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Molecular Imprint of Exposure to Naturally Occurring Genetic Variants of Human Cytomegalovirus on the T cell Repertoire

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Exposure to naturally occurring variants of herpesviruses in clinical settings can have a dramatic impact on anti-viral immunity. Here we have evaluated the molecular imprint of variant peptide-MHC complexes on the T-cell repertoire during human cytomegalovirus (CMV) infection and demonstrate that primary co-infection with genetic variants of CMV was coincident with development of strain-specific T-cell immunity followed by emergence of cross-reactive virus-specific T-cells. Cross-reactive CMV-specific T cells exhibited a highly conserved public T cell repertoire, while T cells directed towards specific genetic variants displayed oligoclonal repertoires, unique to each individual. T cell recognition foot–print and pMHC-I structural analyses revealed that the cross-reactive T cells accommodate alterations in the pMHC complex with a broader foot-print focussing on the core of the peptide epitope. These findings provide novel molecular insight into how infection with naturally occurring genetic variants of persistent human herpesviruses imprints on the evolution of the anti-viral T-cell repertoire.

Infection with human cytomegalovirus (CMV) is the most common cause of infectious complications in newborns, causing deafness and other developmental abnormalities[1,2]. CMV also leads to serious complications in patients actively undergoing immunosuppression following both solid organ and hematopoietic stem cell transplantation[3]. Whilst pre-existing immunity in both settings plays a central role in reducing disease burden, intrauterine transmission of CMV and subsequent hearing loss in infants has been reported from women with pre-conceptional immunity[4–7]. It is now well established that exposure to heterologous strains of CMV in immunocompetent individuals can alter the humoral response to CMV, leading to the emergence of new non-crossreactive neutralizing antibodies recognising surface glycoproteins[8]. In a solid organ transplant setting, donor seropositivity increases the risk of CMV antigenemia, even in seropositive recipients[9]. Recent genotypic analysis has revealed the complex nature of CMV infection in transplant recipients, whereby the presence of multiple genotypically distinct CMV strains has been observed and the presence of multiple genotypes has been associated with increased viral load and delayed viral clearance[10,11].

Despite emerging evidence that exposure to genotypically distant variants of CMV is a common phenomenon that can lead to CMV-associated disease, little is known about the potential impact of exposure to genetic variants of CMV on the T cell repertoire in humans. Therefore to explore this we analysed the impact of sequence variation within the immunodominant immediate-early (IE) 1 protein of CMV on the T cell response. Using a combination of functional avidity analysis, major histocompatibility complex (MHC) multimer staining, T cell repertoire analysis, biophysical and structural analysis, we provide insight into the complex dynamics of the T cell repertoire generated in response to heterologous strains of CMV. We demonstrate that exposure to heterologous strains of

CMV shapes the peripheral blood T cell repertoire, which is reflected in both the functional profile of virus-specific T cells and the biophysical interactions between peptide-MHC (pMHC) and pMHC-T cell antigen receptor (TCR).

Results

Longitudinal evolution of anti-viral CD8$^+$ T cell responses following primary co-infection with genetic variants of CMV. Previous studies have shown that exposure to genetic variants of human herpesviruses in persistently infected individuals can impact on the selection of the anti-viral T cell repertoire[12,13]. However, very little is known on how these T cell responses evolve following primary infection with distinct genetic variants and their impact on the establishment of memory/effector anti-viral T cell repertoire. To address this issue we focused on an immunodominant HLA B8-restricted IE-1 epitope for which four distinct genetic variants have been identified[14,15]. These include ELRRKMMYM (referred to as ELR_MYM), ELKRKMIYM (referred to as ELK_IYM), ELKRKMMYM (referred to as ELK_MYM) and ELNRKMIYM (referred to as ELN_IYM)[14,15]. These epitope variants contain a conserved mutation (R → K) or a non-conserved (R → N) mutation at position 3, a known HLA B8 anchor residue[16], and a mutation at position 7 (M → I). We initially investigated the impact of co-infection with CMV genetic variants in a seronegative HLA B8+ transplant recipient who received a kidney transplant from a seropositive donor. This transplant recipient developed acute primary CMV infection six months after transplant and continued to show recurrent viral reactivation for a prolonged period. Sequence analysis of viral DNA from the peripheral blood revealed that this patient was co-infected with two distinct genetic variants of CMV encoding HLA B8-restricted IE-1 epitopes ELR_MYM and ELK_IYM. To assess the impact of these genetic variants on anti-viral T cell immunity, we first co-stained peripheral blood CD8$^+$ T cells with pMHC multimers specific for ELR_MYM and ELK_IYM epitopes. These analyses revealed that this patient generated two distinct T cell populations recognizing the ELR_MYM or ELK_IYM epitopes simultaneously around sixteen months post-transplant (Fig. 1a). The frequency of these T cells progressively increased with ELR_MYM-specific T cells dominating overall expansion. Interestingly, analysis of CMV-specific CD8$^+$ T cells at 54 months post-transplant showed the emergence of a cross-reactive T cell population, which recognized both ELR_MYM and ELK_IYM, suggestive of the evolution of a cross-reactive T cell population over time (Fig. 1a).

To further characterize the broader reactivity of CMV-specific CD8$^+$ T cells against other IE-1 genetic variants, we conducted *ex vivo* intracellular cytokine assays using ELR_MYM, ELK_IYM, ELK_MYM and ELN_IYM synthetic peptides. Consistent with the data obtained with pMHC multimers, a gradual predominance of ELR_MYM-specific T cells early post-reactivation was observed (Fig. 1b). More importantly, the broader cross-recognition by IE-1 specific T cells not only extended to the ELK_IYM epitope but also ELK_MYM and ELN_IYM as well. We next expanded CMV-specific CD8$^+$ T cells *in vitro* using ELR_MYM and ELK_IYM peptide epitopes and assessed their reactivity against four different genetic variants of the IE-1 epitope. Data presented in Figure 1c shows that the T cells expanded with ELK_IYM peptide showed broad cross-reactivity against ELR_MYM, ELK_IYM, ELK_MYM and ELN_IYM epitopes, while stimulation with ELR_MYM peptide generated T cells which only recognized ELR_MYM and ELK_MYM epitopes. Taken together these preliminary analyses suggested that primary exposure to distinct genetic variants of CMV can have a significant impact on the evolution of T cell responses and their ability to recognize variant epitope sequences.

***Ex vivo* profiling of IE-1-specific T cells from individuals infected naturally occurring genetic variants of CMV.** To extend our preliminary analyses described above, we recruited a cohort of 18 HLA B8+ individuals including eight solid organ transplant recipients and ten healthy virus carriers. Five transplant recipients were seropositive before engraftment, while three patients underwent primary infection following transplant from seropositive donors. *Ex vivo* analysis using the dual MHC-multimer staining revealed four distinct patterns of pMHC multimer staining. Representative analyses of these distinct patterns of staining are shown in Figure 2a. In both transplant recipients and healthy individuals, the majority of individuals showed preferential binding of either ELR_MYM (represented by Tx4 and H6) or ELK_IYM (represented by Tx7 and H4) multimers. Interestingly, some individuals cross-recognized both ELR_MYM and ELK_IYM variants (represented by Tx2 and H1), while others (represented by Tx9 and H5) bound the pMHC multimers independently of each other, similar to that seen in the transplant patient in Figure 1.

To further profile the T cell cross-recognition of distinct genetic variants, we next performed *ex vivo* intracellular IFN-γ assays using ELR_MYM, ELK_IYM, ELK_MYM and ELN_IYM peptides. Representative data from five transplant recipients and five healthy virus carriers is shown in Figure 2b. While consistent with the dual MHC-multimer analysis, these observations revealed distinct patterns of recognition that were differentially influenced by the amino acid substitutions at positions 3 and 7. Individuals whose T cells cross-recognised both of the MHC-multimers (Tx2 and H1) responded efficiently to the ELR_MYM, ELK_IYM, and ELK_MYM variants, but failed to recognise the ELN_IYM, suggesting that while their T cells could accommodate the conserved R→K mutation at position 3, and the M → I mutation at position 7, they could not accommodate the R→N mutation at position 3. Whereas individuals displaying differential binding of the multimers could only accommodate the amino acid substitution at position 3, whereby reactivity to the ELK_MYM variant was evident in donors who preferential bound the ELR_MYM multimer (Tx4, Tx9, H5 and H6), and reactivity to the ELN_IYM variant was also evident in donors who recognised ELK_IYM (Tx7, Tx9, H4 and H5). Interestingly, one individual, H8, preferentially recognised the ELN_IYM variant, with reduced reactivity to the ELK_IYM variant.

To confirm the preferential recognition of different variants by individual donors we expanded T cells specific for the ELR_MYM, ELK_IYM or ELN_IYM epitopes following *in vitro* stimulation with cognate peptide, and tested these T cells for recognition of all four variant peptides. Representative analysis from eight populations is shown in Figure 3. Consistent with our ex vivo analysis T cells from individuals that cross-recognised the ELR_MYM, ELK_IYM and ELK_MYM variants could be expanded from some individuals (represented by H1). Conversely T cells from other donors, who did not efficiently cross-recognise both multimers, displayed preferential recognition of either ELR_MYM and ELK_MYM (Tx9, H5 and H6), following expansion with ELR_MYM, ELK_IYM following expansion with ELK_IYM (Tx9, H5 and H4), or ELN_IYM (H8). These observations also confirmed that in some donors (Tx9 and H5) co-infection with genetic variants can lead to distinct variant specific non-cross reactive T cell populations.

We next sought to explore the ability of the different IE-1 specific T cell populations to recognise the endogenously processed epitope variants in a viral infection setting. HLA B8+ fibroblasts were infected with either the TB40E or Merlin strains of CMV, which encode the ELK_IYM and ELR_MYM variants, respectively. IE-1 specific T cells from 4 donors: 1 cross-reactive (H1), 1 ELR_MYM specific (Tx9) and 2 ELK_IYM specific (Tx9 and H4), were then incubated with the infected fibroblasts and assessed for IFN-γ production (Fig 4). Although the cross-reactive T cells were capable of recognising both the Merlin and TB40E strains, these effector cells displayed reduced recognition of the ELR_MYM expressing Merlin strain-infected cells (Fig. 4a). This reduced recognition of endogenously processed

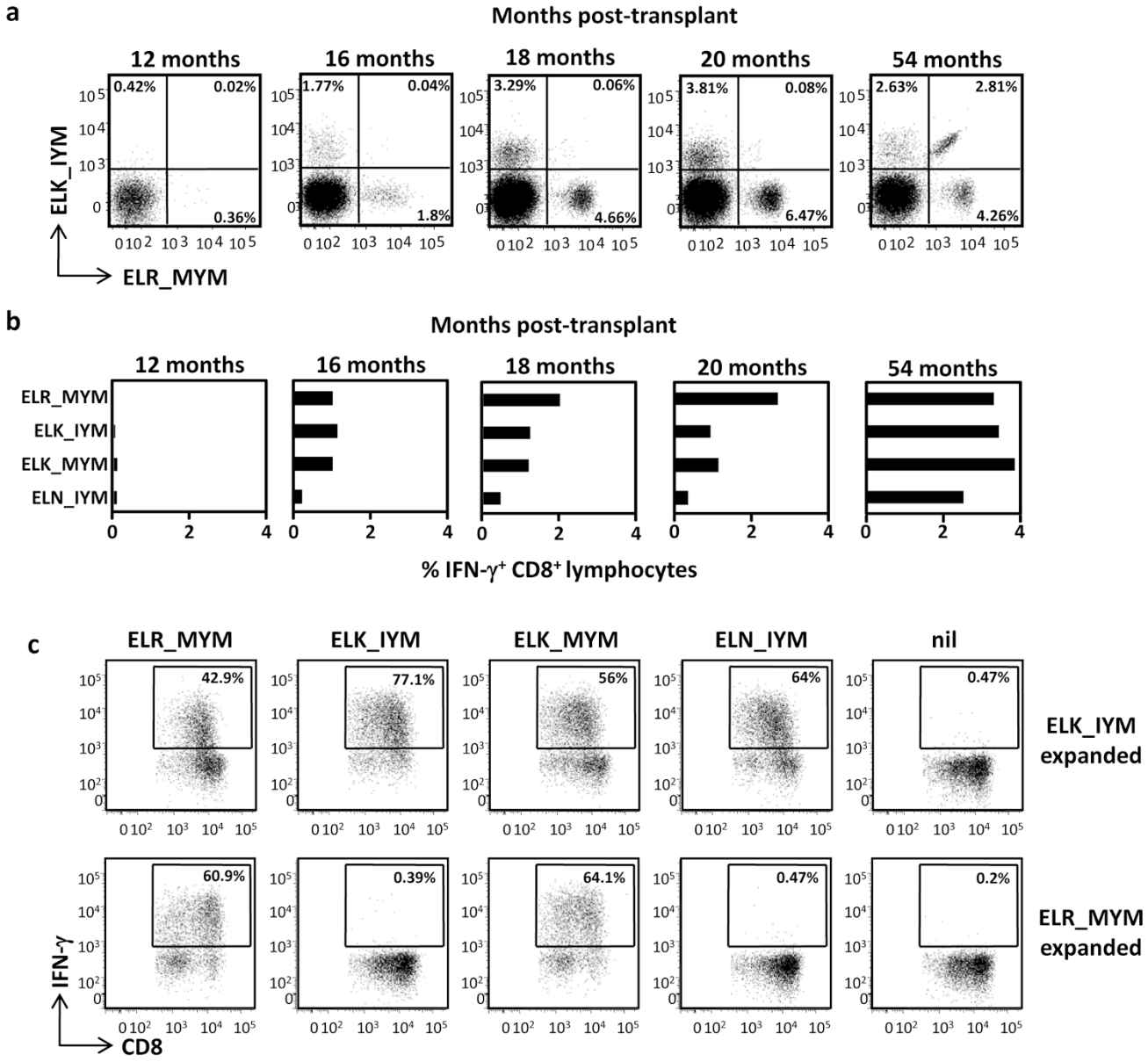

Figure 1 | The kinetics of variant specific T cell activation following viral reactivation in a seronegative transplant recipient. (a) PBMC from a transplant patient (Tx1) at different stages post-transplant were co-stained with a PE-conjugated HLA B8/ELK_IYM dextramer and an APC conjugated HLA B8/ELR_MYM dextramer, then labeled with anti-CD8. (b) PBMC from Tx1 were incubated for five hours with the HLA B8-restricted IE-1 encoded peptide epitope variants, labeled with anti-CD8 and then assessed for intracellular expression of IFN-γ. Data shows the percentage of IFN-γ producing $CD8^+$ T cells reactive against each of the epitope variants. (c) Following in vitro expansion for two weeks in the presence of cognate peptide and IL-2, ELR_MYM or ELK_IYM specific T cells were incubated for four hours with all four peptide variants, then IFN-γ expression was assessed using an intracellular cytokine assay. FACS plots show the proportion of ELR_MYM or ELK_IYM expanded $CD8^+$ T cells responding to the peptide epitope variants.

ELR_MYM variant epitope was not due to poor infectivity, since target cells infected with the Merlin strain were efficiently recognized by ELR_MYM-specific T cells from Tx9 (Fig. 4b). The ELR_MYM and ELK_IYM-specific T cells displayed exquisite specificity for cognate variant epitope and only recognised either Merlin or TB40E infected cells, respectively (Fig. 4b-d).

Clonotypic analysis of the TCR repertoire for $CD8^+$ T-cells specific for different variants of IE-1 epitopes. To examine the impact of exposure to naturally occurring CMV genetic variants on the TCR repertoire architecture of the virus-specific CD8+ T-cells, we employed a novel protocol which allows single-cell paired analysis of TRAV and TRBV sequences[17]. To compare the TCR repertoire in T cells displaying different levels of cross-reactivity, we enriched $CD8^+$ T cells which bound to both ELR_MYM-HLA B8 and ELK_IYM-HLA B8 multimers and compared the TCR architecture to T cells which bound specifically to ELR_MYM-HLA B8, ELK_IYM-HLA B8 or the ELN_IYM-HLA B8 multimers. These enriched antigen-specific $CD8^+$ T cells were then analysed for TRAV and TRBV usage. TCR CDR3 usage confirmed the unique nature of the T cell repertoires generated in response to the different epitope variants (Table 1). CMV-specific clonotypes which cross-recognized the ELR_MYM and ELK_IYM epitopes showed a high level of conservation at the nucleotide level, which has been described as a 'Type 3 bias', whereby both the TRBV usage and CDR3 sequence was identical. Furthermore, when the CDR3 region from the TRAV chain was sequenced,

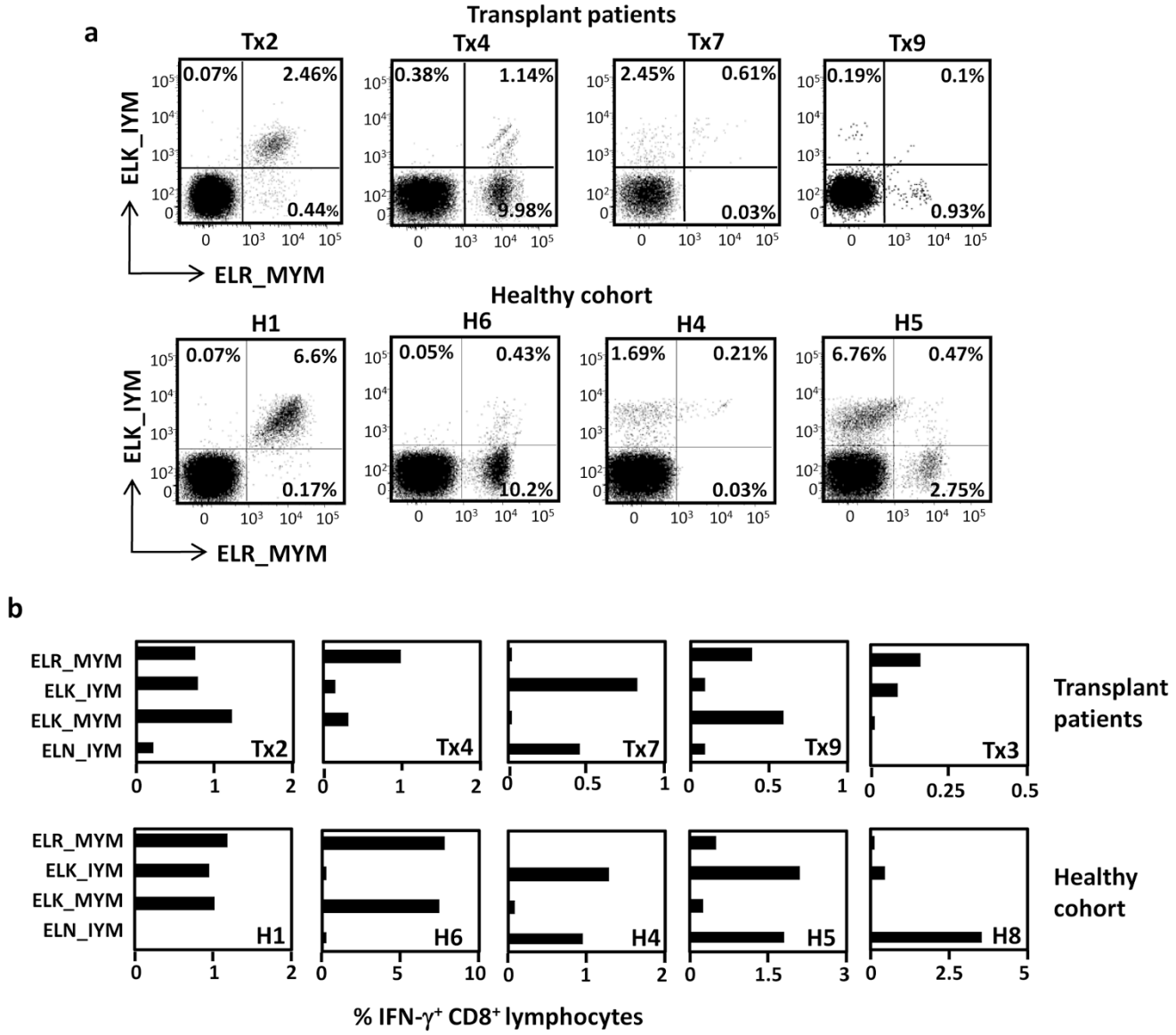

Figure 2 | Recognition of IE-1 peptide variants by HLA B8 transplant recipients and healthy volunteers. (a) PBMC from HLA B8 CMV-seropositive individuals were co-stained with a PE-conjugated HLA B8/ELK_IYM dextramer and an APC conjugated HLA B8/ELR_MYM dextramer, then labeled with anti-CD8. The frequency of MHC multimer positive $CD8^+$ T cells from individuals displaying the four distinct patterns of dextramer binding is shown. (b) PBMC from HLA B8-positive transplant recipients and healthy volunteers were incubated for five hours with the HLA B8-restricted IE-1 encoded peptide epitope variants, labeled with anti-CD8 and then assessed for intracellular expression of IFN-γ. Data shows the percentage of IFN-γ producing $CD8^+$ T cells reactive against each of the epitope variants in a total of 5 transplant recipients and 5 healthy volunteers.

it too showed high levels of bias. These observations were confirmed following TRBV analysis in other individuals who generated a cross-reactive T cell response. Conversely, $CD8^+$ T cells recognizing ELK_MYM or ELK_IYM showed oligoclonal TCR architecture with a clear bias in CDR3 usage with a single dominant clonotype in each individual representing 45-80% of CDR3 sequences. Of note, one minor clonotype (TRBV5-6/TRAV19) was detected in both the ELK_IYM and ELR_MYM multimer populations sorted from one individual who showed evidence of distinct ELR_MYM and ELK_IYM specific T cell populations, suggesting the presence of a small cross-reactive clonotype in this individual. Type-I TCR bias[18,19] was observed in the ELR_MYM response with two separate individuals deploying T cells bearing TRBV27 and TRAV19 TCR architecture. Analysis of T cells directed towards the ELN_IYM variant epitope also showed an oligoclonal TCR repertoire with distinct TRAV and TRBV usage when compared to the T cells that recognized both ELR_MYM and ELK_IYM epitopes. These observations indicate that virus-specific T cells with broader cross-reactivity display highly-biased monoclonal TCR repertoire, while effector cells directed towards specific genetic variants displayed an oligoclonal repertoire which was uniquely altered in each virus-infected individual.

TCR interaction with HLA B8-ELR_MYM and HLA B8-ELK_IYM complexes. To gain further insight into TCR recognition of these variant epitopes, single amino acid substitutions were introduced into the ELR_MYM and ELK_IYM peptides and the analogue peptides were tested for T cell recognition by three different IE-specific T cell lines. These T cells either cross-recognized both ELR_MYM and ELK_IYM peptides or reacted exclusively against the ELR_MYM or ELK_IYM peptides. The T cells which cross-recognized both ELR_MYM and ELK_IYM peptides displayed a broad footprint on the MHC-bound peptide as replacement of any residue from P3-P8 with alanine dramatically impacted on immune

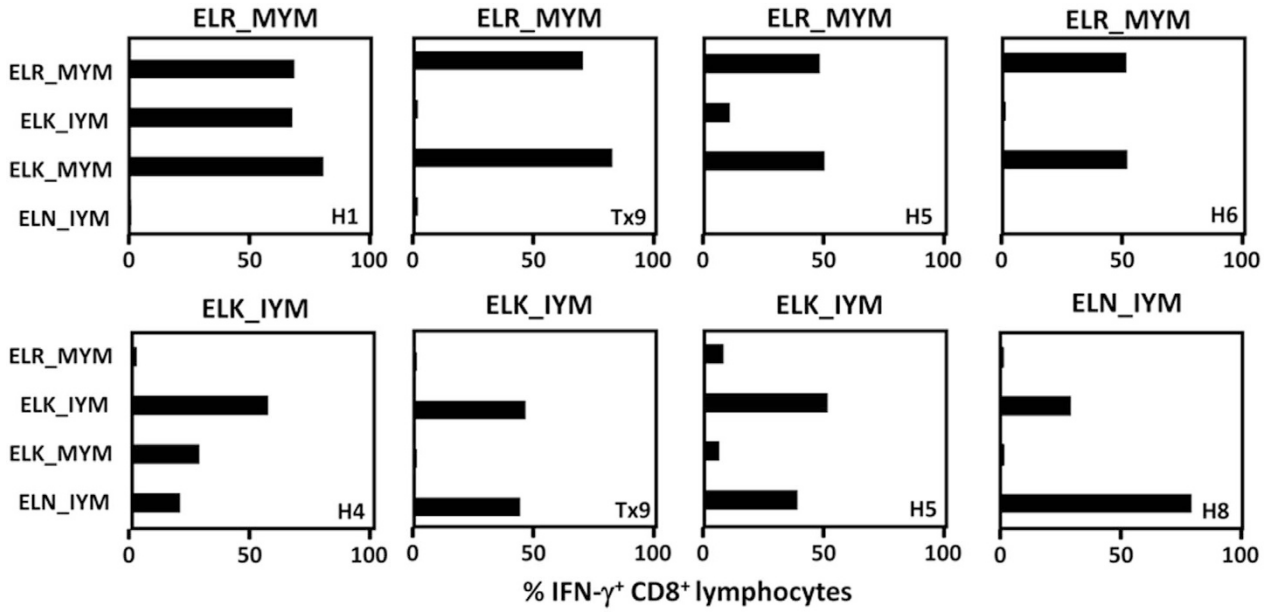

Figure 3 | **Recognition of epitope variants following in vitro expansion.** Following in vitro expansion for two weeks in the presence of cognate peptide and IL-2, ELR_MYM ELK_IYM and ELN_IYM specific T cells were incubated for four hours with all four peptide variants, then IFN-γ expression was assessed using an intracellular cytokine assay. Data represents the proportion of $CD8^+$ T cells responding to the peptide epitope variants.

recognition (Fig. 5). In contrast, T cells exclusively recognizing the ELR_MYM or ELK_IYM epitopes showed greater flexibility as residues P1, P2, P3, P9, and/or P5 and P7 could be replaced with alanine with minimal impact on the immune recognition. It is important to note that although the P7 residue was crucial for the immune recognition by ELK_IYM-specific T cells, substitution of this residue with alanine had minimal impact on the immune recognition by ELR-MYM-specific T cells (Fig. 5).

Structural characterization of HLA B8-ELR_MYM, HLA B8-ELK_IYM and HLA B8-ELN_IYM complexes. Considering the impact of pMHC structural constraints on the binding of the TCR and its selection within an immune repertoire, we next determined whether the structural landscape of the HLA B8-ELR_MYM, HLA B8-ELK_IYM and HLA B8-ELN_IYM complexes correlated with the pattern of T cell recognition. Thermal stability (Tm) analysis of the HLA B8-ELR_MYM, HLA B8-ELK_IYM and HLA B8-ELN_IYM complexes revealed that the ELR_MYM (Tm: 63.0±1.0°C) and ELK_IYM (Tm: 63.2± 0.8°C) peptides bound the HLA B8 molecules with more stability when compared to the ELN_IYM peptide (Tm: 58.2±0.8°C). Crystal structures of the HLA B8-ELR_MYM, HLA B8-ELK_IYM and HLA B8-ELN_IYM revealed that although the N-terminus of the three peptides adopted a similar conformation in the peptide-binding cleft of HLA B8, important differences were observed in the interaction of other peptide residues with the HLA molecule (Fig. 6a). Of particular interest was the P3 residue, which acts as an anchor residue for HLA B8-binding peptides[16]. Namely, the P3-Arg side chain of the ELR_MYM peptide pointed into the antigen binding cleft of HLA B8, and was stabilized by salt bridges and hydrogen bonds to Asp156, Asp114 and Tyr116 (Fig. 6b). However, the shorter side chains of the Lys and Asn, in the ELK_IYM and ELN_IYM peptides respectively, failed to contact Tyr116 (Fig. 6B). In the ELK_IYM-HLA B8 structure, Tyr116 shifted its hydroxyl group by 1.7 Å compared to the ELR_MYM structure. Whereas in the ELN_IYM-HLA B8 structure, we observed multiple discrete conformations of Tyr116, one of which interacted with the P5-Lys (Fig. 6b). The conformation of the solvent exposed residues P6, P7 & P8, which are likely

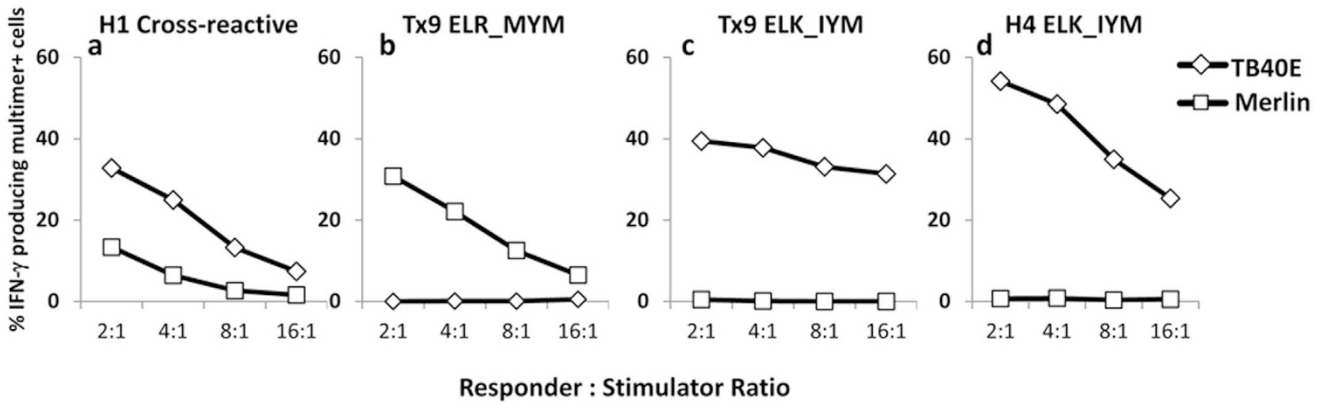

Figure 4 | **Recognition of endogenously expressed variant epitopes.** ELR_MYM/ELK_IYM (a), ELR_MYM (b) or ELK_IYM (c & d) specific T cells were incubated for five hours with HLA B8+ fibroblasts infected with the ELR_MYM encoding Merlin or ELK_IYM encoding TB40E strains. IFN-γ expression was then assessed using an intracellular cytokine assay. Data represents the proportion of ELR_MYM or ELK_IYM specific $CD8^+$ T cells recognising the CMV-infected cells at the indicated responder to stimulator ratio.

Table 1 | TCR usage of IE-1 variant specific T cell populations

Specificity	Donor	TRAV	CDR3	TRAJ	TRBV	CDR3	TRBJ	TRBD	TRBV Freq.
ELR_MYM/ELK_IYM	H1	19*01	CALGANFGNEKLTF	48*01	27*01	CASSQGLAGLEETQYF	2-5*01	2*01	35/35
	H7	No Data			27*01	CASSQGLAGLQETQYF	2-5*01	2*01	9/9
	H9	No Data			27*01	CASSLGLAGLTDTQYF	2-5*03	2*01	10/10
ELK_IYM	H2	38-2	CAFGGSQGNLIF	42*01	2	CASSFGLSSYNSPLHF	1-6*02	2*01	15/21
		38-2	CAFGGSQGNLIF	42*01	12-3	CASGLTHVPQHF	1-5*01	2*01	1/21
		38-2	CAYRSGNFNKFYF	21*01	6-4	CASSSRDTLGEYTEAFF	1-1*01	1*01	2/21
		21	CAVYNNNDMRF	43*01	6-4	CASSSRDTLGEYTEAFF	1-1*01	1*01	1/21
		13-2	CAVPPGASGTYKYIF	40*01	29-01	CSVEGGTIYEQYF	2-7*01	2*01	1/21
		No Data			19*01	CASRGGPQETQYF	2-5*01	1*01	1/21
	H5	27*01	CAGAEGAAGNKLTF	17*01	7	CASSSPGLAGAQEYF	2-7*-01	2*01	24/30
		24*01	CAFIGYSGYALNF	31	30	CAWRETDNYGYTF	1-2*01	1*01	3/30
		12-2	CAVNRYGNKLVF	47*02	20-1	CSARDENQGAEAFF	1-1*01	1*01	1/30
		1-2*01	CAVYSSGGANNLFF	36*01	10-3	CAIRTGQANEQYF	2-7*01	1*01	1/30
		19*01	CAPNARLMF	31*01	5-6	CASTLGGYAGNTIYF	1-3*01	2*01	1/30
ELR_MYM	H10	20*01	CAVQGTGGFKTIF	9*01	7	CASSLDVGQYLGNEQFF	2-1*01	1*01	8/12
		19*01	CAPNARLMF	31*01	5-6	CASTLGGYAGNTIYF	1-3*01	2*01	2/12
		8-1*01	CAVLGAGNMLTF	39*01	19	CASSMAAPFPFDGYTF	1-2*01	2*01	1/12
		13-2	CAEVAEKSSGDKLTF	46*01	27*01	CASSSYRVSLNTEAFF	1-1*01	1*01	1/12
	H5	38-1	CAFRGAGNMLTF	39*01	2	CASIPWQGANGEQYF	2-7*01	2*01	14/24
		19*01	CAQGSNFGNEKLTF	48*01	27*01	CASSSYRTNLNTEAFF	1-1*01	1*01	9/24
		5*01	CAETQPGAGSYQLTF	28*01	27*01	CASSLGPGQEQYF	2-7*01	1*01	1/24
ELN_IYM	H8	12-3	CAGAEGAAGNKLTF	50*01	20-1	CSAKSRDRTGANVLTF	2-6*01	1*02	5/11
		14	CAETQPGAGSYQLTF	28*01	29-1	CSVVGGQSYEQYF	2-7*01	2*01	5/11
		25*01	CAIYGGSQGNLIF	42*01	20-1	CSANEGIHYGYTF	1-2*01	2*02	1/11

important for TCR interaction[20] were also altered. Namely, in the ELK_IYM-HLA B8 and ELN_IYM-HLA B8 structures, the P6-Met was positioned in the middle of the groove, making contact with Thr69 and Thr73, whereas the P6-Met in the ELR_MYM structure leant towards the α2-helix, making contact with residues Ala150, Arg151 and Val152 (Fig. 7a). These collective structural differences impacted on the Ag-binding cleft and hence potential TCR contact surface. Namely, the movement of P6-Met in the ELR_MYM peptide was accompanied by a shift in Tyr116 and Trp147, which resulted in a more "open" antigen binding cleft (a displacement of the α1-helix by 0.5 Å compared to the respective Ag-binding clefts of the ELK_IYM and ELN_IYM structures) (Fig. 7a). The P7-Met side chain of the ELR_MYM peptide contacts the P8-Tyr side chain (Fig. 7b), which adds a structural constraint and influences the

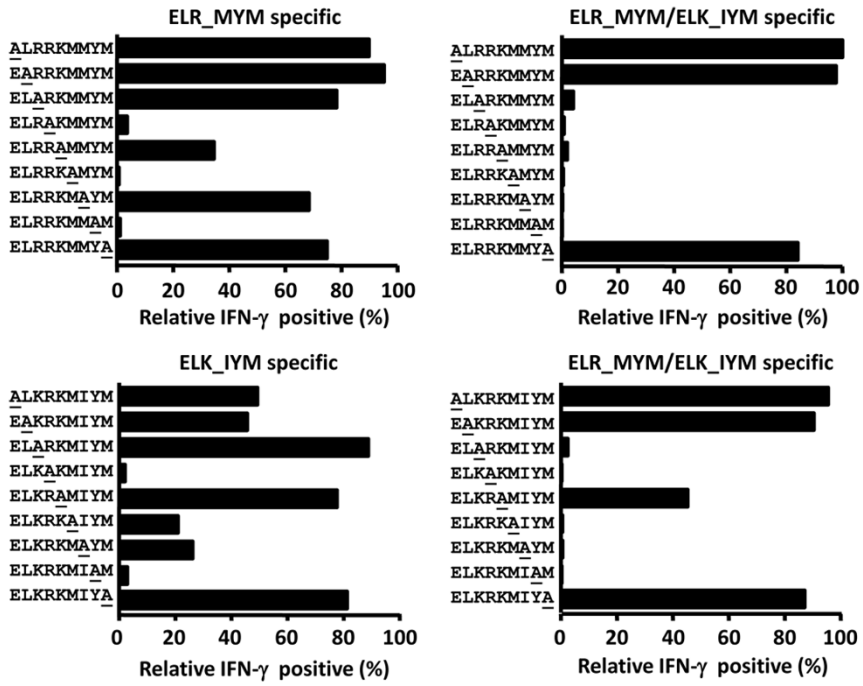

Figure 5 | Recognition of alanine analogs by ELR_MYM and ELK_IYM-specific T cells. ELR_MYM and ELK_IYM specific T cells were incubated for five hours with both wild-type peptide and alanine analogs of ELR_MYM and ELK_IYM peptides, then assessed for intracellular IFN-γ expression. Data presented in each of the subpanels show relative IFN-γ expression in the presence of alanine analogs compared with the wild-type peptide.

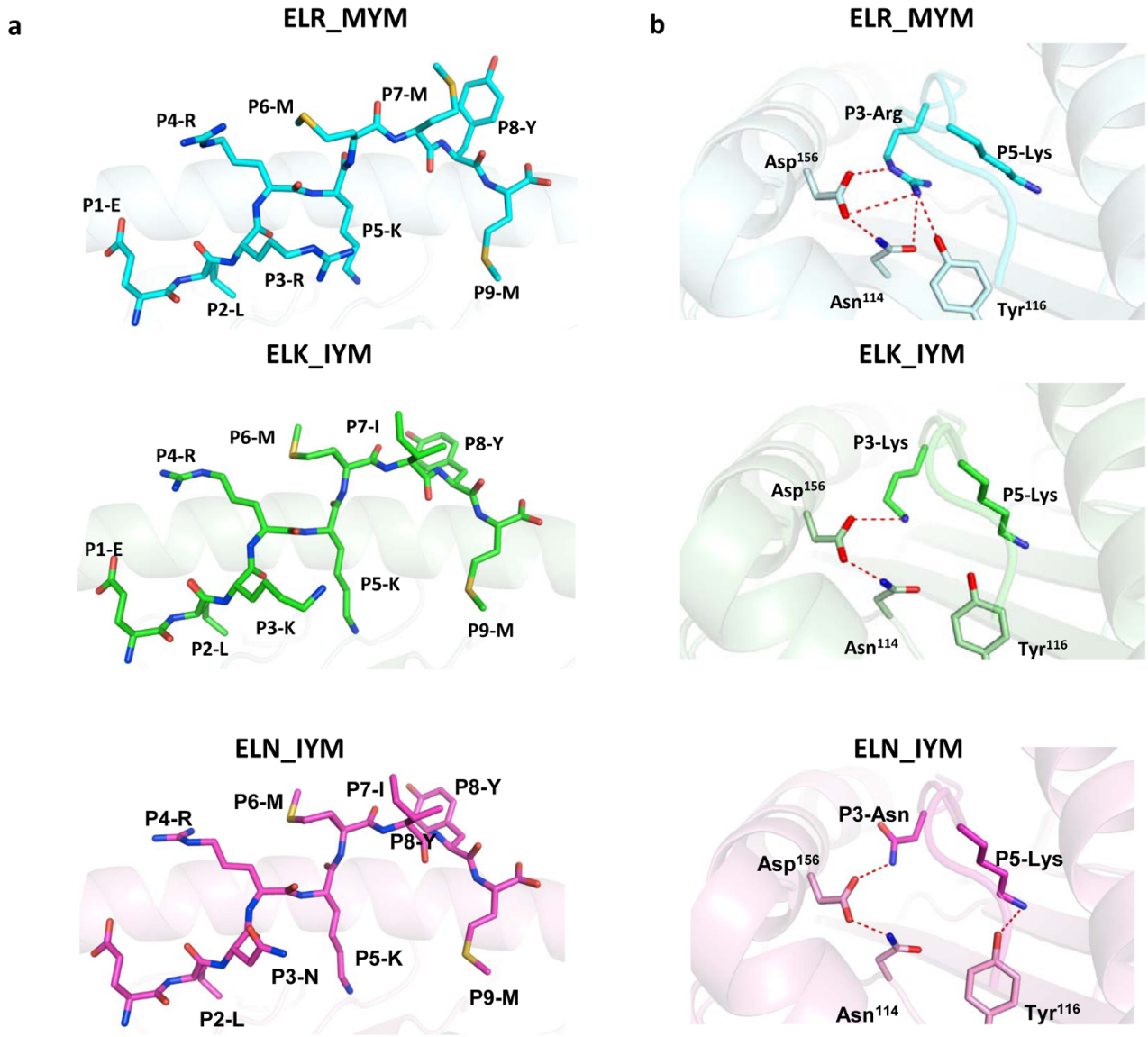

Figure 6 | The structure of the HLA B8 molecule in complex with the IE-1 epitope variants. (a) The panels show a cartoon representation of the HLA B8 molecule with the peptide in stick colour in blue for the ELR_MYM peptide, in green for the ELK_IYM peptide, and pink for the ELN_IYM peptide. (b) The panels show the specific interaction (residue shown in stick) of the peptide P3 and P5 residues with the antigen binding cleft of the HLA B8 molecule. The peptides were coloured according to panel A, and the red dashed lines represent hydrogen bond interactions.

overall shape of the peptide, as seen previously in other MHC molecules[21]. This interaction turns the P8-Tyr side-chain 180° towards the α1-helix in comparison to the ELK_IYM and ELN_IYM peptides structures. It is also important to note that although the P7 residue was crucial for the immune recognition by ELK_IYM-specific T cells, substitution of this residue with alanine had minimal impact on the immune recognition by ELR-MYM-specific T cells (Fig. 5). This contrasting pattern of T cell recognition was supported by the structural analysis which clearly showed that the P7 residue side chain of the ELR_MYM peptide contacts the P8 residue side chain and thus turns this residue 180° towards the α1-helix. This positioning may reduce the interaction of the P7 residue with the TCR for ELR_MYM-specific T cells, while TCR recognizing ELK_IYM epitope can interact with both P7 and P8 residues.

Discussion

The $CD8^+$ T cell response generated following infection with CMV is directed towards a diverse array of antigens and plays a crucial role in preventing the establishment of chronic symptomatic disease[15,22-25]. This is particularly evident in the transplant setting, whereby serostatus of both the donor and recipient have a significant impact upon viral infection[9]. However, it has become increasing evident from studies investigating the presence of genotypically distinct strains of CMV in the transplant setting that although pre-existing immunity is protective against disease it may not necessarily prevent re-infection[9-11]. Observations from immunocompetent individuals have also shown that serial re-infection is likely to occur and that exposure to heterologous strains of CMV can alter the subtype specific antibody response[8,26]. Despite this little is known about the impact exposure to heterologous strains of virus has upon the T cell response. Whilst previous studies have investigated the impact of IE-1 sequence variation on T cell recognition[27,28], the present study delineates how infection with genotypic variants of CMV imprints on the T cell repertoire in exposed individuals.

The parallel emergence of multiple strains encoding the ELR_MYM and ELK_IYM epitope variants in a transplant recipient, and the detection of variant specific T cell populations in other

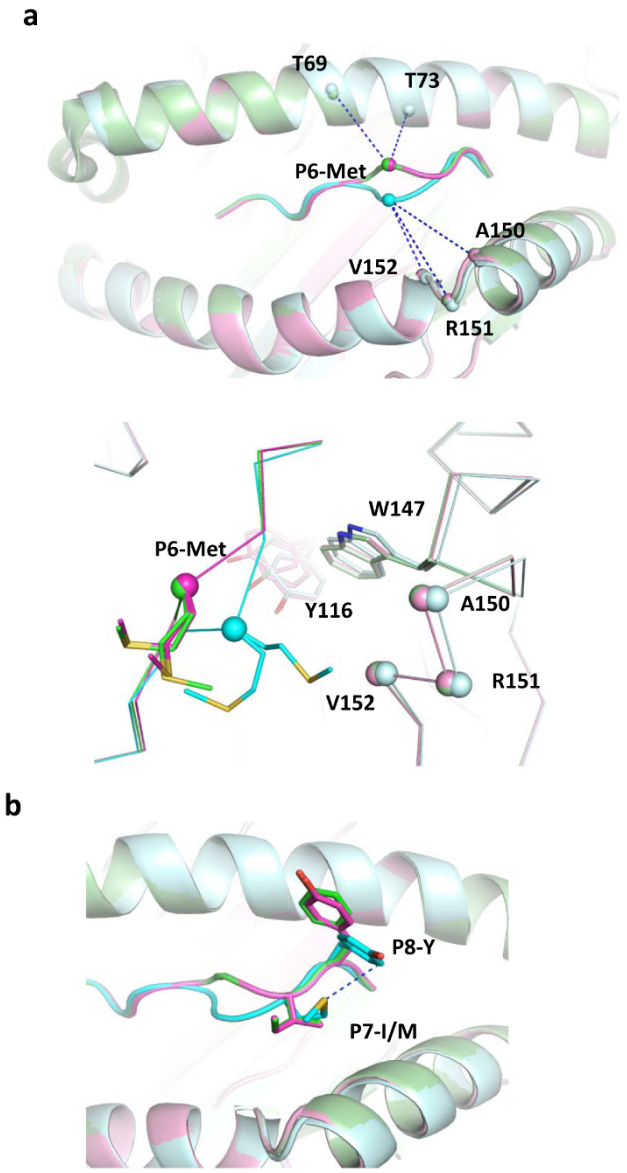

Figure 7 | Structural changes associated with the P6-P8 residues of the IE-1 epitope variants in complex with the HLA B8 molecule. (a) The panels represent a superposition of the three peptide-HLA B8 structures, with the HLA B8 molecules represented in cartoon and the peptide coloured in blue for ELR_MYM, in green for ELK_IYM and pink for ELN_IYM. The spheres represent the Cα atom of the P6 residue on the peptides and of the Ala150, Arg151, Val152, Thr69 and Thr73 on the HLA B8 molecules. The blue dashed lines represent the atomic interaction between the peptide residues and the HLA B8 residues. (b) The panel shows the structural difference in the peptides at position P7 and P8, with the peptides coloured according to panel A, and the blue dashed line represents the interaction between the P7 and P8 residues of the ELR_MYM peptide.

individuals supports previous observations that simultaneous infection with CMV can occur, even in healthy individuals[10,29], and implies that strain specific immunity may play a role in the control viral reactivation, at least in settings of co-infection following organ transplantation. Interestingly, while previous observations have indicated that T cell repertoires following CMV infection are relatively stable during latent infection[30], our observations suggest that co-infection can alter the T cell repertoire and selectively promote the expansion of clonotypes that can cross-react with multiple virus strains. While it is not evident what impact the selection of a cross-reactive clonotype over time has upon the control of reactivating CMV variant viruses, recent evidence in HIV infected individuals has demonstrated that the recruitment of cross-reactive clonotypes recognising HIV variants plays an important role in long-term immunity associated with elite controllers[31,32].

The biophysical and structural landscape of the peptide-MHC complex plays a critical role in TCR selection[18,21,33-36]. Unsurprisingly, given the distinct nature with which the different populations recognise variant peptides, we saw little evidence for highly predictable public TCR usage in the IE-1 variant specific T cell populations. However, these observations did reveal a bias in the repertoire capable of efficiently recognising both immunodominant variants with the selection of a single cross-reactive TCR. Structural observations and alanine substitution of the ELR_MYM and ELK_IYM variants further emphasised the likely influence TCR repertoire selection plays in defining the patterns of variant recognition. The cross-reactive TCR was highly susceptible to alanine substitution at position 3, which completely ablated T cell activation. Although this T cell could accommodate the Arg to Lys substitution at position 3, it could not accommodate the Arg to Asn substitution that is found in the ELN_IYM variant, whereas the ELK_IYM specific T cells that appear to preferentially dock at the C-terminus of the peptide could accommodate this substitution. The structural differences that restricted cross-recognition of the ELR_MYM/HLA B8 and ELK_IYM/HLA B8 complexes by the majority of the variant specific T cells were primarily driven by the residue substitution at position 7, and the impact this had upon residues 6 and 8. Similar observations have previously demonstrated how intra-peptide interactions, or peptide-induced conformational change can influence TCR binding[21,37-40]. Less obvious were the mechanisms that dictate differential recognition of the ELK_IYM and ELN_IYM peptide variants which displayed a very similar structure. Previous studies using the Human Immunodeficiency Virus encoded HLA B8 restricted epitope, GGKKKYKL, and a variant, GGRKKYKL, suggested that a shift in the position of the MHC α1 and α2 helices which flank the peptide binding groove following substitution of the P3-K with the larger arginine residue were responsible for the failure of GGKKKYKL specific T cells to recognize the GGRKKYKL variant[16]. Whilst substitution of K for R at P3 appeared to have no bearing on the recognition of the IE-1 peptide variants, differential interaction of the ELN_IYM and ELK_IYM peptides with the HLA B8 molecule also likely alter TCR recognition. Position 3 is a known HLA B8 anchor residue, therefore the reduce thermostability of the ELN_IYM HLA B8 complex is likely attributable to the less stable interaction of the P3-Asn residue with the HLA B8 molecule. This could also potentially alter the flexibility of the peptide in the MHC groove. It has previously been reported that increased peptide flexibility can alter the responding TCR repertoire[33], although in this instance instability was due to polymorphisms in the MHC molecule rather than the peptide itself.

Overall, these observations provide insight into the impact exposure to heterologous strains of CMV has upon the T cell repertoire. The collective results paint a complex and dynamic picture of the interaction between CMV and the human T cell repertoire, demonstrating the impact exposure to viral variants, co-infection with multiple viral variants and TCR repertoire selection have upon the induction of the cellular immune response to CMV.

Methods

Study Participants. A panel of 9 solid organ transplant recipients (SOT; renal or heart and/or lung) and 10 HLA B8-positive human volunteers were recruited after informed written consent in accordance with the Queensland Institute of Medical Research ethical guidelines. This study was approved by the Human Ethics Committees of the Queensland Institute of Medical Research, the Prince Charles Hospital and the Princess Alexandra Hospital.

Establishment and maintenance of cell lines. Polyclonal T cell lines specific for the HLA B8-restricted $CD8^+$ T cell epitope variants from the IE-1 protein, ELR_MYM, ELK_IYM and ELN_IYM, were generated following stimulation of PBMC with 1μg/ml of cognate peptide. Polyclonal T cell cultures were maintained in growth medium containing recombinant interleukin-2 (IL-2).

pMHC Dextramer staining. MHC-peptide dextramers supplied by Immudex were used to detect epitope-specific $CD8^+$ T cells. For dual dextramer staining, PBMCs were incubated for ten minutes at 4°C with both the APC labelled HLA B8/ELR_MYM dextramer and the PE labelled HLA B8/ELK_IYM dextramer. Cells were then washed and incubated for a further 30 minutes at 4°C with PerCP-Cy5.5 anti-CD8, PE-Cy7 anti-CD3 (BD Biosciences), efluor450 anti-CD19 and Pacific Blue anti CD14 (BD Biosciences). Cells were then acquired using a FACSCanto II with FACSDiva software, and post-acquisition analysis performed using FlowJo software.

Intracellular Cytokine Staining. PBMC or peptide epitope stimulated bulk T cell cultures were stimulated with either 1μg/mL of peptide or with IFN-γ activated HLA-matched fibroblasts infected with the HCMV strains TB40E or Merlin (kind gifts from Dr Barry Slobedman, Westmead Millennium Institute, Sydney, Australia) to assess endogenous antigen recognition. Cells were incubated for four to five hours in the presence of Brefeldin A (BD Biosciences), then incubated with PerCP-Cy5.5 anti-CD8 (eBioscience), fixed and permeabilised using a BD Cytofix/Cytoperm kit, and incubated with PE or AF700 anti-IFN-γ (BD Biosciences). Cell acquisition was performed using a FACS Canto II (BD Biosciences). Post-acquisition analysis was performed using FlowJo software (TreeStar).

TRAV and TRBV Sequence Analysis. Paired analysis of TRAV and TRBV CDR3 usage was performed using a recently described method involving multiplex analysis in single cells[17]. Briefly, PBMC from HLA B8+ individuals were labelled with pMHC dextramer, then dextramer positive T cells were single cell sorted into a 96 well plate using a FACSAria (BDBiosciences). Nested multiplex PCR for TRAV and TRBV was then performed on sorted single cells. PCR products were then sequenced and analysed for TRAV and TRBV CDR3 usage using the IMGT/V-QUEST software[41].

Thermostability assay. To assess the effect of each mutation, either on the HLA molecule or on the peptide stability, a thermal shift assay was performed. The fluorescent dye Sypro orange was used to monitor the protein unfolding. The thermal stability assay was performed in the Real Time Detection system (Corbett RotorGene 3000). Each pMHC complex in 10 mM Tris-HCl pH 8, 150 mM NaCl, at two concentrations (5 and 10 μM) in duplicate, was heated from 25°C to 95°C with a heating rate of 1°C/min. The fluorescence intensity was measured with excitation at 530 nm and emission at 555 nm. The Tm, or thermal melt point, represents the temperature for which 50% of the protein is unfolded.

Protein expression, purification and crystallisation. Soluble class I heterodimers containing the three CMV peptides (ELR_MYM, ELK_IYM and ELN_IYM) were prepared as described previously[42]. Briefly, the truncated forms (residues 1–276) of the HLA B8 heavy chain and full-length β2-microglobulin (β2m) were expressed in *Escherichia coli* as inclusion bodies. The complex of HLA B8-peptides were refolded by diluting the heavy chain and β2m inclusion body preparations into refolding buffer containing a molar excess of peptide ligand. The refolded complexes were concentrated and purified by anion exchange chromatography and gel filtration chromatography. Crystals of the HLA B8 in complex with the three CMV peptides, ELK_IYM, ELR_MYM and ELN_IYM were grown by the hanging-drop, vapour-diffusion method at 20°C with a protein/reservoir drop ratio of 1:1, at a concentration of 10 mg/mL in 10 mM Tris-HCl pH 8, 150 mM NaCl. Large stick-shaped crystals grew using 15-20% Poly Ethylene Glycol (PEG) 4000), 0.2 M ammonium acetate and 0.1 M Na-Citrate pH 5.6.

Data collection and structure determination. The HLA B8-peptide crystals were soaked in a cryoprotectant solution containing mother liquor solution with the PEG concentration increased to 30%(w/v) and then flash frozen in liquid nitrogen. The data were collected on the MX1 beamline for the HLA B8-ELR_MYM, and on the MX2 beamline for the HLA B8-ELK_IYM and HLA B8-ELN_IYM complexes, at the Australian Synchrotron, Clayton using the ADSC-Quantum 210 and 315r CCD detectors respectively (at 100K). Data were processed using the XDS software and scaled using XSCALE software[43]. The HLA B8-peptide crystals belonged to the space group $P2_12_12_1$ with unit cell dimensions (Supplemental Table 1) consistent with one complex in the asymmetric unit. The structures were determined by molecular replacement using the PHASER program[44] with the HLA B8-FLR for the HLA model without the peptide (Protein Data Bank accession number, 1M05)[42]. Manual model building was conducted using Coot software[45] followed by maximum-likelihood refinement with the PHENIX program[46]. Coordinates submitted to the PDB database, pdb codes (to be advised): for the HLA B8-ELK_IYM, HLA B8-ELR_MYM and HLA B8-ELN_IYM complexes. All molecular graphics representations were created using PyMol.

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Author contributions

C.S. and R.K. designed this study and wrote the manuscript with S.G., C.S., S.G., N.L.B., S.A.V., R.M.B., J.B. and K.A.T. conducted various experimental studies. J.R., J.J.M., S.R.B. and K.K. provided critical intellectual input into the design of experiments and contributed in writing the manuscript. D.C., S.B. and S.C. were responsible for recruitment and clinical management of the patients enrolled in this study.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/scientificreports

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