (a) Ribbon diagram of the CD8αα/H-2K^b complex (PDB ID 1BQH⁴), illustrating the relative orientation of the CD8α α1 and α2 subunits. pMHCI is colored magenta, β₂-microglobulin (β₂M) is colored mauve and CD8αα is colored maroon. (b) Combination of the CD8αα/pMHCI and CD8αβ/YTS156.7 Fab structures by superimposition of C^α atoms in CD8α provides two alternative models for the YTS156.7/pMHCI/CD8αβ interaction, dependent upon the relative disposition of CD8β in the pMHCI/CD8αβ complex. If CD8β binds to pMHCI in the upper (α1) position, YTS156.7 would inhibit pMHCI/CD8αβ interaction by a molecular clash with the pMHCI α1 and α2 domains. (c) In the alternate model, CD8β would occupy the lower (α2) position and inhibition would occur through clash between the Fab and the AB loop of pMHCI α3. pMHCI is colored magenta, β₂M is colored mauve, YTS156.7 Fab is colored cyan and CD8α and CD8β are colored green and blue, respectively. (d) An equivalent model, representing the experimental effects of mAb YTS105.18 Fab upon

CD8 $\alpha\beta$ interaction with soluble pMHCI. Models were created using the CD8 α /pMHCI (PDB ID 1BQH⁴) and CD8 α /YTS105.18 Fab (PDB ID 2ARJ⁶) structures. Superimposition of C $^{\alpha}$ atoms in the CD8 α component of both models indicates that, if CD8 α binds to pMHCI in the α 1 position, YTS105.18 would inhibit pMHCI/CD8 $\alpha\beta$ interaction by a molecular clash with the pMHCI α 1 and α 2 domains. (e) If CD8 α binds to pMHCI in the lower α 2 position, YTS105.18 would have no effect upon interaction of CD8 $\alpha\beta$ with soluble pMHCI, consistent with the results of our tetramer binding assay.

Table 1
Crystallographic data and refinement

Data Processing	
Resolution range	50.0 - 2.70 Å (2.80 - 2.70 Å)
Unique reflections	44,334 (4,468)
Completeness	97.5% (99.8%)
Redundancy	3.3 (3.4)
R_{sym}	13.1 % (57.9%)
Average I/σ (I)	8.6 (2.2)
Refinement	
Reflections	42,077 (3,110)
Reflections (test)	2,216
R_{cryst}^c	23.5%
R_{free}^d	28.4%
Residues/protein atoms	1,320/10,298
Water molecules	36
Coordinate error ^e	0.33 Å
Rmsd bonds/angles	0.015 Å/1.58°
Average B values	
<i>Complex A (LHAB)</i>	
YTS156.7.7 Fab	68.4 Å ²
CD8αβ	72.0 Å ²
<i>Complex B (CDEF)</i>	
YTS156.7.7 Fab	80.2 Å ²
CD8αβ	71.4 Å ²
Waters	39.0 Å ²
Carbohydrates	116.0 Å ²
Ramachandran statistics	
Most favored	86.0 %
Additionally allowed	12.9%
Generously allowed	0.9%
Disallowed	0.2% ^f

^aValues in parenthesis refer to the highest resolution shell.

^b $R_{\text{sym}} = 100 \cdot \sum_h \sum_i |I_i(h) - \langle I(h) \rangle| / \sum_h I(h)$, where $I_i(h)$ is the i^{th} measurement of the h reflection and $\langle I(h) \rangle$ is the average value of the reflection intensity.

^c $R_{\text{cryst}} = \sum |F_O| - |F_C| / \sum |F_O|$, where F_O and F_C are the structure factor amplitudes for the experimental data and from the calculated model, respectively.

^d R_{free} is R_{cryst} with 5% of the test set structure factors.

^eValues are based on maximum likelihood

^fResidues Thr^{L51} and Thr^{C51} lie within a well-defined γ-turn in the YTS156.7 Fab light chain, as in almost all antibodies.