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SHORT COMMUNICATION

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SARS-CoV-2-specific CD8$^+$ T-cell responses and TCR signatures in the context of a prominent HLA-A*24:02 allomorph

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Abstract

In-depth understanding of human T-cell-mediated immunity in coronavirus disease 2019 (COVID-19) is needed if we are to optimize vaccine strategies and immunotherapies. Identification of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) T-cell epitopes and generation of peptide–human leukocyte antigen (peptide–HLA) tetramers facilitate direct *ex vivo* analyses of SARS-CoV-2-specific T cells and their T-cell receptor (TCR) repertoires. We utilized a combination of peptide prediction and *in vitro* peptide stimulation to validate novel SARS-CoV-2 epitopes restricted by HLA-A*24:02, one of the most prominent HLA class I alleles, especially in Indigenous and Asian populations. Of the peptides screened, three spike-derived peptides generated CD8$^+$IFNγ^+ responses above background, $S_{1208–1216}$ (QYIKWPWYI), $S_{448–456}$ (NYNYLYRLF) and $S_{193–201}$ (VFKNIDGYF), with S_{1208} generating immunodominant CD8$^+$IFNγ^+ responses. Using peptide–HLA-I tetramers, we performed direct *ex vivo* tetramer enrichment for HLA-A*24:02-restricted CD8$^+$ T cells in COVID-19 patients and prepandemic controls. The precursor frequencies for HLA-A*24:02-restricted epitopes were within the range previously observed for other SARS-CoV-2 epitopes for both COVID-19 patients and prepandemic individuals. Naïve A24/SARS-CoV-2-specific CD8$^+$ T cells increased nearly 7.5-fold above the average precursor frequency during COVID-19, gaining effector and memory phenotypes. *Ex vivo* single-cell analyses of TCR$\alpha\beta$ repertoires found that the A24/$S_{448}$$^+$CD8$^+$ T-cell TCR$\alpha\beta$ repertoire was driven by a common TCRβ chain motif, whereas the A24/$S_{1208}$$^+$CD8$^+$ TCR$\alpha\beta$ repertoire was diverse across COVID-19 patients. Our study provides an in depth characterization and important insights into SARS-CoV-2-specific CD8$^+$ T-cell responses associated with a prominent HLA-A*24:02 allomorph. This contributes to our knowledge on adaptive immune responses during primary COVID-19 and could be exploited in vaccine or immunotherapeutic approaches.

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has, as of June 2021, infected more than 170 million people, caused at least 3.5 million deaths[1] and paralyzed economies globally. International research efforts have led to the development of successful COVID-19 vaccine candidates, which have proven to be safe and effective in phase 2/3 clinical trials, especially against severe hospitalization and fatal disease outcomes.[2,3] While rolling out the vaccination programs globally, it is still important to develop an in-depth understanding on immune responses to SARS-CoV-2 infection so that immunopathology can be managed, immunotherapies optimized and universal next-generation vaccines designed rationally.

CD8$^+$ T cells play an important part in antiviral immunity by killing virally infected cells, producing antiviral cytokines such as interferon gamma (IFNγ), tumor necrosis factor (TNF) and interleukin-2 and establishing long-term and generally broadly cross-reactive immunological memory. Previous studies demonstrated that CD8$^+$ T cells become activated prior to recovery from COVID-19,[4,5] and survive into convalescence,[6,7] indicating an involvement of CD8$^+$ T cells in SARS-CoV-2 clearance and recovery. Both *in vitro* peptide stimulation assays and peptide–major histocompatibility complex-I tetramer approaches provided further evidence on activation, IFNγ production and clonal proliferation of SARS-CoV-2-specific CD8$^+$ T cells.[8-10] Activated CD69$^+$CD137$^+$CD8$^+$ T cells directed toward overlapping megapeptide pools derived from spike (S), membrane (M), nucleocapsid (N) and ORF proteins could be detected in nearly 70% of acute and convalescent COVID-19 patients. Cross-reactive CD8$^+$ T-cell responses toward peptides derived from circulating human coronaviruses (hCoVs) have also been proposed in some individuals.[11,12]

To provide a better understanding of SARS-CoV-2-specific CD8$^+$ T cells in acute COVID-19, their persistence into long-term memory and subsequent recall following vaccination and/or infection, specific CD8$^+$ T-cell epitopes need to be identified. Several SARS-CoV-2 CD8$^+$ T-cell epitopes have recently been defined for human leukocyte antigen (HLA) class I alleles such as HLA-A*01:01, A*02:01, A*03:01, A*11:01, HLA-B*07:02, B*27:05, B*40:01 and B*44:03.[8,11,13,14] Identification of these SARS-CoV-2 CD8$^+$ T-cell specificities allowed us to determine the precursor frequency of SARS-CoV-2 tetramer-specific CD8$^+$ T cells in prepandemic samples, acute COVID-19 and convalescence.[8,9] *Ex vivo* phenotypic analysis revealed that immunodominant B7/$N_{105}$$^+$CD8$^+$ T-cell responses originated from high-frequency naïve pools found across HLA-B*07:02-expressing individuals.[9] By contrast, A2/$S_{269}$$^+$CD8$^+$ T cells were of suboptimal frequency and phenotypes.[8] The question remains whether SARS-CoV-2-specific CD8$^+$ T-cell responses generally reflect the immunodominant B7/$N_{105}$$^+$CD8$^+$ or subdominant A2/$S_{269}$$^+$CD8$^+$ T-cell responses. To answer this question, a broad range of SARS-CoV-2 CD8$^+$ T-cell specificities restricted across several dominant HLAs need to be identified and analyzed directly *ex vivo*.

In our study, we utilized a combination of peptide prediction and *in vitro* peptide stimulation to identify novel SARS-CoV-2 epitopes restricted by one of the most frequent HLA class I, especially in Indigenous and Asian populations, HLA-A*24:02. Of the peptides screened, three spike-derived peptides generated CD8$^+$IFNγ$^+$ responses above background, $S_{1208–1216}$ (QYIKWPWYI), $S_{448–456}$ (NYNYLYRLF) and $S_{193–201}$ (VFKNIDGYF), with S_{1208} generating the strongest CD8$^+$IFNγ$^+$ responses. Using peptide–HLA-I tetramers, we performed direct *ex vivo* tetramer enrichment for A24/$S_{1208}$$^+$CD8^{+9} and A24/$S_{448}$$^+$CD8$^+$ T cells in HLA-A*24:02-expressing COVID-19 patients and prepandemic controls. We found that CD8$^+$ T cells directed at both HLA-A*24:02-restricted epitopes had similar frequencies and activation phenotypes in COVID-19 patients, whereas the frequencies in prepandemic samples were comparable to those of A2/$S_{269}$$^+$CD8$^+$ T cells.[8] A24/$S_{448}$$^+$CD8$^+$ T-cell and A24/$S_{1208}$$^+$CD8$^+$ T-cell responses had contrasting T-cell receptor αβ (TCRαβ) repertoires, where the A24/$S_{448}$$^+$CD8$^+$ TCRαβ repertoire was driven by a common TCRβ chain motif, whereas the A24/$S_{1208}$$^+$CD8$^+$ TCRαβ repertoire was diverse across COVID-19 patients.

RESULTS

CD8$^+$ T cells respond to HLA-A*24:02-restricted SARS-CoV-2 epitopes

In this study, we recruited eight HLA-A*24:02-expressing individuals: four HLA-A*24:02$^+$ convalescent COVID-19 patients from community infections (median age 63 years, range 52–75, 100% male, range 71–217 days postsymptom onset) and four HLA-A*24:02$^+$ prepandemic healthy controls (median age 36 years, range 24–59, 100% male).

Using the COVID-19 donors, we probed for SARS-CoV-2-specific CD8$^+$ T cells recognizing predicted HLA-

A*24:02-binding peptides from the SARS-CoV-2 S, N and M proteins based on the NetCTLpan prediction algorithm (Figure 1a; accessed May 2020). Peripheral blood mononuclear cells from four convalescent HLA-A*24:02$^+$ COVID-19 patients were expanded *in vitro* with a pool of either 12 S (pool 1) or 10 M or N (pool 2) predicted A24/SARS-CoV-2 peptides for 12 days, then restimulated with individual peptides in an intracellular cytokine staining assay to determine peptide immunogenicity (Figure 1b). Of the 12 S peptides screened, three peptides generated CD8$^+$IFNγ^+ responses above background (dimethyl sulfoxide control): $S_{1208–1218}$ (QYIKWPWYI), $S_{448–456}$ (NYNYLYRLF) and $S_{193–201}$ (VFKNIDGYF) (Figure 1c). S_{1208} generated the strongest CD8$^+$IFNγ^+ response in three of four donors tested (mean 9.73%), with S_{448} and S_{193} responses observed in two (mean 3.85%) and one (3.37%) donors, respectively (Figure 1d). Only one donor showed responses to the M and N pool, with very small IFNγ production (~1%) to four of the six M peptides tested that was not able to be narrowed to a single epitope.

Peptide sequence conservation analysis for the S-derived SARS-CoV-2 immunogenic peptides was extended to previously circulating coronaviruses. SARS-CoV-2 $S_{1208,}$ S_{448} and S_{193} peptide sequences were compared with reference protein sequences for the hCoV strains NL63, 229E, HKU1 and OC43 (Figure 2a). The SARS-CoV-2/S_{1208} epitope shared 66% sequence identity with all of the hCoV strains. All hCoV strains displayed differences at position 1 and 9 (anchor residue), in addition to variation at either position 3 (HKU1 and OC43) or 8 (NL63 and 229E). Sequence conservation was lower for S_{448} and S_{193} peptides, where sequence identity ranged between 44% and 55%. More of these amino acid variations occurred at position 2 (anchor residue) and we therefore predict that analogous hCoV peptides are unlikely to be HLA-A*24:02 ligands, though this is yet to be formally determined.

A24/SARS-CoV-2-specific CD8$^+$ T cells are increased in COVID-19 patients compared with prepandemic donors

To further analyze the SARS-CoV-2-specific A24/CD8$^+$ populations, tetramer-associated magnetic enrichment[15,16] was performed to determine the *ex vivo* frequencies of A24/$S_{1208}$$^+$CD8$^+$[9] and A24/$S_{448}$$^+$CD8$^+$ T cells in HLA-A*24:02$^+$ COVID-19 patients and prepandemic controls (Figure 2b). The precursor frequency of A24/$S_{448}$$^+$CD8$^+$ T cells in COVID-19 convalescent donors (mean 6.3×10^{-5}, $n = 4$) was significantly higher than that observed in prepandemic healthy individuals (mean 8.44×10^{-6}, $n = 4$, $P = 0.0286$) (Figure 2c, left panel). The precursor frequencies of A24/$S_{448}$$^+$CD8$^+$ T cells were comparable to the previously observed frequencies of A24/$S_{1208}$$^+$CD8$^+$ T cells (mean 7.71×10^{-5} for COVID-19 convalescents and 9.50×10^{-6} for prepandemic donors[9]), with most donors having similar frequencies of both epitopes (Figure 2c, right panel). Furthermore, the precursor frequencies for both SARS-CoV-2 HLA-A24 epitopes were within the range previously observed for other SARS-CoV-2 epitopes for both COVID-19 patients and prepandemic individuals.[8,9] Finally, the percentage of both the A24/$S_{448}$$^+$CD8$^+$ and A24/$S_{1208}$$^+$CD8$^+$ T cells increased, though not significantly, after 12 days of stimulation with the S peptide pool in 2 and 3 COVID-19 donors, respectively (Figure 2d). Evidently, the SARS-CoV-2-specific CD8$^+$ T cells were primed by SARS-CoV-2 in the COVID-19 individuals and are thus, at least under *in vitro* conditions, capable of clonal expansion. Overall, our data suggest that naïve A24/SARS-CoV-2-specific CD8$^+$ T cells can be expanded nearly 7.5-fold following COVID-19.

Different activation profiles of SARS-CoV-2-specific A24/$S_{448}$$^+$CD8$^+$ T cells in COVID-19 and healthy individuals

The activation profiles of A24/$S_{448}$$^+$CD8$^+$ T cells tested directly *ex vivo* from COVID-19 patients and prepandemic healthy individuals were assessed by CD27, CD45RA and CD95 staining to determine the prevalence of the naïve (CD27$^+$CD45RA$^+$CD95$^-$), stem cell memory (CD27$^+$CD45RA$^+$CD95$^+$), central memory-like (CD27$^+$CD45RA$^-$), effector memory-like (CD27$^-$CD45RA$^-$) and effector memory CD45RA (CD27$^-$CD45RA$^+$) T-cell subsets (Figure 2b). The phenotype of the A24/$S_{448}$$^+$CD8$^+$ T cells was highly varied across the convalescent COVID-19 donors, with the majority of the tetramer-positive CD8$^+$ T cells being stem cell memory ($n = 2$), effector memory CD45RA ($n = 1$) or naïve ($n = 1$) (Figure 2e). Conversely, the prevalence of the naïve phenotype (mean of 73%) was significantly enriched in prepandemic individuals compared with convalescent COVID-19 patients ($P = 0.0008$). This phenotypic pattern in both the COVID-19 patients and prepandemic healthy individuals aligns with what has been previously observed for other SARS-CoV-2-specific CD8$^+$ T cells, including A24/$S_{1208}$$^+$CD8$^+$ T cells.[9] The prepandemic individuals displayed a mainly naïve profile (mean of 61%), whereas the A24/S_{1208}-specific CD8$^+$ T cells in the COVID-19 donors were more skewed toward naïve ($n = 2$), central memory ($n = 1$) or effector memory CD45RA ($n = 1$) T-cell phenotypes.[9]

(a)

	Peptide Sequence	Parent Origin	Start	End	Affinity* (nM)	CTL % Rank$	# of patients (n=4)^	Published
1	QYIKWPWYI	S	1208	1216	13.22	0.05	3	Ferretti, 2020 Kared, 2021 Nelde, 2021
2	YYHKNNKSW	S	144	152	223.53	0.15	0	
3	YFPLQSYGF	S	489	497	41.54	0.15	0	
4	VYSTGSNVF	S	635	643	19.05	0.2	0	
5	IYQTSNFRV	S	312	320	124.60	0.4	0	
6	PYRVVVLSF	S	507	515	152.38	0.4	0	
7	TFEYVSQPF	S	167	175	337.12	0.8	0	
8	VFKNIDGYF	S	193	201	2033.45	0.8	1	
9	YNSASFSTF	S	369	377	252.42	0.8	0	
10	NYNYLYRLF	S	448	456	28.87	0.8	2	Kared, 2021
11	IAIPTNFTI	S	712	720	695.29	0.8	0	
12	GTITSGWTF	S	880	888	3135.84	0.8	0	
13	YFIASFRLF	M	95	103	48.15	0.05	0	
14	LWLLWPVTL	M	54	62	108.22	0.2	1	
15	RYRIGNYKL	M	198	206	401.09	0.2	1	
16	LWPVTLACF	M	57	65	65.55	0.3	1	
17	SYFIASFRL	M	94	102	27.75	0.3	1	
18	LFARTRSMW	M	102	110	944.10	0.8	0	
19	YYRRATRRI	N	86	94	74.89	0.8	0	
20	KAYNVTQAF	N	266	274	4511.77	0.8	0	
21	KHWPQIAQF	N	299	307	2031.28	0.8	0	
22	KHIDAYKTF	N	355	363	556.43	0.8	0	

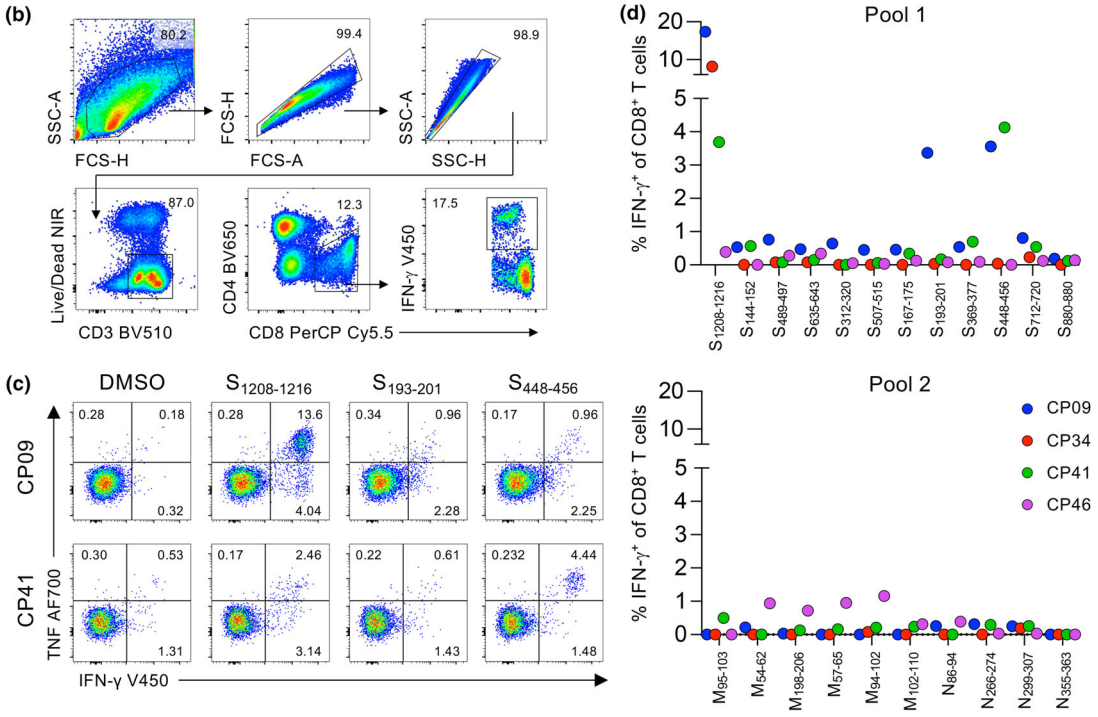

Figure 1. Identification of SARS-CoV-2-specific HLA-A*24:02-restricted CD8$^+$ T-cell epitopes. **(a)** List of peptides predicted to bind HLA-A*24:02. *Affinity (equilibrium dissociation constant) predicted using NetMHCpan. $CTL percentage rank predicted using NetCTLpan. ^Number of COVID-19 donors functionally responding by ICS in this study. See also Kared *et al.*[10], Ferretti *et al.*[11] and Nelde *et al.*[23] **(b)** Gating strategy of IFNγ-producing CD8$^+$ T cells following ICS. **(c)** Representative flow cytometry plots of CD8$^+$ IFNγ/TNF staining after stimulation with the SARS-CoV-2 peptides $S_{1208–1216}$, $S_{193–201}$ and $S_{448–456}$ presented by C1R.A*24:02 cells. **(d)** Frequencies of IFNγ$^+$ of CD8$^+$ T cells for each SARS-CoV-2 peptide, with background (DMSO) staining subtracted (n = 4). Peptide screen was performed in convalescent COVID-19 PBMCs after 12-day expansion *in vitro* with pooled S (pool 1) or M and N peptides (pool 2). CTL, cytotoxic T lymphocyte; COVID-19, coronavirus disease 2019; DMSO, dimethyl sulfoxide; FCS-A, forward scatter–area; FCS-H, forward scatter–height; HLA, human leukocyte antigen; ICS, intracellular cytokine staining; IFN, interferon; PBMCs, peripheral blood mononuclear cells; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SSC-A, side scatter–area; SSC-H, side scatter–height; TNF, tumor necrosis factor.

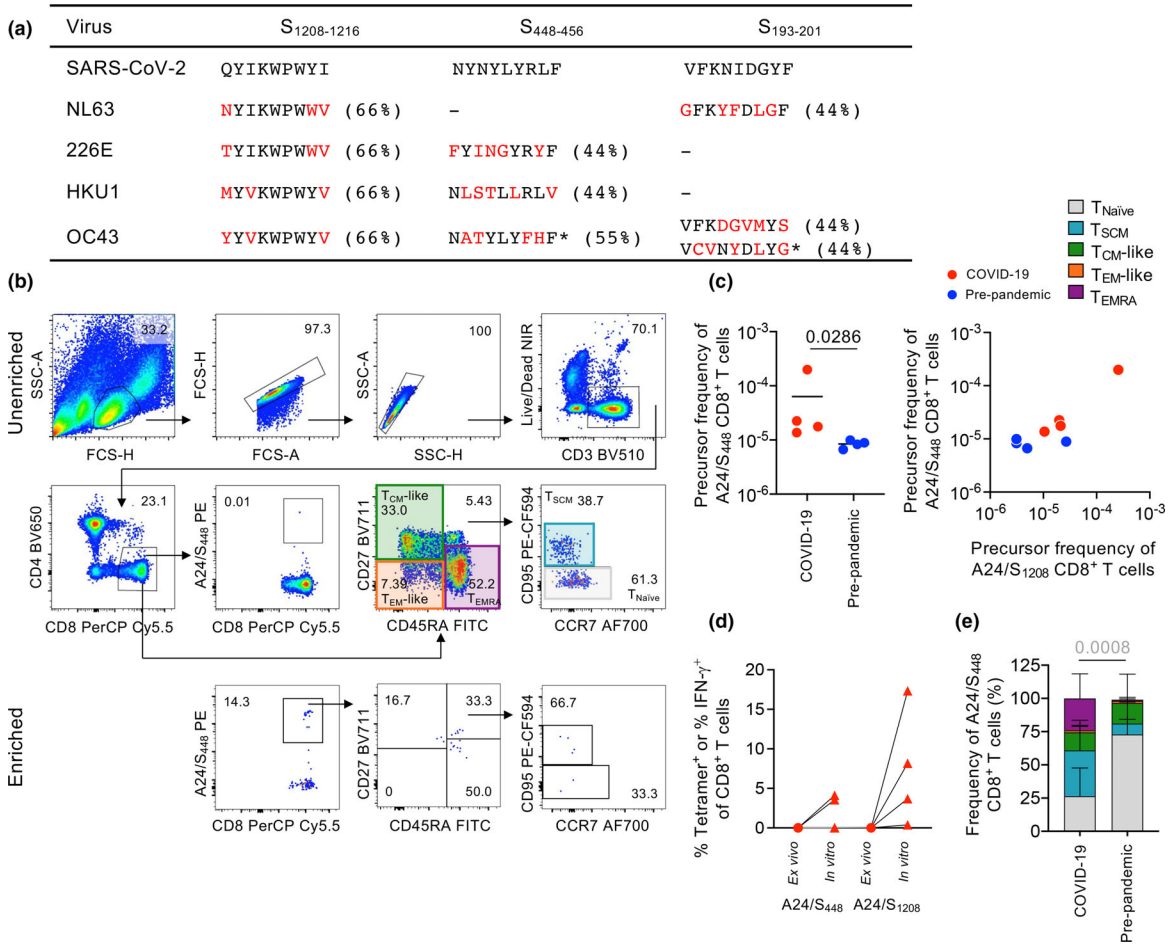

Figure 2. Phenotypes of SARS-CoV-2-specific A24/CD8+ T cells. **(a)** List of SARS-CoV-2 peptide sequences and their homology with hCoV. Variable amino acids shown in red; *unlikely to be HLA-A*24:02 ligand; – no significant homology. **(b)** Gating strategy for phenotype analysis of tetramer+ CD8+ T cells in the unenriched fraction (top panels), which was used to gate enriched tetramer+ CD8+ T cells (bottom panels). **(c)** Precursor frequency of A24/$S_{448–456}$ CD8+ T cells (of CD8+ T cells) between COVID-19 and prepandemic individuals (left panel) and against A24/$S_{1208–1216}$ CD8+ T-cell frequencies (right panel). **(d)** Frequencies of A24/SARS-CoV-2+CD8+ T cells in the CD8+ population in convalescent COVID-19 donors on day 0 and day 12 of expansion. *Ex vivo* frequency measured by tetramer+ staining and *in intro* frequency determined by IFNγ+ staining. **(e)** Stacked graphs of each phenotype subset within A24/$S_{448–456}$ CD8+ T cells (of CD8+ T cells) between COVID-19 and prepandemic individuals (mean with SD is shown). Exact *P*-values are shown where statistical significance was determined with the two-tailed **(c)** Mann–Whitney *U*-test and **(e)** Šídák's multiple comparisons test. COVID-19, coronavirus disease 2019; FCS-A, forward scatter–area; FCS-H, forward scatter–height; FITC, fluorescein isothiocyanate; HLA, human leukocyte antigen; IFN, interferon; SSC-A, side scatter–area; SSC-H, side scatter–height; PE, phycoerythrin; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; T_{CM}, central memory T cells; T_{EM}, effector memory T cells; T_{EMRA}, effector memory CD45RA T cell; T_{SCM}, stem cell memory T cells.

A24/S_{448}+CD8+ TCRαβ repertoire is driven by a common TCRβ chain

CD8+ T-cell immunodominance, functionality and protection are all impacted by the molecular signature of the TCR repertoire.[17-19] Thus, using direct *ex vivo* tetramer staining and human single-cell TCRαβ multiplex reverse transcription-PCR,[15,20] we determined the TCRαβ clonal composition and diversity. We dissected the TCRαβ repertoires for A24/S_{448} and A24/S_{1208}-specific CD8+ T cells in peripheral blood mononuclear cells from four HLA-A*24:02-expressing COVID-19 patients, examining the TCR sequence for a total of 48 SARS-CoV-2-specific CD8+ T cells (Figure 3a;Table 1).

The overall diversity of the TCRαβ sequences was analyzed, first focusing on the TRBV region (Figure 3b). For A24/S_{448}+CD8+ T cells, all donors had TRBV2 gene usage where one key TCRβ motif within the CDR3β loop was found across the COVID-19 patients, despite the fact that a limited number of A24/S_{448}-specific sequences were

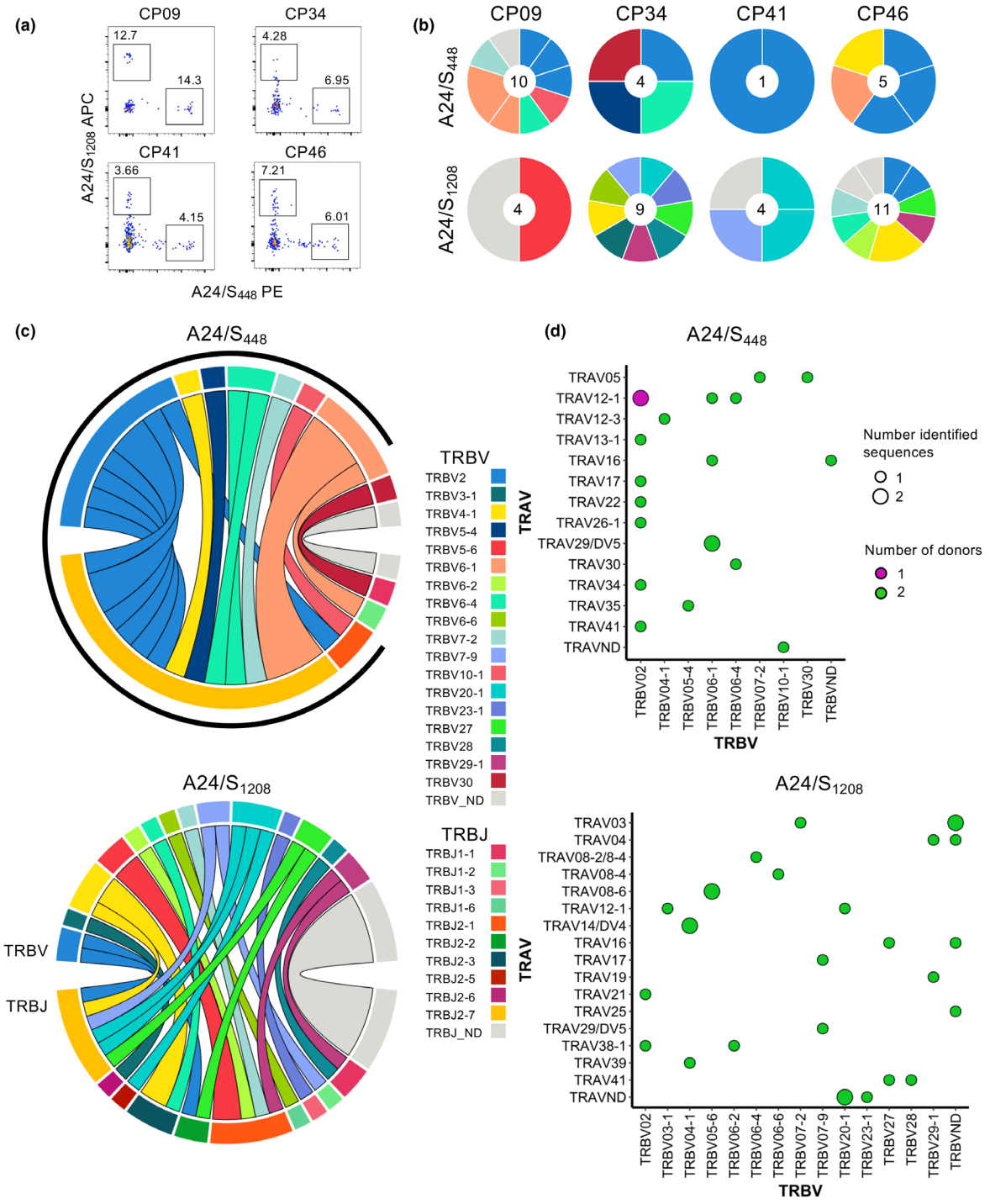

Figure 3. SARS-CoV-2-specific A24/CD8$^+$ T cells have different TCRαβ repertoire diversity. **(a)** FACS profiles of enriched A24/$S_{448–456}{}^+$ and A24/$S_{1208–1216}{}^+$CD8$^+$ T cells from four COVID-19 donors, which were single-cell index sorted for TCRαβ analysis. **(b)** Pie charts of TRBV gene usage. Segments shown by the same color represent TCRβ clonotypes with the same TRBV segment usage but different TRBJ segments or CDR3 sequences. **(c)** Circos plots of TRBV and TRBJ segment linking; top arch and segment color indicate TRBV usage and bottom outer arch color depicts TRBJ usage. Surrounding black arc depicts the common CDR3β motif for A24/$S_{448}{}^+$CD8$^+$ T cells. **(d)** Bubble plot showing the distribution (number of donors and sequences) of TRBV/TRAV gene usage in COVID-19 patients. APC, allophycocyanin; COVID-19, coronavirus disease 2019; FACS, fluorescence-activated cell sorting; PE, phycoerythrin; SARS-CoV-2, severe acute respiratory syndrome coronavirus; TCR, T-cell receptor.

Table 1. Full list of paired α and β TCR gene usage and CDR3 amino acid sequence.

	TRBV	TRBJ	CDR3b	TRAV	TRAJ	CDR3a	09	34	41	46
A24/S_{448}	2	2-7	CASSETGGYEQYF	22	13	CAPRD#NSGGYQKVTF	1			
	2	2-7	CASSESQGYEQYF	12-1	12	CVVVRMDSSYKLIF	1			
	2	2-1	CASSEGQGYEQFF	26-1	4	CIVYNKLIF	1			
	2	2-7	CASSEGQGYEQYF	13-1	15	CAARPEPTSTGTALIF				1
	2	2-7	CASASGQGYEQYF	12-1	33	CVVNDLVDSNYQLIW				1
	2	2-7	CASASGQGYEQYF	41	33	ND				1
	2	2-7	CASSEFRSYEQYF	34	3	CGAA*R#ASKIIF		1		
	2	2-7	CASSRGSGVSYEQYF	17	4	CAXFFWW#YNKLIF			1	
	10-1	2-1	CASSEGQGYEQFF	ND	ND	ND	1			
	6-4	2-7	CASSEGQGYEQYF	12-1	28	ND	1			
	6-4	2-7	CASSERTGYEQYF	30	3	CGTES##YSSASKIIF		1		
	5-4	2-7	CASSLLGGYEQYF	35	42	CAGQ#GSQGNLIF		1		
	6-1	2-7	CASSETGGYEQYF	12-1	53	CVVNMLYSGGSNYKLTF	1			
	6-1	2-7	CASSETGGYEQYF	29/DV5	27	CAARH##AGKSTF	2			
	7-2	2-7	CASSLASGYEQYF	5	17	CAESMMEAAGNKLTF	1			
	4-1	2-7	CASSQGPGPYEQYF	12-3	39	XAFFNKAGNXLXF				1
	6-1	1-2	CASSEWGQGVAYGYTF	16	52	FAPXAGXXXXGKXTF				1
	30	1-1	CAWSVTGVTEAFF	5	11	CAEMNSGYSTLTF		1		
	ND	ND	ND	16	54	CALKDPLMAVQGAQKLVF	1			
A24/S_{1208}	2	2-2	CAGKSTGELFF	21	27	CAVRHTNAGKSTF				1
	2	2-7	ND	38-1	49	CAFMKINQFYF				1
	3-1	2-6	CASCPSWGSGANVLTF	12-1	9	CVPNTGGFKTIF		1		
	4-1	2-7	CASSVGATGAYEQYF	39	33	ND		1		
	4-1	2-3	CASSQDYWGGGADTQYF	14/DV4	39	CAMREGQGNAGNMLTF				2
	5-6	2-1	CASSQSGGGEQFF	8-6	17	CAVSEVGNKLTF	2			
	6-2	2-1	CASSWNGLAGDEQFF	38-1	52	CAFLNAGGTSYGKLTF				1
	6-4	2-3	CASSALVAFSTDTQYF	8-2/8-4	23	CAVYNQGGKLIF				1
	6-6	1-6	CASSFHPGQDRGNSPLHF	8-4	5	CAVSEGR#GRRALTF		1		
	7-2	2-1	CASSSLAGEVNEQFF	3	21	CAVRALYNFNKFYF				1
	7-9	2-7	CASSLVGEGRGREQYF	17	12	CATEARMDSSYKLIF		1		
	7-9	1-2	CASSLGETIYGYTF	29/DV5	29	CAARSGNTPLVF			1	
	20-1	2-7	CSARDQDRAYEQYF	12-1	47	CVVIEGNKLVF		1		
	20-1	2-5	CSALNLD#QETQYF	ND	ND	ND			1	
	20-1	2-7	CSARTISQGAHYEQYF	ND	ND	ND			1	
	23-1	1-3	CASSGTDS#SGNTIYF	ND	ND	ND		1		
	27	2-7	CASSLSTTTASYEQYF	16	18	CALRARGSTLGRLYF		1		
	27	2-2	CASSSYTQRGLDYTGELFF	41	34	CAVESYNTDKLIF				1
	28	1-1	CASTPWGAEAFF	41	44	CAVDTGTASKLTF		1		
	29-1	2-1	CSVDPGQFYNEQFF	19	3	CALSYSSASKIIF		1		
	29-1	1-1	CSVEDPMGVGTEAFF	4	6	ND				1
	ND	ND	ND	3	25	CAVRDIRGQGFSFIF	2			
	ND	ND	ND	25	17	CAGQ#AAGNKLTF			1	
	ND	ND	ND	16	27	CALSGEGG#TNAGKSTF				1
	ND	ND	ND	4	9	CLVGENSGGFKTIF				1
						Total	14	13	5	16

ND = not determined; X = unresolved amino acid; * = stop codon; # = out-of-frame shift.

examined per donor (1–10 cells/donor; Table 1). This motif was dominated by the TRBV2/TRBJ2-7 CAS(S/A) *XXX*GYEQYF (where *X* denotes any amino) sequence found in all COVID-19 patients (40% of the total TCR repertoire). This same CDR3β loop motif was also identified when TRBJ2-7 or 2-1 linked with five different TRBV segments, TRBV5-4, 6-1, 6-4, 7-2 and 10-1 found across two of the four COVID-19 patients (40% of the total TCR repertoire). Similar CDR3β loops were also identified in a third donor, composed of TRBV4-1/TRBJ2-7 gene segments. Overall, the sequences with this CDR3β loop comprised 85% of the A24/S_{448}-specific

repertoire (Figure 3c). The CDR3β loop motif paired with a range of different TRAV/TRAJ gene segments (Figure 3d) and therefore A24/$S_{448}$$^+$CD8$^+$ T-cell specificity was likely driven by the TCRβ chain rather than the TCRα chain.

By contrast, the A24/S_{1208}-specific CD8$^+$ TCRαβ repertoire (4–11 cells/donor) was generally diverse, with almost all sequences identified only once and no TCRαβ clonotypes overlapping between individuals. Each donor had distinct usage of TRAV, TRBV and TRAJ gene segments, with no common motifs within CDR3α and CDR3β sequences. Interestingly, the TRBJ2-7 and 2-1 were enriched in the A24/S_{1208}-specific CD8$^+$ TCRαβ repertoire, represented in 26% and 22% of the sequenced TCRβ chains, respectively (Figure 3c). Overall, the common motif suggests more rigid requirements for TCRαβ clones recognizing the A24/S_{448} epitope compared with the A24/S_{1208} epitope. The lack of TCRαβ plasticity may explain the low naïve precursor frequency observed for A24/S_{448}, similar to what has been previously observed for A2/S_{269}[8]; however, more data may be needed to determine the driving factors for A24/S_{1208} recognition.

DISCUSSION

HLA-A*24:02 is one of the most prominent class I HLA alleles worldwide, especially widely expressed in the Indigenous and Asian populations. In comparison its 10% global distribution, HLA-A*24:02 is of the highest frequency in Oceania (37%), North-East Asia (32.9%), Australia (21.4%) and Central and South America (20.6%).[21] Specifically, HLA-A*24:02 is highly expressed in Indigenous Taiwan Paiwan (96.1%), Papua New Guinea Karimui Plateau Pawaia (74.4%), New Caledonia (60.7%), Alaskan Yupik (58.1%), New Zealand Maori (38%), Chile Easter Island (35.8%), American Samoans (33%) and Australian Aboriginal people (24%),[21] demonstrating its prominence in Indigenous people. As Indigenous people are susceptible to severe respiratory diseases such as influenza and HLA-A*24:02 was associated with influenza-induced mortality during the 2009-pH1N1 outbreak,[22] identification of SARS-CoV-2-specific CD8$^+$ T-cell epitopes restricted by HLA-A*24:02 is essential to understand the relationship between HLA-A*24:02 and disease susceptibility in future studies.

Using peptide prediction tools in combination with an *in vitro* peptide stimulation, we identified SARS-CoV-2 epitopes restricted by HLA-A*24:02, namely, three spike-derived peptides, $S_{1208–1216}$ (QYIKWPWYI), $S_{448–456}$ (NYNYLYRLF) and $S_{193–201}$ (VFKNIDGYF), with S_{1208} generating the strongest CD8$^+$IFNγ$^+$ responses. Both A24/$S_{1208–1216}$ and A24/$S_{448–456}$ have been recently independently identified by other groups.[10,11,23] Although all three SARS-CoV-2-derived peptides shared 44–66% sequence identity with hCoVs, the variations occur in either the anchor residues or likely TCR contact residues, suggesting a potential to escape preexisting CD8$^+$ T-cell immunity and supported by our observation of predominantly naïve A24/S_{1208}- and A24/S_{448}-specific CD8$^+$ T cells in prepandemic individuals. Using peptide–HLA-I tetramers, we performed direct *ex vivo* tetramer enrichment for HLA-A*24:02-restricted CD8$^+$ T cells in COVID-19 patients and prepandemic controls. The precursor frequencies for HLA-A*24:02-restricted epitopes were within the range previously observed for other SARS-CoV-2 epitopes such as HLA-A*02:01-restricted S_{269} for both COVID-19 patients and prepandemic individuals.[8] Thus, it appears that 5–10-fold expansion of SARS-CoV-2-specific CD8$^+$ T-cell responses is prototypical for the primary COVID-19, with the exception of B7/$N_{105}$$^+$CD8$^+$ T-cell responses.[9] Similarly, the phenotypic pattern of the A24/$S_{448}$$^+$ and A24/$S_{1208}$$^+$CD8$^+$ T cells aligns with previous SARS-CoV-2 epitopes, with prepandemic individuals displaying a prototypical naïve phenotype compared with the more varied central memory T-cell phenotype observed in COVID-19 patients.[8,9] A24/$S_{448}$$^+$CD8$^+$ T cell and A24/$S_{1208}$$^+$CD8$^+$ T-cell responses had contrasting TCRαβ repertoires. While A24/$S_{448}$$^+$CD8$^+$ T-cell TCRαβ repertoire was driven by a common TCRβ chain motif, the A24/$S_{1208}$$^+$CD8$^+$ TCRαβ repertoire was diverse across COVID-19 patients. As in our recent study,[9] it appears that TCRαβ diversity might be linked with the prominence of SARS-CoV-2 CD8$^+$ T-cell responses during the primary infection. The common TCRβ chain motif recognizing the A24/S_{448} and the associated lack of TCRαβ plasticity are reminiscent of the TCR requirements for the subdominant A2/S_{269} epitope[9,24] and in contrast to the diverse TCRαβ repertoire and promiscuity in TCRα–TCRβ pairing of the immunodominant HLA-B*07:02-restricted N_{105}-specific CD8$^+$ T cells.[9]

Overall, our study provides important insights into SARS-CoV-2-specific CD8$^+$ T-cell responses associated with a highly frequent HLA-A*24:02 allele and thus contributes to our knowledge on the experimentally verified SARS-CoV-2 CD8$^+$ T-cell epitopes. Assembling a comprehensive data set on the key CD8$^+$ T-cell epitopes restricted by the prominent HLA alleles is needed to depict "universal" HLAs in COVID-19 capable of presenting peptides which elicit broadly cross-reactive immunity across a range of emerging variants. Our previous work defined universal HLA class I alleles (A*02:01, A*03:01, B*08:01, B*18:01, B*27:05 and

B*57:01) in influenza, mounting robust $CD8^+$ T-cell responses against any human influenza A virus circulating over the last century, including the pandemic strains as well as the avian H5N1 and H7N9 viruses.[25] Identification of such universal $CD8^+$ (and $CD4^+$) T-cell epitopes in COVID-19 is needed for the rational design of the next-generation universal COVID-19 vaccines, especially for high-risk groups such as Indigenous people.

METHODS

Study participants and ethics statement

This study recruited four convalescent COVID-19 individuals from community infections (mild: CP09, CP34 and CP46; moderate: CP41) and four prepandemic healthy buffy pack donors obtained from the Australian Red Cross LifeBlood (West Melbourne, VIC, Australia). HLA typing was performed (VTIS, Melbourne, VIC, Australia) and all donors were HLA-$A*24:02^+$. Peripheral blood from COVID-19 donors was collected in heparinized tubes and peripheral blood mononuclear cells from peripheral blood and buffy packs were isolated via Ficoll-Paque separation. Experiments conformed to the Declaration of Helsinki Principles and the Australian National Health and Medical Research Council Code of Practice. Written informed consents were obtained from all blood donors prior to the study with ethics approval from The University of Melbourne (#2056689, #2056761, #1955465 and #1443389) and the Australian Red Cross Lifeblood (ID 2015#8) Human Research Ethics Committees.

Cell lines, peptides and major histocompatibility complex class I tetramers

HLA-A*24:02-transfected class I-reduced B-lymphoblastoid cells (C1R.A*24:02 cells) were maintained in RF-10 medium [Roswell Park Memorial Institute-1640 with 10% heat-inactivated fetal calf serum (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA)] with 0.3 mg mL^{-1} hygromycin-B (Life Technologies, Thermo Fisher Scientific).[21,26] SARS-CoV-2 peptides predicted to bind HLA-A*24:02 using NetCTLpan1.1[27] were purchased from GenScript Biotech (Singapore) and reconstituted in dimethyl sulfoxide (Figure 1a). Tetramers were generated from soluble, biotinylated HLA-A*24:02 monomers. In brief, HLA α-heavy chain with a C-terminal BirA biotinylation motif and β2-microglobulin were expressed and purified as inclusion bodies in *E. coli*, solubilized in 6 M guanidine HCl and refolded with either S_{1208} or S_{448} peptide, as essentially as described.[8] Purified, fully biotinylated HLA-A24 monomers were stored at −80°C and conjugated to fluorescently labeled streptavidins, phycoerythrin (PE)–streptavidin or allophycocyanin (APC)–streptavidin (BD Biosciences, Franklin Lakes, NJ, USA) at an 8:1 monomer to streptavidin molar ratio to form peptide–major histocompatibility complex class I tetramers.

Intracellular cytokine staining

Peripheral blood mononuclear cell samples were stimulated with A24/SARS-CoV-2-predicted peptides from two peptide pools (1 μM) for 12 days in RF-10 medium (+20 U mL^{-1} interleukin-2).[28] Pool 1 contained 12 spike (S) peptides and pool 2 contained 6 membrane (M) and 4 nucleocapsid (N) peptides. On day 12, cells were stimulated with C1R.A*24:02 cells that were pulsed with individual peptides for 6 h in the presence of GolgiPlug and GolgiStop (BD Biosciences) plus 10 U mL^{-1} interleukin-2, and the SARS-CoV-2-reactive $CD8^+$ T cells were quantified using anti-IFNγ-V450 and anti-TNF-AF700 in an intracellular cytokine staining assay.[29]

Ex vivo tetramer-associated magnetic enrichment and TCR analysis

For tetramer-associated magnetic enrichment, cells ($5–32 \times 10^6$) were incubated with FcR Block (Miltenyi Biotec, Bergisch Gladbach, Germany) and anti-NKB1 (BD Biosciences)[21] before staining with A24/S_{448}–PE and A24/S_{1208}–APC tetramers at 1:100 for 1 h in MACS buffer (PBS with 0.5% bovine serum albumin and 2 mM ethylenediaminetetraacetic acid). $Tetramer^+$ cells were enriched with anti-PE and anti-APC microbeads (Miltenyi Biotec) using magnetic separation.[15,16] Unenriched, enriched and flow-through fractions were stained with anti-CD4-BV650, anti-CD27-BV711, anti-CD14-APC-H7, anti-CD19-APC-H7, anti-CD45RA-FITC, anti-CD8-PerCP-Cy5.5, anti-CD95-PE-CF594 (BD Biosciences), anti-CD3-BV510 (BioLegend, San Diego, CA, USA) and Live/Dead near-infrared (Invitrogen, Carlsbad, CA, USA) for 30 min, washed and resuspended in MACS buffer. Prepandemic samples were acquired on a LSRII Fortessa (BD Biosciences). Enriched $tetramer^+$ cells from COVID-19 samples were single-cell index-sorted into empty 96-well twin.tec PCR plates (Eppendorf, Hamburg, Germany) using the FACSAria III (BD Biosciences) for TCR analyses. Plates were kept cold, centrifuged and then stored at −80°C. Amplification of paired CDR3α and CDR3β regions was performed using multiplex-nested reverse transcription-PCR, as described previously.[15,16] TCR sequences were analyzed using IMGT/V-QUEST and flow cytometry data were analyzed using FlowJo (version 10) software (BD, Ashland, OR, USA). Data visualization was performed in R using packages for circular layout[30] and graphics generation.[31]

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTION

Louise C Rowntree: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft. **Jan Petersen:** Formal analysis; Methodology; Supervision; Writing-review & editing. **Jennifer A Juno:** Formal analysis; Resources; Writing-review & editing. **Priyanka Chaurasia:** Investigation; Methodology; Writing-review & editing. **Kathleen M Wragg:** Methodology; Writing-review & editing. **Marios Koutsakos:** Formal analysis; Writing-review & editing. **Luca Hensen:** Formal analysis; Methodology; Writing-review & editing. **Adam K Wheatley:** Formal analysis; Writing-review & editing. **Stephen J Kent:** Methodology; Resources; Writing-review & editing. **Jamie Rossjohn:** Methodology; Resources; Supervision. **Katherine Kedzierska:** Conceptualization; Funding acquisition; Resources; Supervision; Writing-original draft. **Thi HO Nguyen:** Conceptualization; Formal analysis; Investigation; Methodology; Supervision; Writing-original draft.

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