Broad CD8+ T cell cross-recognition of distinct influenza A strains in humans

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Newly-emerged and vaccine-mismatched influenza A viruses (IAVs) result in a rapid global spread of the virus due to minimal antibody-mediated immunity. In that case, established CD8+ T-cells can reduce disease severity. However, as mutations occur sporadically within immunogenic IAV-derived T-cell peptides, understanding of T-cell receptor (TCRαβ) cross-reactivity towards IAV variants is needed for a vaccine design. Here, we investigate TCRαβ cross-strain recognition across IAV variants within two immunodominant human IAV-specific CD8+ T-cell epitopes, HLA-B*37:01-restricted NP338-346 (B37-NP338) and HLA-A*01:01-restricted NP44-52 (A1-NP44). We find high abundance of cross-reactive TCRαβ clonotypes recognizing distinct IAV variants. Structures of the wild-type and variant peptides revealed preserved conformation of the bound peptides. Structures of a cross-reactive TCR-HLA-B37-NP338 complex suggest that the conserved conformation of the variants underpins TCR cross-reactivity. Overall, cross-reactive CD8+ T-cell responses, underpinned by conserved epitope structure, facilitates recognition of distinct IAV variants, thus CD8+ T-cell-targeted vaccines could provide protection across different IAV strains.
Influenza A viruses (IAVs) rapidly evolve and cause significant morbidity and mortality (reviewed in refs 1, 2). Annual epidemics are responsible for >500,000 deaths worldwide3, while pandemic IAVs can cause >50 million deaths (reviewed in ref. 4). Although vaccines are available, they primarily induce neutralizing antibodies directed towards the rapidly mutating surface glycoproteins, rather than cross-reactive CD8+ T cell immunity1,2, mandating that these vaccines are updated and administered annually (reviewed in ref. 5). Furthermore, these vaccines are fallible when the circulating strains do not match the predicted vaccine strains7 or in a scenario when a novel viral subtype enters the population. Thus there is an urgent need to understand correlates of T cell protection towards IAV to provide effective influenza vaccine design.

In the absence of neutralizing antibodies, strain cross-reactive CD8+ T cells can protect against IAVs. Murine studies show that CD8+ T cells correlate with decreased morbidity and mortality following IAV infection9–12 and can provide protection during infection with heterosubtypic IAV strains11,13–15. Human studies are consistent with murine data. Namely, published evidence shows that prominence of influenza-specific CD8+ T cells correlates with lower viral titers16 and decreased disease severity17–19 during IAV infection. Furthermore, CD8+ T cells primed with seasonal circulating IAV strains can cross-react with pandemic H1N1 (pH1N1) or variant seasonal peptides20–22 or virulent H7N9 and H5N1 avian IAV-derived peptides23–26. Together, these data suggest that an IAV-specific CD8+ T cell-mediated vaccine can provide broad cross-reactive immunity across distinct influenza A strains and subtypes for both conserved and variable CD8+ T cell epitopes.

It is well established that CD8+ T cells with diverse T cell receptor (TCR) repertoires are greatly beneficial for disease outcome, contributing to reduced disease severity27, enhanced CD8+ T cell function28, cross-reactivity across different peptide variants29,30, and preventing viral escape31,32. Importantly, although CD8+ TCRs are typically highly specific for their cognate peptide, they can also recognize a broad range of peptide variants, thus allowing CD8+ T cells to have a powerful capacity to recognize not only their cognate peptide but also a range of viral mutants11,30,33–36. In case of highly mutating influenza viruses, such cross-reactive CD8+ T cells are highly desirable as they elicit immune responses towards multiple viral strains and hence provide cross-strain protection.

The precise mechanisms underlying cross-recognition by influenza-specific CD8+ TCRs in humans are unclear. To date, TCRβ repertoires have only been dissected for two immunodominant influenza-specific human epitopes, HLA-A*02:01-restricted M131 and preventing viral escape31,32. Importantly, although CD8+ TCRs are typically highly specific for their cognate peptide, they can also recognize a broad range of peptide variants, thus allowing CD8+ T cells to have a powerful capacity to recognize not only their cognate peptide but also a range of viral mutants11,30,33–36. In case of highly mutating influenza viruses, such cross-reactive CD8+ T cells are highly desirable as they elicit immune responses towards multiple viral strains and hence provide cross-strain protection.

Here we use an ex vivo multiplex reverse transcription polymerase chain reaction (RT-PCR) approach30,37,38 to analyze paired TCRβ repertoires for two additional prominent human CD8+ T cell epitopes, HLA-B*37:01-restricted NP338–346- FEDLRVLSF peptide39,40 and HLA-A*01:01-restricted NP344–352 CTELKLSDY peptide39,40, restricted by alleles that are frequent in the human population (~19% of the cumulative coverage). We identify cross-reactive TCRβ clonotypes capable of recognizing the wild-type (WT) peptide and peptide variants. This is most prominent in HLA-B*37:01-expressing donors, where distinct and cross-reactive NP338-specific TCRβ clonotypes bound each of the NP338 WT, NP338-L7S, and NP338-V6L variants (93–100% of distinct IAV strains), highlighting their potential to provide protection against distinct influenza strains and subtypes. Our structural analysis reveals that the variants adopt a similar conformation than the WT epitope for both HLA-A*01:01 (HLA-A1) and HLA-B*37:01 (HLA-B37) molecules, providing a molecular basis for CD8+ TCRβ cross-reactivity. Structural analysis indicates that molecular similarity may underpin how the HLA-B37-restricted cross-reactive TCRβ clone EM2, can recognize the variants. Thus our data suggest that structural resemblance underpins cross-reactivity of HLA-B37+NP338+CD8+ and HLA-A1+NP44+CD8+ T cells, despite their diverse TCR repertoires between individuals towards those two epitopes.

### Results

Only HLA-B37+ donors elicit a NP338+CD8+ T cell response.

Our previous work identified NP338 as an immunodominant CD8+ T cell epitope in individuals expressing HLA-B3759. However, NP338 was previously reported to be restricted by HLA-B*44:0341. Thus we first investigated whether the NP338 peptide can be presented by other HLA allomorphs using the online SYFPEITHI peptide-binding prediction tool42. A high prediction binding score for the NP338 peptide was obtained for HLA-B*37, HLA-B*44:02, and HLA-B*18:01 (HLA-B18) (Table 1). The SYFPEITHI website contains only the HLA-B*44:02 in its database but not the closely related allomorphs, such as HLA-B*44:03 and HLA-B*44:0543. The high prediction binding score for the NP338 peptide obtained for HLA-B*44:02 is likely to be shared by HLA-B*44:03 and HLA-B*44:05 (HLA-B44), given their known overlapping peptide repertoire44. Both HLA-B44:44–47 and HLA-B18:46–48 display a preference for P2-E and P3-F/Y in their bound peptides, and both residues are present in the IAV-derived NP338 peptide.

We first refolded the NP338 peptide with HLA-B37, HLA-B18, and HLA-B44 molecules and assessed the stability of each peptide–HLA (pHLA) complex. We used HLA-B*44:05 molecule as the refold yield was higher for this allomorph than for the HLA-B*44:02/03. The NP338 peptide could bind each of the three HLA molecules (HLA-B37, -B18, and -B*44:05); however, the stability of HLA-B37-NP338 was superior by 6°C to HLA-B18-NP338 and by 14°C to HLA-B*44:05-NP338 (Supplementary Table 1).

We then solved the binary structures of the NP338 peptide presented by HLA-B37, HLA-B18, and HLA-B44 (Supplementary Table 2, Fig. 1). The NP338 peptide adopted an extended conformation in the cleft of HLA-B37, with the P2-Glu and P9-Arg acting as anchor residues in the B- and F-pockets of HLA-B37, respectively49. In addition to the primary anchor residues, the P5-Arg acted as a secondary anchor residue, fully buried into the F-pocket, and formed a salt bridge with Asp77 from the α1-helix in the HLA-B37 molecule (Fig. 1a). The remaining six residues were...
all solvent exposed and represented potential contacts for the TCR. Comparison of the HLA-B37-NP338 with HLA-B18-NP338 and HLA-B44-NP338 complexes show an overall similar HLA-binding cleft (Fig. 1d), with a root mean square deviation (r.m.s. d.) of 0.2 and 0.4 Å, respectively. Similar to the HLA-B37-NP338 structure, the NP338 peptide was anchored to HLA-B18 and HLA-B44 by P2-Glu and P9-Phe. However, the NP338 peptide adopted a strikingly distinct conformation when bound to HLA-B37 as compared with HLA-B18 (r.m.s.d. of the peptide 1.7 Å) and HLA-B44 (r.m.s.d. of 1.8 Å for the peptide). In HLA-B18, P5-Arg is partially buried in the D-/E-pocket and only exposed its guanidinium group to the solvent, stabilized by a salt bridge with the peptide P3-Asp (Fig. 1b). Polymorphism at position 116 underneath P5-Arg, that only shares Tyr74. The additional anchor residue observed in the B37-NP338 complex could explain the higher stability of this pHLA complex (Supplementary Table 1). These distinct conformations adopted by the NP338 peptide, due to HLA polymorphism, changed the surface presented to the TCR. On one hand, the NP338 peptide revealed a hydrophobic surface when bound to HLA-B37, while on the other, it was an epitope with central positively charged residues in the cleft of HLA-B18 and HLA-B44.

**Fig. 1** NP338 elicits robust CD8+ T cell responses only in the context of HLA-B*37:01. **a-d** Crystal structures of a HLA-B*37:01 (purple), **b** HLA-B*18:01 (blue), and **c** HLA-B*44:05 (yellow) in complex with NP338 WT (represented in black stick). **d** is showing an overlay of the three peptide-HLA complexes in cartoon representation, with NP338 colored accordingly to the bound HLA, namely, purple (HLA-B*37:01), blue (HLA-B*18:01), and yellow (HLA-B*44:05).

**e-g** PBMCs from HLA-B*37:01 donors (n = 5), HLA-B*44:02* donors (n = 5), HLA-B*44:03* donors (n = 5), or HLA-B*18:01 donors (n = 5) were stimulated with a pool of NP338 peptides (10 μM each peptide, 30 μM total), and specificity was assessed in an ICS assay against 3 μM NP338 pool (1 μM each peptide, HLA-B*44:02* donors) directly into the well or 30 μM (10 μM each peptide, all other donors) NP338-pooled pulsed APC lines (HLA-B*37:01, HLA-B*44:03, and HLA-B*18:01). CD8+ T cells are gated on lymphocytes, singlets, live (when stained with the second antibody cocktail only) CD3mid-high, CD8+ T cells; as per Supplementary Fig. 2a. Representative dot plots of IFN-α, IFN-γ, and TNF-α production by pooled CD8+ T cell lines derived from HLA-B*37:01+, HLA-B*44:02+, HLA-B*44:03+, or HLA-B*18:01+ donors, towards a pool of NP338 peptides. No peptide control represented in brackets. Summary of IFN-γ, TNF-α, and CD107a production, minus no peptide controls, against the pool of NP338 peptides (closed circle) or a positive control (ConA open square or PMA closed square) across multiple donors. Statistical analysis using a Dunnett’s two-way ANOVA in which *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001. g Summary of the polyfunctional profiles, minus background, from the four HLA-B*37:01+ donors who responded towards the pool of NP338 peptides.
We next probed memory NP$_{338}$$^{+}$CD8$^{+}$ T cell responses in individuals expressing HLA-B*37:01, HLA-B*44:02, HLA-B*44:03, or HLA-B*18:01 (Fig. 1e–g, Supplementary Table 3) by stimulating their peripheral blood mononuclear cells (PBMCs) with a pool of NP$_{338}$ peptides corresponding to the main variants in the circulating influenza A strains (Table 2). Following 10–16 days of peptide stimulation, we detected robust CD8$^{+}$ T cell responses by interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα) production as well as CD107a expression in the majority (4/5) of HLA-B37$^{+}$ donors (Fig. 1f, g), with a high level of polyfunctionality (Fig. 1g). However, NP$_{338}$$^{+}$ CD8$^{+}$ T cell responses were undetectable in all (5/5) HLA-B*44:02, HLA-B*44:03, or HLA-B*18:01 individuals (Fig. 1f, g), despite their CD8$^{+}$ T cells having responded strongly to non-specific stimulation by phorbol 12-myristate 13-acetate or Concanaavalin A (Fig. 1f).

Overall, these data suggest that HLA-B37, but not HLA-B44 or HLA-B18, represents the HLA restriction for the NP$_{338}$ peptide. The lack of NP$_{338}$$^{+}$CD8$^{+}$ T cell responses in the context of HLA-B44 and HLA-B18 might be due to the distinct conformations, or lower stability, of NP$_{338}$ as compared to HLA-B37.

**Cross-reactive NP$_{338}$$^{+}$CD8$^{+}$ T cells towards NP$_{338}$ variants.** Having shown robust NP$_{338}$$^{+}$CD8$^{+}$ T cell responses in HLA-B37 donors, we next determined the level of cross-recognition towards the main NP$_{338}$ variants occurring in IAVs. Our conservation analysis of the NP$_{338}$ viral peptides across vaccine and circulating IAV strains, including pH1N1, H5N1, and H7N9, found 16 natural variants of NP$_{338}$ peptide (Table 2), with the most common mutations occurring at position (P) 5, 6, and 7 of the peptide. Two major variants; FEDLRVLSF (NP$_{338}$-L7S) and FEDLRLLSF (NP$_{338}$-V6L) were dominant in H1N1, pH1N1, H5N1, and H7N9 or H3N2 strains, respectively, representing 93–100% of the distinct strains and were thus selected in addition to the previously published (WT) peptide for further analysis.

To assess the cross-reactive potential of HLA-B37-NP$_{338}$$^{+}$CD8$^{+}$ T cells, pooled or variant-specific NP$_{338}$ T cell lines were generated using PBMCs from HLA-B37$^{+}$ donors. We also generated HLA-B37-NP$_{338}$ and mutant HLA-B37-NP$_{338}$-V6L and HLA-B37-NP$_{338}$-L7S tetramers. The ability to recognize, and functionally respond, to each of the NP$_{338}$ variant was then assessed using tetramers and an intracellular cytokine staining assay, respectively (Fig. 2). Staining with all three pHLA tetramers showed similar proportions of CD8$^{+}$ T cell-staining/functioning levels in all WT or variant-specific NP$_{338}$-T cell lines (Fig. 2a, c), suggesting that the majority of NP$_{338}$$^{+}$ specific CD8$^{+}$ T cell lines were able to cross-recognize (Fig. 2a, c) and respond to (Fig. 2b, c) each of the three distinct peptides, indicating broad cross-reactivity towards these epitopes (Fig. 2b, c). Interestingly, the NP$_{338}$-V6L tetramer showed a slightly stronger staining than the WT and NP$_{338}$-L7S tetramers (Fig. 2a).

CD8$^{+}$ T cell lines typically recognized and responded most prominently to the peptide they were generated against, with the lowest level of cross-reactivity being observed by the NP$_{338}$-V6L-specific CD8$^{+}$ T cell lines, when stimulated with the NP$_{338}$-WT (21.45 ± 18.86% by tetramer, 20.98 ± 19.80% by IFNγ, and 18.88±18.90% by TNFα) and NP$_{338}$-L7S variant (16.31 ± 14.47% by tetramer, 13.63 ± 12.04% by IFNγ, and 13.04 ± 11.65% by TNFα). These data suggest that a high level of cross-reactivity across different variants exists, although some variant-specific reactivity can be also detected. These data highlight that such cross-reactive CD8$^{+}$ T cells may offer protection against H1N1, H5N1, H7N9, and possibly other novel IAV strains.

To further assess whether the WT NP$_{338}$ peptide elicited CD8$^{+}$ T cell responses of high functional avidity, CD8$^{+}$ T cell lines were generated against the WT NP$_{338}$ peptide (n = 3), and their functional response was assessed towards decreasing concentrations of the WT NP$_{338}$ peptide (Fig. 2d). All data were normalized to the maximal response (when stimulated with maximum peptide). High and stable functional avidity towards NP$_{338}$ (IFNγ, TNFα, and CD107a) was observed in cell lines derived from all three donors (Fig. 2d).

Our data show that HLA-B37$^{+}$ individuals display multifunctional and highly cross-reactive CD8$^{+}$ T cell response to the NP$_{338}$ epitope and its major variants.

Table 2 | Conservation of NP$_{338}$ epitope in distinct IAV strains

| Sequence | Abbreviation | Frequency | IAV—H1N1 | IAV—H3N2 | Other IAV strains |
|----------|-------------|-----------|-----------|-----------|------------------|
|          |             | All       | Aust      | Vac       | All             | Aust | Vac | All | Aust | Vac | All | Aust | Vac |
| FEDLRVLSF | WT          | 0.9       | 2.4       | 6.3       | 98.5            | 95.9 | 100.0 |
| FEDLRVSSF | L7S         | 96.5      | 1.3       | 1.3       | 95.6            | 96.9 | 93.8 |
| FEDLRVSSF | V6L         | 0.8       | 0.1       | 2.3       | 0.1             |
| FEDLRVSSF | L41+L75     | 0.2       | 0.7       | 3.1       | 0.5             | 1.0 |
| FEDLRVSSF | L75+F9L     | 0.1       | 0.7       | 3.1       | 0.5             | 1.0 |
| FEDLRVSSF | R51+L75     | 0.1       | 0.7       | 3.1       | 0.5             | 1.0 |
| FEDLRVSSF | R5K         | 0.1       | 0.7       | 3.1       | 0.5             | 1.0 |
| FEDLRVSSF | L75+F9Y     | 0.2       | 0.7       | 3.1       | 0.5             | 1.0 |
| FEDLRVSSF | L7T         | 0.1       | 0.7       | 3.1       | 0.5             | 1.0 |
| FEDLRVSSF | R5G+V6L     | 0.1       |           |           | 98.5            | 95.9 | 100.0 |
| FEDLRVSSF | R5K+V6L     | 0.1       |           |           | 98.5            | 95.9 | 100.0 |
| FEDLRVSSF | V6L         | 0.1       |           |           | 98.5            | 95.9 | 100.0 |
| FEDLRVSSF | V6L+58N     | 0.1       |           |           | 98.5            | 95.9 | 100.0 |
| FEDLRVSSF | F1S+V6L     | 0.1       |           |           | 98.5            | 95.9 | 100.0 |

Number of sequences: 1155 43 9 1264 159 16 858 97 39

Coverage of selected strains (%): 98.1 93.0 100.0 99.4 100.0 100.0 98.5 95.9 100.0

Sequences were obtained from the NCBI Influenza Database https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database. Full-length sequences of Australian (denoted Aust), vaccine (denoted Vacc) and pH1N1, H5N1 and H7N9 were obtained and were aligned using the influenza database. Underlined are anchor residues for HLA-B*37:01, and mutations are shown in bold. The first three rows in italics represent sequences chosen for further analysis.
NP338 variants are molecular mimics of the WT NP338 epitope. To understand the molecular basis of HLA-B37-NP338+ CD8+ T cell cross-reactivity, we solved the structures of two major NP338 variants, namely, NP338-V6L and NP338-L7S in complex with HLA-B37 (Fig. 3; Supplementary Table 2). Both peptides bound HLA-B37 in an extended conformation with the canonical P2-Glu and P9-Phe, while P5-Arg acted as a secondary anchor residue (Fig. 3a, b). Superimposition of the HLA-B37-NP338 binding cleft with either HLA-B37-NP338-V6L (Fig. 2c) or HLA-B37-NP338-L7S (Fig. 2d) revealed a similar conformation of the HLA-binding cleft (r.m.s.d. of 0.08 Å). In addition, the peptides adopted a similar conformation as the NP338 peptide with an r.m.s.d. of 0.08 Å.

### Table 2

| Donor | Pooled WT | L7S | V6L |
|-------|-----------|-----|-----|
| 1     | 37.2      | 53.8| 47.3|
| 2     | 2.87      | 1.77| 2.36|
| 3     | 40.6      | 66  | 66  |
| 4     | 53.8      | 50.2| 50.2|

| Donor | Pooled WT | L7S | V6L |
|-------|-----------|-----|-----|
| 1     | 53.8      | 47.3| 2.87|
| 2     | 50.2      | 66  | 66  |
| 3     | 47.3      | 2.87| 2.87|
| 4     | 66        | 66  | 66  |

| Peptide concentration (μM) | % Max IFNγ | % Max TNFα |
|---------------------------|------------|------------|
| 0.0625                    | 100        | 100        |
| 0.125                     | 100        | 100        |
| 0.25                      | 100        | 100        |
| 0.5                       | 100        | 100        |
| 1                         | 100        | 100        |
| 2                         | 100        | 100        |
| 4                         | 100        | 100        |
| 8                         | 100        | 100        |

### Graphs

- **Graph a**: HLA-B37:01 NP338 Tetramer
- **Graph b**: NP338 Peptide variant
- **Graph c**: Tetramer, IFNγ, TNFα
- **Graph d**: % Max IFNγ, % Max TNFα, % Max CD107a

*Figures and graphs illustrate the binding and activation of CD8+ T cells in response to NP338 variants.*
s.d. of 0.12 and 0.09 Å, respectively (Fig. 3e, f). The hydrophobic P6-Val residue sits near the a2-helix and interacts with Val152, Gln155, and Asp156 of the HLA (Fig. 3g). The substitution of P6-Val for P6-Leu in the H3N2 was readily accommodated in the HLA-B37 cleft and did not change either the peptide or HLA conformation (Fig. 3h). The P7-Leu residue sits above Trp147, the P6-Val residue sits near the HLA-B37 cleft and did not change either the peptide or HLA conformation (Fig. 3i). The substitution from P7-Leu to P7-Ser did not impact the same conformation as the NP 338 WT peptide and representative (Fig. 6). The EM2 TCR docked centrally onto HLA-B37-NP338 (Supplementary Table 2, Fig. 6). The EM2 TCR docked centrally onto HLA-B37-NP338 with a 66° angle (Fig. 6b), falling in the range previously observed for TCR-pHLA-I (average of 63°)50. The buried surface area (BSA) of the complex was of ~1800 Å², also falling in the previously observed range for TCR-pHLA-I (average of 1900 Å²)50. The EM2 TCR α-chain contributes to 40% of the contact surface (Fig. 6c), while the biased TRBV19 -chain contributes to 60% of the contact surface. The peptide contributed to 25% of the pHLA BSA, with all six solvent-exposed residues contacted by the EM2 TCR. CDR2β (27%), CDR3α (23%), and CDR3β (20%) are the highest contributors to the BSA, followed by CDR1α, CDR2α, and CDR1β (6–8% each), as well as the framework (FW) residues of the β-chain (9%) (Fig. 6c).

All of the CDR loops of the EM2 TCR contacted the HLA-B37 molecule (Fig. 6d), with the addition of two FW residues from the β-chain (Gln67β and Lys83β). From the EM2 α-chain, the CDR1/2 loops contributed to ~10% of the TCR-HLA BSA and 16% for the CDR3α (Supplementary Table 5). The CDR1/2α loops made a contact with the region between the hinges of the HLA a2-helix (residue 151–158, Fig. 6f), while the CDR3α loop contacted the N-terminal part of the HLA a1-helix (residues 58 and 62, Fig. 6g). Within the β-chain, the CDR1 made only few contacts with the HLA (~2% TCR-HLA BSA), and the contacts were driven by CDR2β (34%), CDR3β (14%), as well as FWβ (11%) (Fig. 6d). The CDR3β loop contact with the HLA focused mostly on Gln155, which has its side chain sandwiched by the Tyr30α (CDR1α) and the 110SAMGT114 motif from the CDR3β loop (Supplementary Table 5, Fig. 6h). The FW residue Lys83β formed
structures of HLA-B37 bound to NP338 and its two variants. Structure of the HLA-B37 (white cartoon) presenting either a NP338-V6L (orange) or b NP338-L7S (green). c, d Top view or e, f side view of the structural overlay of HLA-B37 (white cartoon) presenting the NP338 (purple) with either c, e NP338-V6L (orange) or d, e NP338-L7S (green). g-j Zoomed in view on the HLA-B37 interaction with residue at position 6 (g, h) and position 7 (i, j) of the NP338 (purple), NP338-V6L (orange) and NP338-L7S (green) peptides. The red dashed lines represent salt bridges between residues.

EM2 TCR contacts the NP338 peptide via a hydrophobic network. The peptide is predominantly contacted by CDR3α (47%) and CDR3β (26%), as well as by CDR1β (17%) and CDR2β (10%) (Fig. 6b, e, Supplementary Table 5). The motif SGG within the CDR3α loop, with small or no side chain, allow the loop to be in close proximity and lodge itself between the side chains of P1-Phe and P3-Asp (Fig. 7a). The proximity of the loop allows the Gln113α to form hydrogen bonds with P3-Asp of the peptide and hydrophobic interaction with P4-Leu. Therefore, CDR3α loop forms a lid-like structure that covers the N-terminal part of the peptide (Supplementary Table 5). Within the EM2 TCR β-chain, all the CDR loops were contacting the peptide C-terminal section from residue P4-Leu to P8-Ser. P4-Leu was contacted by Gln57β from the CDR2β loop, while the CDR1β loop was interacting with both P7-Leu and P8-Ser that form a hydrogen bond with Asp30β (Supplementary Table 5). P6-Val and P7-Leu were both contacted by the CDR3β loop that sat on top of the central region of the HLA helix spanning from residue 65 to 79 (Fig. 6b, d, j and Supplementary Table 5). The residue Gln67β from the FW, located downstream of the CDR2β loop was also contacting the α1-helix of the HLA-B37 molecule. Gln57β, Asn60β, Asp61β, and Gln67β were forming a large hydrogen network with the HLA-B37 molecule. Overall, CDR2β and FWβ make extensive interaction with the HLA-B37 molecule, providing a basis for the strong bias TRBV19+ usage observed in the donors.

EM2 TCR affinity for NP338-V6L is higher than for NP338-WT. We next evaluated the ability of the EM2 TCR to recognize the NP338 and its two major variants (NP338-V6L and NP338-L7S)
Key TCRs are cross-reactive towards A1-NP44 variants. To investigate whether cross-reactivity and thus protection against distinct IAV strains is NP338-specific or occurs towards other IAV-specific epitopes, we assessed CD8\(^+\) T cell responses towards using surface plasmon resonance (Fig. 7d–f; Supplementary Table 6). The EM2 TCR bound to the HLA-B37-NP338 complex with a \(K_d\) of \(\sim 130\ \text{µM}\) (Fig. 7d), displaying a low affinity for an antiviral CD8\(^+\) T cell, the average being 35 \(\text{µM}\)\(^{30}\). The EM2 TCR bound to NP338-L7S with a similar affinity to the one observed for the NP338 bound to HLA-B37 (\(K_d\sim 150\ \text{µM}\), Fig. 7e). Interestingly, the EM2 TCR affinity was increased by four-fold for the NP338-V6L (\(K_d\sim 30\ \text{µM}\), Fig. 7f) compared to NP338 when bound to HLA-B37.

The structures of each NP338 and variants in complex with the HLA-B37 molecule (Fig. 3, Supplementary Table 2) allowed us to understand the impact of the NP338 mutation onto the EM2 TCR recognition. Overlay of the HLA-B37-NP338-L7S with the EM2 TCR-HLA-B37-NP338 structures showed that the smaller P7-Ser would be readily accommodated by the EM2 TCR without structural changes (Fig. 7g). This might explain the similar affinity observed for the EM2 TCR towards both peptides (\(K_d\sim 130-150\ \text{µM}\)). In a similar fashion, an overlay of the HLA-B37-NP338-V6L with the EM2 TCR-HLA-B37-NP338 structures (Fig. 7h) shows that the larger side chain of the P7-Leu might create additional contact with the CDR3\(^\beta\) loop and might in turn provide a basis for the improved affinity observed for the EM2 TCR towards the HLA-B37-NP338-V6L (\(K_d\sim 30\ \text{µM}\)).

Thus our data demonstrate that CD8\(^+\) T cell cross-reactivity in the context of NP338 peptide and its variants is favored by similar conformations adopted by the epitopes.

**Fig. 4** HLA-B37\(^+\) NP338\(^{-}\)-specific CD8\(^+\) T cells recognize NP338 variants directly ex vivo. PBMCs from HLA-B*37:01\(^+\) donors (\(n = 3\)) were tetramer stained individually with each variant NP338 tetramer conjugated to PE and assessed via flow cytometry. Samples were gated on lymphocytes, singletons, CD3\(^+\)live \(\text{CD}16\text{+}\text{dump}\) cells; as per Supplementary Figure 2c. a Representative dot plots of tetramer staining of CD3\(^+\)CD8\(^+\) T cells (purple) superimposed on unenriched CD8\(^+\) T cells. b Frequency of NP338 variant-specific CD8\(^+\) T cells in HLA-B*37:01\(^+\) donors.
**Fig. 5** Key TCRs cross-recognize NP338 variant peptides directly ex vivo. PBMCs from HLA-B*37:01+ donors (Donors 1–3) were tetramer stained individually with each of the NP338 tetramers conjugated to PE, enriched, and single-cell sorted on lymphocytes, singlets, CD3+ variants of NP338 peptides from HLA-B*37:01 repertoire used by each of three donors for the recognition of variant NP338 peptides. 

| CDR3 Variant | Preferential length(s) | Frequency | Average CDR3 length |
|--------------|------------------------|-----------|---------------------|
| WT           | 9                      | 37.67 ± 11.24 |
| V6L          | 9                      | 27.7 ± 16.77 |

**CDR3α**

| Variant | Preferential length(s) | Frequency |
|---------|------------------------|-----------|
| L7S     | 9                      | 31.93 ± 14.48 |

**CDR3β**

| Variant | Preferential length(s) | Frequency |
|---------|------------------------|-----------|
| L7S     | 10                     | 26.71 ± 23.19 |

**HLA-B*37:01 NP338 Tetramer**

- **a**: Graphical representation of the TCRβ repertoire used by each of three donors for the recognition of variant NP338 peptides. 
- **b**: Bar graph showing the preferential CDR3β length used for the recognition of NP338 from HLA-B*37:01+ donors. 
- **c**: Preferred CDR3α and CDR3β length of NP338-specific cells isolated from HLA-B*37:01+ donors in the recognition of variant NP338 peptides.
the HLA-A1-restricted NP44 peptide (Fig. 8). Conservation analysis revealed that there were two major variants of the peptide, the CTELKLSDY (NP44) and the S7N mutant CTELKLNDY (NP44-S7N), covering 34–100% of H3N2, H7N9, H5N1, and H1N1 virus strains (Supplementary Table 7). To investigate cross-reactivity towards these variant NP44 peptides, PBMCs from healthy HLA-A1 donors \((n=3)\) were tetramer stained with each of the NP44 variants directly ex vivo and magnetically enriched and single-cell sorted (Fig. 8a). The TCR \(\alpha\beta\) repertoire was then determined using multiplex RT-PCR (Fig. 8b). Two of the three donors (Donors 1 and 20) had both NP44- and NP44-S7N-specific CD8\(^+\) T cell populations. Interestingly, Donor 21 had only a NP44-specific CD8\(^+\) T cell population (Fig. 8b). A predominant TRAV8-2\(^+\) TCR\(\alpha\) chain expressing a CDR3\(\alpha\) sequence CVVSDRNFNKFYF paired with multiple distinct TCR\(\beta\)s and was detected in two out of three donors (Supplementary Table 8). Similar to the NP338 system, specific clonotypes were detected (Fig. 8c).
isolated from Donor 1 could recognize both NP₄₄ variants, while the remainder of the repertoire was variant specific (Fig. 8b).

Interestingly, the TCRαβ repertoire of Donor 20 was entirely NP₄₄ variant specific. We previously solved the structures of both NP₄₄ and NP₄₄-S7N peptides in complex with the HLA-A1 molecule. In similar fashion as HLA-B37 and the NP₃₃₈ variants, the NP₄₄ variants adopted similar conformation in the cleft of HLA-A1 molecule. This suggests that here too molecular similarity might underpin CD₈⁺ T cell cross-reactivity and favor the recognition of multiple IAV strains.

Overall, these data show that TCRαβ cross-reactivity is not limited to the recognition of HLA-B37-NP₃₃₈ and highlight the importance of cross-reactive CD₈⁺ T cells in the recognition of virus-infected cells and thus their potential to protect against distinct IAV strains.
Discussion

There is great interest in the development of a universal CD8+ T cell-mediated vaccine towards IAV. However, to realize this, first a thorough understanding of prominent human influenza-specific CD8+ T cell epitopes is needed. We have previously shown that NP is the most immunogenic antigen in both HLA-A*02:01-positive and -negative individuals. However, as the conservation of the known influenza epitopes varies between 16% and 56%, depending on ethnicity, understanding inter-epitope cross-recognition across diverse IAV strains is needed. In this study, we have used the variable NP338 and NP44 peptides to dissect CD8+ T cell recognition of different peptide variants and thus distinct IAV strains.

Fig. 8 TCRαβ clonotypes can recognize HLA-A*01:01-restricted NP44 variants. PBMCs from HLA-A*01:01+ donors (Donors 1, 20 and 21) were tetramer stained individually with each of the NP44 tetramers conjugated to PE. Samples were enriched, surface stained, and single-cell sorted on lymphocytes, singlets, CD3+live−dump− cells, CD8+Tet− cells, as per Supplementary Figure 2c, and the TCRαβ repertoire was determined using a multiplex RT-PCR. Representative dot plots of NP44 tetramer staining of CD3+ T cells following magnetic enrichment. Numbers are represented as a proportion of tetramer+ of CD8+ T cells.

Summary of the TCRαβ repertoire used by each of the three donors for the recognition of the variant NP44 peptides
NP338 has two major variant peptides, covering ~93% (NP338-L7S) and ~96% (NP338-V6L) of H1N1 and H3N2 viruses, respectively. Although CD8+ T cell responses are highly peptide specific34, they can tolerate variations within CD8+ T cell peptides22,29,36,34. We identified functional cross-reactivity towards the variant NP338 peptides, albeit to different levels. Using a multiplex RT-PCR for the detection of the TCRαβ repertoire directly ex vivo, we confirmed that CD8+ T cells were cross-reactive towards these distinct NP338 variants, at a clonotypic level, with ~55% TCRαβ repertoire being capable of recognizing multiple NP338 variants. Interestingly, unique clonotypes with distinct TRAV–TRBV pairings in each individual recognized all three NP338 variants. Furthermore, this cross-reactivity was increased following in vitro amplification, potentially suggesting that activation increases expansion of cross-reactive clones, allowing certain TCRαβ clonotypes to recognize distinct viral variants and potentially prevent viral escape. Additionally, we showed that the importance of cross-reactive TCRαβ repertoire is not limited to the recognition of NP338 and that cross-reactive CD8+ TCRαβ can recognize HLA-A1-restricted NP44 variant peptides.

To understand the molecular basis of cross-reactive TCRαβ recognition, we solved the structure of NP338, and its variants bound to the HLA-B37 molecule, as well as the structure of a cross-reactive TCR in complex with HLA-B37-NP338. First, our data show that the two NP338 variants were structural mimics of the WT peptide. Second, the NP338 peptide conformation was not altered by TCR engagement, and therefore the conserved structure of the NP338 variants favored TCR cross-reactivity, a mechanism that is thought to underpin cross-reactivity and alloreactivity more broadly45,52,53. The structures also demonstrated that only HLA-B37, and not HLA-B18 nor HLA-B44, binds the NP338 with a secondary anchor residue (P5-Arg), revealing the hydrophobic surface of the NP338 peptide, which might help cross-reactivity6,6,54. This hydrophobic surface of the NP338 was disrupted when the peptide was presented by HLA-B44 or HLA-B18 molecules, which exposed the charged P5-Arg, and for which no T cell recognition was observed.

Together, these data imply that HLA-B37+ donors may have a level of protection against H1N1, pH1N1, H3N2, H5N1, H7N9, and possibly novel IAV strains. Furthermore, the findings highlight the potential of inducing inter-strain cross-protection by the inclusion of a single peptide variant in a CD8+ T cell-mediated vaccine, suggesting that vaccine epitope identification will no longer need to focus solely on conserved epitopes for universal inter-strain cross-protection.

Diverse and cross-reactive TCR repertoire are important for viral control and preventing viral escape27,29, and we thus dissected the importance of TCRαβ diversity in the recognition of the variant NP338 peptides. Our data show that cross-recognition of distinct IAV strains is underpinned by a diverse TCR repertoire and surprisingly that no shared clonotypes could be detected between individuals. This highlights that cross-reactivity and TCRαβ repertoire diversity may provide further protection against distinct IAV strains. Furthermore, TCRαβ diversity is likely to aid in limiting viral escape, offering protection against any quasispecies that arise during infection. Additionally, we have shown that cross-reactivity towards distinct IAV strains is not limited to HLA-B37-NP338, and cross-reactive TCRs towards the HLA-A1-restricted NP44 were also observed. In both contexts, we have isolated cross-reactive clonotypes that were not shared between individuals, showing the plasticity of the TCR repertoire whereby multiple TCRs are able to cross-react and provide protection against multiple IAV strains.

Overall, our study provides evidence that inter-epitope cross-reactivity is common for the human influenza epitopes and occurs for prominent HLA-B37-restricted NP338 and HLA-A1-restricted NP44 epitopes, in addition to previously published HLA-A*0201-restricted M13030. CD8+ T cells can recognize variations within CD8+ T cell epitopes and cross-reactive CD8+ TCRαβ repertoire can recognize variant HLA-B37-restricted NP338 and HLA-A1-restricted NP44 peptides across donors.

Methods

Ethics statement. All work was undertaken in line with the Australian National Health and Medical Research Council (NHMRC) Code of Practice, with ethics approval by the University of Melbourne Human Ethics Committee, ethics numbers 0931311 and 1443389. Australian Red Cross Blood donors provided written informed consent on the day of their blood donation. Written informed consent was obtained from all healthy blood donors. Donor information including HLA typing result is reported in Supplementary Table 3.

NP338 conservation analysis. Full-length vaccine, Australian, pH1N1, H5N1, and H7N9 isolates with identical sequences collapsed were obtained from the NCBI Influenza Research Database [https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?gse=database] in February 2018. Sequences were aligned using [https://www.biobyte.org/home/wp-content/decorator=influenza].

Antigen-presenting cell lines (APC lines). APC lines were kindly provided by Professor James McCluskey (University of Melbourne, VIC, Australia; C1R-B44/03) and Dr. Nicole Mifsud (Monash University, VIC, Australia; C1R-B18/01). The A2-20091204 (HLA-B*37:01+) IBLCL cells were provided by Professor Weisen Chen (LaTrobe University, VIC, Australia).

Isolation of PBMCs. Buffy coats were obtained from the Australian Red Cross Blood Service (ARCS) and whole blood donations from healthy volunteers. PBMCs were separated using density gradient separation and were cryopreserved until use. All samples were HLA-typed by the Victorian Transplant and Immunogenetics Service (VTIS, West Melbourne, VIC, Australia) at the ARCS tissue typing Laboratory.

Generation of NP338-specific CD8+ T cell lines. PBMCs were used to generate CD8+ T cell lines at a 1:2 stimulator-to-responder ratio. Stimulator PBMCs were washed in fetal calf serum (FCS)-free media and were pulsed with 10 μM (unless otherwise stated) peptide for 90 min at 37 °C with 5% CO2. Stimulator PBMCs were washed twice and added to the responder PBMCs. Cells were cultured for 10–16 days and supplemented twice weekly with 10 U/mL IL-2, 5 μM Momenin A (Sigma Aldrich, MO, USA) and GolgiStop (BD Biosciences, CA, USA) with aCD107a-FITC/AF488 (1:100–1:200; eBioscience # 53-1079-41, CA, USA) for 5 h. Following activation, cells were surface stained for 30 min with antiCD3-PE (1:25–1:50; BD Pharmingen # 553333, CA, USA), antiCD8-APC (1:50–1:100; BD Biosciences #305485, CA, USA) or with or without Live/Dead-NIR (1:1000; Molecular Probes Cat#L10119, MA, USA), or antiCD4-FITC (1:50–1:100; eBioscience # 25-0038-42), antiCD8-PerCP/Cy5.5 (1:50; BD Biosciences # 340511) and Live/Dead/NIR (1:1000; Molecular Probes #L10119). Cells were then fixed with 1% paraformaldehyde (PFA; Electron Microscopy Sciences, PA, USA) or BD-Fix-Perm buffer (BD Biosciences) for 15–20 min. Cells were intracellularly stained for 4 h with aCD107a-FITC/AF488 (1:100–1:200; eBioscience #45-7319-41) and anti-TNFα-APC (1:100; BD Pharmingen #557647, CA, USA), anti-IFNγ-PE (1:100; BD Biosciences #340542) and anti-TNFα-APC (1:100–1:200; BD Biosciences #340534) or anti-IFNγ-V500 (1:50–1:100; BD Biosciences #154276) in perm wash buffer (BD Biosciences), or 0.3% Saponin (Sigma, MO, USA), respectively. Cells were washed, acquired on the BD FACS Canto II (BD Biosciences) and analyzed using the Flowjo software version 9.7.6 (Treestar, OR, USA).

Tetramer staining of CD8+ T cell lines. CD8+ T cells were stained with tetramers for 1 h at room temperature in the dark. Cells were washed and surface stained for 30 min with aCD3-PerCP/Cy5.5 (1:50–1:100; BD Biosciences #25-0038-42) or antiCD4-FITC (1:100–1:500; Bidegene #300341, SD, USA) with aCD28-PerCP/Cy5.5 (1:50; BD Biosciences #340105), antiCD27-APC (1:50–1:100; BD Biosciences #337169), aCD45RA-FITC (1:50–1:100; BD Pharmingen #555488) and Live/Dead-
Ex vivo magnetic enrichment of PBMCs. In all, 1–2 × 10^7 PBMCs from HLA-B^*37:01^+ donors were FACs blocked in MACS buffers (phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA); Gibco, CA, USA, 0.2 mM EDTA; DJa Finechem, NSW, Australia) at 15 min for 4°C (Miltiényi Biotech, Bergisch Gladbach, Germany) and tetracer stained with variant-specific tetracer conjugated to PE in PBS at room temperature. Cells were washed, a small amount was removed for unrerchored control, and labeled with anti-PE microbeads (Miltiényi Biotech, Bergisch Gladbach, Germany) for 30 min at 4°C. Following washing, cells were enriched by passing twice over LS magnetic columns (Miltenyi Biotec, Bergisch Gladbach, Germany)^35. Bound cells were eluted in MACS buffer. All examples (unenriched, enriched, and flow-through) were surface stained for 30 min with αCD3-PEc-Y7 (E Biosciences), αCD8-PerCP/Cy5.5 (BD Biosciences), Live/Dea-NIR (Molecular Pobercles), αCD14-APCH7 (BD Pharmorgen), αCD4-APCH7 (BD Biosciences), αCD19-APCH7 (Biologend), αCD27-APC (BD Biosciences) and αCD45RA-FTIC (BD Pharmingen) at 4°C in the dark. Lymphocytes were washed and re-suspended in sort buffer (PBS, 0.1% BSA; Gibco, CA, USA), and were acquired on the BD Aria III.

Surface plasmon resonance measurement and analysis. Surface plasmon resonance experiments were conducted at 25°C on the Biacore 3000 instrument with TBS buffer (10 mM Tris-HCl pH 8, 150 mM NaCl, and 0.005% surfactant P20). TBS buffer was supplemented with 1% BSA to prevent non-specific binding. TCRαβ and TCR-specific monoclonal antibody, 12H8 antibody^56, was coupled to research-grade CM5 chips with standard amine coupling. The experiment was conducted with one empty flow cell (use for subtraction as blank) and one flow cell with the EM2 TCR captured to approximately 300 response unit per flow cell onto the 12H8 antibody^56. Then a concentration range of 200 µM maximum of the pcHLA complexes were passed over all the flow cells. The final response was calculated from position 4 and excludes the final xF motifs. TRAV and TRBV nomenclature was derived using the IMGT nomenclature^57. The final models have been validated using the Protein Data Base validation website and the final refinement statistics are summarized in Supplementary table 2. Coordinates were validated by the PDB database. All molecular graphic representations were created using PyMol^57.

Statistical analyses. All statistical analyses were undertaken using Prism 6 (GraphPad, CA, USA). A Dunnett’s two-way analysis of variance was used for all non-parametric non-paired analysis where multiple comparisons were made. Statistical significance was defined as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
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Author contributions

Conceived and designed the experiments: E.J.G., K.K., S.G., M.B., W.C. Performed the experiments: E.J.G., E.B.C., I.L., S.G. Analysed the data: E.J.G., T.J., L.L., S.S., E.B.C., S.G. Wrote the paper: E.J.G., T.J., M.B., J.R., W.C., K.K., S.G.

Additional information

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