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Lack of Heterologous Cross-reactivity toward HLA-A*02:01 Restricted Viral Epitopes Is Underpinned by Distinct $\alpha\beta$ T Cell Receptor Signatures*

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$\alpha\beta$ T cell receptor (TCR) genetic diversity is outnumbered by the quantity of pathogenic epitopes to be recognized. To provide efficient protective anti-viral immunity, a single TCR ideally needs to cross-react with a multitude of pathogenic epitopes. However, the frequency, extent, and mechanisms of TCR cross-reactivity remain unclear, with conflicting results on anti-viral T cell cross-reactivity observed in humans. Namely, both the presence and lack of T cell cross-reactivity have been reported with HLA-A*02:01-restricted epitopes from the Epstein-Barr and influenza viruses (BMLF-1 and M1₅₈, respectively) or with the hepatitis C and influenza viruses (NS3₁₀₇₃ and NA₂₃₁, respectively). Given the high sequence similarity of these paired viral epitopes (56 and 88%, respectively), the ubiquitous nature of the three viruses, and the high frequency of the HLA-A*02:01 allele, we selected these epitopes to establish the extent of T cell cross-reactivity. We combined *ex vivo* and *in vitro* functional assays, single-cell $\alpha\beta$ TCR repertoire sequencing, and structural analysis of these four epitopes in complex with HLA-A*02:01 to determine whether they could lead to heterologous T cell cross-reactivity. Our data show that sequence similarity does not translate to structural mimicry of the paired epitopes in complexes with HLA-A*02:01, resulting in induction of distinct $\alpha\beta$ TCR repertoires. The differences in epitope architecture might be an

obstacle for TCR recognition, explaining the lack of T cell cross-reactivity observed. In conclusion, sequence similarity does not necessarily result in structural mimicry, and despite the need for cross-reactivity, antigen-specific TCR repertoires can remain highly specific.

$\alpha\beta$ T cells recognize peptides (p) bound to the MHC molecule, or human leukocyte antigen (HLA)⁴ in humans, via their $\alpha\beta$ T cell receptor (TCR). Each TCR comprises two chains, α and β , that are composed of variable (V), joining (J), and constant (C) genes for the α -chain and an additional diversity (D) segment for the β -chain. The TCR has three complementarity determining regions (CDRs), which determine pHLA specificity (1). Although CDR1 and CDR2 are germ line encoded and found within the V gene segment, the CDR3 spans the junction between the V(D)J gene segments. During somatic recombination, TCR gene segments are rearranged, and the additional insertion/deletion of nucleotides at each gene junction creates a high level of diversity within the CDR3, termed a hypervariable loop (2). Thus, the CDR3 loops are often used to define epitope-specific $\alpha\beta$ TCR clonotypes.

The clonal selection theory (3) suggests that protective immunity is a “one clonotype, one specificity” system, in which a single TCR recognizes a single pHLA complex. However, this theory was questioned by Mason (4), who hypothesized that 10^{15} T cells would be required to recognize 10^{15} non-self peptides. Because the weight of 10^{15} T cells would be more than 500 kg in one individual, such single specificity of T cells is mathematically impossible. This suggests that each T cell must be able to recognize a vast number of peptides to ensure protective immunity in a process known as cross-reactivity. Thus, T cell cross-reactivity is critical for the efficacy of the immune system,

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The atomic coordinates and structure factors (code 5SWQ) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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⁴ The abbreviations used are: HLA, human leukocyte antigen; TCR, T cell receptor; PDB, Protein Data Bank; EBV, Epstein-Barr virus; HCV, hepatitis C virus; NHMRC, National Health and Medical Research Council; CDR, complementarity determining region; LCMV, lymphocytic choriomeningitis virus; VV, vaccinia virus; Pn, position n; aa, amino acid(s); PBMC, peripheral blood mononuclear cell; ICS, intracellular cytokine staining; PFA, paraformaldehyde; APC, antigen presenting cell; PE, Phycoerythrin.

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

whereby T cells exposed to one epitope can recognize viral variants (5–7) to provide immune protection upon subsequent challenge (5).

T cell cross-reactivity can also occur during viral infections, in which effector or memory CD8⁺ T cells can recognize epitopes from an unrelated virus, a process known as heterologous T cell cross-reactivity. Heterologous T cell cross-reactivity has been extensively characterized in murine models, in which mice primed with one virus can be subsequently challenged with an unrelated virus during the effector phase (8–12). Studies using similar viruses, such as lymphocytic choriomeningitis virus (LCMV) and Pichinde virus (9), or distinct viruses, such as LCMV and vaccinia virus (VV) (10), have shed some light onto the mechanisms of heterologous cross-reactivity. For example, the H-2K^b-restricted NP₂₀₅ epitope from both LCMV (YTVKYPNL) and Pichinde virus (YTVKFPNM) are 75% identical, with differences only at position (P) 5 and P8 (9). Structures of the two H-2K^b-NP₂₀₅ peptides revealed that P5 and P8, which share similar residues, were both buried inside the antigen-binding cleft, thereby enabling a high level of cross-reactivity toward these distinct viral peptides. Conversely, others have shown that heterologous cross-reactivity can occur toward epitopes with lower sequence identity. For example, the LCMV gp₃₄ epitope (AVYNFATM) and the VV A11R₁₉₈ epitope (AIVNYANL) share three identical residues (37% identity). However, previous exposure to LCMV provided a level of protection against VV (13). Despite sharing only three identical residues, the two pMHC complexes adopted similar structures, with the variable amino acids buried within the antigen-binding cleft, thus providing a molecular basis for T cell cross-reactivity (14).

A recent study utilizing a library of peptides containing systematic substitutions showed that TCRs are predominantly cross-reactive because they are tolerant of peptide residue substitutions rather than recognizing multiple distinct peptides (14). Moreover, the “tolerated” substitutions were either not in direct contact with the TCR or were conservative and thus permitted binding by the TCR, in line with the observations from Shen *et al.* (13). This suggests that T cells can cross-recognize distinct pMHC complexes because they are permissive of substitutions and recognize specific pMHC architectures rather than degeneracy in TCR binding. Accordingly, only a handful of studies have shown how a single $\alpha\beta$ TCR could engage highly divergent pMHC complexes (15–18).

Although heterologous immunity in mice is well established, there is controversy regarding heterologous T cell cross-reactivity in humans and its impact on protective anti-viral immunity. For example, studies have described the presence (19, 20) or absence (21) of heterologous CD8⁺ T cell cross-reactivity toward the HLA-A*02:01-restricted influenza-derived (M1₅₈, GILGFVFTL) and Epstein-Barr virus (EBV)-derived (BMLF-1, GLCTLVAML) epitopes. Similarly, other studies have described the presence (22–24) or absence (25, 26) of heterologous CD8⁺ T cell cross-reactivity toward the HLA-A*02:01-restricted influenza-derived (NA₂₃₁, CVNGSCFTV) and hepatitis C virus (HCV)-derived (NS3₁₀₇₃, CINGVCWTV) epitopes. Interestingly, both NS3₁₀₇₃ and NA₂₃₁ epitopes exhibit variations between distinct viral strains, which may explain the vari-

able frequency of cross-reactivity between individuals (22, 26), because a single amino acid substitution can impact the CD8⁺ T cell frequency and cross-reactivity (27).

Based on the previous reports of human heterologous cross-reactivity (20, 23, 24, 28), we focused our current study on the well described and highly prevalent HLA-A*02:01-restricted epitopes, namely M1₅₈/BMLF-1 and NS3₁₀₇₃/NA₂₃₁. These paired peptides share identical (3 and 6, respectively) as well as chemically conserved (2 each) residues, with a sequence homology of 56 and 88%, respectively. Therefore, they provide a good model to determine the molecular basis underlying heterologous T cell cross-reactivity in humans.

In this study, we combined single-cell $\alpha\beta$ TCR repertoire sequencing with biophysical and structural analysis of the four epitopes in complex with the HLA-A*02:01 molecule. We also undertook functional studies (including *ex vivo* and *in vivo* T cell expansion in healthy individuals for both peptide pairs and in HCV-infected individuals for the NS3₁₀₇₃/NA₂₃₁ peptide pair) to determine the frequency and biological relevance of heterologous T cell cross-reactivity toward these HLA-A*02:01-restricted epitopes. Our data show that the sequence similarity between the paired epitopes did not translate to structural mimicry. Namely, the paired epitopes exhibited distinct architectures and mobility within the HLA binding cleft and selected distinct $\alpha\beta$ TCR repertoires. Together, these findings underlie a lack of heterologous cross-reactivity detected directly *ex vivo* by tetramer enrichment and *in vitro* via IFN- γ and tetramer assays. Whereas T cell cross-reactivity is an intrinsic requirement for protective immunity, our data indicate that the sequence similarity of peptides alone is not a reliable indication of CD8⁺ T cell cross-reactivity. In line with previous studies (13, 14), our results highlight that pHLA architecture impacts CD8⁺ T cell cross-reactivity.

Results

Lack of Structural Homology between Paired Epitopes—To understand the mechanisms underpinning human heterologous CD8⁺ T cell cross-reactivity, we selected two pairs of prominent human epitopes containing viral peptides derived from three ubiquitous viruses (influenza, HCV, and EBV) that display high sequence similarity and are restricted by HLA-A*02:01. Conflicting literature reports either the presence (20, 23, 24) or lack (21, 25, 26) of heterologous CD8⁺ T cell cross-reactivity between HLA-A*02:01-restricted epitopes M1₅₈ (GILGFVFTL) and BMLF-1 (GLCTLVAML) epitopes, as well as NA₂₃₁ (CVNGSCFTV) and NS3₁₀₇₃ (CINGVCWTV).

The M1₅₈ and BMLF-1 epitopes share 56% sequence homology, with three identical residues at P1-Gly, P6-Val, and P9-Leu (similarly to the LCMV gp₃₄ and VV A11R₁₉₈ epitopes), as well as chemically conserved P2-I/L and P5-F/L residues. The NA₂₃₁ and NS3₁₀₇₃ peptides share higher sequence homology (88%). To determine whether the sequence variation could impact the stability of each peptide within the HLA-A*02:01 antigen binding cleft, we performed a thermal stability assay. The thermal melting point (T_m) values were very similar for the paired pHLA complexes, with a T_m of $\sim 59^\circ\text{C}$ for M1₅₈ and BMLF-1 and of $\sim 56^\circ\text{C}$ for NA₂₃₁ and NS3₁₀₇₃, suggesting that each pHLA complex displays similar stability.

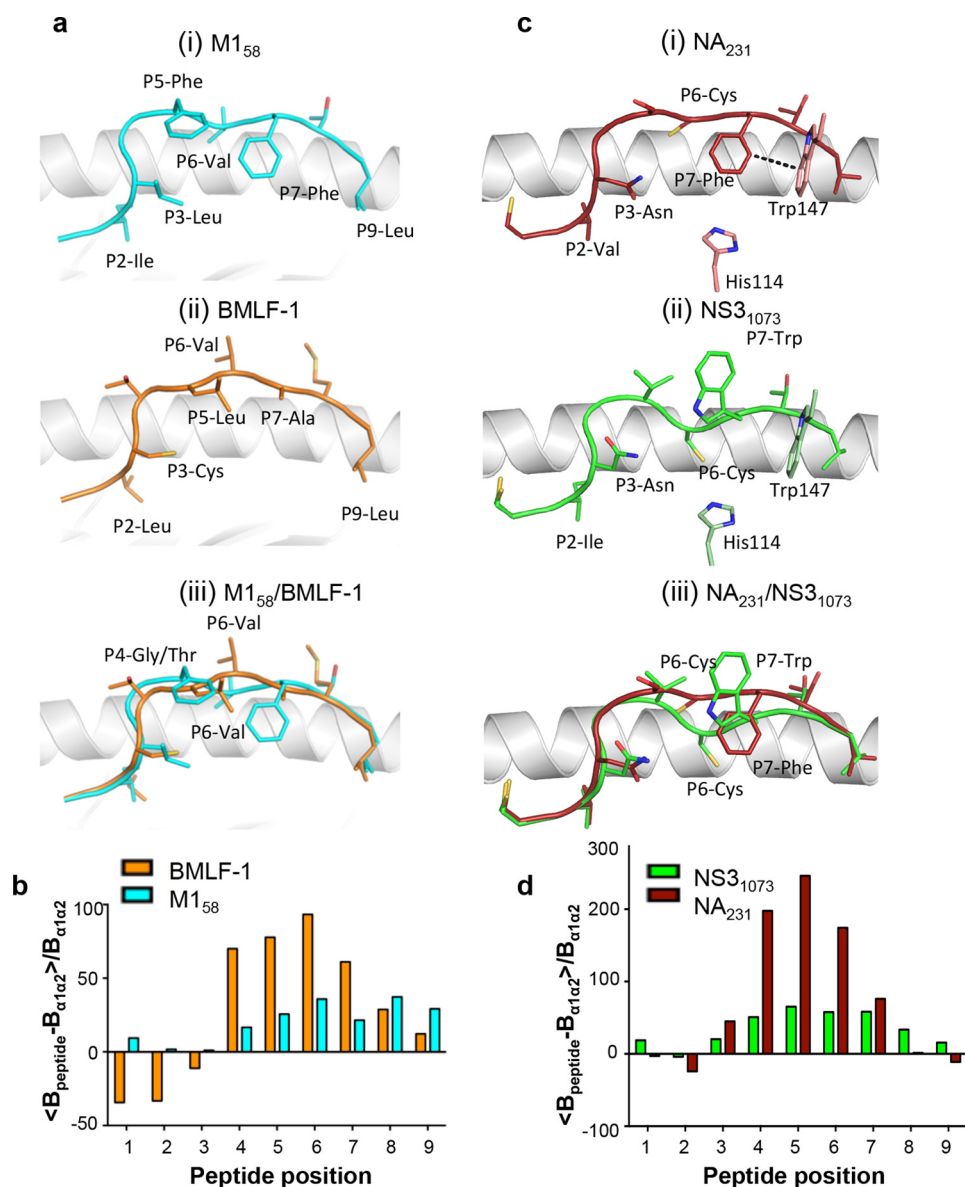


FIGURE 1. Structural differences between peptide pairs bound to the HLA-A*02:01 molecule. The panels represent the HLA-A*02:01 left in *white cartoon*, bound to the four different peptides in *stick format*. *a*, structures of the M1₅₈ peptide in *cyan* (*panel i*), BMLF-1 in *orange* (*panel ii*), and a superposition of the two peptides in complex with HLA-A*02:01 (*panel iii*). *b*, histogram of the relative mean B factors for the M1₅₈ (*cyan*) and BMLF-1 (*orange*) peptides. For each peptide residue, $100 \times ((\langle B_{\text{residue}} \rangle - \langle B_{\text{HLA-A*02:01-}\alpha1\alpha2} \rangle) / \langle B_{\text{HLA-A*02:01-}\alpha1\alpha2} \rangle)$ was calculated, with positive values representing a peptide residue more stable than the overall HLA $\alpha1\alpha2$ domains, and negative values representing a peptide residue more mobile than the overall HLA $\alpha1\alpha2$ domains. *c*, structures of the NA₂₃₁ peptide in *brown* (*panel i*), NS3₁₀₇₃ in *green* (*panel ii*), and a superposition of the two peptides in complex with HLA-A*02:01 (*panel iii*). *d*, histogram of the relative mean B factors for the NA₂₃₁ (*brown*) and NS3₁₀₇₃ (*green*) peptides, calculated as per *b*.

We next compared the four peptide-HLA-A*02:01 structures (Fig. 1). The structures of HLA-A*02:01 in complex with NS3₁₀₇₃ (PDB code 3MRG (27)), BMLF-1 (PDB code 3MRE (27)), and M1₅₈ (PDB code 2VLL (29)) were determined previously, and here we report the HLA-A*02:01-NA₂₃₁ structure to a resolution of 2.0 Å (Table 1). The four pHLA complexes adopted similar overall structures, with an average root mean standard deviation of 0.40 Å on the C α atoms of the $\alpha1\alpha2$ domains. All four peptides adopted an extended conformation within the HLA-A*02:01 cleft, whereby the P2 and P9 residues were used as anchors and were buried inside the cleft, whereas P3 acted as a partial anchor residue.

Structure of M1₅₈ versus BMLF-1 in Complex with HLA-A*02:01—The M1₅₈ peptide has been referred to as a “plain vanilla” peptide, because its structure is featureless in the cleft of HLA-A*02:01; the two large Phe residues at P5 and P7 are buried inside the cleft, and only the small P6-Val side chain is exposed for TCR contact (Fig. 1*a*, *panel i*) (29–31). The P5-Leu and P7-Ala of the BMLF-1 peptide (27) adopted a buried conformation similar to the P5 and P7 of the M1₅₈ epitope (Fig. 1*a*, *panel ii*); however, the smaller P5 and P7 side chains of the BMLF-1 peptide were shifted toward the HLA $\alpha2$ -helix. As a result, the conserved P6-Val adopted a more central and fully solvent-exposed conformation in the BMLF-1 peptide than in the M1₅₈ peptide (C α displacement of 1.6 Å; Fig. 1*a*, *panel iii*).

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

TABLE 1
Data collection and refinement statistics

	HLA-A*02:01-NA ₂₃₁
Data collection statistics	
Temperature (K)	100
Space group	P ₂₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	51.45, 80.06, 55.00
β (°)	111.36
Resolution (Å)	100–2.00 (2.10–2.00)
Total number of observations	87,946 (11,254)
Number of unique observations	26,350 (3398)
Multiplicity	3.3 (3.3)
Data completeness (%)	93.5 (89.4)
<i>I</i> /σ	11.18 (3.57)
<i>R</i> _{merge} (%) ^a	10.0 (39.0)
Refinement statistics	
Non-hydrogen atoms	
Protein	3259
Water	372
<i>R</i> _{factor} (%) ^b	19.4
<i>R</i> _{free} (%) ^b	25.8
Root mean square deviations from ideality	
Bond lengths (Å)	0.008
Bond angles (°)	1.165
Ramachandran plot (%)	
Favored	98.48
Outliers	0

$$^a R_{\text{merge}} = \frac{\sum |I_{\text{hkl}} - \langle I_{\text{hkl}} \rangle|}{\sum I_{\text{hkl}}}$$

$$^b R_{\text{factor}} = \frac{\sum |F_{\text{o}}| - |F_{\text{c}}|}{\sum |F_{\text{o}}|} \text{ for all data except } \approx 5\%, \text{ which were used for } R_{\text{free}} \text{ calculation. The values in parentheses are for the highest resolution shell.}$$

Moreover, as judged by temperature factor analyses (relative B factor of each peptide residue compared with the HLA antigen binding cleft B factor), the central part (P4–P7) of the BMLF-1 peptide was more flexible than the M1₅₈ peptide, with a maximum mobility at the conserved P6–Val (Fig. 1*b*). Peptide mobility can influence peptide conformation, as well as CD8⁺ T cell recognition (32, 33). Therefore, despite a conserved P6–Val and 56% sequence homology, the M1₅₈ and BMLF-1 peptides were presented differently by the HLA-A*02:01 molecule.

Structure of NA₂₃₁ versus NS3₁₀₇₃ in Complex with HLA-A*02:01—Despite the sequence similarity of the NA₂₃₁ and NS3₁₀₇₃ epitopes (27), the structures of both peptides differed (Fig. 1*c*). For example, the conserved aromatic residue at P7 (NS3₁₀₇₃ P7–Trp; NA₂₃₁ P7–Phe) adopted contrasting conformations. The NA₂₃₁ P7–Phe was buried inside the cleft and interacted with Trp¹⁴⁷ and His¹¹⁴ of HLA-A*02:01 (Fig. 1*c*, *panel i*) and was not available for TCR recognition. Conversely, the NS3₁₀₇₃ P7–Trp was solvent-exposed, with its aromatic side chain potentially available for TCR interaction (Fig. 1*c*, *panel ii*). The different P7 conformations changed the central part of the peptide (Fig. 1*c*, *panel iii*), resulting in the conserved P6–Cys sitting higher in the HLA-A*02:01 cleft for the NA₂₃₁ peptide compared with NS3₁₀₇₃ (Cα deviation of 2.4 Å). This resulted in a 20% decrease in TCR accessible surface area for the NA₂₃₁ peptide (270 Å²) compared with NS3₁₀₇₃ (335 Å²) (Fig. 1*c*, *panel iii*). Temperature factor analysis revealed that the NS3₁₀₇₃ peptide was rigid, whereas the NA₂₃₁ peptide showed higher mobility at P4 to P6 (Fig. 1*d*). Taken together, the structural analysis revealed that HLA-A*02:01 presents the two peptide pairs with distinct epitope conformations and differing flexibility.

CD8⁺ T Cells Utilize Distinct αβTCR Repertoires to Recognize the Epitopes Pairs—Next, we asked whether the responding αβTCR repertoires showed any overlap in response to the

paired HLA-A*02:01-restricted M1₅₈/BMLF-1 and NS3₁₀₇₃/NA₂₃₁ epitopes. We investigated the αβTCR clonotypes (Fig. 2) and CDR3αβ usage (Fig. 3) in M1₅₈- and BMLF-1-reactive CD8⁺ T cell lines (Fig. 2*a*) or in NA₂₃₁⁺CD8⁺ and NS3₁₀₇₃⁺CD8⁺ T cells generated directly *ex vivo* from healthy donors following tetramer magnetic enrichment (Fig. 2*b*). In addition, we assessed the recognition of NS3₁₀₇₃ by CD8⁺ T cell lines from donors with chronic HCV infection (Fig. 2*c*). CD8⁺ tetramer⁺ cells were single cell-sorted, and the αβTCR repertoire was determined using human RT-PCR multiplex (34).

The M1₅₈-specific αβTCR repertoire was quite diverse in the three donors (12–19 clonotypes/donor from 27–32 sequences). The M1₅₈-specific αβTCR clonotypes were highly biased toward TRBV19 gene usage (96.3 ± 3.7%) and paired with different TRAV chains (Table 2 and Fig. 2*a*, *panel i*), in line with previously published repertoires (35, 36). Distinct M1₅₈ clonotypes favored a CDR3α length of 9 amino acids (aa) (29.9 ± 17.4%) (Fig. 3*a*, *panel i*) and a short CDR3β of 8 aa (74.6 ± 0.8%) (Fig. 3*a*, *panel ii*). Multiple CDR3α sequences were observed (GGSQGNL, GGTSYGKL, and SSNTGKL) (Table 2) sharing the Z_{3–4}XG motif (Fig. 3*b*, *panel i*), where Z represents a small amino acid (Gly, Ser, or Thr), and X represents any residue, irrespective of the TRAV gene pairing. This motif is known to allow the CDR3α loop to closely contact P4–P5 of the peptide (31). The common CDR3β RS motif was detected in 28, 63, and 74% of total sequences from donors H-1, H-2, and H-3, respectively (Table 2 and Fig. 3*b*, *panel i*), consistent with previously published data (30, 35, 36).

The BMLF-1-specific αβTCR repertoire was relatively more restricted (5–7 clonotypes/donor from 27–32 sequences) (Table 2; Fig. 2*a*, *panel ii*). A bias for the TRAV5/TRBV20 pairing was observed in all donors (100, 9.7, and 40.7% in H-1, H-2, and H-3 donors, respectively) (Table 2), consistent with previously published data (37). Furthermore, the same CDR3α loop sequence (DNNARL) was observed in the H-1 donor (84%) and the H-3 donor (4%), with conserved sequences found in donor H-2 (D(S/Q)NARL, 9%) (Table 2). The distinct CD8⁺ T cell clonotypes from the three donors preferred a short CDR3α of 6 aa (48.4 ± 45.1%) (Fig. 3*a*, *panel i*) with a conserved DXNARL motif (Fig. 3*b*, *panel ii*) and a preferred an 8-aa-long CDR3β (63.5 ± 33.8%) (Fig. 3*a*, *panel ii*) exhibiting shared motif (RXXXGN, Fig. 3*b*, *panel ii*).

There were no evident TRAV or TRBV biases in the NA₂₃₁-specific CD8⁺ T cells (Table 3 and Fig. 2*b*, *panel i*). The most common CDR3α lengths for the distinct clonotypes were 8 or 10 aa (both 28.3 ± 10.4%) (Fig. 3*a*, *panel i*), whereas CDR3β was most commonly 10 aa long (26.7 ± 30.6%) (Fig. 3*a*, *panel ii*). No shared motifs were observed within either of the CDR3 loops (Fig. 3*b*, *panel iii*). The number of NA₂₃₁-specific TCRs available was low because of their low pHLA affinity and the predominant naïve phenotype of the NA₂₃₁-specific CD8⁺ T cells.

The NS3₁₀₇₃ TCRαβ repertoire was characterized by a limited TCRαβ diversity in donors with chronic HCV infection. HCV-1 had seven NS3₁₀₇₃-specific clonotypes, whereas HCV-2 had a more limited repertoire with only four clonotypes (Fig. 2*c*). A TRAV4 bias was observed in the TCR repertoire of both healthy (37.8 ± 20.4%) and HCV-infected donors (59.1 ±

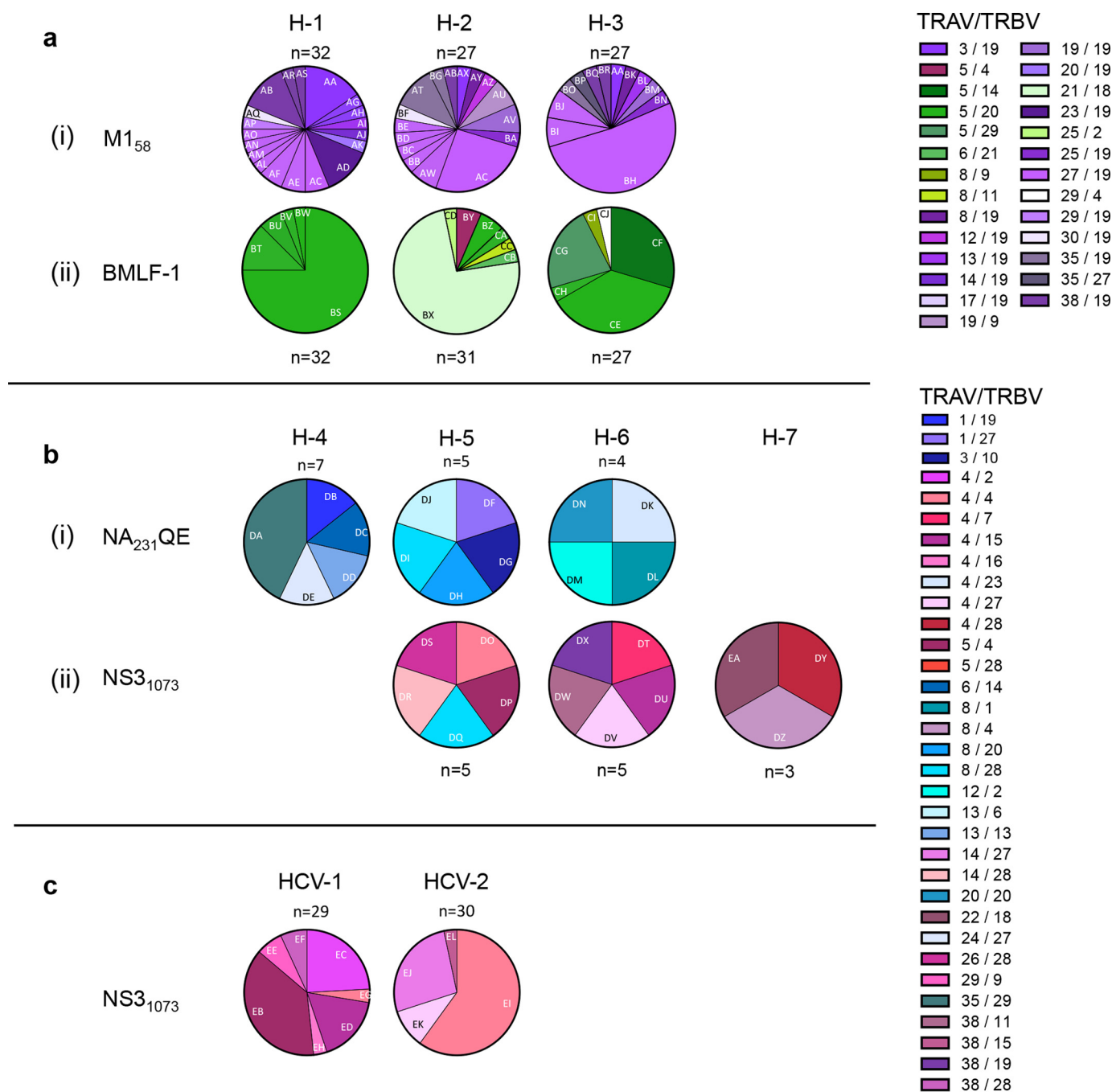


FIGURE 2. Distinct $\alpha\beta$ TCR repertoires recognize each peptide in complex HLA-A*02:01. PBMCs from healthy and HCV-infected donors were stimulated with the M1₅₈ or BMLF-1 peptides (13 days) or the NS3₁₀₇₃ peptide (19–21 days). CD8⁺ T cell lines were stained with the cognate peptide, whereas PBMCs (from healthy donors) were stained with either NA₂₃₁QE or NS3₁₀₇₃ tetramers and were magnetically enriched. The cells were stained as lymphocytes, singlets, CD3^{mid-high}, dump⁻ (PBMCs only), CD8⁺, tetramer⁺ cells, and single-cell sorted. Then the $\alpha\beta$ TCR repertoires were determined using a multiplex RT-PCR. *a*, $\alpha\beta$ TCR repertoire utilized to recognize the M1₅₈ (*panel i*) or BMLF-1 (*panel ii*) tetramers (in H-1, H-2, and H-3 healthy donors). *b*, $\alpha\beta$ TCR repertoire utilized to recognize NA₂₃₁QE (*panel i*) or NS3₁₀₇₃ (*panel ii*) tetramers (in H-4, H-5, H-6, and H-7 healthy donors). *c*, NS3₁₀₇₃ tetramer⁺ $\alpha\beta$ TCR repertoire (HCV1 and HCV-2 are HCV-infected donors).

15.4%), which paired with a range of TRBVs (Fig. 2, *b* and *c*), despite the low number of NS3₁₀₇₃-specific CD8⁺ T cells isolated from healthy donors because of their predominant naïve phenotype. In addition, the HCV-1 donor showed a preference for TRAV5 (~38%) (Fig. 2*c*). The NS3₁₀₇₃-reactive repertoire from both healthy and HCV-infected individuals consisted of distinct clonotypes with different CDR3 lengths, with the most common CDR3 α length of 10 aa (26.4 \pm 18.4%) (Fig. 3*a*, *panel*

i) and the most common CDR3 β length of 9 aa (26.3 \pm 24.5%) (Fig. 3*a*, *panel ii*).

Overall, no common TCR $\alpha\beta$ clonotypes were utilized for the recognition of M1₅₈ and BMLF-1 or the NA₂₃₁ and NS3₁₀₇₃ peptides. On a broader level, only a single common TRAV8/TRBV28 pair was identified in the NA₂₃₁ and NS3₁₀₇₃ TCR $\alpha\beta$ repertoires; however, they were completely distinct clonotypes. Furthermore, no common TRAV/TRBV pairs were detected in

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

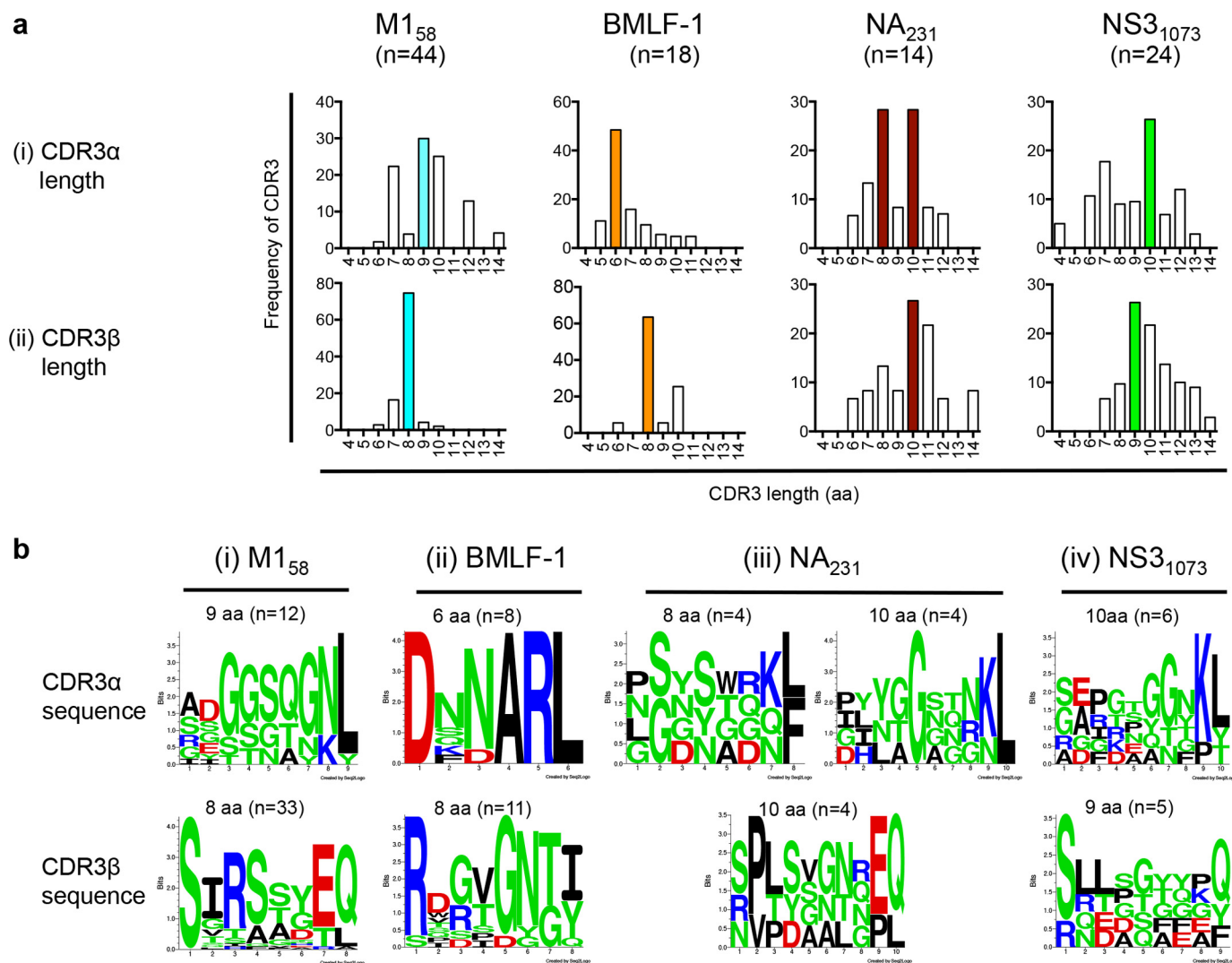


FIGURE 3. Epitope-specific CDR3 $\alpha\beta$ motifs are conserved between donors. *a*, CDR3 α (panel *i*) and CDR3 β (panel *ii*) length used by distinct antigen-specific CD8⁺ T cell clonotypes for recognition of M1₅₈ (cyan), BMLF-1 (orange), NA₂₃₁ (brown), and NS3₁₀₇₃ (green) peptides. The colored bar represents the most frequent CDR3 length for each peptide. *b*, analysis of the CDR3 motifs (of the most common lengths) used by distinct clonotypes in the recognition of M1₅₈ (panel *i*), BMLF-1 (panel *ii*), NA₂₃₁ (panel *iii*), and NS3₁₀₇₃ (panel *iv*). *n* = number of distinct clonotypes.

the M1₅₈ and BMLF-1 $\alpha\beta$ TCR repertoires. These findings further highlight the high level specificity of memory CD8⁺ T cells for their pHLA. Thus, despite high sequence homology, the paired peptides were structurally different and mobilized distinct $\alpha\beta$ TCR repertoires with no overlap.

*Lack of T Cell Cross-reactivity Observed for the Paired HLA-A*02:01-restricted Epitopes as Assessed Directly ex Vivo*—Based on our findings on differing $\alpha\beta$ TCR repertoires, we next investigated heterologous cross-reactivity directly *ex vivo* from human peripheral blood mononuclear cells (PBMCs). We used a magnetic enrichment technique (38, 39) for the unbiased detection of low-frequency epitope-specific CD8⁺ T cells. PBMCs from healthy HLA-A*02:01-positive donors were stained with the specific HLA-A*02:01⁺ tetramer, positively enriched, and surface-stained using α CD3, α CD8, dump (α CD4, α CD14, and α CD19), and phenotypic antibodies (Fig. 4*a*). Using the phenotypic antibodies CD27 and CD45RA, M1₅₈- and NS3₁₀₇₃-specific CD8⁺ T cells from healthy donors were analyzed for their activation phenotype, respectively (Fig.

4*b*). Furthermore, using the CD8 α enhanced (40) HLA-A*02:01-Q115E-NA₂₃₁ mutant tetramer (NA₂₃₁QE), we detected low affinity NA₂₃₁-specific CD8⁺ T cells, which were undetectable with the WT tetramer (Fig. 4*c*). We identified memory M1₅₈- and BMLF-1-specific CD8⁺ T cells across donors with a mean frequency of 5×10^{-5} and 1×10^{-4} , respectively (Fig. 4*d*). Low affinity NA₂₃₁- and naïve CD27⁺CD45RA⁺ NS3₁₀₇₃-specific CD8⁺ T cells were found at lower frequencies comparable with other studies (39), at 3×10^{-6} and 4×10^{-6} , respectively (Fig. 4*d*).

To identify any cross-reactive CD8⁺ T cells directly *ex vivo*, PBMCs from healthy and HCV⁺ donors (Table 4) were co-stained with either M1₅₈ and BMLF-1 or NA₂₃₁QE and NS3₁₀₇₃ tetramers conjugated to different fluorochromes, followed by tetramer magnetic enrichment (Fig. 5). Memory M1₅₈⁺- and BMLF-1⁺-specific CD8⁺ T cell populations were detected at an average of 12.0 ± 7.8 and $20.4 \pm 16.1\%$ of CD8⁺ T cells, respectively (Fig. 5*b*, panel *i*). Similarly, naïve NS3₁₀₇₃- and low affinity NA₂₃₁-specific CD8⁺ T cells were detected in healthy donors

TABLE 2

αβTCR repertoire usage for the recognition of M1₅₈ and BMLF-1

Code represents the individual clonotypes. The colored residues represent the conserved motifs within the CDR3 loops between donors, in which blue is the Z₃₋₄XG motif (Z is a small amino acid, and X is any residue), red is the RS motif of the M1₅₈-specific clones, purple is the DXNARL motif, and green is the RXXXGN motif of the BMLF-1-specific clones. Each clonotype frequency is expressed as the proportion of total sequences. TRAV, variable α-chain gene usage; TRBV, β-chain gene usage; TRAJ, junction α-chain gene usage; TRBJ, junction β-chain gene usage; H, individual donor.

Code	Pairs	TRAV	TRAJ	CDR3α	TRBV	TRBJ	CDR3β	Frequency M1 ₅₈		
								H-1	H-2	H-3
AA	3/19	3	36	CAV-RDGTGANL-FF	19	2-2	CAS-SISSTGEL-FF	16		4
AB	38-1/19	38-1	52	CAF-MKDAGGTSYGKL-TF	19	1-2	CAS-SIGVYGY-TF	13	4	
AC	27/19	27	42	CAG-GGSQGNL-IF	19	2-7	CAS-SIRSSYEQ-YF	6	26	
AD	23/19	23/DV6	42	CAL-AGSQGNL-IF	19	2-7	CAS-SIRASYEQ-YF	13		
AE	27/19	27	42	CAG-ADGGSQGNL-IF	19	2-2	CAS-SGRAAGEL-FF	6		
AF	27/19	27	42	CAA-GGSQGNL-IF	19	2-7	CAS-SVRSYEQ-YF	6		
AG	3/19	3	37	CAA-PPSNTGKL-IF	19	2-3	CAS-STRAADTQ-YF	3		
AH	3/19	3	27	CAV-RDLLTNAGKS-TF	19	2-7	CAS-SGTSGTEQ-YF	3		
AI	13-2/19	13-2	42	CAE-NMGGGSQGNL-IF	19	2-5	CAS-SIRAGETQ-YF	3		
AJ	14/19	14/DV4	42	CAM-RDGGGSQGNL-IF	19	2-7	CAS-SIRSGAEQ-YF	3		
AK	20/19	20	35	CAV-APTGNVL-HC	19	2-2	CAS-SIRSTGEL-FF	3		
AL	27/19	27	42	CAG-AGSQGNL-IF	19	2-7	CAS-SVRSYEQ-YF	3		
AM	27/19	27	42	CAG-GLGGGSQGNL-IF	19	2-7	CAS-SIRAAEQ-YF	3		
AN	27/19	27	42	CAG-ADGGSQGNL-IF	19	2-2	CAS-SISSTGEL-FF	3		
AO	27/19	27	37	CAG-GGSSNTGKL-IF	19	2-7	CAS-SIRSSYEQ-YF	3		
AP	29/19	29/DV5	42	CPG-GSQGNL-IF	19	1-2	CAS-SIGVYGY-TF	3		
AQ	30/19	30	42	CGT-GGSQGNL-IF	19	2-3	CSS-VRSADTQ-YF	3		
AR	38-1/19	38-1	52	CAF-MINAGGTSYGKL-TF	19	1-2	CAS-SIGSYGY-TF	3		
AS	38-2/19	38-2/DV8	52	CAY-GTNAGGTSYGKL-TF	19	1-2	CAS-SIGVYGY-TF	3		
AT	35/19	35	42	CAG-PTHDMNYGGGSQGNL-IF	19	2-3	CAS-SIHSTDTQ-YF		11	
AU	19/9	19	52	CAL-SEVSNAGGTSYGKL-TF	9	2-1	CAS-SVDGGGLDEQ-FF		7	
AV	19/19	19	23	CAL-FGGYNQGGK-IF	19	1-1	CAS-STSGGGTEA-FF		7	
AW	27/19	27	37	CAG-GGSSNTGKL-IF	19	2-7	CAS-SIRSAEQ-YF		7	
AX	3/19	3	36	CAV-RDGTGANL-FF	19	2-7	CAS-SGTSVHEQ-YF		4	
AY	8-6/19	8-6	42	CAV-GGSQGNL-IF	19	2-7	CAS-SIRSSYEQ-YF		4	
AZ	12-2/19	12-2	37	CAV-PPSNTGKL-IF	19	2-3	CAS-STRSADTQ-YF		4	
BA	25/19	25	42	CAG-SSGGSQGNL-IF	19	2-3	CAS-SIRSSDTQ-YF		4	
BB	27/19	27	37	CAG-AQSSNTGKL-IF	19	2-1	CAS-SIRSAEQ-FF		4	
BC	27/19	27	37	CAG-AVGSSNTGKL-IF	19	2-7	CAS-SIRSSYEQ-YF		4	
BD	27/19	27	37	CAG-ASSSNTGKL-IF	19	2-7	CAS-SIRSAEQ-YF		4	
BE	27/19	27	42	CAA-GGSQGNL-IF	19	2-7	CAS-SIRSSYEQ-YF		4	
BF	30/19	30	42	CGT-EEGGGSQGNL-IF	19	2-3	CAS-SSRSTDTQ-YF		4	
BG	35/19	35	42	CAG-RPEVDSQGNL-IF	19	2-7	CAS-SIFSVSNEQ-YF		4	
BH	27/19	27	42	CAG-AEGGSQGNL-IF	19	2-2	CAS-SIRSTGEL-FF			52
BI	27/19	27	42	CAG-AEGGSQGNL-IF	19	2-2	CAS-SGRSTGEL-FF			7
BJ	27/19	27	42	CAG-GGSQGNL-IF	19	2-7	CAS-STRSSYEQ-YF			7
BK	8 / 19	8-1	8	LKE-NTGFQKL-VF	19	2-7	CAS-SGTGAGEQ-YF			4
BL	13-2/19	13-2	42	CAE-NRGGGSQGNL-IF	19	2-7	CAS-SQRASYEQ-YF			4
BM	19/19	19	40	CAL-IITSGTYKY-IF	19	1-5	CAS-SVGQPQ-HF			4
BN	25/19	25	42	CAG-SSGGSQGNL-IF	19	2-1	CAS-SIRSTNEQ-FF			4
BO	35/19	35	42	CAG-SYGGGSQGNL-IF	19	2-1	CAS-SVRSSEGEQ-FF			4
BP	35/27	35	37	CAG-PDGSSNTGKL-IF	27	1-1	CAS-SLIFPGRA-FF			4
BQ	38-1/19	38-1	52	CAF-MVNAAGGTSYGKL-TF	19	1-2	CAS-SIGSYGY-TF			4
BR	38-2/19	38-2/DV8	52	CAY-SPASGGTSYGKL-TF	19	1-2	CAS-SIGLYGY-TF			4
Total # sequences								32	27	27

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

TABLE 2—continued

Code	Pairs	TRAV	TRAJ	CDR3 α	TRBV	TRBJ	CDR3 β	Frequency BMLF-1		
								H-1	H-2	H-3
BS	5 / 20	5	31	CAE-DNNARL-MF	20-1	1-3	CSA-RVGVGNTI-YF	75		
BT	5 / 20	5	31	CAE-DFNARL-MF	20-1	1-3	CSA-RTGVGNTI-YF	13		
BU	5 / 20	5	31	CAE-DNNARL-MF	20-1	1-2	CSA-RDRTGNGY-TF	6		
BV	5 / 20	5	31	CAE-DNNARL-MF	20-1	1-2	CSA-RDGTGNGY-TF	3		
BW	5 / 20	5	31	CAE-DKDARL-MF	20-1	1-3	CSA-RDRIGNTI-YF	3		
BX	21 / 18	21	41	CAL-YSNSGYAL-NF	18	1-2	CAS-RPDSYDGY-TF		74	
BY	5 / 4	5	24	CAD-GSGTDSWGKL-QF	4-1	1-1	CAS-SQNQDMGTEA-FF	6		
BZ	5 / 20	5	31	CAE-DSNARL-MF	20-1	1-3	CSA-RSGVGNTI-YF	6		
CA	5 / 20	5	31	CAE-DQNARL-MF	20-1	1-3	CSA-RWGVGNTI-YF	3		
CB	6 / 21	6	3	CAL-DSTYSSASKI-IF	20-1	1-2	CSA-RDRTGNGY-TF	3		
CC	8 / 11	8-3	20	CAV-GASRDYKL-SF	11-2	2-3	CAS-SFSSGTTDTQ-YF	3		
CD	25 / 2	25	42	CAY-GGSQGNL-IF	2	2-5	CAS-SQGDVAPGTQ-YF	3		
CE	5 / 20	5	40	CAE-VSGTYKY-IF	20-1	1-1	CSA-WDREVVGTEA-FF			37
CF	5 / 14	5	16	CAE-LGQKL-LF	14	2-5	CAS-SQSPGGTQ-YF			30
CG	5 / 29	5	37	CAE-STGKL-IF	29-1	1-4	CSV-GTGGTNEKL-FF			22
CH	5 / 20	5	31	CAE-DNNARL-MF	20-1	1-3	CSA-RIGVGNTI-YF			4
CI	8 / 9	8-3	39	CAD-PIEDAGNML-TF	9	2-2	CAS-SDGQLTAGEL-FF			4
CJ	29 / 4	29/DV5	34	CAA-TDNTDKL-IF	4-1	1-2	CAS-SRLGDY-TF			4
Total # sequences								32	31	27

with mean frequencies of 2.2 ± 1.4 and $2.2 \pm 1.2\%$ of CD8⁺ T cells, respectively (Fig. 5*b*, panel *ii*). Memory NS3₁₀₇₃-specific CD8⁺ T cell populations were detected at a higher frequency in donors with chronic HCV infection ($13.0 \pm 3.0\%$, Fig. 5, *c* and *d*). No discernable cross-reactive population was observed for either set of epitopes, of which only 0.19 ± 0.16 or $0.09 \pm 0.08\%$ of CD8⁺ T cells in healthy donors were M1₅₈⁺BMLF-1⁺ or NA₂₃₁⁺NS3₁₀₇₃⁺, respectively. Similarly, only $0.10 \pm 0.14\%$ of CD8⁺ T cells were NA₂₃₁⁺NS3₁₀₇₃⁺ in HCV-infected donors (Fig. 5*d*).

Lack of Heterologous Cross-reactivity after *in Vitro* Amplification—To determine whether the reported heterologous cross-reactivity could be a consequence of an *in vitro* amplification, epitope-specific CD8⁺ T cell lines from healthy (Fig. 6) or HCV-infected donors (Fig. 7 and Table 4) were assessed for cross-reactivity by tetramer co-staining and an IFN γ ⁺ intracellular cytokine staining (ICS) assay. M1₅₈⁺ and BMLF-1⁺ CD8⁺ T cell lines specifically responded to their cognate peptide, with 22.4 ± 16.8 and $17.9 \pm 7.1\%$ of the tetramer⁺ CD8⁺ T cells (Fig. 6, *a* and *b*) and 25.0 ± 12.3 and $9.7 \pm 7.5\%$ being IFN γ ⁺TNF α ⁺, respectively (Fig. 6, *c* and *d*). The frequency of CD8⁺ T cells able to bind both tetramers and produce IFN γ ⁺TNF α ⁺ was just above background, showing the absence of CD8⁺ T cells able to cross-react on both the M1₅₈ and BMLF-1 epitopes (Fig. 6).

Unlike previous studies (24), CD8⁺ T cells did not expand in the presence of NA₂₃₁, and only $0.06 \pm 0.04\%$ and $0.19 \pm 0.17\%$ of CD8⁺ T cells were NA₂₃₁-tetramer⁺ or IFN γ ⁺TNF α ⁺ in an ICS assay, respectively (Fig. 7, *a* and *c*). Conversely, NS3₁₀₇₃-specific CD8⁺ T cells could be detected at 14.3 ± 11.7 and $4.0 \pm 3.5\%$ in the tetramer and ICS assays, respectively (Fig. 7, *b* and *d*). No cross-reactivity was detected in NA₂₃₁ or NS3₁₀₇₃-stimulated CD8⁺ T cell lines either by tetramer co-staining ($0.02 \pm$

0.01 and $0.09 \pm 0.14\%$ of CD8⁺ T cells) (Fig. 7, *a* and *b*) or in the ICS assay (0.15 ± 0.11 and $0.60 \pm 0.99\%$ of CD8⁺ T cells were IFN γ ⁺TNF α ⁺), respectively (Fig. 7, *c* and *d*). Together, these *ex vivo* and *in vitro* data highlight the lack of heterologous cross-reactivity toward M1₅₈ and BMLF-1 or NA₂₃₁ and NS3₁₀₇₃ epitopes, even following extended *in vitro* amplification.

No Cross-reactivity between Different NA₂₃₁ Variant Peptides—Based on the low number of NA₂₃₁ tetramer⁺ CD8⁺ T cells detected in our study, and because the NA protein is highly variable, we investigated whether the donors may have been exposed to other NA variants. We first examined the conservation of the NA₂₃₁ peptide in the pH1N1, recent trivalent seasonal influenza vaccine strains, and Oceania-originated isolates (Table 5). A total of 28 variant peptides were identified. Mutations were common in the anchor P2 and P9 residues (buried residues; Fig. 1*c*), which are unlikely to affect TCR binding (Table 5). The P2 mutation, V2I, was common to the vaccine (71%) and Oceania (~67%) isolates and thus further increased the sequence homology to the NS3₁₀₇₃ peptide. Three major variants from the NA₂₃₁ WT CVNGSCFTV were identified and chosen for further analysis, namely CINGTCTVV (P2-Ile-P5-Thro-P7-Thr-P8-Val), CVNGSCFTI (P9-Ile), and CINGSCFTI (P2-Ile-P9-Ile) (Table 5).

To assess whether our HCV-infected donors had been exposed to a different NA₂₃₁ variant (Table 5), CD8⁺ T cell lines were established against each or a pool of the peptides containing WT NA₂₃₁ and three NA₂₃₁ variants, as well as WT NS3₁₀₇₃ and a single NS3₁₀₇₃ variant. No NA₂₃₁-specific CD8⁺ T cells were expanded, despite extended *in vitro* amplification of up to 18 days (Fig. 8*a*). CD8⁺ T cells were successfully expanded against the NS3₁₀₇₃ peptides in both donors (Fig. 8*b*),

TABLE 3

αβTCR usage for the recognition of NA₂₃₁ and NS3₁₀₇₃

Code represents the individual clonotypes. TRAV, variable α-chain gene usage; TRBV, β-chain gene usage; TRAJ, junction α-chain gene usage; TRBJ, junction β-chain gene usage; H, individual donor.

Code	Pairs	TRAV	TRAJ	CDR3α	TRBV	TRBJ	CDR3β	Frequency							
								Naïve-NA ₂₃₁			Naïve-NS3 ₁₀₇₃			Memory-NS3 ₁₀₇₃	
								H-4	H-5	H-6	H-5	H-6	H-7	HCV-1	HCV-2
DA	35/29	35	52	CAG-QSNAGGTSYGKL-TF	29-1	2-7	CSV-EDGYEQ-YF	43							
DB	1/19	1-2	33	CAP-MDSNYQL-IW	19	2-6	CAS-SNSEGSGANVL-TF	14							
DC	6/14	6	38	CAL-PYNAGNNRKL-IW	14	2-7	CAS-SPLYVALREQ-YF	14							
DD	13/13	13-2	42	CAE-IHYGGSQGNL-IF	13	2-2	CAS-TSGNTGEL-FF	14							
DE	24/27	24	22	CAS-LSGSARQL-TF	27	2-1	CAS-SPTVAGEQ-FF	14							
DF	1/27	1-2	10	CAV-DILTGGGNKL-TF	27	1-5	CAS-NPLSAGNQPF-HF		20						
DG	3/10	3	26	CAV-PGNYGQNF-VF	10-1	2-7	CAS-SEDQGLTATYEQ-YF		20						
DH	8/20	8-1	34	CAV-GSYNTDKL-IF	20-1	2-2	CSA-RVPDSNTGEL-FF		20						
DI	8/28	8-2	43	CAV-SDSYNDM-RF	28	1-2	CAS-SSPRTYGNYGY-TF		20						
DJ	13/6	13-1	39	CAE-NAGNML-TF	6	2-1	CAS-SPTSGGNNEQ-FF		20						
DK	4/23	4	32	CLV-GLYGGATNKL-IF	23-1	1-5	CAS-SQDRGVLVLIDQPQ-HF			25					
DL	8/1	8-6	34	CAV-SRSYNTDKL-IF	1-1	20-1	CSA-RTEGAEA-FF			25					
DM	12/2	12-2	24	CAV-NGDSWGKF-QF	2-5	14	CAS-SYWTGSSAETQ-YF			25					
DN	20/20	20	4	CAV-QAWFSGGYNKL-IF	20-1	2-1	CSA-RTSGDFGEQ-FF			25					
DO	4/4	4	39	CLV-GAGNML-TF	4	2-7	CAS-SQGGQGAPYEQ-YF		20						
DP	5/4	5	42	CAE-SGGSQGNL-IF	4-1	1-1	CAS-SQAENTEA-FF		20						
DQ	8/28	8-2	5	CAV-SSLMDTGRRAL-TF	28	2-2	CAS-SLLGLAGVGEL-FF		20						
DR	14/28	14	52	CAM-REDAGGTSYGKL-TF	28	2-1	CAS-SLTGTGFKQ-FF		20						
DS	26/28	26-2	52	CIL-RDLGGTSGYGKL-TF	28	2-3	CAS-SLTLGGIGTDTQ-YF		20						
DT	4/7	4	36	CPF-QTGANNL-FF	7-2	2-7	CAS-SLGTGAPYEQ-YF			20					
DU	4/15	4	43	CLV-GAPGNTPL-VF	15	1-2	CAT-SNLDSFGGY-TF			20					
DV	4/17	4	43	CLV-GDNNNDM-RF	27	2-7	CAS-SLSTGAPYEQ-YF			20					
DW	38/11	38-2	45	CAY-RSPLSGGGADGL-TF	11-2	2-1	CAS-SLGPDPNEQ-FF			20					
DX	38/19	38-2	40	CAY-TSGTYKY-IF	19	2-1	CAT-SPGQGFYNEQ-FF			20					
DY	4/28	4	9	CLK-AGGFKT-IF	28	2-7	CAS-RLLAGAYEQ-YF					33			
DZ	8/4	8-3	27	CAV-GAGTNAGKS-TF	4	1-1	CAS-SQESGTEAF-FG					33			
EA	22/18	22	47	CAV-ADPREYGNKL-VF	18	1-4	CAS-NRENEKL-FF					33			
EB	5/4	5	13	CAE-SGGYQKV-TF	4	1-1	CAS-SQEKGTEA-FF						38		
EC	4/2	4	28	CLV-GVPVAGASYQL-TF	2	1-4	CAS-TTGSSEKL-FF						24		
ED	4/15	4	26	CLV-VGDNYGQNF-VF	15	1-2	CAT-STTFQTDYAEPYGY-TF						17		
EE	29/9	29	23	CAA-SARGNQGGKL-IF	9	2-2	CAS-SVDEGNTGEL-FF						7		
EF	38/28	38-2	45	CAY-RSATFSGGGADGL-TF	28	1-2	CAS-SRPVGLFNFGY-TF						7		
EG	4/4	4	9	CLV-GEGDTGGFKT-IF	4	1-2	CAS-SQVQTEPSGY-TF						3		
EH	4/16	4	45	CLV-GGGADGL-TF	16	2-7	CAS-SQEVTVPTYEQ-YF						3		
EI	4/4	4	43	CLD-SNDM-RF	4	2-7	CAS-SQAQSGTAPYEQ-YF							60	
EJ	14/27	14	40	CAM-REFTSGTYKY-IF	27	2-5	CAS-SLTLGLAGVETQ-YF							27	
EK	4/27	4	20	CLV-GSPNDYKL-SF	27	2-1	CAS-SSPSGRPTYNEQ-FF							10	
EL	38/15	38-2	17	CAY-SGIKAAAGNKL-TF	15	1-5	CAT-SRDPQYQPQ-HF							3	
<i>Total # of sequences</i>								7	5	4	5	5	3	29	30

and CD8⁺ T cells stimulated with the pool of peptides were able to respond only to both NS3₁₀₇₃ peptides (Fig. 8c). Together, these data show that there is no cross-reactivity between WT and variants NA₂₃₁ with NS3₁₀₇₃ in donors with chronic HCV infection, in line with previously published data (26).

Discussion

Heterologous T cell cross-reactivity is a phenomenon whereby an individual CD8⁺ T cell can recognize and respond to more than one viral antigen derived from unrelated viruses (41). It has the potential to be either beneficial, by increasing the chances that any given viral peptide is recognized by a CD8⁺ T cell (28), or detrimental, by contributing to the CD8⁺ T cell-mediated immunopathology associated with diseases such as EBV and HCV infection (23, 24). Because the few reports on T cell cross-reactivity in humans have

shown conflicting results (20–25, 41), we investigated four epitopes from ubiquitous viruses (influenza, EBV, and HCV) restricted by the highly prevalent HLA-A*02:01 molecule. We aimed to elucidate the extent (if any) of the proposed heterologous cross-reactivity toward two distinct sets of HLA-A*02:01 restricted peptides: M1₅₈ (29) with BMLF-1 (27) and NA₂₃₁ with NS3₁₀₇₃ (27).

The most likely mechanism for heterologous T cell cross-reactivity would be that an individual CD8⁺ T cell with a single TCR can recognize unrelated but similar pHLA complexes. This suggests that molecular mimicry would underpin heterologous T cell cross-reactivity, in a similar fashion to the interepitope cross-reactivity (5). Structures of the HLA-A*02:01 in complex with each paired peptide showed, however, that despite the sequence similarity of the paired peptides, the pHLA complexes display distinct architectures.

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

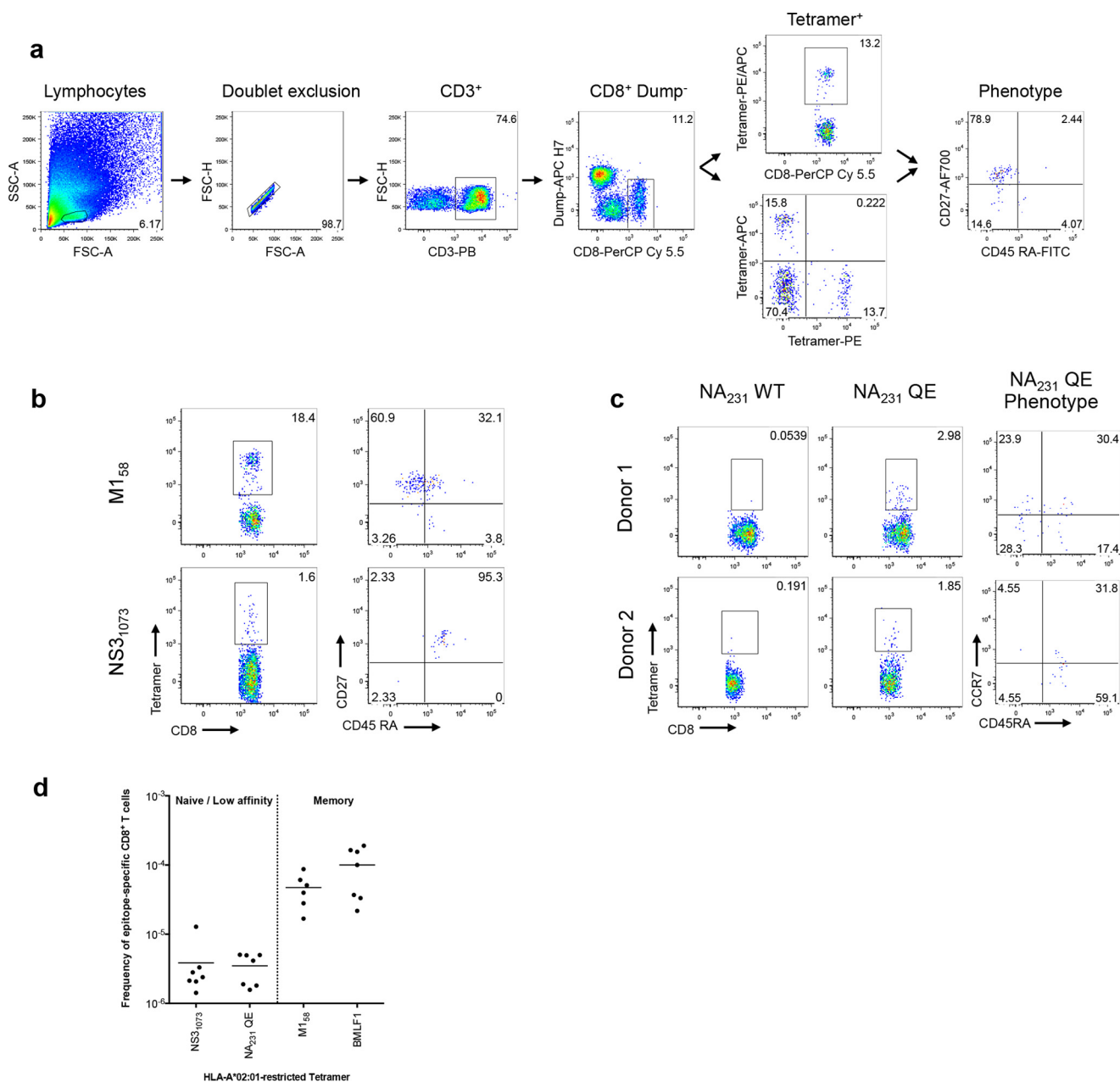


FIGURE 4. Enumeration of naive and memory HLA-A*02:01-restricted CD8⁺ T cell populations. PBMCs from healthy HLA-A*02:01⁺ donors were tetramer-stained or co-stained with either M1₅₈ and BMLF-1 or NA₂₃₁ and NS3₁₀₇₃ conjugated to PE or APC. The samples were magnetically enriched and antibody-stained (as outlined under “Experimental Procedures”) and gated on lymphocytes, singlets, CD3⁺, dump⁻CD8⁺, and tetramer⁺. *a*, representative dot plots describing the gating strategy used for data analysis of tetramer magnetic enrichment experiments. *b*, representative dot plots of naive and memory tetramer positive populations and their respective phenotypes using α CD27 and α CD45 RA phenotypic markers. *c*, representative dot plots of PBMCs from two donors stained with either WT or HLA-A*02:01-QE mutant NA₂₃₁-specific tetramers and their phenotype using α CCR7 and α CD45 RA phenotypic markers. *d*, frequency of naive, low affinity, and memory epitope-specific CD8⁺ T cell populations in healthy HLA-A*02:01⁺ donors after magnetic enrichment.

The M1₅₈ and BMLF-1 peptides share 56% sequence homology, and the conserved P6-Val adopts a different conformation between the two peptides. In BMLF-1, the P6-Val is solvent-exposed and available for TCR contact, whereas it is buried within the HLA-binding cleft in the M1₅₈ structure (29). This solvent-exposed P6-Val is important in the recognition of the BMLF-1 pHLA complex, as demonstrated by the structure of a prototypical public TCR (AS01) in complex with HLA-A*02:01-BMLF-1 (37). The highly conserved CDR3 β loop positions itself atop the P6-Val, and the RXXXGN motif directly interacts with the P6-Val (36). The BMLF-1 peptide does not undergo

structural change upon AS01 TCR binding, and moreover, alanine substitution of the P6-Val dramatically decreased the TCR affinity by 13-fold (37). Therefore, the buried conformation of P6-Val in the M1₅₈ complex would prevent binding by BMLF-1-specific CD8⁺ T cells. From the structure of a conserved prototypical TRBV19⁺ TCR (JM22) in complex with HLA-A*02:01-M1₅₈, it was observed that the M1₅₈ also does not undergo structural change upon TCR binding. Therefore, the solvent-exposed P6-Val of the BMLF-1 pHLA complex would cause steric clashes with the JM22 CDR3 β loop, thus preventing binding (29, 30).

TABLE 4
Donors used in this study

H, healthy donor; HCV, HCV-infected donor.

	Figure												
	HLA-A	HLA-B	2A	2B	2C	4D	5Bi	5Bii	5D	6A-B	6C-D	7	8
H-1	0201, 1101	3501, 3901	Y								Y		
H-2	02:01, 11:01	35:03, 44:02	Y								Y		
H-3	02:01, 03:02	18:01, 35:08	Y								Y		
H-4	02:01, 2501	0702, 1801		Y		Y	Y	Y					
H-5	02, 24	07, 40		Y		Y	Y	Y		Y	Y		
H-6	02, 02	18, 18		Y		Y	Y	Y					
H-7	02, 25	18, 44		Y									
H-8	02:01, 68:01	15:01, 40:01				Y							
H-9	02, 02	18, 27				Y							
H-10	0201, 0201	1501, 1801				Y	Y	Y					
H-11	0201, 2402	1302, 4403				Y	Y	Y					
H-12	0201, 0301	1401, 4002				Y	Y						
H-13	0101, 0201	0801, 4402								Y	Y		
H-14	0201, 0301	1402, 5801								Y	Y		
HCV-1	02:01, 03:01	08:01, 40:23			Y							Y	
HCV-2	02:01, 03	38:01, 45:01			Y							Y	Y
HCV-3	01:01, 02:01	35:02, 44:02							Y				
HCV-4	02:01, 31	18:01, 40							Y				
HCV-5	02:01, 32:01	15:01, 41:01										Y	Y
Total # of donors			3	4	2	8	5	5	2	3	6	3	2

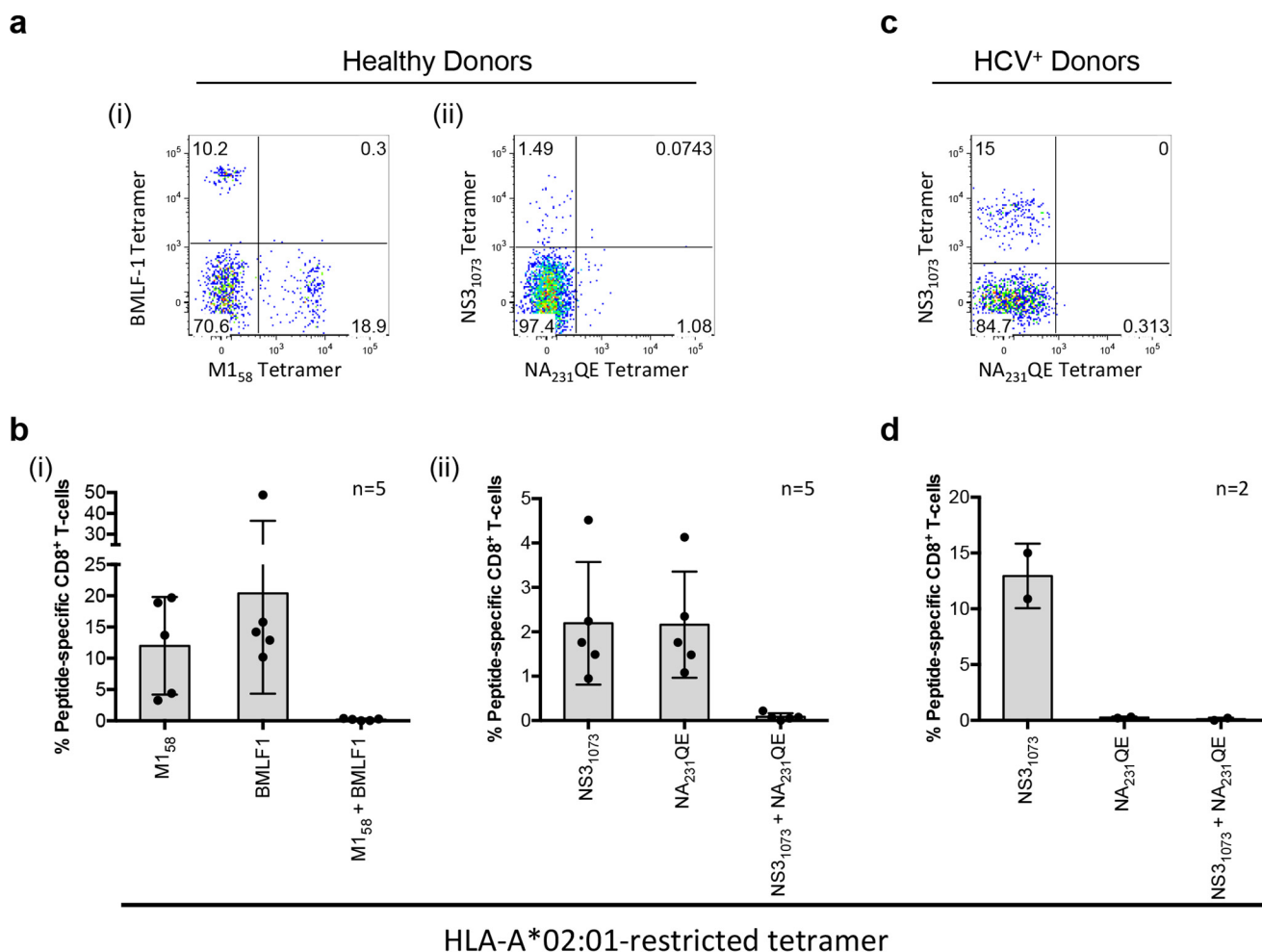


FIGURE 5. Lack of heterologous T cell cross-reactivity between influenza-M1₅₈ and EBV-BMLF-1 or influenza-NA₂₃₁ and HCV-NS3₁₀₇₃ detected directly *ex vivo*. PBMCs from healthy and HCV-positive HLA-A*02:01⁺ donors were tetramer co-stained with either M1₅₈ and BMLF-1 or NS3₁₀₇₃ and NA₂₃₁QE tetramers. Following magnetic enrichment, PBMCs were surface-stained (as described under "Experimental Procedures") and gated on lymphocytes, singlets, CD3⁺, and dump⁻ CD8⁺. *a* and *b*, representative dot plots (*a*) and summary (*b*) of (panel *i*) memory M1₅₈⁺ and BMLF-1⁺ or (panel *ii*) low affinity NA₂₃₁⁺ and naïve NS3₁₀₇₃⁺ tetramer⁺ CD8⁺ T cells derived from healthy donors (*n* = 5). The bar charts represent the means with the error bars representing standard deviation. *c* and *d*, representative dot plot (*c*) and summary (*d*) of low affinity NA₂₃₁⁺ and memory NS3₁₀₇₃⁺-specific tetramer⁺ CD8⁺ T cells found in donors with chronic HCV infection (*n* = 2). The bar charts represent the means with the error bars representing standard deviation.

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

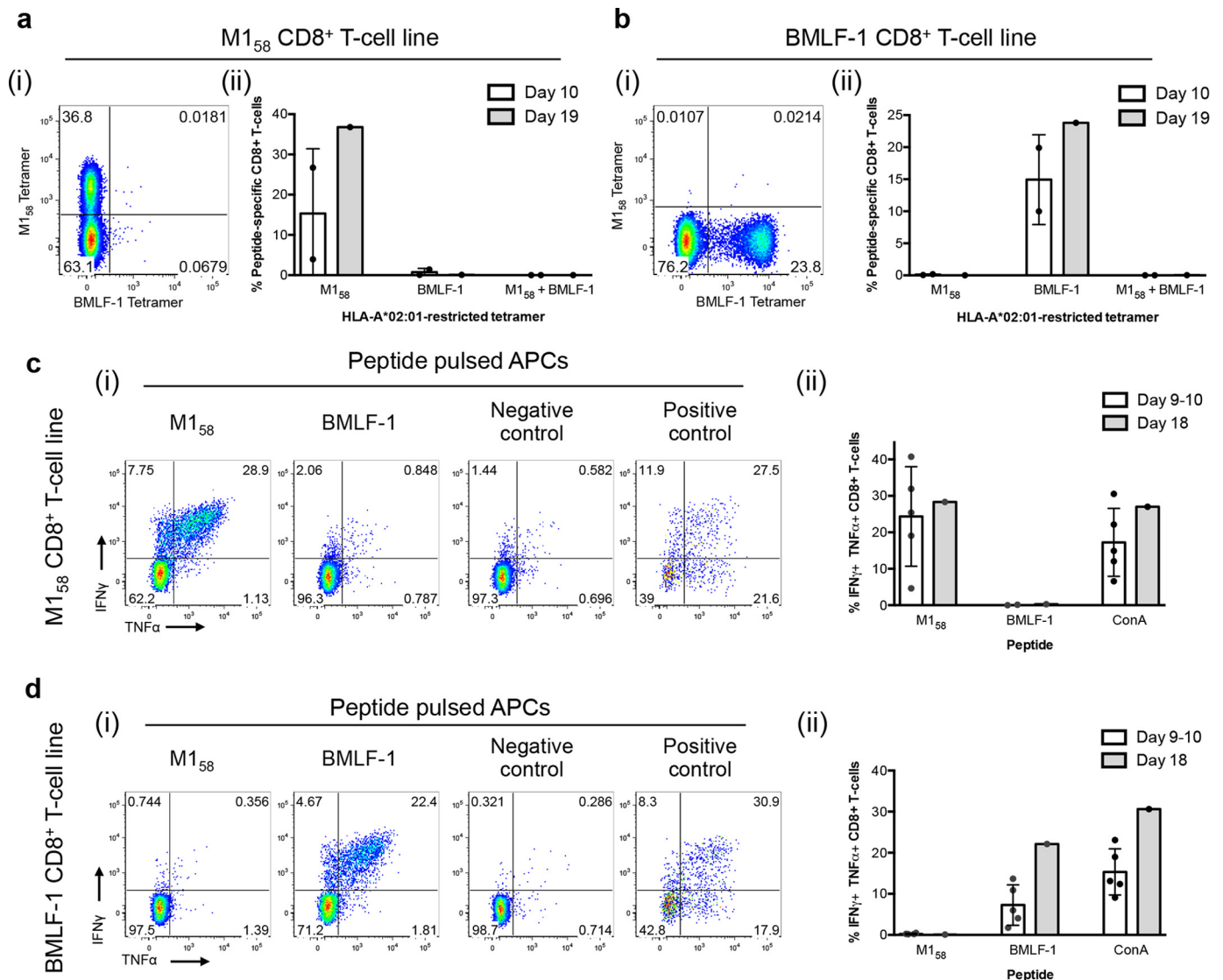


FIGURE 6. Lack of heterologous T cell cross-reactivity was observed following *in vitro* culture of PBMCs with either the M1₅₈ or BMLF-1 peptides. M1₅₈ and BMLF-1-specific CD8⁺ T cell lines (stimulated for 9–19 days) from healthy donors were assessed for heterologous cross-reactivity by tetramer co-staining (with PE and APC) and an IFN γ ⁺TNF α ⁺ ICS assay. All CD8⁺ T cell lines were antibody-stained (as described under “Experimental Procedures”), and the samples were gated on lymphocytes, singlets, CD3^{mid-high}, and CD8⁺ T cells. *a*, representative dot plot (donor H-13) (*panel i*) and summary (*panel ii*) of epitope-specific tetramer⁺ CD8⁺ T cells in M1₅₈-specific CD8⁺ T cell lines ($n = 3$). *b*, representative dot plot (donor H-13) (*panel i*) and summary (*panel ii*) of epitope-specific tetramer⁺ CD8⁺ T cells found in BMLF-1₂₈₀-specific CD8⁺ T cell lines ($n = 3$). *c*, representative dot plots (donor H-13) (*panel i*) and summary (*panel ii*) of IFN γ ⁺TNF α ⁺ production (no-peptide control subtracted) derived from M1₅₈-specific CD8⁺ T cell lines (APCs; $n = 6$) in response to each peptide and controls. *d*, representative dot plots (donor H-13) (*panel i*) and summary (*panel ii*) of IFN γ ⁺TNF α ⁺ production (no-peptide control subtracted) made by BMLF-1-specific CD8⁺ T cell lines (APCs; $n = 6$) in response to each peptide and controls. The *bar charts* represent the means with the *error bars* representing standard deviation.

highlight that the paired peptides are presented differently by HLA-A*02:01 and consequently elicit very different α BTCCR repertoires. Thus, it is unlikely that heterologous T cell cross-reactivity could be occurring at a high frequency in HLA-A*02:01-positive donors between those two peptide pairs.

Using a combination of *ex vivo* and *in vitro* techniques, including high sensitivity tetramer magnetic enrichment, we were unable to detect any heterologous memory CD8⁺ T cell cross-reactivity in healthy ($n = 12$) or HCV-infected ($n = 5$) donors. The prominent M1₅₈ and BMLF-1 CD8⁺ T cell subsets were identified in all donors directly *ex vivo* and expanded to high levels following *in vitro* amplification. Despite this, no heterologous T cell cross-reactivity was detected even following extensive *in vitro* amplification. In addition, because the NA

protein is variable between influenza strains, we tested a panel of common NA₂₃₁ variants in HCV-infected donors for heterologous T cell cross-reactivity with the NS3₁₀₇₃ peptide. However, no responses were detected against the WT and variant NA₂₃₁ peptides. Together, these data imply that heterologous T cell cross-reactivity is not occurring frequently toward these epitopes in both healthy and HCV-infected donors.

The precise reasons for the conflicting reports on human heterologous T cell cross-reactivity are unknown. However, differences in methodology of T cell culture, donor cohorts, directionality, and strain specificity of the viral infection might influence the results. In our study, we have chosen to avoid prolonged stimulation of CD8⁺ T cells or exposure to high concentration of numerous cytokines, to reproduce as closely

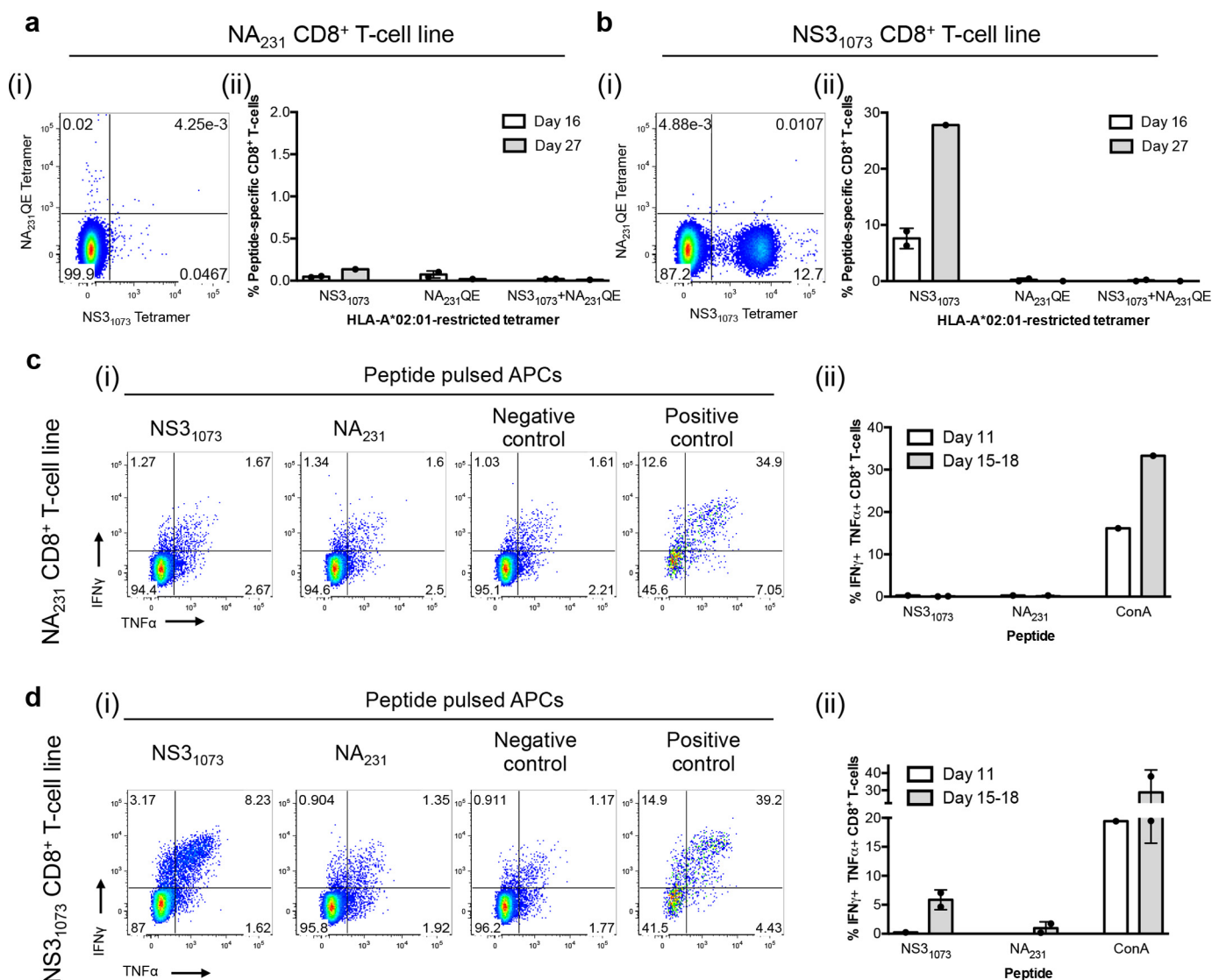


FIGURE 7. Lack of heterologous T cell cross-reactivity following *in vitro* culture of PBMCs from donors with chronic HCV infection stimulated with either NS3₁₀₇₃ or NA₂₃₁ peptides. PBMCs from HCV-infected individuals were stimulated (for 11–27 days) with NA₂₃₁ or NS3₁₀₇₃ peptides, and heterologous cross-reactivity was evaluated by tetramer co-staining and an IFN γ ⁺TNF α ⁺ ICS assay. The samples were stained (as described under “Experimental Procedures”) and gated on lymphocytes, singlets, CD3^{mid-high}, and CD8⁺ T cells. *a*, representative dot plot (donor HCV-2) (*panel i*) and summary (*panel ii*) of epitope-specific tetramer⁺ CD8⁺ T cells found in NA₂₃₁-stimulated CD8⁺ T cell lines ($n = 3$). *b*, representative dot plot (donor HCV-2) (*panel i*) and summary (*panel ii*) of epitope-specific tetramer⁺ CD8⁺ T cells found in NS3₁₀₇₃-specific CD8⁺ T cell lines ($n = 3$). *c*, representative dot plots (donor HCV-2) (*panel i*) and summary (*panel ii*) of IFN γ ⁺TNF α ⁺ production (no-peptide control subtracted) made by NA₂₃₁-stimulated CD8⁺ T cell lines (APCs; $n = 3$) in response to each peptide and controls. *d*, representative dot plots (donor HCV-2) (*panel i*) and summary (*panel ii*) of IFN γ ⁺TNF α ⁺ production (no-peptide control subtracted) derived from NS3₁₀₇₃-specific CD8⁺ T cell lines (APCs; $n = 3$) in response to each peptide and controls. The *bar charts* represent the means with the *error bars* representing standard deviation.

as possible a physiological CD8⁺ T cell response. If heterologous T cell cross-reactivity occurs only in extreme circumstances of prolonged and potent T cell stimulation *in vitro*, it is therefore unlikely to happen frequently *in vivo* in humans. As shown in the present study, NS3₁₀₇₃-specific CD8⁺ T cells were frequent in HCV⁺ donors but rare in healthy donors and with a naïve phenotype, and no cross-reactivity with NA₂₃₁ was observed. Conversely, Urbani *et al.* (22) were able to detect T cell cross-reactivity for the NA₂₃₁/NS3₁₀₇₃ pair, but only in a few HCV⁺ donors with severe liver pathology. The directionality of viral infection, viral strains, and acute infection could thus impact on the presence or absence of heterologous CD8⁺ T cell cross-reactivity.

Our data fit within the current published literature (22, 25, 26) and the lack of heterologous CD8⁺ T cell cross-reactivity

observed between the HLA-A*02:01-restricted epitopes (M1₅₈ with BMLF-1 and NA₂₃₁ with NS3₁₀₇₃) highlights that sequence similarity does not translate to structural mimicry and therefore does not lead to T cell cross-reactivity. In addition, limited sequence homology can lead to molecular mimicry, whereby different pMHC complexes can share structural features that will in turn be recognized by cross-reactive T cells (17, 42). Our data also fit with a recent study showing that TCRs are cross-reactive because of their “tolerance” to peptide changes (substitution with a similar residue or at a position not involved with TCR contact), leading to an overall molecular conservation (14). In conclusion, CD8⁺ T cells can display a high level of specificity for their pHLA complex because of their $\alpha\beta$ TCR, ensuring that autoimmunity is still a rare event within the population.

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

TABLE 5

NA₂₃₁ sequences found in human influenza viruses isolated from 1935 to 2014

Sequences for conservation analysis were obtained from the NCBI Influenza Research Database. Full-length sequences were obtained and were aligned using the IEDB Analysis Resource. All vaccine strains, sequences from viruses isolated within Oceania, and all pH1N1 sequences were chosen for analysis. The frequency of each variant is shown in the table; the underlined residues are those that vary from the WT NA₂₃₁ epitope.

Sequence	Mutant	Frequency		
		Vaccine	Oceania	pH1N1
CVNGSCFTV	WT	4	11	51
CINGTCTIVV	V2I + S5T + F7T + T8V	71	63	
CVNGSCFTI	V9I	21	20	47
CMNGSCFTI	V2M + V9I	4	3	>1
CINGSCFTI	V2I + V9I		>1	>1
CINGTCAVV	V2I + S5T + F7A + T8V		2	
CINRTCTIVV	V2I + G4R + S5T + F7T + T8V		>1	
CITYGTCTVV	V2I + N3Y + S5T + F7T + T8V		>1	
CVNGSCFTL	V9L			>1
CINGSCFTV	V2I			>1
CVNGSCFI	T8I + V9I			>1
CLNGSCFTV	V2L			>1
CVNGSCFIV	T8I			>1
CVNGSCFTM	V9M			>1
CANGSCFTV	V2A			>1
CVDGSCFTV	N3D			>1
CVHGSCFTV	N3H			>1
CVKGCSCFTV	N3K			>1
CVNGACFTV	S5A			>1
CVNGECFTV	S5F			>1
CVNGSYFTV	C6Y			>1
CVNGSCFAV	T8V			>1
CVNGSCFTT	V9T			>1
CANGSCFTI	V2A + V9I			>1
CVDGSCFTI	N3D + V9I			>1
CVNGECFTI	S5F + V9I			>1
CVNGPCFTI	S5P + V9I			>1
CLNGSCFTI	V2L + V9I			>1
Total # of sequences		28	376	2430
Total strain coverage		96%	95%	98%

Experimental Procedures

Protein Purification, Crystallization, and Structure Determination—Soluble HLA-A*02:01 containing the M1₅₈, BMLF-1, NA₂₃₁, or NS3₁₀₇₃ peptides were prepared as described previously (43, 44). Crystals of the HLA-A*02:01 in complex with the NA₂₃₁ peptide 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 using 20% PEG 6K, 0.2 M NaCl, 0.1 M sodium citrate, pH 6.5. Prior to being flash frozen in liquid nitrogen, the crystals were soaked in a cryoprotectant solution containing mother liquor solution with 30% (w/v) PEG. The data were collected at the Australian Synchrotron (Clayton, Australia) on the 3BM1 Beamline (ADSC-Quantum 210 CCD detector) (45), processed with XDS software (46), and scaled using XSCALE software (46). The molecular replacement was performed with the PHASER program (47) using a previously solved HLA-A*02:01 peptide structure as the search model (PDB code 3GSO (43)). Manual model building was conducted using the Coot software (48) followed by maximum-likelihood refinement with the PHENIX program (49). The final HLA-A*02:01-NA₂₃₁ model has been validated using the Protein Data Base validation web site (Table 1), and the coordinates have been submitted under the PDB code 5SWQ. All molecular graphics representations were created using PyMOL (50). Peptide sequence alignment was performed using the Clustalw

server, and the sequence homology encompasses identical and chemically similar residues.

Thermal Stability Assay—To assess the stability of each peptide within the HLA-A*02:01 molecule, a thermal shift assay was performed as previously described (30).

Human Samples—Buffy packs from healthy blood donors (seronegative for HIV, HBV, and HCV) were obtained from the Australian Red Cross Blood Service (West Melbourne, Australia). Blood from HCV-infected subjects was collected as a part of social network study of intravenous drug users (Burnet Institute, Melbourne, Australia (51)). HCV status was assessed via serology and the presence of viral RNA at the Victorian Infectious Diseases Reference Laboratory (Parkville, Australia). Samples were HLA-typed by the Victorian Transplant and Immunogenetics Service (West Melbourne, Australia) at the Australian Red Cross Blood Service tissue-typing laboratory. HLA-A*02:01-positive, HBV-negative, HIV-negative injected-drug users were selected. Donors used in this study are listed in Table 4. The experiments conformed to the NHMRC Code of Practice and were approved by the University of Melbourne Research Human Ethics Committee.

Separation of PBMCs from Whole Blood—PBMCs were separated from peripheral blood by density gradient centrifugation over Ficoll-Paque Plus (Sigma-Aldrich). Diluted blood was layered onto 15 ml of Ficoll-Paque Plus and centrifuged. PBMCs were collected from the interface of the Ficoll, washed three times, resuspended in freezing medium (10% dimethyl sulfoxide; Sigma-Aldrich) in heat-inactivated FCS (Bovogen Biological, Keilor East, Australia), and stored in liquid nitrogen.

Peptides and Tetramers—The following HLA-A*02:01-restricted peptides were purchased from Genscript: HCV-NS3_{1073–1081} and variant peptides (CINGVCWTV; CVNGVCWTV), influenza-NA_{231–239} WT and variant peptides (CVNGSCFTV; CVNGSCFTI; CINGSCFTI; CINGTCTVV), influenza-M1_{58–66} (GILGFVFTL), and BMLF-1 (GLCTLVAML). Wild type HLA-A*02:01 and CD8 β -binding HLA-A*02:01 mutant (Q115E; QE) (40) monomers were purchased from the Immuno ID Facility (Department of Microbiology and Immunology, University of Melbourne, Melbourne, Australia).

CD8⁺ T Cell Lines—PBMCs were thawed, washed, and rested for 1 h in RF10 (RPMI (Gibco) supplemented with 10–20% FCS, 5 mM HEPES (MP Biomedicals), 100 μ g/ml streptomycin (Gibco), 100 units/ml benzylpenicillin (CSL, Parkville, Australia), 2 mM L-glutamine (MP Biomedicals), 55 μ M β -mercaptoethanol (Sigma-Aldrich), and 100 mM non-essential amino acids (Gibco)). Samples were split into stimulators and responders at a ratio of 1:2 for healthy donors or 1:1 for HCV-infected individuals. Stimulators were pulsed with 10 μ g/ml peptide for 90 min, washed, and added to the responders. Media half changes were done twice weekly with RF10 supplemented with 10–20 units/ml of IL-2 with or without 50% T cell growth factor (supernatant from MLA44 cell). Cultures derived from HCV-infected individuals were supplemented with 25 units/ml IL-7 on day 3. The cultures were restimulated on day 7 and then weekly for CD8⁺ T cell lines at a 1:10–15 stimulators to responders ratio with C1R-HLA-A*02:01-expressing cells pulsed with 10 μ g/ml peptide for 60 min and gamma-irradiated at 8,000 rad.

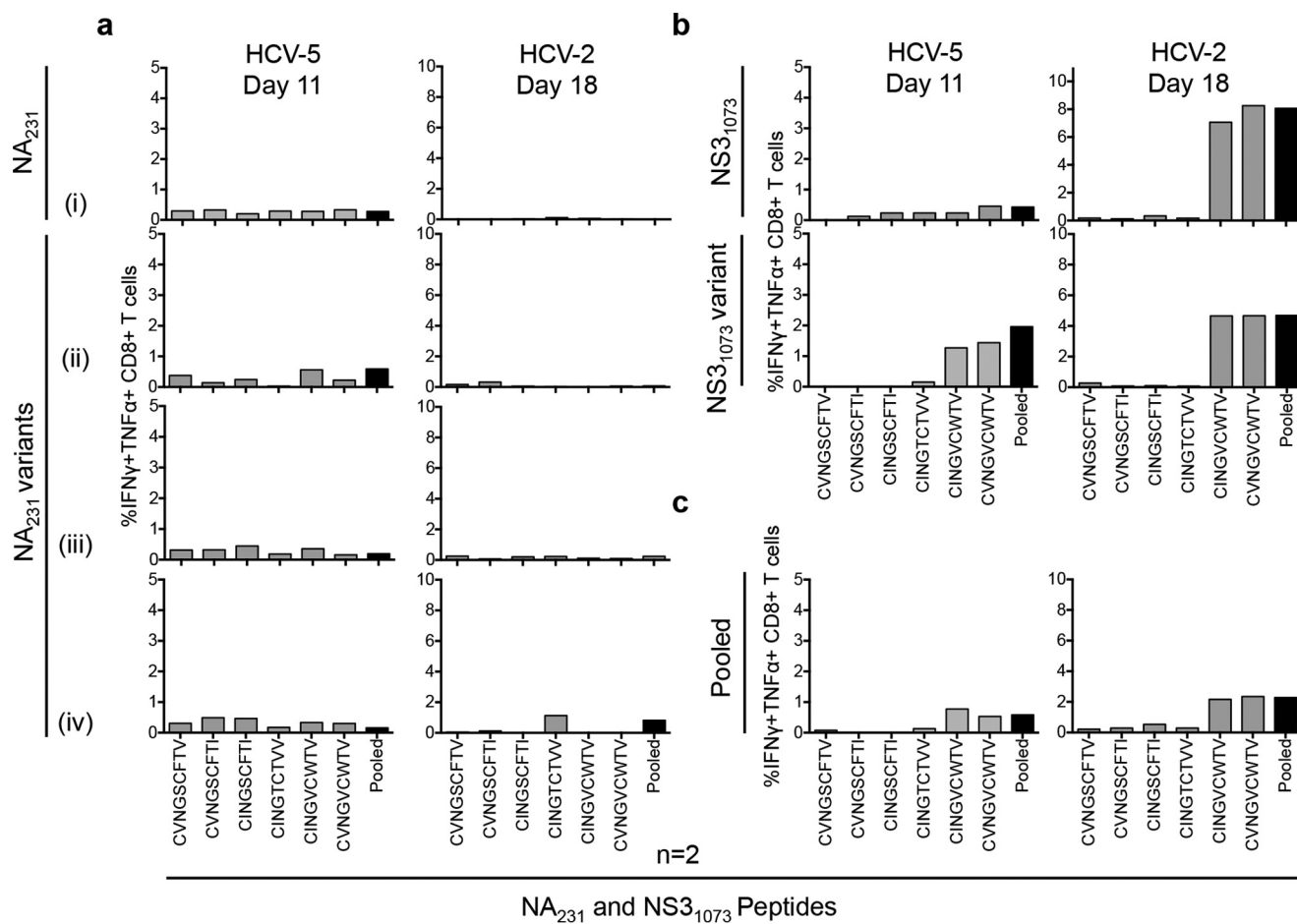


FIGURE 8. **PBMCs from individuals chronically infected with HCV do not expand to NA₂₃₁ variant peptides *in vitro*.** CD8⁺ T cell lines from donors with chronic HCV infection were raised against WT and variant NA₂₃₁ peptides, and WT or variant NS3₁₀₇₃ peptides, or a pool of all peptides for 11–18 days ($n = 2$). CD8⁺ T cell responses toward each individual peptide were assessed in an IFN γ ⁺TNF α ⁺ ICS assay. The cells were stained (as described under “Experimental Procedures”) and gated on lymphocytes, singlets, CD3^{mid-high}, and CD8⁺ T cells. *a*, proportion of IFN γ ⁺TNF α ⁺ production (no peptide control subtracted) by CD8⁺ T cells lines raised against NA₂₃₁-WT (CVNGSCFTV) (*panel i*), NA₂₃₁-I9 (CVNGSCFTI) (*panel ii*), NA₂₃₁-I2-I9 (CINGSCFTI) (*panel iii*), and NA₂₃₁-I2-T5-T7-V8 (CINGTCTVV) (*panel iv*) in response to each peptide. *b*, proportion of IFN γ ⁺TNF α ⁺ production toward each peptide (no peptide control subtracted) by CD8⁺ T cells lines raised against WT NS3₁₀₇₃ (CINGVCWTV) and NS3₁₀₇₃-V2 (CVNGVCWTV). *c*, proportion of IFN γ ⁺TNF α ⁺ production (no peptide control subtracted) by CD8⁺ T cells raised against pooled peptides, in response to each peptide.

Intracellular Cytokine Staining—CD8⁺ T cell lines were stimulated with C1R-HLA-A*02:01-expressing cells pulsed with 10 μ g/ml of peptide for 1 h at a ratio of 1:2 stimulators to responders in the presence of IL-2. The cells were incubated for 1 h and then supplemented with 10 μ g/ml brefeldin A (Sigma-Aldrich) and incubated for a further 5 h. Cells were then surface-stained with α CD3-PeCy7 and α CD8-PerCP (BD Biosciences) for 30 min and fixed in 1% paraformaldehyde (PFA; Electron Microscopy Sciences, Hatfield, PA) for 15 min. The cells were permeabilized with 0.3% saponin (Sigma) diluted in PBS and stained for internal cytokines α IFN γ (PE or FITC) and α TNF α (FITC or PE) overnight (BD Biosciences). The samples were acquired on the BD FACS Canto II (BD Biosciences) and analyzed using Flowjo software (Treestar).

Tetramer Staining—CD8⁺ T cell lines were stained with tetramers for 1 h at room temperature. The cells were subsequently surface-stained with α CD3-PeCy7, α CD8-PerCP/PerCPCy5.5, α CD27-APCH7, and α CD45RA-FITC (BD Biosciences) in PBS for 30 min, fixed in 1% PFA, acquired on the BD FACS Canto II, and analyzed using Flowjo software. The markers CD27, CCR7, and CD45RA were utilized to determine the

phenotype of epitope-specific cells. Naïve cells are CD27⁺ or CCR7⁺CD45RA⁺, whereas cells of all other combinations represent memory CD8⁺ T cells.

Magnetic Enrichment of Peptide-specific CD8⁺ T Cells *ex Vivo*—PBMCs were incubated in 20 μ l/1 \times 10⁷ cells FcR block (Miltenyi Biotech) for 15 min after thawing. Tetramer staining of 1 \times 10⁷ cells (for memory CD8⁺ T cells) or 5 \times 10⁷ cells (for naïve or low affinity CD8⁺ T cells) was performed at a 1:100 dilution of PE- or APC-conjugated tetramers in sorter buffer (PBS, 0.5% BSA (Gibco), 2 mM EDTA (Ajax Finechem)) for 1 h. The cells were incubated with 100 μ l of α PE and 100 μ l of α APC microbeads (Miltenyi Biotech) in 300 μ l of sorter buffer for 30 min. The cells were thoroughly washed, positively enriched by running twice over a LS magnetic column (Miltenyi Biotech), and then surface-stained with α CD3-PB α CD8-PerCP Cy5.5, α CD4-APCH7, and α CD45RA-FITC in combination with α CD27-AF700, α CD14-APCH7, and α CD19-APCH7 or α CCR7-APC and α CD14-PeCy7 (BD Biosciences, CA, USA) for 30 min. The samples were fixed with 1% PFA, acquired on the BD FACS LSR II (BD Biosciences), and analyzed using Flowjo software.

Heterologous CD8⁺ T Cell Cross-reactivity in Humans

Single-cell Sorting and Multiplex PCR—Epitope-specific CD8⁺ T cells were antibody-stained, and CD3⁺CD8⁺-tetramer⁺ cells were single-cell sorted directly into 96-well PCR plates (Eppendorf, Hamburg, Germany) using the BD FACS Aria II (BD Biosciences). cDNA was synthesized using the VILO RT kit (Invitrogen). Multiplex nested PCR was performed as previously described (34, 52). Briefly, the external round was performed with 40 V α -external and 27 V β -external primers and the C α -external and C β external primers. The internal PCR rounds were performed individually with the 40 V α -internal and C α -internal or the 27 V β -internal and the C β -internal primers (34, 52). PCR products were detected on a 2% agarose gel and purified using Exosap (Affymetrix) or Exostar (GE Healthcare). The sequencing reaction was performed using the C-internal primer and BigDyeV3.1 (Applied Biosystems) and cleaned on DyeEx sequencing plates (Qiagen). Sequencing was performed by the Pathology Department at the University of Melbourne (Melbourne, Australia). The sequences were analyzed using FinchTV, and V and J region usage was identified by IMGT query (53). CDR3 amino acid sequences described within the manuscript are productive (without stop codons and with an in-frame junction) $\alpha\beta$ TCR pairs and start from CDR3 position 1, as determined by the IMGT software. CDR3 length and motifs are from position 4. Samples with two α or β chains were dissected by performing an internal round of PCR with the specific individual primers and then sequenced as above. Where possible, both chains were resolved; however, in a few instances only the dominant chain could be resolved. Samples with TRBV19 (M1₅₈⁺CD8⁺ T cell repertoire) had the internal round of PCR repeated with TRBV19 and were subsequently sequenced with the TRBV19 forward primer. IMGT nomenclature was used (54). CDR3 motif conservation analysis (Fig. 8) was generated using the Seq2Logo 2.0 server (55) using the Shannon logo type, no clustering, 0 weight on prior, and seq2logo default amino acid color settings.

Author Contributions—E. J. G., T. M. J., S. A. V., J. R., M. B., S. G., and K. K. designed the research; E. J. G., T. M. J., S. A. V., S. G., and K. K. performed the research; M. H., M. B., L. W., and J. R. contributed new reagents/analytic tools; E. J. G., T. M. J., S. A. V., S. G., and K. K. analyzed the data; and E. J. G., T. M. J., J. R., S. G., and K. K. wrote the paper.

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References

1. Chothia, C., Boswell, D. R., and Lesk, A. M. (1988) The outline structure of the T-cell $\alpha\beta$ receptor. *EMBO J.* **7**, 3745–3755
2. Rossjohn, J., Gras, S., Miles, J. J., Turner, S. J., Godfrey, D. I., and McCluskey, J. (2015) T cell antigen receptor recognition of antigen-presenting molecules. *Annu. Rev. Immunol.* **33**, 169–200
3. Jerne, N. K. (1955) The natural-selection theory of antibody formation. *Proc. Natl. Acad. Sci. U.S.A.* **41**, 849–857
4. Mason, D. (1998) A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunol. Today* **19**, 395–404
5. Valkenburg, S. A., Gras, S., Guillonnet, C., La Gruta, N. L., Thomas, P. G., Purcell, A. W., Rossjohn, J., Doherty, P. C., Turner, S. J., and Kedzierska, K. (2010) Protective efficacy of cross-reactive CD8⁺ T cells recognising mutant viral epitopes depends on peptide-MHC-I structural interactions and T cell activation threshold. *PLoS Pathogens* **6**, e1001039
6. Kedzierska, K., Guillonnet, C., Gras, S., Hatton, L. A., Webby, R., Purcell, A. W., Rossjohn, J., Doherty, P. C., and Turner, S. J. (2008) Complete modification of TCR specificity and repertoire selection does not perturb a CD8⁺ T cell immunodominance hierarchy. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 19408–19413
7. Wooldridge, L., Ekeruche-Makinde, J., van den Berg, H. A., Skowera, A., Miles, J. J., Tan, M. P., Dolton, G., Clement, M., Llewellyn-Lacey, S., Price, D. A., Peakman, M., and Sewell, A. K. (2012) A single autoimmune T cell receptor recognizes more than a million different peptides. *J. Biol. Chem.* **287**, 1168–1177
8. Brehm, M. A., Pinto, A. K., Daniels, K. A., Schneck, J. P., Welsh, R. M., and Selin, L. K. (2002) T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nat. Immunol.* **3**, 627–634
9. Chen, A. T., Cornberg, M., Gras, S., Guillonnet, C., Rossjohn, J., Trees, A., Emonet, S., de la Torre, J. C., Welsh, R. M., and Selin, L. K. (2012) Loss of anti-viral immunity by infection with a virus encoding a cross-reactive pathogenic epitope. *PLoS Pathogens* **8**, e1002633
10. Chen, H. D., Fraire, A. E., Joris, I., Brehm, M. A., Welsh, R. M., and Selin, L. K. (2001) Memory CD8⁺ T cells in heterologous antiviral immunity and immunopathology in the lung. *Nat. Immunol.* **2**, 1067–1076
11. Kim, S. K., Cornberg, M., Wang, X. Z., Chen, H. D., Selin, L. K., and Welsh, R. M. (2005) Private specificities of CD8 T cell responses control patterns of heterologous immunity. *J. Exp. Med.* **201**, 523–533
12. Selin, L. K., Varga, S. M., Wong, I. C., and Welsh, R. M. (1998) Protective heterologous antiviral immunity and enhanced immunopathogenesis mediated by memory T cell populations. *J. Exp. Med.* **188**, 1705–1715
13. Shen, Z. T., Nguyen, T. T., Daniels, K. A., Welsh, R. M., and Stern, L. J. (2013) Disparate epitopes mediating protective heterologous immunity to unrelated viruses share peptide-MHC structural features recognized by cross-reactive T cells. *J. Immunol.* **191**, 5139–5152
14. Birnbaum, M. E., Mendoza, J. L., Sethi, D. K., Dong, S., Glanville, J., Dobbins, J., Ozkan, E., Davis, M. M., Wucherpfennig, K. W., and Garcia, K. C. (2014) Deconstructing the peptide-MHC specificity of T cell recognition. *Cell* **157**, 1073–1087
15. Colf, L. A., Bankovich, A. J., Hanick, N. A., Bowerman, N. A., Jones, L. L., Kranz, D. M., and Garcia, K. C. (2007) How a single T cell receptor recognizes both self and foreign MHC. *Cell* **129**, 135–146
16. Garcia, K. C., Degano, M., Pease, L. R., Huang, M., Peterson, P. A., Teyton, L., and Wilson, I. A. (1998) Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science* **279**, 1166–1172
17. Macdonald, W. A., Chen, Z., Gras, S., Archbold, J. K., Tynan, F. E., Clements, C. S., Bharadwaj, M., Kjer-Nielsen, L., Saunders, P. M., Wilce, M. C., Crawford, F., Stadinsky, B., Jackson, D., Brooks, A. G., Purcell, A. W., et al. (2009) T cell allorecognition via molecular mimicry. *Immunity* **31**, 897–908
18. Yin, L., Huseby, E., Scott-Browne, J., Rubtsova, K., Pinilla, C., Crawford, F., Marrack, P., Dai, S., and Kappler, J. W. (2011) A single T cell receptor bound to major histocompatibility complex class I and class II glycoproteins reveals switchable TCR conformers. *Immunity* **35**, 23–33
19. Clute, S. C., Naumov, Y. N., Watkin, L. B., Aslan, N., Sullivan, J. L., Thorley-Lawson, D. A., Luzuriaga, K., Welsh, R. M., Puzone, R., Celada, F., and Selin, L. K. (2010) Broad cross-reactive TCR repertoires recognizing dissimilar Epstein-Barr and influenza A virus epitopes. *J. Immunol.* **185**, 6753–6764
20. Clute, S. C., Watkin, L. B., Cornberg, M., Naumov, Y. N., Sullivan, J. L., Luzuriaga, K., Welsh, R. M., and Selin, L. K. (2005) Cross-reactive influenza virus-specific CD8⁺ T cells contribute to lymphoproliferation in Epstein-Barr virus-associated infectious mononucleosis. *J. Clin. Invest.* **115**, 3602–3612
21. Odumade, O. A., Knight, J. A., Schmeling, D. O., Masopust, D., Balfour, H. H., Jr, and Hogquist, K. A. (2012) Primary Epstein-Barr virus infection does not erode preexisting CD8⁺ T cell memory in humans. *J. Exp. Med.* **209**, 471–478

22. Urbani, S., Amadei, B., Fiscaro, P., Pilli, M., Missale, G., Bertoletti, A., and Ferrari, C. (2005) Heterologous T cell immunity in severe hepatitis C virus infection. *J. Exp. Med.* **201**, 675–680
23. Kasprócz, V., Ward, S. M., Turner, A., Grammatikos, A., Nolan, B. E., Lewis-Ximenez, L., Sharp, C., Woodruff, J., Fleming, V. M., Sims, S., Walker, B. D., Sewell, A. K., Lauer, G. M., and Klennerman, P. (2008) Defining the directionality and quality of influenza virus-specific CD8⁺ T cell cross-reactivity in individuals infected with hepatitis C virus. *J. Clin. Invest.* **118**, 1143–1153
24. Wedemeyer, H., Mizukoshi, E., Davis, A. R., Bennink, J. R., and Rehermann, B. (2001) Cross-reactivity between hepatitis C virus and Influenza A virus determinant-specific cytotoxic T cells. *J. Virol.* **75**, 11392–11400
25. Neveu, B., Debeauvais, E., Echasserieau, K., le Moullac-Vaidye, B., Gassin, M., Jegou, L., Decalf, J., Albert, M., Ferry, N., Gournay, J., Houssaint, E., Bonneville, M., and Saulquin, X. (2008) Selection of high-avidity CD8 T cells correlates with control of hepatitis C virus infection. *Hepatology* **48**, 713–722
26. Pasetto, A., Frelin, L., Brass, A., Yasmeen, A., Koh, S., Lohmann, V., Bartenschlager, R., Magalhaes, I., Maeurer, M., Sällberg, M., and Chen, M. (2012) Generation of T-cell receptors targeting a genetically stable and immunodominant cytotoxic T-lymphocyte epitope within hepatitis C virus non-structural protein 3. *J. Gen. Virol.* **93**, 247–258
27. Reiser, J. B., Legoux, F., Gras, S., Trudel, E., Chouquet, A., Léger, A., Le Gorrec, M., Machillot, P., Bonneville, M., Saulquin, X., and Housset, D. (2014) Analysis of relationships between peptide/MHC structural features and naive T cell frequency in humans. *J. Immunol.* **193**, 5816–5826
28. Selin, L. K., Brehm, M. A., Naumov, Y. N., Cornberg, M., Kim, S. K., Clute, S. C., and Welsh, R. M. (2006) Memory of mice and men: CD8⁺ T-cell cross-reactivity and heterologous immunity. *Immunol. Rev.* **211**, 164–181
29. Ishizuka, J., Stewart-Jones, G. B., van der Merwe, A., Bell, J. I., McMichael, A. J., and Jones, E. Y. (2008) The structural dynamics and energetics of an immunodominant T cell receptor are programmed by its V β domain. *Immunity* **28**, 171–182
30. Valkenburg, S. A., Josephs, T. M., Clemens, E. B., Grant, E. J., Nguyen, T. H., Wang, G. C., Price, D. A., Miller, A., Tong, S. Y., Thomas, P. G., Doherty, P. C., Rossjohn, J., Gras, S., and Kedzierska, K. (2016) Molecular basis for universal HLA-A*0201-restricted CD8⁺ T-cell immunity against influenza viruses. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 4440–4445
31. Stewart-Jones, G. B., McMichael, A. J., Bell, J. I., Stuart, D. I., and Jones, E. Y. (2003) A structural basis for immunodominant human T cell receptor recognition. *Nat. Immunol.* **4**, 657–663
32. Liu, Y. C., Chen, Z., Neller, M. A., Miles, J. J., Purcell, A. W., McCluskey, J., Burrows, S. R., Rossjohn, J., and Gras, S. (2014) A molecular basis for the interplay between T cells, viral mutants, and human leukocyte antigen micropolymorphism. *J. Biol. Chem.* **289**, 16688–16698
33. Miles, J. J., Borg, N. A., Brennan, R. M., Tynan, F. E., Kjer-Nielsen, L., Silins, S. L., Bell, M. J., Burrows, J. M., McCluskey, J., Rossjohn, J., and Burrows, S. R. (2006) TCR α genes direct MHC restriction in the potent human T cell response to a class I-bound viral epitope. *J. Immunol.* **177**, 6804–6814
34. Nguyen, T. H., Rowntree, L. C., Pellicci, D. G., Bird, N. L., Handel, A., Kjer-Nielsen, L., Kedzierska, K., Kotsimbos, T. C., and Mifsud, N. A. (2014) Recognition of distinct cross-reactive virus-specific CD8⁺ T cells reveals a unique TCR signature in a clinical setting. *J. Immunol.* **192**, 5039–5049
35. Lehner, P. J., Wang, E. C., Moss, P. A., Williams, S., Platt, K., Friedman, S. M., Bell, J. I., and Borysiewicz, L. K. (1995) Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V β 17 gene segment. *J. Exp. Med.* **181**, 79–91
36. Moss, P. A., Moots, R. J., Rosenberg, W. M., Rowland-Jones, S. J., Bodmer, H. C., McMichael, A. J., and Bell, J. I. (1991) Extensive conservation of α and β chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8987–8990
37. Miles, J. J., Bulek, A. M., Cole, D. K., Gostick, E., Schauenburg, A. J., Dolton, G., Venturi, V., Davenport, M. P., Tan, M. P., Burrows, S. R., Woodruff, L., Price, D. A., Rizkallah, P. J., and Sewell, A. K. (2010) Genetic and structural basis for selection of a ubiquitous T cell receptor deployed in Epstein-Barr virus infection. *PLoS Pathogens* **6**, e1001198
38. Valkenburg, S. A., Gras, S., Guillonau, C., Hatton, L. A., Bird, N. A., Twist, K. A., Halim, H., Jackson, D. C., Purcell, A. W., Turner, S. J., Doherty, P. C., Rossjohn, J., and Kedzierska, K. (2013) Preemptive priming readily overcomes structure-based mechanisms of virus escape. *Proc. Natl. Acad. Sci. U.S.A.* **110**, 5570–5575
39. Alanio, C., Lemaitre, F., Law, H. K., Hasan, M., and Albert, M. L. (2010) Enumeration of human antigen-specific naive CD8⁺ T cells reveals conserved precursor frequencies. *Blood* **115**, 3718–3725
40. Woodruff, L., Lissina, A., Vernazza, J., Gostick, E., Laugel, B., Hutchinson, S. L., Mirza, F., Dunbar, P. R., Boulter, J. M., Glick, M., Cerundolo, V., van den Berg, H. A., Price, D. A., and Sewell, A. K. (2007) Enhanced immunogenicity of CTL antigens through mutation of the CD8 binding MHC class I invariant region. *Eur. J. Immunol.* **37**, 1323–1333
41. Selin, L. K., Włodarczyk, M. F., Kraft, A. R., Nie, S., Kenney, L. L., Puzone, R., and Celada, F. (2011) Heterologous immunity: immunopathology, autoimmunity and protection during viral infections. *Autoimmunity* **44**, 328–347
42. Adams, J. J., Narayanan, S., Birnbaum, M. E., Sidhu, S. S., Blevins, S. J., Gee, M. H., Sibener, L. V., Baker, B. M., Kranz, D. M., and Garcia, K. C. (2016) Structural interplay between germline interactions and adaptive recognition determines the bandwidth of TCR-peptide-MHC cross-reactivity. *Nat. Immunol.* **17**, 87–94
43. Gras, S., Saulquin, X., Reiser, J. B., Debeauvais, E., Echasserieau, K., Kissenpennig, A., Legoux, F., Chouquet, A., Le Gorrec, M., Machillot, P., Neveu, B., Thielens, N., Malissen, B., Bonneville, M., and Housset, D. (2009) Structural bases for the affinity-driven selection of a public TCR against a dominant human cytomegalovirus epitope. *J. Immunol.* **183**, 430–437
44. Gras, S., Kedzierski, L., Valkenburg, S. A., Laurie, K., Liu, Y. C., Denholm, J. T., Richards, M. J., Rimmelzwaan, G. F., Kelso, A., Doherty, P. C., Turner, S. J., Rossjohn, J., and Kedzierska, K. (2010) Cross-reactive CD8⁺ T-cell immunity between the pandemic H1N1–2009 and H1N1–1918 influenza A viruses. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 12599–12604
45. Cowieson, N. P., Aragao, D., Clift, M., Ericsson, D. J., Gee, C., Harrop, S. J., Mudie, N., Panjikar, S., Price, J. R., Riboldi-Tunncliffe, A., Williamson, R., and Caradoc-Davies, T. (2015) MX1: a bending-magnet crystallography beamline serving both chemical and macromolecular crystallography communities at the Australian Synchrotron. *J. Synchrotron Radiat.* **22**, 187–190
46. Kabsch, W. (2010) XDS. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 125–132
47. Read, R. J. (2001) Pushing the boundaries of molecular replacement with maximum likelihood. *Acta Crystallogr. D Biol. Crystallogr.* **57**, 1373–1382
48. Emsley, P., and Cowtan, K. (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 2126–2132
49. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., et al. (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 213–221
50. DeLano, W. L. (2002) *The PyMOL Molecular Graphics System*, DeLano Scientific, Palo Alto, CA
51. Aitken, C. K., Lewis, J., Tracy, S. L., Spelman, T., Bowden, D. S., Bharadwaj, M., Drummer, H., and Hellard, M. (2008) High incidence of hepatitis C virus reinfection in a cohort of injecting drug users. *Hepatology* **48**, 1746–1752
52. Wang, G. C., Dash, P., McCullers, J. A., Doherty, P. C., and Thomas, P. G. (2012) T cell receptor $\alpha\beta$ diversity inversely correlates with pathogen-specific antibody levels in human cytomegalovirus infection. *Sci. Trans. Med.* **4**, 128ra142
53. Brochet, X., Lefranc, M. P., and Giudicelli, V. (2008) IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. *Nucleic Acids Res.* **36**, W503–W508
54. Lefranc, M. P. (2011) IMGT, the International ImmunoGeneTics Information System. *Cold Spring Harbor Protocols* **2011**, 595–603
55. Thomsen, M. C., and Nielsen, M. (2012) Seq2Logo: a method for construction and visualization of amino acid binding motifs and sequence profiles including sequence weighting, pseudo counts and two-sided representation of amino acid enrichment and depletion. *Nucleic Acids Res.* **40**, W281–W287

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