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An Extensive Antigenic Footprint Underpins Immunodominant TCR Adaptability against a Hypervariable Viral Determinant

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Mutations in T cell epitopes are implicated in hepatitis C virus (HCV) persistence and can impinge on vaccine development. We recently demonstrated a narrow bias in the human TCR repertoire targeted at an immunodominant, highly mutable, HLA-B*0801–restricted epitope (1395HSKKKCDEL1403 [HSK]). To investigate if the narrow TCR repertoire facilitates CTL escape, structural and biophysical studies were undertaken, alongside comprehensive functional analysis of T cells targeted at the natural variants of HLA-B*0801–HSK in different HCV genotypes and quasispecies. Interestingly, within the TCR–HLA-B*0801–HSK complex, the TCR contacts all available surface-exposed residues of the HSK determinant. This broad epitope coverage facilitates cross-genotypic reactivity and recognition of common mutations reported in HCV quasispecies, albeit to a varying degree. Certain mutations did abrogate T cell reactivity; however, natural variants comprising these mutations are reportedly rare and transient in nature, presumably due to fitness costs. Overall, despite a narrow bias, the TCR accommodated frequent mutations by acting like a blanket over the hypervariable epitope, thereby providing effective viral immunity. Our findings simultaneously advance the understanding of anti-HCV immunity and indicate the potential for cross-genotype HCV vaccines. The Journal of Immunology, 2014, 193: 5402–5413.

Hepatitis C virus (HCV) is a major cause of chronic hepatitis infecting an estimated 170 million people worldwide. About 75% of infected individuals develop persistent infection that can lead to liver cirrhosis and hepatocellular carcinoma (1). Strong and broadly directed CD8+ T cell responses are associated with HCV clearance (2–5); however, the mechanisms contributing to the decline in the immune function during chronic HCV infection are still not fully understood. Viral evolution over the course of infection can contribute to persistence by enabling immune escape via mutation of key epitopes targeted by T lymphocytes. For hypervariable viruses, a diverse Ag-specific T cell repertoire is proposed to reduce the potential for immune escape (6, 7), whereas a narrow TCR repertoire is thought to catalyze instances of viral escape and disease progression (6, 8). Despite hypervariability of the viral genome leading to different HCV genotypes and documented evidence of circulating quasispecies in individuals with persistent infection, the effect of TCR repertoire diversity on viral escape and disease outcome remains unclear for HCV infection.

Natural variants of an immunodominant, HLA-B*0801–restricted CD8+ T cell Ag, 1395HSKKKCDEL1403 (HSK), derived from the highly immunogenic HCV nonstructural protein 3, have been reported previously (9–11). HLA-B*0801 is a common allele, prevalent in 10–18% of Caucasian populations (5). More than 90%
of HCV-infected, HLA-B*0801+ subjects respond to HSK, but it is noteworthy that HLA-B*0801 is associated with HCV persistence (12). In addition to the frequent intergenotypic variations, K > R at position 3 of the peptide (P3) in HSK-G2 and L > I at P9 in HSK-G3, other mutations have been reported (5, 10, 11) in viral isolates (HCV quasispecies) circulating in chronically infected subjects.

G3, other mutations have been reported (5, 10, 11) in viral isolates a broad TCR repertoire was recruited against HLA-A*0101-1435 matched subjects with diverse disease outcomes (natural HCV P4K and P9 L narrowly focused, with a strong bias towards the TCR characteristics of the peptide bound in the HLA groove, convergent recombination, thymic selection bias, Ag load, and clonal compe-
tition (14–18).

To understand the repercussions of a narrowly biased TCR repertoire on the recognition of hypervariable but immunodominant HCV Ags, structural and functional studies were conducted on T cells directed at HSK, its intergenotypic variants, and other reported mutants. The structure of the TCR complexed with HLA-B*0801–HSK revealed that the antigenic footprint of the TCR was extensive, covering all the peptide solvent exposed resi-
dues. Functional assays revealed that except for some rare/transient

Materials and Methods
Ethics statement
PBMCs from HCV-infected participants of the Networks cohort were used in this immunological study after approval from the Department of Health’s Human Research Ethics Committee. This study was also registered at the University of Melbourne. Written informed consent was obtained from each subject in accordance with the ethical guidelines of the Declaration of Helsinki.

Subjects
PBMCs from two HLA-B*0801–positive subjects, D#1 and D#2, known to be previously infected with HCV, but who had naturally cleared the virus, were selected for this study. Both subjects were participants in the Net-
works cohort (19).

Establishment and characterization of HSK-specific T cells
HCV peptide–specific T cell cultures were raised as described previously (20) by in vitro peptide stimulation of PBMCs isolated from HLA-B*0801+. HCV-infected individuals. Briefly, PBMCs from each subject were cocultured with autologous PBMCs pulsed with 0.1 μM HSK pep-
tide. Cultures were supplemented with rIL-2 on day 3 and analyzed for TCR usage on day 10. Peptide-specific T cell lines were generated by weekly restimulation with recombinant human IL-2 (rIL-2) and γ-irradiated (8000 rad) peptide-pulsed C1R-B*08 cell lines. A monoclonal T cell line (DD31) was derived against HSK by repeated rounds of limiting dilu-
tion assay to determine the matching Vα and Vβ TCR heterodimer. Peptides with >90% purity were purchased from Mimotopes (Clayton, VIC, Australia) or GenScript. Peptide-specific T cell activity and avidity was assessed by intracellular cytokine staining (ICS) for IFN-γ and/or TNF-α and in a standard 4-h [3H]release assay as previously described (20). T cells were verified for purity and peptide specificity by tetramer staining. TCR repertoire was determined by costaining T cells with an anti-human CD8 mAb (BD Pharmingen), HLA-B*0801–HSK tetramer, and a panel of TRBV chain–specific mAbs (Beckman Coulter). Cells were washed and analyzed on a FACSCalibur using Cell Quest software (BD Biosciences).

Intracellular cytokine staining
ICS for IFN-γ and TNF-α was performed as previously described (20). Briefly, C1R.B*0801 cells were presensitized with respective synthetic peptide at a concentration of 10 μg/ml for 20 h. Autologous T cells were incubated with expanded HCV-specific T cell cultures at an E:T ratio of 2:1 for 1 h at 37°C. Following incubation, brefeldin A (Sigma-Aldrich, St. Louis, MO) was added at a final concentration of 10 μg/ml and in-
cubated for a further 5 h at 37°C. Cells were washed and stained with Tricolor-conjugated anti-CD8 (Caltag/BD Biosciences) and PE-conjugated anti-CD3 (eBioscience) for 30 min at 4°C in darkness and then fixed for 20 min at room temperature with 1% paraformaldehyde (ProSciTech, Kirwan, QLD, Australia). Fixed cells were permeabilized with 0.3% sa-
pinon (Sigma-Aldrich) and stained with either FITC-conjugated anti-
human IFN-γ (BD Biosciences, San Jose, CA) or FITC-conjugated anti-
human TNF-α (eBioscience) for 30 min at 4°C. The cells were washed and resuspended in staining buffer prior to analysis by flow cytometry using FlowJo software (Tree Star, Ashland, OR). A total of 100,000-200,000 live events were acquired on a FACSCalibur (BD Biosciences). Lymphocytes were gated in a forward light scatter versus side scatter plot and further analyzed for CD8 staining on the x-axis and TNF-α staining on the y-axis. The top right quadrant of each plot represents the percentage of TNF-α+/CD8+ T cells out of the total lymphocyte population. Similar gating strategies were used for the remaining ICS assays for IFN-γ secretion. Results presented in the graphs were expressed as standard mean, and error bars reflected SD. Prism 5.0 (GraphPad Software) was used.

Cytotoxic assays
[51Cr] release assay was performed to assess the cytolytic activity of in vitro–expanded HSK peptide–specific T cells (effectors) as previously described (20). Autologous PHA blasts or C1R-B*0801 (target cells) were presensitized with synthetic HCV peptides, prior to incubation with radioactive [51Cr] for 60 min at 37°C, 5% CO2. After incubation, these cells were washed three times with cold RF10 and resuspended at 1 × 106 cells/ml. Target cells were then seeded into a 96-well plate in triplicates and cocultivated with the ef-
tector T cells at E:T ratios of 10:1, 5:1, and 1:1. After 5 h, the cytol-

T cell repertoire analysis of the HSK-specific clone
Total RNA was extracted from the T cell clone using TRIZol reagent (Invitrogen). Reverse transcription was performed with Superscript III (Invitrogen) and antisense TRCα- and TRCβ-chain primers (21). PCR was performed using a TCR Cα constant primer and 1 of 34 TCR Vα family–specific primers or a TCR Cβ constant primer and 1 of 26 TCR Vβ family–specific primers (21). PCR products were purified and cloned into the pGEM-T vector system (Promega) and sequenced using the ABI PRISM Big Dye Termination Reaction kit (Applied Biosystems). The International ImMunoGeneTics information system TCR gene nomenclature was used throughout (22), and CDR3 length was determined according to previous criteria. The DD31 TCR CDR3 sequences for both the α- and the β-chains are shown in Table I.

Generation of DD31 TCR–HLA-B*0801–HSK complex
RNA was extracted from the HSK-specific monoclonal T cell line DD31. cDNA was obtained by reverse transcription and further amplified using gene-specific primers for TRAV9.2*01/TRAJ4*01 and TRBV11.2*01/
TRBD1*01/TRBJ1.5*01 by PCR and cloned into pGEM-T (Promega). Clones carrying insert with correct sequences were subcloned into pEF expression vectors (Novagen), and soluble TRAV2.9 and TRVB11.2 proteins were expressed in Bl21 Escherichia coli expression strains. The DD31 TCR was expressed, refolded, and purified with an engineered disulfide linkage in the constant domains between the TCRα constant and TCRβ constant. Both the TCRα- and TCRβ-chains of the DD31 TCR were expressed as inclusion bodies and then resuspended in 8 M urea, 20 mM Tris-HCl (pH 8), 0.5 mM sodium-EDTA, and 1 mM DTT. Inclusion body proteins were refolded in the refolding buffer, dialyzed to remove urea, and the protein solution was purified using DEAE anion exchange, S75 gel filtration, and Mono-Q anion exchange chromatography (GE Healthcare) (23). Soluble class I heterodimers consisting of HLA-B*0801 H chain (aa residues 1–276), bound noncovalently to full-length β2-microglobulin and complexed to the HSK peptide, were prepared as described previously (24). Purified HLA-B*0801–HSK was treated with 10 mM DTT to prevent dimerization of P6-C, and the treated protein was subjected to gel filtration to remove the DTT. The eluted HLA-B*0801–HSK complex was mixed with an excess of purified DD31 TCR, and the ternary complex was purified by gel filtration chromatography using Superdex 200 16/60 column (GE Healthcare).

Crystallization

Crystals of the HLA-B*0801–HSK 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) and 150 mM NaCl. Large stick-shaped crystals grew using 20–24% polyethylene glycol (PEG) 4000, 0.2 M ammonium acetate, and 0.1 M trisodium-citrate (pH 5.6). Prior to crystallization of the DD31 TCR in complex with the HLA-B*0801–HSK, both proteins were copurified over a gel-filtration column to ensure a 1:1 ratio. Rhomboid-shaped crystals of the DD31 TCR–HLA-B*0801–HSK complex were obtained following the same technique, with a concentration of 10 mg/ml, in 100 mM Tris-HCl (pH 8) and 150 mM NaCl. The crystals grew using 13–18% PEG 3350, 0.1 M sodium citrate at pH 6, and 0.2 M ammonium iodide.

Data collection and structure determination

The crystals of HLA-B*0801–HSK and the DD31 TCR/HLA-B*0801–HSK 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 MX1 and MX2 beamlines at the Australian Synchrotron, using the ADSC-Quantum 210r and 315 charge-coupled device detectors for the HLA-B*0801–HSK and the DD31 TCR/HLA-B*0801–HSK, respectively (at 100 K). Data were processed using either the XDS software or scaled using XSCALE software (25). The HLA-B*0801–HSK crystal belonged to the space group P21_P21_P21 with unit cell dimensions (Table III), consistent with one complex in the asymmetric unit. The DD31 TCR–HLA-B*0801–HSK crystal belonged to the space group P21 with unit cell dimensions (Table III), consistent with two complexes and one unliganded DD31 TCR in the asymmetric unit. The structures were determined by molecular replacement using the PHASER program (26) with the HLA-B*0801/FLR (Protein Data Bank [PDB] accession number 1M05; http://www.rcsb.org/pdb/home/home.do) for the HLA model without the peptide (24) and the LC13 TCR as the search model for the TCR (PDB accession number 1KG0) (27). Manual model building was conducted using the Coot software (28) followed by refinement with the PHENIX program (29). The TCR was numbered according to the ImMunoGeneTics unique numbering system (22) in which the CDR1 loops start at residue number 27, the CDR2 loops start at 53, and the CDR3 loops start at 105. The final models have been validated using the PDB validation Web site, and the final refinement statistics are summarized in Table III.

PDB accession number

Coordinates are submitted to the PDB under the PDB identification number 4QRQ for HLA-B*0801–HSK and 4QRP for the DD31 TCR–HLA-B*0801–HSK complex. All molecular graphic representations were created using PyMol (30).

Surface plasmon resonance measurement and analysis

All surface plasmon resonance (SPR) experiments were conducted at 25°C on the Biacore 3000 instrument (Biacore) with the HEPA-filtered saline buffer supplemented with 1% buried surface area (BSA) to prevent nonspecific binding. Human TCR-specific mAb 12H8 (31) was coupled to research-grade CMS chips with standard amine coupling and DD31 TCR was passed over the flow cell until ~200–400 response units were captured by the Ab. Then either HLA-B*0801 in complex with the native or variant peptides were injected over all four flow cells at a rate of 20 μl/min with a concentration range of 0–100 μM. The final response was calculated by subtracting the response of the Ab alone from that of the Ab and the DD31 TCR–HLA-B*0801–HSK complex. The Ab surface was regenerated between each analyte injection with Actisep (Sterogene). The HLA-B*0801–peptide complexes were treated with DTT prior to SPR analysis to avoid dimerization due to the P6-C of the peptide. All experiments were conducted in duplicate at least.

BIAspecific binding was determined using PyMol (30).

Circular dichroism spectroscopy was measured on a Jasco 815 spectropolarimeter (JASCO) using a thermostatically controlled cuvette. A far-UV spectra was collected from 190 to 250 nm. The UV minimum was determined as 219 nm for pHLA. The measurements for the thermal melting experiments were made at the minimum for pHLA, at intervals of 0.1°C and at a rate of 2°C/min from 25 to 85°C. The Jasco Spectra Manager software (JASCO) was used to view and smooth the traces, and then GraphPad Prism (GraphPad Software) was used to plot temperature versus percent unfolded. The midpoint of thermal denaturation for each protein was determined as the point at which 50% unfolding was achieved. The measurements were done in duplicate at two concentrations (5 and 10 μM) in a solution of 10 mM Tris-HCI (pH 8) and 150 mM NaCl.

Results

HSK and its natural variants stabilize HLA-B*0801 equally

To determine the effect of mutations of HSK on the stabilization of the HLA-B*0801 molecule, thermal melts assays were undertaken. Each of the peptide variants, P3K > R, P4K > R, P7D > A, P7E > E, and P9L > I, were refolded with HLA-B*0801, and thermal stability of each pHLA complex was determined. The variation in the thermal melting point, or midpoint of thermal denaturation, between all peptides was no more than 3°C compared with the native peptide HSK (Supplemental Table I), indicating that potential differences in the reactivity of the T cells to these peptide variants were not attributable to altered stability of the Ag-presenting molecule.

Peptide residues P4, P6, and P7 are critical for T cell recognition and function

Our previous observation of biased TRBV11 usage against HLA-B*0801–HSK (13) suggested a potential for CTL escape by HCV mutating key TCR contacts within the epitope. To understand the molecular basis of this narrow bias and to assess if it predisposes CTL escape, we undertook an integrated structural, functional, and biochemical analysis of this TCR–pHLA interaction. The structural study was based on an archetypal T cell line designated as D#1 generated from an HCV-infected subject (D#1) who primarily deployed TRBV11 clonotypes to engage HLA-B*0801–HSK and the functional study used HSK-specific polyclonal T cell lines from the same donor (D#1) and another HCV-infected, HLA-B*0801 subject (D#2). The overall HSK-specific TCR repertoire has been previously published in Miles et al. (13), in which D#1 and D#2 are designated as D#2 and D#5, respectively.

To investigate the contribution of each amino acid of the HSK Ag in TCR recognition, a series of peptides incorporating individual alanine substitutions at each position of HSK were synthesized. HSK-specific polyclonal T cell lines derived from donor D#1 (TRBV usage: TRBV4, TRBV5, TRBV7, and TRBV11) were used to assess the reactivity of the T cells to alanine substituted peptides by ICS and [51Cr] release assay (Fig. 1A–C). Further, to investigate if subtle differences in TRBV usage could affect T cell reactivity towards the peptide variants, an HSK-specific T cell line derived from...
another donor D#2 (TRBV usage: TRBV4, TRBV5, TRBV 14, and TRBV11) was used in a [51Cr] release assay (Fig. 1D). ICS assays performed using T cells from D#1 revealed a comparable number of cytokine-producing T cells against native HSK and the alanine-substituted peptides, whereas (B) summarizes the IFN-γ-secreting T cells. (C) and (D) depict the cytolytic activity against these variants in a standard [51Cr] release assay at E:T ratios of 10:1, 5:1, and 1:1 (D#1) and E:T ratio of 10:1 (D#2), respectively. ICS was performed on duplicate samples and [51Cr] release assay on triplicate samples. Each bar depicts the mean, and error bars reflect the SD. HSK-specific cytokine secretion and cytolytic activity are nearly abolished by alanine substitution at P4, P6, and P7.

![FIGURE 1](http://www.jimmunol.org)

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other donor D#2 (TRBV usage: TRBV4, TRBV5, TRBV 14, and TRBV11) was used in a [51Cr] release assay (Fig. 1D).

ICS assays performed using T cells from D#1 revealed a comparable number of cytokine-producing T cells against native HSK and peptides with alanine substitution at P2, P8, and P9, a 40–50% reduction was observed against P1, P3, and P5 substitutions, whereas substitution at P4, P6, and P7 abrogated the peptide-specific cytokine production (Fig. 1A, 1B). [51Cr] release assays (Fig. 1C) also reflected a similar trend, with alanine substitution at P4 resulting in significant reduction in cytolytic potential and with a total lack of cytolysis against P6 and P7 alanine substitutions. In contrast, comparable cytolyis was observed against native HSK and peptides with alanine substitution at P2, P8, and P9 and with a marginal decrease against P1, P3, and P5 substituted peptides. [51Cr] release assays performed with a T cell line from donor D#2 (Fig. 1D) demonstrated a similar pattern, indicating that minor changes in TRBV usage did not drastically impact T cell reactivity. Overall, the data suggested that P4-K, P6-C, and P7-D were crucial for T cell reactivity.

**HSK-specific T cells are cross-reactive to intergenotypic variants and common HCV quasispecies mutants**

Naturally occurring variations in HSK include: intergenotypic variations P3K > R (HCV-G2) and P9L > I (HCV-G3); common mutations P8E > D, P9L > F/V (5, 10); and rare mutations at P1H > R, P2S > T (5, 11), P3K > K > Q, P4K > S, P5K > g (11), P6C > Y (10), P7D > G (11), P7D > A (10), P9L > F (5), and P9L > M (13). To assess the effect of these intergenotypic and quasispecies mutants on T cell recognition and function, peptides corresponding to the mutants were tested in functional assays using the HSK-specific T cell lines derived from D#1.

As shown in Fig. 2A, the IFN-γ secretion against target cells presenting HSK, P3K > R (HSK-G2), or P9L > I (HSK-G3) was comparable, demonstrating efficient cross-recognition of the intergenotypic variants by the biased TRBV11 TCR. Similarly, IFN-γ secretion against the common mutants P8E > D and P9L > F/V was also comparable to that of HSK (Fig. 2B), whereas cytokine secretion against P4K > R was significantly reduced even though K > R is a conservative mutation. Interestingly, IFN-γ secretion against peptides corresponding to rare mutations (P3K > Q, P4K > S, P6C > Y, P7D > G, and P7D > A) was drastically reduced (Fig. 2C).

This trend was also observed in the cytolytic activity against the variants in which the cytotoxic effect against HSK and its intergenotypic variants HSK-G2 and HSK-G3 (Fig. 2D) was comparable, substitution at P8 and P9 did not affect cytolytic activity, but substitution at P4K > R decreased the cytolytic activity by ~50% (Fig. 2D). This observation is supported by previous findings by Timm et al. (5) that the P4K > R variant (HSKRKCDEL) is less...
efficient in stimulating IFN-γ secretion and cytotoxicity than the parental sequence (HSKKKCDEL).

Altogether, the data demonstrated that HSK-specific T cells could not tolerate variations at positions P4, P6, or P7 located centrally within the peptide, leading to partial or total abrogation of recognition of such variants. However, these T cells accommodated mutations at position P3K<sup>R</sup> and P9L<sup>I</sup>, thus efficiently cross-recognizing the intergenotypic variants. Analysis of T cell avidity through a peptide titration of HSK and some of its variants (P3K<sup>R</sup>, P4K<sup>R</sup>, P6C<sup>Y</sup>, and P7D<sup>A</sup>) confirmed that differences in T cell reactivity were not due to differences in T cell avidity (data not shown).

**HSK mutants have a variable effect on the binding affinity for DD31 TCR**

To understand the basis of cross-genotypic reactivity demonstrated above, further study was conducted to elucidate the structural interactions between HSK and its specific TCR. For this, HSK-specific monoclonal T cell line DD31 was derived from an HCV-infected HLA-B*0801<sup>+</sup> donor and the TCR CDR3 sequences of the TCR<sup>a</sup> variable (TRAV) and TRBV chains determined to be TRBV11.2 TRBD2*01 TRBJ1.5, TRAV9.2 TRAJ43 (Table I). The soluble purified DD31 TCR thus obtained from this monoclonal T cell line was used to elucidate the TCR–pHLA interactions and binding affinities.

SPR analysis was conducted to study the binding affinity and kinetics of the DD31 TCR interaction with the native HSK peptide and selected variants bound to HLA-B*0801<sup>+</sup>. Peptides corresponding to the native peptide (HSKKKCDEL) and its variants (HSKG3-P9-HSKKKCDEI, HSKG2-P3-HSRKKCDEI, HSKQKCDEL, P4-HSKKKCDEI, HSKSKCDEL, P6-HSKKKYDEL, P7-HSKKKCAEL, HSKKKCDEL, P8-HSKKKCDDL, and P9-HSKKKCDEV, HSKKKCDEF, and HSKKKCDEM) and tested in an ICS assay (for IFN-γ secretion) using HSK-specific T cell line derived from D#1. Figures show percentage of CD8<sup>+</sup> T cells producing IFN-γ against HSK and its intergenotypic variants (A), HSK and common variants (B), and HSK and rare variants (C). Each bar depicts the mean cytokine secretion from two replicates. (D) shows the cytolytic activity against some of these variants in a standard [<sup>51</sup>Cr] release assay at three E:T ratios (10:1, 5:1, and 1:1). Error bars represent SD.

**FIGURE 2.** T cell specificity to HSK and its intergenotypic variants (HCV-G2 and G3) is comparable, whereas mutations at P4, P6, or P7 are recognized to varying degrees. APCs (C1RB*08) were pulsed with peptides corresponding to the native peptide (HSKKKCDEL) and its variants (HSKG3-P9-HSKKKCDEI, HSKG2-P3-HSRKKCDEI, HSKQKCDEL, P4-HSKKKCDEI, HSKSKCDEL, P6-HSKKKYDEL, P7-HSKKKCAEL, HSKKKCDEL, P8-HSKKKCDDL, and P9-HSKKKCDEV, HSKKKCDEF, and HSKKKCDEM) and tested in an ICS assay (for IFN-γ secretion) using HSK-specific T cell line derived from D#1. Figures show percentage of CD8<sup>+</sup> T cells producing IFN-γ against HSK and its intergenotypic variants (A), HSK and common variants (B), and HSK and rare variants (C). Each bar depicts the mean cytokine secretion from two replicates. (D) shows the cytolytic activity against some of these variants in a standard [<sup>51</sup>Cr] release assay at three E:T ratios (10:1, 5:1, and 1:1). Error bars represent SD.

**Table I. DD31 TCR CDR3 sequences**

<table>
<thead>
<tr>
<th>TRBV</th>
<th>CDR3&lt;sup&gt;3+&lt;/sup&gt;</th>
<th>TRBJ</th>
</tr>
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<tbody>
<tr>
<td>11-2</td>
<td><strong>C A S S L R G R G D Q P Q H F</strong>&lt;br&gt;tg t gcc agc agc t ty agg gga cga gga gat cac ccc cag cat ttt</td>
<td>1-5</td>
</tr>
<tr>
<td>9-2</td>
<td><strong>C A L S D P V N D R F</strong>&lt;br&gt;tg t gct cty gat cac ccg gtc aat gac atg cgc ttt</td>
<td>TRAJ 43</td>
</tr>
</tbody>
</table>

*Bold and italicized letters designate germline origin.*
to that of HSK-G3 (P9, > 1) with Kd equilibriums (Kd eq) of 2.5 and 3.2 µM, respectively, whereas the affinity of HSK-G2 (P3K > K) was similar (Kd eq of 6.6 µM) (Supplemental Fig. 1B, Table II). Also, the binding kinetics of the DD31 TCR to HSK, HSK-G2, or HSK-G3 were comparable with a k on of 21,500 M^-1s^-1 and a k off of 0.34 s^-1 for the wild-type peptide, a range that is typical for previously observed TCR–pHLA interactions (32). In contrast, the affinity of DD31 TCR for the P4K > K mutant (no binding) and P7 D > E mutant (>100 µM) indicated that even subtle changes at P7 could have a drastic impact on the TCR binding (Supplemental Fig. 1D).

Crystal structure of the DD31 TCR–HLA-B*0801–HSK complex

Next, crystal structures of HLA-B*0801–HSK and its complex with DD31 TCR were determined. The crystal structure of HLA-B*0801–HSK was solved to 1.8 Å resolution and to an R factor and R free of 18.6 and 23.8%, respectively (Table III). The electron density of the bound HSK and the interacting residues was unambiguous, with the exception of the P4-K side chain that was ambiguous, with the exception of the P4-K side chain that was mobile. HSK was anchored to the HLA-B*0801 molecule via the residues at positions P2-S, P3-K, P5-K, and P9-L, and thus their side chains were not available for TCR interaction. The central region of the peptide, P4–P6, adopted a constrained conformation (33) in which the solvent exposed residues were P1-H, P4-K, P6-C, P7-D, and P8-E, (Fig. 3A), and thus, these residues were potential TCR contact points.

The structure of the DD31 TCR in complex with HLA-B*0801–HSK was then solved at a resolution of 2.9 Å (Fig. 3B, Table III). The asymmetric unit contained two DD31 TCR–HLA-B*0801–HSK complexes and, surprisingly, one unliganded DD31 TCR. The DD31 TCR docked 75˚ across the long axis of the HLA-B*0801 Ag-binding cleft, consistent with previous TCR–pHLA docking modes (34, 35) (Fig. 3B, 3C). The center of mass of the Vα- and the Vβ-chains showed that the TCR docked over the N-terminal end of the Ag-binding cleft; nevertheless, the 13-residue–long CDR3β loop stretched out to make contact with the C-terminal of the peptide as well (Fig. 3C).

The total BSA at the interface was ≈2050 Å^2, falling within the range generally observed for TCR–pHLA structures (36, 37). The TCR β-chain contributed 55% BSA at the interface, thereby providing initial insight into the repeated TRBV11 usage in unrelated individuals. The CDR2α and CDR1β loops did not contribute to the interaction. The germline-encoded CDR1α and CDR2β loops contributed equally to the interaction (18% BSA each), both contacting HLA-B*0801 exclusively. Further, two residues from the framework region of the β-chain (residues Val66β and Asp67β), downstream of the CDR2β loop, contributed 12% BSA to the interface. As such, the CDR3α and CDR3β loops were the major contributors (27 and 25% BSA, respectively) to the interface, interacting with both the HSK epitope and the HLA-B*0801 molecule.

### Table II. Binding affinities and kinetics of HLA-B*0801–HSK and its variants to the DD31 TCR

<table>
<thead>
<tr>
<th>Analyte</th>
<th>K_d eq (µM)</th>
<th>K_m (10^3/Ms)</th>
<th>K_off (1/s)</th>
<th>t_1/2 (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSKKKCDEL</td>
<td>2.48 ± 0.11</td>
<td>2.15 ± 0.05</td>
<td>0.34 ± 0.06</td>
<td>2.10 ± 0.36</td>
</tr>
<tr>
<td>HSRRKCDDEL</td>
<td>6.59 ± 0.62</td>
<td>2.21 ± 0.76</td>
<td>0.55 ± 0.01</td>
<td>1.26 ± 0.02</td>
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<tr>
<td>HSKKCDEL</td>
<td>3.18 ± 0.01</td>
<td>1.23 ± 0.15</td>
<td>0.36 ± 0.03</td>
<td>1.93 ± 0.16</td>
</tr>
<tr>
<td>HSUKCDEL</td>
<td>15.80 ± 0.40</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>HSUKCDEL</td>
<td>&gt;100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NB, no binding.</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

Bold and italicized letters indicate mutations at the corresponding positions in the peptide sequence of the wild-type HSK peptide.

NB, no binding.

### Table III. Data collection and refinement statistics

<table>
<thead>
<tr>
<th></th>
<th>HLA-B*0801–HSK</th>
<th>DD31 TCR–HLA-B*0801–HSK</th>
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</thead>
<tbody>
<tr>
<td><strong>Data collection statistics</strong></td>
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</tr>
<tr>
<td>Temperature</td>
<td>100 K</td>
<td>100 K</td>
</tr>
<tr>
<td>Space group</td>
<td>P2_12_12</td>
<td>P2_1</td>
</tr>
<tr>
<td>Cell dimensions (a, b, c) (Å)</td>
<td>50.82, 81.34, 110.79</td>
<td>74.16, 252.19, 79.44</td>
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<td>Resolution Å</td>
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<td>1.70–2.90 (3.00–2.90)</td>
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<tr>
<td>Total no. of observations</td>
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<td>262,440 (23,663)</td>
</tr>
<tr>
<td>No. of unique observations</td>
<td>51,111 (7,046)</td>
<td>62,701 (5,396)</td>
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<tr>
<td>Multiplicity</td>
<td>5.0 (3.5)</td>
<td>4.3 (5.3)</td>
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<tr>
<td>Data completeness (%)</td>
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<td>96.3 (88.8)</td>
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<tr>
<td>I/</td>
<td>I</td>
<td>29.6 (4.8)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
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<td>12.4 (50.3)</td>
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<tr>
<td>Re s (%)</td>
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</tr>
<tr>
<td>Rfactor (%)</td>
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</tr>
<tr>
<td>Rfree (%)</td>
<td>20.9</td>
<td>24.8</td>
</tr>
<tr>
<td>rmsd from ideality</td>
<td>Bond lengths (Å)</td>
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</tr>
<tr>
<td>Bond angles (°)</td>
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<td>0.99</td>
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<tr>
<td>Ramachandran plot (%)</td>
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<td>Allowed region</td>
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<td>0.5</td>
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<tr>
<td>Disallowed region</td>
<td></td>
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DD31 TCR–HLA-B*0801 interactions

The CDR1α loop made a few hydrophobic contacts with the α2-helix of HLA-B*0801, in which Thr10α and Tyr20α of the TCR contacted Glu166 and Glu161, respectively (Fig. 4A, Supplemental Table II). The CDR3α loop spanned across the Ag-binding cleft, in which the non–germline-encoded residue Pro109α was positioned between Gln155 and Thr163 of HLA-B*0801 in a peg-notch fashion (Fig. 4B). Additionally, the TRAJ43-encoded residues, Asn111α and Asp112α from the CDR3α loop, formed polar interactions with Arg62 and Gln65 of the HLA molecule (Fig. 4B).

Many of the TCR–HLA-B*0801 interactions were mediated via the CDR2β loop that sat atop of the α1-helix, interacting with residues spanning from Lys68 to Gln76. In addition to the CDR2β loop, two framework residues, Val66β and Asp67β, were found to extend the footprint on the α1-helix, interacting with Gln65, covering a stretch of ~17 Å on the HLA. The core of the contacts mediated by the CDR2β loop and framework regions were hydrophobic, with two H-bonds (Gln72 and Asn99β and Gln65 and Asp67β) at the periphery of the CDR2β–HLA-B*0801 interface (Fig. 4C). The extensive interaction between the germline-encoded CDR2β, which involved all of the residues within CDR2β (except Phe56β), and HLA-B*0801 provided a basis for the biased usage of the TRBV11 gene by the HLA-B*0801-HSK–specific T cell clones. The CDR3β also interacted with HLA-B*0801, with the Arg111β contacting the residues Ala190 and Arg191 at the hinge of the α2-helix and also the non–germline-encoded residue Asp113β contacting the Gln155 (Fig. 4D).

Interestingly, the TRBV11 of the DD31 TCR is shared with another HLA-B*0801–restricted TCR, namely CF34 (23). The CF34 TCR recognizes HLA-B*0801 bound to the EBV epitope named FLRGRAYGL (FLR). The CF34 TCR made very few interactions with the HLA-B*0801 molecule via its CDR2β loop, in which only Gln57 was involved. The CF34’s Gln57β contacted Thr73, an interaction not shared with DD31 TCR, and Thr69, which was seen in the DD31 TCR–HLA-B*0801–HSK complex. Surprisingly, however, most of the contacts mediated by the framework residues Val66β and Asp67β were conserved between the CF34 and DD31 TCR ternary complexes.

Peptide-mediated contacts

The DD31 TCR contacted all of the solvent accessible residues of HSK, thereby displaying maximal coverage of this epitope. The HSK peptide interacted only with the CDR3 loops, with the CDR3α loop contacting P1-H, P2-S, and P4-K and CDR3β contacts P4-K, P6-C, P7-D, and P8-E (Fig. 5A). CDR3α made contacts with HSK via Pro109α, which was seen in the DD31 TCR–HLA-B*0801–HSK complex. Surprisingly, however, most of the contacts mediated by the framework residues Val66β and Asp67β were conserved between the CF34 and DD31 TCR ternary complexes.
CDR3 loops. The main chain of Arg\(^{109}\) interacted with P6-C and stretched its side chain over the peptide to make a salt bridge with P8-E (Fig. 5C). Arg\(^{111}\) contacted P4-K with its main chain, P6-C with both main and side chains, and moreover stretched its side chain toward the C-terminal part of the HSK peptide to make a salt bridge with P7-D. P7-D interacted with Arg\(^{111}\) by a salt bridge, and its smaller side chain fitted between the epitope backbone and the \(\alpha_2\)-helix of the HLA molecule without pushing away the CDR3 loop. The blue dashed lines represent the hydrophobic interactions and the red dashed lines the hydrogen bonds.

**Conformational changes upon ligation of DD31 TCR to HLA-B*0801–HSK**

The comparison of the unliganded structure of HLA-B*0801–HSK and its complex with the DD31 TCR permitted an analysis of the structural changes upon TCR ligation. The HLA-B*0801 molecule did not change conformation appreciably upon TCR ligation (root mean square deviation [rmsd] of 0.60 Å on the C\(\alpha\) atoms of the \(\alpha_1\)-\(\alpha_2\) domain) (Fig. 6A). A few residues of HLA-B*0801 rearranged their side chains upon TCR ligation to avoid steric clashes with the CDR loops. In addition to Arg\(^{62}\) shifting its side chain (Fig. 6A), Gln\(^{155}\) reoriented 180° as a result of the presence of Arg\(^{111}\) from the DD31 TCR, which slotted between the peptide and the tip of the \(\alpha_2\)-helix.

The peptide backbone did not undergo major conformational changes upon TCR ligation (rmsd of 0.33 Å between liganded and unliganded) (Fig. 6A), although three solvent accessible side chains changed their conformation. The P1-H flipped its side chain due to interactions mediated by the CDR3\(\alpha\) loop, whereas P6-C and P8-E reoriented due to CDR3\(\beta\) loop-mediated interactions. This conformation of the P6-C further enabled the CDR2\(\beta\) loop to contact this residue.

The crystals of the DD31 TCR–HLA-B*0801–HSK complex contained an unliganded DD31 TCR alone for which the CDR loops were not involved in crystal contacts. Thus, this unliganded DD31 TCR structure allowed the analysis of the conformational changes that occurred in the TCR upon ligation. The superposition of the liganded and unliganded DD31 TCR structures showed a very similar overall conformation (rmsd of 0.72 Å) (Fig. 6B).
The CDR loop backbone conformations were very similar with the exception of the CDR3α loop (rmsd 2Å), with a maximum displacement of 4 Å for the residues Asp108, Val110, Asn111, and Asp112 (10, DPVN111) (Fig. 6C). This conformational change allowed the TCR to dock its CDR3α loop on top of the N-terminal part of the HSPE peptide and also avoid steric clashes with the Arg62 of the α-helix of the HLA-B*0801 (Fig. 6D). Thus, apart from some limited mobility in the CDR3α loop, a relatively rigid lock-and-key interaction underscored the ligation to the HLA-B*0801–HSK epitope.

**Discussion**

Previous studies have established that biased TCR use may reflect the selection of TCRs with optimal structural characteristics that impart optimal specificity for a given pHLA molecule topography (23, 38–43). In the current study, T cells specific for HLA-B*0801–HSK preferentially used a TRBV11 chain. We have previously reported (13) that a clear genetic bias was observed for this bias showing that this arginine formed salt bridge interaction with P8-E of the HSK peptide, supporting the hypothesis that any mutation at this position could pose a viral fitness cost. The rare P7D > A disrupts the interaction of P7-D and Arg111β. Despite these mutations having a drastic impact on T cell recognition, they are rare, and further studies are required to define the extent to which fitness cost constrains the development of escape mutations within HSK and other immunodominant HCV epitopes.

P3K > R is a conservative mutation and, not surprisingly, was cross-recognized by HSK-specific T cells. Moreover, the lysine cluster at residues P3-K to P5-K enables water-mediated interaction with nucleotides of the RNA (45), and that substitution with arginine impairs this interaction to a smaller extent. Salloum et al. (10) have reported that viral replication was affected up to 50% by the P3K > R mutation. In contrast, P3K > R mutation is frequently reported, it is a prototype sequence for HCV-G2, has also been reported in HLA-B*08–negative subjects (10), and hence it is proposed that factors other than HLA-B*0801–mediated immune pressure could select this substitution. The L > I mutation at P9 is also conservative, and the additional methyl group is well accommodated in the F-pocket of the HLA-B*0801 molecule. The common mutations in HSK occur at P4, P8, and P9, with P4K > R being the most common. Both P4-K and P4-R are positively charged; however, the head group of P4-R is larger than P4-K. Even if the P4-K mimicked a peg–nottch interaction with both the CDR3 loops, only two-thirds of its side chain would be surrounded by the TCR, leaving just enough space to accommodate a larger side chain residue such as tyrosine. Interestingly, so far there is only one report of a rare C > Y mutation at P6 in the Los Alamos database (10), suggesting that any mutation at this position could pose a viral fitness cost. The rare P7D > A disrupts the interaction of P7-D and Arg111β. Despite these mutations having a drastic impact on T cell recognition, they are rare, and further studies are required to define the extent to which fitness cost constrains the development of escape mutations within HSK and other immunodominant HCV epitopes.

**FIGURE 5.** DD31 TCR–HSK peptide interactions by the CDR3 loops. (A) CDR3α (yellow) contacted the N-terminal part of the peptide (P1-H, P2-S, and P4-K), and CDR3β (orange) contacted the C-terminal part of the peptide P4-K, P6-C, P7-D, and P8-E. (B) Figure shows the specific interaction of the CDR3α with the peptide through residues Pro109α, Val110α, and Asp112α. (C) Figure shows the specific interaction of the CDR3β loop with the peptide by the non–germline-encoded residues Arg109β, Arg111β, and Gly112β from the TRBD segment. The blue dashed lines represent the hydrophobic interactions and the red dashed lines the hydrogen bonds.
Previous studies on TCR–pHLA cocomplexes suggest that in general, most TCRs usually contact only one or two solvent-exposed residues to achieve specificity (32, 35). For example, despite an array of solvent-exposed residues (P4, P6, P7, or P8) in the EBV epitope HLA-B*0801/FLR, its recognition by LC13 TCR is dependent on P7-Y, which resides within the bulged and exposed region of the peptide (41). Even for bulged epitopes, such as the EBV/HLA-B*3501–EPLPQGQLTAY, in which the 11-aa long peptide contains 7 highly exposed side chains, the interaction with the specific ELS4-TCR at the interface remains within the range (18–34%) normally observed for standard nonamer peptides (43).

In contrast, the DD31 TCR made contacts with all of the solvent-exposed residues for its interaction with the peptide. Four residues (Pro109α, Val110α, Asn111α, Asp112α) from the CDR3α interacted with the side chains of the P1-H, P2-S, and P4-K, thus covering N-terminal segment of the peptide. Pro109α, Asn111α, and Asp112α extensively made van der Waals’ interactions with P4-K. Arg109β, Gly110β, and Arg111β from CDR3β interacted with the C-terminal segment of the peptide from residues P4-K to P8-E through salt bridge and van der Waals’ interactions. Hence, the TCR spans the entire peptide like a wide blanket. However, these interactions are not completely perfect, as certain escape variants can still occur at the TCR contact sites, some of them completely cross-recognized like P8E_D, others less so such as P4K_R that resulted in suboptimal T cell function, whereas some transient mutations such as P6C_Y that totally abrogated CTL recognition are rarely observed. Overall, the structure clarifies the mechanism by which the narrowly biased TCR repertoire accommodates common variations in HSK while maintaining exquisite specificity.

We conclude that the extensive TCR contacts across the immunodominant HSK Ag overcomes the constraints of the narrow TCR repertoire deployed against this viral determinant, facilitating the recognition of intergenotypic and most of the common variants. Indeed, recent studies into hypervariable animal viruses have demonstrated that TCR repertoire diversity does not always correlate with functional advantage against viral mutants (46, 47) with the presence of preset public TCR in a cellular response even bestowing superior biological outcome (48, 49). Our observations

**FIGURE 6.** Structural changes upon ligation. The four panels show the differences between the liganded structures represented in pink, the unliganded state with HLA-B*0801–HSK structures represented in white, and that of DD31 TCR represented in green. (A) Superposition of the HLA-B*0801 Ag-binding cleft, and the structures are represented in cartoon form with the HSK peptide represented in stick form. The residues from the HLA-B*0801 that changed conformation due to the DD31 TCR binding are represented in stick form. (B) Superposition of the variable domains of the DD31 TCR structures in liganded (pink cartoon) and unliganded (green cartoon) states. (C) Superposition of the CDR3α of the DD31 TCR between the liganded and unliganded states showing the shift of the loop upon ligation with the HLA-B*0801–HSK complex. (D) Same as (C) in a different orientation showing the peptide and HLA-B*0801 (white cartoon) with Arg62 of HLA-B*0801 in stick form.
make it unlikely that CTL escape through rare mutants of HSK contributes significantly to HCV persistence. Similar studies on other highly mutated immunodominant HCV epitopes are required to determine whether CTL escape plays a major role in chronic HCV infection or other factors such as T cell exhaustion or anergy are more often responsible for the lack of T cell reactivity observed in chronically infected subjects. It is also proposed that different outcomes of HCV infection may not solely depend on viral escape mutations per se, but on the potency and breadth of the T cell response during acute infection, which may indirectly drive CTL escape mutations that become the result, rather than the cause, of viral persistence (50). Furthermore, despite the reported hyper-variability in some of the immunodominant T cell epitopes, our work supports the notion of developing cross-strain vaccines against HCV.

Acknowledgments

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Disclosures

The authors have no financial interests of interest.

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