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A Structural Basis for Varied αβ TCR Usage against an Immunodominant EBV Antigen Restricted to a HLA-B8 Molecule

Stephanie Gras,* Pascal G. Wilmann,* Zhenjun Chen,† Hanim Halim,* Yu Chih Liu,* Lars Kjer-Nielsen,‡ Anthony W. Purcell,‡ Scott R. Burrows,§ James McCluskey,‡ and Jamie Rossjohn*

EBV is a ubiquitous and persistent human pathogen, kept in check by the cytotoxic T cell response. In this study, we investigated how three TCRs, which differ in their T cell immunodominance hierarchies and gene usage, interact with the same EBV determinant (FLRGRAYGL), bound to the same Ag-presenting molecule, HLA-B8. We found that the three TCRs exhibit differing fine specificities for the viral Ag. Further, via structural and biophysical approaches, we demonstrated that the viral Ag provides the greatest energetic contribution to the TCR–peptide-HLA interaction, while focusing on a few adjacent HLA-based interactions to further tune fine-specificity requirements. Thus, the TCR engages the peptide-HLA with the viral Ag as the main glue, such that neighboring TCR–MHC interactions are recruited as a supportive adhesive. Collectively, we provide a portrait of how the host’s adaptive immune response differentially engages a common viral Ag. The Journal of Immunology, 2012, 188: 000–000.

Epstein-Barr virus is a ubiquitous human pathogen, with ~90% of the population infected; once infected, the virus is rarely cleared from the host (reviewed in Refs. 1, 2). EBV infection, although typically asymptomatic in immunocompetent individuals, is the causative agent of infectious mononucleosis and has been linked to the development of several cancers, including Burkitt’s lymphoma, immunoblastic lymphoma, and Hodgkin’s disease; certain forms of T cell lymphoma; and some epithelial tumors, including nasopharyngeal carcinoma and a proportion of gastric cancers. Viral infection and persistence are achieved through a balance of lytic (productive) and latent (non-productive) infections that are controlled by a series of lytic and latent proteins, respectively. Primary infection with EBV is associated with the expression of a large number of lytic proteins that can be classified into three temporal phases: immediate early, early, and late. The latent proteins, of which the nuclear Ags predominate (termed Epstein-Barr virus nuclear Ag [EBNAs]), enable the virus to colonize the B cell pool through latent growth-transforming infection and, thereby, establish a reservoir of virus genome-positive cells upon which long-term virus persistence depends. Subsequent reactivation of the B cell reservoir from latency into the lytic cycle can then initiate secondary foci of virus replication at pharyngeal sites, leading to low-level shedding of infectious virus throughout life.

EBV is contained by a vigorous cellular immune response that is directed against a specific subset of latent epitopes. The EBV-replicative lesions are subject to direct CTL control, in which the immediate early and early proteins are frequently the immunodominant targets (1). To gain a greater understanding of CTL immunity and Ag presentation, it is important to investigate specific elements relating to the protective immune response to EBV. Indeed, EBV is an important natural infection that provides an ideal model for investigating the antiviral immune response in humans because of its ubiquity in human populations and because it is the best-known and most widely studied herpes virus as a result of its clinical and oncogenic importance.

The cytotoxic T cell response toward viruses is directed toward class I MHC (MHC-I) molecules complexed to viral peptide Ags (pMHC-I). These complexes are expressed on the surface of infected cells and are specifically recognized by clonally distributed αβ TCRs on CD8+ T lymphocytes, a crucial cellular component of the immune system. Typically, the antigenic peptide is tethered within the Ag-binding cleft of the MHC molecule, such that the peptide makes a number of specificity-determining contacts with the MHC, whereas the “upward-pointing” amino acids represent potential TCR contact points (3). Appropriately armed and activated CD8+ T cells can eliminate infected cells and prevent viral replication. The specificity in the cellular immune response arises from an extensive TCR repertoire, as well as from...
HLA polymorphism that controls the size and diversity of the peptide repertoire presented. Indeed, the HLA locus is the most polymorphic region of the genome, whereby single amino acid polymorphisms between closely related HLA allomorphs can dictate the functional outcome in viral immunity (4–6), as well as levels of cross-reactivity and alloreactivity (7, 8). TCR diversity is generated via gene rearrangement within the V domains of the TCR, in which the V and J gene segments make up the Vα-chain, whereas the Vβ-chain is composed of V, D, and J genes (9). Further, there is diversity (N) at the junctions of these gene segments. This TCR diversity is critical to accommodate the myriad of potential peptide-MHC (pMHC) complexes comprising pathogen-derived peptides. Despite the potentially vast TCR repertoire, some immune responses, such as the CTL response to EBV, display strong biases in TCR selection. Although our understanding of the underlying mechanisms of TCR bias is far from complete, pMHC-I structure is considered to play a role. Some-what surprisingly, there is relatively little information on whether or not particular TCR bias and diversity profiles are functionally important during infectious diseases. Defining what determines an optimal peptide-HLA I determinant and TCR response is essential during infectious diseases. Defining what determines an or not particular TCR bias and diversity profiles are functionally complete, pMHC-I structure is considered to play a role. Some-

Previously, we explored the in vivo memory CTL response to an immunodominant HLA-B8–restricted epitope FLRGRAYGL from the latent EBV Ag EBNA3A. In HLA-B8* individuals, the immune response to this peptide is characterized by biased TCR usage, in which the archetypal TCR, LC13, is focused upon the P7-Tyr residue of the FLRGRAYGL determinant (10–12). The LC13 TCR is alloreactive toward HLA-B44; therefore, to avoid self-reactivity in HLA-B8–B44 heterozygotes, the public LC13 CTLs are deleted from the mature T cell repertoire (13, 14). Nevertheless, presumably to keep the EBV infection in check, the HLA-B8– restricted CTL response toward FLRGRAYGL still persists but at a slightly lower precursor frequency. These CTLs, which express different TCR gene combinations, exhibit fine specificity shifts either toward the P1-Phe residue or the P8-Gly residue of FLRGRAYGL (13, 15). This system, which has evolved naturally in humans, allows us to address how TCRs specific for distinct aspects of the same peptide-HLA (pHLA) complex apportion the energetic contributions from the MHC H chain contacts with respect to the peptide Ag, thereby providing fundamental insight into the basis of MHC restriction. Further, the comparison of these subdominant and immunodominant complexes will be highly informative and will reveal how self-tolerance is differentially established by shifting of fine specificity at the atomic level due to the presence of a trans-HLA B44 allele.

Materials and Methods

Cytotoxicity assay

The RL42 CTL clone was described previously (13) and was generated using initial stimulation of PBMCs with the FLRGRAYGL peptide, followed by biweekly restimulation with γ irradiated (8000 rad) HLA-B8* lymphoblastoid cell lines. This CTL clone was tested in duplicate in the standard 5-h chromium-release assay for cytotoxicity against 51Cr-labeled target cells that had been treated for 1 h with 0.1 μM peptide. The target cells were HLA-B8* PHA-stimulated blasts that were raised by stimulating PBMCs with PHA, followed by propagation in IL–2–containing medium for up to 8 wk. Peptides were synthesized by Mimotopes. A β-scintillation counter (Topcount Microplate; Packard Instrument) was used to measure 51Cr levels in assay-supernatant samples. The mean spontaneous lysis for targets in culture medium was always <20%, and variation about the mean specific lysis was <10%.

Protein expression, purification, and crystallization

The RL42 TCR was expressed, refolded, and purified using an engineered disulfide linkage in the constant domains between the α- and β-chains. Both the α- and β-chains of the RL42 TCR were expressed separately as inclusion bodies in BL21 E. coli strain. Inclusion bodies were resuspended in 8 M urea, 20 mM Tris-HCl (pH 8), 0.5 M NaCl, 0.2 M Na-EDTA, and 50 mM DTT. The RL42 TCR was refolded by flash dilution in a solution containing 5 M urea, 100 mM Tris (pH 8), 2 mM Na-EDTA, 500 mM L-arginine–HCl, 0.5 mM oxidized glutathione, and 5 mM reduced glutathione. The refolding solution was then diazylized to eliminate the urea. The resulting protein solution was then purified by gel filtration and HiTrap Q anion exchange chromatography. The LC13 and CF34 TCRs were produced, as previously described (12, 17). Soluble class I heterodimers containing the FLRGRAYGL peptide (or the peptide variant) were prepared, as described previously (18, 19). Briefly, the truncated forms (amino acid residues 1–276) of the HLA-B8 H chain (or mutated H chain) and full-length β2-microglobulin were expressed in E. coli as inclusion bodies. The HLA-B8 peptide complex was refolded by diluting the H chain and β2-microglobulin inclusion body preparations into refolding buffer containing a molar excess of peptide ligand. The refolded complexes were concentrated and purified by anion exchange chromatography. The correct fold of the pMHC and TCR proteins was assessed by conformational Abs binding W6/32 and 12H8, respectively (10).

Crystals of the unliganded RL42 TCR were obtained by hanging-drop, vapor diffusion at 20˚C with a protein concentration of 5 mg/ml. Large cube-shaped crystals grew using 13% polyethylene glycol (PEG) 3350 and 0.1 M Na acetate buffer. Crystals of the RL42–HLA-B8–FLRGRAYGL complex were grown by the hanging-drop, vapor-diffusion method at 20˚C, with a protein/reservoir drop ratio of 1:1, at a concentration of 10 mg/ml in 10 mM Tris-HCl (pH 8), 150 mM NaCl. Large stick-shaped crystals grew using 14% PEG 3350, 0.2 M ammonium tartrate, and 7% ethylene glycol. Crystals of HLA-B8–I66A in complex with the FLRGRAYGL peptide and HLA-B8 in complex with the P8-valine variant of the epitope were both obtained by hanging-drop, vapor diffusion at 20˚C, with a protein concentration of 10 mg/ml. Large rod-shaped crystals grew using 20% PEG 4000, 0.2 M ammonium acetate, and 0.1 M trisodium citrate (pH 5.6).

Data collection and structure determination

The RL42 TCR and RL42–HLA-B8–FLRGRAYGL crystals were soaked in a cryoprotectant solution containing mother liquor solution, with the PEG concentration increased to 30% (w/v), and then flash-frozen in liquid nitrogen. Data were collected on the protein crystallography beamlines at the Australian Synchrotron (Clayton, Australia) using the ADSC-Quantum 210 CCD on MX1 detector and an ADSC-Quantum 315r CCD on MX2 detector (at 100k). Data were processed using XDS software and scaled with XSCALE (20). The RL42 TCR crystal belonged to the space group P1 with unit cell dimensions (Supplemental Table I), consistent with four TCRs in the asymmetric unit. The RL42–HLA-B8–FLRGRAYGL crystal belonged to the space group P212121 with unit cell dimensions (Supplemental Table I), consistent with four complexes in the asymmetric unit. The structures were determined by molecular replacement using the PHASER (21) program, with the LC13 TCR as the search model for the TCR (Protein Data Bank accession number, 1KGC; http://www.pdb.org/pdb/home/home.do) (22) and HLA-B8–FLRGRAYGL for the HLA minus the peptide FLRGRAYGL as model (Protein Data Bank accession number, 1M05; http://www.pdb.org/pdb/home/home.do) (11). HLA-B8–I66A in complex with the FLRGRAYGL peptide and HLA-B8 in complex with the P8-valine variant of the epitope crystals belong to the space group P212121 with unit cell dimensions (Supplemental Table I) consistent with one complex in the asymmetric unit. The structures were determined by molecular replacement using the PHASER (21) program with HLA-B8–FLRGRAYGL for the MHC model without the peptide (Protein Data Bank accession number, 1M05; http://www.pdb.org/pdb/home/home.do) (11).

Manual model building was conducted using Coot software (23), followed by maximum-likelihood refinement using PHENIX (24). “Translation, liberation, and screw-rotation” displacement refinement was used to model anisotropic displacements of defined domains. The TCR was numbered according to the International Immunogenetics Information System unique numbering system (25) whereby the CDR1 loops start at residue number 27, the CDR2 loops start at residue number 56, and the CDR3 loops start at residue number 105. The final models were validated using the Protein Data Bank validation Web site, and the final refinement statistics are summarized in Supplemental Table I. Coordinates submitted to Protein Data Bank database (http://www.rcsb.org/pdb/home/home.do) code: 3SKN (RL42 TCR), 3SJV (RL42–HLA-B8–FLRGRAYGL), 3SKO (HLA-B8–A66–FLRGRAYGL), 3SKM (HLA-B8–FLRGRAYGL). All molecular graphics representations were created using PyMol (26).
Surface plasmon resonance measurement and analysis

All surface plasmon resonance (SPR) experiments were conducted at 25°C on the BIAcore 3000 instrument with HEPES buffer salt buffer (10 mM HEPES [pH 7.4], 150 mM NaCl, and 0.005% surfactant P20). The HBS buffer was supplemented with 1% BSA to prevent nonspecific binding. The human TCR-specific mAb, 12H8 (10), was coupled to research-grade CM5 chips with standard amine coupling. For each experiment, CF34, RL42, and LC13 were passed over the flow cell until ~200–400 response units were captured by the Ab. One of the four flow cells was left empty and used as control for each experiment. HLA-B8 (or mutant), in complex with the peptide (or variant), was injected over all four flow cells, at a rate of 20 μl/min, with a concentration range of 0.78–200 μM. The final response was calculated by subtraction of the response of the Ab alone from that of the Ab CF34, RL42, or LC13 complex. The Ab surface was regenerated between each analyte injection with Actisep (Sterogene). All experiments were conducted in duplicate (at least). BIAevaluation Version 3.1 was used for data analysis; the 1:1 Langmuir binding model, modified to include an additional parameter for the drifting baseline for the TCR capture by 12H8, was used to calculate the kinetic constants.

Thermal-stability assay

A thermal-shift assay was performed to assess the effect of each mutation, either on the HLA molecule or on the peptide, on the stability of the pHLA complex. The fluorescent dye Sypro orange was used to monitor the protein unfolding. The thermal-stability assay was performed in the Real Time Detection system (Corbett RotorGene 3000), originally designed for PCR. Each pHLA complex was in 10 mM Tris-HCl (pH 8) and 150 mM NaCl, at two concentrations (5 and 10 mM) in duplicate, and was heated from 25 to 95°C at a rate of 1°C/min. The fluorescence intensity was measured with excitation at 530 nm and emission at 555 nm. The thermal melt point, representing the temperature at which 50% of the protein is unfolded, was determined its structure in the nonliganded state at 2.9 Å resolution to an Rfactor of 19.4 and 29.1%, respectively (Supplemental Table I). Next, we purified the RL42 TCR–HLA-B8FLRGRAYGL complex and solved the structure at 3.1 Å resolution to an Rfree of 25.3 and 32.1%, respectively (Fig. 1). The RL42 TCR exhibited markedly different gene usage (TRAV12/1/TRAJ23, TRBV6/2/ TRBJ2-4/1) compared with the LC13 and CF34 TCRs. How does the subdominant RL42 TCR dock onto HLA-B8FLRGRAYGL with specificity requirements for the P1, P4, P6, P7, and P8 positions of the FLRGRAYGL peptide?

To address this, we expressed and refolded the RL42 TCR and determined its structure in the nonliganded state at 2.9-Å resolution to an Rfactor and Rfree of 19.4 and 29.1%, respectively (Supplemental Table I). Next, we purified the RL42 TCR–HLA-B8FLRGRAYGL complex and solved the structure at 3.1 Å resolution to an Rfree of 25.3 and 32.1%, respectively (Fig. 2A, Supplemental Table I). The initial experimental phases clearly showed unbiased electron density for the FLRGRAYGL peptide (data not shown), and the electron density at the RL42 TCR–HLA-B8FLRGRAYGL interface was unambiguous, thereby permitting structural analysis to be undertaken and compared with previously published LC13 TCR and CF34 TCR ternary complexes.

The RL42 TCR docked at ~65° across HLA-B8FLRGRAYGL, which was similar to the LC13 TCR and CF34 TCR docking modes (60 and 58°, respectively, Fig. 2). However, although the CF34 TCR and LC13 TCR docked above the N- and C-terminal end of the HLA-B8 Ag-binding cleft (12, 13). In HLA-B8*B44+ individuals, different subdominant TCRs are deployed against HLA-B8*FLRGRAYGL, indicating that the T cell repertoire contains considerable redundancy in receptors with appropriate specificity for HLA-B8*FLRGRAYGL. The CF34 CTL clone (TRAV14*01- TRAJ49, TRBV11-2*03-TRBD2*01-TRBJ2-3) represents the prototype of the TCRs in HLA-B8*B44+ individuals who exhibit exquisite sensitivity to substitutions at P1-Phe. To account for this P1-Phe specificity, the CF34 TCR focuses upon the prominent P7-Tyr and the C-terminal end of the FLRGRAYGL peptide.

Unrelated HLA-B8+ individuals virtually all make a biased CTL response to the peptide FLRGRAYGL from the latent EBV Ag EBNA 3A. These CTLs, of which LC13 is the archetype, all use the same TCR α- and β-chains derived from identical Vα, Jα (TRAV26-2*01, TRAJ52*01), with the same CDR3 loops sequences and identical TCR Vβ, Dβ, Jβ (TRBV7-8*03, TRBD1/D2, TRBJ2-7*01) (27) and N-region sequences (28, 29). The LC13 TCR focuses upon the prominent P7-Tyr and the C-terminal end sequences of the FLRGRAYGL peptide?
end of HLA-B8^{FLRGRAYGL}, respectively, the RL42 TCR sat centrally over HLA-B8^{FLRGRAYGL}, thereby providing immediate insight into how this TCR exhibits sensitivity toward both the P1 and P8 positions of the peptide (Fig. 2A, Supplemental Table II). The total buried surface area (BSA) upon ligation was similar among the three TCR–pMHC complexes: 2100, 2020, and 2180 Å² for the RL42, LC13, and CF34 TCR complexes, respectively. Accordingly, the RL42 TCR contacted HLA-B8^{FLRGRAYGL} to a similar extent as did the LC13 and CF34 TCRs (RL42: 116 vdw contacts, 14 hydrogen bonds [H-bonds], and one salt bridge; CF34: 127 vdw contacts and 12 H-bonds; and LC13: 135 vdw contacts, 14 H-bonds, and one salt bridge). In fact, the affinity of the RL42–HLA-B8^{FLRGRAYGL} interaction (K_D 31 μM) was lower than was the affinity of LC13 TCR and CF34 TCR, the subdominant RL42 TCR did not exhibit superior docking features, despite being centrally located above the HLA-B8 Ag-binding cleft.

Divergent footprints
The V_α and V_β domains of the RL42 TCR contributed 64 and 36%, respectively, to the BSA at the interface, indicating a bias toward the α-chain in mediating contacts with HLA-B8^{FLRGRAYGL}, in which CDR1_α, 2_α, and 3_α loops contributed 20, 16, and 16% BSA to the interface, respectively (Fig. 2D, Supplemental Table II). Notably, 12% BSA arose from framework residues within the V_α-chain. Although the β-chain played a lesser role at the interface, there was a relatively even distribution of contacts among the CDR loops, with the CDR1_β, 2_β, and 3_β loops contributing 10, 9, and 17% BSA to the interface, respectively. Thus, the relative interplay of the CDR loops of the RL42 TCR contrasted with that of the CF34 TCR and the LC13 TCR (Fig. 2E, 2F), in which the CDR3 loops played a dominant role in contacting the epitope (58 and 45% BSA, respectively, versus 33% BSA for the RL42 TCR).

RL42 TCR interacted with a stretch of the α2-helix, spanning from Arg151 to Ala158 (Fig. 3A). The region was contacted solely by the CDR2_α loop and the surrounding V_α framework residues: the Arg151 side-chain was positioned toward the CDR2_α loop and contributed most of the contacts, interacting with Tyr57_α (CDR2_α), Ser55_α, and Asn66α (Fig. 3A). The neighboring Glu154 also contacted Tyr57_α and Ser55_α of the CDR2_α loop. The interactions between CDR2_α and the α2-helix were further stabilized by an intricate network of polar interactions, which included an H-bond between Asn66α and Ser55_α, as well as a salt bridge between Arg151 and Glu154. The RL42 TCR made more extensive contact with the α1-helix of HLA-B8, involving five of the CDR loops, which contacted a large area that virtually spanned the length of the Ag-binding cleft (from Arg62 to Arg79) (Fig. 3B). Asn27_α of the CDR1_α loop bonded to Arg62, an H-bond residue that reoriented upon ligation. The three CDR_β loops interacted with the HLA-B8 α1-helix, spanning from Thr69 to Arg79 (Fig. 3C, 3D). Glu30_β salt bridged to Arg79, whereas Tyr31_β inserted its aromatic ring between Glu76 and Thr73, H-bonded to Glu76, and formed additional contacts with Gln72 (Fig. 3C). Val57 from the CDR2_β loop formed vdw contacts with Gln72 and H-bonded via its main chain to Arg79, whereas Gly58_β made vdw contacts with Arg79 (Fig. 3D). The only residue from the CDR3_β loop to interact with the HLA-B8 molecule was the nongermline-encoded Asn110_β, which interacts with Thr69, Gln72, and Thr73.
The RL42 footprint on HLA-B8<sup>FLRGRAYGL</sup> contrasted with the corresponding footprints of the LC13 TCR and the CF34 TCR: although the CF34 TCR and the LC13 TCR adopted an N-terminal and C-terminal docking mode, the center of mass of the RL42 TCR aligned with the central region of the HLA-B8 Ag-binding cleft (Fig. 2D). Further, in contrast to the interactions mediated by the LC13 and CF34 TCRs, the RL42 TCR exhibited a marked bias toward interacting with the α1-helix. The contrasting footprints were suggestive of the structural focus of the HLA-B8–restricted TCRs being dictated by the initial focus on the peptide derived from the EBV Ag, with MHC interactions governing the additional fine-specificity features.

Viral Ag-mediated contacts

The CDR1<sub>α</sub>, CDR2<sub>α</sub>, CDR3<sub>α</sub>, and CDR3<sub>β</sub> loops from the RL42 TCR contacted the FLRGRAYGL EBV peptide, which contributed 20% BSA of the pMHC interface in the RL42 complex, and was similar to that observed in both the LC13 TCR and the CF34 TCR ligated complexes (Fig. 2D–F). The viral Ag side-chains that were exposed for TCR contact were P1-Phe, P6-Ala, and P7-Tyr; the RL42 TCR contacted all three positions, as well as the P4-Gly main chain (Fig. 4A). The extent of peptide contacts contrasted with that of the LC13 TCR, which contacted positions P6-Ala, P7-Tyr, and P8-Gly only (Fig. 4B), and was less extensive than that of the CF34 TCR, which contacted P1-Phe, P4-Gly, P5-Arg (main chain), P6-Ala, and P7-Tyr (Fig. 4C).

P1-Phe, an important position for RL42 TCR recognition (Fig. 1), was only partially buried, in that it was solely contacted by Ala<sub>29</sub> of the CDR1<sub>α</sub> loop (Figs. 4A, 5A), contrasting with the CF34 TCR–P1-Phe–mediated interaction, which was shielded fully by the CDR3<sub>α</sub> loop. Thus, the sensitivity of the RL42 TCR to substitutions at the P1 position may be more reflective of perturbing the local environment, which included Arg<sub>62</sub>, a residue that also contacted the CDR1<sub>α</sub> loop. P4-Gly H-bonded to the side-chain of Gln<sub>31α</sub> (Fig. 5A) and was contacted by Arg<sub>107α</sub>, a residue derived from the N region of the CDR3<sub>α</sub> loop (Fig. 5B). Both CDR3 loops of the RL42 TCR surrounded P6-Ala, whose small size permitted close interactions with Arg<sub>107α</sub>, as well as with Gly<sub>109β</sub> and Asn<sub>110β</sub> from the CDR3<sub>β</sub> loop (Fig. 5B). The RL42 TCR interacted with P7-Tyr, mostly via its CDR3<sub>β</sub> loop.
with its aromatic ring being flanked by Gln108\textsubscript{b} and Asp112\textsubscript{b}, and was also contacted by Tyr51\textsubscript{a} of the CDR2\textsubscript{a} loop (Fig. 5C). Further, the P7-Tyr\textsubscript{OH} group was stabilized by an H-bond with Asp112\textsubscript{b} (Fig. 5C). Analogous to the CF34 complex, P7-Tyr was partially buried (\sim 70\%) by the RL42 TCR, contrasting with the LC13 TCR, which fully encapsulates P7-Tyr (Fig. 4). Nevertheless, although there were differences in the mode and extent of binding to P7-Tyr, all three TCRs formed an H-bond to the P7-Tyr\textsubscript{OH} group. Interestingly, although the RL42 and CF34 TCRs did not contact P8-Gly, the former is nevertheless sensitive to substitutions at this position, which contrasts with the CF34 TCR. The presence of bulky side-chains at the P8 position is likely to impact on the conformation of Glu76 from the \alpha\textsubscript{1}-helix of HLA-B8 (Fig. 3C). Thus, the central location resulted in the RL42 TCR exhibiting sensitivity to all of the solvent-exposed residues of the FLRGRAYGL peptide: P1-Phe, P4-Gly, P6-Ala, P7-Tyr, and P8-Gly.

Conformational plasticity

Having determined the structure of subdominant RL42 TCR and HLA-B8\textsuperscript{FLRGRAYGL} in the nonliganded and liganded state, we were able to establish the extent of plasticity required for RL42 TCR-HLA-B8\textsuperscript{FLRGRAYGL} ligation. Upon RL42 TCR ligation, there were very few structural changes observed in HLA-B8\textsuperscript{FLRGRAYGL}, with limited movements in the peptide (root-mean-square deviation [r.m.s.d.] of 0.38 \text{"A\textsuperscript{2}}) and in the Ag-binding cleft (r.m.s.d. of 0.69 \text{"A\textsuperscript{2}}). Nevertheless, some side-chain movement was observed upon ligation, principally concerning three arginine residues: Arg62, Arg79, and Arg151. Arg62, located at the N-terminal region of the HLA-binding cleft, was pushed away to avoid steric clashes with the CDR3\textsubscript{a} loop and H-bonded to Asn27\textsubscript{a} of the CDR1\textsubscript{\alpha} loop, whereas Arg 79, located at the C-terminal end of the Ag-binding cleft, flattened against the \alpha1-helix upon RL42 TCR ligation, forming contacts with CDR1\textsubscript{\alpha} and CDR2\textsubscript{\beta} loops; Arg151 reoriented 180\degree to contact Tyr57\textsubscript{a} and the V\textsubscript{\alpha} framework residues. With respect to the RL42 TCR, there was a 9\degree reorientation between the V\textsubscript{\alpha}-V\textsubscript{\beta} domains upon ligation (data not shown). The major difference between the unliganded and liganded counterpart resided within the germline-encoded CDR2\textsubscript{\alpha} loop, which shifts 2.4 \text{"A\textsuperscript{2}} upon ligation, whereby Tyr57\textsubscript{a} exhibited the largest structural change (3.7 \text{"A\textsuperscript{2}}) (Fig. 5D). The conformational change of the CDR2\textsubscript{\alpha} loop was not a result of the avoidance of steric clashes per se, but was attributable to specificity-governing contacts, enabling the aromatic ring of Tyr57\textsubscript{a} to pack against P7-Tyr of the FLRGRAYGL peptide. The limited extent of conformational changes made by the RL42 TCR in binding to HLA-B8\textsuperscript{FLRGRAYGL} is in marked contrast to that of the LC13 TCR, in which the CDR3\textsubscript{\alpha}, CDR3\textsubscript{\beta}, CDR1\textsubscript{\alpha}, and CDR2\textsubscript{\alpha} loops were remodeled upon HLA-B8\textsuperscript{FLRGRAYGL} ligation (12). Accordingly, although the dominant LC13 TCR and subdominant RL42 TCR bind to HLA-B8 complexed to the same EBV determinant, differing degrees of conformational change underpin the recognition event.

High specificities for the viral Ag

Previously, we demonstrated the impact of the FLRGRAYGL mutations at P1, P7, and P8 on both the LC13 and CF34 TCRs (16); therefore, we undertook SPR analysis with the same peptide variants (P1-T, P1-Y, P7-F, and P8-V) and observed their effect on RL42 TCR recognition. In addition, an alanine-scanning mutagenesis study of the peptide residues (P1, P4, P6, P7, and P8, with P6 being mutated to a Gly) contacting at least one of the three TCRs was undertaken (Fig. 6). The effects of the FLRGRAYGL residue substitutions were grouped into three categories: no effect, a mod-
erate effect representing a 3–5-fold loss of TCR-pMHC affinity, and a drastic effect of >5-fold loss of affinity. The peptide variants were refolded with HLA-B8, and all were able to bind to the mAb W6/32 (data not shown). The effect of the peptide mutations on HLA-B8 thermal stability was assessed; with the exception of the mutation at position P1, all of the peptide variants showed the same stability as the wild-type HLA-B8^{FLRGRAYGL} (Supplemental Table III). The effect of substitutions at the P1 position (3–6°C) is indicative of perturbing the architecture around the P1 pocket.

The systematic substitution of the FLRGRAYGL-immunodominant EBV Ag showed that the RL42 CTLs were sensitive to all solvent-exposed residues of the peptide (Fig. 1). The RL42 TCR affinity was dramatically decreased when the P1 residue was mutated, confirming the N-terminal sensitivity of this TCR (Fig. 6A). Interestingly, the P1-Tyr substitution, which dramatically decreased the CF34 TCR affinity (Fig. 6B), improved the affinity of the RL42 interaction by ~6-fold, suggesting that the additional hydroxyl group of the P1-Tyr formed stabilizing interactions with the RL42 TCR (Fig. 5A). The P7-Phc mutation dramatically decreased the RL42 TCR affinity, similar to that previously observed for the LC13 and CF34 TCRs (16). The P8-Val mutation abrogated LC13 TCR and RL42 TCR binding; moreover, the RL42 CTLs were very sensitive to most substitutions at the P8 position (Fig. 1), despite the fact that the RL42 TCR did not directly contact this position. To address this, we solved the structure of HLA-B8 in complex with the P8-Val variant of the FLRGRAYGL peptide (Supplemental Table I). Although the HLA-B8 Ag-binding cleft did not change conformation appreciably (r.m.s.d. 0.59 Å), the conformation of the peptide itself was altered (i.e., superposition of the wild-type peptide and the P8-Val mutant showed an r.m.s.d. of 1.58 Å, with a maximum displacement of 2.6 Å for the P6-Ala Ca atom and 5.8 Å for the hydroxyl group of the P7-Tyr [Supplemental Fig. 1A]). Accordingly, the P8-Val mutation induced a structural change of the central part of the peptide, which shifted closer to the α2-helix of HLA-B8, thereby providing a basis for understanding the impact of P8 substitutions on the RL42 TCR interaction.

Next, we undertook SPR analysis of the LC13, CF34, and RL42 TCRs against the alanine mutants of the solvent-exposed peptide residues (Fig. 6A). The RL42 TCR affinity was moderately decreased by the P1-Ala and P8-Ala mutations, and it was dramatically decreased by substitutions at P4, P6, and P7. The location of the key positions of the FLRGRAYGL peptide correlated very well with the central docking of the RL42 TCR. In comparison, the CF34 TCR was extremely sensitive to substitutions at the P1, P4, P6, and P7 positions of the FLRGRAYGL peptide (Fig. 6B). The LC13 TCR was unaffected by the P1-Ala substitution, but it was adversely impacted by substitutions at P4, P6, P7, and P8 (Fig. 6C). Although P4-Gly was not contacted by the LC13 TCR (12), the P4-Ala substitution likely impacted on the conformation of the closely abutting CDR3α loop, thereby affecting the LC13 TCR affinity. Collectively, the substitution on the FLRGRAYGL peptide showed a heightened peptide dependency by the three HLA-B8–restricted TCRs, with each TCR exhibiting differing specificities toward the same viral Ag.

**The energetic hot spots on HLA-B8 are driven by the EBV determinant**

To evaluate which HLA-B8 residues are required energetically for the three TCR–HLA-B8^{FLRGRAYGL} interactions, we undertook an alanine-scanning mutagenesis study across the HLA-B8 Ag-binding interface. The HLA-B8 residues targeted for substitution were selected based on the previously determined crystal structures (12, 16) and the RL42 TCR ternary complex reported in this article (i.e., alanine mutants were made for the HLA-B8 residues that contacted, via their side-chain, at least one of the three TCRs). Collectively, 20 single-site HLA-B8 mutants were selected and assessed for binding to the three TCRs using SPR. The W6/32 mAb was used to confirm that all of the mutant HLA-B8^{FLRGRAYGL} complexes were correctly folded (data not shown).

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**FIGURE 6.** SPR on the FLRGRAYGL mutants. SPR data performed on the FLRGRAYGL peptide mutants bound to the HLA-B8 molecule against the three TCRs: RL42 (A), CF34 (B), and LC13 (C). The y-axis represents ΔAGeq (kcal/mol). The black line indicates a 3-fold increase when comparing the mutant to the wild-type, and the dashed black line represents a 5-fold increase. Experiments were conducted in duplicate; error bars indicate the SD for each duplicate.
The effects of the HLA-B8 substitutions were grouped into three categories: no effect, a moderate effect representing a 3–5-fold loss of affinity, and a drastic effect of >5-fold loss of affinity (Fig. 7).

We also tested the effect of these mutants on the thermostability of the pHLA complex (see Materials and Methods). As judged by the thermal melt point, 5 of the 20 mutations affected the stability of the pHLA complex (i.e., Ile66, Arg79, Lys146, Trp167, and Arg170), indicating that the mutation possibly impacted on the conformation of the HLA-B8^{FLRGRAYGL} complex (Supplemental Table III). With the exception of Ile66, which was contacted by the three TCRs, the mutations did not impact on the affinity of the TCRs, because they were not involved in the TCR–MHC interaction. To assess the impact of the Ile66Ala mutation, we solved the structure of HLA-B8–A66 in complex with the FLRGRAYGL peptide (Supplemental Table I). Superposing the HLA-B8 Ag-binding cleft of the wild-type and HLA-B8–A66 mutant (r.m.s.d. of 0.75 Å) showed a shift in the juxtapositioning of the α1-helix in the HLA-B8–A66 structure, which resulted in a narrower HLA-B8 Ag-binding cleft. Further, the peptide moved slightly in the A66 mutant, in which P4-Gly moved toward Ala66 to fill the void caused by the missing side-chain at position 66 of HLA-B8 (Supplemental Fig. 1B). The impact of the Ile66Ala mutant can be attributed, in part, to altering the structure of the local environment of HLA-B8^{FLRGRAYGL}.

For the LC13 TCR–HLA-B8^{FLRGRAYGL} interaction, 10 single-site HLA-B8 mutations had no impact, 3 had a moderate effect, and 7 positions exhibited a marked impact on the affinity of the interaction (Fig. 7A). Of the 13 positions that had no/moderate impact, 7 directly contacted the LC13 TCR. The mutated positions that had minimal impact on the affinity were clustered mostly around the N-terminal region of the peptide, whereas the hot spot positions were mostly clustered around the C-terminal region of the Ag-binding cleft, most notably on the α1-helix that abutted the P7-Tyr residue (Fig. 7D). Previously, we conducted an alanine-scanning mutagenesis study on the LC13 TCR (10), thereby providing us an opportunity to compare the effect of the reciprocal interface-disrupting mutations. The majority of HLA-B8 residues that correspond to the hot spot area are contacted by the CDR3α and CDR3β, two loops that we previously showed to be important in driving the energetics of the LC13 TCR–HLA-B8^{FLRGRAYGL} interaction (10).

For the CF34 TCR–HLA-B8^{FLRGRAYGL} interaction, 10 single-site HLA-B8 mutations had no impact, 4 had a moderate effect, 4 positions notably reduced the affinity of the interaction, and 2 mutations, Gln155Ala and Glu166Ala, resulted in a 3- and 8-fold factor improvement in affinity, respectively (Fig. 7B). Upon ligation, Gln155 was pushed down by the CDR3α loop, and the Gln155Ala mutant resulted in a slower on-rate (wild-type $k_{on} =$
6.74 ± 1.18 10^4 M\(^{-1}\)s\(^{-1}\), Gln155Ala \(k_{\text{on}} = 3.93 ± 0.11 10^4 M\(^{-1}\)s\(^{-1}\)) and a longer off-rate (wild-type \(k_{\text{off}} = 0.26 ± 0.01 s\(^{-1}\), Gln155Ala \(k_{\text{off}} = 0.09 ± 0.01 s\(^{-1}\)) for the interaction (data not shown). All of the residues affecting the affinity of the interaction contacted the CF34 TCR, with the four hot spot residues (Arg62, Ile66, Thr73, and Trp167) clustering around the critical Ag residues P1-Phe, P4-Gly, and P6-Ala. Trp167 and Arg62 both surrounded the P1-Phe of the Ag (Fig. 7E); Arg62 was engulfed by the CDR3\(\alpha\) and CDR3\(\beta\) loops, interacting with residues corresponding to the J and N segments, respectively. Trp167 was contacted by the CDR1\(\alpha\) and the CDR3\(\alpha\) via Pro30\(\alpha\) and Asp109\(\alpha\) (N segment), respectively. Thr73, in close proximity to P6-Ala, was contacted by Gln57 of the CDR2\(\beta\) loop. Finally, Ile66, which contacted P4-Gly, a critical determinant on the FLRGRAYGL peptide, was contacted by the nongermline-encoded residue Trp110 of the CDR3\(\beta\) loop. Accordingly, the HLA-B8 residues that were critically important for the CF34 TCR–HLA-B8\(^{FLRGRAYGL}\) interaction were engaged mostly by nongermline-encoded residues of the CF34 TCR.

For the RL42 TCR–HLA-B8\(^{FLRGRAYGL}\) interaction, 12 single-site HLA-B8 mutations had no impact, 2 had a moderate effect, and 6 positions (Ile66, Thr69, Asn80, Lys146, Glu154, and Gln155) were recognized to represent hot spots, because mutation of each notably reduced the affinity of the interaction (Fig. 7C, 7F). In comparison with the LC13 TCR and CF34 TCR hot spots, the critical HLA-B8 residues for the RL42 TCR interaction were more extensive and diffuse, located adjacent to the P1 pocket, centrally, and at the C-terminal end of the Ag-binding cleft. Thr69 and Ile66, the latter of which was also a hot spot for the LC13 and CF34 TCRs, interacted with the CDR3\(\alpha\) loop of the RL42 TCR. In the central region of the \(\alpha\)-helix, Glu154\(\alpha\) and Gln155\(\alpha\) mutations had a dramatic effect on the affinity of the RL42 TCR, which is attributable to loss of contacts with the CDR2\(\beta\) loop. Further, the alanine-scanning mutagenesis study indicated that the C-terminal end of the binding cleft on the \(\alpha\)-helix was important for RL42 TCR recognition, with two positions having a dramatic effect (Lys146 and Asn80) and two having a moderate effect (Glu76 and Arg79). Although the side-chains of Glu76 and Arg79 were contacted by the CDR1\(\beta\) and CDR1/2\(\beta\) loops, respectively, Asn80 and Lys146 did not contact the RL42 TCR. Both of these residues are involved in binding to the peptide; thus, the effects of mutating these residues to alanine were attributable to local structural perturbations that impact on RL42 TCR recognition. Interestingly, however, the Asn80\(\alpha\)Ala and Lys146\(\alpha\)Ala mutations did not impact on CF34 TCR recognition, and they moderately impacted on LC13 TCR recognition, indicating a heightened sensitivity of RL42 TCR to this region of the Ag-binding cleft.

Accordingly, our data suggested that EBV determinant drives the energetic contribution to the TCR–Ag–HLA interaction, whereas the energetically important HLA-based interactions colocalized the peptide hot spots (Figs. 7D–F, 8).

**Discussion**

EBV is a ubiquitous human pathogen, contained by a vigorous cellular immune response that is directed against a number of immunodominant EBV determinants. To gain a greater understanding of CTL immunity and Ag presentation, we investigated specific elements relating to the protective immune response to EBV and have provided fundamental insight into HLA restriction and a portrait of immunodominant versus subdominant TCR usage.

With regard to MHC restriction, an appealing conceptual framework suggests that the germline-encoded regions of the TCR dictate MHC bias. In line with this view, there is some evidence that MHC reactivity is inherent in the germline-encoded portions of the TCR (30–34). Moreover, conserved pair-wise interactions between closely related V\(\beta\),\(2\) TCRs and peptide-MHC-II (pMHC-II) molecules have led to the concept of interaction motifs between the CDR2\(\beta\) loop of a TCR and a given MHC molecule (35–38).

However, other studies do not support this view in that structural and mutagenesis studies have clearly shown a wide array of TCR-pMHC docking modes, in which the CDR3 loops often play a prominent role in interacting with the MHC, and the germline-encoded loops frequently contact the viral Ag (39, 40). A central question that arises from these observations is whether the viral Ag and/or the MHC H chain principally determines productive TCR engagement. A two-step binding model proposes that the TCR binds the MHC first, then the peptide (41). Moreover, MHC-centric alloreactivity further suggests that docking orientation is governed by TCR–MHC contacts (42). However, T cell alloreactivity can also be peptide centric, and TCR recognition of unusually long MHC-I–restricted viral Ags demonstrates minimal MHC contacts and indicates an Ag-biased focus by the TCR (6, 7, 43).

Further, the mutagenesis studies reported in this article showed that nongermline-encoded residues of the TCRs make important energetic contributions to HLA-B8 binding: the LC13 TCR used two nongermline-encoded CDR3\(\beta\) residues (Gly97 and Gln98) to interact with three HLA-B8 hot spot residues (Gln72, Ala73, and Gly76) (Fig. 8A); the nongermline-encoded residues of the CF34 CDR3 loops (Asp109\(\alpha\) and Trp110\(\beta\)) contact the three

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**FIGURE 8.** Nongermline-encoded TCR residue interaction with the HLA-B8 hot spot. *A.* The LC13 CDR3\(\beta\) (green) nongermline-encoded Gly97 and Gln98 interacting with the HLA-B8 Glu76 and the Gln72 residues, both critical for the interaction (red). *B.* The nongermline CF34 residues from the CDR3\(\alpha\) (Asp109 in orange) and CDR3\(\beta\) (Trp110 in green) loops interacting with the energetically important residues of the HLA-B8 molecule: Arg62, Ile66, and Trp167 (red). *C.* Asn110 of the RL42 CDR3\(\beta\) loop (green), also a nongermline-encoded residue, inserting its side-chain between two hot spots on HLA-B8, Thr73, and Thr69 (red). Red dashed lines represent hydrogen bonds, and blue dashed lines represent the hydrophobic interactions.
HLA-B8 hot spot residues (Arg62, Ile66, Trp167) (Fig. 8B); and the nongermline-encoded residue Asn110β from the CDR3β loop of the RL42 TCR interacts with two HLA-B8 hot spot residues (Thr69 and Thr73) (Fig. 8C).

These observations question the relative importance of the viral determinant versus the MHC in mediating TCR contacts and, in particular, whether the TCR has a virally encoded peptide-centric or MHC-centric view? Viral Ag peptide centricity is suggested by the observations that displacement of the self-peptide with the foreign Ag triggers an immune response and that the viral peptides are considered to represent structural mimics of the self-peptide; TCR–pMHC interactions are very sensitive to the peptide itself, because altered peptide ligands can markedly alter the immune outcome, yet they do not alter MHC docking (44); TCRs bind to superbulged pMHC complexes, such that they form a minimal footprint on the MHC (6, 43); protective immunity can be governed by a germline-encoded TCR polymorphism, which interacts exclusively with a peptide encoded by an EBV Ag (45); and alloreactivity can be governed by peptide-centric molecular mimicry (7, 46). Further, although the TCR–MHC contact is much more extensive compared with TCR–peptide interactions, HLA-mutagenesis studies showed that the HLA energetic hot spot can be small and variable between various TCR–MHC systems (47). Previous studies primarily focused on TCR mutagenesis, and the relative importance/sensitivity to alterations in peptide versus MHC H chain residues in the TCR–pMHC interaction was unclear. Such TCR-centric mutagenesis studies include the 2C TCR system (48), autoreactive TCRs (49), and biased antiviral TCRs (10) (50). Two studies observed the effect of the mutation on the HLA-A2 molecule in two different systems: in the context of the tax peptide recognized by the A6 TCR and with the p1049 peptide recognized by the AHHIII TCR (47, 51). These studies revealed that only three HLA residues (Arg65, Lys66, and Ala69) define the energetic hot spot for the A6 TCR (47).

To address this issue of the viral Ag versus MHC H chain in driving the TCR interaction, we examined the same Ag-presenting molecule, bound to a common EBV determinant, and compared recognition against one immunodominant and two subdominant TCRs that varied in gene usage. These TCRs exhibited differing fine specificities for the viral determinant; together with the HLA-B8 mutagenesis studies, it allowed us to address the energetic landscape of recognition (12, 19). Our data demonstrated that, although the TCRs recognize the same viral determinant, the structural footprints vary between the three TCRs; moreover, the corresponding energetic footprints on the MHC differ between each TCR. In the case of the immunodominant LC13 and subdominant CF34 TCRs, the energetic footprint is clustered around the site of the peptide sensitivity; for the CF34 and LC13 TCRs, the energetic hot spot is around the P1 pocket and the P7 pocket, respectively. However, for the subdominant RL42 TCR, which adopts a central docking mode, the hot spot is more extensive and diffuse and is reflective of the sensitivity at a number of positions within the peptide. Thus, our studies indicated that the energetic requirement of HLA-B8 residues is contingent on the nature and localization of the broadly peptide-centric nature of the interaction. Thus, in contrast to the two-step TCR–pMHC binding mode previously proposed (41), our data indicated that the viral Ag acts as the molecular glue for the TCR–pMHC interaction, whereas the nearby MHC interactions stabilize TCR ligation and modulate the fine-specificity requirements of the TCR for the viral Ag itself. Further, given that the subdominant RL42 TCR adopts a central docking topology on the pMHC, our data indicated that the factors shaping dominant versus subdominant T cell repertoire selection are not governed by favorable docking topologies. Instead, other factors, such as affinity for the TCR–pMHC interaction, precursor frequency, or signaling capacity, contribute to T cell immunodominance (52–54). Collectively, we provided insight into understanding how varied αβ TCR usage against an identical HLA leads to contrasting views of the common viral determinant.

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Disclosures

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