Homologous peptides derived from influenza A, B and C viruses induce variable CD8+ T cell responses with cross-reactive potential

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Objective. Influenza A, B and C viruses (IAV, IBV and ICV, respectively) circulate globally, infecting humans and causing widespread morbidity and mortality. Here, we investigate the T cell response towards an immunodominant IAV epitope, NP265-273, and its IBV and ICV homologues, presented by HLA-A*03:01 molecule expressed in ~ 4% of the global population (~300 million people). Methods. We assessed the magnitude (tetramer staining) and quality of the CD8+ T cell response (intracellular cytokine staining) towards NP265-IAV and described the T cell receptor (TCR) repertoire used to recognise this immunodominant epitope. We next assessed the immunogenicity of NP265-IAV homologue peptides from IBV and ICV and the ability of CD8+ T cells to cross-react towards these homologous peptides. Furthermore, we determined the structures of NP265-IAV and NP323-IBV peptides in complex with HLA-A*03:01 by X-ray crystallography. Results. Our study provides a detailed characterisation of the CD8+ T cell response towards NP265-IAV and its IBV and ICV homologues. The data revealed a diverse repertoire for NP265-IAV that is associated with superior anti-viral protection. Evidence of cross-reactivity between the three different influenza virus strain-derived epitopes was observed, indicating the discovery of a potential vaccination target that is broad enough to cover all three influenza strains. Conclusion. We show that while there is a potential to cross-protect against distinct influenza virus lineages, the T cell response was stronger against the IAV peptide than IBV or ICV, which is an important consideration when choosing targets for future vaccine design.

Keywords: CD8+ T cell, cross-reactivity, HLA, immune response, immunodominant epitope, Influenza
INTRODUCTION

Influenza viruses cause significant morbidity and mortality, with an estimated 650,000 individuals succumbing to infection annually. Individuals who are young, elderly, immunocompromised and pregnant or have other co-morbidities such as diabetes or asthma are particularly susceptible to severe influenza disease and death. The influenza viruses are classified into types A to D. Of these, influenza A virus (IAV) and influenza B virus (IBV) are in constant circulation and the most threatening to human health as they are responsible for annual epidemics. Additionally, IAV has been responsible for 4 global pandemics since 1918. Comparatively, influenza C virus (ICV) typically causes mild infection; however, it can be severe in young children, and influenza D (IDV) is not yet known to infect humans.

Although a vaccine is available against influenza, it typically induces a humoral response, targeting the rapidly mutating haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins. These mutations are predominately responsible for seasonal epidemics and often render previous vaccines ineffective, meaning they must be updated, manufactured and administered annually. Additionally, vaccine effectiveness can vary widely, 10–60%, and is particularly low in individuals aged over 65 years old (10–35%).

Furthermore, vaccines offer limited to no protection in the face of novel reassortments or avian-derived influenza viruses, which continually pose a threat to human health. As such, there is an urgent need to develop novel and effective therapeutics to combat this deadly disease.

CD8+ T cells are critical in the control and clearance of many viral infections, including influenza virus infections. It is well known that memory CD8+ T cells reduce disease severity and symptom scores following influenza virus challenge, and CD8+ T cell numbers positively correlate with milder influenza disease. Unlike antibodies, CD8+ T cells also recognise internal proteins, such as nucleoprotein (NP), that are typically more conserved than the HA and NA surface glycoproteins, making CD8+ T cells an attractive target for vaccination. Indeed, there is a lot of interest in the development of CD8+ T cell-mediated vaccines against influenza.

There are several factors to consider when thinking about targets for a CD8+ T cell-mediated vaccine to maximise its protective potential. Firstly, it is important to select human leukocyte antigen (HLA) molecules expressed at high frequency in the human population. CD8+ T cells recognise viral peptides presented by HLA class I (HLA-I) molecules on the surface of virally infected cells. HLA molecules are extremely polymorphic, with over 24,000 HLA-I molecules described to date. Additionally, HLAs are genetically encoded, resulting in distinct expression profiles in different ethnicities and geographical locations.

Secondly, despite being more conserved than surface glycoproteins, internal proteins are subject to mutations that can lead to viral escape. To avoid this, one could target conserved peptides, such as the universal IAV peptides previously described. Thirdly, the response elicited should activate cross-reactive CD8+ T cells that may offer superior protection against mutations and distinct influenza virus strains.

The ILRGSHAHK273 (NP265-IAV) peptide derived from IAV was shown to be immunogenic with NP265-IAV-specific CD8+ T cells isolated from HLA-A*03:01 donors. Subsequently, it was confirmed that NP265-IAV could be presented and stabilised by the HLA-A*03:01 molecule. Of note, HLA-A*03:01 is the 5th most expressed HLA allele in humans, expressed by ~4% of the global population, or approximately 300 million individuals worldwide.

NP265-IAV has been classified as a universal IAV peptide, demonstrating 100% conservation within circulating IAV strains, even including those from avian H7N9 viruses. However, despite these features making it an attractive vaccine candidate, the immune response towards this peptide has not been well characterised.

In this study, we investigated the universal NP265-IAV peptide and characterised the resulting immune response including the T cell receptors (TCR) used by HLA-A*03:01 individuals in the recognition of NP265-IAV. Furthermore, we identified homologues of the NP265-IAV peptide in both IBV and ICV...
Here, we characterise the CD8+ T cell response towards these peptides and demonstrate that CD8+ T cells expanded against one peptide are capable of cross-reacting towards the other peptides. We also observed that despite the presence of CD8+ T cells able to recognise each peptide in some donors, the T cell response was stronger against NP265-IAV peptide. Together, our results provide an in-depth characterisation of the universal NP265-IAV peptide and demonstrate the presence of cross-reactive CD8+ T cells able to recognise NP-derived homologous peptides from IAV, IBV and ICV. This further highlights the potential for T cells to effectively contribute to vaccines, by eliciting an immune response in a significant proportion of the global population across different types of influenza viruses.

RESULTS

The NP265-IAV-specific CD8+ T cell response has a highly diverse TCR repertoire

The CD8+ T cell response to HLA-A*03:01-restricted NP265-IAV has been previously reported21,28,32,42,44, however, data regarding polyfunctionality and TCR repertoire associated with the CD8+ T cell response are limited. The CD8+ T cell response towards NP265-IAV has been shown to be quite variable between donors,21 so we firstly wanted to assess the CD8+ T cell response towards this peptide in HLA-A3+ donors (n = 5, Table 1). CD8+ T cell lines were generated against the NP265-IAV peptide, and specificity was determined using an intracellular cytokine staining (ICS) assay (Figure 1). We observed that the CD8+ T cell response towards NP265-IAV was variable in our HLA-A3+ donors, with 3/5 donors producing IFNγ and/or TNF in response to the NP265-IAV peptide (Figure 1a and c). The majority of NP265-IAV-specific CD8+ T cells were able to produce both IFNγ and TNF (double-positive cells) (Figure 1c). While tetramer staining of our ICS-positive CD8+ T cell lines confirmed our results, there was no correlation between the strength of the T cell response and the level of tetramer+/CD8+ T cell observed (Figure 1b and d).

We then determined the phenotypic profile of NP265-IAV-specific CD8+ T cells directly ex vivo and assessed the TCR repertoire specific for the NP265-IAV peptide. We used NP265-IAV tetramer-associated magnetic enrichment (TAME) to stain T cells from peripheral blood mononuclear cells (PBMCs) isolated from HLA-A*03:01+ donors (Figure 2a and b). Tetramer-positive CD8+ T cells were single-cell sorted, and the TCR repertoire was determined using a reverse transcriptase-multiplex-PCR37,47 (Figure 2c, Table 2). A large tetramer+/CD8+ T cell population spanning a large range of mean fluorescence intensities (MFIs) was observed in all donors (Figure 2a). Unexpectedly, the majority of tetramer+/CD8+ T cells displayed a CD27+/CD45RA+ phenotype (Figure 2b), typically consistent with naive cells (average of 36.9 ± 23.9%). Naïve-like epitope-specific CD8+ T cells have been observed previously at high proportions in individuals with a poor response to the peptide of interest.37,48 For comparison, we performed TAME with a M1-58-66 tetramer using PBMCs from an HLA-A*02:01+ individual and observed that the majority of tetramer+/CD8+ T cells were of central memory phenotype (CD27+/CD45RA−), Supplementary figure 2). By comparison, the remaining NP265-IAV+specific CD8+ T cells displayed a memory phenotype consistent with other previously published peptide-specific CD8+ T cells,37,48,49 with 22.7 ± 11.2%, 29.1 ± 22.2%, 11.3 ± 4.8% displaying an effector memory-like (CD27+/CD45RA−), central memory-like and terminally differentiated phenotype (CD27+/CD45RA+), respectively (Figure 2b). Although CD8+ T cells from SG5 donor are capable of producing IFNγ and TNF towards the positive control (PMA-I), no specific IFNγ or TNF production was observed upon NP265-IAV peptide stimulation, despite the presence of HLA-A*03:01- NP265-IAV tetramer+/CD8+ T cells from SG5 donor’s sample, with some cells staining with a high MFI (Figure 2a).

To our knowledge, there is only a single report of an NP265-IAV-specific TCR repertoire.46 The NP265-IAV-specific TCR repertoire was determined using TAME on human lung tissue from a single

| Donor ID | HLA-A     | Age (years) | Sex |
|---------|-----------|-------------|-----|
| SG4     | 03:01, 68:01 | 27          | F   |
| SG5     | 03:01, 01:01 | 45          | F   |
| SG11    | 03:01, 68:01 | 35          | F   |
| SG12    | 03:02, 02:01 | 41          | F   |
| SG17    | 03:01, 02:11 | 21          | F   |
| SG27    | 03:01, 31:01 | 28          | F   |
| SG29    | 03:01, 02:01 | 24          | M   |
donor and was very diverse, with only a few clonotypes observed at a higher frequency than the others. Similarly, our analysis showed that the TCR repertoire utilised in the recognition of NP265-IAV was diverse in all three donors, with only a single clonotype observed more than once in donors SG4 and SG5 (Table 2), resulting in a high Simpson diversity index of 0.9989, 0.9523 and 1.0000 in donors SG4, SG5 and SG27, respectively. The TCR repertoire was also entirely private, with no shared clonotypes between individuals (Table 2). Some TRAV and TRBV gene usage biases were observed across the distinct clonotypes (Figure 2c); however, these were predominantly different between individuals. Shared TRAV19 biases were observed for SG5 (20%) and SG27 (23%), and shared TRBV9 and TRBV20-1 biases were seen in SG4 (12% and 14%, respectively) and SG5 (21% and 14%, respectively) (Figure 2c). A shared TRBV27 bias was also observed in SG5 (14%) and SG27 (12%) (Figure 2c). Interestingly, these were different to the TRAV25 and TRBV14/DV4 biases observed by Pizzolla et al. further highlighting the diversity and private nature of the NP265-IAV-specific CD8\(^+\) TCR repertoire. The TCR repertoire showed a preferred CDR3\(_a\) length of 12 (~23\%/C6\%), 14 (19\%/C6\%) or 15 (19\%/C6\%) amino acids and a CDR3\(_b\) length of 14 (24\%/C6\%) or 15 (19\%/C6\%) residues across the distinct clonotypes (Supplementary figure 3a). These CDR3 loops are slightly longer than those reported for other immunodominant IAV epitopes such as HLA-B\(^*\)37:01-restricted NP338 peptide, the HLA-A\(^*\)02:01-restricted M158 peptide, and the HLA-B\(^*\)35:01-restricted NP418 peptide. However, no obvious CDR3 motifs were observed within these preferred lengths (Supplementary figure 3b).

Overall, we have shown that the NP265-IAV peptide induces variable CD8\(^+\) T cell responses in our cohort of HLA-A\(^*\)03:01 donors, and ex vivo analysis revealed that a high proportion of NP265-IAV-specific CD8\(^+\) T cells display a naive like phenotype and utilise a private and highly diverse TCR repertoire for the recognition of the NP265-IAV peptide.
NP265-IAV homologous peptides are present in IBV and ICV

The NP265-IAV peptide was described in 2013 as a universal epitope due to its high conservation among influenza A virus strains, including the then-emerging avian H7N9 virus. To assess the conservation of the NP265-IAV peptide and determine whether it is still highly conserved, we aligned 4328 strains of IAV and assessed the conservation within the NP265-IAV peptide. We found that the NP265-IAV peptide is still highly conserved, displaying 98% conservation within our 4328 isolates (Table 3). Interestingly, it has been published that the NP265-IAV peptide most frequently has an isoleucine at position 1 (P1) and rarely a Valine at P1, even though both are immunogenic epitopes. Given the high conservation of the NP265-IAV peptide, we wondered if this peptide could also be conserved across different influenza virus types. Therefore, we aligned the NP sequences from a representative strain of influenza A, B and C virus. The IAV-NP protein is 34.5% identical in protein sequence with IBV-NP and 19.0% identical with ICV-NP. NP265-IAV peptide homologues were identified in both IBV with the NP323-IBV peptide (323VVRPSVASK331) and ICV with the NP 270-ICV peptide (270LLKPQITNK278). The three peptides all shared canonical anchor residues characteristic of the HLA-A*03:01 molecule, with a hydrophobic residue at position 2 (P2-V/L) and a C-terminal lysine (P2-K). The conservation of these residues indicated that all three peptides might be able to bind to the HLA-A*03:01 molecule and potentially activate CD8 T cells. The NP265-IAV and NP323-IBV shared five identical residues (55% identity and 78% similarity) and only two identical residues with NP270-ICV (22% identity and 78% similarity).
| TRAV | TRAJ | CDR3A               | Length | TRBV | TRBJ | CDR3B | Length | SG4 | SG5 | SG27 |
|------|------|---------------------|--------|------|------|-------|--------|-----|-----|------|
| 20*1/02/03/04 | 37*0/2 | CAVQAIRRSSNTGKL | 17 | 19*0/1 | 1-1*0/1 | CASSVVYGEAFF | 14 | 1 |
| 38-2/DV8*01 | 42*0/1 | CAVENYGGSGGNUL | 15 | 28*0/1 | 2-3*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 20*0/12/03/04 | 53*0/1 | CAVLSSRSNYYKLT | 15 | 12-3*0/1 or 12-4*0/1/02 | 2-7*0/1 | CASSVAVYEQYF | 12 | 1 |
| 13-1*0/1 | 10*0/1 | CAATSTGQGKLN | 14 | 27*0/1 | 2-1*0/1 | CASSASSRTRWSHEQFF | 16 | 1 |
| 17*0/1 | 34*0/1 | CATDAEADKLI | 12 | 13*0/0/2 | 2-3*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 25*0/1 | 34*0/1 | CAVNLYGNYKL | 12 | 13*0/0/2 | 2-3*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 8-6*0/2 | 21*0/1 | CAVQYNFKTYF | 12 | 20-1*0/1,2/03/04/05 | 2-7*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 1-2*0/1,3 | 29*0/1 | CARRGNTLFLV | 11 | 14*0/0/2 | 2-1*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 27*0/1 | 28*0/1 | CAGPGAGSYSQIL | 14 | 20-1*0/1,2/03/04/05 | 2-3*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 21*0/1,2 | 48*0/1 | CAYLSQNEKLT | 13 | 9*0/1 | 2-2*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 5*0 | 34*0/1 | CAERDTDKL | 11 | 9*0/1 | 1-1*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 29/DV5*0 | 44*0/1 | CAARALYYTGASKLTF | 18 | 5-1*0/0/2 | 2-7*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 20*0/12/03/04 | 34*0/1 | CAVQ5RYNTDKLIF | 14 | 19*0/1 | 1-6*0/0/2 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 12-1*0/1 | 4*0/1 | CVTSSEGNYKLI | 13 | 6-5*0/1 | 2-5*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 21*0/1,2 | 52*0/1 | CAVQGNAQGTSYKLT | 18 | 7-2*0/1,2/2/03/04/05 | 2-5*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 29/DV5*3 | 52*0/1 | CAVPAGGNTSYKLT | 16 | 5-1*0/1,2/2/03/04/05 | 2-5*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 27*0/1 | 31*0/1 | CADQDNARLUMF | 11 | 20-1*0/1,2/03/04/05 | 2-7*0/1 | CASSPLGSDPDKTDQYF | 18 | 1 |
| 27*0/1 | 28*0/1 | CAGPPAGSYSQL | 14 | | | | | |
| 17*0/1 | 8*0/1 | CATDASFOKLF | 12 | | | | | |
| 20*1/02/03/04 | 15*0/1 | CAVQASSQGT | 13 | | | | | |
| 12-2*0/1,0/2/03 | 10*0/1 | CAVNNTGGNKL | 13 | | | | | |
| 6*0/1,0/2,05/07 | 42*0/1 | CAYLGGQSNQJU | 13 | | | | | |
| 13-2*0/1,0/2 | 9*0/1 | CAVNTGGKF | 12 | | | | | |
| 1-2*0/1,3 | 16*0/2 | CAVSVQGQKL | 12 | | | | | |
| 26-2*0 | 54*0/1 | CILRGPSIQAOQFL | 16 | | | | | |
| 1-2*0/1,3 | 43*0/1 | CAVHPGYNNNMRF | 14 | | | | | |

(Continues)
| TRAV | TRAJ | CDR3A | Length | TRBV | Length | TRBJ | CDR3B | Length | SG4 | SG5 | SG27 |
|------|------|-------|-------|------|-------|------|-------|-------|-----|-----|-------|
| 29/DV5*01 | 26*01 | CAASAKNYGQNFVF | 14 | 19*01 | 15 | 1 | CASSPYREYTEAFF | 15 | 1 |
| 4–3*01/02/03/04 | 1–1*01 | | | | | | | | | | |
| 12–2*01/02/03/04 | 13*02 | CAVNQGYQKVTF | 12 | 4–2*01/02 | 1–2*01 | | CASSQEGGYGGYTF | 14 | 1 |
| 13–2*01/02 | 26*01 | CAEAVNFVF | 9 | 7–8*01/03 | 1–5*01 | | CASSQSITGLNQPOHF | 16 | 1 |
| 19*01 | 23*01 | CALSDLNYQGGKLF | 15 | 12–3*01 | 2–5*01 | | CASSXSGGGGAETFQYF | 15 | 5 |
| 41*01 | 45*01 | CAVRGGGGLADQLTIF | 14 | 3–1*01/02 | 2–1*01 | | CASSLVLNQEGYF | 14 | 1 |
| 20*01/02/03/04 | 16*01 | CAVRSDQGKLLF | 14 | 3–2*01/02/03/04 | 2–2*01 | | CASSPPLSGGQETQYF | 17 | 1 |
| 21*01/02 | 50*01 | CAVRITSYDKVIF | 13 | 27*01 | 1–6*01 | | CASSQRSNPLHF | 12 | 1 |
| 19*01 | 22*01 | CALSEALRRSGARQLTF | 16 | | | | | | | | |
| 29/DV5*01 | 26*01 | CAAXXXXYGQNFVF | 14 | 28*01 | 2–5*01 | | CASSLLAQETQYF | 14 | 1 |
| 12–3*01/02 | 33*01 | CAMSAPEEGNYQLW | 15 | 6–5*01 | 2–1*01 | | CASSYGGGQEQF | 12 | 1 |
| 26–2*01 | 41*01 | CILRQNSSGYSYLW | 16 | 4–2*01/02 | 1–2*01 | | CASSQAEAGRQYTF | 14 | 1 |
| 19*01 | 54*01 | CALSDPQGAQKLVF | 14 | 9–01/02/03 | 2–5*01 | | CASSXSGGGGAEQTQYF | 15 | 1 |
| 14/DV4*03 | 33*01 | CAMRVRTDSNQYLLW | 15 | 20–1*01/02/03/04 | 2–1*01 | | CASSGQPRYNEHQFF | 15 | 1 |
| 19*01 | 54*01 | CALSGLQQAQKLVF | 15 | 27*01 | 1–6*01 | | CASSQNSNPLHF | 12 | 1 |
| 24*01 | 37*02 | CAFGSSTNGKLF | 13 | 6–5*01 | 2–7*01 | | CASSVGTQKQEQYF | 14 | 1 |
| 38–1*01/02/03/04 | 40*01 | CAFMKQAYKYIF | 12 | 28*01 | 1–1*01 | | CASSAIVNTEAFF | 13 | 1 |
| 36/DV7*04 | 31*01 | CAILNNLRLMF | 12 | 24–1*01/02 | 1–4*01 | | CASSQERSSYNEQFF | 15 | 1 |
| 38–2/DV8*01 | 33*01 | CSDLSNQYLV | 11 | 21*01/02 | 1–4*01 | | CATSRSQGSGSLNQYF | 18 | 1 |
| 13–2*01/02 | 42*01 | CAESGYGGQSOQNLIF | 15 | 24–1*01/02/03/04 | 2–7*01 | | CASSFQPGYSNQPOHF | 16 | 1 |
| 39*01 | 49*01 | CAVDYTQGQYFY | 12 | 28*01 | 2–1*01 | | CASSQGERGDFWNEQFF | 16 | 1 |
| 19*01 | 40*01 | CAXSGTIXYQYF | 12 | 29*01 | 2–1*01 | | CASSQGERDFWNEQFF | 16 | 1 |
| 38–1*01/02/03/04 | 34*01 | CAFHYNTDKLF | 13 | 30*01/05 | 2–5*01 | | CASSISAGQGEGETQYF | 15 | 1 |
| 28*01 | 2–7*01 | | | | | | | | | |
| 30*01/05 | 2–5*01 | | | | | | | | | |
| 27*01 | 2–1*01 | | | | | | | | | |
| 7–9*01/03 | 2–3*01 | | | | | | | | | |
| 7–2*01/02/03/04 | 2–1*01 | | | | | | | | | |
| 28*01 | 1–5*01 | | | | | | | | | | (Continues)
To confirm that our selected virus strains and identified homologous peptides were representative, we determined the conservation of these peptides within circulating viruses IBV and ICV strains from the Influenza Research Database. We found that the NP323-IBV peptide was 98.1% conserved between 1710 unique IBV-NP sequences, and NP270-ICV was determined to be 100% conserved in all 66 unique ICV-NP sequences (Table 3), demonstrating that they are universally conserved within their influenza virus type.

IBV and ICV-derived NP265-IAV homologous peptides can stabilise HLA-A*03:01 but are less immunogenic than IAV-derived one

Firstly, we wanted to assess the stability of NP265-IAV in complex with HLA-A*03:01 and whether these IBV- and ICV-derived homologous could also stabilise the HLA-A*03:01 molecule, which would suggest that they can be presented by HLA-A*03:01 at the cell surface for CD8+ T cell recognition. To do this, we refolded HLA-A*03:01 with each of the homologous peptides and assessed their stability using Differential Scanning Fluorimetry. All three peptides stabilised the HLA-A*03:01 molecule; however, NP265-IAV and NP323-IBV both showed a ~7°C higher melting point (Tm) than NP270-ICV (53.9°C and 53.3°C, respectively, versus 46.9°C) (Table 4). Since all three homologous peptides can bind and stabilise the HLA-A*03:01 molecule, we sought to determine whether NP323-IBV and NP270-ICV could activate CD8+ T cells. To this end, we generated NP323-IBV- and NP270-ICV-specific CD8+ T cell lines and assessed their specificity using an intracellular cytokine staining (ICS) assay (Figures 3 and 4, respectively). Interestingly, we found that NP323-IBV was immunogenic in two out of four donors (Figure 3a and b), whereas the NP270-ICV generated minimal CD8+ T cell specificity (Figure 4a and b). The specificity of the CD8+ T cells towards NP323-IBV was further confirmed using HLA-A*03:01-NP323-IBV tetramers on the remaining NP323-IBV-specific CD8+ T cell lines (Figure 3c and d). Similarly, using NP270-ICV tetramer, we observed a small proportion of tetramer+/CD8+ T cells (Figure 4c and d) in line with the minimal CD8+ T cell response observed upon NP270-ICV stimulation (Figure 4a and b).

Altogether, these data show that NP323-IBV and NP270-ICV represent novel immunogenic epitopes...
eliciting CD8\(^+\) T cell recognition and activation, albeit with a lower proportion of CD8\(^+\) T cells responding to NP\(_{323}\)-IBV and NP\(_{270}\)-ICV than NP\(_{265}\)-IAV.

**Table 3.** Sequence conservation of NP\(_{265}\)-IAV and its IBV and ICV homologues

| Peptide      | Influenza A | Influenza B | Influenza C |
|--------------|-------------|-------------|-------------|
| ILRGSVAHK    | 4328        | 1710        | 66          |
| VVRPSVASK    | 98.24%      | 98.13%      | 100.00%     |
| LLKPQITNK    |             |             |             |

IBV, Influenza B virus; ICV, Influenza C virus.

**Table 4.** Thermal stability of peptide-HLA complexes

| pHLA-A*03:01 complex | Tm (°C) ± sem |
|----------------------|--------------|
| NP\(_{265}\)-IAV      | 53.9 ± 0.1   |
| NP\(_{323}\)-IBV      | 53.3 ± 0.3   |
| NP\(_{270}\)-ICV      | 46.9 ± 0.1   |

Tm, thermal midpoint temperature.

**Figure 3.** Select HLA-A*03:01\(^+\) individuals have a response to the NP\(_{323}\)-IBV peptide. Peptide-specific CD8\(^+\) T cells from HLA-A*03:01\(^+\) donors (n = 4) were expanded in vitro through stimulation with the NP\(_{323}\)-IBV peptide and cultured for 10 days with IL-2. Specificity was assessed using an ICS assay or tetramer staining. Samples are gated as per Supplementary figure 1. (a) FACS plots showing IFN\(\gamma\) and TNF production (n = 4) in response to the NP\(_{323}\)-IBV peptide. (b) Summary of the proportion of IFN\(\gamma\), TNF\(^+\) and IFN\(\gamma\)/TNF\(^+\) production by CD8\(^+\) T cells in response to the NP\(_{323}\)-IBV peptide, minus the no-peptide control. (c) FACS plots showing NP\(_{323}\)-IBV tetramer staining (n = 3 donors). (d) Summary of the proportion of CD8\(^+\)Tet\(^+\) T cells (n = 3).
observed when CD8\(^+\) T cell lines were generated against the NP 270-ICV peptide (Figure 5b).

Overall, here we demonstrate that CD8\(^+\) T cell responses are typically strongest against their cognate peptide; however, some level of cross-reactivity can be observed.

**NP\(_{265\text{-IAV}}\) and NP\(_{323\text{-IBV}}\) epitopes are presented in a similar conformation**

To further understand the difference in stability and potentially immunogenicity, we aimed to solve the structure of each of the three peptides in complex with the HLA-A*03:01 molecule using X-ray crystallography (Table 5). We successfully solved the crystal structures for NP\(_{265\text{-IAV}}\) and NP\(_{323\text{-IBV}}\) in complex with HLA-A*03:01 with a well-defined electron density for the peptides but could not crystallise HLA-A*03:01-NP270-ICV (Supplementary figure 4). The NP\(_{265\text{-IAV}}\) peptide adopted a canonical conformation within the cleft of HLA-A*03:01 anchored by P2-Leu and P9-Lys (Figure 5c), as well as secondary anchor P3-Arg that forms a salt bridge with Glu152 (Supplementary figure 5a). The P9-Lys is buried deep in the F pocket of HLA-A*03:01 interacting via a salt bridge with the Asp116 and with a hydrophobic patch formed by Leu81, Ile95 and Ile97 (Supplementary figure 5b). The exposed surface of the NP\(_{265\text{-IAV}}\) is relatively flat and hydrophobic, with small residues such as the P4-Gly, P5-Ser, P6-Val and P7-Ala, with the only large and exposed residue being the P8-His (Figure 5c). Interestingly, this lack of featured side chains, and a rather flat conformation of the peptide, is similar to one observed for HLA-A*02:01-restricted influenza A virus M1 58-66 peptide.\(^{24,53}\)

Superimposition of the HLA-A*03:01-NP\(_{265\text{-IAV}}\) and HLA-A*02:01-M1\(_{58\text{-66}}\) structures reveals a similarly flat and central conformation of the peptide backbone, favored by a shared P4-Gly residue that introduces a kink at the start of the central region of the peptides (Supplementary figure 5c).

The NP\(_{323\text{-IBV}}\) peptide differs from the NP\(_{265\text{-IAV}}\) peptide at four positions (Figure 5d), namely P1 (Val to Ile), P2 (Val to Leu), P4 (Pro to Gly) and P8 (Ser to His). The anchor residues P3 and P9 are identical for both peptides (Figure 5c and d), but P2-Val (NP\(_{323\text{-IBV}}\)) has a shorter side chain anchoring into the B pocket. Additionally, P4-Pro

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**Figure 4.** NP\(_{270\text{-ICV}}\) elicits weak CD8\(^+\) T cell response in select HLA-A*03:01 donors. Peptide-specific CD8\(^+\) T cells from HLA-A*03:01 donors (n = 4) were expanded in vitro through stimulation with the NP\(_{270\text{-ICV}}\) peptide and cultured for 10 days with IL-2. Specificity was assessed using an ICS assay or tetramer staining. Samples are gated as per Supplementary figure 1. (a) FACS plots showing IFN\(_\gamma\) and TNF production (n = 4) in response to the NP\(_{270\text{-ICV}}\) peptide. (b) Summary of the proportion of IFN\(_\gamma\), TNF\(^+\) and IFN\(_\gamma\)/TNF\(^+\) production by CD8\(^+\) T cells in response to the NP\(_{323\text{-IBV}}\) peptide, minus the no-peptide control. (c) FACS plots showing NP\(_{270\text{-ICV}}\) tetramer staining (n = 3 donors). (d) Summary of the proportion of CD8 Tet\(^+\) T cells (n = 3).
creates a more constrained kink than P4-Gly. Despite this, superimposition of the HLA-A*03:01 structures presenting the NP 265-IAV and NP 323-IBV peptides and cultured for 10 days with IL-2. Specificity and cross-reactivity were assessed by stimulating CD8+ T cell lines with each of the homologous peptides separately in an ICS assay. (a) Representative FACS plots of IFNγ and TNFα production in the ICS assay. (b) Summary heatmap representing IFNγ production by CD8+ T cells, minus the no-peptide control, in the ICS assay. (c) HLA-A*03:01 (white ribbon) binding to NP 265-IAV (stick) coloured in salmon. (d) HLA-A*03:01 (white ribbon) binding to NP 323-IBV (stick) coloured in teal. (e) Overlay of NP 265-IAV (salmon) and NP 323-IBV (teal) presented by HLA-A*03:01 (white ribbon) with the differences in peptide sequence represented as sticks and the shared residues in the drawing below.

**Figure 5.** Cross-reactivity of CD8+ T cells towards the three NP peptides and similar presentation of NP 265-IAV and NP 323-IBV peptides by HLA-A*03:01 molecule. Peptide-specific CD8+ T cells from HLA-A*03:01 donors (n = 3) were expanded in vitro through stimulation with one of NP 265-IAV, NP 323-IBV or NP 270-ICV peptides and cultured for 10 days with IL-2. Specificity and cross-reactivity were assessed by stimulating CD8+ T cell lines with each of the homologous peptides separately in an ICS assay. (a) Representative FACS plots of IFNγ and TNFα production in the ICS assay. (b) Summary heatmap representing IFNγ production by CD8+ T cells, minus the no-peptide control, in the ICS assay. (c) HLA-A*03:01 (white ribbon) binding to NP 265-IAV (stick) coloured in salmon. (d) HLA-A*03:01 (white ribbon) binding to NP 323-IBV (stick) coloured in teal. (e) Overlay of NP 265-IAV (salmon) and NP 323-IBV (teal) presented by HLA-A*03:01 (white ribbon) with the differences in peptide sequence represented as sticks and the shared residues in the drawing below.

Overall, the two pHLA complex structures show that the NP 265-IAV and NP 323-IBV peptides were presented in a similar fashion by HLA-A*03:01. The structural similarity is likely to contribute to the cross-reactive potential of the CD8+ T cell observed, as well as been favored by a diverse TCR repertoire.

**DISCUSSION**

Cytotoxic CD8+ T cells play an important role in anti-viral immunity by limiting viral replication and clearing infected cells that display viral peptides presented by HLA molecules at the cell surface.14–16 Since influenza viruses are constantly mutating, CD8+ T cells that recognise highly
conserved peptides, such as NP265-IAV, can ensure the recognition of distinct virus strains. Therefore, it is important to understand and identify these conserved epitopes and their associated immune responses, as they may be of interest in the generation of future CD8+ T cell-mediated influenza vaccines.

Here, we characterised the CD8+ T cell response towards the ‘universal’ HLA-A*03:01-restricted NP265-IAV peptide, which was conserved in 98% of distinct IAV isolates analysed. The CD8+ T cell response varied in our five donors, from no activation to up to 17.9% of IFNγ/TNF+CD8+ T cells, similar to previously published studies. Interestingly, there was no NP265-IAV-specific CD8+ T cell response in SG12 (Figure 1a), a donor that expressed the HLA-A*03:02 allomorph rather than HLA-A*03:01 allomorph (Table 1). This allomorph differs from HLA-A*03:01 by two residues located within the HLA cleft (HLA-A*03:01: Asp151/Leu155, HLA-A*03:02: Val151/Gln155) that will impact both peptide presentation and TCR interaction and could explain the lack of CD8+ T cell response observed. We determine the TCR repertoire directly ex vivo from three donors, which expanded on the previously published NP265-IAV-specific CD8+ T cell repertoire from a single donor. We showed that NP265-IAV-specific CD8+ T cells utilise a private and diverse TCR repertoire. Conversely, the published HLA-A*02:01-restricted M158-66-specific TCR repertoire is extremely biased with public clonotypes shared between multiple individuals. Interestingly, the crystal structure of the NP265-IAV peptide in complex with HLA-A*03:01 revealed a featureless conformation of the peptide similar to the one observed for the immunodominant M158-66 peptide. The featureless conformation of the M158-66 peptide was proposed as the molecular basis for the biased TCR repertoire in HLA-A*02:01+ individuals containing public TCRs. This is in contrast with the diverse and private NP265-IAV-specific TCR repertoire observed here for HLA-A*03:01+ individuals, despite having a similarly featureless conformation. Diverse TCR repertoires are known to offer superior anti-viral protection further highlighting the interest in NP265-IAV as a potential CD8+ T cell-mediated vaccine candidate.

Additionally, we assessed the phenotype of NP265-IAV-specific CD8+ T cells directly ex vivo. Interestingly, the majority of the NP265-IAV-specific

### Table 5. Data collection and refinement statistics

|                      | HLA-A*03:01-NP265-IAV | HLA-A*03:01-NP323-AIV |
|----------------------|-----------------------|-----------------------|
| Data collection      |                       |                       |
| Space group          | P 6 2 2               | P 6 2 2               |
| Cell dimensions (a, b, c (Å)) | 155.57, 155.59, 170.76 | 155.26, 155.26, 85.33 |
| Resolution (Å)       | 48.82–1.95(1.98–1.95)  | 44.82–2.20(2.27–2.20)  |
| Total number of observations | 2 027 076 (104 909)   | 1 259 531 (108 430)   |
| Number of unique observations | 88 833 (4468)         | 31 267 (2662)         |
| Multiplicity         | 22.8 (23.5)           | 40.3 (40.7)           |
| Data completeness (%) | 100 (100)             | 100 (100)             |
| I0 (%)               | 13.5 (2.2)            | 16.5 (2.5)            |
| Mn(I) half-set correlation CC(1/2) | 0.998(0.781)         | 0.987(0.916)          |
| Rmerge (%)           | 4.8 (49.1)            | 4.4 (55.0)            |
| Refinement statistics |                       |                       |
| Non-hydrogen atoms   |                       |                       |
| Protein              | 6412                  | 3202                  |
| Water                | 1060                  | 301                   |
| Rfactor (%)          | 19.3                  | 21.7                  |
| Rmerge (%)           | 22.4                  | 26.6                  |
| Rms deviations from ideality |               |                       |
| Bond lengths (Å)     | 0.010                 | 0.010                 |
| Bond angles (°)      | 1.04                  | 1.10                  |
| Ramachandran plot (%)|                       |                       |
| Allowed region       | 97.6                  | 95.0                  |
| Generously allowed region | 2.2                  | 5.0                   |
| Disallowed region    | 0.2                   | 0.0                   |

*a Rmerge = Σhkl [1/(N–1)][Σ |Ihkl,i– Ihkl| / Σ Ihkl] / Σ Ihkl

*b Rfactor = Σhkl [ |Fo| – |Fc| / |Fo|] for all data except 5%, which were used for Rmerge calculation.

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CD8+ T cells displayed a naïve-like (CD27+/CD45RA+) phenotypic profile in two out of three donors. This is unusual for peptide-specific CD8+ T cells towards circulating viruses such as influenza, which typically display a higher proportion of cells with a memory phenotype.\textsuperscript{37,48,49} Such a naïve-like phenotype has been described before in the HLA-A*68:01-restricted NP\textsubscript{265}-specific CD8+ T cells that likewise resulted in minimal and variable activation in HLA-A*68:01+ individuals.\textsuperscript{50} It may be possible that the naïve-like T cells found in some donors, despite binding to NP\textsubscript{265} tetramer, are not or weakly activated by the peptide.\textsuperscript{59,60} which would explain the variability of response observed towards this epitope between donors.

We identified two NP\textsubscript{265} homologous peptides derived from IBV and ICV, and both were able to form a stable complex with the HLA-A*03:01 molecule; however, the NP\textsubscript{265} and NP\textsubscript{323} were more stable than NP\textsubscript{270} complex. We characterised the immune response towards these homologous peptides as few peptides derived from IBV or ICV have been defined and are therefore generally understudied.\textsuperscript{51} CD8+ T cell responses towards NP\textsubscript{323} and NP\textsubscript{270} were variable between donors, with NP\textsubscript{323} stimulating a strong CD8+ T cell response in a single donor, while NP\textsubscript{270} stimulated limited CD8+ T cell responses in all donors in both tetramer staining and ICS assays. Despite this variability, these results suggest that CD8+ T cells may display a preference for NP\textsubscript{265} followed by NP\textsubscript{323} and then NP\textsubscript{270}; however, which viruses the individual has been exposed to, and when, are likely to influence this preference. These data also show that NP\textsubscript{323} and NP\textsubscript{270} are immunogenic and represent new influenza CD8+ T cell epitopes.

Moreover, despite the variable and sometimes minimal CD8+ T cell responses, CD8+ T cells stimulated with one of the homologous peptides could cross-react towards the other peptides in some donors. This was most evident in CD8+ T cell lines generated against the NP\textsubscript{265} peptide. CD8+ T cells capable of cross-reacting towards homologous peptides could provide a level of protection towards distinct virus strains. Indeed, cross-reactive CD8+ T cells have been shown to recognise and respond to distinct influenza virus strains\textsuperscript{21,28,31-37} further supporting that these homologous peptides, and perhaps specifically the NP\textsubscript{265} peptide, could be a good target in vaccination strategies.

The structures of NP\textsubscript{265} and NP\textsubscript{323} epitopes in complex with HLA-A*03:01 show the peptides adopting a similar conformation, lying flat across the binding cleft, with a minor difference being a shorter anchor residue at P2-V for NP\textsubscript{323} verses that of P2-L for NP\textsubscript{265}. The similarity between the NP\textsubscript{265} and NP\textsubscript{323} conformation likely provides the basis for the CD8+ T cell cross-reactivity observed. The side chain of the P8 residue in NP\textsubscript{265} (P8-H) is larger than that of NP\textsubscript{323} (P8-S), and this might be responsible for the stronger T cell response observed towards NP\textsubscript{265} peptide. T cell cross-reactivity is an essential mechanism for CD8+ T cells to be able to recognise and respond to a wide array of pathogens and their mutations using a limited number of cells. This is critically important, in the face of new viruses, such as SARS-CoV-2,\textsuperscript{62} where prior exposure to other pathogens may generate CD8+ T cells capable of cross-reacting with novel viruses.\textsuperscript{39-41} We described here a thorough characterisation of the CD8+ T cell response towards NP\textsubscript{265} including the level, quality, phenotype, TCR repertoire used and peptide presentation. We have identified and characterised the CD8+ T cell response towards novel homologous peptides derived from IBV and ICV. We show that these three peptides are conserved and can be presented by HLA-A*03:01, the 5th most common HLA-A molecule worldwide. They can induce a CD8+ T cell response capable of cross-reacting with the homologous peptides. This cross-reactivity, stronger towards the NP\textsubscript{265} and NP\textsubscript{323} peptides, is underpinned by similar peptide conformations revealed by the crystal structures of the peptide-HLA complexes.

Together, these features would make these peptides, or perhaps more specifically, NP\textsubscript{265}, a candidate that could be included in vaccine strategies. Furthermore, this study demonstrated the potential of CD8+ T cell cross-reactivity in the protection against viral mutations, distinct virus strains, as well as newly emerging viruses.

METHODS

Ethics, donors and HLA typing

All work was undertaken according to the principles of the Declaration of Helsinki. The Human Research Ethics Committee at Monash University approved this work (HREC #19079). Whole blood donations were received from healthy volunteers who provided written and informed consent at the time of donation. Buffy coats were obtained from the Australian Red Cross Lifeblood who provide written and informed consent that their unused blood products be used for research at the time of donation. Donors were HLA typed at the Victorian Transplantation
and Immunogenetics Service (VTIS, Melbourne, VIC, Australia) or using AlloSeq Tx17 (CareDx Pty Ltd, Fremantle, Australia), summary of HLA-A locus typing of the donors used is found in Table 1.

**Peripheral blood mononuclear cells**

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood oruffy coats using density gradient centrifugation, as previously described.37,63 PBMCs were cryogenically stored until required.

**Generation of peptide-specific CD8\(^+\) T cell lines**

CD8\(^+\) T cell lines were generated as previously described.37 In summary, 1/3 of PBMCs were pulsed with 10 \(\mu\)M of individual peptides, washed twice, added to the remaining 2/3 PBMCs and cultured for 10–14 days in RPMI-1640 (Gibco, Carlsbad, USA) supplemented with 1x Non-essential amino acids (NEAA; Sigma-Aldrich, St Louis, USA), 5 mM HEPES (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 1x penicillin/streptomycin/Glutamine (Life Technologies, Clayton, Australia). Cultures were cryogenically stored for subsequent analysis.

**Intracellular cytokine staining assay**

Intracellular cytokine staining (ICS) assay was completed as previously described.37 Briefly, CD8\(^+\) T cell lines were co-cultured with peptide-pulsed C1R-HLA-A*03:01 at a 1:2 stimulators: responders (APC: T cell) ratio and incubated for 5 h in the presence of GolgiPlug (BD Biosciences, Franklin Lakes, USA) and GolgiStop (BD Biosciences). Cells were stained with anti-CD3-BV480 (BD Biosciences), anti-CD8-PerCP-Cy5.5 (BD Biosciences, Franklin Lakes, USA), anti-CD4-BUV395 (BD Biosciences) and live/dead fixable near-IR dead cell stain (Life Technologies) for 30 min, fixed and permeabilised using BD Cytofix/Cytoperm solution (BD Biosciences). Cells were then stained with PE-conjugated tetramer in MACS buffer for 1 h at room temperature, washed and labelled with anti-PE microbeads (Miltenyi Biotec) at 4°C for 30 min. Epitope-specific cells were enriched by passing twice over a LS magnetic column (Miltenyi Biotec) and surface stained with \(\alpha\)CD3-CD8-PerCP-Cy5.5 (BD Biosciences), Live/Dead-NIR (Life Technologies), \(\alpha\)CD14-APC7 (BD Biosciences), \(\alpha\)CD4-APC7 (BD Biosciences), \(\alpha\)CD19-APC7 (BD Biosciences), \(\alpha\)CD27-BV711 (BD Biosciences), \(\alpha\)CD45RA-FITC (BD Biosciences), \(\alpha\)CCR7-PECy7 (BD Biosciences) and \(\alpha\)CD59-BV421 (BD Biosciences) at 4°C in the dark. Cells were resuspended in sort buffer (PBS, 0.1% BSA; Gibco, CA, USA), and tetramer\(^\text{high}\)/CD8\(^+\) T cells were single-cell sorted directly into Twin-Tech PCR plates (Eppendorf, Hamburg, Germany) on an Aria Fusion (BD Biosciences) and were stored at −80°C until used. The gating strategy is shown in Supplementary figure 1c.

**Ex vivo tetramer magnetic enrichment and single-cell sorting**

Tetramer magnetic enrichment and single-cell PCR were undertaken as previously described.37 Briefly, PBMCs from HLA-A*03:01\(^*\) individuals were FCR blocked (Miltenyi Biotec) in MACS buffer (phosphate-buffered saline (PBS)), 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and 0.2 mM EDTA (Sigma-Aldrich) for 15 min at 4°C. PBMCs were then stained with PE-conjugated tetramer in MACS buffer for 1 h at room temperature, washed and labelled with anti-PE microbeads (Miltenyi Biotec) at 4°C for 30 min. Epitope-specific cells were enriched by passing twice over a LS magnetic column (Miltenyi Biotec) and surface stained with \(\alpha\)CD3-CD8-PerCP-Cy5.5 (BD Biosciences), \(\alpha\)CD3-PerCP-Cy5.5 (BD Biosciences), \(\alpha\)CD3-PerCP-Cy5.5 (BD Biosciences), \(\alpha\)CD27-BV711 (BD Biosciences), \(\alpha\)CD45RA-FITC (BD Biosciences), \(\alpha\)CCR7-PECy7 (BD Biosciences) and \(\alpha\)CD59-BV421 (BD Biosciences) at 4°C in the dark. Cells were resuspended in sort buffer (PBS, 0.1% BSA; Gibco, CA, USA), and tetramer\(^\text{high}\)/CD8\(^+\) T cells were single-cell sorted directly into Twin-Tech PCR plates (Eppendorf, Hamburg, Germany) on an Aria Fusion (BD Biosciences) and were stored at −80°C until used. The gating strategy is shown in Supplementary figure 1c.

**Single-cell multiplex PCR**

Single-cell multiplex PCR was carried out as previously described.37 Briefly, CD8\(^+\) T cell lines were co-cultured with peptide-pulsed C1R-HLA-A*03:01 at a 1:2 stimulators: responders (APC: T cell) ratio and incubated for 5 h in the presence of GolgiPlug (BD Biosciences, Franklin Lakes, USA) and GolgiStop (BD Biosciences). Cells were stained with anti-CD3-BV480 (BD Biosciences), anti-CD8-PerCP-Cy5.5 (BD Biosciences, Franklin Lakes, USA), anti-CD4-BUV395 (BD Biosciences) and live/dead fixable near-IR dead cell stain (Life Technologies) for 30 min, fixed and permeabilised using BD Cytofix/Cytoperm solution (BD Biosciences) for 20 min and then intracellularly stained with anti-IFN-\(\gamma\)-BV421 (BD Biosciences) as well as anti-TNF-PECy7 (BD Biosciences) for 30 min. Samples were acquired on a BD Fortessa and analysed using FlowJo v10 (BD Biosciences). The gating strategy is shown in Supplementary figure 1a.

**Tetramer staining**

p-HLA tetramers were prepared by conjugating purified biotinylated p-HLA monomers to streptavidin at a 8:1 monomer to streptavidin molar ratio. Streptavidin-PE (Invitrogen, Waltham, USA) was added slowly onto the monomer at 1/10 of the volume required and incubated for 10 min at room temperature, 10 times. CD8\(^+\) T cell lines were tetramer stained for 1 h at room temperature. Cells were washed and surface stained with anti-CD3-BV480 (BD Biosciences), anti-CD8-PerCP-Cy5.5 (BD Biosciences), anti-CD4-BV395 (BD Biosciences) and live/dead fixable near-IR dead cell stain (Life Technologies). Cells were fixed with 1% paraformaldehyde and acquired on the BD LSR Fortessa and were analysed using Flowjo v10 (BD Biosciences). The gating strategy is shown in Supplementary figure 1b.

**Protein expression, refold and purification**

DNA plasmids encoding HLA-A*03:01 \(\alpha\)-chain and \(\beta\)-2-microglobulin were transformed separately into a BL21 strain of Escherichia Coli, as previously described.63 Recombinant proteins were expressed individually, where inclusion bodies were extracted and purified from the transformed E. coli cells. Soluble pHLA complexes were produced by refolding 30 mg of HLA-A*03:01 with 10 mg of \(\beta\)-2-microglobulin and 5 mg of either NP\(_{265-IAV}\), NP\(_{323-IBV}\) or NP\(_{370-ICV}\) peptide (Genscript, Piscataway, USA) into a buffer of 3 M Urea (Univar
solutions, USA), 0.5 M L-Arginine (Sigma-Aldrich), 0.1 M Tris–HCl pH 8.0 (Fisher Bioreagents), 2.5 mM EDTA pH 8.0 (Sigma-Aldrich, St Louis, USA), 5 mM Glutathione (reduced) (Goldbio, St Louis, USA) and 1.25 mM Glutathione (oxidised; Goldbio) for 3 h. The refold mixture was dialysed into 10 mM Tris–HCl pH 8.0 (Fisher Bioreagents), and soluble pHLA was purified using anion exchange chromatography using a HiTrapQ column (GE Healthcare).

**Differential Scanning Fluorimetry**

Differential scanning fluorimetry was performed using a Qiaqen R66 real-time PCR machine by heating up pHLA samples in 10 mM Tris–HCl pH 8.0 (Fisher Bioreagents), 150 mM NaCl (Merck, Darmstadt, Germany), at two concentrations (5 and 10 mM) in duplicate, from 30 to 95°C at a rate of 0.5°C/min, with the emission channel set to yellow (excitation of ~530 nm and detection at ~557 nm). All samples contained a final concentration of 10X SYPRO Orange Dye (Invitrogen). Data were plotted using GraphPad Prism 9 (version 9.0.0) and normalised, and the Tm value was determined at the 50% point of maximal fluorescence intensity and summarised in Table 4.

**Crystallisation and structural determination**

Crystals of pHLA complexes were grown via sitting-drop, vapour diffusion method at 20°C with a protein: reservoir drop ratio of 1:1, at a concentration of 7 mg mL⁻¹ or 10 mg mL⁻¹ in 10 mM Tris–HCl pH 8.0 (Fisher Bioreagents), 150 mM NaCl (Merck), 0.1 M Bis-Tris propane pH 6.5 (Hampton), 22% PEG 3350 (Sigma-Aldrich). HLA-A*03:01-NP₂₅₅₋₁₈₅ were grown in 1 M trisodium citrate (Hampton) and 0.1 M sodium cacodylate pH 6.5 (Hampton). These crystals were soaked in a cryoprotectant containing the mother liquor supplemented with 20% EG (Sigma-Aldrich) and then flash-frozen in liquid nitrogen. The data were collected on the MX2 beamline at the Australian Synchrotron, part of ANSTO, Australia. The data were processed using XDS, and the structures were determined by molecular replacement using the PHASER programme. Manual model building was conducted using COOT followed by refinement with BUSTER. The final model has been validated using the wwPDB OneDep System with the accession numbers of 7UC5 for HLA-A*03:01-NP₂₅₅₋₁₈₅ and 7MILE for HLA-A*03:01-NP₂₅₅₋₁₈₅. The final refinement statistics are summarised in Table 5. All molecular graphics representations were created using PyMOL.

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**AUTHOR CONTRIBUTIONS**

Andrea T Nguyen: Data curation; formal analysis; investigation; methodology; supervision; validation; visualization; writing – original draft; writing – review and editing. Hiu Ming Peter Lau: Data curation; formal analysis; investigation. Hannah Sloane: Formal analysis; visualization. Dhilshan Jayasinghe: Investigation; methodology. Nicole Mifsud: Methodology; resources. Demetra SM Chatzileontiadou: Investigation; methodology; writing – review and editing. Emma Grant: Formal analysis; funding acquisition; methodology; supervision; validation; writing – original draft; writing – review and editing. Christopher Sztoto: Formal analysis; funding acquisition; investigation; methodology; supervision; validation; visualization; writing – original draft; writing – review and editing.

**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.