Structural basis for clonal diversity of the human T-cell response to a dominant influenza virus epitope

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Xinbo Yang‡§, Guobing Chen*, Nan-ping Weng†, and Roy A. Mariuzza‡§

From the ‡University of Maryland Institute for Bioscience and Biotechnology Research, W. M. Keck Laboratory for Structural Biology, Rockville, Maryland 20850, the §Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742, and the †Laboratory of Molecular Biology and Immunology, National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224

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Influenza A virus (IAV) causes an acute infection in humans that is normally eliminated by CD8+ cytotoxic T lymphocytes. Individuals expressing the MHC class I molecule HLA-A2 produce cytotoxic T lymphocytes bearing T-cell receptors (TCRs) that recognize the immunodominant IAV epitope GILGFVFTL (GIL). Most GIL-specific TCRs utilize α/β chain pairs encoded by the TRAV27/TRBV19 gene combination to recognize this relatively featureless peptide epitope (canonical TCRs). However, ~40% of GIL-specific TCRs express a wide variety of other TRAV/TRBV combinations (non-canonical TCRs). To investigate the structural underpinnings of this remarkable diversity, we determined the crystal structure of a non-canonical GIL-specific TCR (F50) expressing the TRAV13-1/TRBV27 gene combination bound to GIL–HLA-A2 at 1.7 Å resolution. Comparison of the F50–GIL–HLA-A2 complex with the previously published canonical TCR (JM22) revealed that F50 and JM22 engage GIL–HLA-A2 in markedly different orientations. These orientations are distinguished by crossing angles of TCR to peptide–MHC of 29° for F50 versus 69° for JM22 and by a focus by F50 on the C terminus rather than the center of the MHC α1 helix for JM22. In addition, F50, unlike JM22, uses a tryptophan instead of an arginine to fill a critical notch between GIL and the HLA-A2 α2 helix. The F50–GIL–HLA-A2 complex shows that there are multiple structurally distinct solutions to recognizing an identical peptide–MHC ligand with sufficient affinity to elicit a broad anti-IAV response that protects against viral escape and T-cell clonal loss.

CD8+ T cells play a critical role in the immune response to viruses by recognizing and eliminating infected cells (1). Recognition is mediated by αβ T-cell receptors (TCRs),2 which bind viral peptides presented by major histocompatibility complex (MHC) class I molecules on infected cells. TCRs engage peptide–MHC (pMHC) through their six complementarity-determining region (CDR) loops, three from the variable α (Vα) domain, and three from Vβ. The first and second CDRs (CDR1 and CDR2) are encoded within the Vα and Vβ gene segments. CDR3 is formed by DNA recombination involving juxtaposition of Vα and Jα segments for the α chain genes and of Vβ, D, and Jβ segments for the β chain genes. Estimates of TCR diversity in humans have placed the number of unique structures in the range of 105–108 (2–5).

The human CD8+ T-cell response to influenza A virus (IAV) has been studied extensively (6–11). The dominant epitope in individuals expressing the MHC class I molecule HLA-A*0201 (HLA-A2) corresponds to residues 58–66 of matrix protein M1 (GILGFVFTL; referred to as GIL) (12). Initial studies of GIL-specific CD8+ T-cell responses in HLA-A2+ subjects indicated that the Vβ repertoire is highly biased toward usage of the TRBV19 gene (up to 98%) with a highly conserved CDR3β motif, 97XRSX100 (6–9). The Vα gene segment, although not as strongly selected as Vβ, showed a strong preference for TRAV27 (up to 80%) (6, 7, 13). This canonical TRAV27/TRBV19 gene combination is observed in multiple HLA-A2-matched but otherwise genetically unrelated individuals. More recently, however, high-throughput sequencing and single-cell TCR analysis have revealed substantially greater repertoire diversity than previously realized, with only ~60% of GIL-specific TCRs expressing the TRBV19 gene and ~20% expressing TRAV27 (14). These non-canonical TCRs utilize a wide variety of TRAV and TRBV genes, including TRAV13-1, TRAV17, TRAV29, TRAV38-2, TRBV14, TRBV24-1, TRBV27, and TRBV29-1, among others. Indeed, 2,406 unique TCRα and 2,437 TCRβ sequences have been identified for TCRs recognizing the GIL peptide presented by HLA-A2 (14). These sequences include 461 Vα4/5-Jα and 359 Vβ1-Jβ combinations, as well as dozens of distinct CDR3α and CDR3β consensus motifs. Broad TCR repertoire diversity has also been documented for other defined viral antigens, including ones from cytomegalovirus, Epstein–Barr virus, and HIV (14–17). Such diversity ensures robust T-cell responses to single viral epitopes that would not be possible if a single epitope could only elicit a few
TCR clonotypes (18). Moreover, TCR diversity provides protection against viral escape (19–21).

In most pMHC structures, one or more residues in the central portion of the antigenic peptide (P4–P6) feature solvent-exposed side chains that facilitate TCR binding (22). However, the GIL peptide is unusual in that only the side chain of P8 Thr, at the C-terminal end of the peptide, is substantially exposed to solvent, making GIL a challenging target for TCR recognition (23). The crystal structure of a canonical GIL-specific TRAV27/TRBV19 TCR (JM22) bound to GIL–HLA-A2 revealed what appears to be the most efficient solution to recognizing the featureless GIL peptide with sufficient affinity to permit selection of engaging T cells. In the JM22–GIL–HLA-A2 complex, the side chain of the conserved Arg98 residue of the CDR3 consensus motif fills a notch between the peptide and the HLA-A2 α2 helix (23). This structural solution is also adopted by other GIL-specific TCRs expressing TRBV19 and the CDR3β motif, even when TRBV19 is paired with chains different from TRAV27 (14, 24). However, ~40% of GIL-specific TCRs do not use the canonical TRBV19 gene, and not all TRBV19-expressing TCRs contain the CDR3β motif (14, 24). Hence, multiple solutions exist for binding the featureless GIL peptide. Here we investigated the structural basis for the surprising diversity of the TCR response to this dominant IAV epitope by determining the structure of a GIL-specific TCR (F50) expressing a completely different, non-canonical TRAV/TRBV gene combination (TRAV13-1/TRBV27) in complex with GIL–HLA-A2.

**Results**

**Interaction of TCR F50 with GIL–HLA-A2**

The IAV GIL-specific TCR F50 was isolated from CD8+ T cells from the peripheral blood of an HLA-A2+ healthy male donor following in vitro stimulation with the GIL peptide as described under “Experimental procedures.” F50 utilizes gene segments TRAV13-1 and TRAJ54 for the chain, and TRBV27 and TRBJ1-1 for the chain, whereas JM22 utilizes TRAV27 and TRAJ37 for the chain and TRBV19 and TRBJ7–2 for the β chain. We used surface plasmon resonance (SPR) to measure the affinity of TCR F50 for HLA-A2 loaded with the GIL peptide (Fig. 1A). To characterize the interaction of F50 with GIL–HLA-A2, we expressed these proteins by in vitro folding from inclusion bodies produced in Escherichia coli. Biotinylated GIL–HLA-A2 was directionally coupled to a streptavidin-coated biosensor surface, and different concentrations of F50 were sequentially flowed over the immobilized pMHC ligand. A dissociation constant ($K_D$) of 76 ± 4 μM was obtained by fitting equilibrium data to a 1:1 binding model (Fig. 1B). This affinity is 25-fold weaker than that of JM22 for GIL–HLA-A2 ($K_D = 3.2$ μM) (25), with the caveat that we did not independently measure the affinity of JM22 in our experimental system. It is, however, well within the range of 0.5–500 μM for natural TCR-pMHC interactions (26). Moreover, it is consistent with the much lower representation among GIL-specific TCRs of the TRBV27 β chain expressed by F50 than the dominant TRBV19 β chain expressed by JM22 (14, 24), in agreement with the concept of affinity-driven clonal expansion of T cell repertoires.

![Figure 1. Surface plasmon resonance analysis of the binding of TCR F50 to GIL–HLA-A2.](image-url)
Overview of the F50–GIL–HLA-A2 complex

To understand how TCR F50 recognizes GIL–HLA-A2 and to compare its recognition mode with that of JM22, we determined the structure of the F50–GIL–HLA-A2 complex to 1.7 Å resolution (Table 1 and Fig. 2a). The interface between F50 and GIL–HLA-A2 was in unambiguous electron density for the single complex molecule in the asymmetric unit of the crystal (Fig. 2b). Of note, the resolution of the F50–GIL–HLA-A2 complex is one of the highest reported for any TCR-pMHC class I or II complex (21). Indeed, the 1.7 Å resolution of the F50–GIL–HLA-A2 complex is second only to that of the JM22–GIL–HLA-A2 complex itself at 1.4 Å (23). The comparably high resolutions of these two structures justify detailed comparisons between them.

F50 docks symmetrically over GIL–HLA-A2 in a diagonal orientation, with a crossing angle of TCR to pMHC (27) of 29° compared with 69° for JM22 (Fig. 2c). Upon binding GIL–HLA-A2, F50 buries 73% (215 Å²) of the peptide solvent-accessible surface. This percentage is significantly less than the 85% (258 Å²) of peptide surface buried by JM22 (23), which may contribute to the lower affinity of F50 compared with JM22. As shown by the footprint of F50 on the pMHC surface (Fig. 2d), F50 establishes contacts with the N-terminal half of the GIL peptide.
mainly via the CDR1α and CDR3α loops, whereas the CDR1β and CDR3β loops mostly contact the C-terminal half. F50 utilizes all six CDR loops to interact with the MHC molecule, with CDR1α and CDR2α positioned over the HLA-A2 α2 helix, and CDR1β and CDR2β positioned over the HLA-A2 α1 helix (Fig. 2c).

**Interaction of TCR F50 with HLA-A2**

The F50–GIL–HLA-A2 complex buries a total solvent-accessible surface of 1760 Å², comparable with that in other TCR-pMHC complexes (22). The Vα and Vβ domains bury 46% (333 Å²) and 54% (391 Å²) of HLA-A2 surface area, respectively. This roughly equal contribution of Vα and Vβ is typical of TCR-pMHC complexes (22). In sharp contrast to the F50–GIL–HLA-A2 complex, MHC recognition in the JM22–GIL–HLA-A2 complex is dominated by Vβ, which accounts for 67% of the buried surface on HLA-A2 (23). Thus, F50 and JM22 use different strategies to engage an identical pMHC ligand.

Table 2

| Hydrogen bonds | van der Waals contacts | Hydrogen bonds | van der Waals contacts |
|----------------|------------------------|----------------|------------------------|
| **HLA-A2**    | F50                    | JM22           | JM22                   |
| R65H          |                         | N98α           | D56β                   |
| K66H          |                         | G99β           | D56β                   |
| K68H          |                         | M50β           | I53β                   |
| A69H          |                         | G99β           | I53β                   |
| Q72H          |                         |                 |                        |
| T73H          |                         |                 |                        |
| R75H          |                         |                 |                        |
| V76H          |                         |                 |                        |
| T80H          | A80H (O°) N51β (N°)    | M50β           | I53β                   |
| T84H          | Y84H(OH) E30β (O°)     | E30β           |                       |
| A146H         |                         | W99β           | V51α, R98β, Y101β      |
| A150H         | R52α                   | A150H(O) R98ββ (N°) | R98β, Y101β |
| H151H         |                         | I53β           |                        |
| V152H         | E154H (O°) N54α (N°)   | W99β           | V51α                   |
| E154H         |                         | N54α           | R98β                   |
| Q155H         | Q155H (N°) N32α (N°)   |                 | S31α                   |
| Q155H         | Q155H (O°) S33α (O°)   |                 |                        |
| Hydrogen bonds | van der Waals contacts | Hydrogen bonds | van der Waals contacts |
| **HLA-A2**    | F50                    | JM22           | JM22                   |
| R65H          |                         | N98α           | D56β                   |
| K66H          |                         | G99β           | D56β                   |
| K68H          |                         | M50β           | I53β                   |
| A69H          |                         | G99β           | I53β                   |
| Q72H          |                         |                 |                        |
| T73H          |                         |                 |                        |
| R75H          |                         |                 |                        |
| V76H          |                         |                 |                        |
| T80H          | A80H (O°) N51β (N°)    | M50β           | I53β                   |
| T84H          | Y84H(OH) E30β (O°)     | E30β           |                       |
| A146H         |                         | W99β           | V51α, R98β, Y101β      |
| A150H         | R52α                   | A150H(O) R98βγ (N°) | R98β, Y101β |
| H151H         |                         | I53β           |                        |
| V152H         | E154H (O°) N54α (N°)   | W99β           | V51α                   |
| E154H         |                         | N54α           | R98β                   |
| Q155H         | Q155H (N°) N32α (N°)   |                 | S31α                   |
| Q155H         | Q155H (O°) S33α (O°)   |                 |                        |

Asn51 makes two side-chain–side-chain hydrogen bonds linking F50 to residues Glu154H and Gln155H of the HLA-A2 α2 helix (Table 2 and Fig. 3a). These hydrogen bonds are reinforced by 16 germline-encoded van der Waals contacts with CDR1α and CDR2α that further anchor Vα to helix α2. Because of the acute crossing angle of F50 to GIL–HLA-A2 (29° compared with 69° for JM22), CDR3α of F50 contacts the HLA-A2 α1 helix instead of the α2 helix contacted by CDR3α of JM22 (Table 2) (23). Indeed, interactions between CDR3α and the MHC α1 helix are characteristic of TCRs docking on pMHC ligands with acute crossing angles (22).

In addition to CDR3α, F50 engages the HLA-A2 α1 helix through CDR1β, CDR2β, and CDR3β (Table 2). Notably, these interactions focus on the C terminus of the α1 helix, as illustrated by the positions of CDR1β and CDR2β on the pMHC surface (Fig. 2c and d). This C-terminal site is rarely targeted by other TCRs, including JM22 (Fig. 2, c and e), which typically dock more toward the center of the MHC α1 helix (22). In particular, F50 residues βGlu30 and βAsn51 make two side-chain–side-chain hydrogen bonds with C-terminal HLA-A2 α1 residues Tyr84H and Thr80H, respectively (Fig. 3b). Neither of these HLA-A2 residues contacts JM22, which instead targets the central portion of helix α1 via its CDR2β (Fig. 2c and Table 2). Interestingly, the HLA-A2–restricted HIV-specific TCR T36-5 (28), which utilizes the same Vβ (TRBV27) as F50, nevertheless engages the MHC α1 helix via a different set of germline-encoded interactions. For example, whereas βAsn51...
Peptide specificity is conferred mainly by shape complementarity, because the F50-GIL interface features only two hydrogen bonds with the main chain of P6 Val, but using different CDR residues: JM22 Trp99 occupies a notch between the GIL peptide (magenta) and the HLA-A2 α2 helix (orange). Superposed onto CDR3 of F50 is CDR3 of JM22 (pink). In the JM22–GIL–HLA-A2 complex (23), the pocket between GIL and the HLA-A2 α2 helix is filled by the side chain of βArg98, which makes hydrogen bonds with HLA-A2 Ala150H and Gln155H (beige).

Table 3

| Hydrogen bonds | van der Waals contacts | Hydrogen bonds | van der Waals contacts |
|----------------|------------------------|----------------|------------------------|
| GIL F50        | F50                    | JM22           | Q52β (Nε2)             |
|                |                        |                | JM22                   |
|                | G4P                    |                | S95a                   |
|                |                        |                | Q96a                   |
|                |                        |                | Q52β                   |
|                | F5P                    |                | S95a                   |
|                |                        |                | G97e                   |
|                |                        |                | Q52β                   |
|                |                        |                | R98β                   |
|                | V6P                    |                | S100β                  |
|                | V6P(N) Q101α(Oε2)      |                | V6P(N) Q52β(Oε2)       |
|                | V6P(O) W99ζ(Nε2)       |                | V6P(O) S99θ(Oε2)       |
|                | W99β                   |                | S99β                   |
|                |                        |                | T8P (O2) D32β (Oε2)    |
|                |                        |                | D32β                   |
|                |                        |                | L3β                    |

Figure 3. Interactions of TCR F50 with HLA-A2 and the GIL peptide. a, interactions between CDR2α (cyan) of F50 and the HLA-A2 α2 helix (orange). The side chains of contacting residues are drawn in stick representation with carbon atoms in cyan (CDR2α) or orange (HLA-A2), nitrogen atoms in blue, and oxygen atoms in red. Hydrogen bonds are indicated by yellow dashed lines. b, interactions of CDR1β and CDR2β (green) with the HLA-A2 α1 helix (orange). c, interactions of CDR1α and CDR3α with the GIL peptide. A bridging water molecule is depicted as a red sphere. d, interactions of CDR1β and CDR3β with the GIL peptide, including bridging water molecules (red spheres). e, interactions between CDR3β (green) of F50 and GIL–HLA-A2. The side chain of βTrp99 occupies a notch between the GIL peptide (magenta) and the HLA-A2 α2 helix (orange). Superposed onto CDR3β of F50 is CDR3β of JM22 (pink). In the JM22–GIL–HLA-A2 complex (23), the pocket between GIL and the HLA-A2 α2 helix is filled by the side chain of βArg98, which makes hydrogen bonds with HLA-A2 Ala150H and Gln155H (beige).
binding of F50 to GIL–HLA-A2, as determined by SPR (not shown), demonstrating that these residues are not functionally interchangeable.

The side chain of HLA-A2 Gln155H adopts different rotamer conformations in the F50–GIL–HLA-A2 and JM22–GIL–HLA-A2 structures (Fig. 3e). In the JM22–GIL–HLA-A2 complex, the Gln155H side chain shifts by 3.6 Å compared with its position in unbound GIL–HLA-A2 to open the notch between the peptide and the /H2 helix, into which /H9251Arg98 docks (23). In the F50–GIL–HLA-A2 complex, the gatekeeper Gln155H side chain shifts an additional 1.8 Å from its unbound position to further open this notch to avoid steric clashes with the bulky /H9252Trp99 side chain and optimize shape complementarity and hydrogen bonding with pMHC (Fig. 3e).

Comparison with GIL-specific TCRs LS01 and LS10

A recent crystallographic study of two GIL-specific TCRs (LS01 and LS10) expressing the canonical TRBV19 gene, but different TRAV genes showed that both TCRs maintain the same overall docking orientation on pMHC as JM22, because of the preservation of specific contacts between the conserved CDR1β and CDR2β loops and HLA-A2 (Fig. 4A) (24). This orientation is distinct from that found in the non-canonical F50–GIL–HLA-A2 complex, which features a much more acute crossing angle, as described above.

The CDR3β motifs of LS01 (97XFX99) and LS10 (97XGY100) differ from the dominant 97XRSX100 motif of other GIL-specific TCRs expressing TRBV19 (24). Consequently, LS01, LS10, JM22, and F50 employ different strategies to recognize the critical notch between GIL and the MHC α2 helix near P5 Phe (Fig. 4B). Whereas JM22 and F50 use Arg98 and Trp99 of CDR3β, respectively, to fill this common pocket, LS01 uses Phe98. By contrast, binding of LS10 induces a conformational change in GIL, whereby the P5 Phe side chain moves into the notch. This shift creates a new notch that is occupied by /H9251Ala98 and /H9251Gly99 and covered by /H9251Tyr103.

Water interactions at TCR-pMHC interfaces

Bound water molecules have been localized in the interfaces of many antigen–antibody (and other protein–protein) complexes, where they act as molecular adaptors to bridge the protein partners and improve the fit between them (31). However, the contribution of bound waters to mediating TCR-pMHC interactions has not been examined in detail, in large measure because relatively few TCR-pMHC crystal structures have been determined at sufficiently high resolution (≤2.5 Å) to permit the identification of ordered waters with a reasonable degree of accuracy.

The high resolution of the F50–GIL–HLA-A2 complex allowed the inclusion of many bound water molecules, includ-
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Water bridges between TCR and GIL peptide in the F50–GIL–HLA-A2 and JM22–GIL–HLA-A2 complexes

| GIL | Water       | F50 factor | JM22 factor |
|-----|-------------|------------|-------------|
| G4(O) | S58 | A31αααααααα(α) | 22.3 | 23.2 |
| T8(N) | S122 | G97ββββββββ(β) | 23.9 | 21.6 |
| T8(O) | S450 | L95ββββββββ(β) | 30.1 | 23.2 |
| T8(O) | S812 | G97ββββββββ(β) | 35.9 | 28.6 |
|       |       |            |            |

Discussion

The application of high-throughput sequencing and single-cell TCR analysis to interrogate CD8⁺ T cell repertoires to single viral epitopes has revealed far greater TCR sequence diversity than previously appreciated for immune responses to IAV, cytomegalovirus, HIV, and other viruses (14, 15, 17, 24). Given this diversity, understanding the structural basis for recognition of an identical pMHC complex by thousands of different TCRs represents a considerable challenge.

To date, ~25 structures of TCRs with different Vα and/or Vβ gene usage bound to an identical (or nearly identical) pMHC ligand have been reported, involving both MHC class I and class II molecules (14, 22, 24, 34). These complexes may be divided into three categories: 1) those in which the TCRs use the same Vα gene segment but different Vβs (30), 2) those in which TCRs use the same Vβ but different Vαs (14, 24, 35–38), and 3) those in which the TCRs use different Vαs and Vβs (34, 39). The third category, which includes TCR F50, has considerably fewer examples than the first two categories, where the TCRs use the same Vα or Vβ region to engage the same pMHC. Nevertheless, some general conclusions may be drawn regarding Vα/Vβ gene usage and TCR docking orientation. Thus, TCRs that use the same Vβ but different Vαs typically bind pMHC in the same overall orientation because of the preservation of most (but not necessarily all) germline-encoded interactions between the conserved CDR1β and CDR2β loops and the MHC α-helices. That is, use of a different Vα has not been observed to reposition Vβ on pMHC in an appreciably different way, although small adjustments do occur. Conversely, TCRs that use the same Vα but different Vβs engage pMHC in very similar orientations because most germline-encoded interac-

Table 4

Water bridges between TCR and GIL peptide in the F50–GIL–HLA-A2 and JM22–GIL–HLA-A2 complexes

| GIL | Water       | F50 factor | JM22 factor |
|-----|-------------|------------|-------------|
| G4(O) | S58 | A31αααααααα(α) | 22.3 | 17.2 |
| T8(N) | S122 | G97ββββββββ(β) | 23.9 | 18.4 |
| T8(O) | S450 | L95ββββββββ(β) | 30.1 | 20.5 |
| T8(O) | S812 | G97ββββββββ(β) | 35.9 | 19.0 |
|       |       |            |            |

Table 5

Shape complementarity (Sα) of TCR–pMHC class I complexes in the presence and absence of interfacial water molecules

| TCR–pMHC class I complex | PDB | Sα with waters | Sα without waters | ΔSα | Number of interfacial waters | Reference |
|--------------------------|-----|----------------|-------------------|-----|-----------------------------|----------|
| F50–GIL–HLA-A2          | 5TEZ | 0.65           | 0.65              | 0.00 | 4 (0 MHC; 4 peptide)        | Ref. 14  |
| JM22–GIL–HLA-A2         | 1GGA | 0.77           | 0.64              | 0.13 | 8 (5 MHC; 5 peptide)        | Ref. 23  |
| F6–GIL–HLA-A2           | 5EUO | 0.61           | 0.54              | 0.07 | 8 (4 MHC; 4 peptide)        | Ref. 14  |
| LS01–GIL–HLA-A2         | 5I5Z | 0.74           | 0.71              | 0.03 | 11 (6 MHC; 5 peptide)       | Ref. 24  |
| LS10–GIL–HLA-A2         | 5JHD | 0.68           | 0.66              | 0.02 | 2 (2 MHC; 2 peptide)        | Ref. 24  |
| B7–Tax–HLA-A2           | 1BD2 | 0.64           | 0.64              | 0.00 | 3 (3 MHC; 3 peptide)        | Ref. 35  |
| AHIII–p1049–HLA-A2      | 1LP9 | 0.74           | 0.71              | 0.03 | 8 (6 MHC; 3 peptide)        | Ref. 49  |
| LC13–EBV–HLA-A8         | 1MIS | 0.61           | 0.61              | 0.06 | 6 (4 MHC; 3 peptide)        | Ref. 50  |
| 1G4–ESO 9V–HLA-A2       | 2BNQ | 0.78           | 0.75              | 0.03 | 9 (5 MHC; 4 peptide)        | Ref. 51  |
| 1G4–ESO 9C–HLA-A2       | 2BNR | 0.75           | 0.75              | 0.00 | 7 (5 MHC; 2 peptide)        | Ref. 51  |
| 2C–Q9–H12Iα             | 2DL2 | 0.67           | 0.64              | 0.03 | 2 (1 MHC; 1 peptide)        | Ref. 52  |
| AGA1–KF11–HLA-B57       | 2YPL | 0.77           | 0.81              | 0.04 | 3 (3 MHC; 0 peptide)        | Ref. 48  |
| H27–14–Nef–HLA-A2       | 3VXR | 0.76           | 0.75              | 0.01 | 7 (5 MHC; 3 peptide)        | Ref. 28  |
| C25–NLV–HLA-A2          | 5D2N | 0.70           | 0.70              | 0.00 | 8 (6 MHC; 2 peptide)        | Ref. 34  |

a Only TCR–pMHC class I structures of at 2.5 Å resolution or better were considered in this analysis.

b The first value is the total number of water molecules in the corresponding TCR–pMHC interface. In parentheses are the numbers of waters bridging TCR and MHC or TCR and peptide in each complex. These numbers may in some cases add up to more than the total number of interfacial waters because a single water molecule can sometimes bridge TCR to both MHC and peptide.
tions between CDR1\(\alpha\) and CDR2\(\alpha\) and the MHC ligand are maintained, irrespective of the V\(\beta\) partner.

By contrast, TCRs using different V\(\alpha\) and V\(\beta\) regions can employ very different strategies to bind an identical pMHC, as seen here and in previous studies (34, 39). For example, a comparison of two TCRs expressing unrelated V\(\alpha\)/V\(\beta\) gene combinations in complex with a bulged peptide from Epstein–Barr virus presented by HLA-B8 revealed two distinct binding modes: one in which the TCR straddles the bulged peptide but makes few contacts with MHC and one in which the TCR is positioned toward the N-terminal end of the peptide binding groove of HLA-B8, thereby largely bypassing the bulged peptide (39). In another case, human cytomegalovirus-specific TCRs C7 (TRAV24/TRBV7-2) and C25 (TRAV26-2/TRBV7-6) were found to dock over MHC with crossing angles of 29° and 61°, respectively, with C7-HLA-A2 interactions dominated by V\(\alpha\) and C25-HLA-A2 interactions dominated by V\(\beta\) (34). Here we have shown that F50 (TRAV13-1/TRBV27) engages GIL–HLA-A2 in a decidedly different orientation than do canonical TCRs JM22 (TRAV27/TRBV19), F6 (TRAV27/TRBV19), LS01 (TRAV24/TRBV19), and LS10 (TRAV38–2/TRBV19) (14, 23, 24). This binding mode is characterized by a more acute crossing angle and focus on the C terminus rather than the center of the MHC a1 helix. In addition, the critical notch between the peptide and MHC a2 helix is occupied by a tryptophan rather than arginine residue as in most canonical GIL-specific TCRs. These TCR-pMHC structures, together with the remarkable diversity of TCRs expressing non-canonical TRAV/TRBV combinations in GIL-specific repertoires (14), demonstrate that there are many ways for TCRs to bind even a featureless peptide such as GIL with sufficient affinity to elicit broad anti-viral responses that provide protection against T-cell clonal loss and viral escape.

**Experimental procedures**

**Isolation of GIL-specific TCR F50**

To obtain TCR F50, GIL-specific CD8\(^+\) T cells were isolated from the peripheral blood of a HLA-A2\(^+\) healthy male donor (71 years old) after 14 days of in vitro stimulation with GIL–HLA-A2 using an artificial antigen presenting system as previously described (14). Briefly, peripheral blood mononuclear cells were separated from leukopheresis cells by Ficoll gradient centrifugation. CD8\(^+\) T cells were isolated from peripheral blood mononuclear cells by immunomagnetic enrichment (34, 40). GIL-specific CD8\(^+\) T cells were expanded in an artificial antigen-presenting system using GIL peptide (GILGFVFTL) (BioMer Technology) (41). The expanded cells were stained with APC- and FITC-conjugated GIL dextramer (Immudex). FITC and APC double-positive cells were sorted by flow cytometry. Single-cell analysis was used to identify the paired \(\alpha\) and \(\beta\) chains of GIL-specific TCRs, including F50 (14, 34).

**Expression and purification of TCR F50**

Soluble TCR F50 for affinity measurements and structure determination was prepared by in vitro folding from inclusion bodies produced in *E. coli*. The V\(\alpha\) and V\(\beta\) regions of F50 (residues 1–209 and 1–244, respectively) were cloned into the expression vector pET26b (Novagen) containing Ca and CB regions. An interchain disulfide (CaCys\(^{163–171}\)) was engineered to increase yield of the TCR a\(\beta\) heterodimer (42). The F50 \(\alpha\) and \(\beta\) chains were expressed separately as inclusion bodies in BL21(DE3) *E. coli* cells (Agilent Technologies). Bacteria were grown at 37 °C in LB medium to \(A_{\text{opt}}^600 = 0.6–0.8\) and induced with 1 mM isopropyl-\(\beta\)-D-thiogalactoside. After incubation for 3 h, the bacteria were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 2 mM EDTA; cells were disrupted by sonication. Inclusion bodies were washed extensively with 50 mM Tris-HCl (pH 8.0) and 5% (v/v) Triton X-100 and then dissolved overnight in 8 M urea, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM DTT. For in vitro folding, the TCR \(\alpha\) and \(\beta\) chains were mixed in a 1:2:1 molar ratio for 30 min prior to dilution into ice-cold folding buffer containing 5 M urea, 0.4 M L-arginine HCl, 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 3.7 mM cystamine, and 6.6 mM cysteamine to a final protein concentration of 80 mg/liter. The folding mixture was dialyzed against 10 mM Tris-HCl (pH 8.0) for 72 h at 4 °C. The mixture was concentrated 20-fold and dialyzed against 25 mM Tris-HCl (pH 8.0). Disulfide-linked TCR F50 heterodimers were purified using sequential Superdex 200 GL and Mono Q columns (GE Healthcare).

**Production of GIL–HLA-A2**

Soluble HLA-A2 loaded with GIL peptide (GILGFVFTL) (GenScript) was prepared by in vitro folding. The HLA-A\(^*\)0201 heavy chain (residues 1–275) and \(\beta\)_2-microglobulin (residues 1–99) were produced separately as inclusion bodies in BL21(DE3) *E. coli* cells transformed by pET26b containing the corresponding genes. Inclusion bodies were dissolved in 8 M urea, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 10 mM DTT. For in vitro folding, the HLA-A\(^*\)0201 heavy chain (30 mg), \(\beta\)_2m (30 mg), and GIL peptide (20 mg) were mixed and added drop-wise to 1 liter of a folding solution containing 5 M urea, 0.4 M L-arginine HCl, 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 3.7 mM cystamine, and 6.6 mM cysteamine. The folding solution was dialyzed against distilled water for 24 h and then against 10 mM Tris-HCl (pH 8.0) for 48 h at 4 °C. After 20-fold concentration and further dialysis against 20 mM Tris-HCl (pH 8.0) and 20 mM NaCl, correctly folded GIL–HLA-A2 was purified using a Mono Q column.

**Crystallization and data collection**

TCR F50 was mixed with GIL–HLA-A2 in a 1:1 molar ratio and concentrated to 10 mg/ml. Crystals of the F50–GIL–HLA-A2 complex grew in 10–15% (w/v) polyethylene glycol 3350, 0.1 M imidazole (pH 8.0), and 0.2 M sodium malonate. For data collection, crystals were transferred to a cryoprotectant solution of mother liquor containing 25% (v/v) glycerol prior to flash cooling in a nitrogen stream. X-ray diffraction data for the F50–GIL–HLA-A2 complex were collected at Beamline 24ID-E of the Advanced Photon Source of the Argonne National Laboratory with an ADSC Q315 CCD detector. Diffraction data were indexed, integrated, and scaled with the program HKL2000 (43). The data collection statistics are summarized in Table 1.
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Structure determination and refinement
The structure of the F50–GIL–HLA-A2 complex was solved by molecular replacement with the program Phaser (44). An HIV-specific TCR (Protein Data Bank accession code 3VXU) (28) and GIL–HLA-A2 (Protein Data Bank accession code 1OGA) (23) were used as search models with the CDRs and peptide removed, respectively. One complex molecule was located in the asymmetric unit. Structure refinement was performed using rigid body and simulated annealing via Phenix (45). The model was further refined by manual model building with Coot (46) based on 2\( F_o \) – \( F_c \) and 2\( F_c \) – \( F_o \) maps with the GIL peptide omitted in the initial refinement. The final \( R_{\text{work}} \) and \( R_{\text{free}} \) values for the F50–GIL–HLA-A2 complex are 19.1 and 21.3%, respectively. Refinement statistics are presented in Table 1. Stereochemical parameters were evaluated by PROCHECK (47).

Surface plasmon resonance analysis
The interaction of TCR F50 with GIL–HLA-A2 was assessed by SPR using a BIAcore T100 biosensor at 25 °C. Biotin-tagged GIL–HLA-A2 (NH Tetramer Core Facility) was immobilized on a streptavidin-covered BIAcore SA chip (GE Healthcare) at 1000 resonance units (RU), followed by blocking the remaining streptavidin sites with 20 μM biotin solution. An additional flow cell was injected only with free biotin to serve as a blank control. For analysis of TCR binding, solutions containing different concentrations of F50 were flowed sequentially over the chips immobilized with GIL–HLA-A2 and the blank. Injections of TCR were stopped at 30 s after SPR signals reached a plateau. Equilibrium data were fitted with a 1:1 binding model using BIAevaluation 3.1 software to obtain the \( K_D \).

Protein Data Bank accession code
Coordinates and structure factors for the F50–GIL–HLA-A2 complex have been deposited in the Protein Data Bank under accession code 5TEZ.

Author contributions—X. Y. determined the crystal structure. G. C. isolated TCR genes. X. Y., G. C., N.-P. W., and R. A. M. analyzed the data and wrote the manuscript.

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