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ORIGINAL ARTICLE

Towards identification of immune and genetic correlates of severe influenza disease in Indigenous Australians

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Indigenous populations, including Indigenous Australians, are highly susceptible to severe influenza disease and the underlying mechanisms are unknown. We studied immune and genetic factors that could predicate severe influenza disease in Indigenous Australians enrolled in the LIFT study: looking into influenza T-cell immunity. To examine CD8⁺ T-cell immunity, we characterised human leukocyte antigen (HLA) profiles. HLA typing confirmed previous studies showing predominant usage of HLA-A*02:01, 11:01, 24:02, 34:01 and HLA-B*13:01, 15:21, 40:01/02, 56:01/02 in Indigenous Australians. We identified two new HLA alleles (HLA-A*02:new and HLA-B*56:new). Modelling suggests that variations within HLA-A*02:new (but not HLA-B56:new) could affect peptide binding. There is a relative lack of known influenza epitopes for the majority of these HLAs, with the exception of a universal HLA-A*02:01-M1₅₈ epitope and proposed epitopes presented by HLA-A*11:01/HLA-A*24:02. To dissect universal CD8⁺ T-cell responses, we analysed the magnitude, function and T-cell receptor (TCR) clonality of HLA-A*02:01-M1₅₈⁺CD8⁺ T cells. We found comparable IFN- γ , TNF and CD107a and TCR $\alpha\beta$ characteristics in Indigenous and non-Indigenous Australians, suggesting that the ~15% of Indigenous people that express HLA-A*02:01 have universal influenza-specific CD8⁺ T-cell immunity. Furthermore, the frequency of an influenza host risk factor, IFITM3-C/C, was comparable between Indigenous Australians and Europeans, suggesting that expression of this allele does not explain increased disease severity at a population level. Our study indicates a need to identify novel influenza-specific CD8⁺ T-cell epitopes restricted by HLA-A and HLA-B alleles prevalent in Indigenous populations for the rational design of universal T-cell vaccines. *Immunology and Cell Biology* (2016) 94, 367–377; doi:10.1038/icb.2015.93

Indigenous populations are at substantially higher risk of hospitalisation and morbidity from influenza infection. Up to 10–20% of Indigenous Australians died from pandemic influenza in 1919 compared with <1% of non-Indigenous Australians.¹ Similarly, although Indigenous Australians comprise 2.5% of the Australian population, they accounted for 16% of patients hospitalised with pandemic (p) H1N1 and 9.7% of those admitted to ICU.² Studies from the Northern Territory, Queensland and Western Australia have found that Indigenous Australians are 3–12 times more likely to be hospitalised than non-Indigenous Australians. Similar patterns have been observed in Indigenous populations from New Zealand, Canada and the United States.^{3–5} Such greater risk of hospitalisation could reflect higher infection rates due to crowded living conditions, and

also increased rates of chronic disease and comorbidities that lead to more severe outcome. In this setting, pre-emptive vaccination is clearly of significant benefit. However, prior administration of the currently available vaccines confers no protection against newly emerged unpredicted influenza viruses. Ultimately, the capacity to clear influenza virus and recover depends on the immune status of the individual.

Immune factors and host genetics can lead to increased severity of influenza disease in some ethnicities. This is exemplified by the expression of an interferon-induced transmembrane protein 3 (IFITM3) single-nucleotide polymorphism (SNP) rs12252, with the IFITM3 rs12252-C/C genotype (versus C/T or T/T) being predictive of early hypercytokinemia and severe influenza-induced disease.

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The IFITM3 C/C genotype is highly prevalent in the Asian population and correlates with severe pH1N1 and H7N9 disease.^{6,7} The prevalence of the IFITM3-C/C genotype in Indigenous Australians has not yet been documented.

Differences in influenza-specific T-cell immunity, especially the protective CD8⁺ T-cell responses,^{8–10} can be affected by distinct human leukocyte antigen (HLA) profiles (HLA restriction) found across different ethnicities.¹¹ Our recent work showed that Indigenous populations, including Indigenous Australians and Alaskans, are at greater risk from severe influenza disease caused by newly emerged influenza viruses due to a lack of CD8⁺ T cells directed at universal influenza epitopes.¹¹ Thus, prolonged and more severe influenza infection in the Indigenous population might reflect differences in CD8⁺ T-cell immunity associated with specific HLA profiles expressed. Indeed, the computational data suggest a strong correlation between pH1N1 influenza-induced mortality and the expression of HLA-A24,¹² an allele highly prevalent in Indigenous Australians and Alaskans.¹³ Previous studies also suggest that some HLA molecules of Indigenous Australians differ from those that predominate in non-Indigenous Australians.^{14–16} Thus, HLAs expressed in Indigenous populations may bind different viral peptides and induce distinct CD8⁺ T-cell responses in comparison with non-Indigenous individuals. To date, there are no data on influenza-specific CD8⁺ T-cell responses, in the context of HLA restriction, in Indigenous Australians. Given the recent emergence of new influenza viruses capable of infecting humans (H7N9, H6N5 and H10N8), there is an urgent need to understand the

immune correlates, and especially the effectiveness of CD8⁺ T-cell immunity within the vulnerable Indigenous communities.

Here we studied influenza-specific CD8⁺ T-cells epitopes in a cohort of Indigenous Australians enrolled in the LIFT study: looking into influenza T-cell immunity. Our HLA analysis verified previous reports of predominant usage of HLA-A*02:01, 11:01, 24:02, 34:01 and HLA-B*13:01, 15:21, 40:01/02, 56:01/02 in Indigenous Australians, and identified new HLA-A*02 and HLA-B*56 alleles. We showed a relative lack of known epitopes for these highly represented HLAs and analysed the magnitude, quality and clonality of CD8⁺ T cells directed at a universal HLA-A*02:01-M1₅₈ epitope. We found comparable characteristics of HLA-A*02:01-M1₅₈⁺CD8⁺ T cells in Indigenous and non-Indigenous Australians. Further, we determined low population frequency of IFITM3-C/C alleles in Indigenous Australians. We propose that identification of novel immunodominant influenza-specific CD8⁺ T cell epitopes restricted by HLA alleles prevalent in the Indigenous populations should be a priority for the rational design of new influenza vaccine strategies.

RESULTS

'Looking into influenza T cell immunity' (LIFT) cohort

We recruited 82 Australian Indigenous donors (LIFT01-LIFT083; Supplementary Table S1) in Darwin, Northern Territory, Australia. Participants included Indigenous patients at the Royal Darwin Hospital admitted with non-influenza related diagnoses and healthy volunteers in the Darwin region. The study was explained to potential

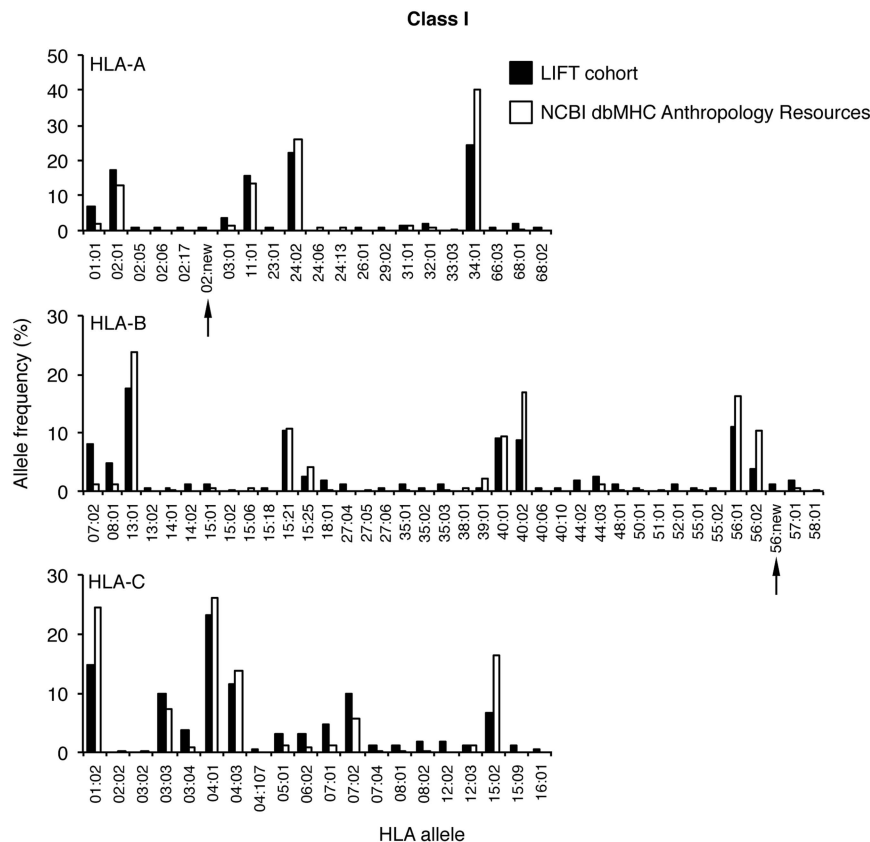


Figure 1 HLA class I allele expression in Indigenous Australians from the Top End. DNA typing of HLA-A, -B and -C alleles was performed for 82 Indigenous Australian individuals from the Top End of the Northern Territory in Australia (LIFT cohort; $2n=164$). The range and frequency of HLA class I allele expression are shown compared against previous studies of HLA class I expression in Indigenous Australians obtained from NCBI dbMHC Anthropology Resources (available online at www.ncbi.nlm.nih.gov/gv/mhc/ihwg.cgi?cmd=page&page=AnthroMain) (HLA-A, $2n=810$; HLA-B, $2n=812$; HLA-C, $2n=764$). The sample size was $2n$ as each individual donor contributed two separate alleles to the analysis.

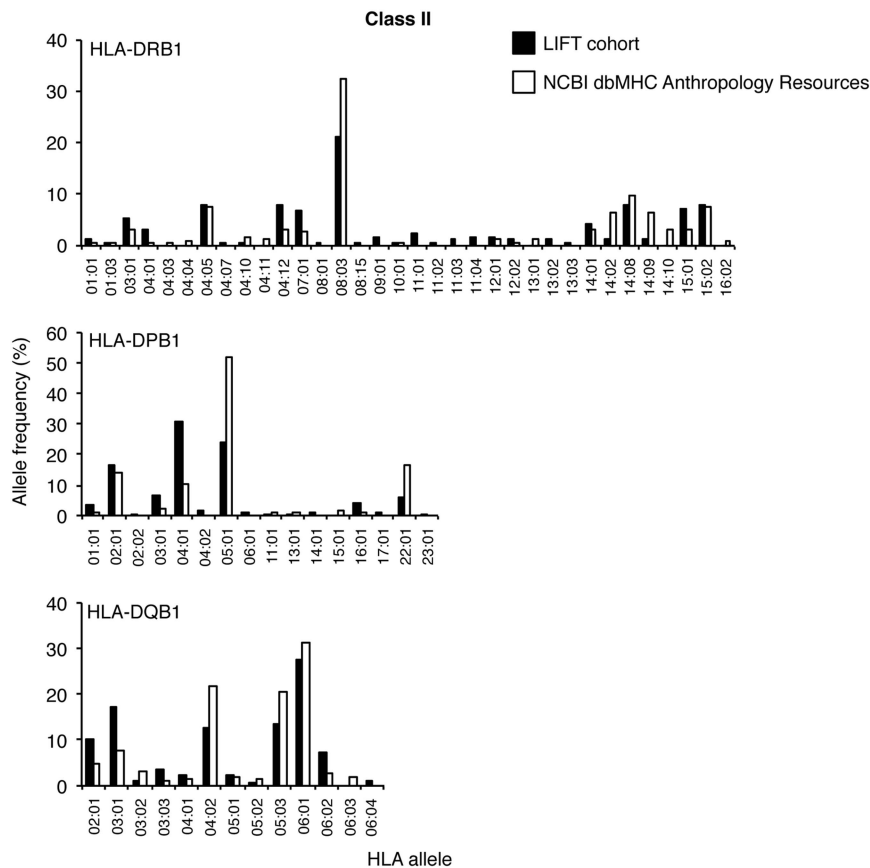


Figure 2 HLA class II allele expression in Indigenous Australians from the Top End. DNA typing of HLA-DRB1, -DPB1 and -DQB1 was performed for Indigenous Australian individuals (LIFT cohort; $2n=164$). Allele expression is shown as described in Figure 1. Data from NCBI dbMHC Anthropology Resources represent: HLA-DRB1, $2n=280$; HLA-DPB1, $2n=268$; HLA-DQB1, $2n=280$.

participants by Indigenous research staff with the use of culturally appropriate flipcharts and all participants provided written informed consent. The study was approved by the Human Research Ethics Committee (HREC) of the Northern Territory Department of Health and Menzies School of Health Research, and included review by the Aboriginal Ethics Sub-committee of the HREC. The median age of participants was 45 years (interquartile range 30, 55) and 42 (52%) were male.

Restricted HLA allele expression in Indigenous Australians from the Top End

Our analysis of 82 LIFT donors showed that HLA allele expression in Top End Indigenous Australians is relatively restricted for both HLA class I (Figure 1) and HLA class II (Figure 2). LIFT donors expressed a more restricted range of HLA alleles compared with Australian Caucasians. The four main HLA-A alleles in LIFT Indigenous donors (HLA-A*02:01, 11:01, 24:02 and 34:01) accounted for 79% of HLA-A alleles, whereas the four main HLA-B alleles (HLA-B*13:01, 15:21, 40:01/02, 56:01/02) accounted for 47% of HLA-B alleles (Figure 1). The main HLA-C alleles were HLA-C*01:02, 04:01/02, 07:02 and 15:02.

Similarly, expression of HLA class II alleles was largely limited (Figure 2). The most dominant HLAs were HLA-DRB1*06:03, HLA-DPB1*102:01, 04:01, 05:01 and HLA-DQB1*03:01, 04:02, 05:03 and 06:01. Our data are in accordance with previous reports.¹⁷ Overall, HLA distribution in our LIFT cohort was similar to those found in previous Indigenous cohorts from Cape York, Groote

Eylandt, Kimberley and Yuendumu regions (NCBI dbMHC Anthropology Resources; Supplementary Table S2). Thus, there appears to be a HLA conservation between geographically distinct Indigenous groups that were unlikely to have had extensive interaction.

New HLA-A*02 and HLA-B*56 alleles in Indigenous Australians

Molecular sequencing of donors LIFT026 and LIFT053 identified two new HLA alleles thus far unique to Indigenous Australians. In LIFT053, the new HLA-A*02 allele differed from HLA-A*02:35:01 at four nucleotide positions affecting three codons. The first nucleotide substitution occurs at codon 62 (GGG to CCG) and results in an amino-acid (aa) change of glycine to arginine. The second and third nucleotide substitutions at codon 63 (GAG to AAC) result in an aa change from glutamic acid to asparagine. A change in a nucleotide at codon 90 (GCC to GAC) results in an aa change from alanine to aspartic acid. Sequencing was verified on two occasions by the analysis of two independent samples. Position 90 is located in one of the loops outside the antigen-binding cleft of the HLA molecule, and therefore is unlikely to change the bound peptide repertoire of the new HLA-A*02 allele. On the other hand, positions 62 and 63 are located within the antigen binding cleft (Figure 3a), with position 62 exposed to the solvent and readily available for TCR contact, whereas position 63 is buried within the cleft and likely interacts with the residue at position 1 of the peptide. The larger arginine 62 of the new HLA-A*02 allele (Figure 3b) will most likely affect TCR interaction. In the HLA-A*02:35:01 molecule, Glu 63 makes a hydrogen bond with the main chain of the first residue of the peptide, stabilizing the bound

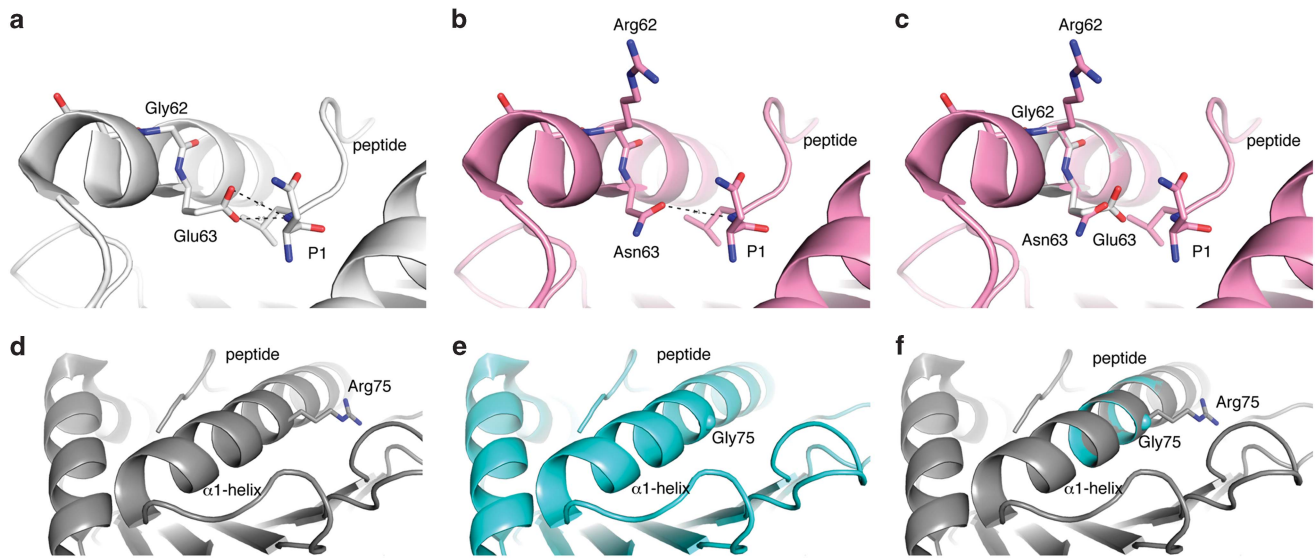


Figure 3 Modelling of new HLA class I alleles identified in Top End Indigenous Australians. The top panels show (a) the HLA-A*02:01 cleft, (b) HLA-A*02:new and (c) a superposition of both HLAs. The HLA cleft is represented in cartoon and the key residues as stick, coloured in white for HLA-A*02:01 and pink for HLA-A*02:new. The dashed lines represent the interaction between the HLA residue 63 and the peptide residue 1. The bottom panels represent the (d) HLA-B*56:01 in grey, (e) HLA-B*56:new in cyan and (f) a superposition of both HLAs. The sphere represents the C α atom of the glycine 75.

peptide. In the new HLA-A*02 allele, the Glu 63 is replaced by an Asn 63, which might not be as suited to strongly bind position 1 of the peptide owing to a smaller side chain (Figure 3c). The change at position 63 might potentially impact on peptide binding and change the peptide repertoire of the HLA-A*02:new compared with that of the HLA-A*02:35:01 molecule. Further studies will determine the peptide repertoire presented by this new HLA-A*02 allele and test their immunogenicity.

HLA-B*56:new was identified in donor LIFT026. This new allele differs from B*56:01:01G or B*56:02 at codon 75 (CGA to GGA) and results in an aa change from arginine to glycine. The Arg75 of HLA-B*56:01 is located on the α 1-helix of the cleft (Figure 3d), outside the cleft and therefore the change to glycine (Figure 3e) should not impact T-cell recognition. The HLA residue 75 does not contact the bound peptide, and the substitution from arginine to glycine (Figure 3f) is unlikely to impact the peptide repertoire. Therefore, the HLA-B*56:new and HLA-B*56:01 alleles are likely to bind the same peptide repertoire.

Dissection of influenza epitopes restricted by HLA-A and HLA-B alleles prevalent in Indigenous Australians

To understand the breadth of potential CD8⁺ T-cell epitopes restricted by HLAs expressed in Indigenous Australians, we analysed epitopes corresponding to the immunogenic influenza proteins: nucleoprotein (NP), matrix 1 (M1) and polymerase basic 1 (PB1),¹⁸ as outlined in the Immune Epitope Database (www.immuneepitope.org). We found that influenza-specific CD8⁺ T-cell epitopes were previously proposed for four HLAs common to non-indigenous populations and identified within our LIFT cohort, including HLA-A*02:01 (40 epitopes), HLA-A*11:01 (20 epitopes), HLA-A*24:02 (10 epitopes) and HLA-B*40:02 (5 epitopes; Table 1). In contrast, no influenza-specific epitopes have been identified for the remaining HLAs (HLA-A*02:new, HLA-A*15:25, HLA-A*34:01, HLA-B*13:01, HLA-B*15:21, HLA-B*40:02, HLA-B*56:01, HLA-B*56:02, HLA-B*56:new) prominent in our LIFT cohort or described as distinct to Indigenous Australians (Table 1). Thus, no influenza-specific CD8⁺ T-cell epitopes have been yet

proposed for 71% of class I HLA alleles identified in Indigenous Australians. This suggests a need to identify prominent influenza epitopes restricted by HLAs associated with Indigenous populations in order to accurately determine the extent of CD8⁺ T-cell immunity in this population. Furthermore, our findings suggest that the immunodominant epitopes in Indigenous Australians are likely to differ from dominant CD8⁺ T-cell specificities in Caucasian populations.

Universal CD8⁺ T-cell immunity directed at the universal HLA-A*02:01-restricted M1₅₈₋₆₆ epitope

To the best of our knowledge, there are currently no data published on influenza-specific CD8⁺ T-cell responses in the Indigenous Australian population. It is, however, well documented that HLA-A*02:01-positive individuals generate prominent CD8⁺ T-cell responses towards the viral M1₅₈₋₆₆ peptide, highly conserved amongst distinct influenza strains and subtypes.^{11,19} To understand how robust and functional M1₅₈⁺CD8⁺ T cells are in the ~15% of Indigenous donors who express HLA-A*02:01, we analysed peripheral blood mononuclear cells (PBMCs) from HLA-A*02:01-positive LIFT donors stimulated with the M1₅₈ peptide, a method used routinely to amplify influenza-specific memory CD8⁺ T cells.^{20,21} M1₅₈⁺CD8⁺ T-cell numbers and their functional capacities were assessed on d10 after the *in vitro* stimulation. Analysis of 7 LIFT individuals of various ages revealed the presence of prominent M1₅₈⁺CD8⁺ T-cell responses in 6 HLA-A*02:01 donors (LIFT07, LIFT09, LIFT011, LIFT022, LIFT027, and LIFT029), but not LIFT03, who expressed HLA-A*02:05 (rather than HLA-A*02:01) positive (Figure 4a). Staining with the A2-M1₅₈ tetramer showed robust expansion of influenza-specific CD8⁺ T cells on d10 after *in vitro* stimulation (mean of 4.2% of CD8⁺ T cells; range 1.23–9.48%).

We next determined the qualitative characteristics of M1₅₈⁺CD8⁺ T cells in the LIFT cohort. As high-quality CD8⁺ T cells simultaneously produce multiple cytokines and display killing capacity, we assessed the production of interferon (IFN)- γ and tumour necrosis factor (TNF) together with CD107a degranulation following short-term stimulation (5 h) with the M1₅₈ peptide and detection by

Table 1 Influenza epitopes presented by HLA-A and -B alleles that are highly prevalent and/or display an ethnic association with Indigenous Australians

Allele	Frequency (LIFT cohort/ NCBI database)	Number of Influenza epitopes identified ^a
<i>Highly prevalent:^b</i>		
A*02:01	17%/13%	40
A*11:01	16%/14%	21
A*24:02	22%/26%	10
A*34:01	24%/40%	0
B*13:01	18%/24%	0
B*15:21	10%/11%	0
B*40:02	8%/17%	5
B*56:01	11%/16%	0
B*56:02	4%/10%	0
<i>Ethnic association:^c</i>		
A*02:new	1%/0%	0
A*24:06	0%/1%	0
A*24:13	0%/1%	0
B*15:25	2%/4%	0
B*56:new	1%/0%	0

Abbreviation: LIFT, looking into influenza T-cell immunity.
^aNumber of different Influenza epitopes defined in the Immune Epitope Database and Analysis Resource (available online at http://www.iedb.org/home_v2.php).
^bFrequency $\geq 10\%$ within the LIFT cohort or NCBI database (for Indigenous Australians).
^cAlleles that are unique to Indigenous Australians or particularly associated with this ethnic group (according to the IPD IMGT/HLA Database available online at <http://www.ebi.ac.uk/ipd/imgt/hla/>).

an intracellular cytokine secretion (ICS) assay. Our data showed that M1₅₈⁺CD8⁺ T cells in the LIFT cohort were polyfunctional and 56.68% \pm 16.3% produced both INF- γ and TNF (Figure 4b). A high proportion of those cells (66.8% \pm 18.3%, representing 40.1 \pm 15.7% of all M1₅₈⁺CD8⁺ T cells) also expressed CD107a (Figure 4c).

Clonal characteristics of the universal CD8⁺ T cells directed at HLA-A*0201-restricted M1₅₈₋₆₆ epitope

TCR usage has an important role in determining the quality of the CD8⁺ T-cell response to viruses and the outcome of infection.²²⁻²⁵ Previous analyses of M1₅₈⁺CD8⁺ T cells, using mainly T-cell clones, have described the M1₅₈-specific TCR repertoire as highly conserved^{26,27} and identified clones shared across different HLA-A*02:01⁺ individuals. Using *in vitro* cultures of three Indigenous and three non-indigenous donors, we dissected the TCR $\alpha\beta$ repertoire utilised by M1₅₈⁺CD8⁺ T cells using a novel single-cell multiplex PCR for simultaneous amplification of the TCR α and TCR β chains (Figure 5).^{21,28} The repertoires were compared in terms of TCR $\alpha\beta$ gene segment usage, CDR3 $\alpha\beta$ loop characteristics (important for the peptide-HLA specificity) and clonal diversity.

Similar to previous reports and our non-indigenous donors (Figures 5c and d), HLA-A*02:01-restricted M1₅₈⁺CD8⁺ T cells isolated from our LIFT cohort were characterised by a strong bias for *TRBV19* gene segment in TCR β chain (frequency of 94.49 \pm 2.11%) and a bias for TRAV27 in the TCR α chain (39.55 \pm 11.30%, Figures 5a and b, Table 2). As seen in non-indigenous donors, individuals in the LIFT cohort also displayed

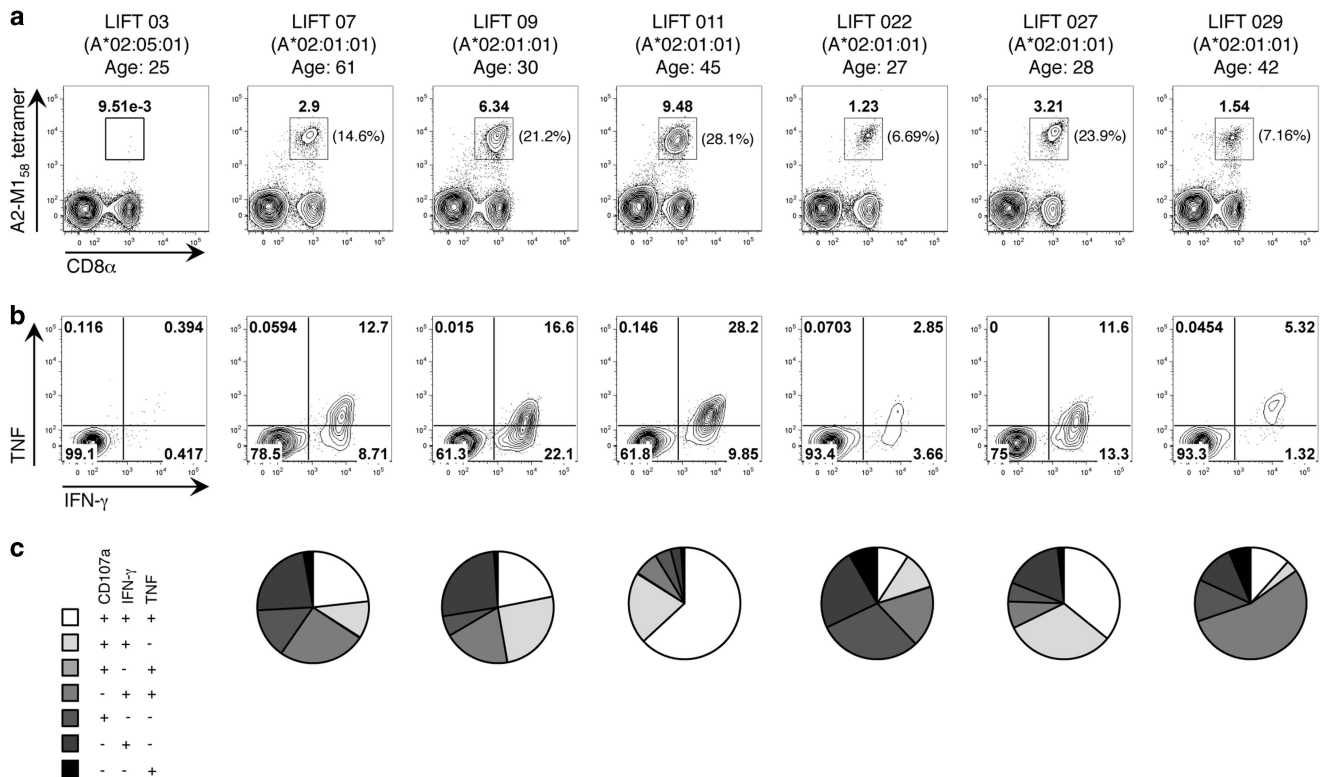


Figure 4 Establishment of robust A2-M1₅₈-specific CD8⁺ T-cell responses in A*02⁺ Indigenous Australians. A2-M1₅₈-specific CD8⁺ T-cell lines were generated by pulsing PBMCs from A*02⁺ donors with M1₅₈₋₆₆ peptide for 10 days in the presence of rIL-2. Plots (a) show A2-M1₅₈ tetramer-PE and anti-CD8-PerCPy5.5 staining of CD3⁺ T cells. Values in parenthesis indicate the proportion of CD8⁺ T cells that bound A2-M1₅₈ tetramer. The function of A2-M1₅₈-specific CD8⁺ T cells was assessed following peptide stimulation in an ICS assay examining expression of IFN- γ , TNF and CD107a (b, c). Plots (b) show anti-IFN- γ -V450 and anti-TNF-APC staining of CD3⁺CD8⁺ T cells. The polyfunctional profile of M1₅₈-specific CD8⁺ T cells is compared in c.

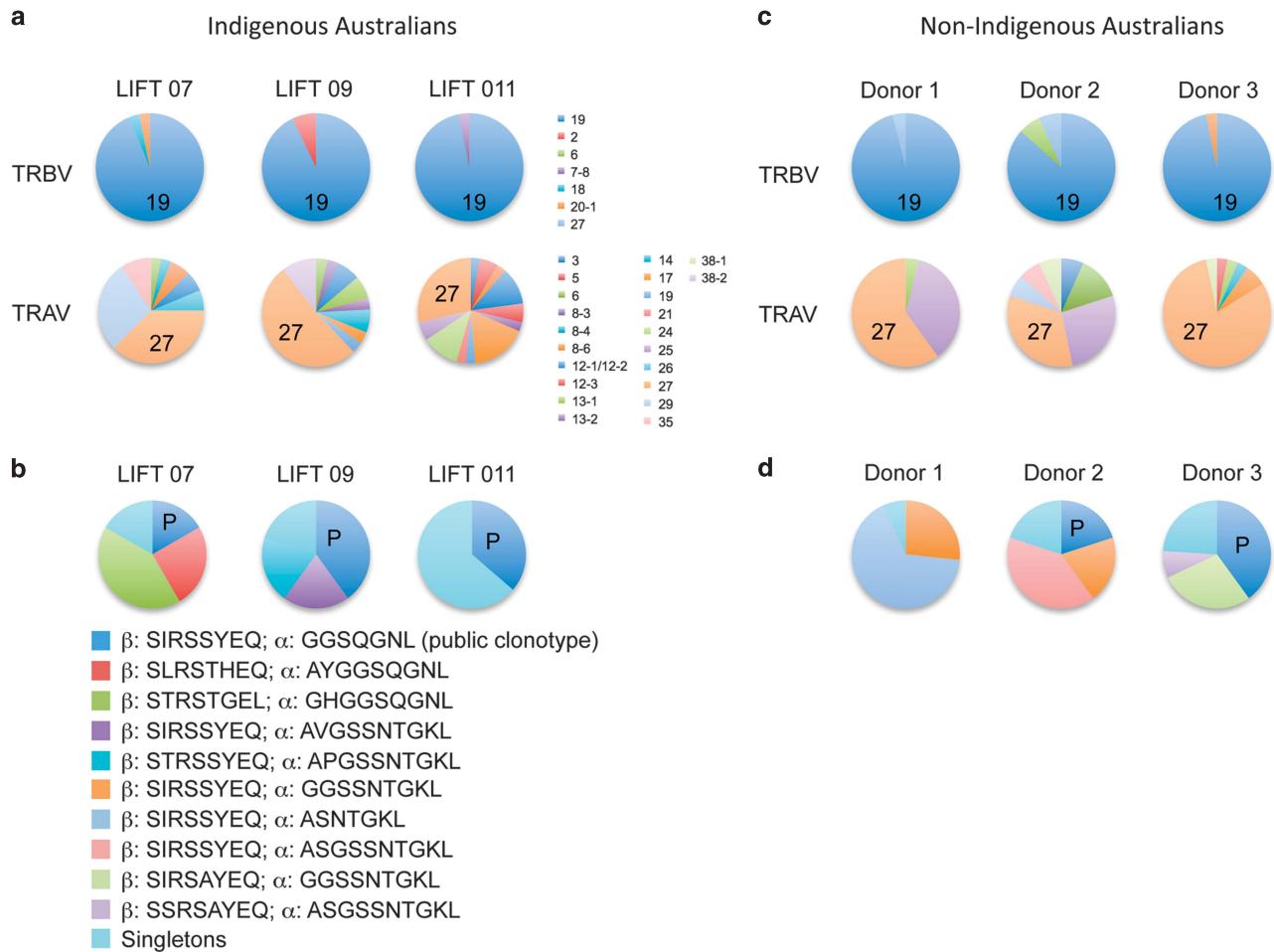


Figure 5 A2-M158-specific CD8⁺ T cells from Indigenous Australians display characteristic TCR $\alpha\beta$ repertoire usage. Single A2-M158-specific CD8⁺ T cells from (a, b) three representative Indigenous and (c, d) three representative non-Indigenous donors were sorted from day 10–19 *in vitro* cultures for analysis of the TCR $\alpha\beta$ repertoire using a multiplex PCR and sequencing protocol. (a, c) *TRBV* and *TRAV* gene usage was compared across donors and (b, d) clonotype usage with the prominent BV19/AV27⁺ subset was further dissected to reveal common usage of a well-defined public A2-M158-specific TCR $\alpha\beta$ clonotype.

a preference for TRBJ2-7 segment within the TCR β chain ($41.96 \pm 18.14\%$ of clones) and TRAJ42 within the TCR α chain ($54.53 \pm 12.59\%$ of clones). The predominant length of the complementarity-determining region 3 of TCR β chain (CDR3 β loop), generally positioned over the centre of the antigenic peptide bound to HLA class I was of 8 aa ($80.21 \pm 9.02\%$ of clones).

Analysis of specific CDR3 β and CDR3 α sequences showed that SIRSSYEQ and GGSQGNL were the most common signatures within the TCR β –TRBV19 and TCR α –TRAV27, respectively, in accord with previous reports. Conversely, LIFT donors displayed a preference for CDR3 α lengths of 7 aa ($28.97 \pm 14.76\%$ of clones), 9 aa ($19.04 \pm 8.33\%$ of clones) and 10 aa ($19.99 \pm 9.27\%$ of clones) in comparison to non-indigenous individuals, which preferentially selected CDR3 α loops of 9 aa in length (41.75 ± 17.24 of clones), followed by 7 aa (28.46 ± 18.88 of clones) and 10 aa (23.20 ± 16.63 of clones).

Interestingly, there was a trend towards a higher level of TCR diversity in Indigenous donors as compared with non-indigenous donors, shown by an increased number of unique CDR3 α (18.33 ± 2.31 versus 10.33 ± 4.73 clonotypes) and CDR3 β clones (15.67 ± 2.52 versus 8 ± 2.65 clonotypes) and CDR3 $\alpha\beta$ pairs (17.67 ± 4.04 versus 11.33 ± 4.62 pairs) per donor. However, these trends were not significant ($P > 0.05$ by a Mann–Whitney test and a non-parametric *t*-test), most likely owing to a limited number of donors.

Overall, our data from Indigenous LIFT donors suggest that in HLA-A*02:01-positive donors A2-M158-specific CD8⁺ T cells are numerically, functionally and clonally comparable to those providing universal immunity to distinct influenza strains and subtypes in non-Indigenous populations.^{11,19,29}

Similar pattern of Rs12252-IFITM3 distribution in Indigenous Australians and Europeans

Rsl2252 in interferon-induced transmembrane protein 3 (IFITM3) can prevent endocytosed virus particles from entering the host cytoplasm and thus, to some extent, control the viral infection.⁶ The SNP rs12252-C variant (C/C genotype), on the other hand, leads to a 21 aa truncation of IFITM3 and is associated with severe outcome of viral diseases, including influenza A virus, HCV and VSV infections.^{6,7,30} High prevalence (30.1%) of rs12252-C/C in East Asia correlates with influenza disease severity^{6,7} and indicates that East Asian populations historically experienced different infection disease patterns from European people who have an rs12252-C/C frequency of <1% (Figure 6). Thus, it was of importance to understand the patterns of SNP rs12252 in Indigenous Australians. Our analysis of rs12252 distribution patterns in Indigenous people showed similar patterns in Europeans and Indigenous people (Figure 6), suggesting that IFITM3-C/C genotype is an unlikely immune correlate for high

Table 2 A2-M1₅₈-specific TCR α β repertoire of Indigenous and non-Indigenous Australians

TRBV	CDR3 β	Aa length	TRBJ	TRAV	CDR3 α	Aa length	TRAJ	Indigenous (%)			Non-Indigenous (%)		
								LIFT 7	LIFT 9	LIFT 11	Donor 1	Donor 2	Donor 3
19	SIRSSYEQ	8	2-7	27	GGSQGNL	7	42	6.3	17.9	9.4	6.7	32.3	
19	SQRSTDTQ	8	2-3	17	DGGGSQGNL	10	42			3.1		6.5	
19	SIRSSYEQ	8	2-7	27	AGSQGNL	7	42			3.1		3.2	
19	SIRSSYEQ	8	2-7	27	AVGSSNTGKL	10	37			3.1		3.2	
19	SIRSSYEQ	8	2-7	27	GGSSNTGKL	9	37				16.0		
19	SMFGGAEA	8	1-1	35	PDRPNGSSQGNL	12	42	25				6.7	
19	STRSTGEL	8	2-2	27	GHGGSQGNL	9	42	15.6					
19	SLRSTHEQ	8	2-1	27	AYGGSQGNL	9	42	9.4					
19	SIGVYGY	7	1-2	38-1	MSPFGTSGYKGL	12	52	9					
19	SIRSSYEQ	8	2-7	8-6	GGSQGNL	7	42	6.3					
19	SIAGGAARPQ	10	1-5	26-2	RDGSSNTGKL	11	37	6.3					
19	STRSGWEQ	8	2-1	27	AGSSNTGKL	9	37	3.1					
20	RGAPGPQ	7	1-5	6	HTRDDKI	8	30	3.1					
18	SLVGGPDEQ	10	2-1	8-4	ARL	3	31	3.1					
19	SSRSAYEQ	8	2-7	12-1	NIGGGSQGNL	10	42	3.1					
19	SIRSSYEQ	8	2-7	12-1	GGDGSQGNL	10	42	3.1					
19	SSRSAYEQ	8	2-7	27	ALGSSNTGKL	10	37	3.1					
19	SITRNEQ	7	2-1	35	FFRDDKI	7	30	3.1					
19	SVRSSEYEQ	8	2-7	27	GGSQGNL	7	42	14.3					
2	SDWDRVKGPDQ	12	2-3	14	REDNGGKL	9	23	7.1					
19	STRSSYEQ	8	2-7	27	APGSSNTGKL	10	37	7.1					
19	SIRSSYEQ	8	2-7	27	AVGSSNTGKL	10	37	7.1					
19	SIGTYGY	7	1-2	38-2	SVNAGGTSYGKL	12	52	7.1					
19	SIRAADTQ	8	2-3	6	DMGGGSQGNL	10	42	3.6					
19	STRSGVEQ	8	2-7	8-3	GGSQGNL	7	42	3.6					
19	SMRSSYEQ	8	2-7	12-2	NDQGGKL	7	23	3.6					
19	SMAINEQ	7	2-1	12-2	SVSKTSYDKV	10	50	3.6					
19	SGRSGETQ	8	2-5	17	SRGEGAQKL	9	54						
19	STRSLEPQ	8	1-5	13-1	QNQGGKL	7	23	3.6					
19	SQRSSYEQ	8	2-7	13-1	TYNQGGKL	8	23	3.6					
19	SIHSGSNNEQ	10	2-1	19	NGGSLGRL	10	18	3.6					
19	SIRSDYTL	8	1-6	27	VGGSYIP	7	6	3.6					
19	SLRSDGEL	8	2-2	27	AGGGSQGNL	9	42	3.6					
19	SIGLYGY	7	1-2	38-2	ASGGSQGNL	9	42	3.6					
19	SGLSNQPPQ	8	1-5	24	ATNAGGTSYGKL	12	52	3.6					
19	SLRSDYEQ	8	2-7	12-2	YGGSNYQL	8	33			12.5			
19	SIRAGTEA	8	1-1	12-3	SSNTGKL	7	37	9.4					
19	SIRSGVEQ	8	2-7	5	SGDGSQGNL	10	42	6.3					
19	SVYSNQPPQ	8	1-5	17	SIRGGSQGNL	10	42	6.3					
19	SSRSYEQ	8	2-7	8-6	DEYGNYSQGNL	13	42	6.3					
19	SSRSAYEQ	8	2-7	12-2	SSNTGKL	7	37	3.1					
19	GPLSTDTQ	8	2-3	3	GGSQGNL	7	42	3.1					
19	STRSTGEL	8	2-2	25	RDGTGANL	9	36	3.1					
19		8			SGGGSQGNL	9	42	3.1					

Table 2 (Continued)

TRBV	CDR3β	Aa length	TRBJ	TRAV	CDR3α	Aa length	TRAJ	Indigenous (%)			Non-indigenous (%)			
								LIFT 7	LIFT 9	LIFT 11	Donor 1	Donor 2	Donor 3	
19	SIRSSYEQ	8	2-7	27	PGSNTGKL	8	37			3.1				
7-8	SLVWVGADSNQPQ	13	1-5	13-2	INSGNTPL	8	29			3.1				
				17	RENGGKL	8	23							
19	SMRSTDTQ	8	2-3	17	DGGGSGQNL	10	42			3.1				
19	SPFGGPNEQ	10	2-1	17	DAWGYGSGQNL	12	42			3.1				
19	SRRSTDTQ	8	2-3	21	L	1	20			3.1				
				25	SGGSGQNL	9	42							
19	SIRSAYEQ	8	2-7	27	GSSNTGKL	8	37			3.1				
19	SARSADTQ	8	2-3	27	AGGSGQNL	9	42			3.1				
19	SVRSSYEQ	8	2-7	27	AVGSSNTGKL	10	37			3.1				
19	SILTPRTEA	10	1-1	27	APRTSGTYKY	10	40			3.1				
19	SIRSSYEQ	8	2-7	27	ASNTGKL	7	37			40.0				
19	SGRSTDTQ	8	2-3	25	TYGGSQNL	9	42			32.0				
19	SVRSQETQ	8	2-5	13-1	SGGSGQNL	9	42			4.0				
27	SLYGPMTSGEL	11	2-2	25	TYGGSQNL	9	42			4.0				
19	SIRSAYEQ	8	2-7	27	THGSSNTGKL	10	37			4.0				
19	SIRSSYEQ	8	2-7	27	ASGSSNTGKL	10	37						13.3	
19	SGTAVEKL	8	1-4	3	RDGTGANL	9	36						6.7	
19	SRRSTDTQ	8	2-3	6	DIGGSGQNL	10	42						6.7	
19	SIFGSSGNTI	10	1-3	6	DNTNAGS	8	27						6.7	
19	SARSTDTQ	8	2-3	25	NYGGSQNL	9	42						6.7	
19	STRSTDTQ	8	2-3	25	SSGSGQNL	9	42						6.7	
19	STRSTDTQ	8	2-3	25	VGGSGQNL	9	42						6.7	
19	SLRSTDTQ	8	2-3	25	SYGGSQNL	9	42						6.7	
19	SIRSSYEQ	8	2-7	27	GGHGSQNL	10	42						6.7	
6	SYSAAAGTSLDIQ	13	2-4	29	SDPNQGGKL	9	23						6.7	
27	SLIFPSGEQ	9	2-7	35	PDGSSNTGKL	10	37						6.7	
19	SIGSYGY	7	1-2	38-1	MNDAGGTSYGKL	12	52						6.7	
19	SIRSAYEQ	8	2-7	27	GGSSNTGKL	9	37						22.6	
19	SSRSAYEQ	8	2-7	27	ASGSSNTGKL	10	37						6.5	
19	SVRSSYEQ	8	2-7	27	SGSQNL	7	42						3.2	
19	SIRSAYEQ	8	2-7	27	ASGSSNTGKL	10	37						3.2	
19	SIRSSYEQ	8	2-7	27	AIGSSNTGKL	10	37						3.2	
19	SVRSSYEQ	8	2-7	27	AIGSSNTGKL	10	37						3.2	
20-1	RTSGDFGEQ	9	2-1	13-1	RGYGGATNKL	10	32						3.2	
19	STRSTDTQ	8	2-3	12-3	TGDDGSGQNL	10	42						3.2	
19	SIYGNTA	8	1-1	14	REDPGGTSYGKL	12	52						3.2	
19	SIGVYGY	7	1-2	38-1	MIGAGGTSYGKL	12	52						3.2	
					Total number of sequences	32	28			15			31	

Abbreviations: Aa, amino acid; LIFT, looking into influenza T-cell immunity. A2-M1₅₈ tetramer-binding CD8⁺ T cells were single-cell sorted. Populations were gated as viable Dump⁺CD3⁺CD8⁺A2-M1₅₈ tetramer⁺ cells. Paired amino-acid CDR3α/β diversity analysis was performed for non-Indigenous donors (n=3) and Australian Indigenous LIFT donors (n=3). Data show TRBJ and TRAV gene usage and the frequency of CDR3α-CDR3β clonotypes. The abundance of particular CDR3α/CDR3β clonotypes is given in bold. TCRαβ repertoires were performed using a TCRαβ multiplex protocol.

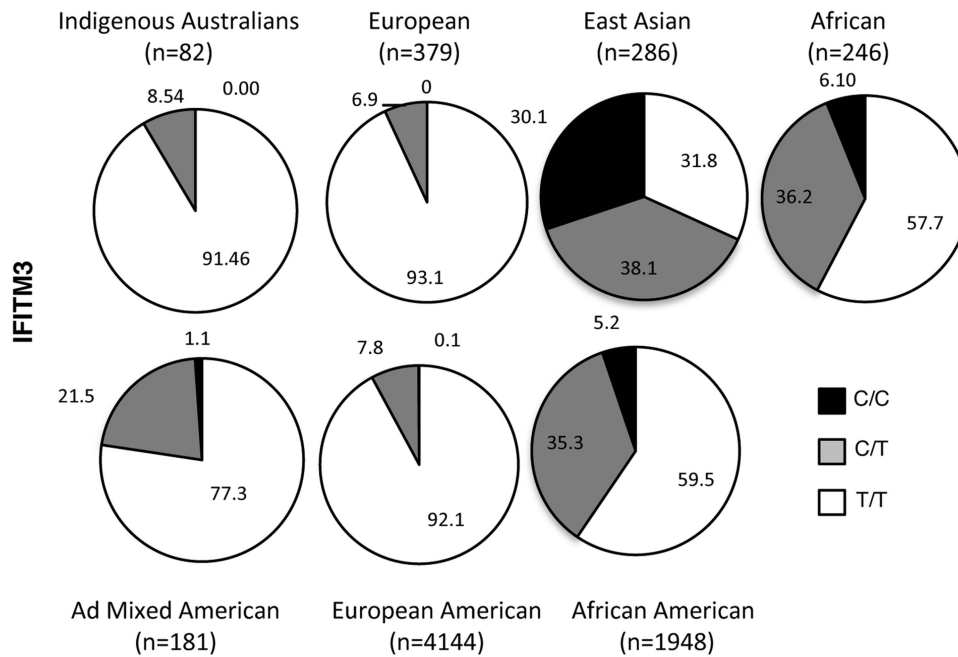


Figure 6 Expression of IFITM3 genotype associated with increased influenza disease severity across different ethnicities. Sequencing of the exon 1 region of IFITM3 containing rs12252 was performed for Indigenous Australians to determine allele frequencies of the SNP rs12252-C variant (in contrast to the WT rs12252-T variant) associated with increased influenza disease severity. Occurrence of the C/C, T/C and T/T genotypes was determined for this study population and compared with data for other ethnicities obtained from the 1000 genomes project (available on line at www.1000genomes.org).

influenza disease severity in Indigenous Australians. Furthermore, it is interesting that while our HLA results (Figures 1–3) showed that Indigenous people share some similarity in HLA patterns with Asian populations, which suggests an ancestral link, the IFITM3 genotypes (Figure 6) are distinct. Considering that African Americans (AFR_AMR, 5.2%, C/C) have similar rs12252-C/C patterns to their ancestor Africans (AFR, 6.1%, C/C), it seems that mixing of AFR and European populations in America two centuries ago did not impose significant changes in rs12252-C/C distribution. Perhaps distinct disease burdens have selectively shaped rs12252-C/C distribution in Asian and Indigenous Australian populations, whereas similar HLA patterns have been retained.

DISCUSSION

Indigenous populations, including Indigenous Australians and Alaskans, are at high risk of severe influenza disease.¹¹ In the case of an emergence of a new influenza viral strain, including avian-derived influenza viruses such as A/H7N9,⁹ CD8⁺ T cells can have an important role in host recovery. However, pre-existing influenza-specific CD8⁺ T-cell responses recognising distinct influenza strains and subtypes vary greatly across ethnicities¹¹ and HLA profiles. We found that while ~15% of Indigenous Australians (HLA-A*02:01-positive) would have robust universal CD8⁺ T-cell pools, epitopes associated with other HLA types prominent in Indigenous people are unknown.

Our analysis of the LIFT cohort found that Indigenous Australians display a restricted and distinct HLA profile in accordance with previously published serological studies.^{14–16} Our molecular HLA typing verified the predominant usage of HLA-A*02:01, 11:01, 24:02, 34:01 and HLA-B*13:01, 15:21, 40:01/02, 56:01/02. Such restriction in HLA diversity and HLA usage is likely to have arisen from an evolutionary bottleneck that established a small ancestral pool with limited HLA diversity. As diversity of HLA alleles evolves rapidly, it is intriguing that there is a high degree of conservation in Indigenous

Australians. This could be partly explained by limited mixing with other populations, long-term adaptation to local pathogens, and minimal exposure to new pathogens that might drive selection and/or emergence of new variants. Limited or no exposure to influenza prior to European contact in the eighteenth century may explain a low prevalence of protective HLA variants for influenza.

In addition, we identified two new HLA alleles, HLA-A*02:new and HLA-B*56:new, in 2 out of 82 LIFT donors. The aa changes in HLA-A*02:new could potentially impact the repertoire of bound epitopes. In HLA-A*02:01, Glu 63 is buried in the cleft and likely interacts with the first residue of the peptide via a network of hydrogen bonds. Asn 63 of the HLA-A*02:new appears to be too short to form the same network and may contact the peptide residue 1 via hydrophobic interaction only. Potentially, the HLA-A*02:new might not be able to bind, or not as stably, the M1₅₈ epitope compared with the HLA-A*02:01 molecule.

Establishment of robust HLA-A*02:01-M1₅₈-specific CD8⁺ T-cell responses with a typical magnitude, function and TCRαβ repertoire structure in HLA-A*02:01-positive Indigenous Australians suggests that at least ~15% of Indigenous Australians would have cross-strain protective CD8⁺ T-cell immunity. Once established, M1₅₈⁺CD8⁺ T cells can recognise any influenza strain and subtype owing to the high level of conservation of this epitope.^{11,19,29} We thus provide the first data on influenza-specific T-cell immunity in the Indigenous population. Our findings suggest that where there are shared HLA profiles between Indigenous Australians and non-Indigenous populations, they display similar CD8⁺ T-cell responses to known, well-characterised influenza epitopes.

With the exception of HLA-A*02:01, little is known about the peptides presented by HLAs specific to Indigenous Australians (Table 1). Further studies are required to identify and characterise epitopes restricted by these unique HLA alleles, in particular HLA-A*34:01 (frequency of 24% of HLA-A alleles), HLA-B*13:01, 15:21,

56:01 and 56:02 (together accounting for 43% of HLA-B alleles), to provide insights into the effectiveness of CD8⁺ T-cell immunity in this population. Furthermore, as there is a strong correlation between the expression of HLA-A*24 and pH1N1 influenza-induced mortality,¹² analyses of CD8⁺ T-cell responses to epitopes restricted by HLA-A*24 alleles in Indigenous Australians are a priority.

Apart from HLA distribution, the expression of IFITM3-C/C SNP rs12252 represents the only other host factor known to be associated with increased influenza disease severity across different ethnicities. Following influenza A virus infection, the expression of IFITM3-C/C SNP rs12252 is related to an early hypercytokinemia, especially in the Asian population.^{6,7} The rs12252-C genotype is reasonably infrequent in Indigenous Australians, suggesting that compromised IFITM3 function appears not to be linked to the increased susceptibility to severe influenza disease in this population.

To support the rational design of an effective, broad-spectrum universal vaccine, it is essential to define the dominant influenza-specific T-cell responses focused on the allelic HLA variants characteristic of Indigenous Australians.^{15,16} Identification of immune and host factors underlying severe influenza disease in Indigenous Australians, highly susceptible to influenza, is of an enormous importance. Understanding which individuals within the Indigenous populations (globally) are at risk of developing severe influenza disease (for example, HLA-A24-expressing individuals¹²) will inform our future prognostic strategies for the treatment and management of severe influenza pneumonia and for designing the vaccination programmes that target specific groups for routine influenza immunisation.

METHODS

Human ethics

All the experiments conformed to the NHMRC Code of Practice and were approved by the University of Melbourne Research Human Ethics Committee (Applications #1441452.1 and #0931311.5) and the Human Research Ethics Committee of Northern Territory Department of Health and Menzies School of Health Research (Application # HREC-2012-1928).

Recruitment of Indigenous Australian donors (LIFT cohort)

To understand influenza-specific responses in the Indigenous population, participants ≥ 18 years of age were recruited from the Royal Darwin Hospital and also from healthy volunteers in Darwin as a 'looking into influenza T-cell immunity' (LIFT) cohort. We ensured representation of different age groups across the main regions of the Top End (Darwin urban, Darwin rural, West Arnhem/Daly, Tiwi Islands, East Arnhem, Katherine). This permitted sampling from a range of language and people groups so as not to bias the population HLA distribution. For hospital inpatients, permissions were requested from the treating clinical team for the research team to approach potential participants. Patients were then approached to discuss the study and seek informed consent for access to medical and immunisation records and to obtain a 50-ml venous blood sample. Participants were excluded if they had a diagnosis of Systemic Inflammatory Response (SIRS) (defined as satisfying ≥ 2 SIRS criteria—temperature $< 36^\circ\text{C}$ or $> 38^\circ\text{C}$; heart rate > 90 b.p.m.; respiratory rate > 20 b.p.m.; white blood cell count $< 4 \times 10^9$ per l or $> 12 \times 10^9$ per l) or a haemoglobin below the normal range. Participant recruitment, sample collection, isolation by Ficoll Paque density centrifugation (GE Healthcare, Uppsala, Sweden)²⁰ and storage of PBMCs were performed at the Menzies School of Health Research. PBMCs were cryopreserved for further use at University of Melbourne. DNA was isolated from granulocytes using a QIAGEN QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions.

PBMC isolation from buffy packs

PBMCs were isolated also from buffy packs (obtained from the Blood Bank, Melbourne, VIC, Australia) for HLA-A*02:01⁺ non-Indigenous donors. PBMCs

were isolated by Ficoll Paque density centrifugation,²⁰ then cryopreserved for future use. Viability of the PBMCs derived from the Indigenous donors was $> 80\%$ (80–95%) upon thawing out. Furthermore, a viability stain (Live/dead stain) was used in all ICS assays to confirm that all detected responses are from viable CD8⁺ T cells. A $> 85\%$ viability was detected in all CD8⁺ T-cell lines derived from Indigenous donors following the ICS assay.

HLA typing and IFITM3 genotyping

HLA class I and class II molecular genotyping was performed from genomic DNA by the Victorian Transplant and Immunogenetics Service (Parkville, Melbourne, VIC, Australia). For IFITM3 sequencing, the *exon 1* region of IFITM3 containing rs12252 was amplified from genomic DNA by PCR with forward (5'-GGAAACTGTTGAGAAACCGAA-3') and reverse (5'-CATACG CACCTCACGGAGT-3') primers, as previously described.⁷

Generation of M1⁵⁸-specific CD8⁺ T-cell lines

To amplify influenza-specific CD8⁺ T cells directed at the immunodominant HLA-A*02:01-restricted M1₅₈₋₆₆ epitope, PBMCs from HLA-A*02 donors were stimulated with the M1₅₈₋₆₆ (GILGFVFTL) peptide and then cultured for 10 to 19 days, as previously described.^{20,31} Cultures were supplemented twice weekly with 10 U ml⁻¹ rIL-2 and CD8⁺ T-cell lines from non-indigenous donors were restimulated once weekly with gamma-irradiated M1₅₈-pulsed C1R-A*02:01 cells.

Intracellular cytokine staining (ICS)

At d10, HLA-A*02-M1₅₈⁺CD8⁺ T cells from Indigenous donors were assessed by an IFN- γ /TNF/CD107a ICS assay. C1R-A*02:01⁺ were used as antigen presenting cells (APCs) in an ICS assay to restimulate PBMCs. APCs (at $1-3 \times 10^7$ cells per ml) were pulsed with 10 μM peptide in 100 μl serum-free media RPMI for 60 min at 37°C. Subsequently, 1×10^5 peptide-pulsed APCs were co-cultured with 2×10^5 restimulated PBMC samples for 5 h at 37°C in U-bottom 96-well plates in the presence of IL-2 (10 U ml⁻¹), 2 μl GolgiPlug (BD Biosciences, Franklin Lakes, NY, USA; final dilution of 1:1000) and 1.33 μl Golgi-Stop (BD Biosciences, final dilution of 1:1500) and 1 μl anti-CD107a-AF488 (eBiosciences, San Diego, CA, USA). Following stimulation, cells were washed with FACS buffer (1% bovine serum albumin (Gibco, Waltham, MA, USA) and 0.02% sodium azide (Sigma, St Louis, MO, USA) in phosphate-buffered saline (PBS)) and stained with anti-CD3-PeCy7 (eBiosciences) and anti-CD8 α -PerCP-Cy5.5 (BD Biosciences) and Live/Dead-NIR (Invitrogen, Carlsbad, CA, USA) in PBS, and then washed twice. Cells were fixed and permeabilised using the BD Cytofix/Cytoperm Plus Fixation/Permeabilisation Kit (BD Biosciences), and intracellularly-stained with anti-IFN- γ -V450 (BD Horizon, Franklin Lakes, NY, USA), anti-TNF-APC (BD Biosciences) in perm-wash buffer. Samples were washed and acquired by flow cytometry on a BD FACS Canto II (BD Biosciences) and analysed by FlowJo software (Treestar, Ashland, OR, USA). Cytokine production was calculated by subtracting background fluorescence using 'no peptide' C1R-A*02:01⁺ controls.

Tetramer staining

D10-19 M1₅₈ cultures were stained with HLA-A*02:01-M1₅₈ tetramer conjugated to PE or APC at a 1:100 dilution in FACS buffer (PBS with 0.1% bovine serum albumin) or PBS. Cells were then washed twice with cold FACS buffer and stained with a cocktail of antibodies including anti-CD3-PB (Biolegend) or anti-CD3-PeCy7 (eBiosciences), anti-CD8-PerCP-Cy5.5 or anti-CD8-PerCP (both BD Biosciences), CD27-APC or APC-Cy7 (BD Biosciences), CD45RA-FITC (BD Pharmingen, San Diego, CA, USA) and Live/Dead-NIR (Invitrogen) (Indigenous donors only), washed twice with FACS buffer or PBS. Cells were then resuspended in 200 μl of sort buffer and passed through a 40- μm sieve prior to flow cytometric analysis or sorting.

Single-cell multiplex RT-PCR for paired CDR3 β and CDR3 α analysis

CD8⁺ T cell lines were tetramer-stained as above and single HLA-A*02:01-M1₅₈-tetramer⁺ CD3⁺dump⁻CD8⁺tetramer⁺ cells were single-cell sorted on a FACS Aria III (BD Biosciences) into 80 wells of a 96-well twin-tec plate (Eppendorf, Hamburg, Germany). The CDR3 $\alpha\beta$ regions were determined by a

novel single-cell multiplex reverse transcription PCR (RT-PCR) protocol.^{21,28} mRNA transcripts were reverse-transcribed to cDNA, using a VILO kit (Invitrogen). For the internal round of PCR, 2.5 µl of the external product was used as template, with either a set of TRAV or TRBV internal primers.²⁸ The internal PCR reaction included the two different primers sets at 5 pmol ml⁻¹ (TRAV and TRAC, or TRBV and TRBC). Positive PCR products were purified with Exo-SAP-IT (Affymetrix, Santa Clara, CA, USA) and sequenced using TRAC or TRBC internal primers with BigDye v3.1 (Applied Biosciences, Foster City, CA, USA). Sequences were cleaned using DyeEx sequencing plates (QIAGEN) and sequencing was performed at the Sequencing and Genotyping facility within the Department of Pathology at the University of Melbourne. Sequences were analysed using FinchTV, and V and J region usage was identified by IMGT query (www.imgt.org/IMGT_vquest).

New HLA class I modelling

The HLA-A*02:new model was made using the published HLA-A*02:01 structure (PDB code: 3GSO²⁰) and mutating the corresponding residues: glycine 62 to arginine, glutamic acid 63 to asparagine. The HLA-B*56:new was modelled using the HLA-B*57:01 structure (PDB code: 3VRI³²) and mutating the residue 75 from arginine to glycine. All the mutations have been generated in Pymol.³³

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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